ANALYTICAL BIOCHEMISTRY 163, 263–269 (1987)

Cation-Exchange High-Performance Liquid Chromatography: Separation of Highly Basic Proteins Using Volatile Acidic Solvents

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Received October 27, 1986

The chromatographic behavior of a number of globular proteins was studied on a Bio-Sil TSK CM-2-SW weak cation exchange HPLC column under acidic conditions. A linear gradient of 0–1 M NH_4Ac in 1 M HOAc, inducing a convex pH gradient from 2.4–4.8, resulted in an excellent separation of highly basic proteins. For these proteins a linear relationship between isoelectric point and retention time was determined experimentally. The effect of pH and the ion composition of the eluting buffer system on this linear correlation was studied. Although the exact basis for protein separation on the CM-2-SW column at low pH is not clear yet, both the pH-dependent net positive charge per unit surface area and most likely the relative percentage of arginine in the total number of basic residues contribute to this separation. Because of the high resolving power and the high protein recovery obtained in a system using only acidic volatile buffer solutions, the cation exchanger is particularly suitable for the purification of nanogram amounts of acid-stable basic growth factors. The present sterile conditions (1 M HOAc/NH_4Ac system, pH < 4) and the easy removal of salt by lyophilization facilitate the detection of these proteins by biological assays.

KEY WORDS: cation exchange HPLC; low pH; volatile salts; protein purification; basic proteins; polypeptide growth factor.

INTRODUCTION

The separation of similar proteins requires the use of high-resolution techniques. Besides methods of protein separation based on molecular weight, hydrophobicity, and ligand specificity, i.e., high-performance gel permeation chromatography (HPGPC),[1] reverse phase HPLC and, affinity chromatography, respectively, isoelectric focusing and ion-exchange HPLC techniques are capable of separating proteins differing only slightly in charge. While isoelectric focusing is a powerful tool for the analysis of a complex protein mixture, large-scale preparation of proteins fractionated by this method is rather laborious (1). In addition, highly basic proteins with isoelectric points above 9.5 cannot be separated well because of the limited pH gradient range generated by the commercially available ampholytes. Although at present cation-exchange HPLC is not frequently used for the separation of highly basic proteins, the present paper shows that the disadvantages described above can be overcome by this technique.

The choice of the type of matrix (cationic or anionic) in ion-exchange chromatography depends on the isoelectric point (p*I*) and the stability of the proteins within a certain pH range (2). For the fractionation of acidic proteins an anion-exchange column should be used while a cation exchanger is most suitable for basic proteins. In most cases the

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[1] Abbreviations used: HPGPC, high-performance gel permeation chromatography; p*I*, isoelectric point; As, accessible area; PDGF, platelet derived growth factor.

0003-2697/87 $3.00

proteins can be separately eluted from the column by choosing the right combination of pH and ionic gradient. For the separation of basic proteins on a cation exchanger the starting pH and the ionic strength are normally chosen so that the protein(s) of interest is just bound to the matrix. Lampson and Tytell (3) have shown that proteins begin to dissociate from ion exchangers at about 0.5 pH units from their p*I* values at an ionic strength of 0.1. However, because of the instability of most proteins in the pH range above 9.0 chromatographic experiments in the high alkaline pH range are not suitable for the purification of highly basic proteins (p*I* > 9.0). A second problem is the difficulty in establishing satisfactory conditions for the separation of highly basic proteins with similar charge and size properties via cation-exchange chromatography. Dunkley and Carnegie (4) for instance have shown that the use of a sulphoethyl–Sephadex 50 column at pH 7.5 according to the method of Hirshfeld *et al.* (5) and Barton *et al.* (6) results in a very poor separation of myelin basic protein (p*I* ~ 11.8) from contaminating histones. In a study of Ohe *et al.* (7) on the fractionation of human spleen histone, broad overlapping peaks of the different histones were obtained after chromatography of the crude preparation on a CM-cellulose column according to Senshu and Iwai (8). As these methods all employed standard (low-resolution) cation exchange, the separation of highly basic proteins at neutral or alkaline pH could be somewhat improved by HPLC techniques.

We were interested in a column system for the separation of highly basic proteins under sterile conditions. In this paper we report the chromatographic properties of a silica-based weak cation-exchange CM-2-SW HPLC column (Bio-Rad) at acidic pH. Eluting buffer systems are described that result in the separation at the baseline of highly basic proteins differing only slightly in p*I* value. The general principles of protein separation in these systems are studied. The advantages of volatile acidic buffer solutions during chromatography and the applications of the ion exchanger for the separation of basic proteins are discussed.

MATERIALS AND METHODS

The proteins used in this study were purchased from the following sources: chymotrypsinogen A from Boehringer–Mannheim and trypsinogen, ribonuclease A, cytochrome *c* (type II-A), lysozyme, ovalbumin, and bovine serum albumin from Sigma. Ammonium acetate was obtained from Merck.

Cation-exchange high-performance liquid chromatography (HPLC) was carried out on equipment from Millipore–Waters comprising a Model 680 automated gradient controller supplied with two 510 solvent pumps and a 481 variable wavelength detector linked to a BD 41 chart recorder from Kipp. A combination of a Bio-Sil TSK HPLC guard column (75 × 7.5 mm, Bio-Rad) and a Bio-Sil TSK CM-2-SW column (250 × 4.6 mm, Bio-Rad) was used. Protein samples of 2 ml were applied to the column using a loop injector (U6K, Millipore–Waters). Elution was performed at a constant flow rate of 0.8 ml/min. A linear ionic gradient of 0–1 M NH_4Ac in 1 M HOAc was used over a 100-min period at room temperature (0.01 M NH_4Ac/min). This salt gradient resulted in a convex pH gradient from 2.4 to 4.8. For elution at constant pH, the starting and limiting buffers of the gradient were adjusted to the pH required by the addition of an aqueous 1 M NH_4Ac (or 1 M NaAc) solution. A linear gradient of 0–1 M NH_4Cl (or NaCl) was then applied to the column. Absorbance was monitored at 280 nm.

RESULTS AND DISCUSSION

In order to develop a general method for the purification of highly basic proteins from complex mixtures, the chromatographic behavior of a number of different proteins (Table 1) was studied on a HPLC cation exchanger at low pH. The proteins were dis-

solved in 1 M HOAc (pH 2.4) and injected onto a silica-based Bio-Sil CM-2-SW cation-exchange HPLC column equilibrated in this solvent. Figure 1a shows the elution profile of a mixture containing five basic proteins (p*I* 9.3–11.8) by applying a linear gradient of NH_4Ac in 1 M HOAc (0.01 M NH_4Ac/min). The proteins emerged as well-separated sharp peaks. The retention times of the proteins varied from 27.5 min for trypsinogen to 56.2 min for lysozyme.

During the linear NH_4Ac gradient the pH is not constant. In Fig. 1a (insert) the pH is plotted against the molarity of NH_4Ac in the eluting buffer. The pH of the starting buffer (1 M HOAc) is 2.4. Continuous addition of increasing amounts of NH_4Ac results in a rapid increase in pH from 2.4 to 3.6 followed by a gradual increase to pH 4.8 (1 M NH_4Ac/1 M HOAc). We investigated the possible importance of the convex pH gradient for the resolution and separation of proteins on the cation-exchange column. For this reason the basic proteins (as in Fig. 1a) were run on the CM-2-SW HPLC column at a pH remaining constant during the salt gradient. In Fig. 1b the elution pattern of these proteins is shown during a linear gradient of 0–1 M NH_4Cl in 1 M HOAc at a constant pH of 3.5. Compared with the profile in Fig. 1a, all proteins are eluted from the column earlier. Ribonuclease A (p*I* 9.6) and chymotrypsinogen A (p*I* 9.5) are hardly separated in this system whereas these proteins are almost separated at the baseline when a combined pH and salt gradient is used (Fig. 1a). Furthermore, the elution position of the two proteins is interchanged at constant pH. These results indicate that a continuous nonlinear change in pH during the salt gradient results in the efficient separation of basic proteins.

Upon initial examination of our data we noted that proteins with a high isoelectric point elute later from the column than do proteins with a relatively lower p*I* value (cf. lysozyme, p*I* 11.0, R_t = 46.5 min and trypsinogen, p*I* 9.3, R_t = 24 min; Fig. 1b). For highly basic proteins, the p*I* value and retention time turn out to be linearly related. This is shown in Fig. 2a for the data obtained with the NH_4Cl system at pH 3.5 (also see Fig. 1b). Changing the counterion in the eluting buffer from NH_4^+ to Na^+ leads to a delay in

TABLE 1

POLYPEPTIDE STANDARDS

Protein	Source	M_r ($\times 10^{-3}$)	$\frac{A^a}{A+L+H}$ %	p*I*[b]
Basic				
Trypsinogen	Bovine	24	10.0	9.3
Chymotrypsinogen A	Bovine	23.2	20.0	9.5 (14)
Ribonuclease A	Bovine	13.7	22.2	9.6 (15)
Cytochrome *c*	Horse	12.3	8.3	10.6 (16)
Lysozyme	Chicken	14.4	61.1	11.0 (17)
Myelin basic protein	Bovine	18	43.9	11.8
Acidic				
Ovalbumin	Chicken	43	35.7	4.6
Serum albumin	Bovine	68	25.2	4.9

[a] A = arginine, L = lysine, and H = histidine.

[b] The p*I* values of trypsinogen, ovalbumin, and serum albumin were obtained from the supplier, the Merck Index (9th ed.), and the Handbook of Biochemistry (2nd ed.), respectively. The p*I* value of myelin basic protein was calculated from the amino acid composition and the p*K* values of the individual amino acids (13).

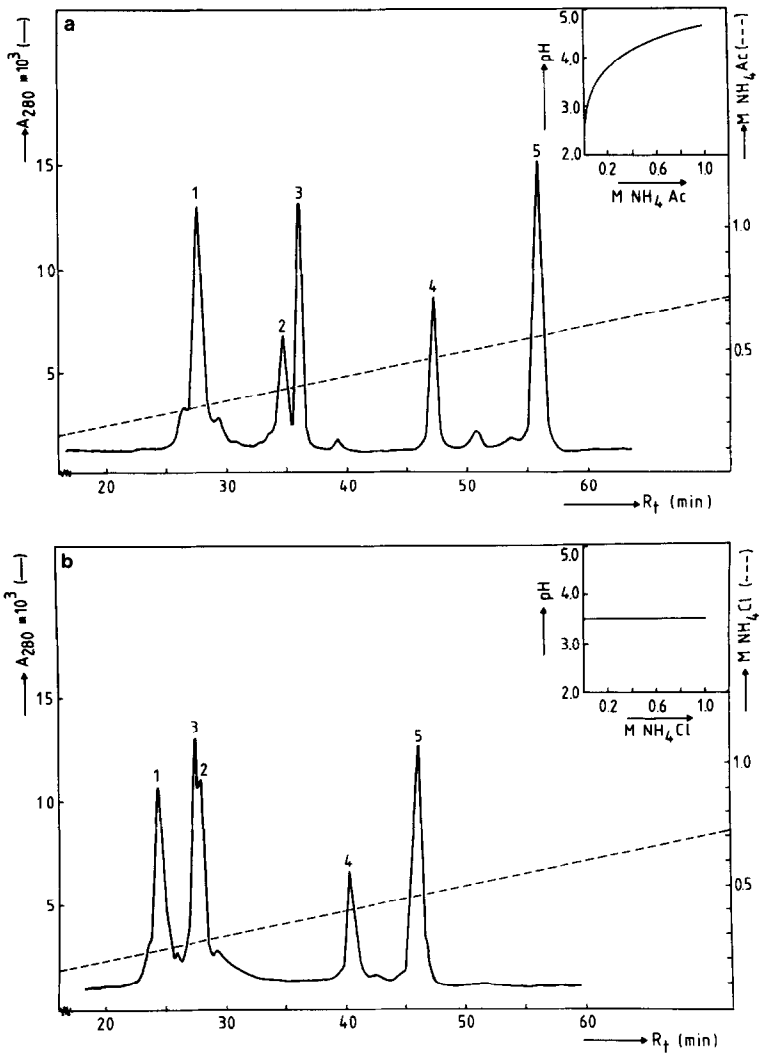

FIG. 1. Separation of five basic proteins on a Bio-Sil TSK CM-2-SW cation-exchange HPLC column. (a) Linear gradient of 0–1 M NH_4Ac in 1 M HOAc (0.01 M/min). (b) Linear gradient of 0–1 M NH_4Cl in 1 M HOAc (0.01 M/min). Both starting and limiting buffer solutions are adjusted to pH 3.5 with aqueous 1 M NH_4Ac. Inserts: Relationship between pH and molarity of ammonium salt in the eluting buffer during the linear salt gradient. (1) Trypsinogen (126 μg), (2) chymotrypsinogen A (50 μg), (3) ribonuclease A (100 μg), (4) cytochrome *c* (50 μg), and (5) lysozyme (50 μg).

elution of the proteins from the column and to an improvement of their separation (Fig. 2b). This results in a decrease in the slope of the regression line. On the other hand by lowering the pH in the NH_4^+ system from 3.5 to 2.4 the linear correlation between p*I* and retention time has completely disappeared (Fig. 2c). All proteins, even the most basic protein tested (myelin basic protein, p*I* 11.8), elute below a salt concentration of 0.2 M.

As already shown in Fig. 1 the best separation between highly basic proteins is achieved using a linear gradient of 0–1 M NH_4Ac in 1 M HOAc. In this system the proteins tested elute exactly according to their isoelectric point (Fig. 2d). Proteins like chy-

motrypsinogen A and ribonuclease A with p*I* values that differ by only 0.1 unit are very well separated. Furthermore, the use of NH_4Ac facilitates the easy removal of salt from the final protein solution by lyophilization. Both the high resolving power and the volatility of the ammonium salt make the NH_4Ac system preferable to the NH_4Cl and NaCl systems described above.

Under all conditions tested acidic proteins like ovalbumin (p*I* 4.6) and bovine serum albumin (p*I* 4.9) deviate from the correlation plots shown in Fig. 2a, 2b, and 2d. These proteins elute from the column later than would expected on the basis of their p*I* value. For this reason CM-2-SW chromatography at low pH should not be considered as a method for estimating isoelectric points of proteins. A possible explanation for the chromatographic behavior of the acidic proteins might be found in the difference in net positive charge per unit surface area between the different proteins. The accessible area (As) of monomeric globular proteins has been shown to vary as the $\frac{2}{3}$ power of the molecular weight: As = 11.12 $M_r^{2/3}$ (9). Figure 3 shows a plot of $\Delta P/M_r^{2/3}$ versus the retention time (R_t), where R_t is determined in the NH_4Cl system at constant pH 3.5 and ΔP is the net number of positive charges per protein molecule at this pH. ΔP is calculated from the amino acid composition of the proteins and the p*K* values of the individual amino acids (13). For most of the proteins tested a linear correlation is observed between $\Delta P/M_r^{2/3}$ and R_t. This correlation might explain not only the deviation of acidic proteins from the plot of p*I* versus R_t (Fig. 2) but also the difference in R_t between ovalbumin and bovine serum albumin in spite of their similar p*I* values. Bovine serum albumin, chymotrypsinogen A, and particu-

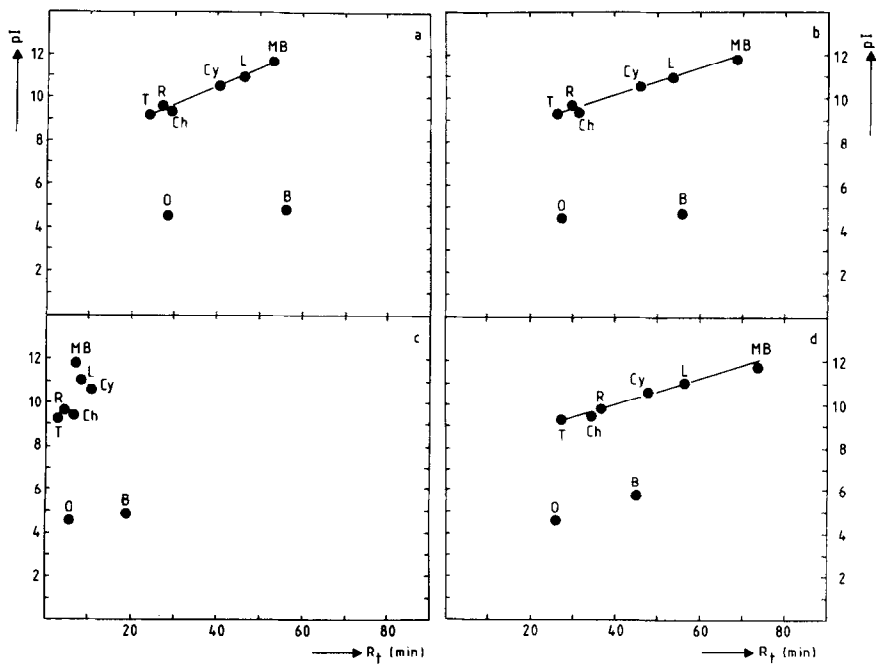

FIG. 2. Relationship between isoelectric point (p*I*) and retention time (R_t) on a Bio-Sil TSK CM-2-SW HPLC column using as mobile phase a linear gradient (0.01 M/min) in 1 M HOAc of (a) 0–1 M NH_4Cl, pH 3.5, (b) 0–1 M NaCl, pH 3.5, (c) 0–1 M NH_4Cl, pH 2.4, and (d) 0–1 M NH_4Ac, pH 2.4–4.8. T = trypsinogen, Ch = chymotrypsinogen A, R = ribonuclease A, Cy = cytochrome *c*, L = lysozyme, MB = myelin basic protein, O = ovalbumin, and B = bovine serum albumin.

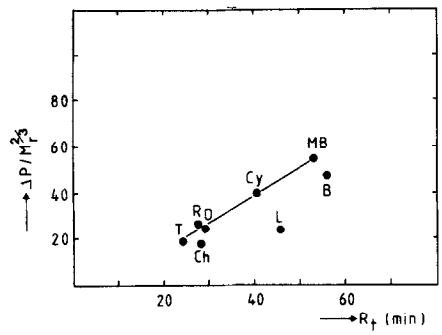

FIG. 3. Relationship between net positive charge per unit surface area ($\Delta P/M_r^{2/3}$) and retention time (R_t) on a Bio-Sil TSK CM-2-SW HPLC column using a linear gradient of 0–1 M NH_4Cl, pH 3.5 (0.01 M/min). ΔP is the net number of positive charges on the protein at pH 3.5 and M_r is the molecular weight of the protein. Abbreviations as in Fig. 2.

larly lysozyme deviate to a certain extent from the linearity seen in Fig. 3. This is probably due to the fact that not only the positive surface charge density of the protein but also the composition of the basic residues are involved in the interaction of proteins with the cation exchanger at low pH (Fig. 2a, 2b, and 2d). Arginine has a higher affinity for the negatively charged matrix groups than lysine (cf. R_t for Arg and Lys in conventional cation-exchange amino acid analysis). This may explain why proteins with the same value for $\Delta P/M_r^{2/3}$ elute from the column according to the proportion of arginine residues in the total number of basic residues (cf. R_t for ovalbumin with 35% Arg and lysozyme with 61% Arg; Fig. 3). Although the exact basis for the chromatographic behavior of acidic and basic proteins is still unknown, we suggest from these results that both the pH-dependent net positive surface charge density and the relative percentage of Arg among the positively charged groups (Table 1) contribute to the separation of proteins on the CM-2-SW column at pH < 4. In practice, highly basic proteins (pI > 9.0), including lysozyme, elute according to their isoelectric point rather than to their net positive charge.

The use of equilibrating and eluting buffers with a pH value below 4 results in a high percentage of unionized matrix carboxymethyl groups (pK ~ 4). For this reason the experiments described in this paper seem to be done under conditions that are suboptimal for the interaction between positively charged proteins and matrix groups. However, the positively charged proteins might displace H^+ ions from the carboxymethyl groups of the column resulting in a strong protein–matrix interaction and a high capacity of the column for these proteins. The use of 1 M HOAc in the starting and limiting buffer solutions that are used to obtain the proper salt gradient minimizes aspecific absorption of proteins to the column. This results in a high protein recovery being of great importance in the purification of proteins. For the highly basic protein cytochrome *c* a recovery of 90–95% was obtained using microgram amounts of this protein.

The method for using the CM-2-SW cation-exchange column is ideally suited for the preparation and identification of some acid-stable basic proteins like transforming growth factor β (10) and platelet derived growth factor (PDGF)-like polypeptides (11). Detection of nanogram amounts of these proteins in column fractions is only possible by testing them in a biological assay under sterile conditions (10, 11). For this reason the use of the 1 M HOAc/NH_4Ac system (pH < 4) is very suitable during the chromatographic steps. The use of volatile buffer solutions like NH_4Ac facilitates the testing of large column fractions without the side effects of high salt concentrations. The CM-2-SW weak cation-exchange column has proven to be very efficient in the purification of a PDGF-like growth factor from mouse neuroblastoma cells (van den Eijnden-van Raaij *et al.*, in preparation) and in the identification of a heparin-binding growth factor from PC13 embryo carcinoma cells as the basic form of fibroblast growth factor (12). As these highly basic, probably nonglobular growth factors do not run according to their pI values (unpublished data) the possible effect of protein conformation as

an additional factor influencing the chromatographic behavior of proteins on the cation exchanger needs to be investigated.

Evaluating the results of our experiments a linear gradient of NH_4Ac in HOAc inducing a convex pH gradient appears to be the optimal condition for the separation of acid-stable basic proteins on a Bio-Sil CM-2-SW cation-exchange HPLC column. Highly basic proteins with p*I* values close to each other can be separated especially well. In this regard the CM-2-SW column might be a powerful tool for the fractionation of the basic histones and the separation of histones from myelin basic proteins (see the introduction). Considering the high recoveries of the proteins from the column, even of the sticky PDGF-like growth factor (75–80%; van den Eijnden-van Raaij *et al.*, in preparation), as well as the advantages of volatile acidic buffer solutions, this HPLC technique should find particular applications in the purification of highly basic, acid-stable, biologically active proteins. The conditions determined in the present study for optimal separation of proteins on an analytical/semi-preparative column can probably also be applied to preparative CM-2-SW cation exchangers.

ACKNOWLEDGMENTS

The authors wish to thank Dr. A. Teelken from the University Hospital Groningen, Department of Neurology, for the generous supply of myelin basic protein and for helpful discussions.

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