AN APPROACH TO AMPHOLYTE-DISPLACEMENT CHROMATOGRAPHY

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SUMMARY

An experimental approach to ampholyte-displacement chromatography is described in which a linear pH gradient is obtained by ampholyte displacement on DEAE-Sepharose CL-6B. The results show that a pH gradient is obtained whatever the initial pH of the ion exchanger. Ampholytes are bound selectively to the ion exchanger according to their respective pI values and to their charge in solution. This experimental approach to ampholyte-displacement chromatography demonstrates the applicability of this technique for the separation of closely related proteins.

INTRODUCTION

Ion-exchange chromatography is a technique in which particles are separated according to their charge differences. On the other hand, isoelectric focusing permits the separation of proteins according to their isoelectric points (pI). Ampholyte-displacement chromatography is a new technique which results in the establishment of a natural pH gradient for the elution of adsorbed proteins without the use of an electric current.

Carrier ampholytes were first synthesized for isoelectric focusing, allowing the formation of a pH gradient in which proteins could be separated according to their pI[1].

Leaback and Robinson[2] and Pagé and Belles-Isles[3] have previously reported the formation of a natural pH gradient when eluting a column of an ion exchanger with a solution of carrier ampholytes. Sluyterman and co-workers[4,5] have also recently described a new technique, chromatofocusing, in which a pH gradient may be obtained by taking advantage of the buffering action of the ion exchanger. In the chromatography of proteins, a focusing effect is obtained by running a buffer at a given pH through a column initially adjusted at a different pH. This technique is however different from ampholyte-displacement chromatography in which carrier ampholytes of various pI values are used for the elution of proteins. We have studied the nature

of this pH gradient, the interactions of ampholytes with the ion exchanger and the mode of action of the latter in the separation of proteins.

We report the effects of ionic strength and the initial pH of the ion exchanger, of the carrier ampholyte concentration and of adsorbed proteins on the pH gradient profile. The distribution of carrier ampholytes in the gel column is also described.

EXPERIMENTAL AND RESULTS

Materials

Carrier ampholytes (Pharmalytes), pH 4.0–6.5, and DEAE-Sepharose CL-6B were purchased from Pharmacia (Montreal, Canada) and carrier ampholytes (Ampholine), pH 2.5–4.0, from Fisher Scientific (Ste-Foy, Canada). Chromatographic columns (4 × 0.7 cm and 20 × 0.7 cm) were obtained from Bio-Rad Labs. (Mississauga, Canada).

A solution of human liver ferritin (5 mg/ml) was used for adsorption on the ion exchanger when needed[6]. Ferritin was measured by an enzyme immunoassay developed in our laboratory[6].

In all experiments the DEAE-Sepharose CL-6B gel was washed with a volume of buffer twenty times greater than the bed volume before use; 0.3-ml fractions were collected at the rate of 5.4 ml/h.

Elution at constant ionic strength

The gel was first equilibrated with a 25 mM Tris–Acetate buffer at pH 7.0, and four column volumes of 2 % Pharmalyte (pH 4.0–6.5) in deionized water were applied; the ampholyte solution was comparable in tonicity to the starting buffer. The natural pH gradient obtained was linear between pH 7 and pH 5 (Fig. 1). This is consistent with previous results[2,3] but not with those obtained by Young and Webb[7,8]. The last workers used a solution of carrier ampholytes prepared in buffer rather than in water.
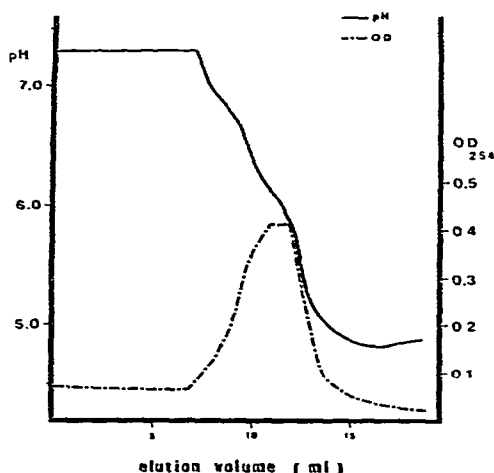

Fig. 1. Elution of a DEAE-Sepharose CL-6B column (pH 7.0) with 2 % carrier ampholytes, pH 4.0–6.5.

Elution at various initial pH values

Different ion-exchange columns were equilibrated at pH values varying from 4.0 to 8.0 with the appropriate buffers. Ferritin was absorbed and eluted with 2% Pharmalyte, pH 4.0–6.5, in deionized water. As shown in Fig. 2, whatever the initial pH of the gel, the elution with carrier ampholytes gave a pH gradient. Differences in the pH gradient profiles (Fig. 3) seem to be caused by the presence of desorbed proteins yielding a plateau in the pH gradient.
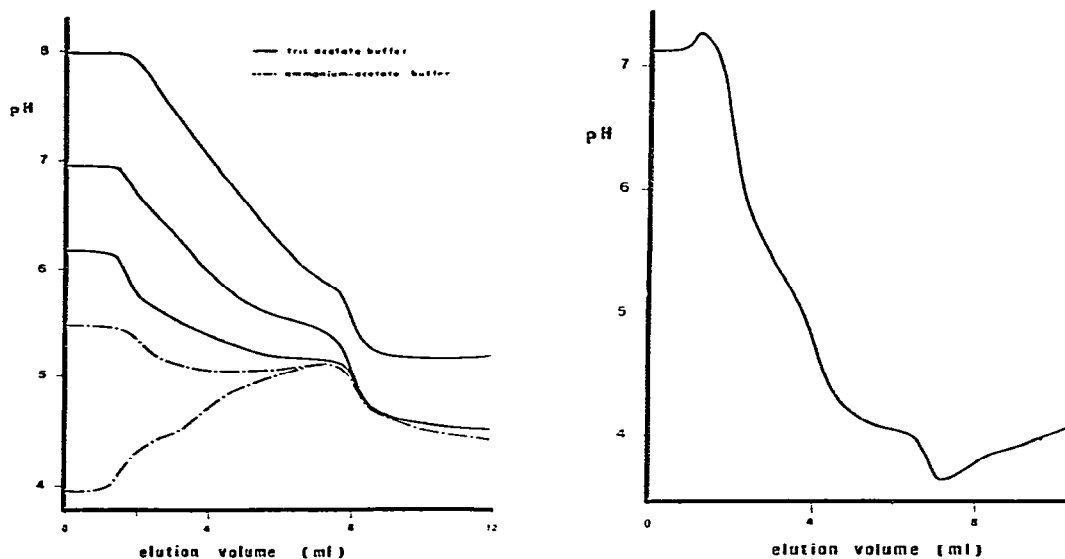

Fig. 2. Elution of different DEAE-Sepharose CL-6B columns at various initial pH values with 2% carrier ampholytes, pH 4.0–6.5.

Fig. 3. Elution of a DEAE-Sepharose CL-6B column (pH 7.0) with 2% carrier ampholytes, pH 2.5–6.5.

When we extended the range of the carrier ampholytes to pH 2.5 instead of pH 4.0 with one volume of 2% Ampholine (25 mM), pH 2.5–4.0, a significant extension in the pH gradient from pH 7.3 to pH 3.6 (Fig. 4) was obtained.

As can be seen in Figs. 2–4, the pH gradient never reached the limits for the range of carrier ampholytes used.

Elution with a carrier ampholyte gradient

Elution with a carrier ampholyte gradient (0–6% Pharmalyte, pH 4.0–6.5) was performed with DEAE-Sepharose CL-6B. It is interesting to note that a pH gradient was also obtained using this system. The Pharmalyte concentration was determined spectrophotometrically by recording the absorbance at 254 nm (Fig. 5). The absorbance of eluted ferritin did not influence significantly the measurement of Pharmalyte as shown in the figure, because the concentration of ferritin applied on the gel (10 μg) was low as compared to the concentration of Pharmalyte. Moreover, we observed a plateau in the Pharmalyte gradient corresponding to the fractions in which ferritin was eluted.
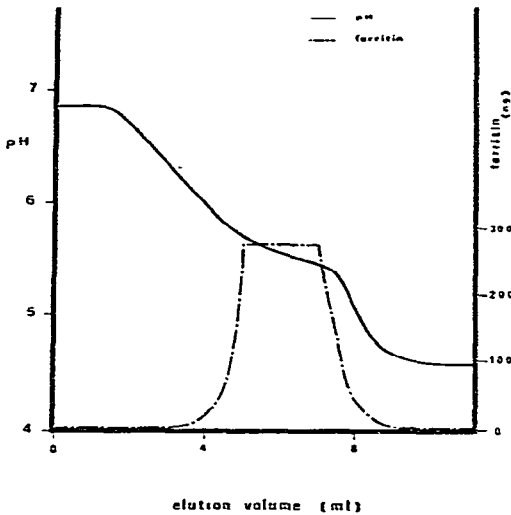
Fig. 4. Elution of absorbed ferritin from a DEAE-Sepharose CL-6B column (pH 7.0) with 2% carrier ampholytes, pH 4.0–6.5.
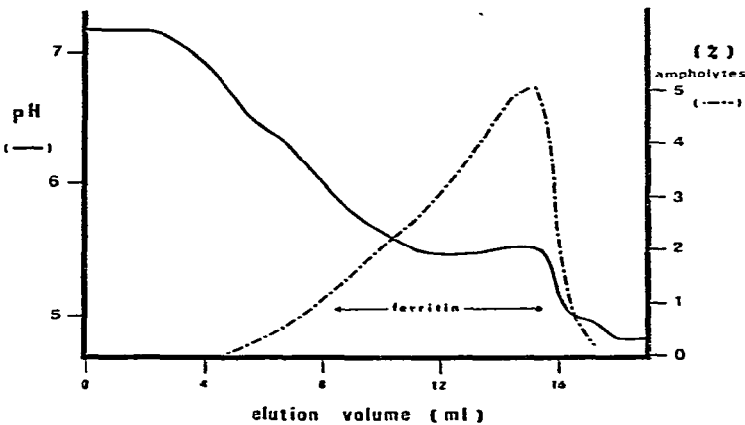

Fig. 5. Elution of a DEAE-Sepharose CL-6B column with a 0–6% carrier ampholyte (pH 4.0–6.5) gradient. Elution of ferritin is shown.

Interaction of Pharmalyte with the ion exchanger

For this purpose, a column of DEAE-Sepharose CL-6B was prepared in a Pharmacia K9/15 column (7.7 × 0.9 cm of gel) after equilibration as described earlier. The column was eluted with 5 ml of 2% Pharmalyte, pH 4.0–6.5, corresponding to the total bed volume (V_t). Elution was then stopped, the gel pushed out of the column from the top with a capillary (0.9 cm O.D.) and the pH of the gel was measured every 5 mm using a surface electrode. After addition of 1 ml of deionized water to each fraction (5 mm of gel per fraction) and centrifugation of these, the pH values of the eluent (supernatant) and of the gel (pellet) were measured. The results expressed in Fig. 6 indicate that carrier ampholytes seem to bind with the ion exchanger, result-
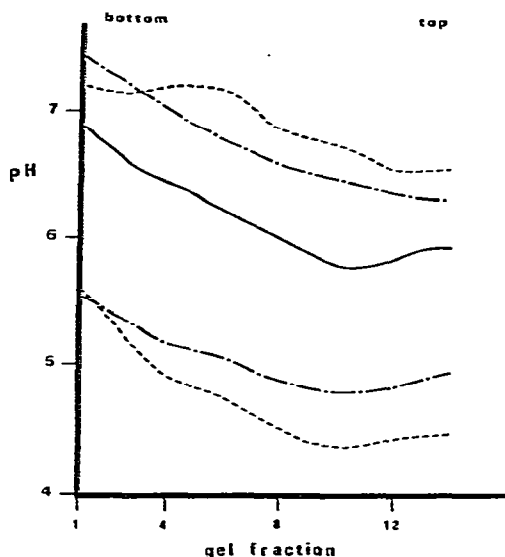
Fig. 6. Study of the pH of the DEAE-Sepharose CL-6B gel fractions from the bottom of the column to the top (full line). (. – .), pH of gel (upper) and eluent (lower) after the first addition of water to gel fraction; (– – –), pH of gel (upper) and eluent (lower) after the second addition of water.

ing in an increase in the acidity of the gel up the column. This supports the idea that carrier ampholytes bind selectively to the ion exchanger depending on their pI value. The pH of the surrounding eluent gives a gradient because of the continuous displacement of ampholyte from the solid phase by the ampholytes which have more affinity. Moreover, these results indicate that elution of the gel with deionized water causes desorption of the less charged carrier ampholytes. An equilibrium seems to be established between the carrier ampholyte concentration in the liquid phase and in the solid phase. Several volumes of water are required for the complete elution of Pharmalytes.

DISCUSSION

A solution of carrier ampholytes in deionized water has a pH slightly below 6. Consequently, each carrier ampholyte will have a different charge in water, since those with a pI lower than the pH of the solution will be predominantly negatively charged, whereas those with a pI higher than the pH of the solution will be positively charged. Therefore, on applying a very small volume of this solution on a DEAE-ion exchanger, the negatively charged carrier ampholytes will bind to the gel and interact with the oppositely charged groups. The more acidic the carrier ampholytes, the more negatively charged they will be and the more important will be the electrostatic interaction between these and the positive groups of the gel. The less negatively charged ampholytes will occupy the void volume or be adsorbed later in the column.

As the column is eluted with another small volume of carrier ampholyte solution the more acidic ampholyte will displace the less acidic. The latter will be retained further down the gel on the next free positive groups, as illustrated in Fig. 7.
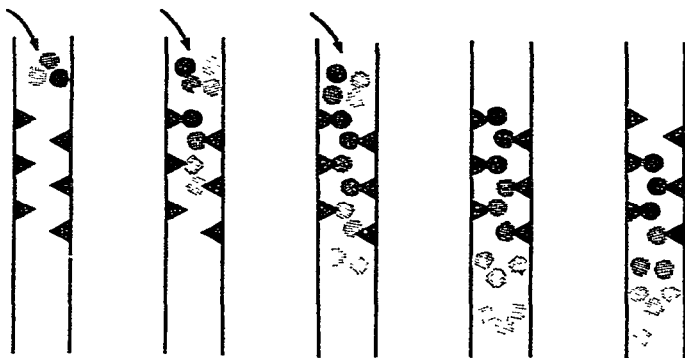
Fig. 7. Illustration of the ampholyte-displacement phenomenon in an ion-exchange gel. The filled triangles represent the ionic groups of the gel. The darkness of the circles used for the carrier ampholytes is proportional to their charge.

On addition of several small volumes of the carrier ampholyte solution the process of ampholyte displacement will continue to the bottom of the gel. The ampholyte displacement may be visualised as a retardation phenomenon in which ampholytes, depending on their charge in the aqueous solution, are retarded by the ionic groups of the gel. As a consequence, the ampholytes which carry the largest opposite charge are retarded the most and, as expected from Fig. 7, the weakest acidic carrier ampholytes will constitute the front of eluent.

Consequently, the elution will be achieved through the increase in the acidity of the carrier ampholytes. Due to their relatively low molecular weight, it is possible that some molecules of carrier ampholytes of related pI may displace an adsorbed protein by either mass action or by affinity displacement which favours the ampholytes because of the diminished steric hindrance. As the electrostatic force between the ion exchanger and the protein is weakened, the latter is eluted and a plateau is observed in the pH gradient.

In such a system, proteins are not necessarily eluted at their respective pI. However, because the net charge of the protein approaches zero when the pH of its surroundings approaches its pI, the protein will be eluted near its pI. As shown on Fig. 4, ferritin was not eluted at the pH value equal to its pI. Human normal liver isoferritins have pI ranging from 5.0 to 5.39. However the elution of ferritin at pH 5.6 was relatively near the pI. The fact that proteins are not necessarily removed from the ion exchanger according to their pI seems to be confirmed by Young and Webb[8]; however, their results corresponded to an elution with a solution of carrier ampholyte diluted in buffer rather than in water. Chromatofocusing, a technique recently described by Sluyterman and co-workers[4,5], takes advantage of the buffering action of the ion exchanger and uses a buffer of a pH different from the initial pH of the column. In ampholyte-displacement chromatography a natural pH gradient is obtained by a continuous displacement in which the more acidic ampholytes constitute the front of the eluent.

This new technique has some similarities with preparative isoelectric focusing, but it offers more versatility since it may be accomplished using various ion exchangers. Since the separation is not directly related to the pI of the eluted proteins, this technique provides a new separation which would be in many cases different from

that obtained after isoelectric focusing. In addition, since smaller columns may be used in the final steps of purification, lower amounts of carrier ampholytes are required.

Because of its simplicity and reproducibility, this new technique can be applied to the separation of proteins and isoenzymes[2]. It is now being applied to the separation of human isoferritins.

ACKNOWLEDGEMENTS

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