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Capillary zone electrophoresis of α-helical diastereomeric peptide pairs with anionic ion-pairing reagents

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Abstract

The present study uses an unique capillary electrophoresis (CE) approach, that we have termed ion-interaction capillary zone electrophoresis (II-CZE), for the separation of diastereomeric peptide pairs where a single site in the centre of the non-polar face of an 18-residue amphipathic α-helical peptide is substituted by the 19 L- or D-amino acids. Through the addition of perfluorinated acids at very high concentrations (up to 400 mM), such concentration levels not having been used previously in chromatography or CE, to the background electrolyte (pH 2.0), we have been able to achieve baseline resolution of all 19 diastereomeric peptide pairs with an uncoated capillary. Since each diastereomeric peptide pair has the same sequence, identical mass-to-charge ratio and identical intrinsic hydrophobicity, such a separation by CZE has previously been considered theoretically impossible. Excellent resolution was achieved due to maximum advantage being taken of even subtle disruption of peptide structure/conformation (due to the presence of D-amino acids) of the non-polar face of the amphipathic α-helix and its interaction with the hydrophobic anionic ion-pairing reagents. In addition, due to the excellent resolution of diastereomeric peptide pairs by this novel CZE approach, we have also been able to separate a mixture of these closely-related α-helical peptides.

Keywords: Ion-interaction capillary zone electrophoresis; Capillary electrophoresis; Diastereomer separation; Peptides

1. Introduction

The importance of efficient separation of peptide diastereomers should not be underestimated, since pharmaceutical applications of peptides are rapidly expanding, with approximately 35 peptides already being commercialized worldwide, 150–300 in the development stage and 20 in late development [1]. Concerning regulation of stereochemistry issues, the detection (and quantitation) of diastereomeric and enantiomeric impurities are required by various regulatory agencies for pharmaceutical peptides. While reversed-phase high-performance liquid chromatography (RP-HPLC) is generally the favored mode of separation for peptide mixtures [2,3], including peptide diastereomers [2,4–6], we believe that capillary electrophoresis (CE) may also have general utility for separation of such peptides.

In order to examine the ability of CE to resolve peptide diastereomers, we have now applied an unique capillary zone electrophoresis (CZE) approach, which we have termed ion-interaction CZE or II-CZE [7,8], to the separation of 18-residue amphipathic α-helical diastereomeric peptide pairs employing aqueous solutions of perfluorinated acid ion-pairing reagents at high concentrations (up to 400 mM) as background electrolyte (BGE). This is in distinct contrast to their employment as anionic ion-pairing reagents for RP-HPLC, where acid concentrations are very low (generally ca. 10 mM; [2,3,9]). The separation of such peptides with single diastereomeric substitutions of L- or D-amino acids in the centre of the non-polar face of the amphipathic α-helix is a challenging task for CE. Indeed, since each diastereomer not only has the same sequence but also identical mass-to-charge ratio and intrinsic hydrophobicity, such a separation by conventional CZE has been considered theoretically impossible [10]. Our previous results demonstrated the importance of this bidimensional separation mechanism we have proposed, whereby the CZE mode produced a separation of identically charged peptides with negligible secondary structure; within each charged group of peptides, the addition of perfluorinated acids at high concentration allowed resolution of the peptides through differences in peptide hydrophobicity [7,8]. Such a mechanism is unique to CZE, since the separations all occur within the mobile phase

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(BGE) in an uncoated capillary. We now wished to extend this novel CE approach to the separation of amphipathic α-helical peptide diastereomers. Although we have previously reported some success in separating such peptides with more traditional CE approaches [11], baseline resolution of all diastereomeric peptide pairs by a single CE approach was not achieved and was now a goal of the present study.

In addition to demonstrating the potential of this unique CE approach for separation of peptide diastereomers, we also wished to determine whether CE could, in a similar manner to RP-HPLC [3,4,12–15], provide useful information for protein structure/function studies, de novo design of amphipathic α-helical antimicrobial peptides, and proteomics applications in general. For example, modulation of the amphipathicity/stability of α-helices by substitution of L/D-amino acids into the centre of the hydrophobic face enables a quantitative assessment of the effects of such substitutions on such properties as molecular self-association, a key issue both for structure/function [13–16] and for the efficacy of potential peptide antimicrobials [13–16]. The peptide models described in the present study are particularly suited for such applications, as has already been demonstrated via their application in RP-HPLC [4].

2. Experimental

2.1. Materials

Trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA) and heptafluorobutyric acid (HFBA) were obtained from Sigma–Aldrich. Lithium hydroxide was obtained from J.T. Baker (NJ, USA).

2.2. Solutions

BGE solutions were prepared from the corresponding acids neutralized to pH 2.0 with lithium hydroxide.

2.3. Peptides

The amphipathic α-helical peptide diastereomers were synthesized by standard solid-phase synthesis methodology as described previously [4]. A synthetic peptide standard with the sequence Arg–Gly–Gly–Gly–Gly–Leu–Gly–Leu–Gly–Lys–amide (+3 net charge) (denoted S_2) was obtained from the Alberta Peptide Institute (University of Alberta, Edmonton, Canada).

2.4. CE instrumentation and run conditions

All CE runs were carried out on a Beckman–Coulter P/ACE capillary electrophoresis system controlled by MDQ software (version 2.3). Uncoated capillaries (50 μm i.d.) were provided by Beckman–Coulter; in all cases, the shorter aperture (100 μm × 200 μm) was used. The total capillary length, L_t, was 60.2 cm and the effective length (L_d, the length from the injection point to the detection point) was 50 cm. The capillary was thermostated at 15 °C, the lowest value allowed by the instrument. Peptides were detected at 195 nm by UV absorption with photodiode array detection (DAD).

Analyte apparent mobility was calculated by software according to Williams and Vigh's correction [17] for voltage ramp (the time for the voltage to reach the programmed separation voltage; the voltage ramp time was 5 min). As a general rule, we recommend the increase of voltage ramp time until no further increase in current is registered at the end of ramp time. Resolution (R_s) was calculated according to USP (United States Pharmacopoiea) rules.

For sample preparation, peptides were dissolved in water and maintained at 6 °C in the instrument storage compartment. Peptide concentration, injection time and pressure were adjusted to produce a signal of 10–30 mAU. The sample volume was 50–100 μl, with evaporation loss minimized by adding water to the vial supporting the sample microvial. The sample plug was bracketed by a pre-sample plug of water (0.5 p.s.i. for 5 s, p.s.i. = 6894.76 Pa) and a post-sample plug of buffer (0.5 p.s.i. for 15 s). The water plug implemented an on-line sample-preconcentration mechanism [18] and the post-sample plug (together with the voltage ramp [19]) prevented sample loss due to thermal expansion of the sample plug. High current and associated Joule heat is generated during these separations, especially in the presence of perfluorinated acids. Bubble formation and subsequent loss of resolution is avoided by a suitable length of voltage ramp time and by a pressurized run, 50 p.s.i. generally being sufficient. In this way, we were able to perform successful runs even at a current of 300 μA (the maximum limit recommended by the instrument manufacturer). Injection conditions were always below 5 p.s.i., 8 s, such conditions already demonstrated to be well below conditions which may cause sample overloading effects [7].

3. Results and discussion

3.1. Synthetic model peptides

The 18-residue model peptide used in the present study was based on the well-characterized sequence: Ac–EAEKAAKEAEKAAKEAEK–amide, known to exhibit a highly amphipathic α-helical structure (Fig. 1) [20,21]. Alanine was selected to form the hydrophobic face of the helix since it contains the minimum side-chain hydrophobicity required to create an amphipathic α-helix and because of its high intrinsic helical propensity and stability contributions [20,22]. Lysine and glutamic acid allow a potential for α-helix stabilizing intrachain electrostatic attractions at the $i \rightarrow i + 3$ and $i \rightarrow i + 4$ positions at pH 7 (Fig. 1) [23]. All substituted model peptides were synthesized with N^{α}-acetylated and C^{α}-amidated termini

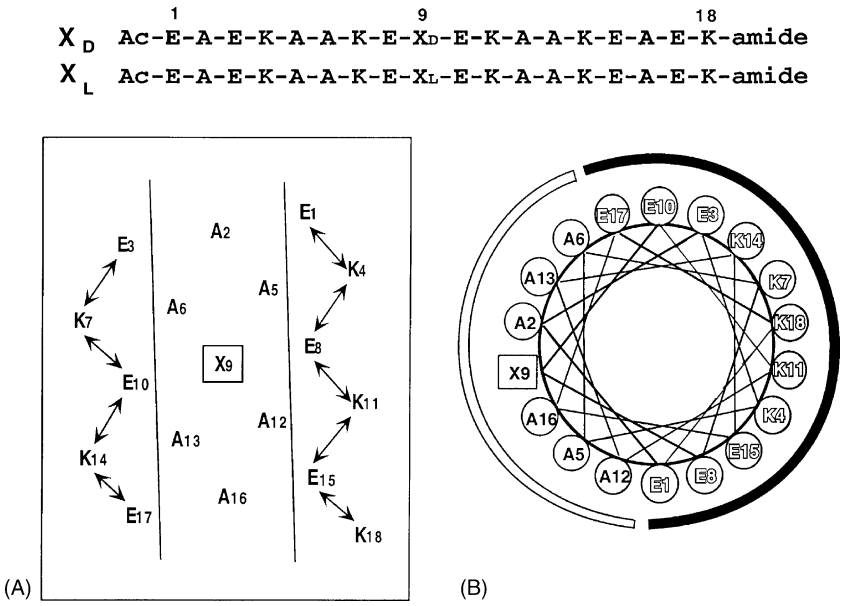

Fig. 1. (A) Helical net and (B) helical wheel representations of the "host" peptide (sequence shown at top). The hydrophobic face (made up of alanine residues) is indicated between parallel lines in (A) and as an open arc in (B). All 19 L- and D-amino acids were substituted at the "guest" site at position 9 (boxed) of the hydrophobic face. In the peptide sequences, X_L and X_D represent L- and D-amino acid residues, respectively. The arrows in (A) indicate potential intra-chain ionic interactions at pH 7. Ac denotes N^{α}-acetyl and amide denotes C^{α}-amide. Peptides are denoted by the one-letter code of the substituted amino acid, e.g., A_L denotes peptide with L-alanine substituted at position 9, P_D denotes peptide with D-proline substituted at position 9, etc.

to reduce the unfavorable dipole interactions of α-helical structure [24].

It has previously been shown [20] that this amphipathic α-helical model exhibits the following features: the helix is single-stranded and non-interacting, enabling determination of the effect of different amino acid substitutions in the non-polar face; there is a uniform environment created by alanine residues surrounding the substitution site in the centre of the non-polar face (position 9; denoted position X in Fig. 1); the small size of the alanine side-chain methyl group ensures minimal interactions with the "guest" amino acid residues; and, finally, the small size of the peptide maximizes the effects of single amino acid substitutions. Two series of model peptide analogues were synthesized, where position 9 in the centre of the non-polar face was substituted either by each of the 19 L-amino acids (X_L series, e.g., I_L denotes L-isoleucine substituted at position 9) or by the 19 D-amino acids (X_D series, e.g., I_D denotes D-isoleucine substituted at position 9). It is important to note that the intrinsic hydrophobicity of the non-polar face of the amphipathic helix is identical for each enantiomeric peptide pair. Despite the inherent helix destabilizing properties of D-amino acids substituted into an α-helix made up entirely of L-amino acids [25–28], previous circular dichroism studies of the peptides used in the present study in both benign medium and in the presence of 50% trifluoroethanol (TFE; a helix-inducing solvent for peptides with potential α-helical conformation [29]) have shown that the presence of the D-amino acids still allows full folding in a hydrophobic environment (with the sole exception of L- or D-proline [4].

The 10-residue peptide standard (S2) described above represents a reference cationic peptide (+3 net charge) with negligible secondary structure (i.e., a "random coil" peptide).

3.2. Approaches to CE separation of model diastereomeric peptide pairs

In a previous study [11], some success in separating these diastereomeric peptide pairs was achieved, particularly through the addition of 3-[(cholamidopropyl)dimethylamino]-1-propanesulfonate (CHAPS) to the BGE and using an uncoated capillary (micellar electrokinetic chromatography, MEKC) or by using a C_8-coated capillary in the presence of 25% TFE or 25% ethanol (open-tubular capillary electrochromatography, OT-CEC). However, no one method was able to separate, to baseline, all 19 peptide pairs. Nonetheless, even the limited success of this previous study suggested an explanation for the separation of specific diastereomeric peptide pairs may lie in conformational differences in the analogues effected by a substitution of an L-amino acid by its D-amino acid counterpart [4]. Any disruption of the amphipathic α-helix in this way would also disrupt the hydrophobicity of the non-polar face, possibly allowing a separation of the peptide pair via the introduction of an hydrophobic mechanism.

An important observation from this previous study [11] was that the separation of specific diastereomeric peptide pairs in the absence of hydrophobic media (e.g., CZE) showed the same general trend as their separations in the

presence of hydrophobic media (e.g., MEKC and OT-CEC), i.e., diastereomers generated by hydrophobic amino acid substitutions were generally better separated than those generated by hydrophilic amino acids. In addition, the CE separation of cationic random coil peptide standards was practically the same in the presence or absence of organized hydrophobic media, the migration order being dictated by peptide hydrophobicity [7,8]. This separation of both helical [11] and random coil [7,8] peptides in the absence of organized hydrophobic media (CZE), coupled with the lack of improvement of the separation of the cationic random coil peptides by the addition of alcohols [7], supported the idea that an hitherto unidentified hydrophobically-mediated mechanism located in the BGE was operating in both cases. The possibility that such a mechanism may be introduced *via* the interaction of ions in the BGE arose from consideration of the mechanism of phase transfer catalysis, where the ion-pairing strength of the catalyst depends on this reagent's hydrophobicity and/or polarizability. For example, hydrophobic quaternary ammonium salts (specifically the quaternary ammonium cation) transport anions from the aqueous to the organic phase; the more hydrophobic the ion-pairing quaternary ammonium salt (such as octyltrimethylammonium > butyltrimethylammonium > tetramethylammonium), the more efficient for specific catalyzed reactions. Adapting this concept to CE, if an ion-pairing (or, more generally, ion–ion interaction) effect was the unidentified hydrophobically-mediated mechanism which resulted in the aforementioned α-helical [11] and random coil [7,8] separations, presumably it would be optimized by an increase in hydrophobicity and/or concentration of an ion-pairing reagent. We set out to test this speculation through the addition of perfluorinated acids to the BGE. Thus, TFA, PFPA and HFBA represent an homologous series of increasingly hydrophobic (TFA < PFPA < HFBA) anionic ion-pairing reagents [2,3,9].

3.3. CE of diastereomeric peptide pairs

Figs. 2 and 3 show the effect of increasing hydrophobicity and concentration of ion-pairing reagent on the separation of representative diastereomeric peptide pairs: A_D/A_L (Fig. 2) and D_D/D_L (Fig. 3). For these two peptide pairs, there is a general trend of improved separation of the peptide pairs with increasing hydrophobicity (TFA < PFPA < HFBA) and concentration of perfluorinated acid. Similar results were obtained, to a greater or lesser extent, with all pairs of peptide diastereomers (with the exception of the Pro analogues). The migration times of the peptides with L-substitutions were greater than the peptides with the corresponding D-substitutions. In RP-HPLC, this can be explained by the disruption of the non-polar face by the D-amino acid substitution which decreases the retention time. Similarly, in CE the disruption of conformation likely affects BGE–analyte interactions, leading to a decrease in migration time. Under optimum conditions (300 mM HFBA), all 19 peptide pairs were separated to baseline (Table 1). Clearly the best results are obtained by the simultaneous increase in magnitude of both hydrophobicity and concentration. It should be noted that, at the concentrations used in the present study (100–400 mM), the perfluorinated acids are present essentially as salts, their pK_a values being ∼0.5; this is in

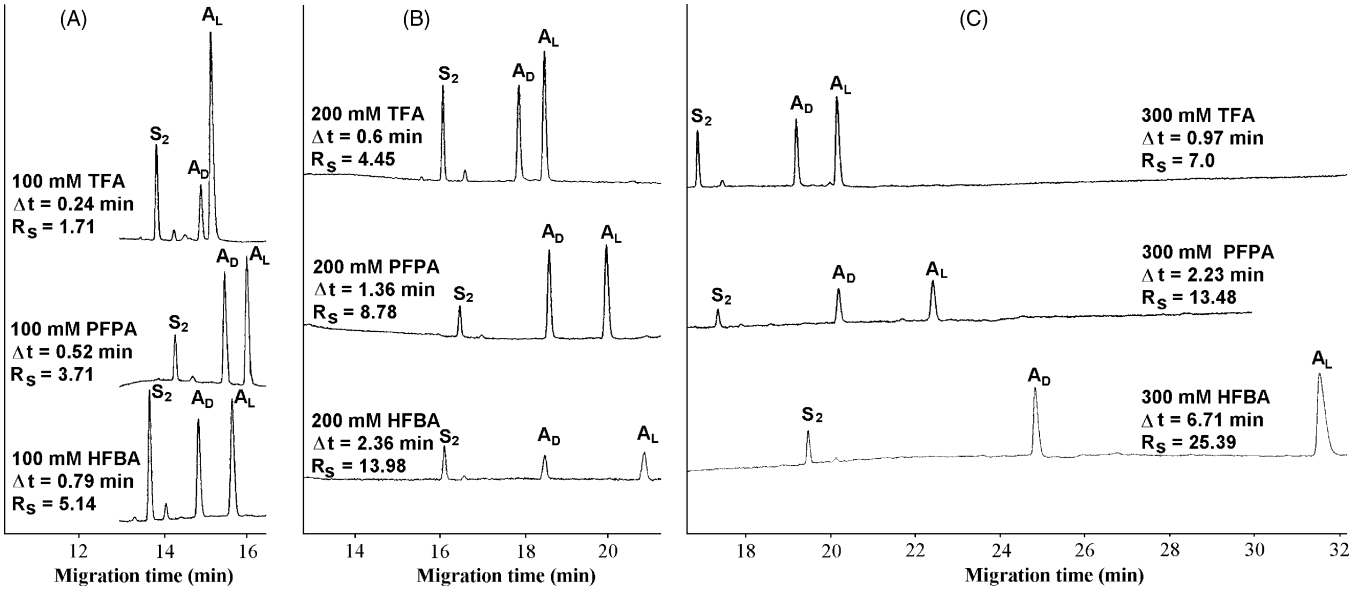

Fig. 2. Effect of hydrophobicity and concentration of perfluorinated acids on CE separation of A_D/A_L diastereomeric peptide pair. Conditions: capillary, uncoated 60.2 cm (50 cm) × 50 μm i.d.; background electrolyte (BGE), various concentrations of aq. TFA, PFPA and HFBA, adjusted to pH 2.0 with lithium hydroxide; applied voltage, 25 kV (direct polarity) with 5 min voltage ramp; temperature, 15 °C; detection; UV absorption at 195 nm. The sequences of A_D and A_L are shown in Fig. 1. S2 represents a 10-residue random coil peptide standard. R_s and Δt denote resolution and difference in migration time, respectively, between the diastereomeric peptide pair.

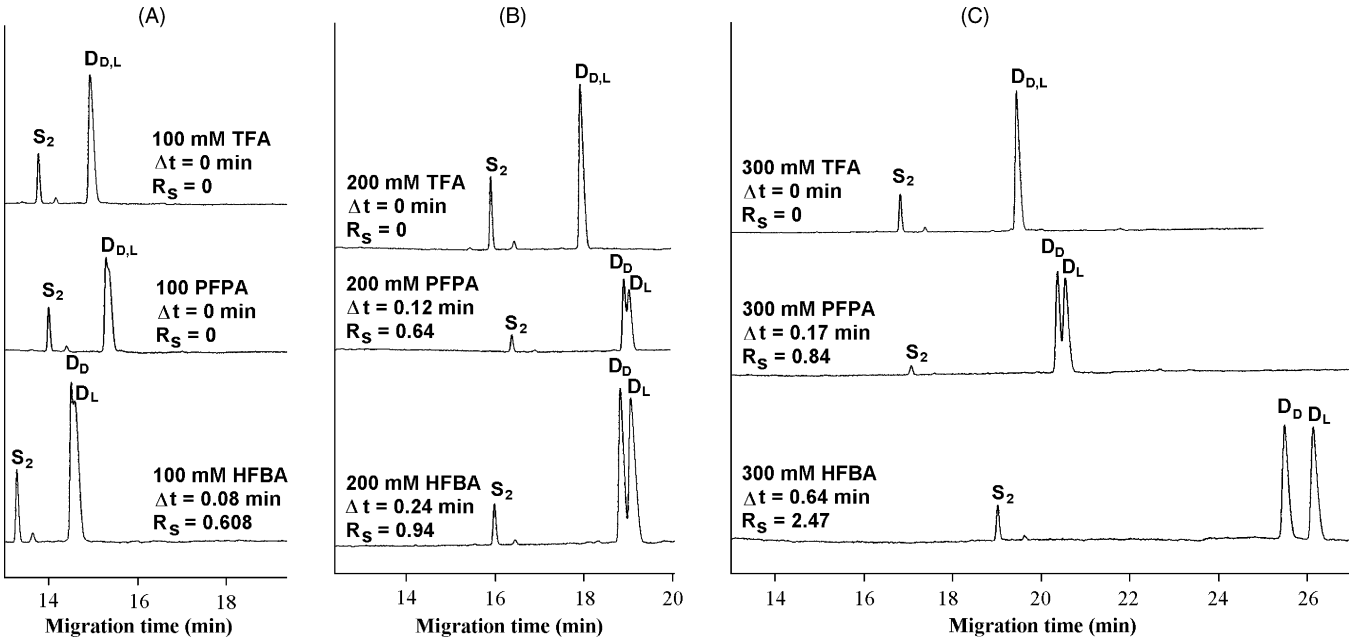

Fig. 3. Effect of hydrophobicity and concentration of perfluorinated acids on CE separation of D_D/D_L diastereomeric peptide pair. Conditions and other details same as Fig. 2. The sequences of D_D and D_L are shown in Fig. 1.

contrast to RP-HPLC where the acid is traditionally employed at much lower concentrations (10 mM–20 mM). It should also be noted that in a previous study [11], no significant differences in migration behavior of these peptides was observed over a pH range of 2–4, indicating that intrachain ion-pairing did not play a role in the separation (due to protonation of the Glu side-chains). In the present study, we have chosen pH 2 to maximize the positive charge (and ion–ion interactions). In addition, this low pH also eliminates alternative explanations for our results, such as the aforementioned intrachain ion-pairing or variability in side-chain dissociation constants potentially caused by hydrophobically-induced peptide conformational changes.

Table 1
Separation of $X_{D,L}$ diasteromers by CZE using an uncoated capillary with 300 mM HFBA as BGE

Uncoated capillary BGE: 300 mM HFBA pH 2 with LiOH		Migration time (min)			Migration time difference $\Delta t = (t_L - t_D)$ (min)	Resolution	Apparent selectivity × $100 = 2(t_L - t_D)/(t_L + t_D)$
#	$X_{D,L}$	S_2 reference (t_R)	Analyte (t_L), X_L	Analyte (t_D), X_D			
1	I	19.51	29.88	23.71	6.17	23.233	23.03
2	V	19.53	31.19	24.73	6.46	24.252	23.1
3	L	19.88	32.41	25.27	7.14	18.907	24.76
4	M	19.66	31.42	25.52	5.9	10.852	20.72
5	C	19.67	28.41	25.14	3.27	12.208	12.21
6	A	19.52	31.59	24.88	6.71	25.388	23.76
7	W	19.51	29.80	25.08	4.72	19.049	17.2
8	F	19.32	29.32	25.13	4.19	15.566	15.39
9	Y	19.42	29.22	25.02	4.2	11.005	15.49
10	T	19.27	26.95	24.39	2.56	9.016	9.97
11	S	19.16	27.16	24.32	2.84	8.757	11.03
12	Q	19.05	29.23	24.45	4.78	16.669	17.81
13	N	19.25	26.79	25.44	1.35	4.552	5.17
14	P	19.13	23.28	22.61	0.67	2.125	2.92
15	H	19.12	27.36	24.84	2.52	8.716	9.21
16	R	18.97	28.32	22.90	5.42	20.052	21.16
17	K	19.07	26.97	21.66	5.31	19.032	21.84
18	E	18.98	29.44	24.48	4.96	16.783	18.4
19	D	19.00	26.12	25.48	0.64	2.467	2.48

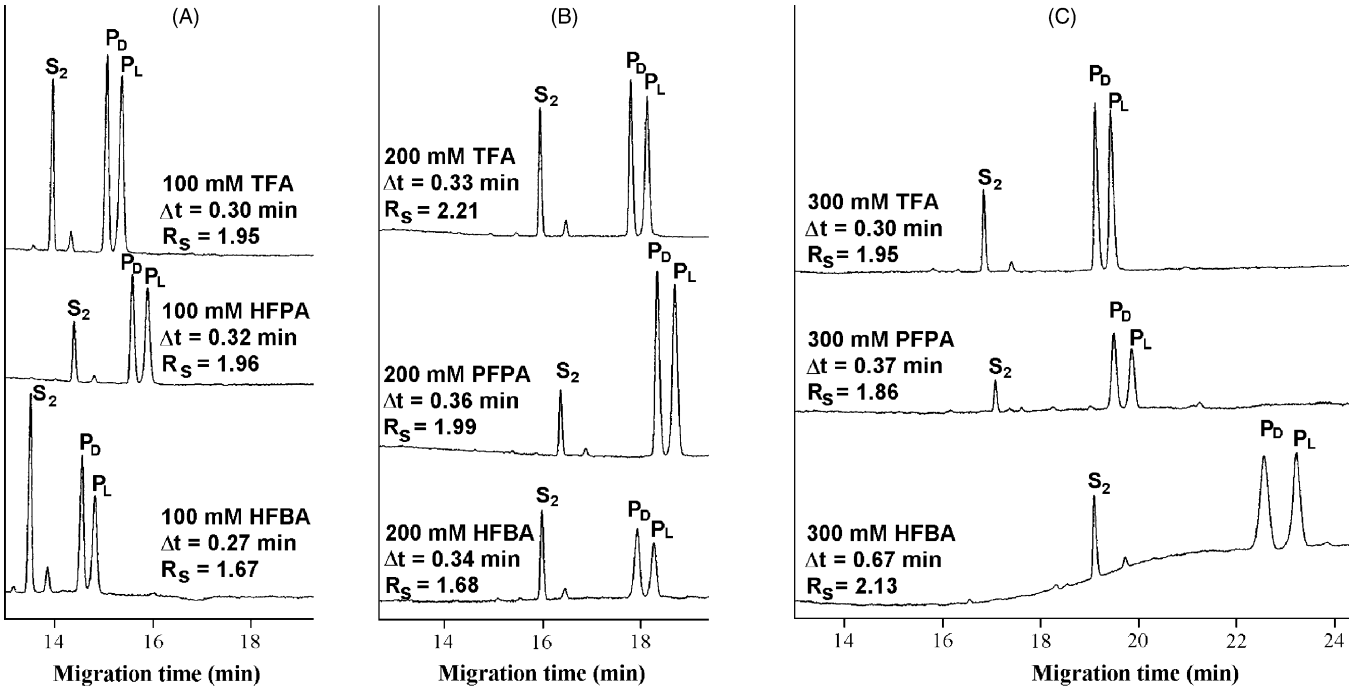

Fig. 4. Effect of hydrophobicity and concentration of perfluorinated acids on CE separation of P_D/P_L diastereomeric peptide pair. Conditions and other details same as Fig. 2. The sequences of P_D and P_L are shown in Fig. 1.

Fig. 2, for the $A_{D,L}$ peptide pair, represents the best results which may be obtained for the majority of the peptide analogues generally and the substituted non-polar residues specifically. In contrast, Fig. 3 ($D_{D,L}$) and Fig. 4 ($P_{D,L}$) represent the most difficult peptide pairs to separate. For the $D_{D,L}$ peptide pair (Fig. 3), satisfactory resolution was only obtained with the most hydrophobic perfluorinated acid (HFBA) at a concentration of 300 mM. The $P_{D,L}$ peptide pair (Fig. 4) represents an interesting phenomenon whereby the separation is practically independent of hydrophobicity and concentration of ion-pairing reagent. In order to examine whether peptide conformational changes potentially induced by the hydrophobic perfluorinated acids could be linked with this CE separation behavior, circular dichroism (CD) spectra of peptides were measured in 150 mM HFBA at pH 2 and 15 °C. For the peptide pairs shown in Figs. 2–4, absolute differences in mean molar ellipticity values between L- and D-diastereomers were in the order $A_{D,L} > D_{D,L} > P_{D,L}$ (both proline analogues exhibited random coil characteristics). Thus, even though some contribution from D- and L-analogue conformational differences may explain the difference in separation of the $A_{D,L}$ and $D_{D,L}$ and the other analogues shown in Table 1, this would be hard to reconcile with the behavior of the P_D and P_L analogues (Fig. 4) which, although both exhibiting negligible secondary structure, are overall more easily separated than D_D and D_L (Fig. 3). However, as noted above, the behavior of the Pro analogues appears to be the sole exception to the overall trend of effects of perfluorinated acids on the separation of the peptide pairs.

3.4. Effect of CE conditions on "apparent selectivity window" of diastereomeric peptide pairs

From Table 1, while the resolution represents a global characterization of separation quality, the selectivity of the separation is reflected in the difference in migration time of a particular diastereomeric peptide pair or, even better, by the apparent selectivity value. Apparent selectivity for the CE separations listed in Table 1 is defined as the ratio between the migration time difference to the mean migration time of the diastereomeric peptide pair (note that the values of apparent selectivity are multiplied by 100 for ease of comparison). Thus, an insight into the general trends of the effectiveness of the perfluorinated acids in the peptide separations may be obtained from a comparison of the apparent selectivities for the separation of all the peptide pairs under all conditions (Fig. 5). We are terming the range of apparent selectivity for all 19 peptide pairs under one set of experimental conditions as the "apparent selectivity window" for those conditions. From Fig. 5, it is clear that the selectivity window is expanding with increasing hydrophobicity and concentration of ion-pairing reagent, although this expansion is not linear, reflecting the different sensitivity of the peptide pairs to the twin optimization parameters. At first glance, the results of Fig. 5 reflect our earlier observation [11] that peptide pairs substituted with hydrophobic amino acids (e.g., Ile, Val, Leu, Met and Ala) are generally better resolved than hydrophilic residues (e.g., Ser, Thr, His, Asn). However, a closer examination shows that this is not a uniform observation, e.g.,

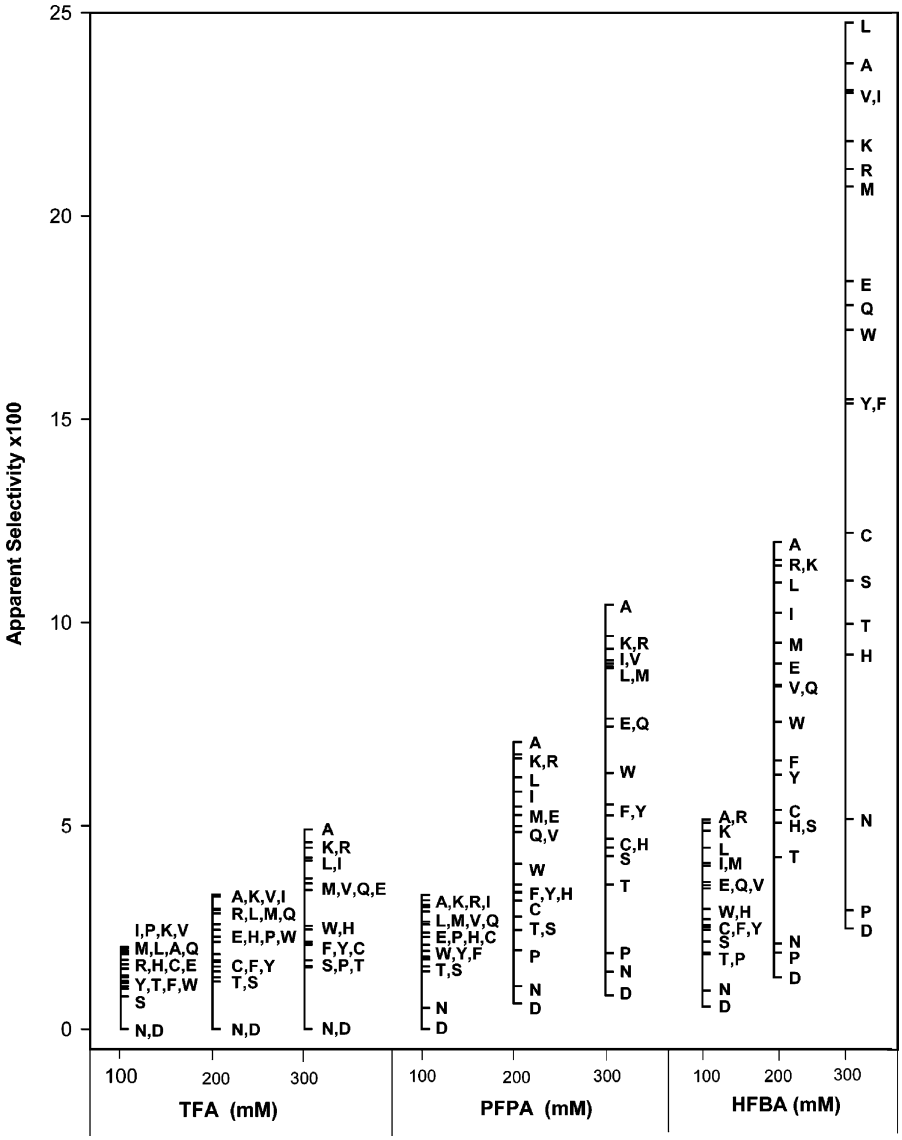

Fig. 5. Effect of hydrophobicity and concentration of anionic ion-pairing reagents on apparent selectivity of CE of diastereomeric peptide pairs. Conditions described in Fig. 2. Amino acid residues substituted into the centre of the hydrophobic face of the model peptides (Fig. 1) are denoted by their one-letter code. Apparent selectivity between diastereomeric peptide pairs is expressed as $2(t_L - t_D)/(t_L + t_D)$, where t_L and t_D are the migration times of the L- and D-amino acid substituted analogues, respectively.

the hydrophilic (and charged) Lys and Arg residues exhibit apparent selectivities (21.84 and 21.16, respectively; Table 1) of a magnitude similar to hydrophobic amino acids listed above, whilst other hydrophobic residues, e.g., Trp, Tyr, Phe, exhibit more moderate, mid-range selectivities (17.2, 15.49, 15.39, respectively; Table 1). In addition, hydrophilic amino acids Gln and Glu also exhibit mid-range selectivities (17.81 and 18.4, respectively; Table 1). Interestingly, from Fig. 5, under optimum conditions (300 mM HFBA), there appear to be distinct groups of diastereomeric peptide pairs as expressed by the magnitude of their apparent selectivities and perhaps based on the character/structure of the side-chain and the ability of a D-amino acid to disrupt the non-polar face of the amphipathic α-helix:

Group 1: amongst side-chains with the highest selectivity are L, A, V, I and M, all non-polar amino acids containing alkyl side-chains; in addition, K and R are positively charged but also contain long, aliphatic side-chains.

Group 2: all three amino acids with aromatic side-chains (Y, W, F) are positioned in this group with the next highest selectivities; also, E and Q are polar, hydrophilic amino acids with the polar functional group attached to the γ-carbon atom.

Group 3: this group is comprised of small, hydrophilic side-chains (C, S, T) or a bulkyl charged group close to the peptide backbone (H) (i.e., side chains attached to the β-carbon).

Group 4: the group with the lowest selectivities includes those amino acids containing polar, hydrophilic groups positioned close to the peptide backbone (N, D) and proline (P) with its cyclic, alkyl side-chain representing part of the peptide backbone.

Overall, it appears that a long *n*-alkyl side-chain should give the best possible separation. Any modification of this side-chain, such as branching or substitutions with charged, uncharged polar, or neutral groups, bulky or otherwise, decreases selectivity. Generally, the closer the modification to the peptide backbone, the more the decrease in selectivity. These observations remained constant even at higher concentrations (e.g., 350–400 mM) of HFBA (data not shown).

It is significant to note that there is a good general correlation between the magnitude of the apparent selectivity of the diastereomeric peptide pairs with the α-helical propensities of the L-analogues as determined by Zhou et al. [20]. For example, the L-analogues of A, R, L, K, M, I of Group 1 (highest selectivities) are amongst the strongest α-helix formers; in contrast, amino acids such as P and D of Group 4 (lowest selectivities) are α-helix disrupters. Thus, it seems reasonable to suggest that substitution of an L-amino acid with strong helix-inducing properties (e.g., alanine) with its D-amino acid counterpart would result in a much more significant disruption of secondary structure (and, hence, the apparent hydrophobicity of the non-polar face of the amphipathic α-helix) than that of a helix disrupter, e.g., D_L to D_D substitution, where the subsequent change in structure would be less. Overall, therefore, the difference in apparent hydrophobicity between the non-polar faces of such analogues as A_L and A_D would be significantly greater than between analogues such as D_L and D_D. Such observations support the view that conformational differences between diastereomeric peptide pairs do, indeed, contribute to the baseline separations presented in this study as well as the ability to resolve more complex mixtures of such peptides (shown below).

3.5. CE of a mixture of diastereomeric peptide pairs

Fig. 6 clearly illustrates the excellent CE separation of a mixture of peptide diastereomers which may be obtained through an increase in HFBA concentration. This particular peptide mixture is comprised of analogues substituted with potentially charged side-chains. However, at pH 2.0, the side-chains of Glu and Asp will be uncharged; (overall net charge of +5 on the peptides), while those of Lys, Arg and His will be charged (overall net charge of +6 on the peptides). In comparison with 300 mM HFBA (Table 1), apparent selectivities between diastereomeric peptide pairs are continuing to increase with increasing HFBA concentration to 400 mM HFBA. These values now are 2.49, 12.66, 25.55, 28.41 and 29.72 for D_D/D_L, H_D/H_L, E_D/E_L, R_D/R_L and K_D/K_L, respectively, compared to 2.48, 9.21, 18.40, 21.16 and 21.84, respectively, in 300 mM HFBA (Table 1). An interesting observation from Fig. 6 is the difference in selectivity of the peptide separation at different HFBA concentrations due to the migration behavior of the His-substituted analogues, which migrate relatively faster with increasing HFBA concentration relative to the other analogues. Thus, at 300 mM HFBA, H_D migrates just prior to E_D as a poorly separated doublet; at 350 mM HFBA, H_D is now baseline resolved from E_D and has a longer migration time than the latter analogue; finally, at 400 mM HFBA, H_D comigrates with D_D, albeit even better separated from E_D. Similarly, at 300 mM HFBA, H_L migrates a little longer than K_L, albeit as a poorly separated doublet; at 350 mM HFBA, H_L is almost baseline resolved from K_L; finally, at 400 mM HFBA, H_L is now well resolved from K_L, its migration time significantly longer than the latter analogue.

3.6. Differences in the mechanism of separation of peptides between RP-HPLC and II-CZE in the presence of anionic ion-pairing reagents

Anionic ion-pairing reagents have seen widespread use for peptide separations in RP-HPLC for over two decades [2,3,9]. Aside from their high hydrophobicities, these reagents have the advantage of low pK_a values, enabling their use at low pH values (e.g., pH 2.0), thus maximizing the positive charge on peptides. Their mode of action is via interactions between the negatively charged anions of these reagents (TFA^-, $PFPA^-$, $HFBA^-$) and positively charged groups, in the peptide sequence (lysine, arginine, histidine side-chains; free α-amino group). The greater the net positive charge on the peptide, the greater the effect of a particular hydrophobic ion-pairing reagent, which subsequently translates into longer RP-HPLC elution times [9]. Increasing concentrations of such ion-pairing reagents also serve to increase RP-HPLC retention times of positively charged peptides [9]. Note that such increases in peptide retention times via increasing hydrophobicity (TFA < PFPA < HFBA) and/or concentration of ion-pairing reagent reflect an increase in effective hydrophobicity of the peptides, i.e., modulation of the overall hydrophobicity of the peptides through manipulation of the mobile phase. Thus, peptides with a high number of positively charged groups become much more hydrophobic than peptides with few charged groups. However, although changes in ion-pairing reagent and/or concentration affects the relative elution order of peptides, it is the hydrophobicity of the reversed-phase matrix that separates the peptides based on differences in overall peptide hydrophobicity, i.e., the more hydrophobic the peptide, the greater the affinity of the peptide for the stationary phase.

Note that, for RP-HPLC separations, two phases are required to achieve peptide separations based on differences in peptide hydrophobicity, i.e., the aqueous mobile phase and the hydrophobic stationary phase. Here, we are suggesting a quite distinct mechanism for II-CZE (which remains a

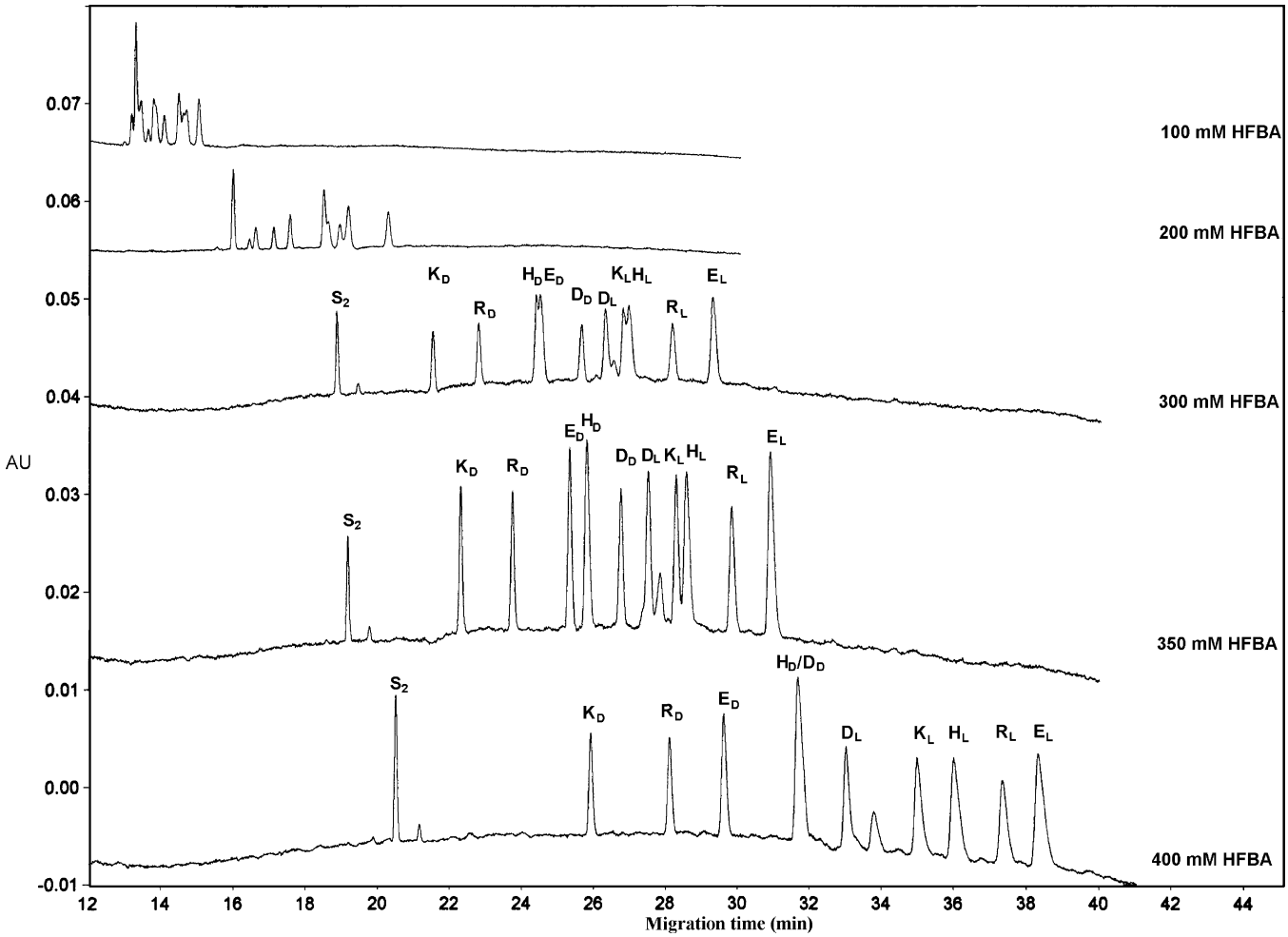

Fig. 6. Effect of HFBA on CE separation of a mixture of diastereomeric peptide pairs. Conditions same as Fig. 2, with BGE represented by various concentrations of aq. HFBA, adjusted to pH 2.0 with lithium hydroxide. The sequences of the peptide are shown in Fig. 1. S2 represents a 10-residue random coil peptide standard. The peak between D_L and K_L is an impurity.

working hypothesis for further studies), where we are able to exploit the hydrophobicity differences between peptides within a single phase, i.e., the BGE, since CZE separations are taking place within an uncoated capillary. We believe the key to success of the II-CZE approach lies in providing an hydrophobic medium within the BGE substantial and uniform enough to separate peptides based on their different affinities for this hydrophobic environment. Thus, the use of hydrophobic anionic ion-pairing reagents such as HFBA increases the hydrophobic environment of the solution to a level where interaction of the peptides with this hydrophobic BGE is able to separate the peptides based on just subtle differences in peptide hydrophobicity. In other words, the hydrophobicity of the high concentrations of ion-pairing reagents (approximately 40-fold higher than those used in RP-HPLC) replaces the hydrophobic matrix of RP-HPLC, i.e., the separation in II-CZE is effected through the interaction or affinity of the peptides for the hydrophobic environment created in the BGE by the high concentrations of perfluorinated acids. It is important to note that, although we term this approach "ion-interaction" CZE, this does not imply that the ion-pairing property of the acids represent the only critical factor in successful peptide separations by this method. Rather, it refers to interactions between the peptides and perfluorinated anions within the single phase BGE, albeit the critical interactions are between the hydrophobic groups in the peptides and the hydrophobic component of the anions. Certainly, the negatively charged anions are able to interact electrostatically with the positively charged cationic groups of the peptides; however, at the very high concentrations of acids used in II-CZE, this ion-pairing factor is maximized, but negligible compared to the overwhelming influence of the hydrophobic environment of the BGE on peptide separations engendered by such concentrations. Support for this view lies in our previous success in using II-CZE to separate three groups (+1, +2 or +3 positive charges) of random coil peptides with identical substitutions of hydrophobic amino acids within their sequences [8]. Thus, for

example, a substitution of a Val for the less hydrophobic Ala produced the same increase in migration time for the former analogue compared to the latter no matter within what charged group of peptides such a substitution had been made, i.e., the affinity for the hydrophobic solution phase produced by the high concentrations of anionic ion-pairing reagents is simply greater for the Val-substituted peptide compared to the Ala-substituted analogue, with no or negligible impact of ion-pairing between the peptides and the ion-pairing reagents. In a similar fashion, in the present study, separations between diastereomeric peptide pairs are achieved by differing affinities of the non-polar faces of the amphipathic peptides (resulting from the change in apparent overall hydrophobicity of this face following an L- to D-amino acid substitution) for the hydrophobic environment of the BGE.

4. Conclusions

The present study demonstrates a novel CE approach to separate, in the absence of organic solvents, peptides of identical sequence, mass-to-charge ratio and inherent hydrophobicity, differing only in a single L- to D-amino acid substitution. We attribute this success primarily to an hydrophobic mechanism effected through the use of extremely high concentrations of perfluorinated acid anionic ion-pairing reagents and suggest terming this CE mode as ion-interaction capillary zone electrophoresis (II-CZE). In addition, we also believe that conformational differences between diastereomeric peptide pairs also contribute significantly to the successful separations, where the differences in hydrophobicity of the non-polar face of the amphipathic α-helix and its interaction with the hydrophobic anionic ion-pairing reagent is effecting the separation. Although small racemic or diastereomeric peptides have been previously separated by CE methodology, such approaches generally depend on additives to the BGE where bulky substituents form a complex with the peptides, such complexes then being separated, or where peptides are derivatized (i.e., chemically modified) prior to separation (references 30 and 31 represent useful introductions to such methods) [30,31]. In contrast, the present study represents the first instance where such manipulations are unnecessary, requiring only a selective interaction of unmodified peptides with the counterion provided by the BGE. Indeed, we believe that the present results represent a starting point for developing CE methods for the pharmaceutical and life sciences fields, including proteomic applications. In addition, the separation of diastereomers allows a rigorous assessment of the resolving power of CE in general as well as that of specific CE instrumentation.

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References

[1] L. Sutherland, Gen. Eng. News 13 (2003) 1.
[2] C.T. Mant, R.S. Hodges (Eds.), HPLC of Peptides and Proteins: Separation, Analysis and Conformation, CRC Press, Boca Raton, FL, 1991.
[3] C.T. Mant, R.S. Hodges, in: K.M. Gooding, F.E. Regnier (Eds.), HPLC of Biological Macromolecules, 2nd ed., Marcel Dekker, New York, 2002, p. 433.
[4] Y. Chen, C.T. Mant, R.S. Hodges, J. Peptide Res. 59 (2002) 18.
[5] M. Gazdag, K. Mihályfi, S. Görög, Anal. Chim. Acta 352 (1997) 231.
[6] D. Koval, V. Kašicka, J. Jirácek, M. Collinsová, J. Sep. Sci. 26 (2003) 653.
[7] T.V. Popa, C.T. Mant, R.S. Hodges, Electrophoresis 24 (2003) 4197.
[8] T.V. Popa, C.T. Mant, R.S. Hodges, Electrophoresis 25 (2004) 1219.
[9] D. Guo, C.T. Mant, R.S. Hodges, J. Chromatogr. 386 (1987) 205.
[10] M. Thorsteinsdóttir, J. Beijersten, D. Westerlund, Electrophoresis 16 (1995) 564.
[11] T.V. Popa, C.T. Mant, R.S. Hodges, Electrophoresis 25 (2004) 94.
[12] L.H. Kondejewski, M. Jelokhani-Niaraki, S.W. Farmer, B. Lix, C.M. Kay, B.D. Sykes, R.E. Hancock, R.S. Hodges, J. Biol. Chem. 274 (1999) 13181.
[13] D.L. Lee, C.T. Mant, R.S. Hodges, J. Biol. Chem. 278 (2003) 22918.
[14] C.T. Mant, Y. Chen, R.S. Hodges, J. Chromatogr. A 1009 (2003) 29.
[15] C.T. Mant, B. Tripet, R.S. Hodges, J. Chromatogr. A 1009 (2003) 45.
[16] L.H. Kondejewski, D.L. Lee, M. Jelokhani-Niaraki, S.W. Farmer, R.E. Hancock, R.S. Hodges, J. Biol. Chem. 277 (2002) 67.
[17] B.A. Williams, G. Vigh, Anal. Chem. 67 (1995) 3079.
[18] M. Albin, P.D. Grossman, D.E. Moring, Anal. Chem. 65 (1993) 489A.
[19] J.H. Knox, K.A. McCormack, Chromatographia 38 (1994) 279.
[20] N.E. Zhou, O.D. Monera, C.M. Kay, R.S. Hodges, Protein Peptide Lett. 1 (1994) 114.
[21] T.J. Sereda, C.T. Mant, F.D. Sönnichsen, R.S. Hodges, J. Chromatogr. A 676 (1994) 139.
[22] O.D. Monera, T.J. Sereda, N.E. Zhou, C.M. Kay, R.S. Hodges, J. Peptide Sci. 1 (1995) 319.
[23] J.M. Scholtz, H. Qian, V.H. Robbins, R.L. Baldwin, Biochemistry 32 (1993) 9668.
[24] K.R. Shoemaker, P.S. Kim, E.J. York, J.M. Stewart, R.L. Baldwin, Nature 326 (1987) 563.
[25] M.I. Aguilar, S. Mougos, J. Boublik, J. Rivier, M.T.W. Hearn, J. Chromatogr. 646 (1993) 53.
[26] S. Rothemund, M. Beyermann, E. Krause, G. Krause, M. Bienert, R.S. Hodges, B.D. Sykes, F.D. Sönnichsen, Biochemistry 34 (1995) 12954.
[27] S. Rothemund, E. Krause, M. Beyermann, M. Dathe, M. Bienert, R.S. Hodges, B.D. Sykes, F.D. Sönnichsen, Peptide Res. 9 (1996) 79.
[28] E. Krause, M. Bienert, P. Schmieder, H. Wensehuh, J. Am. Chem. Soc. 122 (2000) 4865.
[29] J.W. Nelson, N.R. Kallenbach, Biochemistry 28 (1989) 5256.
[30] H. Wan, L.G. Blomberg, J. Chromatogr. A 758 (1997) 303.
[31] H. Wan, L.G. Blomberg, J. Chromatogr. A 875 (2000) 43.