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GRADIENT AND ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS ON A NEW AGAROSE-BASED ANION EXCHANGER

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SUMMARY

We describe a new, simple, and mild method for the preparation of anion-exchangers, based on the coupling of alkylamines to epoxy-activated agarose (prepared by the reaction of agarose with butanediol diglycidyl ether). Since a polar OH-group is formed when an epoxide reacts with an OH or NH_2 group, the ion-exchanger did not show any hydrophobic interaction. This is important, since it may be impossible to desorb a protein from an ion exchanger having a hydrophobic character, because increasing the salt concentration of the eluent to decrease the electrostatic binding inevitably strengthens the hydrophobic interaction.

By the method described, 3-diethylamino-2-hydroxy-propyl agarose (DEAHP-agarose) was prepared. High resolution of proteins was obtained by gradient elution at both high and low degrees of substitution. However, isocratic separations required a low degree of substitution, in accordance with a hypothesis previously put forward in connection with a theoretical and experimental study of the conditions for isocratic elution of macromolecules on amphiphilic gels.

A study of the retention times of several proteins at different pH levels and buffer compositions indicated that different pH levels should be tested for maximal resolution and that, in many cases, the best resolution can be obtained if the DEAHP-agarose is operated in a buffer containing sodium acetate instead of sodium chloride.

A quaternary amine agarose, 3-methyldiethylamino-2-hydroxy-propyl agarose (QAE-agarose), can be synthesized easily from DEAHP-agarose by alkylation with methyl iodide. The titration curves of DEAHP-agarose and QAE-agarose showed pK values around 9.5 and 11.3, respectively.

INTRODUCTION

Classical ion-exchange chromatography is a powerful separation technique for proteins. It is therefore natural that considerable effort has been devoted to the de-

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velopment of ion-exchangers for high-performance liquid chromatography (HPLC). The matrices used have been of inorganic as well as organic nature[1–9]. Since agarose is more compatible with macromolecules of biological origin than are most other matrices and can be prepared in the form of rigid beads, we have used this polysaccharide as support for different HPLC methods[10–18], including ion-exchange chromatography[19,20]. In this paper the latter technique is described in more detail.

DEAE (diethylaminoethyl) ion-exchangers have been synthesized by attaching N-2-chloroethyl-N,N-diethylamino groups to amphiphilic gel beads[8]. We have also used this reaction for the preparation of DEAE-agarose beads suitable for high-performance ion-exchange chromatography.

However, we observed some drawbacks of the method when applied to the preparation of DEAE-agarose for high-performance ion-exchange chromatography. Firstly, the maximum flow-rate obtained for a column packed with such a DEAE-agarose was relatively low, even if the gel beads were crosslinked after derivatization. The reason was probably that a great number of hydroxyl groups on the agarose had been used for attachment of DEAE groups, thus decreasing the number of hydroxyl groups available for crosslinking. Secondly, the conditions for the reaction are very harsh (80°C, 2.5 *M* sodium hydroxide, 5 h), which may cause partial hydrolysis of the agarose chains, resulting in decreased mechanical stability (and flow-rate).

However, it is known that the reaction between amino and epoxide groups is very fast and results in an amine with higher degree of substitution[21]. Based on this reaction, a new method for the preparation of anion-exchange agarose suitable for HPLC was developed.

An important purpose of our work was to establish theoretically the necessary conditions for successful isocratic separations of macromolecules by ion-exchange chromatography, since very few such experiments have been reported in the literature. These studies showed that low ligand density is one of the most essential requirements which was also experimentally verified.

MATERIALS AND METHODS

Diethylamine and methyl iodide were obtained from Fluka (Buchs, Switzerland). Sodium borohydrate was from Merck-Schuchardt (Darmstadt, F.R.G.). 2-Amino-2-methyl-1-propanol, myoglobin (horse heart), lactoglobulin (bovine), hexokinase (yeast), pepsin (porcine) and lysozyme (egg white) were purchased from Sigma (St. Louis, MO, U.S.A.). Ovalbumin (chicken) and butanediol diglycidyl ether were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Human serum albumin and transferrin were gifts from L.-O. Andersson, Kabi-Vitrum (Stockholm, Sweden), Russell's viper venom from D. Eaker (this Institute) and cellulase (Endo II fraction from *Trichoderma reesei*) from G. Pettersson (this Institute). Phycoerythrin from *Ceramium rubrum* was prepared as previously described[22].

The chromatographic system, including 2150 HPLC pumps, 2152 HPLC controller, 2138 UV-detector and 2220 recording integrator, was from LKB (Bromma, Sweden) and the loop injector from Rheodyne (Berkeley, CA, U.S.A.).

All of the experiments were performed on 12% agarose beads prepared by a previously described suspension-gelation procedure[23]. They were fractionated before use by elutriation in water, and the fraction containing beads with diameters between

5–10 μm was collected. The crosslinking of the agarose gel beads will be described elsewhere. The Plexiglas column tubes with an inner diameter of 0.6 cm were packed in distilled water at constant pressure to a height of 6–8 cm. The absorbance measurements were made at 280 nm.

EXPERIMENTAL AND RESULTS

Synthesis of agarose-based anion-exchanger

The 12% agarose beads (diameters: 5–10 μm) were activated with butanediol diglycidyl ether according to a method described by Maisano *et al.*[24]. The coupling of the ion-exchange groups was performed as follows. The activated agarose beads (1 g of sedimented gel) were suspended in 1 ml of 0.5 *M* sodium bicarbonate, containing 6 mg of sodium borohydride. Diethylamine was added, and the mixture was stirred at room temperature for various periods of time. The agarose beads were then washed with water by repeated centrifugation or washing on a Büchner funnel. The reactions involved in the preparation of the ion-exchanger are summarized in Fig. 1. Although a secondary amine was used for the coupling, a tertiary amine was formed, as illustrated in the Figure. The ion-exchange properties can therefore be ascribed to the 3-diethylamino-2-hydroxyl-propyl group. The agarose derivative was named DEAHP-agarose.

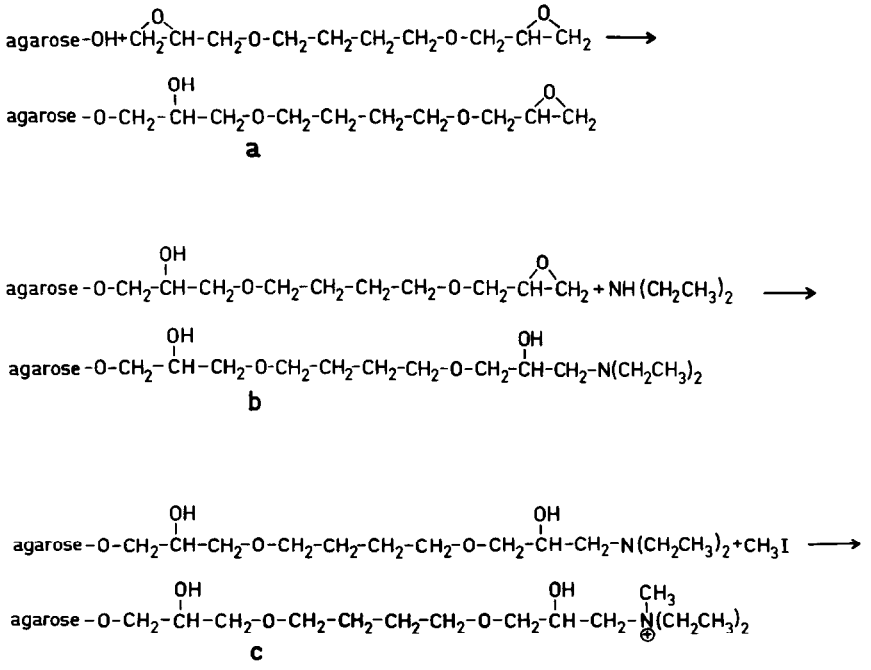

Fig. 1. The reactions involved in the preparation of DEAHP-agarose and QAE-agarose. (a) Activation of agarose with butanediol diglycidyl ether. (b) Coupling of diethylamine to activated agarose. A tertiary amino group, diethylamino-2-hydroxy-propyl- (DEAHP), is formed. (c) The alkylation of the tertiary amine with methyl iodide gives rise to 3-methyldiethylamino-2-hydroxy-propyl agarose (QAE-agarose).

The synthesis of a quaternary amine agarose was performed in the following way. DEAHP-agarose (5 g of sedimented gel) was transferred to dioxane by repeated washing on a Büchner funnel or by centrifugation and re-suspension in 15 ml of dioxane to which 5 ml of methyl iodide had been added. The suspension was stirred for 16 h at room temperature. The gel was then transferred to water by repeated washing on a Büchner funnel. The functional ligand of this quaternary amine agarose (QAE-agarose) is a 3-methyldiethylamino-2-hydroxy-propyl group.

Titration of the synthesized exchangers

The titration was performed according to Peterson and Sober[25]. The distilled water was boiled to remove carbon dioxide. The ion-exchanger (2.5 or 3.5 g of sedimented gel) was suspended in 7.5 ml of 0.5 *M* sodium chloride and titrated with 0.1 *M* hydrochloric acid. The pH was monitored with a Radiometer pH meter. The titration curves shown in Fig. 2 indicate that the p*K* values of DEAHP-agarose and QAE-agarose are about 9.5 and 11.3, respectively.

Determination of protein capacity

A series of DEAHP-columns with a volume of 1 ml were equilibrated with 0.05 *M* Tris–HCl (pH 8.5). One ml of a solution containing 45 mg of hemoglobin in this buffer was applied to one of these columns, which was then washed with 3 ml of the buffer to remove the excess protein. The hemoglobin adsorbed on the column was eluted with 2 ml of 0.05 *M* Tris–HCl (pH 8.5), containing 0.5 *M* sodium chloride.

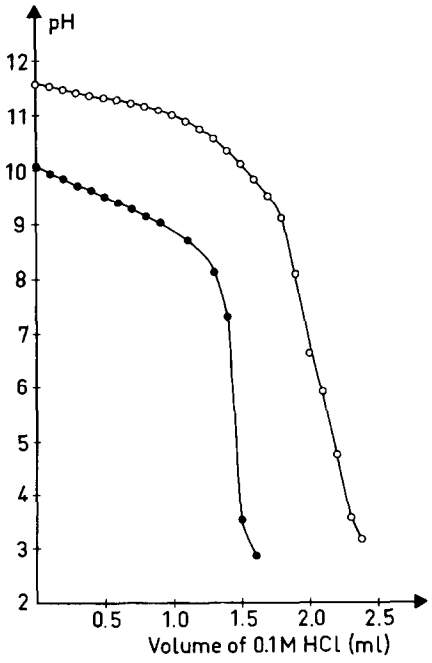

Fig. 2. Titration curves of DEAHP- and QAE-agarose. The amount of DEAHP-agarose (lower curve) and QAE-agarose used were 2.5 g and 3.5 g, respectively.

The protein content in the eluate was measured spectrophotometrically. The protein capacities of the DEAHP-agarose prepared under different conditions are presented in Fig. 3. The protein capacity increases linearly with reaction time for up to 5 h, when the derivatization is carried out with 0.1 ml of diethylamine per g sedimented agarose gel. When the treatment is performed with 0.2 ml diethylamine per g gel, the protein capacity is almost constant for reaction times from 2 to 5 h, indicating that most of the available epoxide groups have reacted within 2 h. Therefore, prolonging the reaction time has no significant influence on the protein capacity. The protein capacities of the DEAHP-agarose beads used for the experiments described below were 28 mg and 2 mg per ml of sedimented DEAHP-agarose. The high- and low-substituted derivatives are called DEAHP-agarose H and DEAHP-agarose L, respectively. From the titration curves (Fig. 2) the capacities of DEAHP-agarose H and QAE-agarose can be estimated at 5.8 and 5.7 mequiv./100 ml gel. (From a similar titration curve for DEAHP-agarose L, its capacity was estimated at 4.4 mequiv./100 ml gel.)

Estimation of the hydrophobicity of DEAHP-agarose and the influence of residence time on the elution pattern

The hydrophobicity of DEAHP-agarose H was estimated with human serum albumin (HSA), because it is one of the most hydrophobic water-soluble proteins. The column was equilibrated with 0.05 *M* Tris–HCl (pH 8.5), containing 0.3 *M* sodium chloride. HSA could be eluted with this solution isocratically. The experiment was then repeated on the same column after increasing the sodium chloride concentration to 0.5 *M*. Two additional experiments were performed at sodium chloride concentrations of 0.7 and 1.0 *M*. The retention time of HSA was the same at all four salt concentrations, which means that the DEAHP-agarose shows no significant hydrophobic interaction with proteins, at least not for sodium chloride concentrations up to 1 *M* (so high a salt concentration is seldom required for desorption of proteins in ion-exchange chromatography).

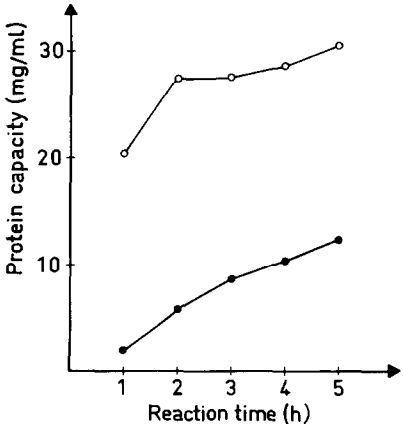

Fig. 3. The protein capacities of DEAHP-agarose, synthesized as outlined in Fig. 1a and b with 0.1 (lower curve) and 0.2 ml diethylamine per gram sedimented gel. The protein capacity was determined with hemoglobin in 0.05 *M* Tris–HCl (pH 8.5).

It has been stated that in ion-exchange chromatography proteins may be first adsorbed on the gel by electrostatic interactions upon which other interactions, *e.g* hydrophobic interactions, may later be superimposed[26]. In such cases, one can expect the chromatographic pattern to change, if the proteins are allowed to remain in the column for some time. Therefore —and also because any kind of interaction with the column may be time-dependent— it was interesting to see whether the residence time of a protein in a DEAHP-agarose column had any influence on the chromatographic pattern. Fig. 4 shows that the chromatogram is almost the same, whether the proteins had been adsorbed at the top of the column for 2 h or were eluted directly. (The proteins were eluted with a linear gradient from 0.05 *M* Tris–HCl (pH 8.5), containing 0.01 *M* sodium chloride to 0.05 *M* Tris–HCl (pH 8.5), containing 0.3 *M* sodium chloride in 40 min.).

Estimation of the recovery

A catalase from *Penicillium chrysogenum* has been highly purified by chromatography on DEAHP-agarose L. The recovery of the enzymatic activity was 97% (ref. 27).

For determination of mass recovery from a DEAHP-agarose H column we used human serum albumin, ovalbumin, human transferrin and phycoerythrin. From measurements of volume and absorption (at 280 nm) of both the applied samples and the eluted fractions the recovery was estimated at 103, 97, 100 and 98%, respectively.

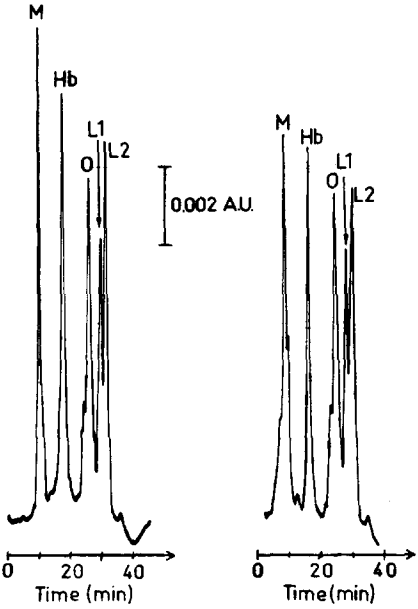

Fig. 4. The influence of the residence time on the appearance of the chromatogram. Column, DEAHP-agarose H. The elution was carried out (a) immediately after the sample application or (b) after the sample had been adsorbed at the top of the column for 2 h. M stands for myoglobin; Hb, human hemoglobin; L, lactoglobulin. For chromatographic conditions see the legend to Fig. 5.

Estimation of the resolving power

The resolving power of the DEAHP-agarose H column was estimated by separation of model proteins by elution with a linear gradient from 0.05 *M* Tris–HCl (pH 8.5), containing 0.01 *M* sodium chloride to 0.05 *M* Tris–HCl (pH 8.5), containing 0.3 *M* sodium chloride in 40 min. The chromatogram presented in Fig. 5 indicates that DEAHP-agarose H gives excellent resolution of the proteins. Isocratic elution of proteins from this column has also been tried, but the result was not satisfactory.

For the same model proteins, similar results could be obtained with DEAHP-agarose L upon gradient elution, although a different gradient was required. Furthermore, the separation of model proteins by isocratic elution was possible (Fig. 6). The proteins were eluted with 0.05 *M* Tris–HCl, pH 8.5, containing 0.07 *M* sodium chloride. Satisfactory resolution was obtained without any indication of tailing.

Influence of pH and organic anions on the protein retention

The experiments were performed only with DEAHP-agarose H because the above parameters should have the same influence on DEAHP-agarose H and DEAHP-agarose L.

The retention of various proteins (in the form of the ratio V_e/V_t; V_e = elution volume, V_t = total volume) was determined by gradient elution at different pH values. A 0.05 *M* Tris–HCl buffer was employed for pH 7.5 and 8.5, and a 0.05 *M* 2-amino-2-methyl-1-propanol–HCl buffer for pH 9.0. A 40-min linear gradient was formed from 0.05 *M* buffer, containing 0.01 *M* sodium chloride, and 0.05 *M* buffer, containing 0.3 *M* sodium chloride. The proteins were chromatographed separately, and V_e/V_t was plotted against pH (Fig. 7).

The effect of organic anions on protein retention was determined at pH 8.5. Acetate, propionate and butyrate were selected for this purpose. The same gradient

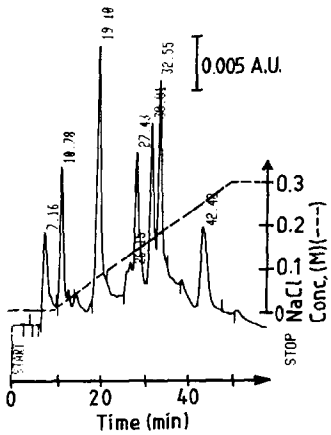

Fig. 5. Separation of proteins by high-performance ion-exchange chromatography on DEAHP-agarose H. The sample contained 15–50 μg of each of the proteins; ribonuclease (t_R = 7.16 min), myoglobin (t_R = 10.78), hemoglobin (t_R = 19.10), ovalbumin (t_R = 27.43), lactoglobulin (t_R = 30.01 and 32.55), and phycoerythrin (t_R = 42.48). Sample volume, 20 μl; column dimensions, 7 cm × 0.6 cm I.D.; flow-rate, 0.2 ml/min. A linear gradient from 0.05 *M* Tris–HCl (pH 8.5), containing 0.01 *M* sodium chloride to 0.05 *M* Tris–HCl, (pH 8.5), containing 0.3 *M* sodium chloride was completed in 40 min.

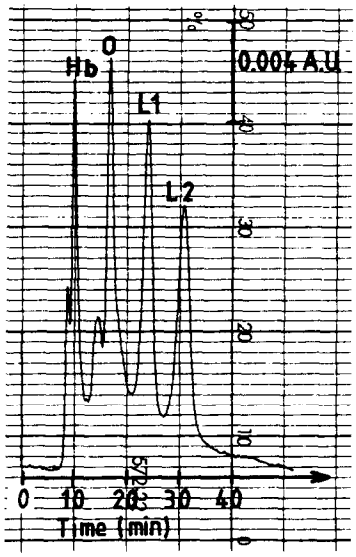

Fig. 6. Isocratic elution of proteins on DEAHP-agarose L. The sample contained 15–50 μg of each of the proteins hemoglobin (Hb), ovalbumin (O), and lactoglobulin (L_1 and L_2). The isocratic elution was performed with 0.05 *M* Tris–HCl (pH 8.5), containing 0.07 *M* sodium chloride. Sample volume, 20 μl; column dimensions, 8 cm × 0.6 cm I.D.; flow-rate, 0.2 ml/min.

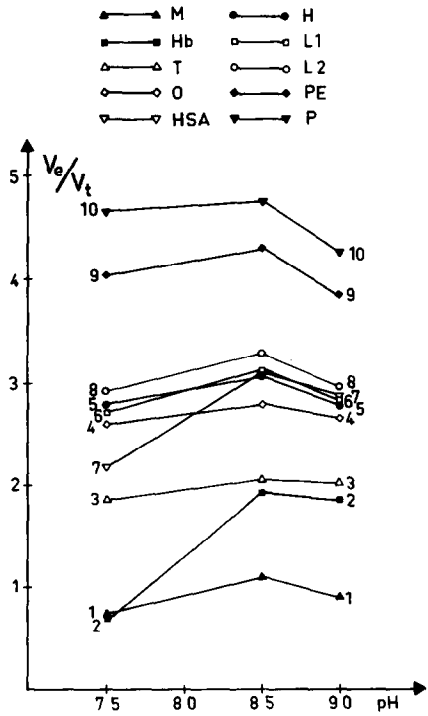

Fig. 7. The pH dependence of the adsorption of proteins on DEAHP-agarose. The V_e/V_t ratio was determined separately for each protein by gradient elution. The model proteins used are listed in the figure, where M denotes myoglobin; Hb, human hemoglobin; T, human transferrin; O, ovalbumin; HSA, human serum albumin; H, hexokinase; L1 and L2, lactoglobulins; PE, phycoerythrin; and P, pepsin.

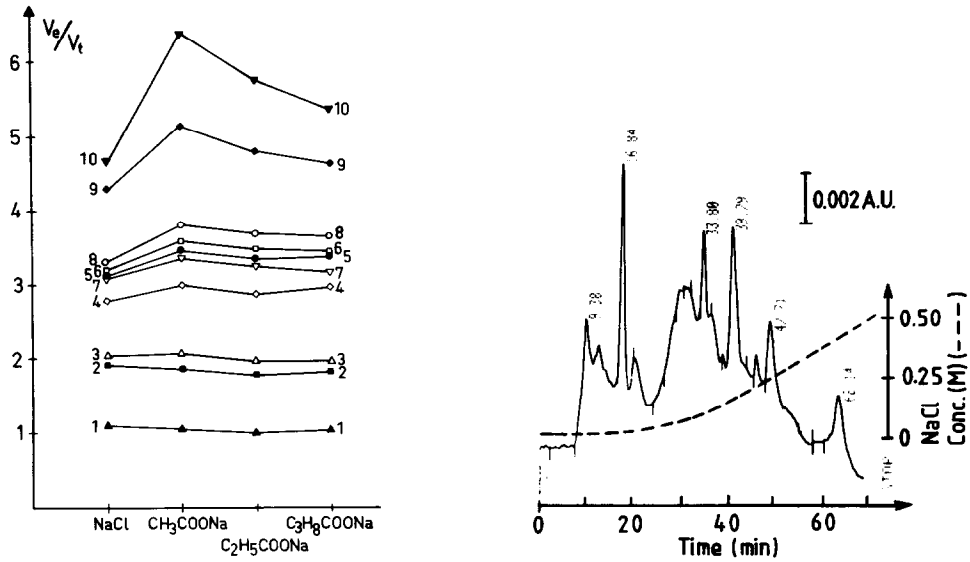

Fig. 8. Influence of different organic salts on protein retention on DEAHP-agarose. The V_e/V_t ratio was determined separately for each protein by gradient elution. The numbers in this figure refer to the same proteins as those in Fig. 7.

Fig. 9. Fractionation of Russel's viper venom by high-performance ion-exchange chromatography on DEAHP-agarose H. The sample contained 100 μg of the venom in 5 μl of 0.04 *M* Tri–HCl (pH 8.5). Column dimensions 7 cm × 0.6 cm I.D.; flow-rate. 0.2 ml/min. The proteins were eluted with a gradient from 0.04 *M* Tris–HCl (pH 8.5) to 0.04 *M* Tris–HCl (pH 8.5), containing 0.3 *M* sodium chloride in 40 min.

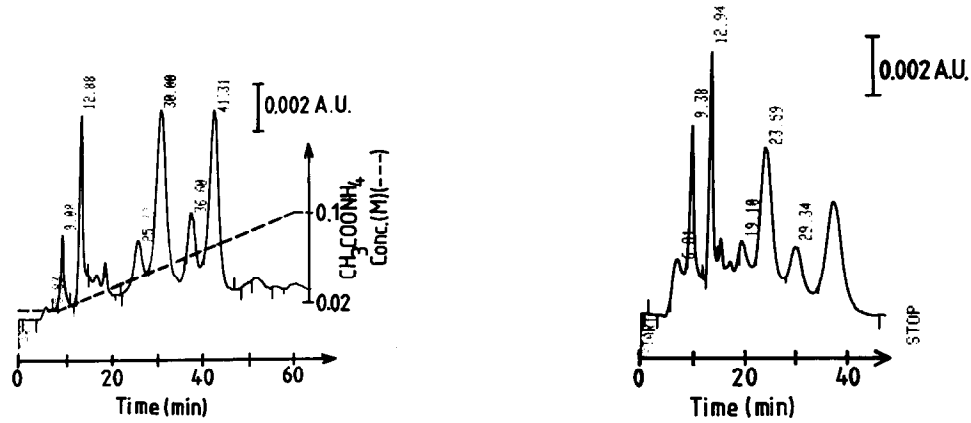

Fig. 10. Gradient high-performance ion-exchange chromatography of a cellulase fraction on DEAHP-agarose L. Sample: 100 μl of cellulase (Fraction Endo II). Column dimensions 8 cm × 0.6 cm I.D.; flow-rate, 0.2 ml/min. The proteins were eluted with a linear gradient from 0.02 to 0.1 *M* ammonium acetate (pH 4.5), in 50 min.

Fig. 11. Isocratic high-performance ion-exchange chromatography of a cellulase fraction on DEAHP-agarose L. The conditions were the same as in the experiment shown in Fig. 10, except that the proteins were isocratically eluted with 0.044 *M* ammonium acetate (pH 4.5).

as that mentioned above was used, except that the chloride ion was replaced by organic anions. The result is presented in Fig. 8.

Applications

Fig. 9 shows the chromatogram of Russell's viper venom on a DEAHP-agarose H column with gradient elution. A similar result was obtained with DEAHP-agarose L, although a lower starting buffer concentration was needed. More informative results were obtained when a defined fraction of cellulase (Endo II p*I* *ca.* 4.5) was chromatographed on a column of DEAHP-agarose L. The resolution on this column (Fig. 10) is better than that obtained with commercial DEAE-Sepharose when the same buffer and nearly the same gradient were used[28]. The chromatogram shown in Fig. 11 presents the isocratic separation of the same cellulase fraction on DEAHP-agarose L. A comparison between Figs. 10 and 11 shows that very similar chromatograms are obtained by gradient and isocratic elution.

DISCUSSION

Fig. 1 shows that the total amount of hydroxyl groups on the column material does not decrease after the activation with butanediol diglycidyl ether, although the position of the new hydroxyl groups formed is somewhat different from the original ones, *i.e.* the hydrophilic character of the agarose is preserved. Another advantage is that a new hydroxyl group is formed (in the vicinity of the nitrogen atom) after the epoxide group has reacted with diethylamine, which also is favorable to decrease the risk of disturbing hydrophobic interactions since an increase in the number of hydroxyl groups makes the gel more hydrophilic. This is extremely important in ion-exchange chromatography, since in this method, desorption, *i.e.* suppression of electrostatic interactions, is achieved by an increase of the ionic strength of the eluent, which increases the tendency for hydrophobic interaction. It may, accordingly, be impossible to desorb a protein from an ion-exchanger with hydrophobic character (or the yield may be low).

The coupling reaction outlined in Fig. 1 is fast and the conditions for the reaction are mild, *i.e.* there is no risk of degradation of agarose leading to low flow-rates. DEAHP-agarose with the desired protein capacity can easily be obtained by using proper amounts of diethylamine (Fig. 3). In a recent paper[18], we discussed the conditions for successful isocratic separation of macromolecules by hydrophobic-interaction chromatography and pointed out that (1) the beads must be small, (2) the non-specific interaction must be negligible, (3) the ligand density must be low, and (4) that the adsorption isotherms must be linear, which is a prerequisite for symmetrical peaks. Referring to the discussions in the above paper, one realizes that the same requirements obtain for isocratic elution of macromolecules in ion-exchange chromatography. For the experiment presented in Fig. 6 we therefore used beads of diameters in the range 5–10 μm showing no hydrophobic interactions. Considering the third condition it was not surprising that satisfactory isocratic separations could be achieved only on the low-ligand-density ion-exchanger, DEAHP-agarose L. The symmetry of the peaks in Fig. 6 shows that the fourth condition also is fulfilled (linear adsorption isotherms).

The titration curve of DEAHP-agarose shows only one p*K*-value, which means

that this ion-exchanger has only one type of electrostatic adsorption site. This is also favorable for isocratic elution (the titration curve thus differs from that of the commercial DEAE-Sepharose CL-6B and DEAE-Sephacel[29]). The main reason why so few isocratic separations of macromolecules are reported in the literature is certainly that the importance of low ligand density has not been pointed out before. Instead, more attention has been paid to increasing the capacity of the adsorbent (equivalent to high ligand density), which also increases the risk of low recovery[26].

For the separation of proteins on anion-exchangers, the pH of the buffer should be above the isoelectric points of the proteins so that they are negatively charged and may become adsorbed. For basic proteins, the eluent must therefore have an alkaline pH. For instance, hemoglobin (pI = 7.4) and myoglobin (pI = 6.8) cannot be separated at pH 7.5, but separate very well at pH 8.5, which is evident from Fig. 7. For similar separations agarose, with its good pH stability, is preferable to silica. However, from the titration curve in Fig. 2 one can conclude that DEAHP-agarose and QAE-agarose should not be used above pH 9 and 10.8, respectively, in order not to have most of the amino groups nonionized with attendent very low capacity of the adsorbent. The diagram in Fig. 7 shows that the selectivity changes somewhat with pH. Therefore, to obtain optimal resolution, it is important to perform experiments at different pH levels. Sodium chloride is the salt that is most commonly used for elution of proteins from anion-exchangers. However, to judge from Fig. 8, acetate, propionate, and butyrate give better resolution, particularly acetate. Therefore, the latter salt should be preferable to sodium chloride. The original reason for the design of the experiments presented in Fig. 8 was to investigate whether organic salts, by virtue of a possible hydrophobic interaction between their non-polar residues and the proteins could change their charge and thus give another separation pattern than that obtained upon elution with sodium chloride.

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