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Journal of Chromatography A, 689 (1995) 219–226

JOURNAL OF CHROMATOGRAPHY A

Recognition of α-helical peptide structures using high-performance liquid chromatographic retention data for D-amino acid analogues: influence of peptide amphipathicity and of stationary phase hydrophobicity

S. Rothemund[a], E. Krause[a,*], M. Beyermann[a], M. Dathe[a], H. Engelhardt[b], M. Bienert[a]

[a]*Forschungsinstitut für Molekulare Pharmakologie, Alfred-Kowalke-Strasse 4, 10315 Berlin, Germany*
[b]*Angewandte Physikalische Chemie, Universität des Saarlandes, 66123 Saarbrücken, Germany*

First received 14 June 1994; revised manuscript received 21 September 1994

Abstract

The reversed-phase HPLC behaviour of double D-amino acid replacement sets of amphipathic and non-amphipathic helix-forming peptides consisting exclusively of leucine, lysine and alanine residues was studied on different polymer-encapsulated silica-based stationary phases. Plotting the retention times versus the position of D-amino acid substitution gives a characteristic pattern showing decreased retention times in the helical region. The retention time profile obtained using an amphipathic α-helix is caused by disturbance of the preferred binding domain of the stationary phase-bound peptide. However, the effect is similar but less pronounced using a non-amphipathic helical peptide that is unable to interact by a preferred binding site. The results demonstrate that reversed-phase HPLC data for peptide analogues provide an indication event of a non-amphipathic helical structure in peptides.

1. Introduction

In recent years, reversed-phase chromatography has become a useful tool for studying the structure of peptides induced by hydrophobic interactions [1]. Peptides consisting of more than 13–15 amino acid residues may form a secondary structure during RPC, influencing the retention behaviour [2]. The formation of an amphipathic α-helical structure with a hydrophobic binding domain causes a considerable increase in retention time in reversed-phase chromatography [3–6]. As incorporation of D-amino acids in α-helical peptides is known to decrease the helical content [7,8], D-amino acid replacement sets of KLALKLALKALKAALKLA-NH_2 and neuropeptide Y (NPY) were synthesized and used for the study of HPLC retention behaviour [9,10]. The results demonstrated that D-amino acid replacements destabilize the amphipathic α-helix leading to a decrease in hydrophobic interaction during reversed-phase HPLC. The effect is enhanced by substitution of two adjacent D-amino acids and correlates well with the circular dichro-

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(94)00909-0

ism (CD)-determined helicity [9]. Thus, the "retention profile" that results from plotting the retention time versus the position of D-amino acid replacement provides an indication of amphipathic α-helical secondary structure in peptides. The effect of D-amino acid substitutions on the retention behaviour explained by disturbance of the hydrophobic binding site has so far been exclusively investigated by examining amphipathic helicies.

This study was carried out to evaluate the relationship between amphipathicity of a helical peptide and the effect of D-amino acid replacements on HPLC retention time. Non-amphipathic and amphipathic model peptides consisting exclusively of leucine, lysine and alanine having the same amino acid composition were synthesized and used in correlation studies of helicity versus HPLC retention time. In an attempt to investigate the influence of the stationary phase on the retention behaviour of the D-amino acid replacement sets, HPLC studies were carried out on polymer-encapsulated silica-based stationary phases with different hydrophobicities.

2. Experimental

2.1. Reversed-phase HPLC

Chromatographic measurements were performed on a Shimadzu LC-10A gradient HPLC system consisting of two LC-10AD pumps, a Sil-10A autoinjector, an SPD-M10A diode-array detector operating at 215 nm and a CLASS-LC10 software package. The sample concentration was 1 mg/ml peptide in 0.1% trifluoroacetic acid (TFA) with an injection volume of 10 μl. Runs were performed at ambient temperature and at a mobile phase flow-rate of 1 ml/min. Mobile phase A was 0.1% TFA in water and B was 0.1% TFA in acetonitrile–water (1:1, v/v), and the retention times of peptides were determined using a linear gradient from 30 to 95% B in 40 min.

2.2. Columns

Analytical runs were carried out on silica encapsulated with butyl acrylate (PolyEncap A300) (250 × 4.6 mm I.D., 5 μm) (Bischoff Analysentechnik, Leonberg, Germany), silica encapsulated with butylacrylamide (BAA) and silica encapsulated with octadecyl acrylate (ODA). The BAA and ODA phases were prepared by copolymerization of vinyl-derivatized silica (Nucleosil 5 μm, 300 Å pore diameter) with the corresponding acrylic acid derivatives as described previously [11]. By varying the acrylic acid derivatives it is possible to prepare different kinds of reversed-phase stationary phases. The relative hydrophobicity of these packings is BAA < PolyEncap < ODA. Columns (250 × 4.6 mm I.D.) were slurry packed using carbon tetrachloride–2-propanole (1:1, v/v).

2.3. Peptide synthesis and purification

The 18-mer model peptides and the corresponding D-amino acid analogues were synthesized automatically (Abimed AMS 422 multiple peptide synthesizer) by solid-phase methods using standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry (TentaGel S RAM resin, 0.21 mmol/g; Rapp Polymere, Tübingen, Germany), 2-(1H - benzotriazol - 1 - yl - oxy - 1,1,3,3 - bispentamethylene-uronium tetrafluoroborate (TBPipU) coupling [12] and deblocking with piperidine–dimethylformamide (20:80). The peptides were N-terminally acetylated with acetic anhydride–dimethylformamide–diisopropylethylamine (20:70:10, v/v/v) and the final cleavage was performed with trifluoroacetic acid–thioanisole–*m*-cresol (90:5:5, v/v/v) for 3 h at ambient temperature. Purification of the crude peptides was carried out by preparative HPLC on PolyEncap A300, 10 μm (250 × 20 mm I.D.) (Bischoff Analysentechnik) to give final products >95% pure by HPLC analysis. The peptides were characterized by matrix-assisted laser desorption ionization mass spectrometry (MALDI II; Kratos, Manchester, UK), which gave the expected $[M+H]^+$ mass

peaks and correct amino acid analyses (Biotronik-Eppendorf LC 3000).

2.4. Circular dichroism measurements

CD measurements were carried out on a Jasco Model 720 spectrometer in trifluoroethanol–water from 185 to 260 nm. Calculation of the percentage helicity content (h) was performed according to Chen et al. [13]:

$$h(\%) = ([\Theta]_{222} - [\Theta]^{0}_{222})/[\Theta]^{100}_{222}$$

where $[\Theta]_{222}$ is the determined mean residue ellipticity at 222 nm. The values of $[\Theta]^{0}_{222}$ and $[\Theta]^{100}_{222}$, corresponding to 0 and 100% helic content, respectively, at 222 nm, are estimated to be -2340 and $-30\,300°cm^2/dmol$, respectively. For precise determination of peptide concentrations, quantitative amino acid analysis was used.

3. Results and discussion

3.1. Design of α-helices with different amphipathicity

Derived from secondary structure-forming peptides which consisted of leucine, lysine and alanine [2], model peptides with 18 residues and identical amino acid composition were synthesized. Differences in the sequence lead to different distributions of hydrophobic and hydrophilic amino acids, as shown in the helical wheel projections in Fig. 1. In the amphipathic peptide KLLK the eight leucines are localized at one site, whereas in the peptide KLAL four leucines are clustered at two different places on the hydrophobic face of the helix. In both amphipathic peptides, KLLK and KLAL, the five hydrophilic lysines are exclusively located at one site of the helix. In contrast, the hydrophilic and hydrophobic amino acid residues of the non-amphipathic peptide KALK are well distributed around the α-helix. However, all peptides synthesized have a strong potential to form an α-helix according to the Chou–Fasman parameter [15]. Since acetylation of the N-terminus and amidation of the C-terminus are able to suppress unfavourable charge–dipole interactions of the helix and therefore increase the helix stability [16], the peptides were N-terminally acetylated and C-terminally amidated.

In order to cause a reliable destabilization along the helical peptide sequence, KLLK, KLAL and KALK analogues having successive replacements of two adjacent amino acids with the corresponding D-amino acids (DD-replacement set) were synthesized.

3.2. Determination of helicity by circular dichroism spectroscopy

The helical potential of the model peptides and of the double D-amino acid replacement sets was determined by circular dichroism. The fractional helicities (h) of KLLK, KLAL and KALK measured in acidic aqueous solutions (0.01 M H_3PO_4) remain less than 15%. As addition of 2,2,2-trifluoroethanol (TFE) is known to induce the formation of helical structures in peptides [17], CD spectroscopy were carried out in 0.01 M H_3PO_4–TFE mixtures. The maximum helical content of KLLK, KLAL and KALK was determined with 50% TFE to reach 79, 82 and 86%, respectively. Further, a change in peptide concentration from $5 \cdot 10^{-6}$ to $1 \cdot 10^{-3}$ M has no influence on CD, indicating that the three peptides consist mainly of monomeric helical structures in the presence of TFE. The CD spectra of some of the KLLK analogues are shown in Fig. 2 and the fractional helicities of the complete DD-replacement sets are summarized in Table 1. The results demonstrate that double D-amino acid substitutions in the amphipathic KLLK and KLAL and also in the non-amphipathic KALK decrease the fractional helicity, especially in the center of the helix. The influence of the position of double D-amino acid substitution on the helicity of the three model peptides is shown in Fig. 3. The obtained helicity profiles of the amphipathic and non-amphipathic peptides are similar and confirm a general destabilization of TFE-induced helical structures by double D-amino acid incorporation.

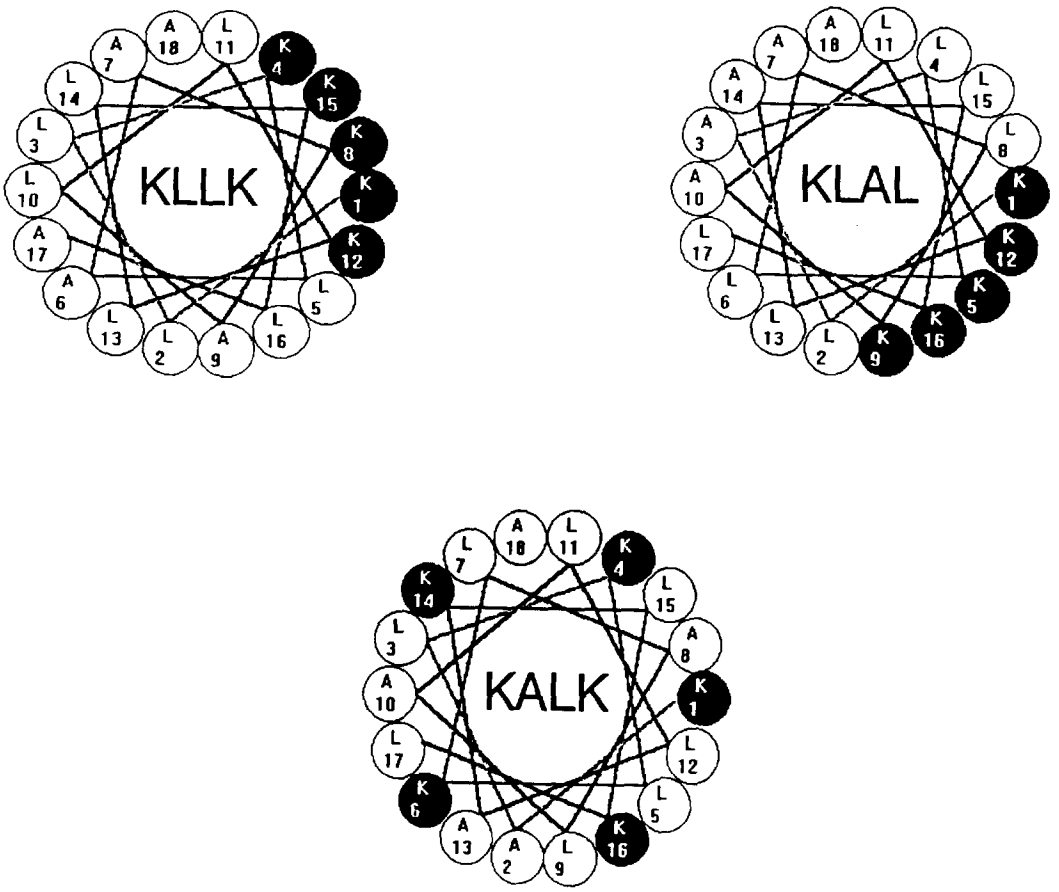

Fig. 1. Helical wheel projections [14] of KLLK, KLAL and KALK. The lysine residues are denoted by black circles.

3.3. Retention behaviour of the double D-amino acid replacement sets

The retention times of the model peptides and of the DD-replacement sets determined using a PolyEncap A300 column are given in Table 1. The order of the retention times of KLLK, KLAL and KALK (31.3, 29.4 and 19.0 min) correlates with the amphipathicity of the helices in line with Zhou et al. [5]. The more amphipathic the peptide, the longer is the retention time. These results indicate that the HPLC conditions are helix inducing and all of the model peptides are bound in a helical arrangement. Next, the effect of double D-amino acid replacement on the retention time was investigated. As shown in Fig. 4, the replacement of two amino acids by their D-isomers decreases the retention time in reversed-phase HPLC. This effect is stronger in the centre of the helix, whereas replacements close to either end have comparatively small effects because the lack of hydrogen bonds decreases the stability at the helix ends. A similar shape of a helix-destabilizing effect was described by Chakrabarty et al. [18] for a series of single alanine–glycine substitutions in a model helix. The results obtained with KLLK, KLAL and KALK confirm that the

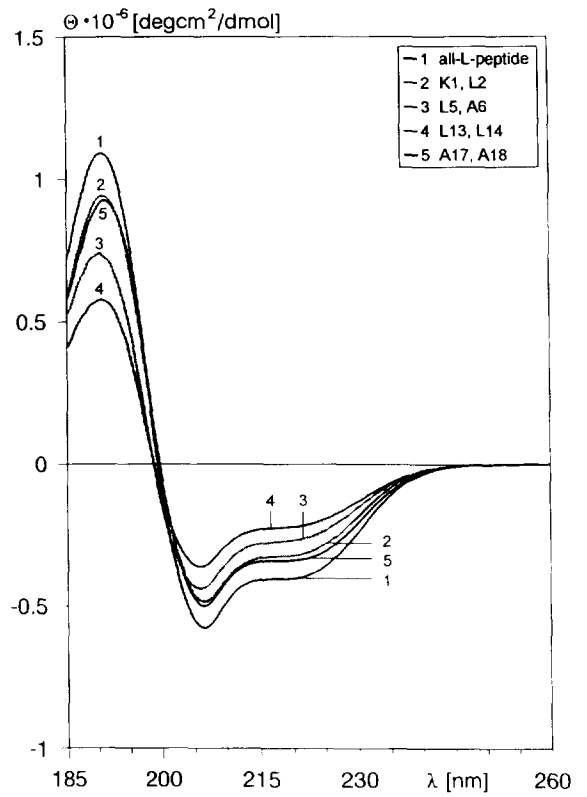

Fig. 2. CD spectra of KLLK and some of its analogues with double D-amino acid substitutions in TFE–0.01 *M* H_3PO_4 (1:1, v/v), $5 \cdot 10^{-5}$ mol/l.

differences in retention behaviour caused by DD-replacement correlate well with the helicity. It is remarkable that the replacement by double D-amino acids also in a non-amphipathic peptide, which is unable to form a preferred hydrophobic domain, leads to a characteristic retention time profile [Δ (retention time) versus substitution position]. Only the magnitude of the differences in retention times between all-L analogues and corresponding D-amino acid analogues is attributed to the disturbance of amphipathicity. Consequently, large differences (8–10 min) were obtained for the amphipathic peptides KLLK and KLAL, whereas the retention time profile of the DD-replacement of the non-amphipathic KALK which is caused exclusively by the destabilization of the helical structure is less pronounced (Fig. 4).

The formation of the energetically favoured helix structure during the chromatographic process depends on the hydrophobicity of the stationary phase [19] and therefore should be considered to examine secondary structure effects of peptides and proteins. Consequently, the retention time profiles of KLLK, KLAL and KALK were determined on different polymer-encapsulated silica-based stationary phases coated with butylacrylamide (BAA), butyl acrylate (PolyEncap A300) and octadecyl acrylate (ODA), as previous studies [20] have demonstrated that this kind of stationary phase is suitable for chromatographic separations of hydrophobic amphipathic peptides. The retention times of the all-L-peptides are given in Table 2. According to the relative hydrophobicity of these stationary phases, all peptides elute much later on ODA than on the less hydrophobic BAA column. Additionally, the retention times correlate on all three phases with the amphipathicity of the model peptides, e.g., the retention orders KLLK > KLAL > KALK were identical.

The retention time profiles resulting from the DD-replacement sets of KLLK, KLAL and KALK are shown in Fig. 5. Comparing the three stationary phases, the typical shapes with a significant decrease in the centre and only a slight effect at the ends are similar. Most interestingly, examining the non-amphipathic KALK, the largest retention time differences between all-L and D-amino acid analogues were obtained by using the less hydrophobic stationary phase BAA. The measurement on BAA leads to a more pronounced retention time profile, in which the retention times of peptides with N-terminal replacements in positions 1–4 are already reduced. An explanation for these findings might be that (i) the high ligand hydrophobicity of both ODA and PolyEncap leads to a stronger capability to induce and/or stabilize the helix and therefore equilize at least in part the effect of the D-amino acid substitutions and (ii) the less hydrophobic BAA is also able to induce a helical structure of the phase-bound KALK, but the

Table 1
Fractional helicity and retention times of D-amino acid analogues

Peptide sequence[a]	D-Amino acid substitution	Retention time[b] (min)	Helicity, h (%)
KLLK			
Ac-KLLKLAAKALLKLLKLAA-NH_2	None	31.3	79
Ac-KLLKLAAKALLKLLKLAA-NH_2	K1, L2	27.8	63
Ac-KLLKLAAKALLKLLKLAA-NH_2	L3, K4	24.9	53
Ac-KLLKLAAKALLKLLKLAA-NH_2	L5, A6	22.3	51
Ac-KLLKLAAKALLKLLKLAA-NH_2	A7, K8	23.1	52
Ac-KLLKLAAKALLKLLKLAA-NH_2	A9, L10	20.7	44
Ac-KLLKLAAKALLKLLKLAA-NH_2	L11, K12	23.7	51
Ac-KLLKLAAKALLKLLKLAA-NH_2	L13, L14	23.9	42
Ac-KLLKLAAKALLKLLKLAA-NH_2	K15, L16	27.7	56
Ac-KLLKLAAKALLKLLKLAA-NH_2	A17, A18	28.6	69
KLAL			
Ac-KLALKLALKALKLALKLA-NH_2	None	29.4	82
Ac-KLALKLALKALKLALKLA-NH_2	K1, L2	27.6	69
Ac-KLALKLALKALKLALKLA-NH_2	A3, L4	24.3	52
Ac-KLALKLALKALKLALKLA-NH_2	K5, L6	21.8	46
Ac-KLALKLALKALKLALKLA-NH_2	A7, L8	22.4	48
Ac-KLALKLALKALKLALKLA-NH_2	K9, A10	20.7	50
Ac-KLALKLALKALKLALKLA-NH_2	L11, K12	19.1	45
Ac-KLALKLALKALKLALKLA-NH_2	L13, A14	21.1	36
Ac-KLALKLALKALKLALKLA-NH_2	L15, K16	22.7	53
Ac-KLALKLALKALKLALKLA-NH_2	L17, A18	27.4	68
KALK			
Ac-KALKLKLALALLAKLKLA-NH_2	None	19.0	86
Ac-KALKLKLALALLAKLKLA-NH_2	K1, A2	19.3	85
Ac-KALKLKLALALLAKLKLA-NH_2	L3, K4	19.6	80
Ac-KALKLKLALALLAKLKLA-NH_2	L5, K6	17.4	51
Ac-KALKLKLALALLAKLKLA-NH_2	L7, A8	18.0	66
Ac-KALKLKLALALLAKLKLA-NH_2	L9, A10	16.3	59
Ac-KALKLKLALALLAKLKLA-NH_2	L11, L12	15.2	61
Ac-KALKLKLALALLAKLKLA-NH_2	A13, K14	16.1	81
Ac-KALKLKLALALLAKLKLA-NH_2	L15, K16	17.9	73
Ac-KALKLKLALALLAKLKLA-NH_2	L17, A18	19.2	74

[a] The one-letter code of the D-amino acids is underlined.
[b] Retention times were determined on PolyEncap A300.

structure induction is not strong enough to compensate for the influence of substitutions.

4. Conclusions

This study has clearly demonstrated the good correlation between retention time profiles and the helical behaviour of the corresponding double D-amino acid replacement sets determined by CD. The decreased retention of analogues was expected for amphipathic peptides owing to reduced hydrophobic binding domains. However, similar characteristic retention time profiles were obtained also for the D-amino acid replacement set of a non-amphipathic peptide. As the non-amphipathic peptide should not interact with the hydrophobic phase by a preferred binding site, the characteristic retention time profile should be caused by disturbances of the

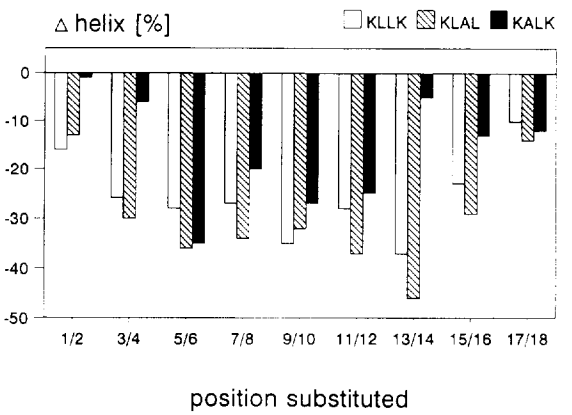

Fig. 3. Influence of the position of D-amino acid substitution on the helicity of KLLK, KLAL and KALK. The helical content of the corresponding all-L-peptides of KLLK, KLAL and KALK were 79, 82 and 86%, respectively. TFE–0.01 *M* H_3PO_4 (1:1, v/v), $5 \cdot 10^{-5}$ mol/l.

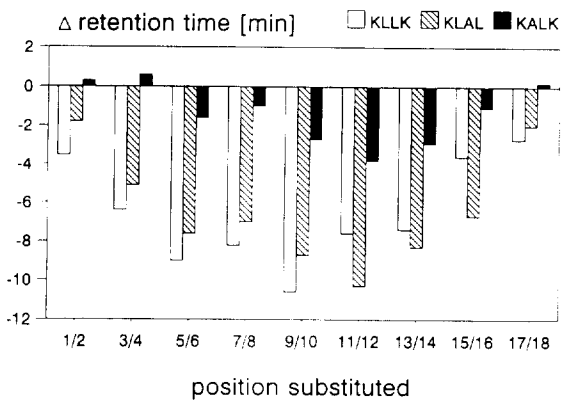

Fig. 4. Influence of the position of D-amino acid substitution on the HPLC retention time of KLLK, KLAL and KALK using PolyEncap A300 as stationary phase. The retention times of the corresponding all-L-peptides of KLLK, KLAL and KALK were 31.3, 29.4 and 19.0 min, respectively.

Table 2
HPLC retention times of KLLK, KLAL and KALK using BAA, PolyEncap A300 and ODA as stationary phases

Peptide	Retention time (min)		
	BAA	PolyEncap	ODA
KLLK	24.5	31.3	39.9
KLAL	23.7	29.4	37.5
KALK	16.5	19.0	24.4

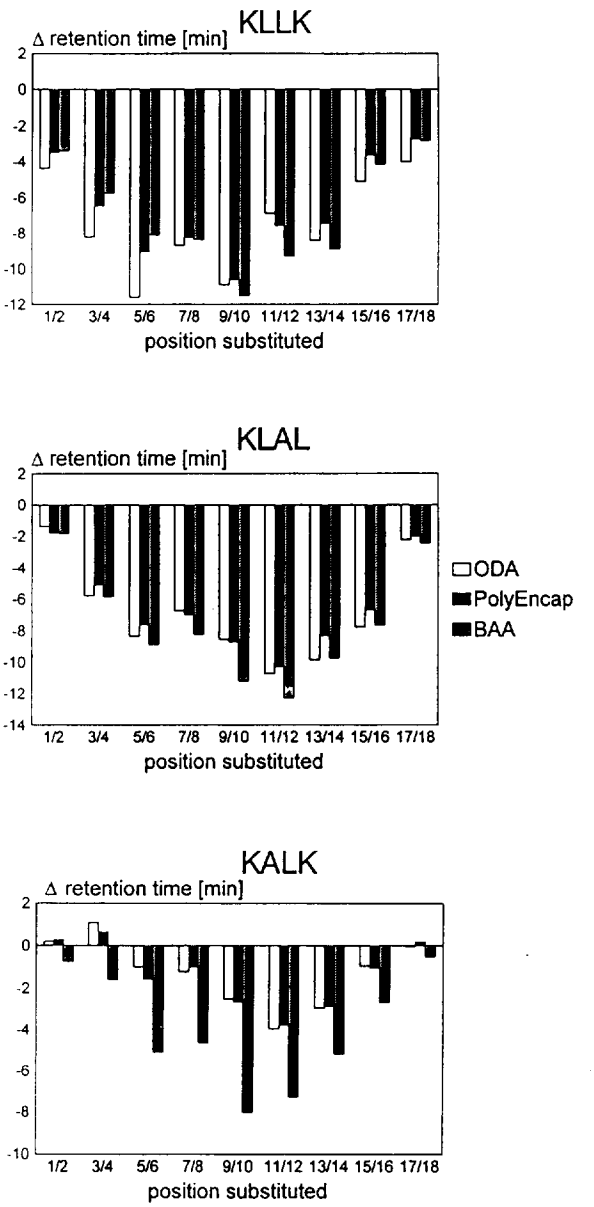

Fig. 5. HPLC retention behaviour of double D-amino acid replacement sets of KLLK, KLAL and KALK on polymer-encapsulated silica-based stationary phases with different hydrophobicities. The retention times of the related all-L-peptides are given in Table 2.

helix formation itself. The finding that BAA shows the influence of D-amino acid replacement more clearly for the non-amphipathic helix indicates that a less hydrophobic phase has a greater

sensitivity for the detection of the helix-destabilizing effect of D-amino acids. Further studies will be necessary to examine the influence of the simultaneous presence of amphipathic and non-amphipathic structure elements in one peptide on the HPLC behaviour of double D-amino acid replacement sets.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft, Kr 1451/2-1. The authors thank Dr. P. Henklein for his assistance with peptide synthesis and H. Nikolenko and B. Pisarz for technical assistance.

References

[1] R. Houghten and S. Blondelle, *Biochemistry*, 31 (1992) 12688.
[2] V. Steiner, M. Schär, K. Börnsen and M. Mutter, *J. Chromatogr.*, 586 (1991) 43.
[3] J. Ostresh, K. Büttner and R. Houghten, in R. Hodges (Editor), *HPLC of Peptides and Proteins: Separation, Analysis and Conformation*, CRC Press, Boca Raton, FL, 1991, p. 633.
[4] K. Büttner, C. Pinilla, J. Appel and R. Houghten, *J. Chromatogr.*, 625 (1992) 191.
[5] N. Zhou, C. Mant and R. Hodges, *Pept. Res.*, 3 (1990) 8.
[6] M. Hanson, K. Unger, R. Hodges and C. Mant, *J. Chromatogr.*, 599 (1992) 65.
[7] H.-C. Chen, J.H. Brown, J.L. Morrel and C.-M. Huang, in J.E. Rivier and G.R. Marshall (Editors), *Peptides: Chemistry, Structure and Biology*, ESCOM, Leiden, 1990, p. 122.
[8] Y. Pounny and Y. Shai, *Biochemistry*, 31 (1992) 9482.
[9] E. Krause, M. Beyermann, M. Dathe, H. Wenschuh, S. Rothemund and M. Bienert, in R.S. Hodges and J.A. Smith (Editors), *Peptides: Chemistry, Structure and Biology*, ESCOM Science, Leiden, 1994, p. 247.
[10] M.I. Aguilar, S. Mougos, J. Boublik, J. Rivier and M.T.W. Hearn, *J. Chromatogr.*, 646 (1993) 53.
[11] H. Engelhardt, H. Löw, W. Eberhardt and M. Mauss, *Chromatographia*, 27 (1989) 535.
[12] P. Henklein, M. Beyermann, M. Bienert and R. Knorr, in E. Girald and D. Andreu (Editors), *Peptides 1990*, ESCOM, Leiden, 1991, p. 67.
[13] Y.H. Chen, J.T. Yang and H.M. Martinez, *Biochemistry*, 11 (1972) 4120.
[14] M. Schiffer and A.B. Edmundson, *Biophys. J.*, 7 (1967) 121.
[15] P.Y. Chou and G.D. Fasman, *Biochemistry*, 13 (1974) 211.
[16] K.R. Shoemaker, P.S. Kim, E.J. York, J.M. Stewart and R.L. Baldwin, *Nature*, 326 (1987) 563.
[17] F.D. Sönnichsen, J.E. Van Eyk, R.S. Hodges and B.D. Sykes, *Biochemistry*, 31 (1992) 8790.
[18] A. Chakrabarty, J.A. Schellman and R.L. Baldwin, *Nature*, 351 (1991) 586.
[19] M. Hanson, K.K. Unger, J. Schmid, K. Albert and E. Bayer, *Anal. Chem.*, 65 (1993) 2249.
[20] E. Krause, H. Wenschuh, M. Beyermann and M. Bienert, in C.H. Schneider and A.N. Eberle (Editors), *Peptides 1992*, Elsevier, Amsterdam, 1993, p. 469.