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Temperature selectivity effects in reversed-phase liquid chromatography due to conformation differences between helical and non-helical peptides

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Abstract

In order to characterize the effect of temperature on the retention behaviour and selectivity of separation of polypeptides and proteins in reversed-phase high-performance liquid chromatography (RP-HPLC), the chromatographic properties of four series of peptides, with different peptide conformations, have been studied as a function of temperature (5–80 °C). The secondary structure of model peptides was based on either the amphipathic α-helical peptide sequence Ac-EAEKAAKE*X*-$_{D/L}$EKAAKEAEK-amide, (position *X* being in the centre of the hydrophobic face of the α-helix), or the random coil peptide sequence Ac-$X_{D/L}$LGAKGAGVG-amide, where position *X* is substituted by the 19 L- or D-amino acids and glycine. We have shown that the helical peptide analogues exhibited a greater effect of varying temperature on elution behaviour compared to the random coil peptide analogues, due to the unfolding of α-helical structure with the increase of temperature during RP-HPLC. In addition, temperature generally produced different effects on the separations of peptides with different L- or D-amino acid substitutions within the groups of helical or non-helical peptides. The results demonstrate that variations in temperature can be used to effect significant changes in selectivity among the peptide analogues despite their very high degree of sequence homology. Our results also suggest that a temperature-based approach to RP-HPLC can be used to distinguish varying amino acid substitutions at the same site of the peptide sequence. We believe that the peptide mixtures presented here provide a good model for studying temperature effects on selectivity due to conformational differences of peptides, both for the rational development of peptide separation optimization protocols and a probe to distinguish between peptide conformations.

Keywords: Temperature effects; Selectivity; Peptides

1. Introduction

Over the past two decades, reversed-phase high-performance liquid chromatography (RP-HPLC) has emerged as the main method in the development of separation protocols for peptide and protein mixtures [1–3]. The resolving power of this technique is reflected by its frequent use in multidimensional separations of polypeptides, including proteomics applications [4–10]. Indeed, the combined desalting/

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purification aspect of RP-HPLC makes it ideal as the final step of a multidimensional separation protocol, notably prior to mass spectrometry of purified solutes.

Clearly, considering the complexity of proteomics applications of liquid chromatography, where the separation of hundreds or even thousands of peptides may be required, e.g. from simultaneous digest of a multi-protein mixture, optimization of the separation protocol is of prime importance. Concerning RP-HPLC, such optimization has traditionally been achieved by mobile phase variations (e.g. changes in organic modifier, ion-pairing reagent or pH) [1–3,11], variations in the organic modifier gradient rate [1,12,13], or even changes in column packing to take advantage of selectivity differences offered by different stationary phase ligands [14,15]. In addition, the introduction in recent years of stationary phases stable to high temperatures has added to the arsenal of RP-HPLC approaches for optimization of the resolution of polypeptide mixtures [15–29].

Many and varied influences will have an impact on the way a particular peptide interacts with a reversed-phase column, not least of which include characteristics of the peptide itself, e.g. amino acid composition [30,31], residue sequence [31,32], peptide length [33,34], and the presence of any secondary structure (α-helix or β-sheet) [31,35–40]; indeed, RP-HPLC of peptides and proteins at varying temperature has also allowed an insight into the role of conformation in the retention behaviour of peptides and proteins [27–29,34,41–45]. The importance of delineating the contribution of α-helical structure (both amphipathic and non-amphipathic) to the selectivity of peptide separations cannot be underestimated, particularly when one considers that peptide fragments from chemical or proteolytic digests of proteins typically contain peptides with α-helical potential. During RP-HPLC, such peptides will be induced into α-helical structure by the non-polar environment characteristic of this technique (hydrophobic matrix and non-polar eluting solvent) [31,38,45,46].

A previous study in our laboratory [13] illustrated the selectivity that may be obtained in a reversed-phase separation based on peptide conformational differences (α-helical versus random coil), highlighted by their retention time behaviour at varying gradients of organic modifier. Thus, the present study examines the effect of temperature on RP-HPLC retention behaviour at pH 2.0 of four series of peptides, based on either the amphipathic peptide sequence Ac-EAEKAAKE*X*EKAAKEAEK-amide (with position *X* in the centre of the hydrophobic face of the α-helix) or the random coil peptide sequence Ac-*X*LGAKGAGVG-amide, where position *X* is substituted by the 19 L- or D-amino acids. We believed that observation of the temperature effect on retention behaviour of such peptide models would have implications, not only for the rational development of separation optimization protocol, but also for the understanding of the hydrophobic interactions between RP-HPLC stationary phases and peptides with conformational differences.

2. Experimental

2.1. Materials

tert.-Butyloxycarbonyl (t-Boc)-protected amino acids were purchased from Advanced ChemTech (Louisville, KY, USA). *o*-Benzotriazol-1-*yl*-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) and 4-methylbenzhydrylamine resin hydrochloride salt (MBHA) (100–200 mesh) were obtained from Advanced ChemTech. Anisole and 1,2-ethanedithiol (EDT) were supplied by Aldrich (Oakville, Canada). Dimethylformamide (DMF) was obtained from FisherBiotech (Fair Lawn, NJ, USA). Trifluoroacetic acid (TFA) was obtained from Halocarbon Products (River Edge, NJ, USA) and diisopropylethylamine (DIEA) was obtained from Caledon (Georgetown, Canada). HPLC-grade acetonitrile was purchased from EM Science (Gibbstown, NJ, USA).

2.2. Peptide synthesis

Synthesis of helical peptides Ac-EAEKAAKE*X*-$_{D/L}$EKAAKEAEK-amide and random coil peptides Ac-$X_{D/L}$LGAKGAGVG-amide were carried out by standard solid-phase synthesis methodology using *t*-Boc chemistry and MBHA resin (0.97 mmol/g) on an Applied Biosystems peptide synthesizer Model 430A (Foster City, CA, USA). The Boc groups were

removed at each cycle with TFA in dichloromethane. Coupling of amino acids were carried out with 0.45 mmol HBTU–0.8 mmol DIEA–DMF at each cycle to activate for 5 min, then added to resin by shaking for 30 min. Finally at the completion of the synthesis, the peptides were acetylated with acetic anhydride–DIEA–dichloromethane (10:20:70, v/v). The peptides were cleaved from the resin by treatment with HF (30 ml/g resin) containing 10% anisole and 2% 1,2-ethanedithiol at −5 to 0 °C for 1 h. The cleaved peptide–resin mixtures were washed with diethyl ether (3×25 ml) and the peptides extracted with neat acetonitrile (3×25 ml). The resulting peptide solutions were then lyophilized prior to purification.

2.3. Instrumentation

The crude peptides were purified by preparative RP-HPLC on a Varian Vista Series 5000 liquid chromatograph (Varian, Walnut Creek, CA, USA).

The analytical HPLC system consisted of an HP 1100 liquid chromatograph (Hewlett-Packard, Avondale, PA, USA), coupled with HP 1100 series diode array detector and thermostatted column compartment, HP Vectra XA computer and HP LaserJet 5 printer.

The correct primary ion molecular masses of peptides were confirmed by VG Quattro electrospray mass spectrometry (Fisons, Pointe-Claire, Canada).

Amino acid analyses of the purified peptides were carried out on a Beckman Model 6300 amino acid analyzer (Beckman, San Ramon, CA, USA).

2.4. Columns and HPLC conditions

Crude peptides were purified on a semi-preparative Zorbax 300 SB-C_8 column (250×9.4 mm I.D.; 6.5-μm particle size, 300-Å pore size; Agilent Technologies, Brockville, Canada), with a linear A–B gradient (0.2% acetonitrile/min) at a flow-rate of 2 ml/min, where eluent A was 0.1% aqueous trifluoroacetic acid (TFA) in water and B was 0.1% TFA in acetonitrile.

Analytical RP-HPLC was carried out on a Zorbax 300 SB-C_8 narrowbore column (150×2.1 mm I.D.; 5-μm particle size, 300-Å pore size) from Agilent Technologies with a linear A–B gradient (0.5% acetonitrile/min) at a flow-rate of 0.25 ml/min, where eluent A was 0.05% aqueous TFA, pH 2.0, and eluent B was 0.05% TFA in acetonitrile. This C_8 column (with SB denoting StableBond) was chosen for this study due to its excellent temperature stability at low pH [15–29].

2.5. Characterization of peptide secondary structure

The mean residue molar ellipticities of the peptide analogues were determined by circular dichroism (CD) spectroscopy, using a Jasco J-720 spectropolarimeter (Jasco, Easton, MD, USA), at 25 °C in 50 m*M* aqueous phosphate–100 m*M* KC1 buffer, pH 7.0 in the presence of an α-helix inducing solvent, 50% 2,2,2-trifluoroethanol (TFE). A 10-fold dilution of a ~500 μ*M* stock solution of the peptide analogs was loaded into a 0.02-cm fused-silica cell and its ellipticity scanned from 190 to 250 nm. The values of molar ellipticity of the peptide analogues at wavelength 222 nm were used to determine the relative α-helical content of each peptide.

3. Results

3.1. Peptide design and designation

The amphipathic α-helix is a very commonly encountered structural motif in peptides and proteins and approximately 50% of all helices in soluble globular proteins are amphipathic [47,48]. In order to study the effect of temperature on selectivity of peptide separations, we believed that the best initial approach was to compare the retention behaviour of peptides with extremes of structure, i.e. either with as close to 100% α-helical conformation as possible or with the complete absence of α-helix. In addition, in a previous study, we showed that α-helical peptides with D-amino acid substitutions exhibited considerably different retention behavior during RP-HPLC compared with L-diastereomeric analogues [49], due to the helix-disrupting characteristics of D-amino acids when substituted into an α-helix made up solely of L-amino acid residues [49–53]. Hence, this study set out to explore whether temperature has a different effect on the separation of D- versus

L-peptide diastereomers. Four series of peptides designed to exhibit markedly different conformational characteristics during RP-HPLC were synthesized, the sequences of which are shown in Fig. 1.

Two series of random coil decapeptide analogues, designed to exhibit negligible α-helical structure, were substituted with a single L- or D-amino acid at position l (random-L and random-D, Fig. 1). The sequence of Ac-$X_{D/L}$LGAKGAGVG-amide, containing four Gly residues, was chosen since it lacks any ability to form any specific secondary structure [13,54]. A 10-residue length for the peptide analogues was chosen to avoid significant effect of chain length on the retention behaviour [33] and to mimic the average sized fragment of a proteolytic digest of a protein. The presence of a lysine residue at position 5 of the peptide analogues ensures sufficient peptide solubility.

The two series of α-helical peptide analogues were synthesized (helical-L and helical-D, Fig. 1), based on the well-characterized sequence of Ac-EAEKAAKEAEKAAKEAEK-amide (also denoted as AA9) [55–63]. L- and D-amino acids were used to substitute the Ala residue at position 9 in the centre of the non-polar face of this amphipathic α-helical sequence (helical wheel, Fig. 1). The use of such amphipathic α-helices was also designed to reflect the common occurrence of such helices in nature, with, as noted above, approximately 50% of all helices in globular proteins being amphipathic [47,48]. Since glycine does not exhibit optical activity, the Gly-substituted analogues in both helical and random coil categories represent useful reference standards during RP-HPLC.

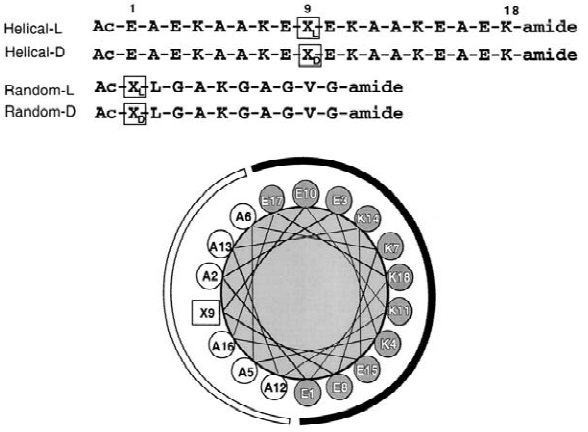

Fig. 1. Model synthetic peptides with conformational differences. Top: sequences of the model amphipathic helical and random coil peptides with L- or D-amino acid substitutions at position *X* (boxed X_L or X_D) *X* represents the substitution site at position 9 in the helical peptides and position 1 in random coil peptides, respectively,. Bottom: helical wheel representation of the model amphipathic α-helical peptide with the substitution site at position 9 (boxed *X*) in the hydrophobic face. The closed arc denotes the hydrophilic face; the open arc denotes the hydrophobic face, Ac denotes N^{α}-acetyl and amide denotes C^{α}-amide. Standard one-letter designations are used for the amino acid residues.

In the present study. since we have a large number (78) peptide analogues (19 L- and D-amino acids substituted at position l of the random coil peptide or position 9 of the α-helical peptide, respectively (plus two glycine-substituted peptides), in order to avoid the complexity of designation of these analogues, the peptides are divided into two main categories as ''random'' and ''helical'' to represent random coil and amphipathic α-helical peptides, respectively; within each category, peptide analogues are named after the substituting amino acid residues at position 1 of random peptides or position 9 of helical peptides. For instance, within helical, L_D or L_L represents the α-helical peptide with amino acid D-leucine or L-leucine substitution at position 9 in the centre of the non-polar face, respectively. However, when comparing temperature effects on L- or D-diastereomeric peptide analogues, we put all the peptides into two groups as ''L-peptides'' and ''D-peptides''; within each category, peptides are named after both the substituting amino acid residue and the peptide structure, e.g. L_R and L_H in D-peptides represent the random coil peptide and the α-helical peptide with a D-leucine substitution at the corresponding position, respectively.

3.2. Conformation of model peptides

The secondary structures of model random and helical peptide categories as represented by L_L and L_D peptides in the presence of the α-helix-inducing solvent 50% TFE at pH 7.0 are shown in Fig. 2. The high helicity of the amphipathic peptide series in the presence of TFE has been previously well documented [49,59,61]. According to our previous study [49] all of the amphipathic peptide analogues with L-/D-amino acid substitutions showed similar molar

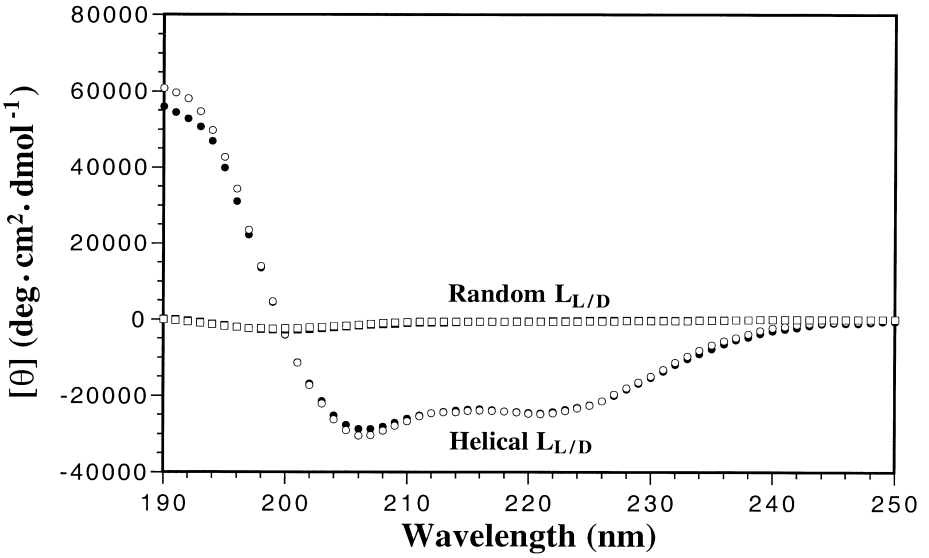

Fig. 2. Circular dichroism (CD) spectra of helical and random coil L_L and L_D peptides. The solution is buffered by 50 m*M* aqueous PO_4 containing 100 m*M* KC1 in the presence of 50% TFE at pH 7.0 and 25 °C. Solid symbols represent the CD spectra of L_D peptides, whereas open symbols represent the CD spectra of L_L peptides. The symbols used are circles for helical peptides and squares for random coil peptides.

ellipticity values at 222 nm in the presence of 50% TFE with over 90% helical content, with the exception of the L-/D-proline substituted peptides. Since TFE is recognized as a useful mimic of the hydrophobic environment characteristic of RP-HPLC [31], as well as being a strong α-helix inducer for potentially helical molecules [64–68], elution of these peptide analogues as α-helices during RP-HPLC is ensured. In addition, the $[\theta]_{220}/[\theta]_{207}$ ratio values of helical L_L and L_D are less than 1, suggesting that, in the presence of 50% TFE, these peptides are single-stranded α-helices [57,58,66]. Taken together, these observations suggest that the helical peptides in this study are bound and eluted in the single-stranded amphipathic α-helical conformation during RP-HPLC. In contrast, the peptides designed as model random coil peptides showed, as expected, no secondary structure, even in the presence of 50% TFE (Fig. 2).

3.3. Temperature effect on RP-HPLC selectivity of amphipathic α-helical peptides

The effect of temperature on the selectivity of amphipathic α-helical model peptides during RP-HPLC is shown in Fig. 3. As noted above, it is known that characteristic RP-HPLC conditions (hydrophobic stationary phase, non-polar eluting solvent) induce helical structure in potentially helical polypeptides [31,38,45,46] in a manner similar to that of the helix-inducing solvent TFE. Polypeptides, such as our model peptides (Fig. 1), which are thus induced into amphipathic α-helices on interaction with an hydrophobic RP-HPLC stationary phase will exhibit preferred binding of their non-polar face with the stationary phase, resulting in considerably more retentive behaviour than non-amphipathic peptides of the same amino acid composition [31]. In Fig. 3, RP-HPLC chromatograms at low temperature (10 °C), intermediate temperatures (25 and 45 °C) and high temperature (80 °C) were chosen as examples to show the effect of temperature on the separation of helical peptides with different L- or D-amino acid substitutions. It is clear that there is a wide range of retention times as would be expected given the differences in side-chain hydrophobicity of the substituted L- or D-amino acids, ranging as they do from the highly non-polar (e.g. Ile, Leu, Phe, Trp) to the polar (e.g. Ser, Thr, Asn, Gln) [49,60,61]. As has previously been observed [49], helical P_L and P_D

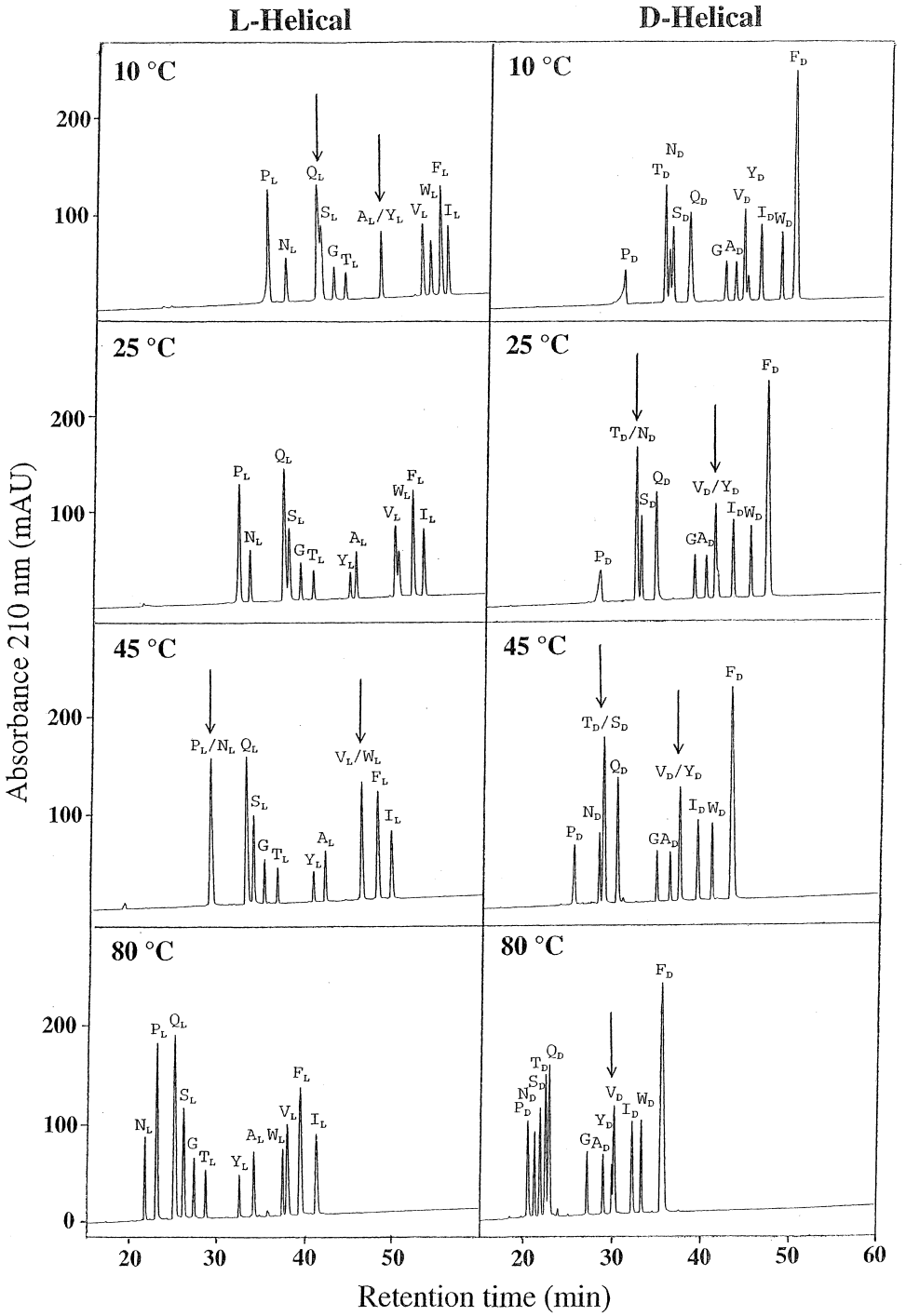

Fig. 3. Effect of temperature on RP-HPLC selectivity for model α-helical peptides with L- or D-amino acid substitutions. Column: 300SB-C_8 column (150×2.1 mm I.D.; 5-μm particle size, 300-Å pore size). Conditions: linear A–B gradient (0.5% acetonitrile/min) at a flow-rate of 0.25 ml/min, where eluent A is 0.05% aqueous TFA, pH 2.0, and eluent B is 0.05% TFA in acetonitrile. Arrows in chromatograms point out the co-eluted or poorly resolved peaks of different peptides. Helical peptides are denoted by the substituting L- or D-amino acid at position 9 in the hydrophobic face as described in Section 3.1 and Fig. 1.

were eluted early, due both to the strong helix-disrupting nature of proline (which also disrupts the amphipathicity of P_L and P_D) and its comparatively low hydrophobicity compared to other non-polar peptide analogues.

From Fig. 3 (left panels), the most obvious phenomenon is that all L- and D-helical peptide analogues became less retentive at higher temperature than those at lower temperature, as expected due to the general effects of increasing temperature resulting in increased solubility of the solute in the mobile phase as the temperature rises [69–72] as well as causing a decrease in solvent viscosity and an increase in mass transfer between the mobile and stationary phases [73]. For L-helical peptides (left column), the profile at 10 °C shows that A_L and Y_L were co-eluted, with Q_L and S_L poorly resolved, as indicated by the arrows. However, with the increase of temperature, these peptide analogues were well resolved 45 °C and resolution improved even more at higher temperatures (80 °C). In contrast, some peptide analogues (e.g. P_L and N_L, V_L and W_L) were well resolved at 10 °C, but were then co-eluted at 45 °C; however, these peptides were again resolved at 80 °C, albeit with a reversal of their elution order compared to 10 °C.

Interestingly, similar phenomena are apparent in the profiles of the D-helical peptides (Fig. 3, right panels). At 10 °C, helical T_D was eluted faster than the adjacent peptide analogues N_D and S_D, but T_D and N_D were co-eluted at 25 °C. Furthermore, at 45 °C, N_D was eluted before T_D In contrast, T_D was co-eluted with S_D, the latest eluted peptide among the three analogues at low temperature. Finally, an elution order change was observed at 80 °C, with N_D being eluted first and T_D eluted last, with all three peptides well resolved. A similar change in elution order can also be seen for helical V_D and Y_D analogues from 10 to 80 °C, albeit to a lesser degree.

From the RP-HPLC elution profiles in Fig. 3, three effects of temperature on the retention behavior of helical peptide analogues are apparent: (i) the retention times of helical peptide analogues decreased with increasing temperature; (ii) temperature affected the retention behaviour of different helical peptide analogues to differing extents; (iii) the overall trend of temperature effect on the helical peptides with either L- or D-amino acid substitutions is without significant difference. Of particular note is the alteration of peak height of peptide analogues during the change of temperature in Fig. 3, where an increase in temperature resulted in increased peak height and decreased peak width during RP-HPLC, which may be important to increase elution resolution.

3.4. Temperature effect on RP-HPLC selectivity for random coil peptides

Random coil peptides used in the present study are molecules without specific secondary structure in a non-polar environment (Fig. 2). Therefore, the separation of random peptides with varying L-/D-amino acid substitutions are merely dependent on side-chain hydrophobicity of the substituting amino acid residue, as the peptides with the less hydrophobic substituting amino acid residues (e.g. Gly, Glu, Pro) are eluted faster and the peptides with the more hydrophobic residues (e.g. Trp, Phe, Leu) are eluted slower (Fig. 4; L-peptides). From Fig. 4, temperature has similar effects on the L-/D-amino acid substituted random coil peptide analogues as was observed for the helical peptides and described above (Fig. 3), i.e. (i) overall, increasing temperature decreased the retention times of the random peptides; (ii) temperature affected the retention behaviour of the random peptides to different degrees; and (iii) the influence of temperature on the L- and D-random peptides during RP-HPLC is similar. For instance, L-Val and L-Tyr-substituted random coil peptides (denoted as V_R and Y_R in Fig. 4, left) were separated, co-eluted and separated once more over the temperature range of 25–75 °C, albeit with reversal of elution order at 75 °C compared to 25 °C.

3.5. Comparison of temperature effect on L- or D-helical and random model peptides

Fig. 4 shows RP-HPLC elution profiles of temperature effect on selectivity of both helical and random peptides with varying L-/D-amino acid substitutions. The temperatures chosen were designed to indicate the alteration of peptide elution profiles with different coeluted peaks (highlighted in bold letters) at different temperatures. Although all peptide analogues helical and random) again exhibit the trend of reducing retention time with increasing temperature,

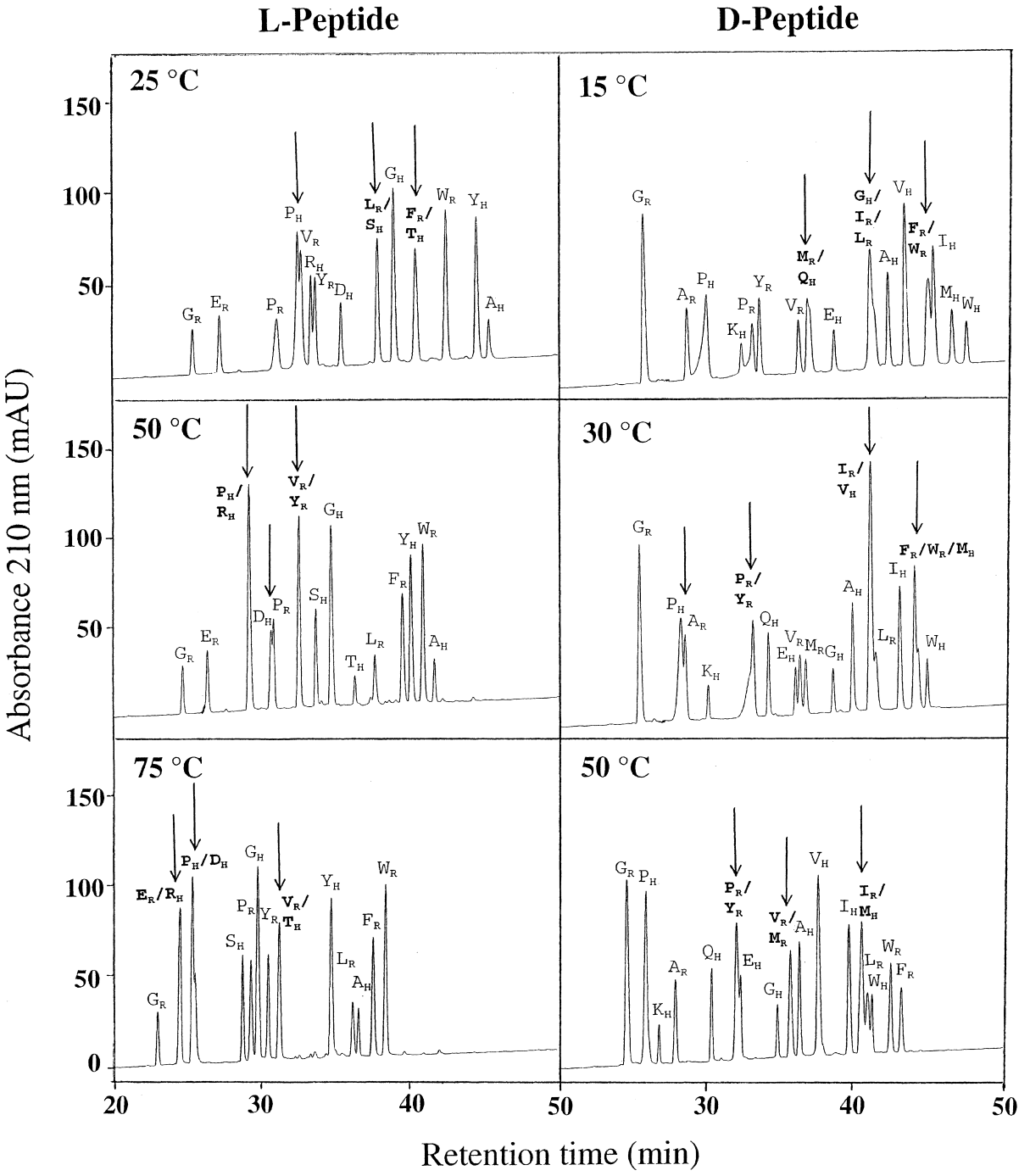

Fig. 4. Effect of temperature on RP-HPLC selectivity for random coil and α-helical peptides. Column and conditions same as for Fig. 3. Arrows in chromatograms point out the co-eluted or poorly resolved peaks of different peptides. Peptide designation is based on the substituting amino acid as described in Section 3.1. Subscripts of letter R and H denote random coil or helical peptide, respectively.

it is also apparent that temperature affects retention behaviour of different peptides to different extents. Thus, co-eluted peaks are composed of different helical and random peptide analogues at different temperatures. Fig. 5 plots the relationship of RP-HPLC retention time versus temperature of helical and random peptide analogues. Temperature has a much greater effect on retention time of the α-helical peptide analogues (both L-amino acid substituted helical peptide analogues in panel A and D-amino acid substituted peptide analogues in panel B) than the random coil peptides. Although not shown here, the slope values of the best fitting lines of peptide retention data within the temperature range 10–75 °C vary from −0.14 to −0.19 for L-helical peptides and −0.02 to −0.07 for L-random peptides (Fig. 5A). Similarly, the slopes vary from −0.11 to −0.18 and −0.02 and −0.08 for D-helical and D-random peptide analogues, respectively (Fig. 5B). The significant difference in magnitude of the slopes of the plots for the helical and random peptides highlights again that temperature has a greater effect on the L-/D-helical peptide analogues compared to the L-/D-random peptide analogues, which can be attributed to differences in peptide structural changes. In addition, the slope variations within helical or random peptides show the subtle differences that temperature can have on peptide analogues in the same structural category with different amino acid substitutions. It is interesting to see that the overall effect of temperature on L- or D-amino acid substituted peptides with the same secondary structure is extremely similar. It is important to note that the linearity of the best fitting lines in Fig. 5 have correlation coefficients greater than 0.97 for most peptides, reflecting the generality of the conclusions.

3.6. Optimum separation of peptide mixtures of α-helical and random coil peptides

From Fig. 5, marked as short black vertical bars, the co-elution points of any two peptides illustrate

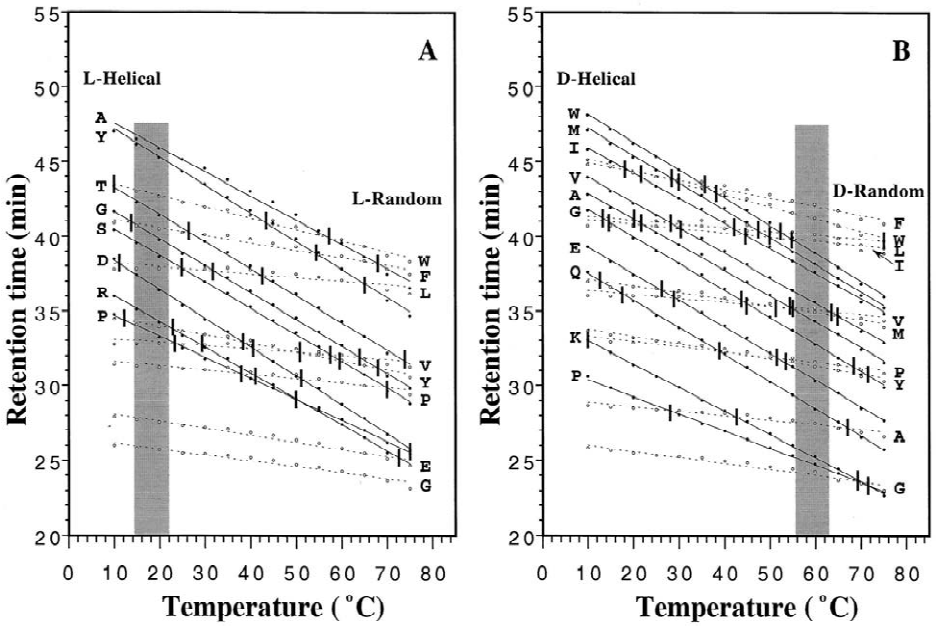

Fig. 5. Effect of temperature on RP-HPLC retention time of helical and random coil peptides. Column and conditions same as for Fig. 3. Panels A and B show the temperature effect on helical and random coil peptides with L-amino acid or D-amino acid substitutions, respectively. In both (A) and (B), solid lines with solid symbols represent helical peptides and dotted lines with open symbols denote random coil peptides. Co-elution or poor resolution of peptide peaks are marked with small black bars. The grey column in each panel shows the temperature zone in which the optimum separation of the peptide mixture can be obtained. Peptide designation is based on the substituting amino acid as described in Section 3.1.

the complexity of RP-HPLC elution in helical and random peptide mixtures within the temperature range used. The prospective optimum separation zones of L- or D-peptide mixtures are denoted as grey columns in Fig. 5, 0.5 °C away (on the *x*-axis) from the nearest co-elution point on both sides of these zones. Hence, in order to obtain the optimum separation of L- or D-peptide mixtures, RP-HPLC was carried out in 0.5 °C increments within the prospective optimum separation zones of the L-peptides (encompassing 16 L-amino acid-substituted helical and random model peptides) and the D-peptides (encompassing 20 D-amino acid helical and random peptides). Fig. 6 shows the RP-HPLC elution profiles of the L- and D-peptide mixtures used in Figs. 4 and 5, with the optimum separation (middle panels) as well as the RP-HPLC profiles obtained at temperatures several degrees lower (upper panels) or higher (lower panels) than the corresponding optimum temperatures. Considering the number of α-helical and random coil peptide analogues in each mixture, excellent separations have been observed at 21 and 62.5 °C for L-peptides and D-peptides, respectively. In addition, the optimum temperatures of L and D-peptides are both in the corresponding empirical prospective optimum zones (Fig. 5), indicating the validity of the temperature-based optimization protocol. In contrast to the optimum elution profiles, the chromatograms at higher or lower temperature represent the sensitivity of the varying temperature approach to influence the selectivity of peptides with conformational differences. It is important to note that, from shifts in temperature in the range of just 2.5 to 4 °C from the optimum temperature, the RP-HPLC profiles clearly show considerably different retention behaviour with different peptides co-eluted (indicated by the arrows), underlining the effectiveness of subtle temperature changes to alter the elution profiles of the peptide models in this RP-HPLC temperature selectivity study.

4. Discussion

Although the retention times of all the helical and random peptides decreased with increasing temperature, it is clear that the overall retention of the α-helical model peptides decreased to a greater extent than that of the random peptide analogues. Since the helical or random model peptides have different conformations, i.e. as single-stranded amphipathic α-helical peptides or random coil peptides, respectively, the varying results of temperature effect on peptide retention behaviour may be mainly attributed to structural differences (Figs. 4 and 5) with the conformation of the α-helical peptides in solution during RP-HPLC strongly influenced by temperature. Indeed, in our previous study [49], we showed that the helical conformation of the model peptides could be denatured to different degrees with a temperature increase in the presence of helix-inducing 40% trifluoroethanol (TFE). In fact, on binding to a reversed-phase column, the high hydrophobicity of the stationary phase stabilizes secondary (α-helical) structure, mimicking the effect of TFE when the peptide is in solution [60]. Therefore, during RP-HPLC, a temperature increase may also induce peptide denaturation and, as a result, disrupt peptide amphipathicity, thereby reducing the retention time of the model peptide. In contrast, the elution of random coil peptides was merely influenced by the general effects of temperature (as described above) with a concomitant lesser effect on retention behaviour compared to the α-helical peptide analogues.

From Fig. 5, the retention behaviour of all L- and D-helical peptides with a change of temperature was similar except for that of L-/D-proline substituted helical peptides. Due to the well-documented helix-disrupting characteristic of proline [49,59,61], proline-substituted model peptides would not be fully helical even in a strong hydrophobic environment, e.g. in the presence of 50% TFE or the hydrophobic conditions of RP-HPLC. As a result, the effect of temperature denaturation on helical P_L and P_D would not be as dramatic as the effect on other α-helical model peptide analogues, since, for all intents and purposes, the helical P_L and P_D analogues are already partially denatured. Interestingly, the slope values of the temperature profiles of the various peptide analogues appear to be related to their the molar ellipticity values, i.e. slope values of helical P_L and P_D during temperature variation are smaller than those of α-helical peptide analogues with other amino acid substitutions, and greater than those of random coil peptides; concomitantly, the molar ellipticity values of helical P_L and P_D in the presence

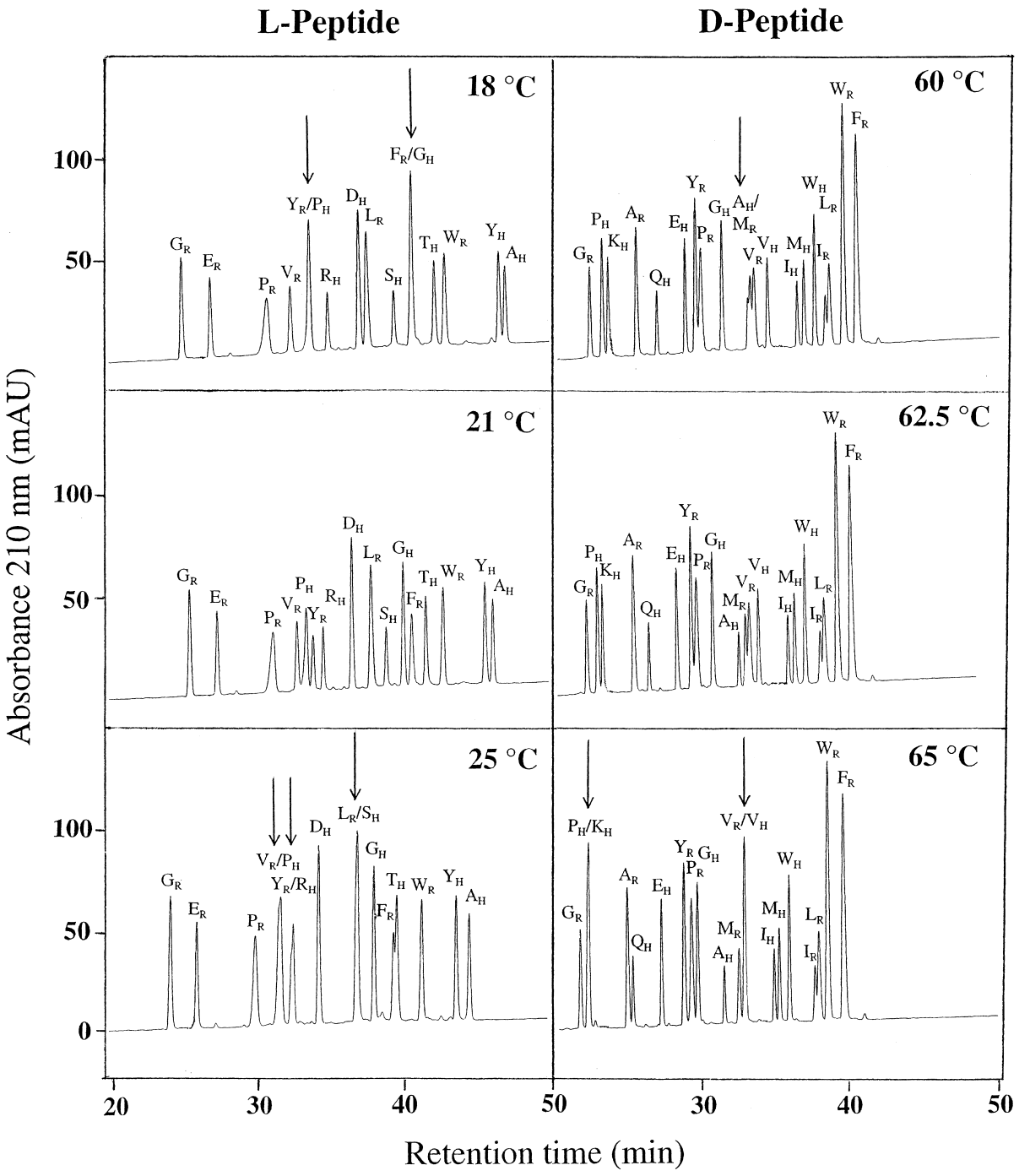

Fig. 6. RP-HPLC separation of random coil and helical L- or D-peptides around the optimum temperatures. Column and conditions same as for Fig. 3. Arrows in chromatograms point out the co-eluted or poorly resolved peaks of different peptides. Peptide designation is based on the substituting amino acid as described in Section 3.1. Subscripts of letter R and H denote random coil or helical peptide, respectively.

of 50% TFE are smaller than those of other α-helical analogues but greater than those of random coil peptides [49] (Fig. 2). Thus, our results support again the premise that temperature can be used as a sensitive probe of peptide conformation during RP-HPLC [27–29,34,41–45].

Fig. 7 shows the temperature effect on RP-HPLC retention behaviour of L-/D-amino acid substituted α-helical model peptides. Thus, the retention behaviour of hydrophobic (Val, Ile, Ala) and hydrophilic (Asn, Gln) L-/D-amino acid substituted helical peptide analogues over a temperature range of 5–80 °C were examined by plotting peptide retention time at a specific temperature minus its retention time at 5 °C versus temperature in order to highlight differences in the elution behaviour of peptides as the temperature is raised. The Gly-substituted helical peptide was also selected as a standard to evaluate the effect of temperature on L-/D-diastereomeric peptide analogues, due to the characteristic non-optical activity of glycine. From Fig. 7, it is clear that L-helical peptides of different amphipathicity/hydrophobicity behave quite similarly during RP-HPLC at different temperatures (panel A), as do the D-amino acid-substituted α-helical diastereomers (panel B). High correlations were obtained with $R=0.985$ for L-helical peptides and $R=0.995$ for D-helical peptides, respectively. In addition, in our previous study [49], D-amino acid substituted peptides generally showed lower helicity in aqueous environment, due to the helix-disrupting characteristics of D-amino acid residues [49–53]; however, the peptides are induced to an highly helical conformation in an hydrophobic environment. In Fig. 7C, temperature has a similar effect on L-/D-helical diastereomeric peptides and the Gly peptide ($R=0.989$), confirming once again that, not only do the hydrophobic conditions of RP-HPLC mimic the helix-inducing properties of TFE, thus inducing helical peptides to fully helical conformation, but also that this temperature-based approach to RP-HPLC is useful for the identification of peptide secondary structures.

Generally, as shown in Fig. 5, temperature is demonstrated to have a similar effect on the retention behaviour of random model peptide analogues with either L- or D-amino acid substitutions. However, subtle differences in the effect of temperature are

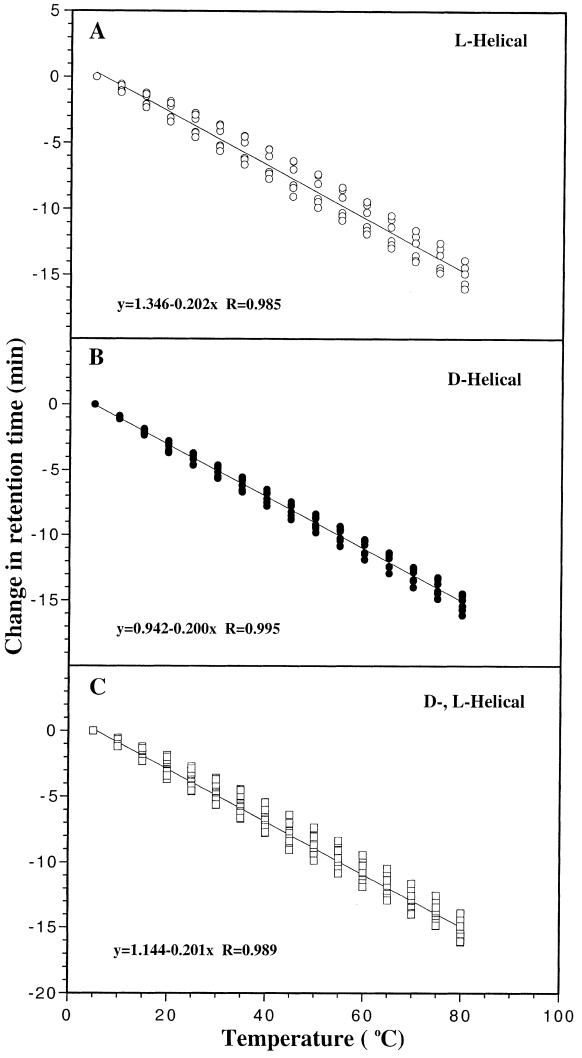

Fig. 7. Comparison of temperature effect on RP-HPLC of L- and D-helical peptides. Column and conditions same as for Fig. 3. (A, B and C) Temperature plotted versus peptide retention time at a specific temperature minus its retention time at 5 °C (change in retention time) for L-helical peptides, D-helical peptides and L- and D-helical diastereomeric peptides, respectively. The symbols used are (○) for L-helical peptides, (●) for D-helical peptides and (□) for both L- and D-helical diastereomeric peptide analogues. Least-squares fit analysis resulted in the correlations shown in each panel. Only L- and D-peptides of Ile, Val, Ala, Gln, Asn and Gly substituted helical peptides were used in this figure.

apparent for different peptide analogues. Thus, Fig. 8 illustrates the surprising results of different temperature effects on aromatic and aliphatic L-/D-amino acid substituted random peptides. Although there is

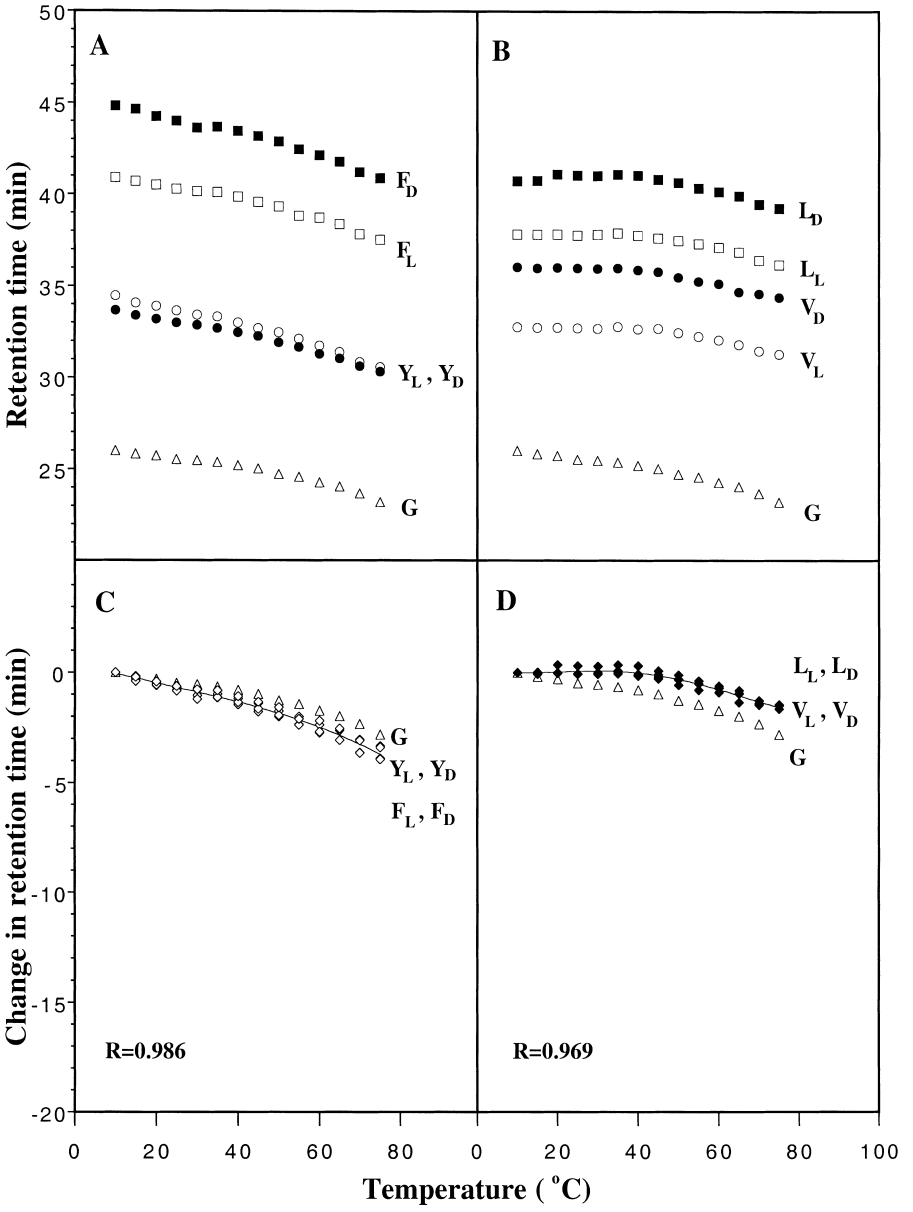

Fig. 8. Comparison of temperature effect on RP-HPLC of random coil peptides. Column and conditions same as for Fig. 3. (A and B) Effect of temperature on the retention time of different random coil peptides. (C and D) Temperature plotted versus peptide retention time at a specific temperature minus its retention time at 10 °C of random coil peptide analogues. In (A) and (B), closed circles and closed squares are used to represent D-random coil peptides; open circles and open squares represent L-random coil peptides; open triangles denote the Gly-substituted random coil peptide. In (C) and (D), open diamonds and closed diamonds represent the aromatic and aliphatic amino acid substituted random coil peptides, respectively; open triangles denote the Gly-substituted random coil peptide. Curvy-linear correlation is shown in (C) and (D). Peptide designation is based on the substituting amino acid as described in Section 3.1.

no dramatic difference in temperature effects (represented by the slopes of the plots) on retention time of individual peptides (Fig. 8A and B), a significant variation in retention behaviour emerged between aromatic and aliphatic L-/D-amino acid substituted random peptides as the temperature is raised incrementally from 10 to 75 °C (Fig. 8C and D), when peptide retention data were presented as peptide retention time at a specific temperature minus its retention time at 10 °C versus temperature. Using the Gly-substituted random peptide as an internal standard, Fig. 8C and D indicate that aliphatic amino acid substituted random peptides (Fig. 8D) are more stably bound to the stationary phase of the Cs column (with plots shallower than that of the standard Gly peptide) than aromatic amino acid substituted analogues (with plots steeper than that of the standard Gly peptide) (Fig. 8C) as the temperature is raised; in other words, temperature is more effective in altering the bound status of aromatic random peptides than that of aliphatic random peptides during RP-HPLC. In addition, since aromatic random F_L and F_D are more hydrophobic (i.e. are eluted later) than aliphatic random peptide analogues during RP-HPLC, while, in contrast, the aromatic Y_L and Y_D are less hydrophobic (i.e. are eluted earlier) than aliphatic random peptide analogues, this alteration of bound status of the model peptides during temperature is independent of peptide hydrophobicity. Calculated by polynomial curve fitting analysis, the excellent curvy-linear correlations shown in Fig. 8C and D also demonstrate the sensitivity and the validity of this temperature approach to distinguish peptides with aliphatic and aromatic amino acid substitutions. Note that the large difference in retention times between random L- and D-substituted peptide pairs (which possess the same inherent hydrophobicity and lack the potential for D-amino acid disruption of secondary structure) could be attributed to nearest-neighbour effects, since the substitution site at position 1 of the sequence is next to a L-Leu residue at position 2 (Fig. 1).

Although, as previously described [27,49], the monomeric status of the α-helical peptide analogues used in this study is ensured, the possibility that random peptide analogues may exhibit a degree of association oligomerization was investigated by comparing the temperature profiles of these peptides with that of the random coil standard, G_R (random Gly-substituted peptide). Thus, the data from Fig. 7C, and Fig. 8C and D were normalized relative to the temperature profile of the G_R, as presented in Fig. 9. Based on previous studies by our laboratory [27–29] which introduced the concept of detecting self-association of peptidic solutes by ‘‘temperature profiling’’ in RP-HPLC, the positive profiles of the aliphatic random coil peptides may indeed be indicative of some degree of association, albeit subtle, compared with aromatic random coil peptides, possibly offering an explanation of different temperature effects between these two kinds of random peptide analogues. From Fig. 9, the negative slopes for the helical peptides are considerably steeper than those of random peptides. Since the data were normalized relative to the random standard G_R, other factors which can influence peptide retention behaviour (the aforementioned effects on mobile phase viscosity and mass transfer effects, for example) are already taken into account in the plots shown in Fig. 9. Thus, as discussed in detail in our previous study [27], the steep negative profiles of the helical peptides indicate considerable unfolding of the α-helices with increasing temperature. Briefly, at low temperature, the bound monomeric α-helices are in equilibrium with the same monomeric folded states free in solution, with their retention times dependent on the hydrophobicity of their nonpolar faces. At high temperature, a considerable amount of the random, disrupted helical forms of these peptides are now present in solution, concomitant with a loss of amphipathicity (note that these peptides are assumed to always be bound to the stationary phase as α-helices [27]). The fast exchange between folded α-helical structure and unfolded form in solution now becomes a major determinant of the observed retention time, i.e. the more random coil present in solution, the greater the decrease in retention time and, hence, the much steeper negative profiles illustrated in Fig. 9 compared to random peptides which possess only negligible secondary structure throughout the entire temperature range.

5. Conclusions

In this study, we report the use of four series of

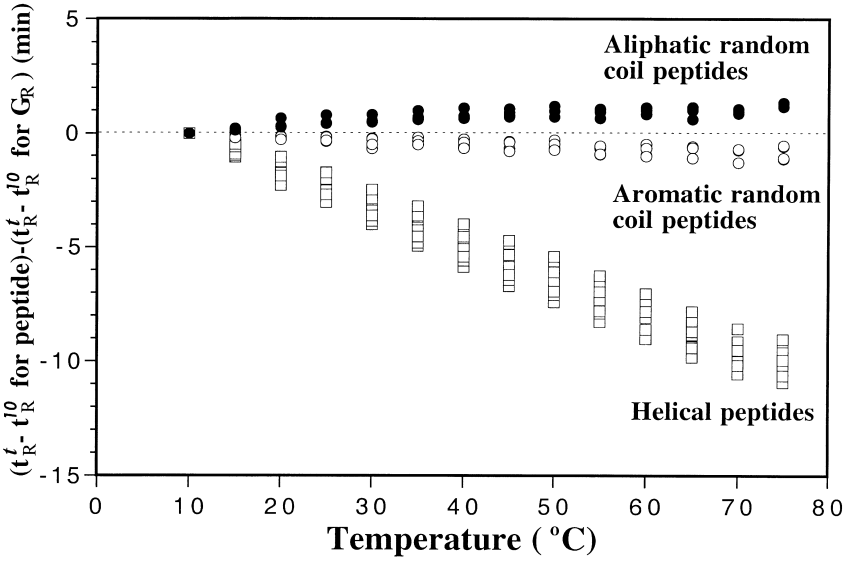

Fig. 9. Effect of temperature on RP-HPLC of helical and random coil peptides: normalization to retention behavior of the random coil Gly peptide. Column and conditions same as for Fig. 3. The retention behaviour of the peptides was normalized to that of random coil Gly peptide through the expression $(t_R^t - t_R^{10})$ minus $(t_R^t - t_R^{10}$ for Gly peptide), where t_R^t is the retention time at a specific temperature of a helical or random coil peptide and t_R^{10} is the retention time at 10 °C.

synthetic model peptides (non-helical and amphipathic α-helical peptides with L-/D-amino acid substitutions) in order to demonstrate the selectivity, based on peptide conformational differences, that may be obtained in a reversed-phase separation during temperature alterations. Temperature variations resulted in dramatically different effects on the retention behaviour of α-helical and random coil peptides. In contrast to random coil peptides, α-helical peptides underwent a temperature-induced unfolding process during RP-HPLC, resulting in considerable selectivity variations. The optimum resolution of mixtures of random coil and α-helical peptide analogues was obtained simply by varying the temperature of the RP-HPLC separation, taking advantage of the different responses to temperature of peptides with secondary structure potential compared to those with no such potential. In addition to aiding the rational development of peptide separation optimization protocols, this study also confirms the value of employing RP-HPLC at varying temperatures as a sensitive and practical probe to distinguish peptide conformations, a role which should prove valuable for peptide/protein structure studies.

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