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SEPARATION OF BASIC PEPTIDES BY CATION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Chromatographic separations of a series of highly basic peptides on commercially available 300 Å pore size CM 300 weak cation-exchange columns have been compared at various loads, pHs and ionic strengths of the eluent. On analytical columns (250 × 4.1 mm I.D.), mixtures of basic peptides containing 7 – 9 nmole of each component were separated with a 50 m*M* KH_2PO_4–KCl gradient (pH 4.5) and under isocratic conditions (pH 4.5 and 6.5). The isocratic conditions demonstrated the effects of pH and ionic strength on retention time and resolving power on the CM 300 column. The load capacity of a CM 300 preparative column (250 × 10 mm I.D.), studied under gradient conditions (50 m*M* KH_2PO_4, 0.2 – 0.4 *M* KCl, pH 4.5 and 6.5), revealed that its capacity is much greater at pH 6.5. Loads up to 10–20 mg (6.6–13.3 μmol) could be applied before peaks in the crude peptide sample tested were seen to fuse.

INTRODUCTION

Our laboratory is actively involved in structure–function relationship studies which require synthetic analogs of the troponin I inhibitory region of the troponin complex of rabbit skeletal fast muscle. We have previously reported the solid-phase synthesis of several highly basic peptides which are excellent mimics of the biological activity of troponin I[1,2]. During solid-phase synthesis, products such as deletion peptides and peptides arising from premature termination of growing chains contaminate the desired product once it is cleaved from the solid support. Purification of products from solid-phase synthesis has relied heavily on open column chromatography based on gel permeation, ion-exchange, adsorption or partition and on counter-current distribution. All these techniques are time-consuming and, depending on how closely the contaminants resemble the product, can have low separation efficiencies.

Recently, a weak cation-exchange resin, CM 300, has become available for use in high-performance liquid chromatography (HPLC). The resin consists of a 6.5 μm silica support with 300–Å pore diameter which has a bonded polymeric coating with carboxylic acid functionalities. In this paper we report methods for the rapid analysis and purification of highly basic peptides by HPLC on CM 300 cation-exchange resin

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columns. The peptides employed to characterize this resin have a known sequence, and vary with respect to their net charge and basic residue composition. Furthermore, the results are not subject to conformational constraints, as these peptides are all known to exist in a random coil conformation. We have examined the effects of sample load and pH and ionic strength of the eluent on the resolving power of the CM 300 resin columns.

EXPERIMENTAL

Reagents

Water was deionized, distilled, and further purified by passage through a SynChropak RP-P column (250 × 10 mm I.D.), supplied by SynChrom, Linden, IN, U.S.A. Potassium chloride was purchased from Fisher Scientific (Pittsburgh, PA U.S.A.) and was A.C.S. certified grade. Peptides A and B were obtained by cyanogen bromide cleavage of troponin I and represent the 21-residue fragment, residues 96–116, prepared as described previously[3]. Peptides C through J are synthetic peptides made by solid-phase methods as described by Talbot and Hodges[2]. Purified peptides were obtained by ion-exchange and reversed-phase chromatography on CM 300 and C_{18} columns, respectively.

Apparatus

Programmed analytical chromatography was performed on a Varian Vista Series 5000 liquid chromatograph interfaced with a Varian CDS 401 data system, and coupled to a Kratos SF769Z variable-wavelength UV spectrometer at 220 nm. Analytical samples were chromatographed on a SynChropak CM 300 column, (250 × 4.1 mm I.D.). Samples were injected with a Hamilton no. 701 10-μl syringe (Hamilton, Reno, NV. U.S.A.) into a 200-μl injection loop (Model No. 7125, Rheodyne, Berkeley, CA, U.S.A.).

Programmed preparative chromatography was carried out with a Spectra-Physics SP8700 solvent delivery system coupled to a Spectra-Physcis SP8750 Organizer pumping system. For detection at 220 nm a Schoeffel GM 770 Monochromator variable UV spectrometer with a 10-mm light path was used. The preparative samples were chromatographed on a SynChropak CM 300 weak cation-exchange column (250 × 10 mm I.D.) followed by final purificaton by chromatography on a SynChropak RP-P reversed-phase column (250 × 10 mm I.D.). Both these columns were purchased from SynChrom. Sample injections were made from a Hamilton no. 1750, 500-μl syringe into a 2.0-ml injection loop (Model No. 7125, Rheodyne). The chromatograms were recorded on a Beckman 10-in recorder. Purified samples were collected manually.

Methods

All chromatography was carried out at room temperature (*ca* 20°C). For preparative chromatography the crude peptides were dissolved in water (Millipore Q grade). The pH of these samples was adjusted to 6.5 by the addition of 1-μl portions of 5 *M* potassium hydroxide. The samples were then centrifuged at 12,000 *g* for 5 min. All columns were equilibrated with the starting buffer for 30 min before each determination. Analytal chromatography was tested on various equimolar mixtures of purified peptides dissolved in water (Millipore Q grade). The pH of these samples was

adjusted with 1-μl portions of 5 *M* potassium hydroxide. Flow-rates were maintained at 1 ml/min (analytical chromatography) and 2 ml/min (preparative runs). Aliquots (5 μl) of stock solutions of the individual purified peptides were hydrolysed in 100 – 200 μl of 6 *M* hydrochloric acid at 110°C for 24 h in evacuated sealed tubes. These hydrolyzates were then analyzed on a Durrum 500 animo acid analyzer to obtain peptide concentrations. Equimolar mixtures of the purified peptides were made up from these stock solutions of known concentration.

RESULTS AND DISCUSSION

Resolution capability

Fig. 2 represents an analytical chromatogram of an equimolar mixture of purified peptides A, B, C, D and E (Fig. 1). This chromatogram was obtained on the CM 300 column (250 × 4.1 mm I.D.) using a linear potassium chloride gradient (8 m*M* KC l/min) in 50 m*M* KH_2PO_4 buffer at pH 4.5. The peptides chosen for this separation vary in net charge at pH 4.5 from +5 to +8. Peptides C and D, however, both have a net charge of +6, but differ by one animo-acid residue. The sequence in peptide C contains one less charged residue (Lys, K). This residue has been replaced by Gly (G) in the sequence (Fig. 1). The two peptides have the same overall net charge, since peptide C contains a free α-NH_2 ($pK_a \approx 9.6$) group at its N-terminus which is protonated under these conditions, while peptide D is N^{α}-acetylated. As can be seen from the chromatogram, the most highly charged species (A and B ; +8 and +7, respectively) are bound most tightly to the resin and are eluted at concentrations of *ca* 0.6 *M* potassium chloride. Good separation is observed to occur between all peptides that have net charge differences of one unit. Though peptides C and D are both +6 spieces, they

Peptide	Net Charge pH 6.5	Sequence
A[1]	+8	N - Q - [K] - L - F - D* - L - [R] - G - [K] - F - [K] - [R] - P - P - L - [R] - [R] - V - [R] - Hse
B[1]	+7	
C	+6	G - (G) - F - [K] - [R] - P - P - L - [R] - [R] - V - [R] - amide
D	+6	Ac - G - [K] - F - [K] - [R] - P - P - L - [R] - [R] - V - [R] - amide
E	+5	Ac - G - (G) - F - [K] - [R] - P - P - L - [R] - [R] - V - [R] - amide
F	+5	Ac - G - [K] - F - [K] - (G) - P - P - L - [R] - [R] - V - [R] - amide
G	+5	Ac - G - [K] - F - (G) - [R] - P - P - L - [R] - [R] - V - [R] - amide
H	+1	S - D* - N - I - P - S - F - [R] - G - amide
I	0	Ac - D* - L - L - A - G - G - [K] - amide
J	-1	Ac - T - D* - L - L - A - G - G - [K] - D* - V - D* - [K] - amide

Fig. 1. Peptides used to study the chromatographic conditions and resolution of CM 300 columns. Asteriks denotes acidic residues ; basic residues are boxed. Circles indicate a substitution of a basic residue (arginine, R or lysine, K) by glycine (G). Hse = homoserine ; Ac = N^{α}-acetyl ; amide = Cα-amide. Aminoacid residues are denoted by the single letter code. Peptides A, B, C and H have free α-NH_2 groups. The charge difference between A and B results from either deamidization or lactonization of Hse.

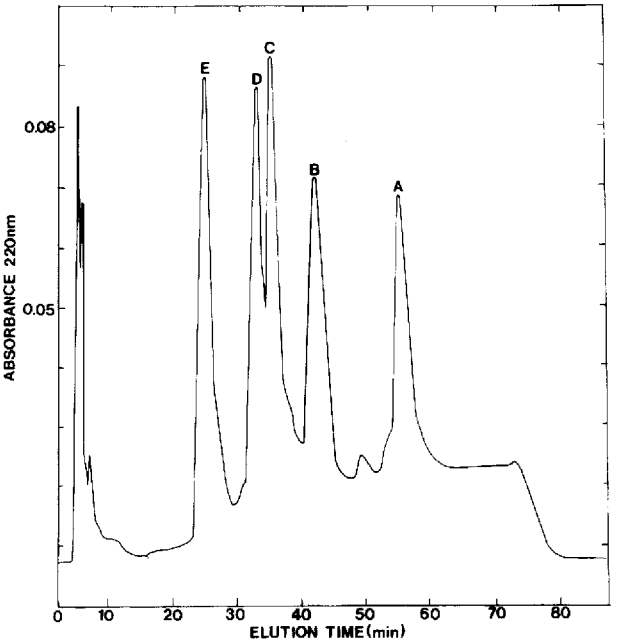

Fig. 2. Analytical chromatogram of the basic peptides A, B, C, D, E (Fig. 1). Column, CM 300 (250 × 4.1 mm I.D.). Buffers : A, 50 m*M* KH_2PO_4, pH 4.5 ; B, 50 m*M* KH_2PO_4, 1 *M* KCl, pH 4.5. A linear KCl gradient (8 m*M* B/min) was applied with the following compositions at the times indicated (min) : (0) 80% A ; (50) 40% A ; (60) 40% A ; (65) 80% A. Flow-rate, 1 ml/min. Chart speed, 6 in./h. Absorbance, 0.1 A.U.F.S. at 220 nm, 10-mm cel. Sample 45 μl, containing 7 – 10 nmole of each of the peptides A, B, C, D and E.

are resolved by this column under these conditions, most likely owing to the pK_a differences associated with the Gly (G) free N^{α}-amino group ($pK_a \approx 9.6$) and the N^{ϵ}-amino group of Lys (K) ($pK_a \approx 10.53$). The peptides C, D and E were also chromatographed at pII 4.5 with a KH_2PO_4 gradient (5 m*M* KII_2PO_4/min) varying linearly from 0.3 *M* KH_2PO_4 to 0.45 *M* KH_2PO_4 over 50 min. The resulting chromatogram (not shown) demonstrated that under these conditions, the column was unable to resolve peptides of similar net charge (D and C), while peptides with one unit net charge difference (E and either D or C) were separated well (*ca* 27 min). Retention times in this gradient system are identical with those of peptides E and D (Fig. 2). The same three peptides were also compared in isocratic elution with a 0.45 *M* KH_2PO_4 buffer at pH 4.5 (chromatogram not shown). Although the retention times for the individual peptide peaks were decreased substantially – peptide E being eluted in 12.5 min and peptides C and D in 20 min – the column was unable to resolve the two closely related structures C and D. In contrast, isocratic potassium chloride (Fig. 3) resolved all three peptides at pH 4.5 (top left) and 0.3 *M* potassium chloride. When the pH was increased to 6.5, the same isocratic solvent system increased the resolution dramatically, but the time necessary to complete the separation more than doubled (bottom). However at pH 6.5, but a higher potassium chloride concentration (top, right) the retention times of all three peptides were reduced, while the separation of E from D, and of C from D was improved.

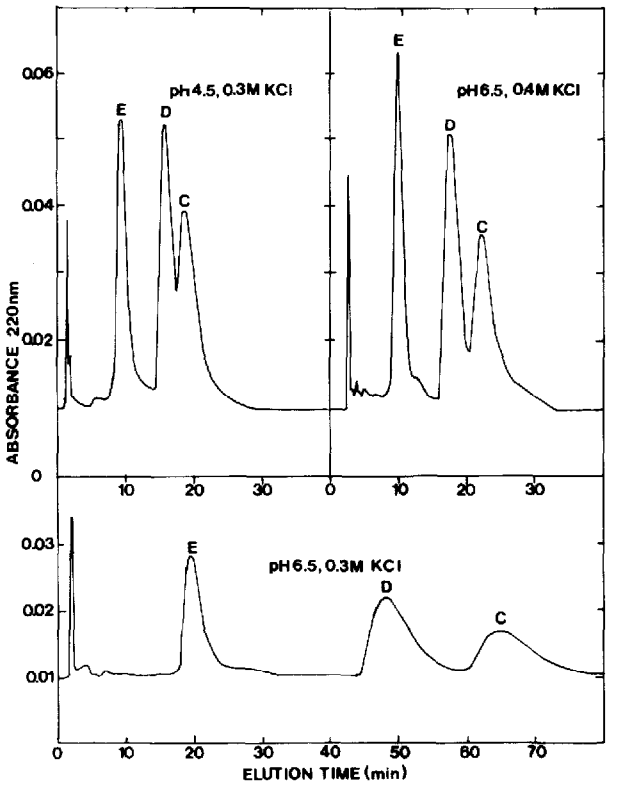

Fig. 3. Effect of pH and ionic strength on the separation of peptides C, D and E under isocratic conditions. Sample, 25 μl, containing 7 – 10 nmole of each of the peptides C, D and E. (Top, left) Eluent composition, 50 m*M* KH_2PO_4, 0.3 *M* KCl, pH 4.5 (Top, right) Eluent composition, 50 m*M* KH_2PO_4, 0.4 *M* KCl, pH 6.5 (Bottom) Eluent composition, 50 m*M* KH_2PO_4, 0.3 *M* Kcl, pH 6.5 (For other conditions, see Fig. 2).

Fig. 4 demonstrates a further application of the resolving capabilities of the CM resin. The mixtures EF and FG contain peptides all carrying the same net charge (+5) but with amino acid composition varying as follows. Peptides E and G each contain one Lys (K) residue and four Arg (R) residues while peptide F contains two Lys (K) residues and three Arg (R) residues. All three peptides are of similar molecular weight and all have acetylated N^{α}-amino groups. The peptide pairs EF and FG were chosen as a means of testing the ability of the resin to discrimitate positional and sequence variation in the EF and FG pairs (Fig. 4, circled residues). Under isocratic conditions (50 m*M* KH_2PO_4, 0.3 *M* KCl, pH 6.5) the CM resin proved capable of resolving each of the peptide pairs (bottom) and the column's ability to resolve both pairs of peptides was increased by lowering the potassium chloride concentration to 0.25 *M* (top) without greatly affecting the overall time necessary to complete the analysis. The results shown in Figs. 2, 3 and 4 demonstrate the importance of choosing the correct peptide counter-ion with which to effect the careful separation of structurally similar spieces. The chromatograms also show that the CM 300 resin is capable of resolving basic peptides containing the same number of positively charged residues but varying in their Arg (R) and Lys (K) content. This separation is most likely due to the pK_a differences associated with Arg (R) and Lys (K) sidechains.

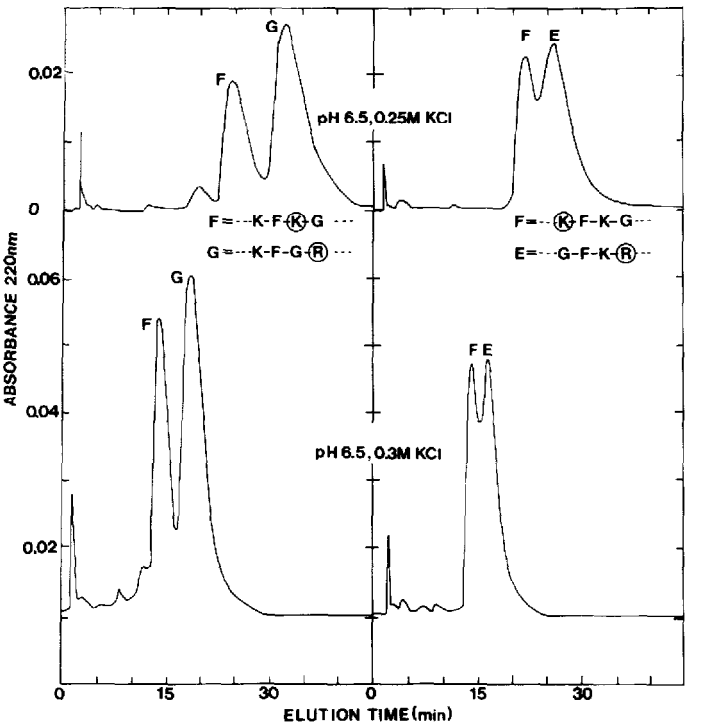

Fig. 4. Demonstration of the resolving capabilities of the CM 300 support. The synthetic peptides E, F and G have an identical number of basic residues but differ in their arginine (R) and lysine (K) compositions (*cf.* Fig. 1). Column, CM 300 (250 × 4.1 mm I.D.). Flow-rate, 1 ml/min. Chart speed, 6 in./h. Absorbance, 0.1 a.u.f.s. at 220 nm, 10-mm cell. Injection volume, 6 μl containing 6 – 8 nmole of each of the peptides E, F and G. (Top) Eluent composition : 50 m*M* KH_2PO_4, 0.25 *M* KCl, pH 6.5 (Bottom) 50 m*M* KH_2PO_4, 0.3 *M* KCl pH 6.5.

Ionic strength and pH effects

A comparison of the plates in Figure 3 (top, left and bottom) indicates that under isocratic conditions (50 m*M* KH_2PO_4, 0.3 *M* KCl) a change in the pH of the eluent from pH 4.5 to 6.5 has a large effect on the resolution and the retention times of the components of the peptide mixture C, D and E. The pK_a values of the Arg (R) and Lys (K) residues (pK_a *ca.* 12.48 and 10.53, respectively) in peptides C, D and E and the N^{α}-amino group ($pK_a \approx 9.6$) of peptide C suggests that the changes in eluent pH do not substantially affect the net charge on these peptides. Deprotonation of these charged groups would neutralize net charge on the peptides and most likely result in a decrease in both the resolution and retention times observed in the chromatogram. Clearly, the observed effects result from an increase in the ability of the carboxymethyl resin to bind these basic components as the pH is increased relative to the pK_a of the carboxyl groups on the support. Increasing the pH from 4.5 to 6.5 increases the number of ionized carboxyl groups present on the CM support, and this, in turn, increases its resolving power. Solutes, such as potassium chloride, can be used to attenuate the effects of pH as is demonstrated in Fig. 3 (top, right). As the potassium chloride concentration is raised from 0.3 to 0.4 *M*, the retention times of the individual peptides

are reduced. However, the separation obtained between the structurally related and similarly charge peptides C and D (+6, Fig. 1) is maintained and enhanced over that obtained at pH 4.5 and 0.3 *M* potassium chloride. All carry similar net charges (+5), but their amino acid compositions vary as described earlier (*cf.* under *Resolution capability* and Fig. 1). In Fig. 4 the separation of peptide pairs EF and FG were chosen to test the resolving power of the CM resin. As expected, a decrease in the concentration of potassium chloride to 0.25 *M* (top) results in increased peptide–resin binding which is reflected in increased retention times and resolution accompanied by peak broadening in both pairs tested.

Load capacity

The results of increased sample load in a preparative CM column (250 × 10 mm I.D.) are shown in Fig. 5. Here crude samples of peptide G (Fig. 1) were applied in 2.5, 5, 10 and 20 mg amounts to determine the maximum load that can be applied to this column without loss of resolution caused by peak fusion. These profiles are representative of peptide mixtures obtained after the synthetic material is cleaved from its solid support by treatment with hydrofluoric acid. The chromatography was carried out using a potassium chloride gradient (5 m*M*/min) in 50 m*M* KH_2PO_4 at pH 6.5. The

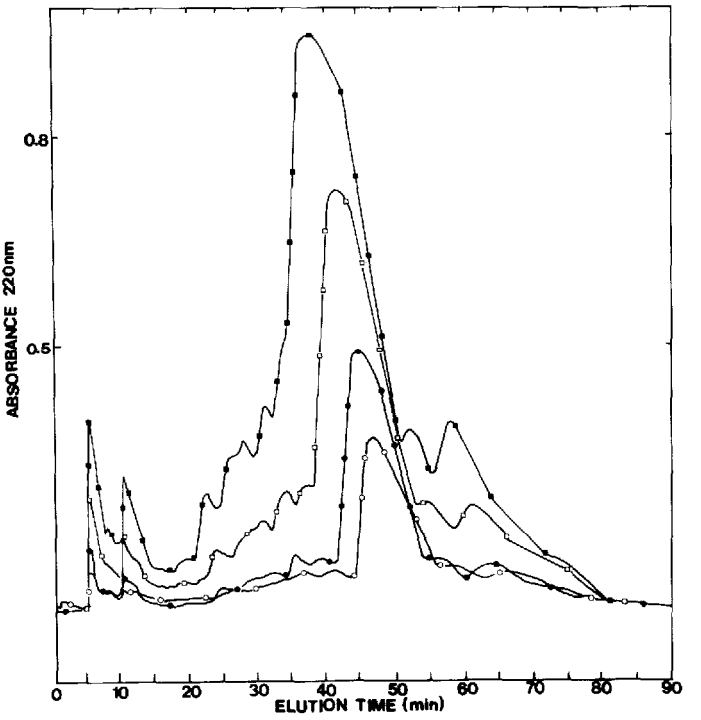

Fig. 5. Effect of load capacity on the resolution of the basic peptide G (Fig. 1). Column, CM 300 (250 × 10 mm I.D.). Flow-rate, 2 ml/min. Chart speed, 0.1 in./min. Absorbance, 1.0 a.u.f.s. at 220 nm ; 10-mm cell. [Peptide] *ca.* 16.6 nmole/μl. Injection volume : ○, 100 μl (2.5 mg) ; ●, 200 μl (5 mg) ; □, 400 μl (10 mg) ; ■, 800 μl (20 mg). Buffers : A, 50 m*M* KH_2PO_4, pH 6.5 ; B, 50 m*M* KH_2PO_4, 1 *M* KCl, pH 6.5. A linear gradient (5 m*M* B/min) was generated with the following compositions at the times indicated (min) : (0) 80% A ; (5) 80% A ; (45) 60% A ; (65) 60% A ; (70) 80% A ; (100) 80% A.

major peak represents the peptide of interest (G) and smaller peaks are those of deletion or termination products. The major peptide peak and the component, which just precedes it, are clearly resolved at loads up to 5 mg. At 10 mg the preceding component still appears as a distinct shoulder but at 20 mg, the two components are no longer distinguishable as peak fusion has occurred at this point. Retention times in all components shown in these profiles are also affected by the amount of peptide sample applied. The major peak undergoes a 10-min shift to shorter retention time as the sample load is increased from 2.5 to 20 mg. Similar amounts of peptide G were also loaded at pH 4.5 under the same solvent conditions (chromatograms not shown). At this lower pH the column load capacity was much reduced and peak fusions occurred on application of 5 mg of peptide. Retention times exhibited on application of 2.5 and 5 mg also varied dramatically, decreasing by 12 min on doubling the concentration of peptide applied. These observations indicate that the binding capacity of the resin in much reduced at this lower pH as fewer carboxylate groups are available for binding, and suggest that for preparative purposes, a pH should be used that is well above the pK_a of the carboxyl group on the support.

Peptides H, I and J (Fig. 1)

These peptides represent examples of weakly basic and acidic molecules having approximate net charges of +1, 0 and –1 at pH 6.5. We have tested the ability of the analytical column to bind these peptides and have found that peptides I and J are not retained on the column in 5 m*M* KH_2PO_4 buffers, pH 4.5 or pH 6.5. Peptide H is bound in the 5 m*M* KH_2PO_4 buffer, pH 4.5 and pH 6.5 for 15 and 10 min, respectively. Peptides of this type are more suited for purification and analysis on either reversed-phase or strong anion-or cation-exchange resins.

CONCLUSIONS

From the results presented, several conclusions may be drawn.

(1) We have selected suitable peptides which act as molecular probes of resolution and load capacity in weak cation-exchange HPLC.

(2) These results have indicated suitable operating conditions (gradient and isocratic) for the separation of basic peptides in both the analytical and preparative weak cation-exchange columns.

(3) The pH of the buffer system can be used as a means of modifying the binding properties of the analytical CM column so as to optimize retention times. In preparative chromatography, however, lowering the pH (4.5) severely limits the load capacity and pH 6.5 is recommended.

(4) In analytical work, ionic strength can be employed to attenuate the effects of pH and thereby to increase the resolving power of the column without significantly affecting efficient retention times.

(5) Under both isocratic and gradient conditions, phosphate buffers containing KCl were more effective in resolving peptides of similar charge than were phosphate buffers alone.

ACKNOWLEDGEMENTS

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