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

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SUMMARY

The effects of pH and gradient conditions on the separation of a series of ten peptides (9–36 residues) and carboxamidomethylated troponin I (CM-TnI, 178 residues) on a new commercially available strong cation-exchange silica based 300-Å column (Synchropak S300) were examined. The elution times of the peptides were linear with respect to their net charge at pH 3.0 and pH 6.5. The basic protein CM-TnI (p$I \approx 9.5$) and peptides with net charges from +2 to +10 were separated with linear AB salt gradients varying from 5 mM to 10 mM B per min (A = 5 mM KH_2PO_4 buffer, pH 6.5 or 3.0; B = 5 mM KH_2PO_4 buffer, pH 6.5 or 3.0, containing 1 M KCl). All peptides and CM-TnI were eluted with KCl concentrations below *ca.* 0.6 M. The advantage of performing cation-exchange chromatography over anion-exchange chromatography was demonstrated for the separation of peptides which, while acidic or weakly basic at neutral pH, through protonation of the acidic functions results in positively charged peptides at pH 3.0.

INTRODUCTION

Our laboratory is involved in structure-function studies of the inhibitory subunit (TnI) of the troponin complex of rabbit skeletal fast muscle[1,2]. Investigative techniques for elucidating the biological activity of troponin I include enzymatic or chemical cleavage of the native protein to localize the site(s) of inhibitory activity and solid-phase synthesis of peptides able to mimic this activity. Both techniques require peptide purification. The much improved separation efficiency of high-performance liquid chromatography (HPLC) over open column chromatography has greatly enhanced the resolution of peptide mixtures in recent years.

The separation of basic peptides on a weak cation-exchange column, CM300, at pH 4.5 and 6.0, was reported recently[3]. The rapid protonation of the carboxylate functionalities as their environment becomes more acidic prohibits the use of this ion exchanger at pH levels low enough to neutralize the negative charge of acidic residues on peptides or proteins. Recently, a strong cation-exchange silica-based column, S300, has become available for use in HPLC at low pH. The ion-exchanger has a particle-size of 6.5 μm with 300-Å pore diameter which is derivatized with sulphonic

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acid functionalities. This paper reports the performance of the Synchropak S300 analytical strong cation-exchange column in resolving several synthetic peptides and protein fragments. The peptides employed have a known sequence and vary with respect to their net charge and basic and acidic residue composition. We have examined the effects of pH of the eluent on the resolving power of the S300 column and demonstrated the value of increasing the net positive charge of a peptide by cation-exchange chromatography under strongly acidic conditions.

EXPERIMENTAL

Materials

Deionized water was purified by passage through a Milli-Q Water Purification System (Millipore, U.S.A.). Potassium chloride (A.C.S. grade) was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Rabbit skeletal CM-TnI (S-carboxamidomethylated TnI) was prepared as described by Chong and Hodges[4]. Peptides 3, 4 and 7–10 (Fig. 1) were obtained by cyanogen bromide cleavage of rabbit skeletal CM-TnI. Peptides 1, 2, 5 and 6 (Fig. 1) are synthetic peptides made by solid-phase

Peptide	pH 3.0 Net Charge	pH 3.0 R_t (min)	pH 6.5 Net Charge	pH 6.5 R_t (min)	
1	+2	nr	+1	nr	S-(D)-N-I-P-S-F-[R]-G-amide
2	+4	13.7	+2	12.1	Ac-S-(D)-Q-(E)-[K]-[R]-[K]-Q-I-S-V-[R]-G-L-amide
3	+4	nd	+3	23.8	L-[K]-A-L-L-G-S-[K]-[H]-[K]-V-C-Hse
4	+5	nd	+4	32.0	L-[K]-A-L-L-G-S-[K]-[H]-[K]-V-C-HseLac
5	+5	20.9	+5	53.1	Ac-G-[K]-F-G-[R]-P-P-L-[R]-[R]-V-[R]-amide
6	+6	24.6	+6	64.8	Ac-G-[K]-F-[K]-[R]-P-P-L-[R]-[R]-V-[R]-amide
7	+9	37.1	+8	82.4	N-Q-[K]-L-F-(D)-L-[R]-G-[K]-F-[K]-[R]-P-P-L-[R]-[R]-V-[R]-HseLac
8	+10	34.7	+1	nr	(D)-L-[R]-A-N-L-[K]-Q-V-[K]-[K]-(E)-(D)-T-(E)-[K]-(E)-[R]-(D)-V-G-(D)-W-[K]-[K]-N-I-(L)-(E)-[K]-S-G-HseLac
9	+6	24.8	-2	nr	L-Q-I-A-A-T-(E)-L-(E)-[K]-(E)-(E)-G-[R]-[R]-(E)-A-(E)-[K]-Q-N-Y-L-A-(F)-[H]-C-P-P-L-S-L-P-G-S-HseLac
10	+5	17.0	-3	nr	A-(E)-V-Q-(E)-L-C-[K]-Q-L-[H]-A-[K]-I-(D)-A-A-(L)-(E)-(L)-[K]-V-(D)-HseLac

Fig. 1. Peptides used in this study. Acidic residues are circled; basic residues are boxed; nd denotes not done; nr denotes not retained. Hse = Homoserine; HseLac = homoserine lactone; Ac = Nα-acetyl; amide = Cα-amide. Amino acid residues are denoted by the single letter code. Peptides 1, 3, 4 and 7–10 have free α-NH_2 groups. Retention times do not include 10-min elution with starting buffer.

methodology as described by Talbot and Hodges[1]. Purified peptides were obtained by ion-exchange (CM300 column[1], peptides 1, 5, 6; S300 column, remaining peptides) and reversed-phase (C_{18}) chromatography.

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. Samples were injected with a Hamilton No. 1710 100-μl syringe (Hamilton, Reno, NV, U.S.A.) into a 500-μl injection loop (Model No. 7125, Rheodyne, Berkeley, CA, U.S.A.) and chromatographed on Synchropak S300 or Q300 analytical ion-exchange columns (250 × 4.1 mm I.D.; SynChrom, Linden, IN, U.S.A.).

Methods

All chromatography was carried out at room temperature (*ca.* 20°C). The peptides were dissolved in the starting buffer (5 m*M* KH_2PO_4) and centrifuged at 12000 *g* for 2 min. CM-TnI was dissolved in starting buffer containing 8 *M* urea, followed by centrifugation at 12000 *g* for 5 min. The columns were equilibrated with the starting buffer for 30 min before each determination. The flow-rate was maintained at 1 ml/min. Aliquots (10 μl) of stock solutions of the individual purified peptides and CM-TnI were hydrolysed in 100–200 μl of 6 *M* hydrochloric acid at 110°C for 24 h in evacuated, sealed tubes. The hydrolysates were subsequently analysed on a Durrum 500 amino acid analyser to obtain peptide and protein concentrations.

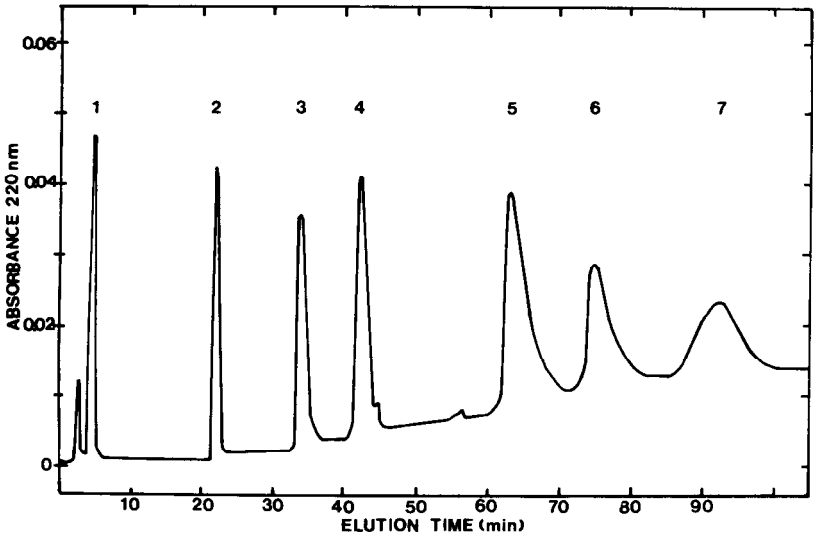

Fig. 2. Analytical chromatogram of the basic peptides 1–7 (Fig. 1). Column, S300 (250 × 4.1 mm I.D.). Buffers: A, 5 m*M* KH_2PO_4 (pH 6.5); B, 5 m*M* KH_2PO_4–1 *M* KCl (pH 6.5). A linear KCl gradient (5 m*M* B/min) was applied, following a 10-min isocratic elution with buffer A. Flow-rate, 1 ml/min. Chart speed, 6 in./h. Absorbance, 0.1 a.u.f.s. at 220 nm, 10-mm cell. Sample 80 μl, containing 8–12 nmole of each peptide.

RESULTS AND DISCUSSION

Resolution capability

Fig. 2. represents a chromatogram of an equimolar mixture of purified peptides 1–7 (Fig. 1) obtained with the S300 column and a linear potassium chloride gradient (5 m*M* KCl/min following 10 min elution with starting buffer) in 5 m*M* KH_2PO_4 buffer at pH 6.5. The peptides vary in net charge from +1 to +8, and under these conditions excellent separation of all seven peptides is observed. The most highly charged species (peptide 7, +8) is bound most tightly to the ion exchanger and is eluted at a KCl concentration of *ca.* 0.4 *M*; the lowest-charged species to be retained by the resin (peptide 2, +2) is eluted at a KCl concentration of *ca.* 0.05 *M*. Peptide 1 (+1) is eluted with the starting buffer. Fig. 3. represents a chromatogram of an equimolar mixture of purified peptides 1, 2, 5, 6 and 7 obtained with the S300 column under the same conditions as above except for a lowering of the pH to 3.0. The peptides vary in net charge from +2 to +9 under these conditions, and excellent separation is observed for all five peptides. The most highly charged species (peptide

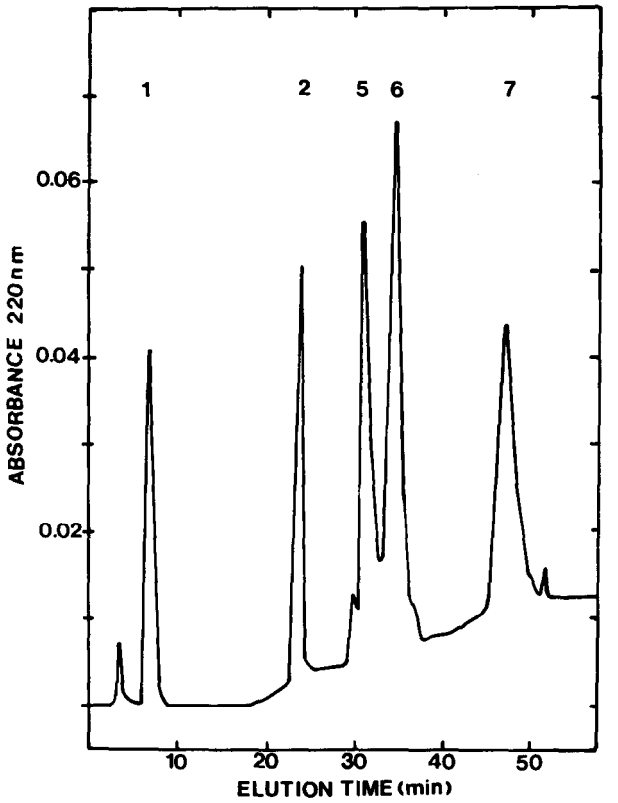

Fig. 3. Analytical chromatogram of the basic peptides 1, 2, 5, 6 and 7 (Fig. 1). Column, S300 (250 × 4.1 mm I.D.). Buffers: A, 5 m*M* KH_2PO_4 (pH 3.0); B, 5 m*M* KH_2PO_4–1 *M* KCl (pH 3.0). A linear KCl gradient (5 m*M* B/min) was applied, following a 10-min isocratic elution with buffer A. Flow-rate, 1 ml/min. Chart speed, 6 in./h. Absorbance, 0.1 a.u.f.s. at 220 nm, 10-mm cell. Sample 60 μl, containing 8–12 nmole of each peptide.

7, +9) is eluted at a KCl concentration of *ca.* 0.18 *M*; the lowest charged species to be retained by the ion exchanger (peptides 2, +4) is eluted at *ca.* 0.05 *M* KCl concentration. A peptide carrying a +3 net charge under these conditions was not available, but peptide 1 (+1) is eluted with the starting buffer. It is noticeable that the drop in pH from 6.5 to 3.0 significantly reduced the retention time of similarly charged peptides.

The relationship of net charge to peptide retention time is demonstrated in Fig. 4. The linearity of both plots (pH 3.0 and 6.5) suggested that the retention time of a particular peptide at either pH is dependent solely on its charge. Of course, the peptides used in this study ranged only from 9–21 residues, and it would not be surprising if conformational effects in larger peptides and proteins resulted in a deviation from strict linearity.

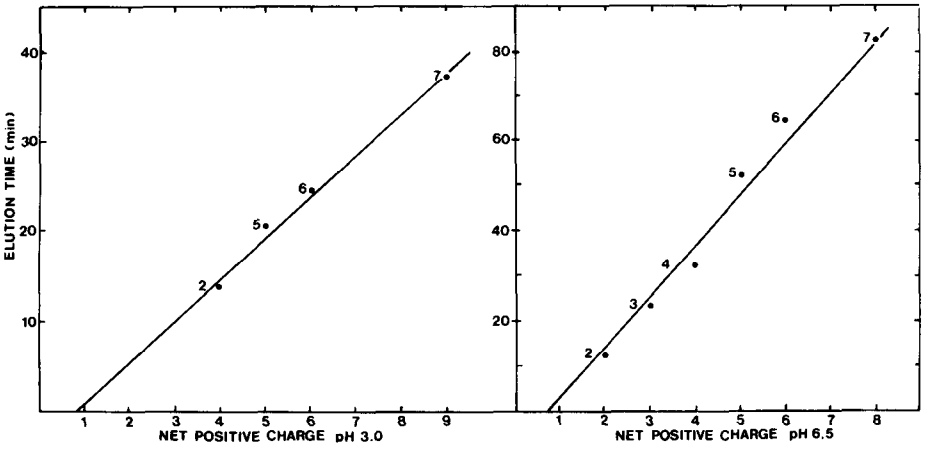

Fig. 4. Relationship of peptide net charge to retention time on the S300 column. The elution times do not include the initial 10-min isocratic elution with the starting buffer (5 m*M* KH_2PO_4). The sequences of peptides 2–7 are shown in Fig. 1.

pH effects on peptide retention

The marked reduction in retention time of similarly charged peptides on lowering the pH from 6.5 to 3.0 (above) prompted further investigation of this column characteristic. Fig. 5 demonstrates very clearly the decreased elution times of peptides 5 and 6 as the buffer pH is decreased from pH 6.5 to 2.2. This decrease appears to be fairly linear from pH 5.0 to pH 2.2. These peptides were chosen because they contained no acidic residues and their terminal carboxyl groups were blocked as amides. This ensured that no complications would arise from carboxyl group protonation as the pH was lowered. Ideally, there should be no variation of elution time of the two peptides with buffer pH. The pK_a values of the arginine (R) and lysine (K) residues (*ca.* 12.48 and 10.53, respectively) suggests that lowering the eluent pH would not substantially affect the net positive charge on peptides 5 and 6 (+5 and +6, respectively) and if anything would increase retention time. The observed effects apparently result from a reduction in column capacity to retain charged species as the pH becomes more acidic. This is unexpected, and undesirable, in a strong cation

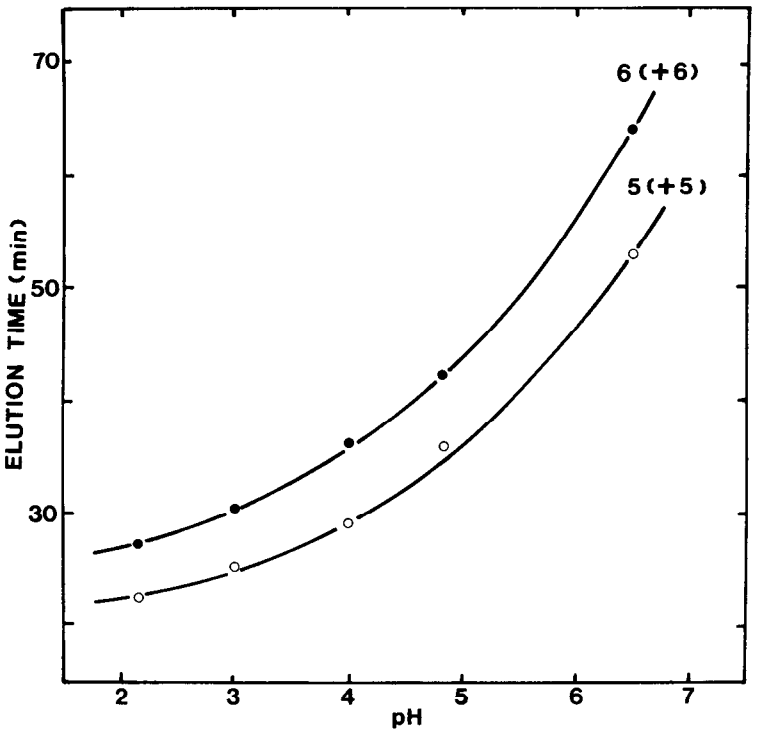

Fig. 5. Effect of pH on S300 column retention of peptides 5 and 6. Buffers: A, 5 m*M* KH_2PO_4; B, 5 m*M* KH_2PO_4–1 *M* KCl; pH 2.2, 3.0, 4.0, 5.0 and 6.5. A linear KCl gradient (5 m*M* B/min) was applied from a starting composition of 100% A. Flow-rate, 1 ml/min. Absorbance measured at 220 nm, 10-mm cell. Sample 30 µl, containing 10 nmole of each peptide.

exchanger where the pK_a of the sulphonate functionalities should be low and uniform throughout the column. The pK_a of the sulphonate group would be expected at < 1.0 and thus, lowering the pH should not significantly affect elution times until the pH of the medium approaches the pK_a. However, the S300 column pH characteristics suggest that the sulphonate groups on the ion exchanger are being blocked by ion-pair formation of underivatized protonated amino groups from the polyamine layer as the pH is lowered. This effect could be easily corrected by acetylation of free amino groups on the support or complete derivatization of the amino groups with the sulphonic acid moiety.

Comparison of strong-cation (S300) and strong-anion (Q300) columns

The advantage of performing cation-exchange chromatography under strongly acidic conditions is demonstrated by the resolution of peptides 8–10. These peptides, obtained by cyanogen bromide cleavage of rabbit skeletal CM-TnI, are weakly basic or acidic at neutral pH and are not retained by the S300 ion exchanger. Lowering the pH to 3.0 results in almost complete protonation of the side chain carboxyl groups of the acidic residues (glutamic acid, E; aspartic acid, D; pK_a values *ca.* 4.0). The now strongly cationic peptides [8 (+10); 9 (+6); 10 (+5), assuming complete protonation of all acidic residues] are all bound to and well resolved on the S300

column (Fig. 6, left) when a linear potassium chloride gradient is applied (5 m*M* KCl/min). The peptides are eluted at KCl concentrations of *ca.* 0.08 *M* (10), 0.12 *M* (9) and 0.17 *M* (8). When applied to a Q300 strong anion-exchange analytical column under the same conditions apart from raising the pH to 6.5 and including a 10-min isocratic elution with buffer A, the three peptides are again well separated (Fig. 6, right) in reverse order of that on the S300 column. Peptides 9 (−2 net charge at pH 6.5) and 10 (−3) are eluted at KCl concentrations of approximately 0.06 *M* and 0.13 *M*, respectively. However, peptide 8 (+1 net charge at pH 6.5) is not bound to the Q300 ion exchanger in the starting buffer.

Retention of rabbit skeletal CM-TnI on the S300 ion exchanger

Fig. 7 represents chromatograms of a mixture of rabbit skeletal CM-TnI and peptide 5 run on the S300 column. Rabbit skeletal TnI is a very basic protein (p*I ca.* 9.5) and serves as a useful probe for a cation-exchange column. Problems associated with TnI solubility were overcome by the addition of 8 *M* urea to the starting buffers when dissolving the samples. At pH 6.5 (Fig. 7, Upper) CM-TnI and peptide 5 are eluted at potassium chloride concentrations of *ca.* 0.18–0.21 *M* and 0.26 *M* respec-

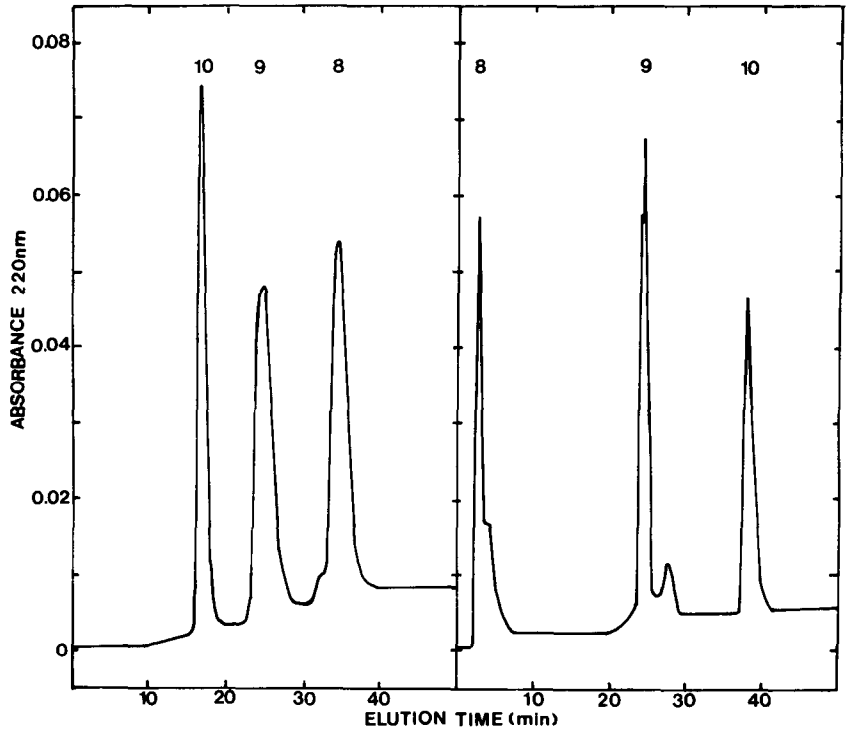

Fig. 6. Comparison of resolution of peptides 8–10 on S300 and Q300 columns. Left: S300 (250 × 4.1 mm I.D.) strong cation-exchange column. Right: Q300 (250 × 4.1 mm I.D.) strong anion-exchange column. Buffers: A, 5 m*M* KH_2PO_4; B, 5 m*M* KH_2PO_4–1 *M* KCl; pH 3.0 (S300) and pH 6.5 (Q300). A linear KCl gradient (5 m*M* B/min) was applied either immediately after sample injection (S300) or following 10-min isocratic elution with buffer A (Q300). Flow-rate, 1 ml/min. Chart speed, 6 in./h. Absorbance, 0.1 a.u.f.s. at 220 nm, 10-mm cell. Sample 50 μl, containing 8–12 nmole of each peptide.

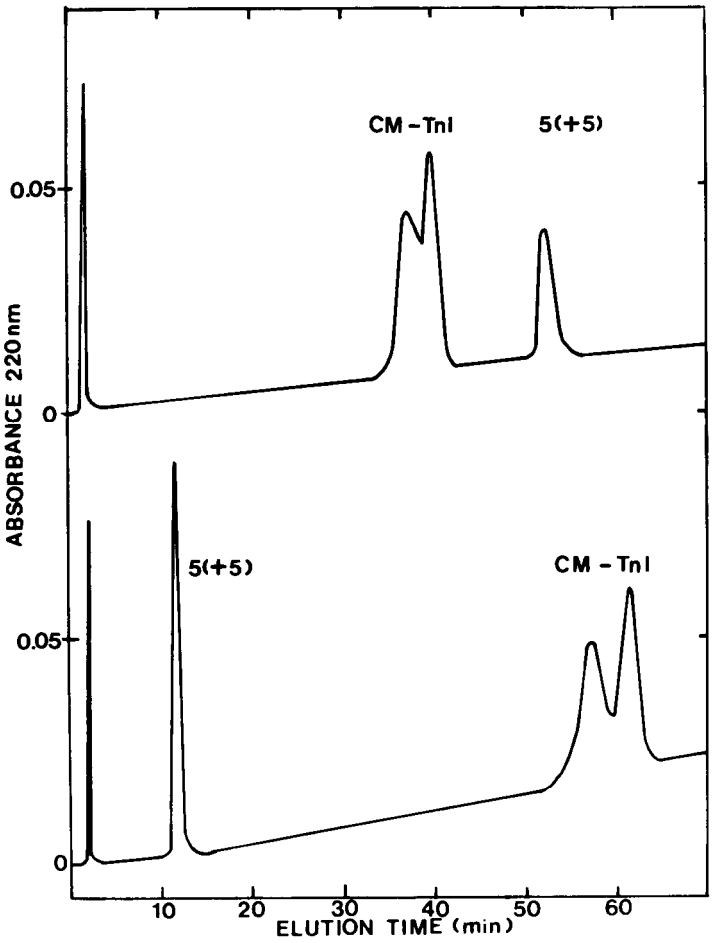

Fig. 7. Resolution of skeletal CM-TnI and peptide 5 on the S300 column. Buffers: A, 5 m*M* KH_2PO_4; B, 5 m*M* KH_2PO_4–1 *M* KCl. Upper: pH 6.5 buffers; a linear KCl gradient (5 m*M* B/min) was applied from a starting composition of 100% A. Lower: pH 3.0 buffers; a linear KCl gradient (10 m*M* B/min) was applied from a starting composition of 100% A. Flow-rate, 1 ml/min. Chart speed, 6 in./h. Absorbance, 0.1 a.u.f.s. measured at 220 nm, 10-mm cell. Samples dissolved in starting buffer plus 8 *M* urea, and 30-μl volumes (0.7 nmole CM-TnI + 10 nmole peptide 5) applied to the column.

tively, following a linear KCl gradient of 5 m*M*/min. At pH 3.0 (Fig. 7, lower) CM-TnI and peptide 5 are eluted at KCl concentrations of *ca.* 0.55–0.63 *M* and 0.12 *M*, respectively, following a linear KCl gradient of 10 m*M*/min. Peptide 5 (+5 at either pH) served as a standard marker and indicated that, at pH 6.5, Cm-TnI was eluted at a KCl concentration typical of peptides with net charges of +3 to +4 (Fig. 2). It may have been expected that, because of its very basic nature, the protein would be bound more tightly to the ion exchanger at pH 3.0 than was in fact observed. The lower retention time demonstrated probably resulted from incomplete neutralization of all side chain carboxyl groups and/or protein conformational effects. The high resolving power of ion-exchange HPLC has separated CM-TnI into two isoforms[5].

This ability of the S300 ion exchanger to resolve CM-TnI at acidic and neutral pH indicated that the column would be extremely useful in resolving both protein and peptide mixtures.

CONCLUSIONS

From the results presented, several conclusions may be drawn.

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

(2) The results have indicated suitable operating conditions for the separation of basic peptides on a strong cation-exchange column at pH 3.0 and pH 6.5.

(3) The elution times of the peptides were linear with respect to their net charge at pH 3.0 and 6.5.

(4) The capacity of the S300 ion exchanger to retain charged species decreased progressively as the pH became more acidic. This is only a limitation for weakly basic molecules (+1, +2) at acidic pH.

(5) The S300 ion exchanger has added another dimension to HPLC ion-exchange chromatography by its very advantageous ability to separate both small peptides and proteins. Improvement of this HPLC cation exchanger to bind weakly basic peptides would greatly enhance this powerful analytical tool.

ACKNOWLEDGEMENTS

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