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Research Article

Nonaqueous *versus* aqueous capillary electrophoresis of α-helical polypeptides: Effect of secondary structure on separation selectivity

The CE separation of α-helical polypeptides composed of 14–31 amino acid residues has been investigated using aqueous and nonaqueous BGEs. The running buffers were optimized with respect to pH. Generally, higher separation selectivities were observed in nonaqueous electrolytes. This may be explained by a change in the secondary structure when changing from water to organic solvents. Circular dichroism spectra revealed a significant increase in helical structures in methanol-based buffers compared to aqueous buffers. This change in secondary structure of the polypeptides contributed primarily to the different separation selectivity observed in aqueous CE and NACE. For small oligopeptides of two to five amino acid residues no significant effect of the solvent was observed in some cases while in other cases a reversal of the migration order occurred when changing from aqueous to nonaqueous buffers. As these peptides cannot adopt secondary structures the effect may be attributed to a shift of the pK_a values in organic solvents compared to water.

Keywords: Circular dichroism spectroscopy / α-Helical peptides / Nonaqueous capillary electrophoresis / Secondary structure DOI 10.1002/elps.200500673

1 Introduction

As hydrophilic compounds peptides can be effectively analyzed by CE using aqueous BGEs [1, 2]. However, the successful separations of peptides by NACE employing solvents such as methanol, ACN, or formamide have also been reported. The advantages of nonaqueous solvents for the analysis of the lipophilic peptide antibiotics gramicidin S and bacitracin have been demonstrated [3]. The separation of enkephalin peptides as anions in the counterelectroosmotic mode could only be achieved by NACE [4, 5]. Lindner *et al.* [6] reported the separation of *N*-protected alanine peptides containing up to six amino acid residues in mixtures of methanol and ethanol with the aid of *tert*-butylcarbamoylquinine as chiral counterion. Generally, the application of nonaqueous media not only is suitable for the separate of solutes that are insouble in water but also often allows the separation of solutes with very similar electrophoretic mobilities in aqueous electrolytes. The different physicochemical properties of organic solvents affect the acid–base behavior and solvation of the analytes resulting in selectivities noticeably different compared to those in aqueous media. Furthermore, organic solvents offer the potential for separation mechanisms based on interactions that cannot take place or are too weak in aqueous media. Hydrophilic interactions such as hydrogen bonding, dipole-related, and ionic interactions can be exploited in the hydrophobic environment in NACE, since these hydrophilic interactions are thermodynamically favored in nonaqueous media relative to aqueous media [7].

Studies on the correlation between the electrophoretic mobility of peptides and their charge and size (M_r) revealed a significant divergence from linearity of plots of mobility *versus* charge-to-size parameter according to several mobility models depending on the secondary structure of the peptides [8]. The "outliers" did not display random coils but rather adopted stable α-helical structures under the low-pH conditions applied. Florance *et al.*

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Abbreviations: ALM F30, alamethicin F30; **AmpA**, ampullosporin A; **CD**, circular dichroism

[9] also demonstrated the influence of the secondary structure of motilin fragments on the migration behavior in CE. Several studies have demonstrated that the addition of organic solvents to aqueous buffers leads to a stabilization of intramolecular hydrogen bonding and ionic interactions resulting in an increased structural order of the peptides. For example, Hirota *et al.* [10] observed unfolding of mellitin, a major component of honeybee venom, in an aqueous environment while the addition of alcohols induced an α-helical structure. All investigated alcohols induced the formation of α-helices but the effectiveness varied with the type of the alcohol. For example, monitoring the ellipticity at 222 nm as a measure of the α-helical state lower concentrations of *tert.*-butanol or 2-propanol were required for the maximal effect compared to ethanol or methanol. Glycerol exhibited only a weak effect. Work by Righetti *et al.* [11] described the transition of the synthetic, branched-chain polymeric polypeptide poly(Lys(Glu-DL-Ala)) with a 50 Lys residue backbone from random coil in water at pH 1.1 to an α-helix upon the addition of methanol. Reaching a 75% v/v methanol concentration the equilibrium was completely shifted toward the helical configuration. Castagnola *et al.* [12] have shown the advantages of the helix-inducing agent 2,2,2-trifluoroethanol for the separation of small polar peptides upon addition to aqueous buffers, concluding that structuring properties of buffer additives which may not be very effective for the separation of small peptides will become a relevant factor for an improvement of separations of large apolar peptides.

The present study was conducted in order to compare the CE separation of amphiphilic α-helical polypeptides in aqueous and nonaqueous BGEs in the context of their secondary structure. Ampullosporin A (AmpA) analogs, the microheterogeneous alamethicin F30 (ALM F30), and the three highly basic amphiphilic peptides magainin 2, cecropin P1, and melittin were used as model compounds (Table 1).

2 Materials and methods

2.1 Chemicals

Methanol and ACN (all of gradient-grade quality), ammonium acetate, acetic acid, formic acid, Tris, 0.1 M sodium hydroxide, sodium dihydrogen phosphate, phosphoric acid, and boric acid were obtained from VWR International. Ammonium acetate was dried overnight in a desiccator over silica before use. The aqueous buffers were prepared in double distilled, deionized water. Magainin 2, cecropin P1, methionine enkephalin, leucine enkephalin, [D-Ala^2] leucine enkephalin, L-Ala-L-Phe, D-Ala-L-Phe, Ala-Phe-Gly, Gly-Ala-Phe, Gly-Gly-Tyr, Tyr-Gly-Gly, Gly-Leu-Tyr, α-Asp-PheOMe, and β-Asp-PheOMe were from Bachem AG (Heidelberg, Germany). Melittin and hexadimethrine bromide were obtained from Sigma-Aldrich (Steinheim, Germany). ALM F30 was isolated from the mold *Trichoderma viride* NRRL 3199 by a method described previously [13]. The AmpA derivatives [des-Leuol] AmpA, [des-Ac] AmpA, and [des-Ac-Trp^1] AmpA were synthesized by solid-phase synthesis according to a method described by Nguyen *et al.* [14] using the Fmoc strategy for the assembly of the peptide on the solid support. The peptides were purified by preparative HPLC and the identity was confirmed by MALDI-MS.

2.2 Apparatus and equipment

2.2.1 CE

CE with UV detection was performed on a Beckman P/ACE 5510 instrument (Beckman Coulter, Krefeld, Germany) equipped with a diode array detector (DAD) at 25°C. UV detection was carried out at 215 nm at the

Table 1. Amino acid sequences and M_r of the investigated α-helical polypeptides. Aib, aminoisobutyric acid; Pheol, phenylalaninol; and Leuol, leucinol

Peptide	Amino acid sequence	M_r
ALM F30[a)]	Ac-Aib-Pro-Aib-Ala-Aib^5-Ala-Gln-Aib-Val-Aib^{10}-Gly-Leu-Aib-Pro-Val^{15}-Aib-Aib-Glu-Gln-Pheol^{20}	1964.3
[des-Leuol] AmpA	Ac-Trp-Ala-Aib-Aib-Leu^5-Aib-Gln-Aib-Aib-Aib^{10}-Gln-Leu-Aib-Gln-OH	1540.7
[des-Ac] AmpA	H-Trp-Ala-Aib-Aib-Leu^5-Aib-Gln-Aib-Aib-Aib^{10}-Gln-Leu-Aib-Gln-Leuol^{15}	1580.5
[des-AcTrp^1] AmpA	H-Ala-Aib-Aib-Leu-Aib^5-Gln-Aib-Aib-Aib-Gln^{10}-Leu-Aib-Gln-Leu-ol	1395.9
Melittin	H-Gly-Ile-Gly-Ala-Val^5-Leu-Lys-Val-Leu-Thr^{10}-Thr-Gly-Leu-Pro-Ala^{15}-Leu-Ile-Ser-Trp-Ile^{20}-Lys-Arg-Lys-Arg-Gln^{25}-Gln-NH_2	2846.5
Cecropin P1	H-Ser-Trp-Leu-Ser-Lys^5-Thr-Ala-Lys-Lys-Leu^{10}-Glu-Asn-Ser-Ala-Lys^{15}-Lys-Arg-Ile-Ser-Glu^{20}-Gly-Ile-Ala-Ile-Ala^{25}-Ile-Glu-Gly-Gly-Pro^{30}-Arg-OH	3338.9
Magainin 2	H-Gly-Ile-Gly-Lys-Phe^5-Leu-His-Ser-Ala-Lys^{10}-Lys-Phe-Gly-Lys-Ala^{15}-Phe-Val-Gly-Glu-Ile^{20}-Met-Asn-Ser-OH	2846.5

a) ALM F30 possesses a microheterogeneous mixture composed of the two major components ALM F30 (amino acid sequence shown) and [Aib^6] ALM F30, and different minor components in concentrations ranging from 0.4 to 4.0% [13].

cathodic end of the capillary (in case of the unmodified capillaries) or at the anodic end of the capillary (in case of the dynamically coated capillaries using the reversed-polarity mode). Sample solutions were introduced by hydrodynamic injections at a pressure of 3.5 kPa for 3 s.

Fused-silica capillaries used for the CE experiments, 50 μm id × 360 μm od, were obtained from Polymicro Technologies (Phoenix, AZ, USA). For aqueous CE analyses the capillaries were conditioned by flushing sequentially with 0.1 M sodium hydroxide for 30 min, with water for 5 min, and with the electrolyte solution for 10 min. Between aqueous CE analyses, the capillary was flushed with 0.1 M sodium hydroxide for 2 min followed by the running buffer for 2 min. For NACE experiments new capillaries were rinsed for 30 min with 0.1 M sodium hydroxide, 5 min with water, and 10 min with methanol followed by the separation medium for 10 min. Between the NACE analyses, the capillary was flushed only with the running buffer for 2 min. When not in use, it was washed with the respective solvent and then dry stored. The pH and the apparent pH (pH_{app}, in case of the nonaqueous electrolyte solutions) were measured with a WTW pMX 3000 potentiometer (WTW, Weilheim, Germany), equipped with a Schott glass electrode N6180. The electrode was calibrated using aqueous standard pH solutions.

2.2.2 Circular dichroism spectroscopy

Circular dichroism (CD) spectra were measured using a Jasco J-710 spectrometer in a rectangular quartz cell of 0.1 cm path length with a scan speed of 20 nm/min and 2 nm bandwidth at 20°C. The far UV CD spectra of the samples were recorded from 260 to 185 nm at a digital resolution of 0.1 nm. Three scans were signal averaged for each wavelength range. The background solvents were determined in the same buffer without peptide and then subtracted. The spectra were measured with samples prepared from stock solutions of 1 mM peptide in methanol resulting in peptide concentrations of 0.1 mM in the respective electrolyte.

The mean residue ellipticity [θ] (deg·cm^2/dmol) was calculated from the observed ellipticity θ (deg) according to

$$[\theta] = \theta \times 100/(cdN) \quad (1)$$

where *c* is the peptide concentration (mol/L), *d* is the path length (cm), and *N* is the number of the amino acid residues in the peptide. Secondary structure estimations were obtained by spectral deconvolution using the CDPro software package [15] which consists of three programs (SELCON3, CDSSTR, and CONTINLL) with a set of 56 proteins. Spectral deconvolutions were analyzed in the wavelength range between 190 and 240 nm with 43–51 data points with corresponding high voltage smaller than 600 V. The fractions of α-helix and β-sheet were obtained by adding the corresponding regular and distorted fractions, for example $\alpha = H(r) + H(d)$. The resulting data are the arithmetic means of these three programs, except for [des-Leuol] AmpA and [des-AcTrp^1] AmpA in aqueous solution. In these two cases the performance of SELCON3 exceeded the 5% range of tolerance. The resulting data for these two samples are the arithmetic means of CDSSTR and CONTINLL.

3 Results and discussion

3.1 Optimization of the NACE and aqueous CE separations

Comparison of aqueous *versus* nonaqueous BGEs for CE separations of peptides was performed with the peptaibol peptides ALM F30, [des-Leuol] AmpA, [des-Ac] AmpA, and [des-Ac-Trp^1] AmpA, and the highly basic peptide antibiotics magainin 2, cecropin P1, and melittin (Table 1). In order to study the effect of the secondary structures on the separation selectivity small oligopeptides which do not assume well-defined secondary structures were included. Methanol and methanol-ACN mixtures, which are frequently used in NACE because of their favorable relative permittivity to viscosity (ε/η) ratio (methanol, ε/η = 61 m·Pa^{-1}s^{-1}; ACN, ε/η = 110 m·Pa^{-1}s^{-1}) [16–18], were evaluated. Trifluoroethanol, which is widely used as helix-inducing solvent for peptides [19], was not investigated due to the unfavorable physicochemical properties, *e.g.*, high viscosity (η = 1.74 mPa·s at 25°C) and a low ε/η ratio of only 15 m·Pa^{-1}s^{-1} which results in very low electrophoretic and electroosmotic mobilities. The separation conditions were optimized with regard to pH. In NACE the apparent pH was modified by changing the composition of the ammonium salt and the acid. Mixtures of ammonium acetate, ammonium trifluoroacetate as well as acetic acid and formic acid were employed. The BGEs which gave the best separations for the α-helical polypeptides (BGE I-VI) and the small oligopeptides (BGE III, VII-XI) are listed in Table 2.

Figure 1 summarizes separations of the polypeptides under pH-optimized aqueous and nonaqueous conditions. Generally, higher separation selectivities were observed in NACE. The acidic peptides ALM F30 and [desLeuol] AmpA could only be analyzed under alkaline conditions (Figs. 1A and B). In NACE, ACN-rich media resulted in low analyte mobilities, and therefore no separation of the components could be achieved. This may be explained by the formation of homo- and/or heteroconjugates of the dissociated Brönsted acids com-

Table 2. Composition and pH of BGEs

BGE	Electrolyte system	Solvent system	pH
I	10 mM ammonium acetate	Methanol	8.0[a)]
II	25 mM sodium borate	Water	11.0
III	25 mM ammonium acetate/1 M acetic acid	Methanol	5.5[a)]
IV	25 mM ammonium acetate/0.5 M acetic acid	Water	3.4
V	25 mM ammonium trifluoroacetate/50 mM TFA, 0.001% hexadimethrine bromide	Methanol/ACN (1:3 v/v)	2.3[a)]
VI	25 mM ammonium acetate/250 mM acetic acid, 0.001% hexadimethrine bromide	Water	3.7
VII	25 mM ammonium acetate/1 M acetic acid	Methanol/ACN (1:1 v/v)	5.4[a)]
VIII	25 mM ammonium acetate/1 M acetic acid	Methanol/ACN (1:3 v/v)	5.3[a)]
IX	25 mM ammonium acetate/1 M acetic acid	Water	3.1
X	25 mM ammonium acetate/0.5 M formic acid	Methanol/ACN (1:1 v/v)	4.6[a)]
XI	25 mM ammonium acetate/0.5 M formic acid	Methanol/ACN (1:3 v/v)	4.8[a)]

a) For organic BGEs the apparent pH is listed.

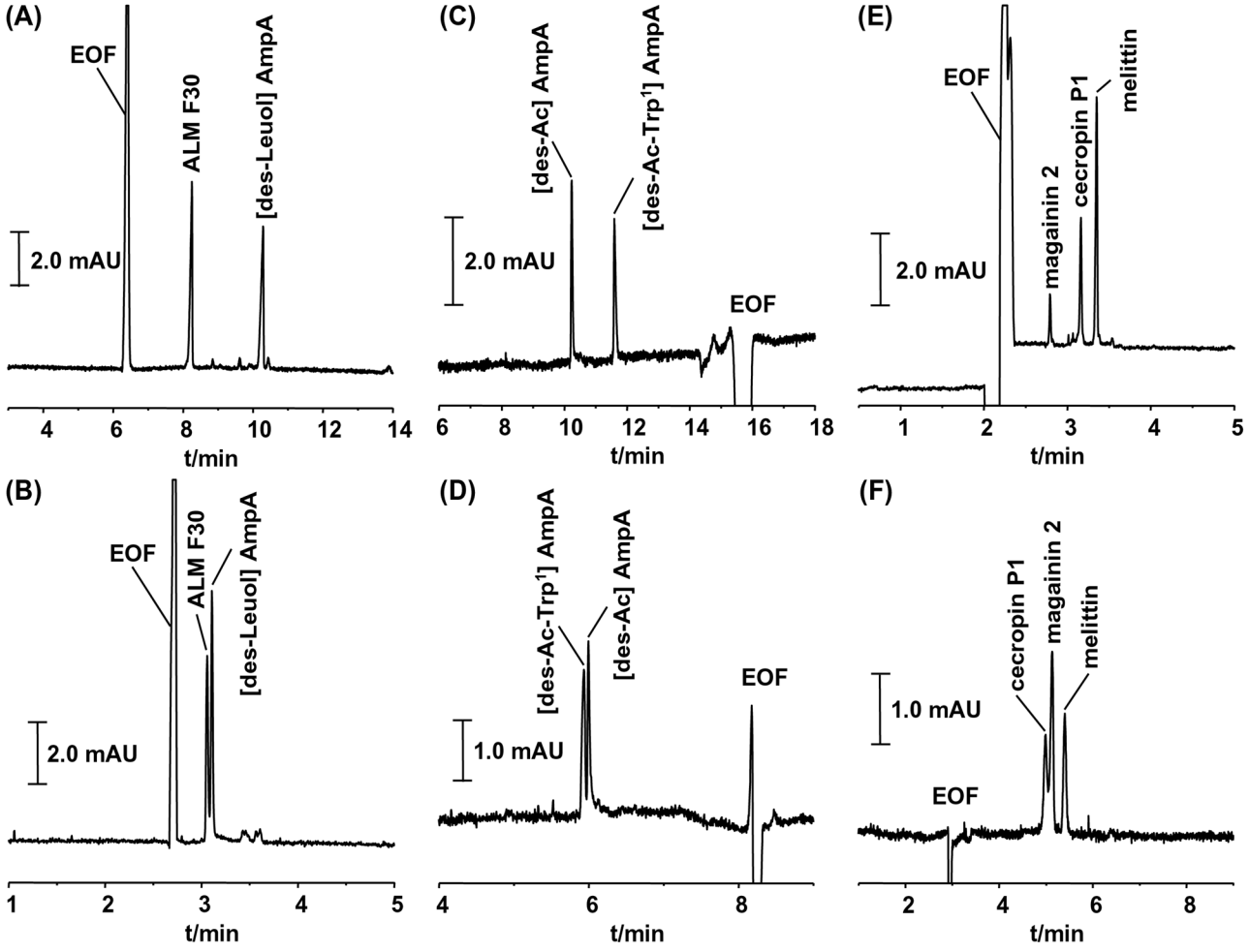

Figure 1. Electropherograms of α-helical polypeptides obtained by NACE (A, C, E) compared to aqueous CE (B, D, F). Experimental conditions: running electrolytes, (A) BGE I, (B) BGE II, (C) BGE III, (D) BGE IV, (E) BGE V, (F) BGE VI; fused-silica capillary, 47/40 cm; separation voltage: (A) +30 kV (9 μA), (B) +25 kV (37 μA), (C) +25 kV (15 μA), (D) +25 kV (26 μA), (E) −25 kV (25 μA), and (F) −25 kV (27 μA).

monly observed with acidic analytes in ACN [20]. The analysis time in methanolic electrolytes is much longer compared to the aqueous borate buffer system due to the higher mobility of the EOF in water compared to methanol. NACE using a methanol-based BGE has been superior for the analysis of minor components of ALM F30 [21].

The peptides [des-Ac] AmpA and [des-Ac-Trp^1] AmpA were separated under acidic pH conditions. Compared to NACE (Fig. 1C) baseline separation could not be achieved in aqueous buffer and the migration order was reversed (Fig. 1D). The strongly basic peptides melittin, magainin 2, and cecropin P1 were also separated under acidic conditions. The CE analysis of melittin from other bee venom components has been performed at pH 1.8 in order to avoid adsorption of the basic peptides to the capillary wall [22]. Alternatively, polyacrylamide-coated capillaries [23] or the application of MEKC [24] have been utilized for the analysis of melittin. The use of bare fused-silica capillaries resulted in peak tailing presumably caused by wall adsorption even in nonaqueous solvents. Therefore, the analyses of these peptides were carried out in dynamically coated capillaries using 0.001% hexadimethrine bromide as EOF modifier. Hexadimethrine bromide also reverses the EOF in ACN/methanol mixtures [25]. As observed for [des-Ac] AmpA and [des-Ac-Trp^1] AmpA a different migration order was observed for the strongly basic peptides when changing from nonaqueous (Fig. 1E) to aqueous buffers (Fig. 1F). The separation selectivities for the BGEs resulting in the highest selectivities under aqueous and nonaqueous conditions are summarized in Table 3.

In contrast to the investigated polypeptides, CE separations of small oligopeptides with two to five amino acid residues showed a different behavior. In most cases no general advantage of NACE was observed (Fig. 2) and comparable separation selectivities were observed for both CE modes (Table 3). However, for some tripeptides the separation was superior in aqueous buffers and, in addition, a reversal of the migration order was observed when changing from aqueous buffers (Fig. 2E) to organic buffers (Fig. 2F). Generally, the separations of the short peptides proceeded faster in nonaqueous buffers.

3.2 CD spectra of the polypeptides

In order to obtain information on the secondary structure of the polypeptides, CD spectra were recorded in aqueous and nonaqueous solutions. CD is generally recognized as a suitable tool for analyzing the conformational state of peptides and proteins. Figure 3 shows the CD spectra of ALM F30 and [des-Leuol] AmpA obtained in aqueous borate buffer compared to methanolic NACE running electrolytes. Similar changes have, for example, also been observed in the CD spectrum of melittin when adding ethanol to a solution of the peptide in 20 mM aqueous hydrochloric acid [10]. Other alkanols including

Table 3. Separation selectivities (*r*) for the analysis of the α-helical polypeptides compared to small oligopeptides obtained with NACE and aqueous CE, respectively

	r_{NACE}[a)]	BGE	r_{ACE}[b)]	BGE	r_{NACE}/r_{ACE}
α-Helical polypeptides (14–31 amino acids)					
[des-Leuol] AmpA/ALM F30	1.69	(I)	1.12	(II)	1.51
[des-Ac] AmpA/[des-Ac-Trp^1] AmpA	1.50	(III)	0.96	(IV)	1.56
Cecropin P1/magainin 2	1.50	(V)	0.96	(VI)	1.56
Melittin/magainin 2	1.71	(V)	1.11	(VI)	1.54
Melittin/cecropin P1	1.14	(V)	1.11	(VI)	1.03
Small oligopeptides (2–5 amino acids)					
Ala-Phe LL/DL	1.29	(VII)	1.19	(IX)	1.08
α-Asp-PheOMe/β-Asp-PheOMe	3.25	(III)	3.56	(IX)	0.91
Ala-Phe-Gly/Gly-Ala-Phe	1.17	(VII)	1.17	(IX)	1.00
Gly-Gly-Tyr/Tyr-Gly-Gly	1.28	(VIII)	0.93	(IX)	1.38
Gly-Ala-Phe/Gly-Gly-Tyr	1.11	(XI)	1.04	(IX)	1.07
Tyr-Gly-Gly/Gly-Leu-Tyr	1.07	(XI)	1.13	(IX)	0.95
Leucine enkephalin/[D-Ala^2] leucine enkephalin	1.14	(VII)	1.07	(IX)	1.07
Methionine enkephalin/leucine enkephalin	1.40	(VII)	1.13	(IX)	1.24

a) Selectivity coefficient using NACE.
b) Selectivity coefficient using aqueous CE (ACE).
For experimental conditions see Figs. 1, 2. Selectivity coefficients were calculated according to $r = \mu_1/\mu_2$, $\mu_1 > \mu_2$, where μ_1 and μ_2 are the effective mobilities of the analytes.

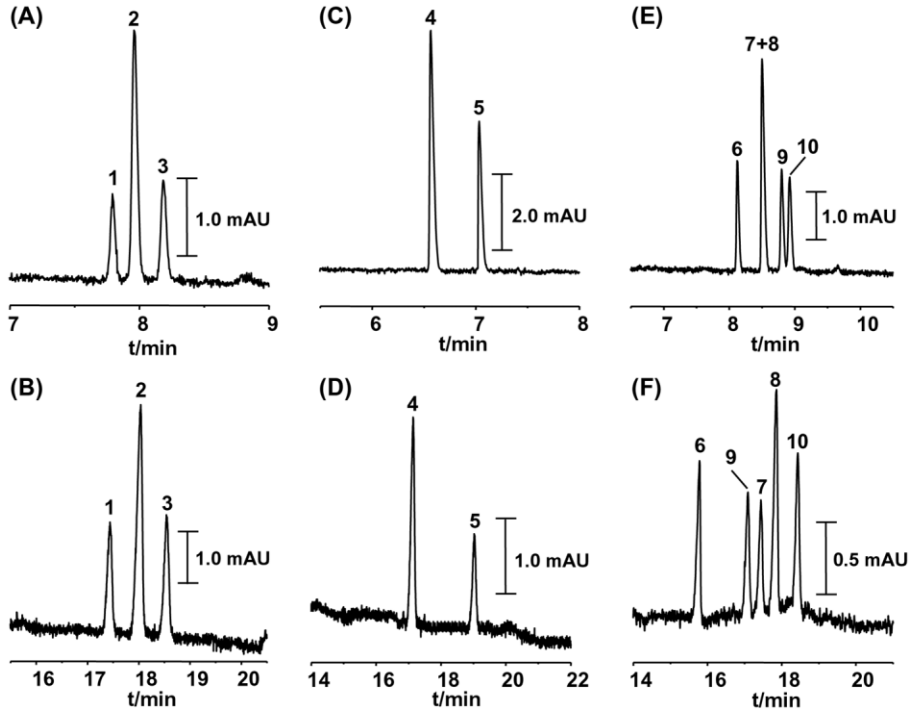

Figure 2. Electropherograms of ten oligopeptides obtained by NACE (A, C, E) compared to aqueous CE (B, D, F). Experimental conditions: running electrolytes, (A, C, E) BGE VII, (B, D, F) BGE IX; fused-silica capillary, 67/60 cm; separation voltage: (A, C, E) +30 kV (16 μA), (B, D, F) +30 kV (22 μA). Peak identification: (1) leucine enkephalin, (2) [D-Ala^2] leucine enkephalin, (3) methionine enkephalin, (4) L-Ala-L-Phe, (5) D-Ala-L-Phe, (6) Ala-Phe-Gly, (7) Gly-Ala-Phe, (8) Gly-Gly-Tyr, (9) Tyr-Gly-Gly, and (10) Gly-Leu-Tyr.

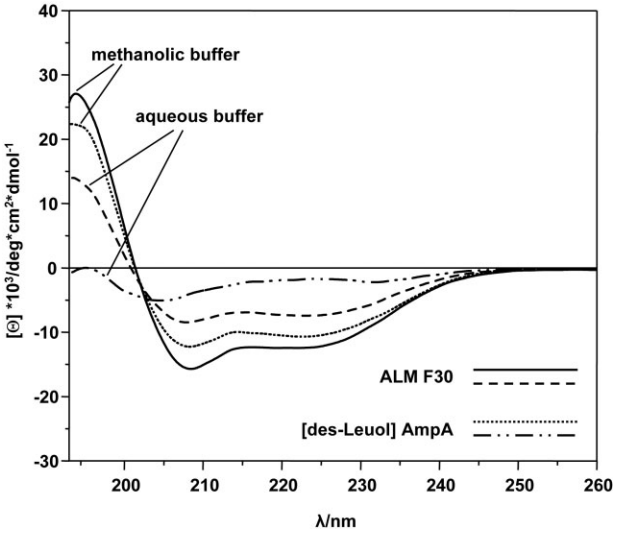

Figure 3. CD spectra of ALM F30 and [des-Leuol] AmpA in aqueous buffer (BGE II) and methanolic buffer (BGE I). For experimental details see Section 2.2.2.

methanol gave similar results. The fractions of the conformation, α-helix, β-sheet, β-turn, and random coil, secondary structures in aqueous compared to nonaqueous media obtained from the CD spectra analysis are summarized in Table 4. The CD spectra of [des-Ac] AmpA and [des-AcTrp^1] AmpA could not be recorded in the CE BGEs because of the high background absorption below 210 nm caused by the high content (0.5–1.0 M) of acetic acid in the CE running buffers. Generally, an increase of the α-helical content was noted when changing from aqueous to nonaqueous media. This effect is most intensely pronounced for the AmpA derivatives, which possess the lowest contents of α-helix (≤7%) in the aqueous environment. The α-helical structure dominates in nonaqueous electrolytes, while a larger fraction of β-sheet and β-turn conformations is observed in water. In most cases a reduction of the fraction of the random coil structure is also found in nonaqueous media. This effect can be explained by a stabilization of intramolecular hydrogen bonds in the hydrophobic environment, which leads to conformational changes in favor of the secondary structure with the lowest conformational energy for the α-helix [25]. The β-sheet structure also has intramolecular hydrogen bonds, but for the investigated polypeptides the conformational energy of an α-helix appears to be significantly lower than that of a β-sheet.

3.3 Effect of the secondary structure on the separation selectivity in NACE compared to aqueous CE

The effect of the solvent on the selectivity coefficient is more pronounced for the α-helical polypeptides (Table 3), which are able to adopt different secondary structures in water and methanol (Table 4). The stabilization of the more structured α-helix conformation in nonaqueous solvents results in different Stokes radii of the analytes compared to the random coil structure in aqueous solu-

Table 4. Secondary structure fractions of the investigated peptides predicted by CD spectra analysis using SELCON3, CDSSTR, and CONTINLL. Estimation procedure is described in Section 2.2.2

Analyte	Solvent	λ-Range (nm)	Fraction of secondary structure			
			α-Helix	β-Sheet	β-Turn	Random coil
ALM F30	BGE I	194–240	0.55	0.10	0.15	0.21
	BGE II	190–240	0.37	0.17	0.24	0.21
[des-Leuol] AmpA	BGE I	194–240	0.46	0.11	0.18	0.24
	BGE II	190–240	0.06	0.38	0.24	0.31
[des-Ac] AmpA	Methanol[a)]	195–240	0.49	0.11	0.16	0.24
	Water[a)]	186–240	0.06	0.40	0.23	0.31
[des-AcTrp[1]] AmpA	Methanol[a)]	195–240	0.49	0.10	0.16	0.24
	Water[a)]	186–240	0.07	0.36	0.24	0.32
Melittin	BGE V	197–240	0.70	0.03	0.09	0.18
	BGE VI	197–240	0.10	0.30	0.25	0.35
Cecropin P1	BGE V	197–240	0.54	0.06	0.15	0.26
	BGE VI	195–240	0.06	0.35	0.22	0.37

a) CD measurements in the corresponding CE BGEs was not possible because of the high background absorption below 210 nm.

tions. Consequently, a higher electrophoretic resolution of the peptides is achieved. On the other hand, organic solvents affect acid–base equilibria and thereby the charge of the solutes as described in several publications [4, 27, 28]. The inversion of the migration order of Gly-Gly-Tyr and Tyr-Gly-Gly in NACE compared to aqueous CE can be explained by a pK_a shift. Moreover, the acid-base equilibria of ionizable groups in peptides and proteins are also affected by the secondary and higher order structures. Overall, the effect of the organic solvents on the peptide dissociation constants should be similar for polypeptides and oligopeptides. Therefore, the selectivity enhancement observed for the α-helical polypeptides can be attributed, at least to a large extent, to differences in the Stokes radii of the analytes. This is caused by the different shapes and frictional drags of such peptides [8, 11]. Using large organic ions with a low charge density Kenndler and co-workers demonstrated for organic solvents that the hydrodynamic friction (Stokes law) appeared to play a major role in the retardation of ionic movements and, *i.e.*, also for solvent-induced selectivity changes [29]. However, additional phenomena such as changes in ion–ion interactions, homo-, and heteroconjugation, changes in the solvation of the analyte ions, or differences in the dielectric frictions [30, 31] may also contribute to an improvement of polypeptide separations in organic *versus* aqueous BGEs.

4 Concluding remarks

Compared to aqueous buffers, higher separation selectivities were observed for the polypeptides when using nonaqueous BGEs. CD spectra recorded in the respective BGEs indicated an increase in the α-helical structure due to the stabilization of intramolecular hydrogen bonds in the organic solvent. Within a series of oligopeptides that are not able to adopt different structures in an organic solvent no significant effect of the solvent was found in some cases but reversal of the migration order in aqueous buffers compared to organic electrolyte systems was observed in one case. For large peptides, in addition to changes of the frictional drag caused by solvent-induced changes of the secondary structure, further phenomena such as pK_a shift of ionizable groups (induced either by the solvent or alterations of the secondary structure), ion-solvation, ion–ion interactions, *etc.* can contribute to the increased separation selectivity observed in the present study when substituting aqueous BGEs by nonaqueous BGEs. Although not investigated in the present study, a pK_a shift appears to be the most likely explanation for the observed reversal of the migration order of some tripeptides. Overall, NACE appears to be an interesting alternative for the analysis of peptides when changes in the secondary structure can be induced in organic solvents.

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