Journal of Chromatography, 206 (1981) 441–447
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,438

CHROMATOFOCUSING

IV. PROPERTIES OF AN AGAROSE POLYETHYLENEIMINE ION EXCHANGER AND ITS SUITABILITY FOR PROTEIN SEPARATIONS

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(Received October 17th, 1980)

SUMMARY

Polyethyleneimine has been attached to epoxide-activated Sepharose. The resulting material exhibits high capacity, fast equilibration on acid–base titration and no maxima in a pH gradient. The resolving power is improved when some of the imino groups are converted into guanidino groups on modification with O-methylisourea. The final material is suitable for separations at pH $\leqslant$9.2.

INTRODUCTION

It was shown in the preceding paper[1] that improved resolution of protein separation is possible on columns of DEAE-Sepharose of high maximum capacity, utilizing the principle of chromatofocusing[2]. This anion exchanger, however, exhibited one drawback: when high protein concentrations in simple buffer systems were used, slight maxima in the pH gradients were observed which had an unfavourable effect on the separations. These maxima were caused by a slow pH equilibration between the ion-exchange beads and the surrounding buffer. In the present paper the preparation and the utilization of an anion exchanger of high maximum capacity which exhibits fast equilibration are described. It consists of polyethyleneneimine attached to agarose beads.

EXPERIMENTAL

The equipment, most materials and procedures were as described previously[2,3].

Polyethyleneimine-Sepharose (PI-Sepharose) was prepared as follows. Epoxide-activated Sepharose was either prepared[4] from Sepharose 6B-CL (Pharmacia, Uppsala, Sweden) and bisoxirane or obtained commercially (Pharmacia). A 100-g amount of this material, briefly sucked dry on a büchner filter, was suspended in a mixture of 200 ml 0.3 *M* Na_3PO_4 and 100 g of polyethyleneimine (Fluka, Buchs, Switzerland). The bottle containing the mixture was slowly rotated on a roller mill for 2 days at

0021-9673/81/0000–0000/$02.50

room temperature. The resulting material was washed extensively with water, 0.1 *M* acetic acid and again with water. If columns containing this material tended to clog, fines were removed by decantation.

PI-Sepharose modified with methylisourea (PI-gu-Sepharose) was prepared by suspending 30 g of PI-Sepharose in 200 ml of 0.05 *M* reagent (Aldrich, Milwaukee, WI, U.S.A.) for 8 h at pH 10.5 and room temperature on a roller mill.

Taurobetaine was prepared from taurine and methyl iodide as described by Brieger[5]. The reaction proceeded readily only in 70–85% (v/v) of methanol and not in dry methanol as solvent.

Pharmalyte buffers were a gift from Pharmacia.

RESULTS

Slow equilibration of protons between DEAE-Sepharose CL and its surrounding medium was evident from direct titration of a suspension of the exchanger, and from the occurrence of a maximum in the pH gradient on a column of the material when a somewhat steep gradient was followed by a weaker one (Fig. 4 of ref. 1). Such sluggishness could be caused by a slow change of the internal structure of the gel matrix. Agar gel consists of bundles of ordered polysaccharide chains interconnected at certain points. In DEAE-Sepharose the amino groups are linked near to the backbone and, hence, are close to each other. When a proportion of the amino groups becomes protonated, the resulting high positive charge density will hamper protonation of neighbouring amino groups. At the same time the mutual charge repulsion will tend to break down the bundles. This may be a slow process, comparable to the ageing processes of freshly prepared gels. Once the chains are a little further apart, the mutual repulsion decreases and some of the remaining amino groups can then take up a proton. This partially increases the mutual repulsion again and tends to increase the separation of the chains, etc., until an equilibrium is established.

If this explanation is correct, this effect could be remedied if the amino groups were attached at a longer distance from the backbone. For this purpose polyethyleneimine was attached to Sepharose 6B-CL by means of bisoxirane. The results confirmed our expectations. Thus virtually immediate equilibration was observed along the whole pH range on titration of a suspension of the new exchanger with sodium hydroxide (Fig. 1, curve a). Furthermore, no maximum was observed (Fig. 2) in the pH gradient, in contrast to the behaviour of DEAE-Sepharose (Fig. 4 of ref. 1). As calculated from Fig. 1 (curve a), the maximum capacity of PI-Sepharose is 0.6 *M* inside the stationary phase. This is three times higher than the maximum capacity of DEAE-Sepharose CL.

In the preceding paper an elution procedure that produces a fairly linear pH gradient and that retains the advantages of the chromatofocusing method was described. We have now applied this procedure to a column of PI-Sepharose for the fractionation of commercial myoglobin. The elution pattern obtained is shown in Fig. 3. Even more bands than before can be distinguished behind the main band. It is striking that the main band emerges at pH 9.6, *i.e.*, at a higher pH than observed on columns of DEAE-Bio-gel and of DEAE-Sepharose (pH 9.1[3] and pH 8.9[1] respectively). In view of the theoretical considerations and the experimental results reported

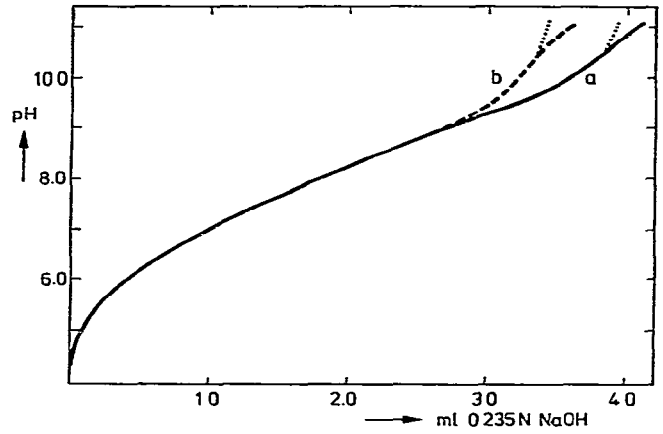

Fig. 1. Titration of 1.5 g of Sepharose derivatives, briefly sucked dry on a büchner filter, in 30 ml of water. ———, PI-Sepharose; – – – –, PI-gu-Sepharose; . . . . . , corrected for base consumption of water alone.

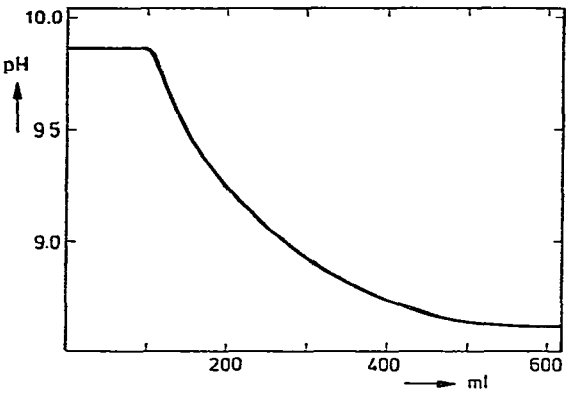

Fig. 2. pH gradient produced on a PI-Sepharose column with a single buffer mixture. Bed size: 15 × 1.6 cm. Pre-equilibration: 15 m*M* ammonia and formic acid down to pH 9.85. Elution: 15 m*M* ammonia and formic acid, pH 8.65. Flow-rate 48 ml/h. Similar results were obtained at 23 and 10 ml/h.

in the preceding paper[1], this suggests that in the high pH range the present exchanger apparently exhibits a low Donnan potential.

In order to increase the charge at high pH, some of the weakly basic imino groups were converted into strongly basic guanidino groups by reaction with O-methylisourea. From the shift of the top of the titration curve (Fig. 1, curve b) it is evident that some of the imino groups which were initially titrated at pH 9–11 had been converted into guanidino groups (p$K \approx 13$) that are not titrated in this pH range. The total charge at pH 9.5, as derived from chloride binding in dilute ammonia, had increased from 110 to 170 μmoles per gram of "dry" PI-Sepharose. The effect of the modification on the elution pattern is shown in Fig. 4, where the peaks emerge at a lower pH. The resolution was also slightly improved. The system was tested with

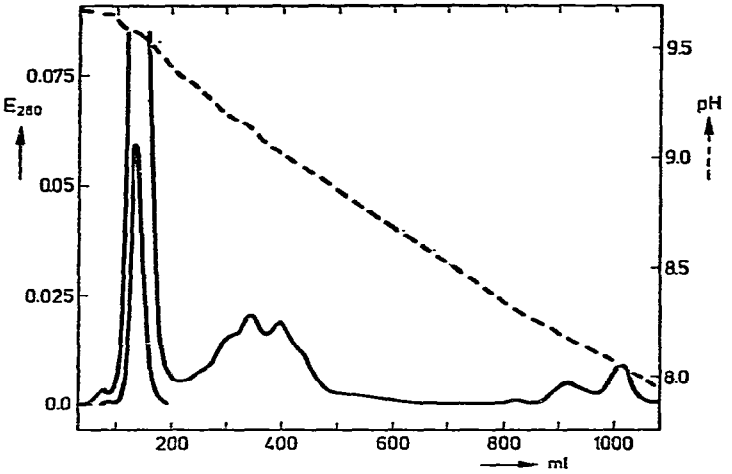

Fig. 3. Fractionation of commercial sperm-whale myoglobin on a column of PI-Sepharose (14 × 1.6 cm). Pre-equilibration: 15 m*M* ethanolamine and propionic acid down to pH 9.68. First elution buffer: 15 m*M* ethanolamine and propionic acid, pH 7.99. Second elution buffer; 15 m*M* Tris and propionic acid, pH 7.99. Gradient mixer adjusted to linear mixing from first to second elution buffer in 16 h, and then with the second buffer only. Flow-rate 45 ml/h. Protein load: 19 mg of myoglobin in 3 ml of first elution buffer, dialyzed against the same buffer. The UV monitor was adjusted to maximum deflections of $\varepsilon = 0.1$ and of $\varepsilon = 1.0$ (lower pattern of the main band).

another protein mixture, a preparation of S-carboxymethylpapain that had been treated with traces of acetic anhydride. The resulting mixture contains protein molecules in which zero, one, two and three amino groups, respectively, have been acetylated, each species carrying one less positive charge than the preceding species. The main species proved to be that carrying one acetyl group, as demonstrated by gel electrophoresis at pH 7.

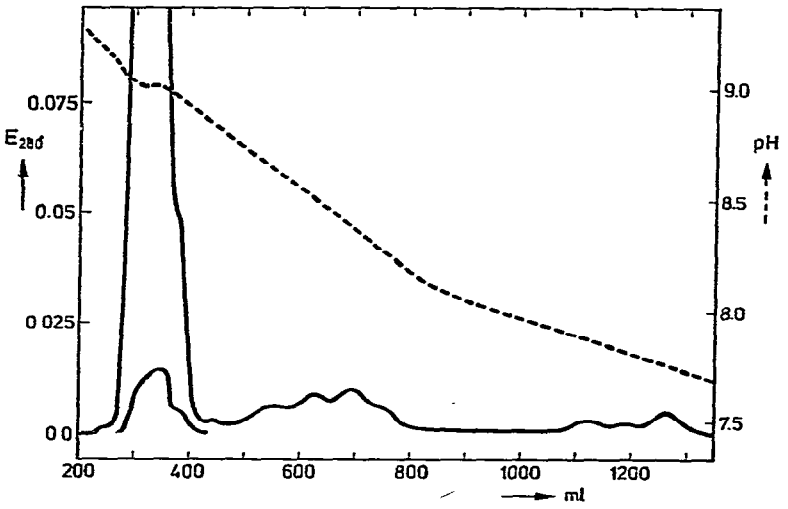

Fig. 4. Fractionation of myoglobin on a column of PI-gu-Sepharose. Protein load: 16 mg of myoglobin in 3 ml of first elution buffer. All other conditions as in Fig. 3.

The lack of solubility at the p*I* of a number of proteins is a general problem in isoelectric focusing methods. Some proteins, the globulins, dissolve in salt solutions. However, such solutions cannot be used in focusing methods. The internal salt taurobetaine was therefore tested for its dissolving power. It was found to increase the solubility of papain at pH 9.0 (Fig. 5, inset). When a 5% (0.3 *M*) solution of taurobetaine was used as solvent for acetylated papain an elution pattern was obtained similar to that shown in Fig. 5, for which 5% dimethyl sulphoxide had been used.

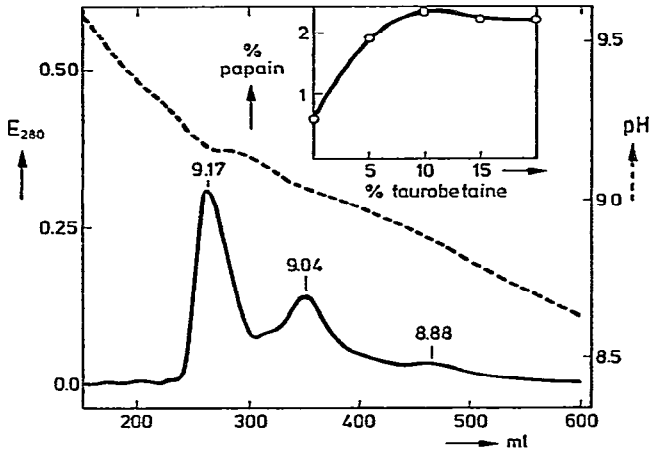

Fig. 5. Separation of partially acetylated papain species on a column of PI-gu-Sepharose 15 × 1.5 cm). Protein load: 22 mg papain dissolved in 3 ml of the first elution buffer. All buffers contained 6% dimethyl sulphoxide. Other conditions as in Fig. 3. Inset: the solubility of native papain at 25°C as a function of taurobetaine concentration. The pH was adjusted to 9.0 with ammonia,

A few preliminary experiments were carried out with ampholyte buffers (Pharmacia). The buffers were utilized for elution without the mixing system. The first part of two elution curves, with two different buffers, is shown in Fig. 6a and b. Good resolution was obtained; in particular the band of slightly different colour, immediately behind the main band, that was hardly separated in Fig. 4, was well separated with the Pharmalytes. It is also apparent that the pH of the main band on emergence is nearly equal (Fig. 6a) or equal (Fig. 6b) to the p*I* value (8.3) of sperm-whale myoglobin, as determined by isoelectric focusing[5].

DISCUSSION

The properties of PI-Sepharose confirm the supposition that sluggishness of the ion exchanger can be avoided by placing the charged groups sufficiently far from the backbone of the matrix.

In spite of the high capacity of PI-Sepharose, the main band of myoglobin emerges at 1.3 pH units higher than its p*I* value. If the pH of emergence is calculated as described in the preceding paper, a difference of only 0.25 is found. The actual difference of 1.3 pH units corresponds to an effective φ value of only half that calculated from the exchanger capacity and the buffer composition, both at pH 9.6. This

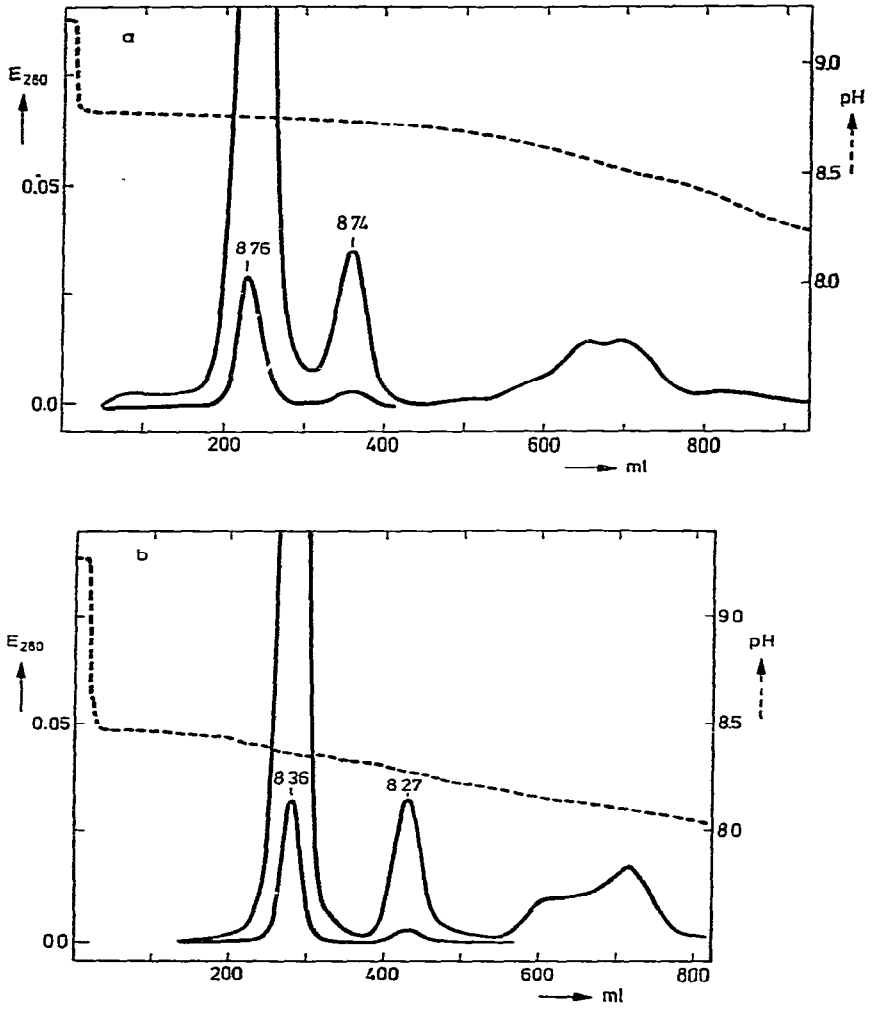

Fig. 6. Fractionation of commercial myoglobin on columns of PI-gu-Sepharose, eluted with Pharmalyte buffers. a, Bed volume: 14.5 × 1.6 cm. Pre-equilibration buffer: 15 m*M* ammonia and propionic acid, pH 9.30. Elution buffer: 7.8 ml Pharmalyte (pH range 8–10.5) in 1100 ml of water, adjusted to pH 7.49. Protein load: 16 mg myoglobin applied in 3 ml of elution buffer. Flow-rate 45 ml/h. b, Bed volume: 16 × 1.6 cm. Elution buffer: 14.9 ml of Pharmalyte (pH range 6.5–9) in 2100 ml of water, adjusted to pH 7.50. Other conditions as in a.

suggests that not all of the charges of the attached polyethyleneimine are accessible to the protein at this pH. Polyethyleneimine is known to be a branched polymer the structure of which is likely to be a closed random coil at low charge, and which opens up when the charge is increased.

The separating power of a column of PI-gu-Sepharose is excellent at pH $\leqslant$ 9.2 (Fig. 5). Above pH 9.2 the buffering capacity of the exchanger tends to diminish (Fig. 1, curve b). This causes a higher gradient than is favourable for good separations

(Fig. 5). It would be desirable, therefore, to devise an exchanger with a higher buffer capacity in the range pH 9–10.

The high resolution at the beginning of the elution with the ampholyte buffers is quite remarkable, and is reflected both in terms of ΔpH, which is proportional to $\sqrt{\mathrm{d\ pH/d}\ V\varphi}$, and of the elution volume ΔV, which is proportional to $\sqrt{1/(\varphi\ \mathrm{d\ pH/d}\ V)}$ (eqn. 26 of ref. 2). Both these quantities can be low only if φ is high. This can be explained in the following manner. The various components of the ampholyte buffer emerge in increasing number as in frontal analysis. Initially, only a few components emerge in almost isoelectric state. The ionic strength is therefore very low and φ is very high, explaining the high resolution and also the small difference between the pH of emergence and pI.

Taurobetaine enhances the solubility of papain. It might therefore be useful to test the solubility of other proteins in the presence of this and similar internal salts.

ACKNOWLEDGEMENT

The authors thank Mr. J. Boven for preparing the taurobetaine.

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