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Hydrophilic interaction/cation-exchange chromatography for separation of cyclic peptides

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Abstract

Mixed-mode hydrophilic interaction/cation-exchange chromatography (HILIC/CEX) is a novel high-performance technique which provides unique selectivities compared to reversed-phase chromatography (RPLC) for peptide separations. Separations by HILIC/CEX are effected by linear increasing salt gradients in the presence of acetonitrile (up to 90%), which promotes hydrophilic interactions overlayed on ionic interactions with the ion-exchange matrix. In the present study, the utility of HILIC/CEX has been extended to the separation of cyclic peptides in the form of synthetic model analogues of gramicidin S: Series 1 comprised six 10-residue cyclic peptide analogues which exhibited amphipathic, rigid β-pleated sheet conformation; Series 2 comprised 14-residue cyclic diastereomeric analogues of gramicidin S, where only the enantiomeric configuration of a single amino acid side-chain is varied from peptide to peptide. Observation of the retention behaviour of these two series of cyclic peptides during HILIC/CEX and RPLC confirmed not only the excellent complementarity of these two chromatographic modes but also highlighted the dramatic separations achievable by the mixed-mode approach.

Keywords: Hydrophilic interaction chromatography; Ion-exchange chromatography; Mixed-mode chromatography; Peptides; Gramicidins; Antibiotics

1. Introduction

Although reversed-phase chromatography (RPLC) is the predominant high-performance liquid chromatography (HPLC) mode applied to peptide separations [1], there is a growing awareness of the potential of mixed-mode hydrophilic interaction/cation-exchange chromatography (HILIC/CEX) as a complementary HPLC mode in situations where RPLC does not offer the required resolution of peptide mixtures [2–5]. Recent reports have also highlighted the potential of HILIC/CEX for protein separations [6,7]. In this novel separation approach, peptides are eluted from an ion-exchange column in the presence of high levels (generally 50–90%) of acetonitrile: at low acetonitrile levels, peptides are eluted mainly by an ionic mechanism; an increase in the acetonitrile concentration leads to a concomitant promotion of hydrophilic interactions overlayed on the ionic interactions, with hydrophilic interactions being dominant at high acetonitrile levels.

The relative popularity of any HPLC approach for peptide separations depends on its ability to resolve mixtures of peptides exhibiting, perhaps, a wide variety of sizes, hydrophobicities and conformations.

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Thus, in a continuing effort to establish the characteristics governing the performance of the mixed-mode HILIC/CEX approach, we have compared RPLC to HILIC/CEX for the separation of two series of synthetic cyclic (i.e., conformationally restricted) peptides representing analogues of the antibiotic peptide gramicidin S: Series 1 is made up of six cyclodecapeptides with amphipathic, rigid β-pleated sheet conformations, the six analogues varying only in the hydrophilic face of the β-sheet; Series 2 is made up of 14 cyclotetradecapeptides, where only the enantiomeric configuration of a single side-chain is varied from peptide to peptide (i.e., amino acid composition and intrinsic hydrophobicity of each peptide remains unchanged). Studies of the RPLC retention behaviour of cyclic peptides related to gramicidin S [8] or other antibiotic peptides such as gratisin [8,9] have been somewhat limited. Thus, separations of these peptide series by RPLC and HILIC/CEX served not only to compare the relative efficiencies of the two modes of HPLC for cyclic peptides but also underlined the excellent potential of the HILIC/CEX approach for general peptide mixtures.

2. Experimental

2.1. Materials

HPLC-grade water and acetonitrile were obtained from BDH (Poole, UK). ACS-grade orthophosphoric acid and triethylamine (TEA, redistilled before use) were obtained from Anachemia (Toronto, Canada). Trifluoroacetic acid (TFA) was obtained from Aldrich (Milwaukee, WI, USA). Sodium perchlorate ($NaClO_4$) and diammonium hydrogenorthophosphate [$(NH_4)_2HPO_4$] were obtained from BDH.

2.2. Instrumentation

The HPLC system consisted of an HP1090 liquid chromatograph from Hewlett-Packard (Avondale, PA, USA), coupled to an HP1040A detection system, HP9000 Series 300 computer, HP9133 disc drive, HP2225A Thinkjet printer and HP7460A plotter.

2.3. Columns

Reversed-phase runs were carried out on a Zorbax 300XDB-C_8 reversed-phase column (150×4.6 mm I.D., 5 μm particle size, 300 Å pore size; void volume, 1.7 ml) from Hewlett-Packard (Little Falls Site, DE, USA).

Hydrophilic interaction/cation-exchange runs were carried out on a poly(2-sulfoethyl aspartamide)-silica (PolySulfoethyl A) strong cation-exchange column (200×4.6 mm I.D., 5 μm, 300 Å; void volume, 1.9 ml) from PolyLC (Columbia, MD, USA).

2.4. Peptide synthesis

Details of the solid-phase peptide synthesis and peptide cyclization protocols have been previously described [10].

3. Results and discussion

3.1. RPLC vs. HILIC/CEX: general principles

Although a detailed discussion comparing RPLC with HILIC/CEX for peptide separations can be found in a companion paper [11], it is important to summarize certain principles which distinguish the two techniques. Thus, hydrophilic interaction chromatography, a term originally coined to describe separations based on solute hydrophilicity [12], depends on hydrophilic interactions between the solutes and a hydrophilic stationary phase, i.e. solutes are eluted in order of increasing overall hydrophilicity (decreasing hydrophobicity); in contrast, of course, peptides are eluted from a RPLC column in order of increasing overall hydrophobicity (decreasing hydrophilicity). Hence, in the absence of any anomalous conformation-dependent retention behaviour, the elution order of HILIC is reversed compared to RPLC.

This laboratory has previously reported [2–5] the efficacy of mixed-mode HILIC/CEX on a strong cation-exchange matrix with the requisite desirable hydrophilic character, namely the PolySulfoethyl A column employed in the present study. Note that the pore size of 300 Å for both this cation-exchange

column and the reversed-phase column used in this study makes such columns generally applicable for separations of both small peptides and larger polypeptides [1]. Typical conditions for mixed-mode HILIC/CEX are a linear increasing $NaClO_4$ gradient (2–20 m*M* $NaClO_4$/min) at pH 3, with both mobile phase buffers containing 15–80% acetonitrile. The cation-exchange column then separates peptides based on net positive charge, this separation mode being overlayed by the presence of acetonitrile overcoming any undesirable hydrophobic interactions with the ion-exchange matrix [13] while promoting favorable hydrophilic interactions.

3.2. HPLC of synthetic model peptides

The model peptides used in this study are analogues based on the cyclic 10-residue peptide antibiotic gramicidin S (GS) [cyclo-(Val–Orn–Leu–D-Phe–Pro)$_2$], first isolated from *Bacillus brevis* over 50 years ago [14]. Both X-ray and NMR studies of GS have shown it forms a two-stranded antiparallel β-sheet structure with the strands fixed in place by two type II′ β-turns [15–17]. The β-sheet described by this symmetric decamer sequence is of an amphipathic nature, with Val and Leu making up the hydrophobic face of the molecule and basic Orn residues comprising the hydrophilic face.

3.2.1. Series 1 model peptides: amphipathic 10-residue GS analogues

Fig. 1 shows the sequence of the cyclic 10-residue analogues of GS as well as the schematic representation of their amphipathic β-sheet structure. Note that the hydrophobic face of the molecule, made up of Leu and Val residues, is constant; only the hydrophilic face, made up of two Dap, Dab, Orn, Lys, His or Arg residues, is varied. In addition, four of these basic residues represent an homologous series, with their side-chains increasing by one methylene group: Dap ($1CH_2$)<Dab ($2CH_2$)<Orn ($3CH_2$)<Lys ($4CH_2$). These peptides were designed to incorporate the alternating hydrophobic–hydrophilic residue pattern found in native GS, where Leu and Val make up the hydrophobic face and basic residues constitute the hydrophilic face. The substitution of D-Tyr for D-Phe was made for purposes of increased peptide solubility [10,18,19]. Previous studies have shown that homologous substitutions such as D-Tyr for D-Phe and Lys for Orn lead to minimal structural changes in GS analogues [10,15,19]. The remainder of the substitutions (Dap, Dab, Arg, His) have been shown not to disrupt the β-sheet conformation (unpublished results); thus the

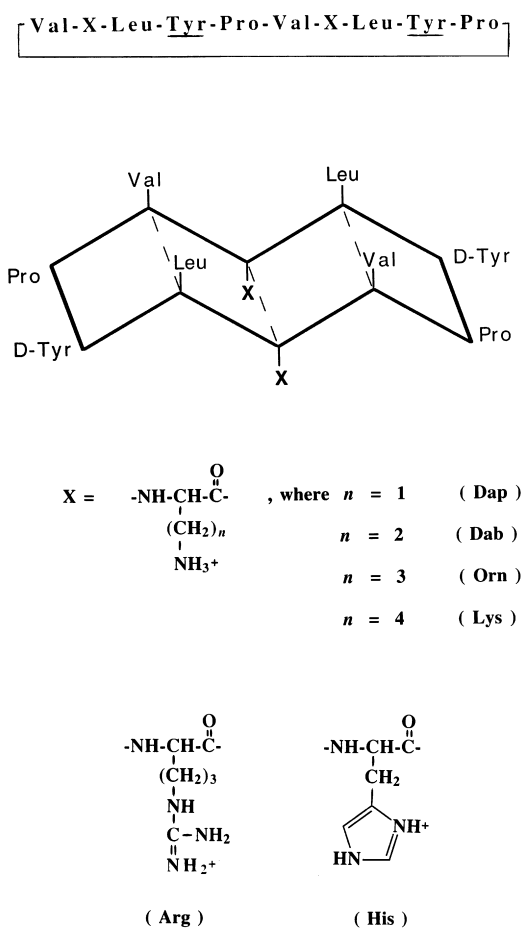

Fig. 1. Series 1 model peptides: amphipathic 10-residue gramicidin S analogues. At top is the linear sequence of the cyclic peptides (underlined residues represent D-amino acids); below is a schematic representation of the β-sheet structure of the peptides. X denotes the substitution site on the hydrophilic face of these amphipathic β-sheet analogues. The peptides are denoted by the three-letter abbreviations of the side-chains at the substitution sites: Dap refers to the peptide analogue with diaminopropionic acid (pK_a 10.9) substituted at both sites; Dab refers to diaminobutyric acid (pK_a 10.7); Orn refers to ornithine (pK_a 10.5); Lys refers to lysine (pK_a 9.6); Arg refers to arginine (pK_a 12.0); and His refers to histidine (pK_a 6.5).

Series 1 analogues retain fully their amphipathic characteristics.

3.2.2. Comparison of HILIC/CEX and RPLC for separation of amphipathic 10-residue GS analogues

In a companion paper to the present study [11], it is hypothesized that side-chain modifications in the hydrophobic face of an amphipathic α-helical peptide are best separated by RPLC; in contrast, side-chain modifications in the hydrophilic face of an amphipathic α-helical peptide are best separated by HILIC/CEX. In the former situation, the preferred binding domain (PBD) of the α-helix is the hydrophobic face interacting with the hydrophobic RPLC stationary face; in the latter situation, the PBD of the α-helix is the hydrophilic face interacting with the hydrophilic/cation-exchange matrix. An amphipathic β-sheet should provide a similar situation. Thus, it might be expected that the amphipathic 10-residue GS analogues shown in Fig. 1 would only be poorly resolved by RPLC, since the hydrophobic PBD of all six analogues is constant. In contrast, since side-chain substitutions are being made on the hydrophilic PBD, HILIC/CEX would appear to have the best potential for complete resolution of the analogues.

From Fig. 2, it can be seen that the six peptides were, indeed, only poorly resolved by RPLC, whether run under conditions of pH 2 (top), pH 3 (middle) or pH 6.5 (bottom). The three different run conditions, together with the relatively shallow gradient rate of 0.5% acetonitrile/min, were designed to maximize any potential of this HPLC mode to resolve the peptides. However, the aforementioned lack of resolution, coupled with unfavourably long elution times, clearly shows this potential to be poor.

Fig. 3 shows the effect of mobile-phase acetonitrile concentration on the elution profiles of the six analogues obtained on the strong-cation-exchange column at pH 3 under conditions of constant gradient rate (2.5 m*M* $NaClO_4$/min). At this pH, all six different basic side-chains should be fully protonated, i.e. positively charged (see pK_a values in the legend to Fig. 1). From Fig. 3, it can be seen that the acetonitrile concentration in the mobile phase has a profound effect on peptide elution behaviour. At a level of 10% acetonitrile, the peptides appear as generally poorly resolved, broad peaks with relatively long retention times. Raising the acetonitrile level to 40% not only leads to much improved resolution, but also narrowed the peaks and reduced considerably the peptide retention times, i.e. the peak broadening and longer retention times observed at a

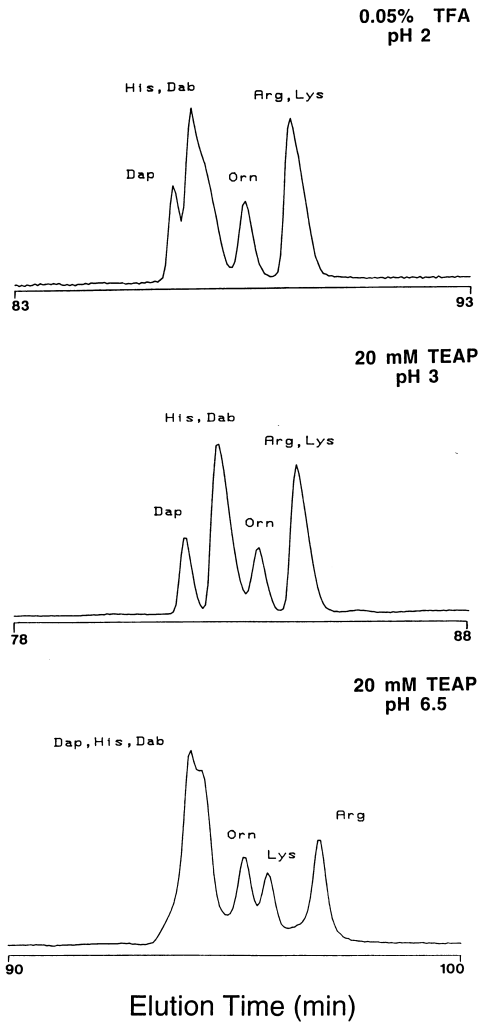

Fig. 2. RPLC elution profiles of cyclic, amphipathic β-sheet peptides. Conditions: linear A–B gradient (0.5% acetonitrile/min) at a flow-rate of 1 ml/min and a temperature of 30°C, where (top) eluent A is 0.05% aqueous TFA and eluent B is 0.05% TFA in acetonitrile (pH 2); (middle) eluent A is 20 m*M* aqueous triethylammonium phosphate (TEAP) and eluent B is 20 m*M* TEAP in 50% aqueous acetonitrile (pH 3), both eluents containing 100 m*M* $NaClO_4$; and (bottom) eluent A is 20 m*M* aqueous TEAP and eluent B is 20 m*M* TEAP in 60% aqueous acetonitrile (pH 6.5), both eluents containing 100 m*M* $NaClO_4$. Peptides were detected by their absorbance at 210 nm. For peptide denotions, see Fig. 1.

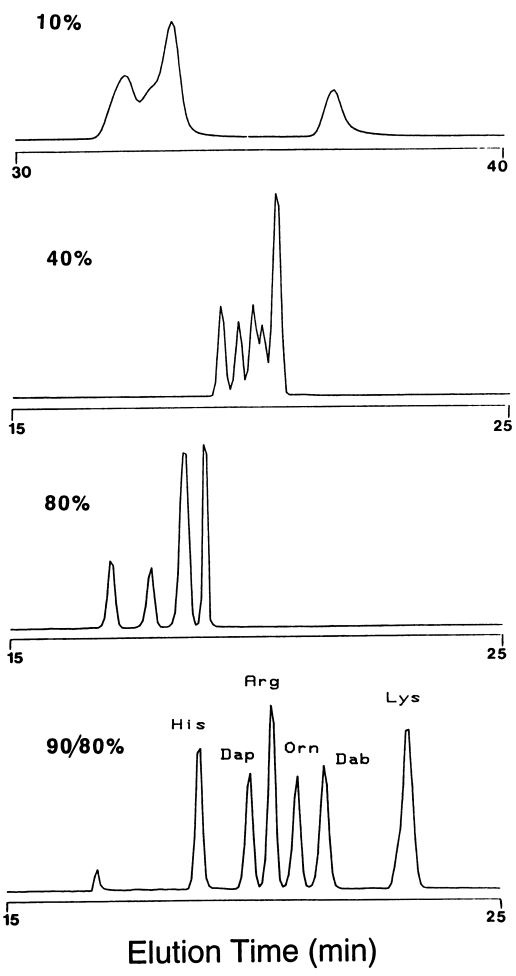

Fig. 3. Effect of acetonitrile concentration on HILIC/CEX elution profiles of cyclic, amphipathic β-sheet peptides. Conditions: linear A–B gradient (2.5 m*M* $NaClO_4$/min, following 5-min isocratic elution with 100% buffer A) at a flow rate of 1 ml/min and a temperature of 30°C, where buffer A is 20 m*M* aqueous triethylammonium phosphate (TEAP), pH 3, and buffer B is buffer A containing 400 m*M* $NaClO_4$; acetonitrile is present in both buffers at concentrations (v/v) of 10, 40 or 80% (denoted as such on the elution profiles), or present at a concentration of 90% (v/v) in buffer A and 80% (v/v) in buffer B (denoted 90%/80%). Peptides were detected by their absorbance at 210 nm. For peptide denotions, see Fig. 1.

level of 10% acetonitrile appeared to be due to hydrophobic interactions between the hydrophobic face of the amphipathic β-sheet peptides and the column packing. Raising the level to 40% acetonitrile served to suppress these hydrophobic interactions. Raising the level to 80% acetonitrile reduced the retention times still further, but the overall separation had worsened slightly at this higher acetonitrile level, with only four peaks visible instead of the five distinguishable at 40% acetonitrile. However, with 90% acetonitrile in buffer A (while maintaining only 80% acetonitrile in buffer B, due to $NaClO_4$ solubility concerns at a 90% acetonitrile concentration) all six peptides were well resolved to baseline, as well as now showing increasing retention times compared to the 80% acetonitrile elution profile. Hence, following suppression of hydrophobic interactions by the addition of acetonitrile, the peptide separation mechanism has become based upon mixed-mode ionic and hydrophilic interactions. The latter interactions dominate at very high (90%) acetonitrile concentration in the mobile phase, leading to the observed excellent separation.

Fig. 4 shows the effect of varying gradient rate of $NaClO_4$ on peptide elution profiles at pH 3 under conditions of constant acetonitrile concentration (90%/80%) in the mobile-phase buffers. Interestingly, optimal separation in this mixed-mode system was achieved at a gradient rate of 2.5 m*M* $NaClO_4$/min. Thus, while shallowing the gradient from 5 m*M*/min (top) to 2.5 m*M*/min (middle) achieved an expected improvement in resolution (Dap and Arg are co-eluted at the higher gradient rate), shallowing the gradient further to 1 m*M*/min (bottom) resulted in a deterioration of the separation, with the Orn and Dab analogues now co-eluted. These results (and, indeed, those shown in Fig. 3) indicate the complexity of peptide retention behavior when responding to variations in run parameters during mixed-mode HILIC/CEX. Nonetheless, it should be noted that all three separations displayed in Fig. 4 showed a distinct improvement over the RPLC profiles shown in Fig. 2, including a considerable shortening of overall run times. Thus, in agreement with the hypothesis stated above, peptides with substitutions in the hydrophilic face of an amphipathic β-sheet are indeed better separated by HILIC/CEX compared to RPLC, even under nonoptimal HILIC/CEX conditions.

In order to underline this point, a comparison was now made between the relative effectiveness of RPLC and HILIC/CEX to separate the linear versions of the six peptides as opposed to the cyclic

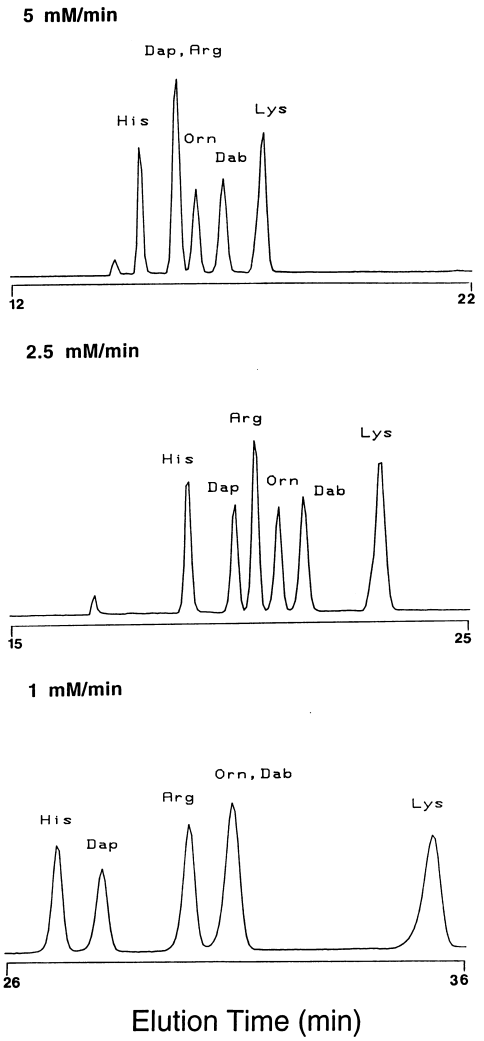

Fig. 4. Effect of salt gradient steepness on HILIC/CEX elution profiles of cyclic, amphipathic β-sheet peptides. Conditions: linear A–B gradient (5 m*M*, 2.5 m*M* or 1 m*M* $NaClO_4$/min, following 5-min isocratic elution with 100% buffer A) at a flow-rate of 1 ml/min and a temperature of 30°C, where buffer A is 20 m*M* aqueous triethylammonium phosphate (TEAP), pH 3, and buffer B is buffer A containing 400 m*M* $NaClO_4$; buffer A and buffer B also contained, respectively, 90% (v/v) and 80% (v/v) acetonitrile. Peptides were detected by their absorbance at 210 nm. For peptide denotions, see Fig. 1.

analogues. The linear analogues do not exhibit the amphipathic nature of their cyclic, amphipathic β-sheet counterparts, i.e. they contain no preferred binding domains. From Fig. 5, the much improved separation of the cyclic amphipathic analogues by HILIC/CEX compared to RPLC is quite clear; the arrows denote relative positions of the peptide analogues between the HILIC/CEX and RPLC runs in order to highlight selectivity differences between the two HPLC modes. In contrast, there is no clear advantage between HILIC/CEX and RPLC for resolution of this particular mixture of linear peptide analogues, reflecting the lack of any preferred binding domain to enhance the separation in either mode. Note also the much reduced RPLC retention times of the linear analogues compared to the amphipathic cyclic peptides due to the lack of a hydrophobic binding domain in the former. The arrows again denote useful selectivity differences between the HILIC/CEX and RPLC runs, thus highlighting the complementarity of these two approaches. While there was very little temperature effect on the cyclic peptides (run at 30°C), the linear peptides showed broad, tailing peaks at 30°C with no resolution, hence the requirement for a high-temperature (70°C) separation.

3.2.3. Series 2 model peptides: 14-residue GS analogues

The series 2 model peptides are based on a synthetic peptide denoted GS14 [cyclo(Val–Lys–Leu–Lys–Val–D-Tyr–Pro–Leu–Lys–Val–Lys–Leu–D-Tyr–Pro)], a 14-residue analogue of GS reported in previous studies by Kondejewski et al. [10,20].

Fig. 6 shows the linear sequence of the cyclic 14-residue analogues of GS, in addition to the schematic representation of the amphipathic β-sheet structure of the ''native'' peptide, denoted GS14. As shown in Fig. 6, GS14 exists in an antiparallel β-sheet conformation and possesses features similar to those present in GS itself, i.e. an amphipathic β-sheet structure, where basic residues (Lys in GS14 and Orn in GS) make up the hydrophilic face of the molecule and hydrophobic residues (Val and Leu) make up the hydrophobic face. Clearly, there are similarities between this peptide series and the Series 1 analogues (Fig. 1), also based on the GS structure. However, for the Series 2 analogues, each residue in the sequence of GS14 has been systematically replaced with its enantiomer, i.e. 14 diastereomers. The peptide analogues are denoted by the position of the substitution, e.g. L3 refers to the analogue with an L-Leu to D-Leu substitution at position 3; Y13 refers

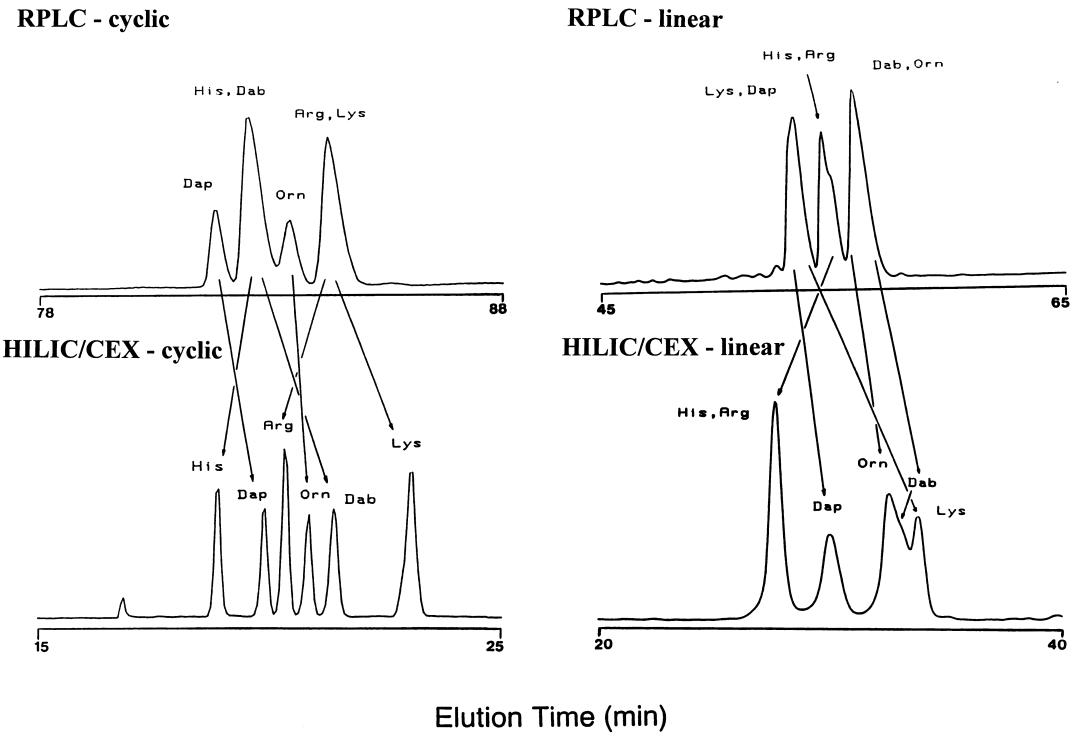

Fig. 5. Comparison of RPLC and HILIC/CEX elution profiles of cyclic, amphipathic β-sheet peptides and their linear, noncyclic analogues. Conditions: RPLC, pH 3 conditions as described in Fig. 2, save the linear peptides were run at 70°C; HILIC/CEX conditions as described in Fig. 4 for 2.5 m*M* $NaClO_4$/min run. Peptides were detected by their absorbance at 210 nm. For peptide denotions, see Fig. 1. Arrows denote selectivity differences between the two HPLC modes.

to the analogue with a D-Tyr to L-Tyr substitution at position 13, etc. Two additional analogues were also included with either double (K2K4) or quadruple (K2K4K9K11) L-Lys to D-Lys substitutions. The result of each enantiomeric substitution within the framework of GS14 was shown by CD and NMR to disrupt β-sheet structure to varying degrees, depending on the position of the substitution [20]. Hence, the 16 analogues of GS14 do not share the very ordered structure of the ''native'' GS14 shown in Fig. 6, with a concomitant reduction or, perhaps, elimination of molecule amphipathicity depending on the degree of disorder.

3.2.4. Comparison of HILIC/CEX and RPLC for separation of diastereomeric 14-residue GS analogues

Fig. 7 compares the RPLC elution profiles of the 16-peptide diastereomeric mixture at 70°C (top) and 30°C (bottom). There is a marked deterioration of the peptide elution profile at the lower temperature, resulting in some peak broadening and overall poor resolution. In contrast, 14 out of a maximum 16 peaks can be clearly identified at 70°C, with a concomitant improvement in peak shapes.

As shown above for the cyclic amphipathic 10-residue β-sheet analogues (Fig. 5), the formation of a hydrophobic binding domain can enhance peptide interactions with hydrophobic reversed-phase matrices compared to linear analogues lacking such a domain. Due to their isomeric nature, GS14 and the GS14 diastereomers have the same intrinsic hydrophobicity. Thus, any differences in RPLC retention times of these cyclic peptides are due to their effective hydrophobicity, i.e. the ability of a particular analogue to form a preferred hydrophobic binding domain and present this hydrophobic face to the reversed-phase matrix. From Fig. 7, there is a

Val-Lys-Leu-Lys-Val-Tyr-Pro-Leu-Lys-Val-Lys-Leu-Tyr-Pro

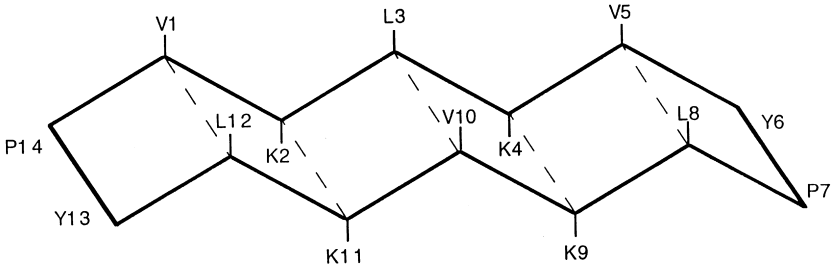

Fig. 6. Series 2 model peptides: diastereomeric 14-residue gramicidin S analogues. At top is the linear sequence of the ''native'' cyclic peptides, GS14 (underlined residues represent D-amino acids); below is a schematic representation of the β-sheet structure of this ''native'' peptide. Peptide analogues (14) were produced by single enantiomeric substitutions at each residue around the ring; an extra two analogues were produced by double or quadruple enantiomeric substitutions of the lysine (K) residues. Amino acids in the β-sheet representation are denoted by their one-letter code; the numbers refer to their position in the ring (see text for denotion of peptide analogues).

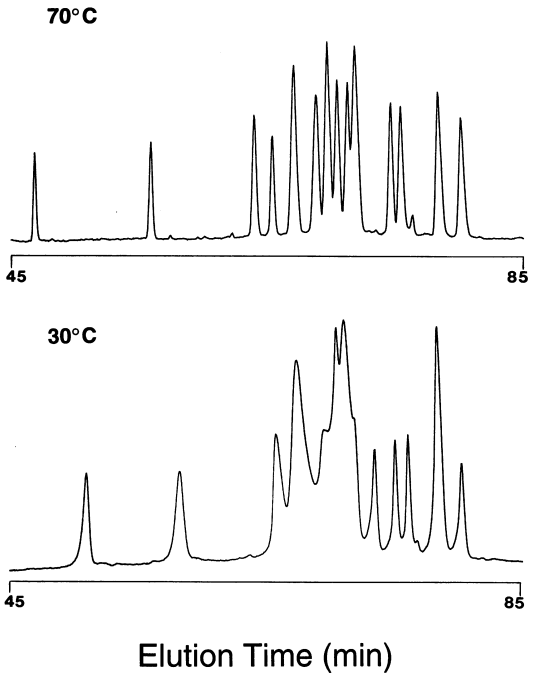

Fig. 7. RPLC elution profiles of cyclic diastereomeric peptides at 70°C (top) and 30°C (bottom). Conditions: linear A–B gradient (0.5% acetonitrile/min) at a flow-rate of 1 ml/min where eluent A is 0.05% aqueous TFA and eluent B is 0.05% TFA in acetonitrile. Peptides were detected by their absorbance at 210 nm.

wide range of retention times observed for the 16 analogues and all have retention times considerably lower than GS14 (although not shown, GS14 was eluted ca. 10 and 9 min after the last eluted analogue at 70 and 30°C, respectively). As seen from the schematic representation of GS14 (Fig. 6), the existence of the parent molecule as an amphipathic β-sheet ensures a large hydrophobic face comprised of six nonpolar side-chains. The analogues with the longer retention times can present a relatively greater hydrophobic face to the reversed-phase matrix compared to analogues with low retention time which cannot present as hydrophobic a face due to more severe disruption of β-sheet structure and, hence, amphipathicity.

Fig. 8 shows the effect of mobile-phase acetonitrile concentration on the elution profile of the 16 analogues obtained on the strong-cation-exchange column at pH 3 using the same gradient rate (2.5 m*M* $NaClO_4$/min). From Fig. 8, the effect on peptide separation of varying acetonitrile concentrations was, in some ways, even more dramatic than that observed for the amphiphatic 10-residue Series 1 analogues (Fig. 3). Thus, at a level of 10% acetonitrile, the peptides were essentially eluted as one major peak, a reasonable observation considering

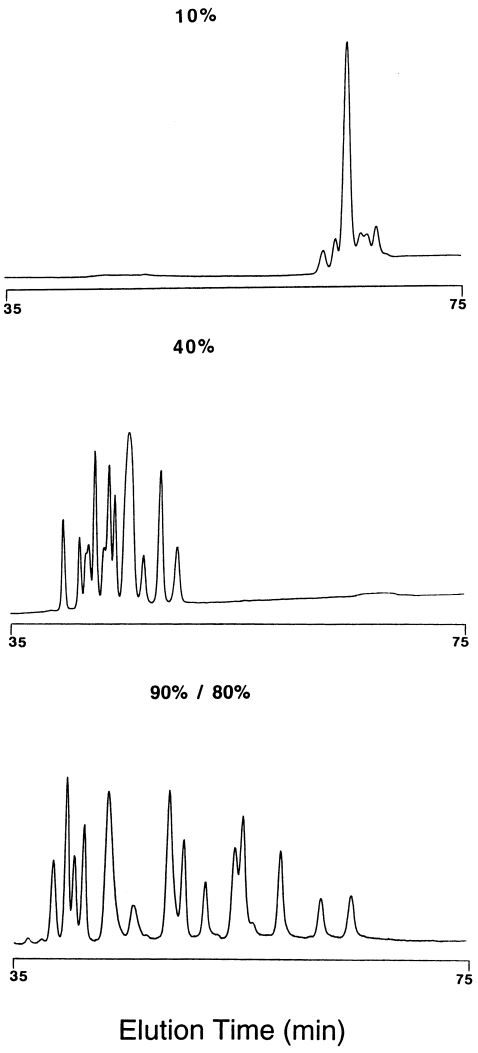

Fig. 8. Effect of acetonitrile concentration on HILIC/CEX elution profiles of cyclic diastereomeric peptides. Conditions: linear A–B gradient (2.5 m*M* $NaClO_4$/min, following 5-min isocratic elution with 100% buffer A) at a flow rate of 1 ml/min and a temperature of 30°C, where buffer A is 20 m*M* aqueous triethylammonium phosphate (TEAP), pH 3, and buffer B is buffer A containing 400 m*M* $NaClO_4$; acetonitrile is present in both buffers at concentrations (v/v) of 10 or 40% (denoted as such on the elution profiles), or present at a concentration of 90% (v/v) in buffer A and 80% (v/v) in buffer B (denoted 90%/80%). Peptides were detected by their absorbance at 210 nm.

they all contain the same net positive charge (+4) at pH 3. Raising the acetonitrile level to 40% reduced the elution time of the peptides considerably, again suggesting suppression of hydrophobic interactions between the peptides and column matrix. Concomitant with this elution time reduction was partial resolution of the peptide mixture, this resolution likely being effected by hydrophilic interactions overlayed over the ionic interactions between peptides and cation-exchange column; alternatively, it is possible that the suppression of hydrophobic interactions is disproportionate from peptide to peptide, also leading to partial separations. Finally, raising the level of acetonitrile to 90% in buffer A (80% in buffer B) produced a much improved separation, with 14 out of a maximum 16 peaks now distinguishable. At this level of acetonitrile, hydrophobic interactions with the column packing are highly unlikely, indicating that achievement of this separation rested solely with differences in hydrophilicity of the analogues. In an analogous manner to the RPLC separation (Fig. 7), since all 16 peptides have the same inherent hydrophilicity, their relative positions in the HILIC/CEX elution order must be dependent on the degree of disruption of the parent GS14 β-sheet structure which, in a similar manner to RPLC, was eluted ca. 10 min after the last eluted GS14 diastereomer.

Fig. 9 compares RPLC with HILIC/CEX for separation of the 16-peptide mixture. The two elution profiles are markedly different, reflecting profound differences in the selectivity of the two HPLC modes as well as in the way disruption of amphipathic β-sheet structure influences peptide elution behaviour between the two modes. Despite the complexity of the peptide elution shifts between RPLC and HILIC/CEX, the more dramatic selectivity shifts (denoted by arrows in Fig. 9) can be readily gleaned. Thus, peptides V1 and L12 are completely co-eluted by RPLC but dramatically separated by HILIC/CEX; peptides L3 and V10 are the last eluted peptides during RPLC, but are eluted towards the middle of the HILIC/CEX elution profile; peptide K2K4K9K11 is the first eluted peptide, by far, during RPLC, but moves into the peptide pack during HILIC/CEX.

4. Conclusions

This study has extended the utility of the mixed-mode HILIC/CEX to the separation of cyclic pep-

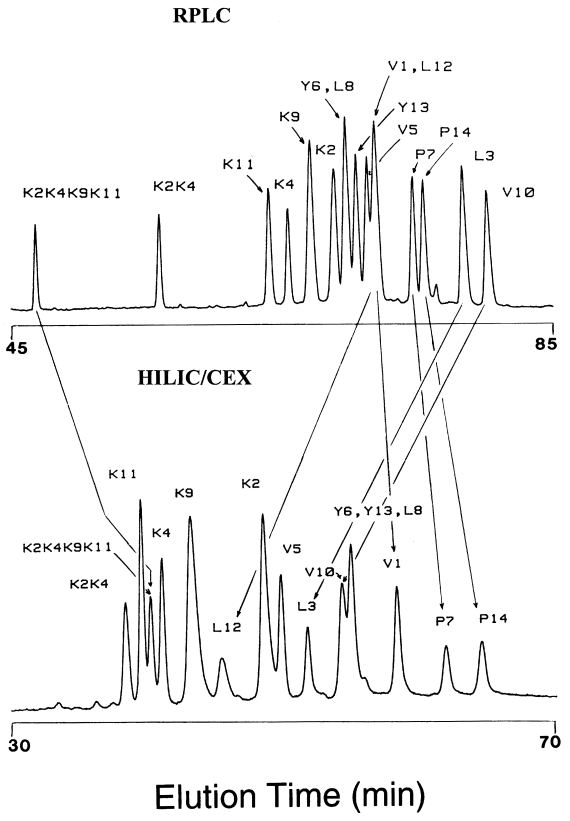

Fig. 9. Comparison of RPLC and HILIC/CEX elution profiles of cyclic peptides. Conditions: RPLC, pH 3 conditions as described in Fig. 7 (70°C run); HILIC/CEX conditions as described in Fig. 8 for 90%/80% run. Peptides were detected by their absorbance at 210 nm.

tides. Such peptides were represented by a model series of amphipathic β-sheet peptides, where the hydrophobic face remained constant while the hydrophilic face was modified, and a second series where disruption of β-sheet structure is effected to varying degrees by enantiomeric side-chain substitution around the ring. Drastically different selectivities between HILIC/CEX and RPLC not only highlighted the exciting potential of the novel HILIC/CEX approach but also further underlined its value as a complementary approach to the ubiquitous reversed-phase mode. In addition, the preparative value of HILIC/CEX should be even further enhanced by the current development (in this laboratory) of completely volatile mobile phases.

Acknowledgements

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