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HIGH-PERFORMANCE CHROMATOFOCUSING OF PROTEINS ON AGAROSE COLUMNS

I. MACROPOROUS 15–20 μm BEADS

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SUMMARY

The synthesis of a polyethyleneimine based anion exchanger is described. The titration capacity was highest at alkaline pH (14–16 mequiv. per 100 g agarose bed) and roughly constant in the interval pH 3–8 (8–10 mequiv. per 100 g agarose bed). The resolution has been determined as a function of flow-rate, concentration of the eluting buffer (Polybuffer™) and sample load. The mass recovery was about 100%, whereas the recovery of the enzymatic activity of β-galactosidase was slightly below 90%. Human serum albumin was isocratically eluted without tailing in 0.05 *M* Tris–HCl, pH 7.5, containing 0.5 *M* sodium chloride, indicating that the beads were hydrophilic. The focusing effect was demonstrated by photographing a separation of haemoglobins A, F, S and C and by application of a sample in different volumes and at different times during the course of the generation of the pH gradient (a sample volume 40-fold larger than the bed volume did not affect the resolution). The resolution increased upon dilution of the Polybuffer up to at least 160-fold; there is accordingly no need to use large amounts of this expensive buffer (a dilution greater than 1:80 is not recommended, since the concentration in the protein zones then becomes low).

INTRODUCTION

Since it was introduced in 1962 as a chromatographic support[1], agarose, a polysaccharide from red seaweed, has probably become the most widely used gel matrix for the fractionation of macromolecules by classical low pressure chromatography, including chromatofocusing. About 20 years later experiments with highly concentrated, cross-linked agarose beads suitable for high-performance liquid chromatography (HPLC) columns, were reported[2]. During the last few years various column packings based on agarose have been prepared and used successfully for HPLC of proteins: for instance, beds for molecular sieving[3–6], hydrophobic inter-

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action chromatography[7], ion-exchange chromatography[8,9], affinity chromatography[10] and boronate chromatography[11].

In this paper we describe the chromatographic properties of a macroporous agarose-based HPLC matrix for chromatofocusing.

MATERIALS AND METHODS

The macroporous, cross-linked 12% agarose beads (diameter: 15–20 μm) were a gift from Dr. Sten Porrvik, Casco Nobel, Sundsvall, Sweden. They were prepared by a modification of a method published previously[12] and were then cross-linked. Polyethyleneimine (molecular weight: 30000) was obtained from Serva (Heidelberg, F.R.G.); Polybuffer™ from Pharmacia (Uppsala, Sweden); β-galactosidase from Boehringer (Mannheim, F.R.G.); haemoglobin A, F, S and C from Isolab (Akron, OH, U.S.A.); and sodium borohydride from E. Merck (Darmstadt, F.R.G.). Human serum albumin and human transferrin were gifts from KabiVitrum (Stockholm, Sweden). Other chemicals, including butanediol diglycidyl ether, Bis-Tris [bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane], ovalbumin and *o*-nitrophenyl-D-galactopyranoside, were obtained from Sigma (St. Louis, MO, U.S.A.).

The Model 2150 HPLC pump, the Model 2152 HPLC controller, the Models 2138 and 2158 detectors and the Model 2210 recorder were from LKB-Products (Bromma, Sweden). The Model 7125 loop injectors were from Rheodyne (Berkely, CA, U.S.A.). The pH-monitor was from Pharmacia. The column tubes, made from Plexiglas, had an inner diameter of 6 mm (their design has been described in ref. 13). They were packed with agarose beads suspended in deionized water at a flow-rate of 1.0 ml/min.

EXPERIMENTAL AND RESULTS

Synthesis of the anion exchanger

The cross-linked agarose beads were treated with 1,4-butanediol diglycidyl ether essentially as described by Maisano *et al.*[14]. By this treatment the agarose chains became not only further cross-linked (in the case when both epoxide groups reacted with the agarose chains) but also activated (in the case where only one epoxide group reacted with the agarose; the other epoxide group was used later for coupling to polyethyleneimine). In brief, the activation (and attendant cross-linking) was performed as follows. Sedimented gel (1 g) was suspended in 1 ml of 0.6 *M* sodium hydroxide and mixed with 0.5 ml of 1,4-butanediol diglycidyl ether and 2 mg of sodium borohydride. After stirring for 15 h at room temperature, the beads were washed with water by centrifugation until the supernatant had a pH of 7–8. The agarose beads, thus activated, were suspended in 1 ml of 0.5 *M* sodium bicarbonate (pH 8.0) containing 6 mg of sodium borohydride[9]. An appropriate amount of polyethyleneimine was added and the suspension was stirred at room temperature for various periods of time. The beads were finally washed with water on a Büchner funnel. The reaction scheme is found in ref. 9.

If not otherwise stated, we used 0.5 ml of polyethyleneimine per gram sedimented agarose and a reaction time of 7 h. After each experiment the columns were cleaned with about 1 ml of a 2 *M* solution of sodium chloride prior to equilibration with the starting buffer.

Titration of the synthesized anion exchanger

The titration was performed essentially as described by Peterson and Sober[15]. A 3-g amount of the sedimented ion exchanger was suspended in 10 ml of a 0.5 *M* solution of sodium chloride prepared from boiled water for the removal of carbon dioxide. The titration capacity in different pH intervals is presented in Fig. 1 along with the total titration capacity as a function of the reaction time and volume of polyethyleneimine used for the synthesis.

Recovery

The column (30 mm × 6 mm I.D.) was equilibrated with 0.025 *M* Bis-Tris, pH 6.5. Ovalbumin (2 mg) was dissolved in 0.1 ml of this buffer and applied to the column. The protein was eluted with Polybuffer 74 (diluted 1:10)–HCl, pH 4, at a flow-rate of 1.0 ml/min. From measurements of the volume and absorption at 280 nm of both the applied sample and the eluted fractions, the mass recovery was estimated at 99%. For human serum albumin (1 mg) the recovery was 100%.

The recovery of enzymatic activity, using β-galactosidase as a sample, was determined in an analogous way to be 89%. When both the starting and eluting buffers contained 1% (w/v) G3707, or 10%, or 20% (v/v) ethylene glycol, the recovery was estimated at 88, 87 and 72%, respectively.

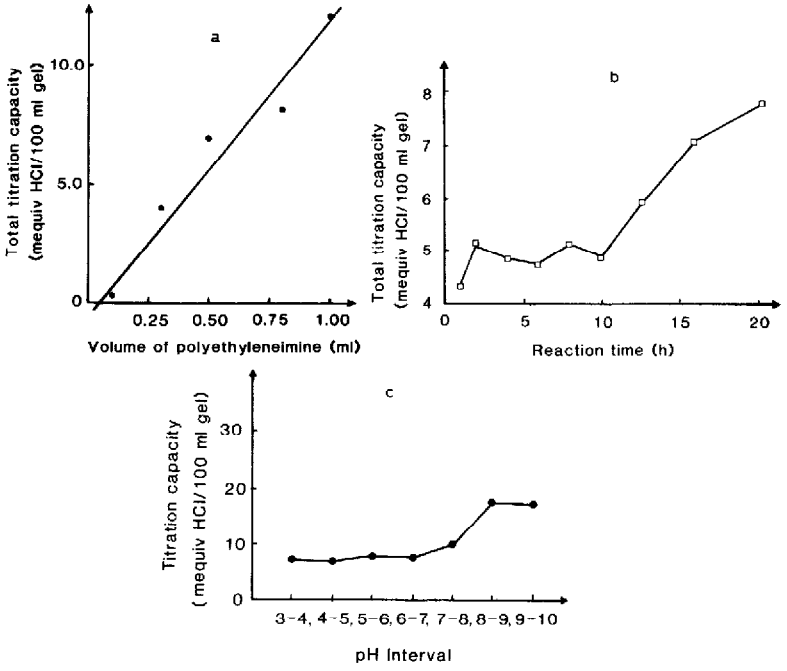


Fig. 1. The total titration capacity as a function of the amount of polyethyleneimine added (a) and as a function of reaction time (b). The titration capacity within different pH intervals is presented in (c). The reaction time in (a) and (c) was 7 h; 0.5 ml of polyethyleneimine per gram agarose were used in (b) and (c).

Resolution as a function of flow-rate

A Plexiglas column with an inner diameter of 6 mm was packed at a flow-rate of 1.2 ml/min to a height of 30 mm with the 12%, cross-linked 15–20 μm agarose beads. The bed was equilibrated with 0.025 *M* Bis-Tris–HCl, pH 6.5. The sample, 0.5 mg of human transferrin, was dissolved in 100 μl of the equilibration buffer (starting buffer). Elution was performed with Polybuffer 74 (diluted 1:20)–HCl, pH 5.0, at a flow-rate of 1.2 ml/min. The experiment was then repeated at the flow-rates 0.9, 0.6 and 0.24 ml/min. The resolution, R_s, between the two transferrin peaks obtained was calculated at the different flow-rates from the relationship.

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

where t_1 and t_2 are the retention times of the tranferrin peaks and t_{w1} and t_{w2} are the peak widths (in time units) at half the peak heights. The results are presented in Fig. 2.

Resolution as a function of Polybuffer concentration

The experimental conditions were similar to those mentioned in the previous section with the exception that the flow-rate was kept constant, 0.6 ml/min, and the dilution of the Polybuffer 74 was varied (1:5, 1:10, 1:20, 1:40, 1:80 and 1:160). Fig. 3a shows a plot of the resolution against dilution. Some of the chromatograms are presented in Fig. 3b in order to give a visual impression of the variations in resolution with the dilution of the Polybuffer.

Resolution as a function of sample load

The column bed (30 mm × 6mm I.D.) was equilibrated with 0.025 *M* triethanolamine–HCl, pH 8.3 (starting buffer). After application of 0.25 mg of a mixture of haemoglobin C, S, A and F, dissolved in 100 μl of the starting buffer, the proteins were eluted at a flow-rate of 0.5 ml/min with Polybuffer 96 (diluted 1:10)–HCl, pH 7.0. The resolution was calculated for haemoglobins C and S according to eqn. 1. After washing with 2 *M* sodium chloride and equilibration of the column, the experiment was

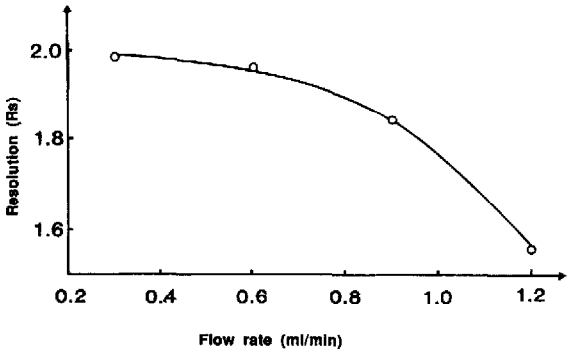
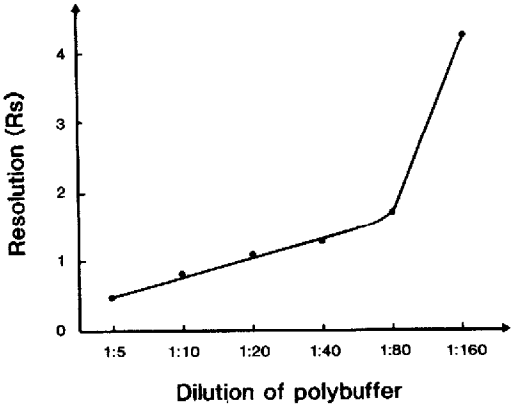
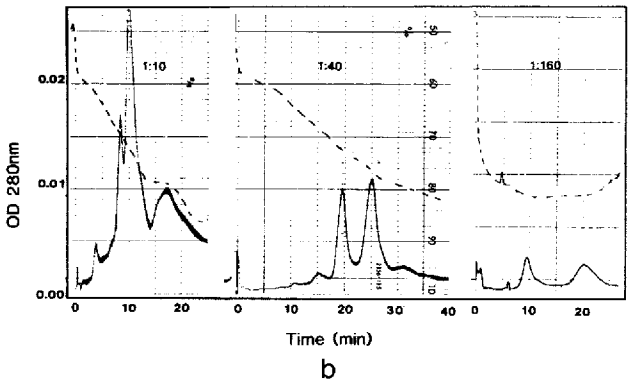


Fig. 2. Resolution as a function of flow-rate. A 0.5-mg amount of transferrin in 100 μl of starting buffer was applied to the column (30 mm × 6 mm) and eluted at flow-rates of 1.2, 0.9, 0.6 and 0.3 ml/min. The resolution between the two transferrin components obtained was calculated from eqn. 1.



a



b

Fig. 3. Resolution as a function of dilution of Polybuffer. Transferrin (0.5 mg), dissolved in 100 μl of the starting buffer, was applied to the column and eluted with different concentrations of Polybuffer at a flow-rate of 0.6 ml/min. (a) A plot of resolution against dilution. The resolution between the two transferrin components obtained was calculated from eqn. 1. (b) Chromatograms obtained at different dilutions of Polybuffer. The dashed curve shows the shape of the pH gradient.

repeated with 0.5 mg of haemoglobin. Similar experiments were then performed with 0.75 and 1.0 mg of haemoglobin. Fig. 4 shows a plot of the resolution against the sample load.

Visual inspection of chromatofocusing of haemoglobins C, S, A and F

The dimensions of the agarose bed were 50 mm × 6 mm I.D. Starting and elution buffers were 0.025 *M* triethanolamine–HCl, pH 8.3 and Polybuffer 96 (diluted 1:20)–HCl, pH 7.0, respectively. The sample consisted of 100 μg of a mixture of haemoglobins A, F, S and C, dissolved in 100 μl of the starting buffer. The flow-rate was 1.0 ml/min. A photograph of the column with the focused haemoglobin zones is presented in Fig. 5.

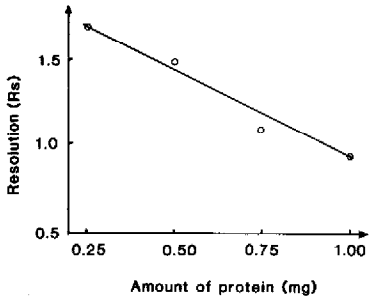


Fig. 4. Resolution as a function of sample load. Samples of Hb C, S, A and F (from 0.25 to 1.0 mg) were dissolved in 100 μl of starting buffer. Elution was performed at a flow-rate of 0.5 ml/min (column dimensions: 30 × 6 mm I.D.). The resolution was calculated for haemoglobins C and S with the aid of eqn. 1.

Fig. 5. Photograph of a separation of haemoglobins C, S, A and F by high-performance chromatofocusing. Conditions: column 50 × 6 mm I.D.; sample, 0.1 mg Hb C, S, A and F in 100 μl of starting buffer; flow-rate, 1.0 ml/min. The picture was taken 15 min after sample application.

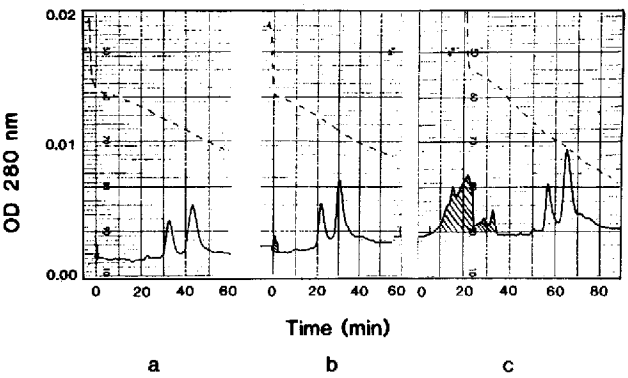


Fig. 6. Demonstration of the focusing effect by application of the sample in different volumes. A 0.5-mg amount of transferrin was dissolved in 0.01 (a), 1.0 (b) and 32 ml (c) of the starting buffer. Column dimensions: 30 mm × 6 mm I.D. Flow-rate: 0.6 ml/min. The shaded peaks are disturbances caused by the sample application. The dashed curves show the shape of the pH gradient. The figure illustrates that the separation pattern is independent of the sample volume.

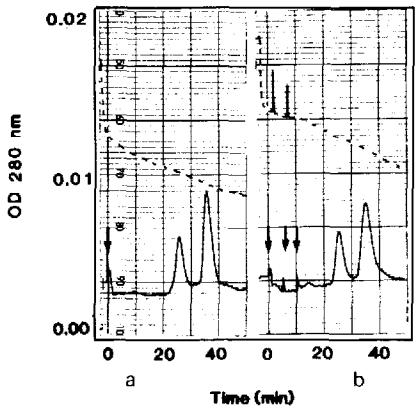


Fig. 7. Demonstration of the focusing effect by application of the sample at different times. (a) The sample (0.5 mg of transferrin) in 100 μl of starting buffer was applied in one injection. (b) The same sample was injected in three 33-μl aliquots at times indicated by arrows. For conditions, see Fig. 6. The dashed curves show the course of the pH gradient. A comparison of the two chromatograms indicates that the focusing pattern is independent of the time of application of the sample.

Demonstration of the focusing effect by application of the sample in different volumes and at different times

The experiments were performed under the same conditions as those given in *Resolution as a function of flow-rate* with the difference that the flow-rate was constant, 0.6 ml/min, and that the sample volumes were 0.01, 1.0 and 32 ml (the latter was injected in 0.6-ml portions every min; the total time for application of this sample was thus about 50 min). The amount of transferrin was 0.5 mg in each case. The chromatograms are presented in Fig. 6. The shaded peaks at the left in the chromatograms do not correspond to proteinaceous material.

The above experiment was then repeated with the exception that the sample was dissolved in 0.1 ml of the starting buffer (Fig. 7a). In another experiment this sample was injected in three aliquots (0.033 ml each) at the times indicated by arrows in the chromatogram (Fig. 7b).

DISCUSSION

Electrofocusing and chromatofocusing, two analogous separation methods

Electrophoresis and chromatography are analogous methods and therefore any electrophoretic method has its chromatographic counterpart and *vice versa*[16]. If more researchers had been aware of this analogy the progress of both these methods would, no doubt, have been faster. For example, electrofocusing of proteins was introduced in 1954 by Kolin[17] and in a more sophisticated version by Svensson (Rilbe) in 1961[18], but it was not until 1978 that chromatofocusing, the chromatographic equivalent of electrofocusing, was first described[19]. Characteristic of both methods is the zone-sharpening effect (Fig. 5), *i.e.*, the final separation pattern is to a great extent independent of the volume of the sample. For chromatofocusing this attractive feature is illustrated in Fig. 6, which shows that a fixed amount of protein gave the same chromatogram when the volume in which the protein was dissolved was varied

3200-fold. This focusing effect (which also can be demonstrated by applying the sample in aliquots; see Fig. 7) has, of course, many advantages, but it may also create problems: the calculated high concentration of proteins in a focused zone is not always attainable because the solubility of many proteins at their isoelectric points is low, resulting in precipitation of the proteins. The presence of such precipitates may decrease the resolution and the recovery considerably and reduce the lifetime of a column. The risk of precipitation can be suppressed if the experiments are performed in the presence of ethylene glycol or a mild neutral detergent such as G3707, heptaoxyethylene lauryl ether[20]. Ethylene glycol has, furthermore, the favourable effect of stabilizing labile proteins (enzymes)[21]. However, the addition of these agents to the buffers did not increase the recovery of activity (89%) of β-galactosidase in the experiments described herein, whereas the mass recoveries of ovalbumin and serum albumin were excellent (about 100%) even in the absence of these agents.

Titration and protein capacity

Sluyterman and Wijdenes[22] found that agarose beads derivatized with polyethyleneimine and used for conventional low-pressure chromatography had a lower titration capacity at basic pH, whereas the column used in this study exhibited the lowest titration capacity in the acidic range (see Fig. 1c). We have no explanation for this discrepancy. In this connection we emphasize that the titration capacity of an ion exchanger is not related to the protein capacity in a simple way, since many of the titrated groups are not available to proteins for steric reasons.

Resolution as a function of the concentration of the eluting buffer

A decrease in the concentration of the eluting Polybuffer affected the resolution favourably (Fig. 3a). Wagner and Regnier[23] did not observe such an effect in their chromatofocusing experiments on Synchropac AX-300. The polyethyleneimine-based anion exchanger used in this study gave a satisfactory separation even when the Polybuffer was diluted as much as 160-fold (see Fig. 3b), which is considerably more than has been reported for other ion exchangers[23]. This is of practical importance, since the high price of Polybuffer limits the usefulness of chromatofocusing, particularly in large-scale operations. However, at a dilution of 1:160 the proteins were eluted at a virtually constant pH (Fig. 3b). The separative mechanism at this extremely high dilution is therefore not based on chromatofocusing but rather on conventional ion-exchange chromatography.

Macroporous vs. deformed non-porous agarose beads

An obvious advantage of conducting chromatofocusing in the HPLC mode as described herein is that columns as short as 3 cm can be used without loss in resolution. The analysis times can accordingly be reduced. However, additional advantages are gained when the macroporous beads used in the experiments described herein are exchanged for non-porous, deformed beads (see the following paper[24]). For instance, the decrease in resolution with an increase in flow-rate demonstrated in Fig. 2 is in accordance with the van Deemter equation[25], but is not observed on columns packed with the non-porous beads.

The residence time

The time between the first and last injection of the sample in the experiment illustrated in Fig. 6c was about 50 min. Since the focusing patterns in Fig. 6a, b and c are very similar one can conclude that the residence time of proteins on the column will not influence the appearance of the chromatograms; in other words, the adsorption is not time-dependent, which is characteristic also of agarose-based hydrophobic interaction and ion-exchange chromatography[7,9].

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