Journal of Chromatography, 476 (1989) 377–389
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1631

STRONG CATION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PEPTIDES

EFFECT OF NON-SPECIFIC HYDROPHOBIC INTERACTIONS AND LINEARIZATION OF PEPTIDE RETENTION BEHAVIOUR

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SUMMARY

Strong cation-exchange chromatography (strong CEX) is probably the most useful mode of high-performance ion-exchange chromatography (IEC) for peptide separations. Although the hydrophobic character of high-performance ion-exchange packings, often giving rise to mixed-mode contributions to solute separations, has long been recognized, a systematic approach to examining the effect and magnitude of the hydrophobicity of these packings during IEC of peptides has so far been lacking.

In the present study, we report the synthesis of three series of positively charged peptide polymers which vary significantly in overall hydrophobicity and polypeptide chain length (5–50 amino acid residues): Ac-(Gly-Lys-Gly-Leu-Gly)$_n$-amide, Ac-(Leu-Gly-Leu-Lys-Ala)$_n$-amide and Ac-(Leu-Gly-Leu-Lys-Leu)$_n$-amide (n = 1, 2, 4, 6, 8, 10). We have examined non-specific hydrophobic interactions of these peptides with both silica- and polymer-based ion-exchange packings, demonstrating how these interactions are overcome by the addition of acetonitrile to the mobile phase. It was also shown that removal of non-specific hydrophobic interactions may be necessary just to elute peptides from the ion-exchange matrix. In addition, from the observed retention times of these three peptide polymer series and other peptides which vary substantially in charge density, net charge, polypeptide chain length and hydrophobicity, we have established a simple approach to linearization and, thus, prediction of peptide retention behaviour in CEX.

INTRODUCTION

High-performance ion-exchange chromatography (IEC) has become increasingly popular for the analysis of both peptides and proteins in recent years[1]. Although, as the name implies, the major separation mechanism of this mode of high-performance liquid chromatography (HPLC) is electrostatic in nature, ion-exchange packings may also often exhibit significant hydrophobic characteristics, giving rise to

0021-9673/89/$03.50

mixed-mode contributions to solute separations[2,3]. As pointed out by Rounds *et al.*[4], a small amount of hydrophobic character in an ion exchanger is not necessarily detrimental to the separation of proteins, and may even enhance resolution by mixed-mode effects. Several researchers have exploited these mixed-mode effects to aid in peptide and protein separations on anion-exchange (AEX)[3–8] and cation-exchange (CEX) columns[9,10]. However, when only the predominant, *i.e.*, ionic, stationary phase–solute interaction is required, the mobile phase must be manipulated so as to minimize non-specific interactions, *e.g.*, by the addition of a non-polar organic solvent such as acetonitrile to the mobile phase buffers to suppress hydrophobic interactions between the solute and the ion-exchange packing. Although the hydrophobic character of high-performance ion-exchange packings has long been recognized, a systematic approach to examining the effect and magnitude of the hydrophobicity of these packings during IEC of peptides has so far been lacking.

Strong cation-exchange chromatography (strong CEX) is probably the most useful mode of IEC for peptide separations[1,9–13]. The utility of strong CEX packings, generally containing sulphonate functionalities, lies in their ability to retain their negatively charged character in the acidic to neutral pH range. At low pH, the side-chain carboxyl groups of acidic amino acid residues are protonated, emphasizing any positively charged character of the peptides. Thus, by manipulating the pH of the mobile phase, the net charge of a peptide may be varied. In addition to overall net charge, other factors which may affect the retention behaviour of peptides during IEC include peptide conformation, polypeptide chain length, charge distribution, and charge density. To understand peptide retention behaviour during IEC completely, it is not sufficient merely to demonstrate that these various factors have an effect on peptide retention, it is also necessary to quantitate the relative contribution each factor makes to retention behaviour.

In the present study, we have synthesized three series of basic peptide polymers (5–50 residues) of varying hydrophobicity and subjected them to strong CEX. From the observed retention behaviour of the polymer sets, we have clearly demonstrated the effects on peptide elution profiles of hydrophobic interactions of peptides with ion-exchange packings. In addition, from the observed retention times of these three peptide polymer series and other peptide mixtures, we have gained a clearer understanding of the effect of both polypeptide chain length and charge density on peptide retention behaviour during strong CEX.

EXPERIMENTAL

Materials

Water (HPLC-grade), acetonitrile (HPLC-grade), and sodium chloride (ACS-grade) were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Four synthetic undecapeptide cation-exchange standards (1–4) were obtained from Synthetic Peptides Inc. (Department of Biochemistry, University of Alberta, Edmonton, Canada). Peptides 1 and 2 were based on the sequence, X^1-X^2-Gly-Leu-Gly-Gly-Ala-Gly-Gly-Leu-Lys, where X^1-X^2 were substituted with Gly^1-Gly^2- (peptide 1) or Lys^1-Tyr^2- (peptide 2); peptides 3 and 4 were based on the sequence, X^1-X^2-Ala-Leu-Lys-Ala-Leu-Lys-Gly-Leu-Lys, where X^1-X^2- were substituted with Gly^1-Gly^2- (peptide 3) or Lys^1-Tyr^2- (peptide 4). Each peptide contained an N^{α}-acetylated N-terminal and a C-terminal amide.

A mixture of five synthetic size-exclusion peptide standards was also obtained from Synthetic Peptides Inc. The sequence of the standards was Ac-(Gly-Leu-Gly-Ala-Lys-Gly-Ala-Gly-Val-Gly)$_n$-amide, where n = 1–5, *i.e.*, 10–50 residues in length.

Peptide synthesis

The peptide polymers described were synthesized on an Applied Biosystems peptide synthesizer Model 430A (Foster City, CA, U.S.A.), using the general procedure for solid-phase synthesis described by Parker and Hodges[14] and Hodges *et al.*[15].

Apparatus

The HPLC instrument consisted of a Varian Vista Series 5000 liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.), coupled to a Hewlett-Packard (Avondale, PA, U.S.A.) HP 1040A detection system, HP 9000 Series 300 computer, HP 9133 disc drive, HP 2225A Thinkjet printer and HP 7440A plotter. Samples were injected with a Model 7125 200-μl injection loop (Rheodyne, Cotati, CA, U.S.A.).

Columns

Peptide mixtures were separated on three strong cation-exchange columns: (1) SynChropak S300, 250 × 4.1 mm I.D. 6.5 μm particle size, 300-Å pore size (SynChrom, Linden, IN, U.S.A.); (2) PolySulfoethyl Aspartamide, 250 × 4.6 mm I.D. 5 μm, 300 Å (PolyLC, Columbia, MD, U.S.A.); (3) Mono S HR 5/5, 50 × 5 mm I.D., 10 μm (Pharmacia, Dorval, Canada).

RESULTS AND DISCUSSION

Design of peptide polymers

In order to examine the effect of peptide chain length on peptide retention behaviour during strong CEX, as well as monitoring any non-specific hydrophobic interactions between solute and column packing, it was necessary to design series of positively charged peptide polymers covering a similar range of chain length, but differing in overall hydrophobicity. Three series of peptide polymers were subsequently synthesized: (a) Ac-(Gly-Lys-Gly-Leu-Gly)$_n$-amide, where n = 1, 2, 4, 6, 8, 10 (5–50) residues, +1 to +10 net charge); (b) Ac-(Leu-Gly-Leu-Lys-Ala)$_n$-amide, where n = 1, 2, 4, 6, 8, 10 (5–50 residues, +1 to +10 net charge); (c) Ac-(Leu-Gly-Leu-Lys-Leu)$_n$-amide, where n = 1, 2, 4, 6, 8, 10 (5–50 residues, +1 to +10 net charge). The hydrophobicity of the polymer series increased in the order, Ac-(Gly-Lys-Gly-Leu-Gly)$_n$-amide ("G" series) < Ac-(Leu-Gly-Leu-Lys-Ala)$_n$-amide ("A" series) < Ac-(Leu-Gly-Leu-Lys-Leu)$_n$-amide ("L" series). In addition, the single lysine residue in each repeating unit of five residues ensured that the overall charge density of every peptide was identical. For the purposes of this study, each peptide is referred to by a number and letter which denote, respectively, the number of residues it contains and to which polymer series it belongs. Thus, 5G refers to the five-residue "G" series peptide; 30A refers to the 30-residue "A" series peptide, etc.

Effect of hydrophobic interactions in strong CEX

The peptide polymers were chromatographed on three different strong cation-exchange columns: (1) the SynChropak S300 was a silica-based column containing

sulphonate groups as the negatively charged functionalities[12,13]; (2) the PolySulfoethyl Aspartamide column also contained a silica-based packing with sulphonate functionalities, but the chemistry of sulphonate attachment to the silica support was different compared to that of the S300[9,10]; (3) the Mono S column contained sulphonate groups attached to a polyether support.

Fig. 1 shows elution profiles of the mixture of the "A" series of peptide polymers on the S300 column. Similar results were obtained on the other two columns. The peptides were chromatographed using a linear sodium chloride gradient (20 m*M* sodium chloride per min, following 10 min elution with starting buffer, at a flow-rate of 1 ml/min) in 5 m*M* KH_2PO_4 buffer at pH 6.5. The mobile phase buffers also contained 0, 10, 20, 30 or 40% acetonitrile (v/v). In the absence of acetonitrile (results not shown), only peptides 5A (+1 net charge) and 10A (+2 net charge) were eluted, with reasonable peak shape, by a sodium chloride gradient up to a concentration of 0.5 *M*. Peptide 20A (+4 net charge) appeared as a late-eluted very broad, badly skewed peak. The more hydrophobic 30A, 40A and 50A peptides (+6, +8 and +10 net charge, respectively) were not eluted by 0.5 *M* sodium chloride. It had previously been shown by Mant and Hodges[13] that a mixture of peptides of average hydrophobicity and a range of net charge from +2 to +8 at pH 6.5 was easily removed from the S300, in the absence of an organic solvent, by a salt gradient up to a concentration of only 0.4 *M*. In fact, in the present study, the "G" series of polymers of the same polypeptide chain length and net charge as the "A" series was easily eluted from the column in the absence of acetonitrile, including the highly charged (+10) 50-residue peptide (50G). Thus, in the present study, it was apparent that, in addition to ionic interactions between the "A" series of peptides and the column packing, non-specific hydrophobic interactions were also affecting the retention behaviour of the polymer series. With the addition of 10% acetonitrile to the mobile phase buffers, designed to help overcome any hydrophobic, as opposed to ionic, interactions, peptides 5A, 10A and 20A (+1, +2 and +4 net charge, respectively) were all now eluted with good peak shape within a concentration range of 0–0.3 *M* sodium chloride. As the concentration of acetonitrile was increased further to 20, 30 and 40%, the more hydrophobic peptides [30A, 40A and 50A (+6, +8 and +10 net charge, respectively)] were also eluted from the column (Fig. 1). In addition, the retention times of all peptides decreased with increasing levels of acetonitrile. At a level of 40% acetonitrile in the mobile phase buffers, the most hydrophobic peptide, 50A (+10 net charge), was eluted at a salt concentration of only *ca.* 0.2 *M*. Raising the level of acetonitrile above 40% in the mobile phase was found to be impractical due to problems associated with salt insolubility at high concentrations of the non-polar solvent. It should be noted that the peptide with a net charge of only +1 (5A) was eluted from the S300 during the initial 10-min isocratic elution with the starting buffer and not by the subsequent gradient. However, the elution time of this peptide also decreased with increasing levels of acetonitrile.

Fig. 2 shows a graphical representation of the results shown in Fig. 1, demonstrating decreasing retention times of peptides 10A–50A with increasing levels of acetonitrile in the mobile phase buffers. The absence of data points for a particular peptide below a certain percentage of acetonitrile means that this peptide was either not eluted by a salt gradient up to 0.5 *M* sodium chloride, or was eluted very late as an extremely broad, poorly defined peak. Although the plots for each peptide are curv-

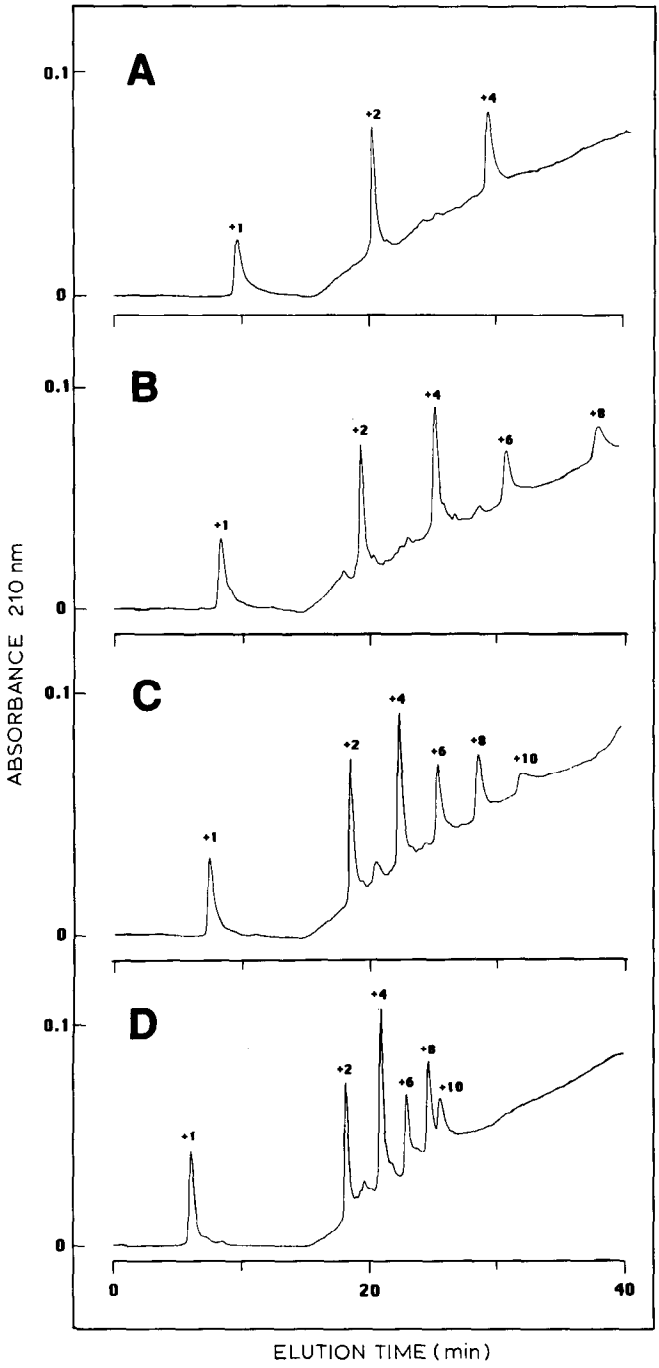

Fig. 1. Strong cation-exchange chromatography of synthetic peptide polymers. Conditions: column, SynChropak S300 (250 × 4.1 mm I.D.); mobile phase, linear A–B gradient (20 m*M* salt per min following 10-min isocratic elution with buffer A), where buffer A is 5 m*M* KH_2PO_4 (pH 6.5) and buffer B is buffer A plus 0.5 *M* NaCl, both buffers containing 10 (A), 20 (B), 30 (C) or 40% (D) acetonitrile (v/v); flow-rate, 1 ml/min; temperature, 26°C. The sequence of the peptides was Ac-(Leu-Gly-Leu-Lys-Ala)$_n$-amide, where n = 1, 2, 4, 6, 8, 10 (+1, +2, +4, +6, +8, +10 net charge, respectively).

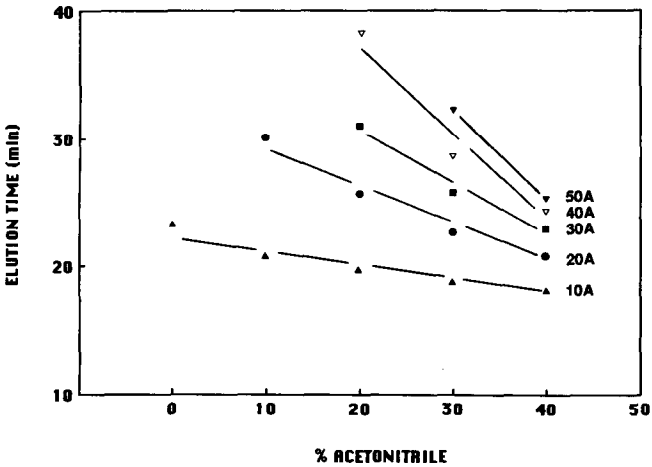

Fig. 2. Plot of peptide elution time *versus* percentage of acetonitrile in mobile phase buffers during strong cation-exchange chromatography of synthetic peptide polymers. Column and conditions as described in Fig. 1. 10A, 20A, 30A, 40A, 50A denote, respectively, 10-, 20-, 30-, 40- and 50-residue peptides of "A" series of peptide polymers (sequences shown in Fig. 1).

ing, they are drawn as best-fit straight lines to highlight the effect of acetonitrile concentration on peptide retention times. From the different slopes of the plots for peptides 10A–50A, it is clear that the effect of increasing acetonitrile concentration on the retention time of a particular peptide is dependent on its hydrophobicity. For instance, in raising the level of acetonitrile from 30 to 40% in the mobile phase, the retention times for 10A, 20A, 30A, 40A and 50A were reduced by 0.7, 1.8, 2.8, 4.2 and 6.8 min, respectively. Thus, the more hydrophobic the peptide (50A > 40A > 30A > 20A > 10A), the greater the comparative effect of increasing acetonitrile concentration in reducing peptide retention time. Similar results were obtained for the less hydrophobic "G" series and more hydrophobic "L" series of peptide polymers.

The relationship between peptide hydrophobicity and mobile phase acetonitrile concentration in strong CEX of peptides is again shown in Fig. 3, which compares the effect of increasing acetonitrile concentration on the retention times of all three series of peptide polymers ("G", "A" and "L"). The plots shown in Fig. 3 were all obtained on the S300 column under the same chromatographic conditions as described in Fig. 1. In Fig. 2, the peptides increased in length (50A > 40A >, etc.) as well as hydrophobicity. In contrast, each panel of Fig. 3 compares the retention times of peptides with varying hydrophobicity but the same chain length, *e.g.*, 50G + 50A + 50L (50-res), 40G + 40A + 40L (40-res), etc. In ideal ion-exchange chromatography, peptides of the same length and net charge should have similar retention times. From Fig. 3, it can be seen that for peptides of the same length and net positive charge, the more hydrophobic the peptide, the greater the retention time due to hydrophobic interactions with the cation-exchange matrix. When these hydrophobic interactions are suppressed (40% acetonitrile in the mobile phase), peptides of the same length and net charge are eluted from the column at similar times. From the varying steepness of the plots, it is again clear that, for each trio of peptides, the more hydrophobic the

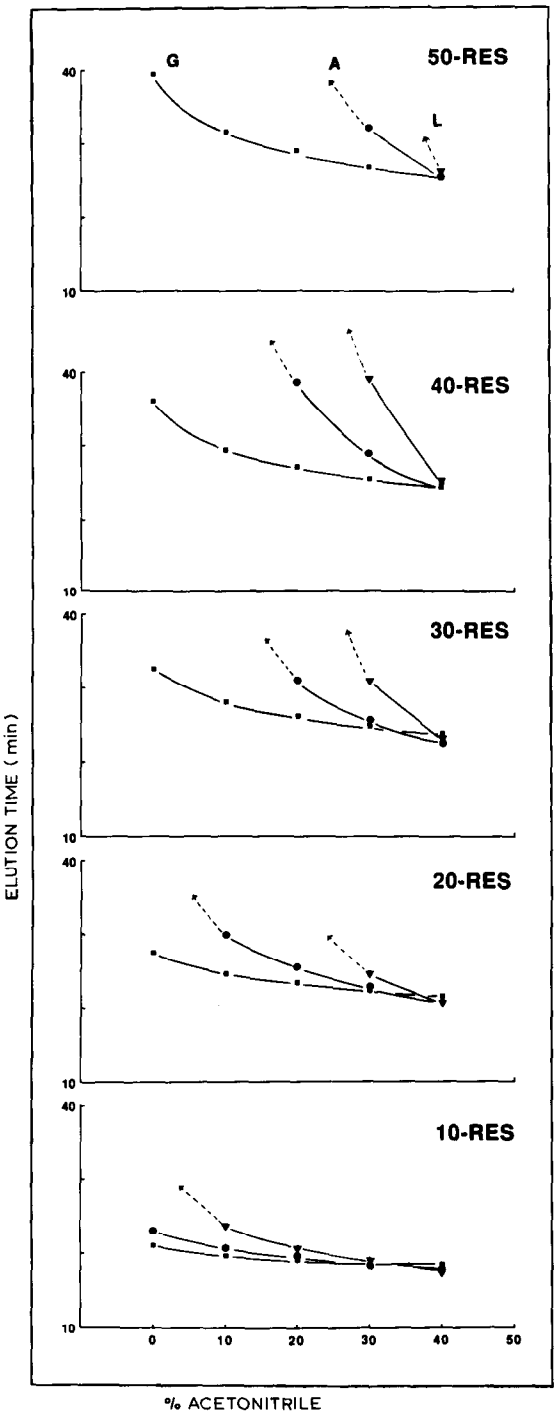

Fig. 3. Plot of peptide elution time *versus* percentage of acetonitrile in mobile phase buffers during strong cation-exchange chromatography of synthetic peptide polymers. Column and conditions as described in Fig. 1. Sequence of the "G", "A" and "L" series of peptide polymers are described in the text. 10-res, 20-res, 30-res, 40-res, 50-res denote 10–50 residues, respectively. A dotted line denotes that a peptide was not eluted, or was eluted as a very broad and/or skewed peak, below a certain level of acetonitrile in the mobile phase.

peptide, the greater the effect of increasing acetonitrile concentration in reducing peptide retention time. For instance, in raising the level of acetonitrile from 30 to 40% in the mobile phase, the retention times for 40G, 40A and 40L were reduced by 1.1, 4.2 and 14.2 min, respectively; the retention times for 30G, 30A and 30L were reduced by 0.9, 2.8 and 8.1 min, respectively, etc.

The differences in hydrophobicity of the three series of peptide polymers and, hence, differences in the intensity of their non-ionic interactions with the ion-exchange packing are also well demonstrated in Fig. 3. All five peptides of the least hydrophobic series (10G–50G) were eluted, with good peak shape, over the entire range of acetonitrile concentrations examined (0–40%). In contrast, only the 10-residue peptide (10A) of the more hydrophobic "A" series of peptide polymers was eluted in the absence of acetonitrile; 20A, 30A, 40A and 50A required at least, respectively, 10, 20, 20 and 30% acetonitrile in the mobile phase to be eluted from the column. In the case of the most hydrophobic "L" series, peptides 10L, 20L, 30L, 40L and 50L required at least, respectively, 10, 30, 30, 30 and 40% acetonitrile in the mobile phase to overcome hydrophobic interactions with the column packing. It should be noted that the five-residue peptides (5G, 5A, 5L) were not included in Figs. 2 and 3, since they were eluted from the S300 column during the initial 10-min isocratic elution with starting buffer and not by the salt gradient.

Effect of polypeptide chain length and charge density on peptide retention times

Fig. 4 shows elution profiles of a mixture of five synthetic peptide size-exclusion standards[1,16]. (Fig. 4A) and a mixture of four synthetic peptide cation-exchange standards (Fig. 4B) on the Mono S strong cation-exchange column. The peptides were eluted with a linear sodium chloride gradient (20 m*M* sodium chloride per min, following 10 min elution with starting buffer) at a flow-rate of 1 ml/min. Since only the effects of polypeptide chain length and/or charge density were being examined, it was important to minimize any non-specific, hydrophobic interactions of peptides with the ion-exchange packing. Thus, both the starting buffer (5 m*M* KH_2PO_4, pH 6.5) and the gradient buffer (5 m*M* KH_2PO_4 + 0.5 *M* NaCl, pH 6.5) contained 40% acetonitrile (v/v). For the purposes of the present study, the five size-exclusion standards (10, 20, 30, 40 and 50 residues; +1, +2, +3, +4 and +5 net charge, respectively) were denoted as the "X" series of peptide polymers; *i.e.*, 10X, 20X, etc.; the four cation-exchange standards were denoted C1–C4 (11 residues in length with +1, +2, +3 and +4 net charge, respectively). The sequences of the two sets of standards are described under Experimental.

From Fig. 4, it can be seen that similarly charged species were not necessarily eluted at similar times. For instance, peptide 50X (+5 net charge) (Fig. 4A) was not retained as long as C3 (+3 net charge) or C4 (+4 net charge) (Fig. 4B). Similarly, peptide 40X (+4 net charge) (Fig. 4A) was eluted prior to C3 (+3 net charge) (Fig. 4B). The two series of peptide standards differed significantly in their range of both peptide chain length (10–50 residues for peptides 10X–50X, respectively; eleven residues each for peptides C1–C4 and charge density (+1 net charge per 10 residues for 10X–50X; +1 to +4 net charge per 11 residues for C1–C4). In order to rationalize the elution profiles shown in Fig. 4, it was necessary to determine the relative contribution that polypeptide chain length and charge density individually make to peptide retention behaviour during strong CEX.

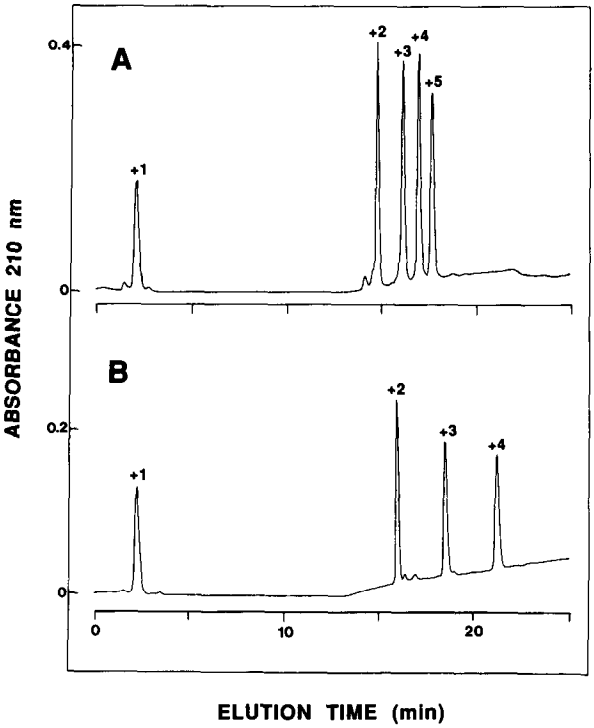

Fig. 4. Strong cation-exchange chromatography of synthetic peptides. Conditions: column, Mono S (50 × 5 mm I.D.); mobile phase, linear A–B gradient (20 m*M* salt per min following 10 min isocratic elution with buffer A), where buffer A is 5 m*M* KH_2PO_4 (pH 6.5) and buffer B is buffer A plus 0.5 *M* NaCl, both buffers containing 40% acetonitrile (v/v); flow-rate, 1 ml/min; temperature, 26°C. (A) Mixture of five synthetic peptide size-exclusion standards (10–50 residues; +1 to +5 net charge). (B) Mixture of four synthetic undecapeptide cation-exchange standards (+1 to +4 net charge). Sequences of the peptides are described under Experimental.

Fig. 5, top panel, demonstrates the relationship between elution time on the S300 column and net charge for the "A" and "X" series of peptide polymers and the cation-exchange peptide standards ("C"). The chromatographic conditions were the same as those described for Fig. 4. The peptides containing a single net positive charge (5A, 10X, C1) were eluted during the initial 10-min isocratic elution with starting buffer and are not included in the plots. The plot for the remaining three cation-exchange standards, C2–C4 (+2 to +4 net charge) demonstrated a linear relationship between peptide elution time and net charge. However, the plots for the two peptide polymer series, "X" and "A", showed a non-linear relationship, with the peptides being eluted earlier than expected with increasing net charge and chain length. Plotting the elution times of the "X" and "A" series of peptides against the logarithm of the number of residues they contained (ln *N*) resulted in the straight-line relationships shown in Fig. 5, middle panel. This exponential relationship between peptide retention time and peptide chain length reflected a similar relationship reported for reversed-phase chromatography of peptides[1,17]. A plot of elution time *versus* ln *N* for C2–C4 (all 11 residues in length) naturally produced a straight, vertical line.

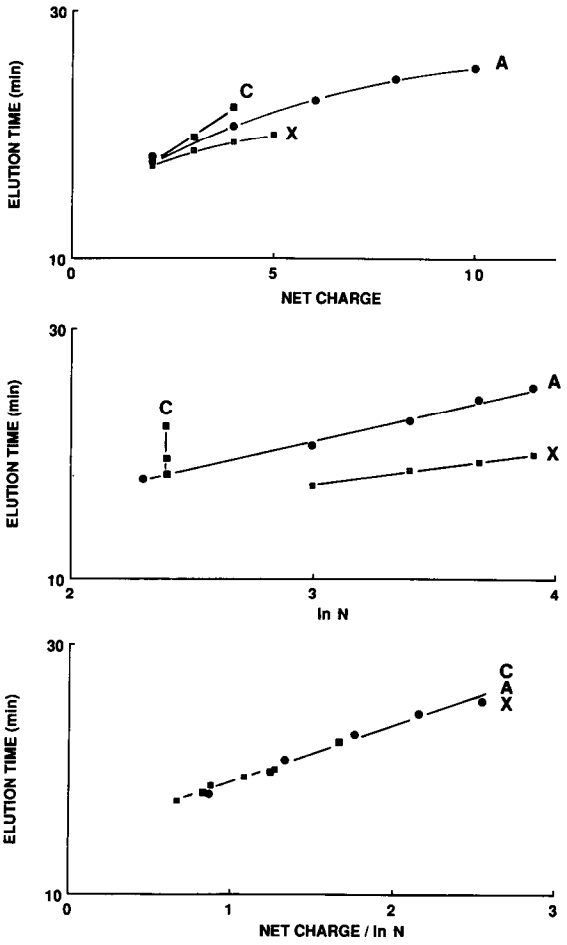

Fig. 5. Relationship of peptide elution time with polypeptide chain length and charge density during strong cation-exchange chromatography of synthetic peptides. Conditions: column, SynChropak S300 (250 × 4.1 mm I.D.; mobile phase, as described in Fig. 4. Top panel: peptide elution time *versus* peptide net charge. Middle panel: peptide elution time *versus* logarithm of the number of residues (ln *N*). Bottom panel: peptide elution time *versus* peptide net charge divided by the logarithm of the number of residues (net charge/ln *N*). The letters C, A and X denote the cation-exchange standards (sequences shown under Experimental), the "A" series of peptide polymers (sequence shown in Fig. 1), and the "X" series of size-exclusion standards (sequence shown under Experimental), respectively.

The divergence of the plots in Fig. 5, top panel, and the difference in slopes in Fig. 5, middle panel, appeared to reflect a difference in the charge densities of the peptides (+1 net charge per 10 residues for the "X" series; +2 net charge per 10 residues for the "A" series; +1 to +4 net charge per 11 residues for C2–C4).

From Fig. 5, bottom panel, it can be seen that dividing the net charge of the peptides from the two polymers series ("X" and "A") and the mixture of cation-exchange standards (C2–C4) by the logarithm of the number of residues they contain (net charge/ln *N*), and plotting this value against the observed elution time resulted in

a single, straight-line plot with a correlation of 0.99 (determined by linear least-squares fitting)[18]. The elution time *versus* net charge/ln N relationship held true for all three columns tested. This simple linearization approach is important for the prediction of retention behaviour of peptides where the net charge is known. The validity of this approach is supported by the diversity of the peptides used in this study. These peptides varied substantially in charge density (+0.1 to +0.4 per residue), net charge (+2 to +10), polypeptide chain length (10–50 residues) and overall hydrophobicity.

Fig. 6 underlines the importance of minimizing non-specific, hydrophobic interactions between peptides and the ion-exchange packing for the elution time *versus* net charge/ln N relationship to remain valid. The "G", "A" and "L" series of peptide polymers were eluted from the S300 column with a linear sodium chloride gradient (20 mM sodium chloride per min, following 10 min elution with starting buffer) at a

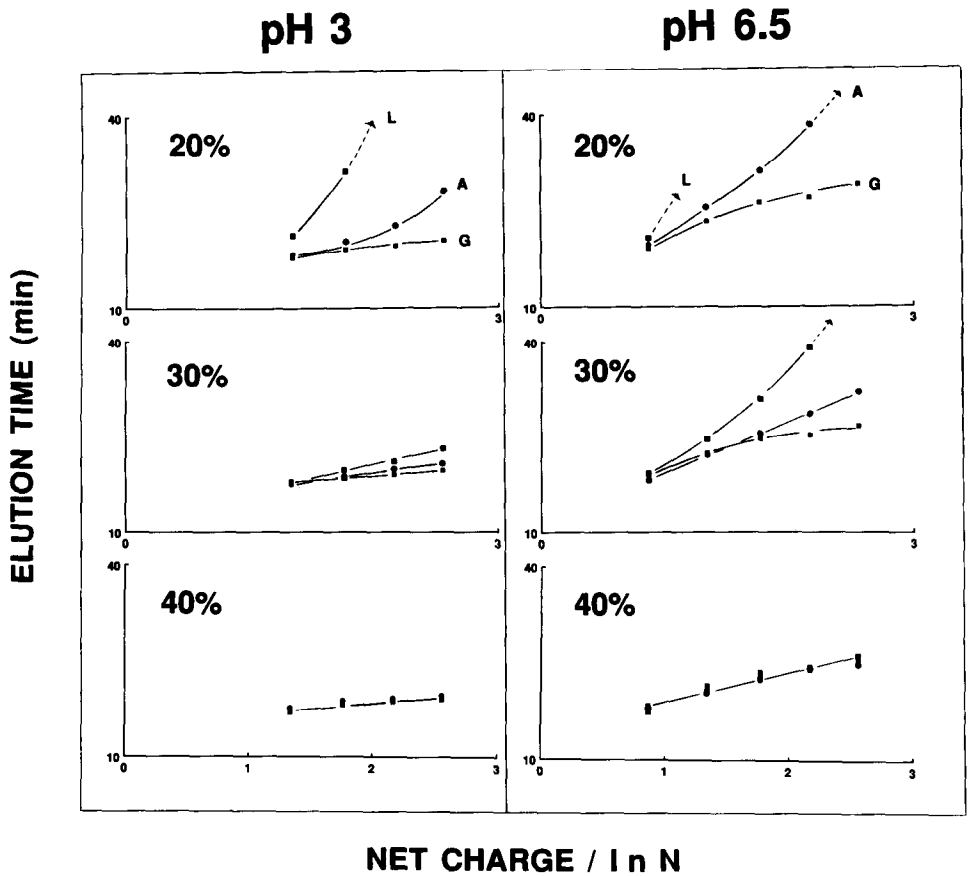

Fig. 6. Plot of peptide elution time *versus* peptide net charge/ln N following strong cation-exchange chromatography of synthetic peptide polymers. Conditions: column, SynChropak S300 (250 × 4.1 mm I.D.); mobile phase, linear A–B gradient (20 mM salt per min following 10 min isocratic elution with buffer A), where buffer A is 5 mM KH_2PO_4 (pH 3.0 or 6.5) and buffer B is buffer A plus 0.5 M NaCl, both buffers containing 20, 30 or 40% acetonitrile (v/v); flow-rate, 1 ml/min; temperature, 26°C. Sequences of the "G", "A" and "L" series of peptide polymers are described in the text.

flow-rate of 1 ml/min. The starting buffer (5 m*M* KH_2PO_4, pH 3.0 or 6.5) and the gradient buffer (5 m*M* KH_2PO_4 + 0.5 *M* NaCl, pH 3.0 or 6.5) contained 20, 30 or 40% acetonitrile (v/v). At either pH, the effect of incompletely suppressed hydrophobic interactions between the peptides and the column packing served to disrupt the linearity of the elution time *versus* net charge/ln *N* relationship. This disruption decreased as hydrophobic interactions were progressively suppressed with increasing levels of acetonitrile in the mobile phase until, at a concentration of 40% acetonitrile, the expected linearity was obtained with correlations of 0.95 and 0.98 at pH 3.0 and pH 6.5, respectively. Similar results were again obtained on both the other columns. As previously reported[13], the S300 column exhibits a pH effect in that peptides with a net positive charge of +2 are retained at pH 6.5, but not at pH 3.0. Hence, while five peptides from each of the polymer series were retained at pH 6.5 (+2, +4, +6, +8 and +10 net charge), only four peptides were retained at pH 3.0 (+4, +6, +8 and +10). In addition, all the retained peptides were eluted by a lower sodium chloride concentration at pH 3.0 than at pH 6.5.

A simple relationship between peptide elution time and net positive charge during strong CEX was reported by Mant and Hodges[13] and later by Crimmins *et al.*[9], *i.e.*, a satisfactory linear relationship was obtained without any correction for peptide chain length. The results shown in Fig. 5 (top panel) suggested that the polypeptide chain length effect on peptide retention times becomes significant only beyond a length of *ca.* 20 residues. The peptides utilized by the previous workers ranged in chain length from only 12–21 residues[13] or 7–13 residues[9]. Thus, for these particular mixtures of peptides, any effect on peptide retention behaviour due to chain length differences was probably fairly small. However, as shown in the present study, it is important to take polypeptide chain length into account for prediction of peptide retention behaviour of peptides >20 residues in length. Interestingly, conformational effects cannot explain the observed divergencies with polypeptide chain length. The peptide polymers used in this study do not have any unique tertiary structure, since the mobile phase conditions used to ensure ideal ion-exchange behaviour (buffers containing 40% acetonitrile) are denaturing to tertiary structures, favouring the exposure of all charged residues. In addition, their secondary structure ranges from random coil to substantial α-helical content (as measured by circular dichroism), yet they still exhibit a similar polypeptide chain length effect.

The importance of overcoming hydrophobic interactions with cation-exchange columns in order to ensure their elution from the column matrix and/or to ensure a linear relationship between peptide elution time and net charge/ln *N* was clearly shown in Fig. 6. The peptides used in this study covered an extreme range of peptide hydrophobicities to values far exceeding those of most peptides encountered and it is not very likely, or desirable, that organic solvent concentrations of as high as 40% (v/v) in the mobile phase buffers will be regularly necessary for strong CEX of average peptide mixtures. However, the results of this study do suggest that, if predictable peptide elution profiles are required, the addition on a regular basis of a low level of acetonitrile [*e.g.*, 10% (v/v)] to the mobile phase buffers would be worthwhile during strong CEX of peptides to suppress any hydrophobic interactions with the ion-exchange packing and, hence, ensure the linearity of the retention *versus* net charge/ln *N* relationship.

Our observations in this study also suggested that the hydrophobic character of

ion-exchange packings has a greater impact on the separation of peptides than those previously reported for proteins[2–4,7,8]. This is probably due to the fact that, under non-denaturating conditions, proteins are folded molecules (tertiary structure) with most of the hydrophobic residues situated in the interior of the molecule and, hence, not available to interact with the column matrix[19]. In contrast, peptides containing less than 50 residues usually exhibit little tertiary structure and all or most of the molecule is available to interact with ion-exchange packings.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council of Canada and equipment grants from the Alberta Heritage Foundation for Medical Research.

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