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Journal of Chromatography A, 735 (1996) 271–281

JOURNAL OF CHROMATOGRAPHY A

Effect of 2,2,2-trifluoroethanol on capillary zone electrophoretic peptide separations

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Abstract

The use of 2,2,2-trifluoroethanol–water mixtures for peptide separations by capillary zone electrophoresis (CZE) displays some advantages over aqueous solutions. First, the increase in viscosity reduces and stabilizes the running current and facilitates heat dispersion, with a consequent improvement in the number of theoretical plates. Second, the decrease in the dielectric constant leads to a modification of the dissociation constants of the ionizable groups. The consequence is a change in selectivity that, for several favourable peptide pairs, provides an increase in resolution. Third, the interaction of trifluoroethanol with the peptide modifies the Stokes radius in a manner strongly dependent on the peptide sequence. This can also be utilized for an increase in CZE performance. Fourth, the structural properties of 2,2,2-trifluoroethanol are particularly useful for an improvement in the separation of large apolar peptides. Finally, the use of trifluoroethanol strongly stabilizes the capillary coating.

Keywords: Capillary electrophoresis; Buffer composition; 2,2,2-Trifluoroethanol; Peptides

1. Introduction

Capillary zone electrophoresis (CZE) is incoming an elective technique for the analytical separation of small polar peptides. With respect to other techniques, CZE is characterized by optimum sensitivity with underivatized samples and good selectivity. In addition, in coated capillaries, and hence in the absence of appreciable electroosmotic flow (f_{eo}), the performance of the electrophoretic separation (in terms of mobility and resolution) as a function of the pH of the separation buffer can be predicted with good efficiency [1]. Recently, interesting studies were dedicated to establishing the effect of various buffer components [2] and of organic solvents on the peptide separations by CZE [3,4]. The addition of an organic solvent to the separation buffer can offer several advantages, such as higher viscosity, lower mobilities and higher voltage. In a study of CZE properties in organic media, Sahota and Khaledi [4] established that formamide exhibits the best characteristics as a non-aqueous solvent with respect to other solvents, including methanol and acetonitrile. In this

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0021-9673/96/$15.00
SSDI 0021-9673(95)00963-9

study, we considered the use of 2,2,2-trifluoroethanol (TFE) as a good candidate for CZE peptide separations in aqueous–organic media. TFE is a widely used structure-inducing cosolvent with oligopeptides [5]. It enhances the solution structure of small protein fragments such as RNase S- and C-peptides [6]. In addition, TFE can induce a stable secondary conformation in peptides that are otherwise unstructured in aqueous solution [7,8]. For this reason, it can contribute to a transition from random to well defined peptide conformations with a consequent decrease in peak dispersion and could offer a good chance for a general improvement in electrophoretic separation.

2. Experimental

2.1. Reagents

All common reagents were of analytical-reagent grade purchased from either Carlo Erba (Milan, Italy) or Merck (Darmstadt, Germany). TFE and horse skeletal muscle myoglobin were obtained from Sigma (St. Louis, MO, USA). ACTH fragments 5–10 and 4–10 were obtained from Serva (Heidelberg, Germany) and GGXA peptides and enkephalins were obtained from Bachem (Bubendorf, Switzerland).

2.2. Instrumentation

The capillary electrophoretic apparatus was a Beckman (Palo Alto, CA, USA) P/ACE System 2100 equipped with automated Gold software. The silica capillary used was provided by Beckman and was coated with a monolayer of acrylamide as described previously [9]. Peptide microtitrations were performed on a Radiometer (Copenhagen, Denmark) VIT90 videotitrator, equipped with an ABU93 triburette and a SAM90 sample station, using the procedure described elsewhere [1]. NMR spectra were measured on a Varian (Palo Alto, CA, USA) Gemini 300 spectrometer. Circular dichroism (CD) spectra were obtained using a Jasco (Tokyo, Japan) instrument equipped with a 1.0 cm path-length cell.

2.3. Methods

The electrophoretic runs were performed with a coated [9] silica capillary [56.5 cm (50.0 cm at the detection window) $\times$ 75 μm I.D.] at a constant applied voltage of 25 kV. The running temperature was 25°C. Buffer solutions in water and in TFE–water mixtures were prepared at the desired pH value from stock solutions in order to obtain a final sodium phosphate concentration of 80 mmol/l. The sample solutions were usually prepared from a stock solution ranging between 2 and 3 mg/ml, diluted with the running buffer in order to obtain a final concentration of about 0.2–1.0 mg/ml in sodium phosphate of concentration 8.0 mmol/l. The injection was performed by pressure for 2 s, corresponding to about 5–8 nl. The detection wavelength was 214 nm.

Tryptic digestion of horse skeletal muscle myoglobin was performed as described previously [9]. The sample of the myoglobin tryptic digest was prepared at an injection concentration of 5 mg/ml. The titration of GGXA peptides and of met- and leu-enkephalin was performed at the chosen TFE concentration as described previously [1].

NMR spectra were measured using a 5 mm diameter tube on samples at a concentration of 3.5 mg/ml. A capillary containing a D_2O–methanol solution (98:2, v/v) was inserted in the tube. The deuterium of D_2O was used to lock the spectrometer. The methanol signal was used as an external standard, its chemical shift being assigned at 49.0 ppm. The measurements were performed at 75.462 MHz and 25°C, using about 2 mg of each peptide in the appropriate CZE solution. The spectra were broad-band decoupled from proton and consisted of 5000 scans, corresponding to 150 min. A 45° pulse, an acquisition time of 0.8 s and a 1-s recovery between pulses were used. A line broadening of 1 Hz was applied before the Fourier transformation. CD spectra were measured using a 0.1 mmol/l peptide concentration in the same solutions as used

for the electrophoretic separations and a TFE concentration ranging from 0 to 90% (v/v).

3. Results

The properties of TFE are reported in Table 1 and are compared with those of other common chromatographic solvents. The UV spectra (not reported) ensure that under isocratic conditions TFE has a cut-off of ca. 200 nm, which permits CZE separations to be performed at all the common wavelengths of analysis. Considering that a good organic cosolvent should modify the viscosity and the dielectric constant, TFE can be regarded as one of the best options. Moreover, its peptide structuring properties could suggest its use as elective in peptide CZE separations.

For this reason, a series of CZE peptide separations were performed at various TFE concentrations, using the selected pairs of standard peptides reported in Table 2. Since the results obtained on the distinct pairs are different, they are presented separately. In addition, the effect of TFE on the separation of peptides deriving from the trypsinization of horse skeletal muscle myoglobin is reported.

Table 2
Sequences of peptides used in the CZE separations

Sequence
GGDA
GGNA
YGGFL (leu-enkephalin)
YGGFM (met-enkephalin)
EHFRWG (ACTH, fragment 5–10)
MEHFRWG (ACTH, fragment 4–10)

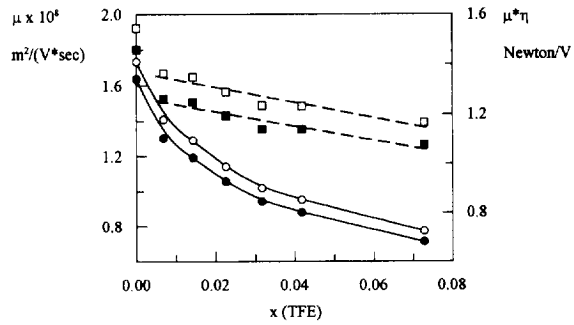

Fig. 1. Mobilities (circles) and values of the product of mobility and viscosity (squares) of GGNA (open symbols) and GGDA (closed symbols) as a function of TFE molar fraction at pH 2.2. The other CZE conditions are reported under Experimental. The viscosity of the TFE–H_2O solutions was calculated according to Ref. [10].

3.1. GGXA peptides

In Fig. 1, the mobility of GGDA and GGNA tetrapeptides is reported as a function of the molar fraction of TFE. With a coated capillary and therefore in the absence of electroosmosis, the decrease of electrophoretic mobility is primarily due to the increase in the solution viscosi-

Table 1
Physical properties of 2,2,2-trifluoroethanol compared with those of some common chromatographic solvents

Solvent	B.p. (°C) (1 atm)	Density (g/ml) (25°C)	n_D (20°C)	ε (25°C)	η (cP) (25°C)
Water	100	0.997	1.333	80	0.89
Formamide	210	1.129	1.447	110	3.30
N,N-Dimethylformamide	153	0.945	1.430	37	0.80
Acetonitrile	82	0.787	1.344	38	0.34
Methanol	65	0.791	1.329	33	0.54
Ethanol	78	0.785	1.361	24	1.08
2-Propanol	82	0.786	1.378	18	1.77
2,2,2-Trifluoroethanol	78	1.371	1.300	26	1.74

ty, according to the basic equation of electrophoresis:

$$\mu = \frac{qZ}{6\pi\eta r_s} \quad (1)$$

where q is the electron charge, Z the peptide charge (valency), η the solution viscosity and r_s the peptide Stokes radius. Nonetheless, since the product of mobility and viscosity as a function of TFE concentration is not constant (Fig. 1), a decrease in the ratio charge to Stokes radius must be postulated. The viscosity values were obtained according to Ref. [10].

To investigate this behaviour in detail, the peptide apparent pK_a at 0.073 TFE molar fraction [corresponding to a concentration of 37.5% (v/v) and to the best separation performances] was measured by microtitration and the values obtained are reported in Table 3. As expected, the decrease in the dielectric constant deriving from the use of TFE, leads to a decrease in the dissociation constant of the carboxyl groups and an increase in that of the ammonium ions. From these apparent pK_a values, the peptide charges can be calculated [1] as a function of apparent pH values. They are reported in Fig. 2 in comparison with those obtainable in aqueous solutions. Since the modification of the apparent pK_a values in TFE solutions leads to an increase in peptide charge at acidic pH values, we assume that the decrease in the product $\mu\eta$ derives from the increase in the Stokes radius of the peptide.

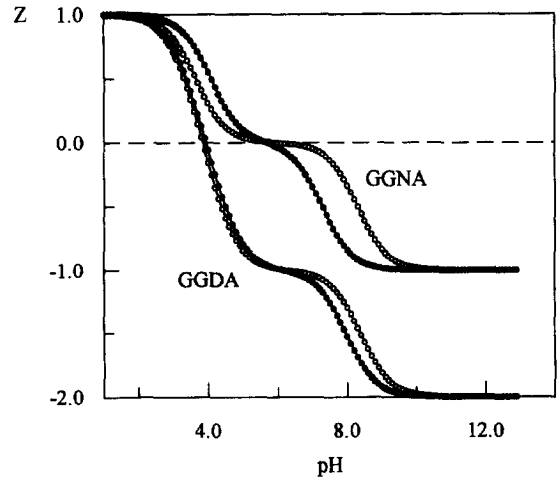

Fig. 2. Theoretical charges (Z) of GGXA peptides in (○) H_2O and (●) TFE–H_2O (37.5:62.5, v/v) as a function of apparent pH. The Z values were obtained from the pK_a values reported in Table 3.

When the mobility is plotted as a function of the peptide charge, in good agreement with Eq. 1, a linear correlation is observed (Fig. 3). From

Table 3
Peptide apparent pK_a values obtained by titration measurements in water and in TFE–water mixtures

Peptide	pK_a	H_2O	TFE–H_2O (37.5:62.5, v/v) (TFE molar fraction 0.0728)
GGNA	pK_{a_1}	3.65 ± 0.01	4.09 ± 0.01
	pK_{a_2}	8.30 ± 0.01	7.28 ± 0.01
GGDA	pK_{a_1}	3.53 ± 0.01	3.56 ± 0.01
	pK_{a_2}	4.16 ± 0.01	4.32 ± 0.01
	pK_{a_3}	8.37 ± 0.02	7.95 ± 0.02
			TFE–H_2O (25:75, v/v) (TFE molar fraction 0.0418)
YGGFM	pK_{a_1}	3.45 ± 0.01	3.66 ± 0.02
	pK_{a_2}	7.36 ± 0.01	7.00 ± 0.03
	pK_{a_3}	10.36 ± 0.02	Not determined[a]
YGGFL	pK_{a_1}	3.69 ± 0.01	3.94 ± 0.02
	pK_{a_2}	7.40 ± 0.01	7.05 ± 0.03
	pK_{a_3}	10.34 ± 0.02	Not determined[a]

[a] Owing to solubility problems these constants were not determined (see text).

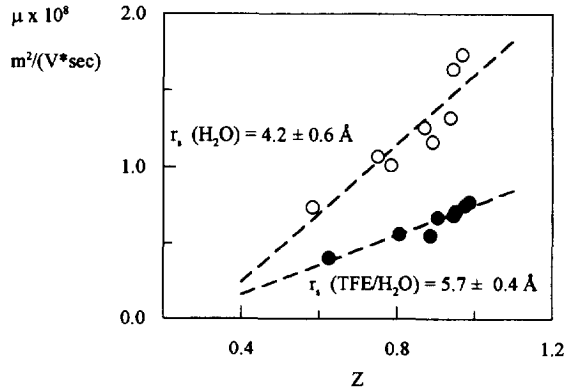

Fig. 3. Mobilities of GGXA peptides as a function of their charge (Z) in (○) H_2O and (●) TFE–H_2O (37.5:62.5, v/v). The Stokes radius was calculated using the slope of the linear regression and Eq. 1.

the slope (A) an acceptable value of the Stokes radius can be obtained according to Eq. 1, with the assumption that it does not change significantly as a function of small peptide charge differences. A Stokes radius of 4.2 ± 0.6 Å was obtained for GGXA peptides in aqueous solution, which increases, as expected, to 5.7 ± 0.4 Å in TFE–H_2O solution.

The spatial resolution (R_s; calculated according to Ref. [1]) and the number of theoretical plates (N), measured in GGXA separations at pH 2.2, are plotted in Fig. 4 and show a strong increase as a function of increasing TFE concentration. According to Ref. [11], the value of N is given by

$$N = \frac{\mu V}{2D} \quad (2)$$

where V is the applied voltage and D the longitudinal diffusion. According to the Stokes–Einstein equation, D is equal to

$$D = \frac{kT}{6\pi\eta r_s} \quad (3)$$

where T is the absolute temperature and k the Boltzmann constant. Introducing Eqs. 1 and 3 into Eq. 2, the following relationship is obtained:

$$N = \frac{qZV}{2kT} \quad (4)$$

Hence, the increase in N as a function of TFE concentration could be principally attributed to a decrease in temperature. In fact, at a constant applied voltage, the use of TFE greatly reduces and stabilizes the current during the electrophoretic run, therefore lowering the heat generated by the Joule effect. Moreover, the peak dispersion is also dependent on the number of molecular conformations of the peptide. When the peptide is blocked in restricted conformations, the structural dispersion is reduced to a minimum. In this respect, the properties of TFE are suitable for stabilizing secondary peptide structures [7,8]. In order to confirm these TFE properties with regard to the peptides under study, a series of ^{13}C NMR spectra were measured under the same conditions as used in CZE separations. As expected, the carbonyl region of the peptide spectrum obtained in the presence of TFE shows resonances broader than those observed in the absence of TFE, in agreement with a reduced molecular motion due to a more limited set of conformers in slow equilibrium [12]. The mean width of the NMR carbonyl peaks reported in Table 4 confirms that the structuring properties of TFE reduce the peptide conformations with respect to aqueous solutions. Further investigation of this effect was made by following the change in CD spectra as a function of the TFE concentration in the solution.

Fig. 5 shows the changes in the CD spectra of

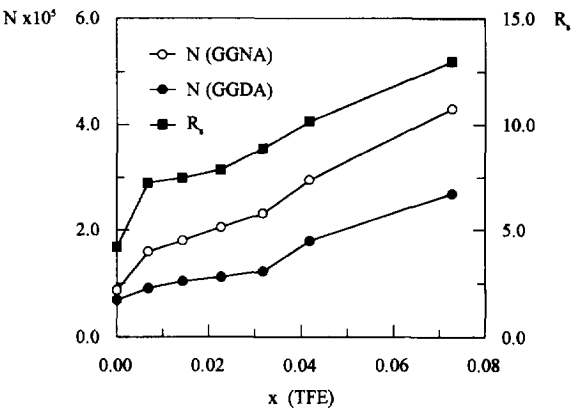

Fig. 4. Number of theoretical plates (N) and spatial resolution (R_s) for CZE separation of GGXA peptides as a function of TFE concentration.

Table 4
Mean width of carbonyl NMR signals measured in H_2O and TFE–H_2O solutions

Peptide	H_2O	TFE–H_2O (37.5:62.5, v/v)[a]	TFE–H_2O (25.0:75.0, v/v)[a]
GGNA	0.774	1.062	–
GGDA	0.766	1.518	–
Leu-enk	0.814	–	0.910
Met-enk	0.850	–	1.032

[a] The apparent pH was 2.2.

the GGXA peptides as a function of TFE concentration. The dichroic profile does not change very much, but the rotational power is clearly affected. The change in the rotational power for the two peptides under study at different concentrations of TFE indicates that also in small peptides which do not assume preferentially a well defined conformation, an increase in TFE concentration changes the distribution of the conformers towards a narrower distribution where the structured forms are highly represented. In other words, among all the possible conformers, some become markedly more populated. This change in populations affects the electrophoretic mobility and the peak dispersion and thus an effect on the peptide CZE separation is observable.

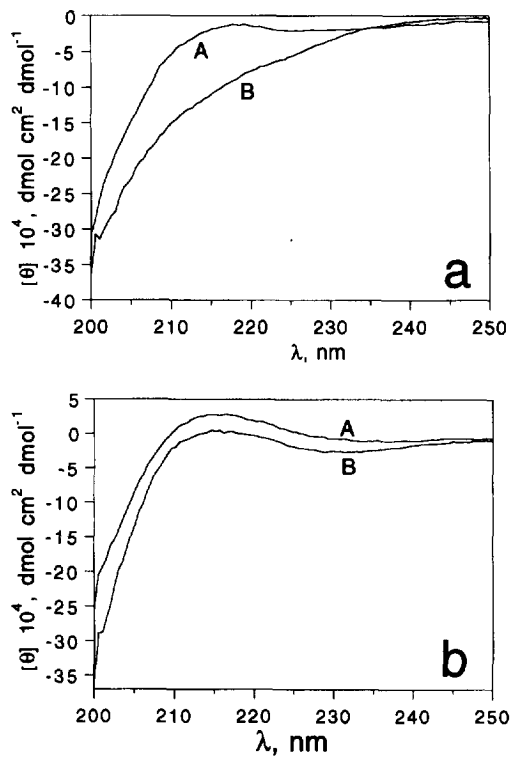

Fig. 5. CD spectra of (a) GGNA and (b) GGDA in solutions containing (A) 0% and (B) 90% TFE. Apparent pH, 2.2; other conditions as reported under Experimental.

The increase in resolution with increasing TFE concentration (Fig. 4) depends not only on the general increase in N but also on the differential modification of the peptide charges since, for peptides of similar Stokes radius (according to Ref. [1]), the following theoretical equation can be applied:

$$R_s = \frac{1}{4} \cdot \frac{\Delta Z}{\sqrt{Z_1 + Z_2}} \cdot \sqrt{\frac{qV}{kT}} \qquad (5)$$

The theoretical resolution calculated through Eq. 5 as a function of pH at 0.073 TFE molar fraction is presented in Fig. 6 and compared with the theoretical resolution in aqueous buffers. The pH range was restricted to 2.0–3.5, since at higher pH values the low mobility gives unacceptable run times. The experimental resolution measured at several pH values is linearly related to the theoretical resolution (inset in Fig. 6), although it corresponds to about 50% its absolute value. The separation obtained at pH 2.8 in the presence and absence of TFE is reported as an example in Fig. 7 and clearly shows the increase in resolution due to the TFE effect.

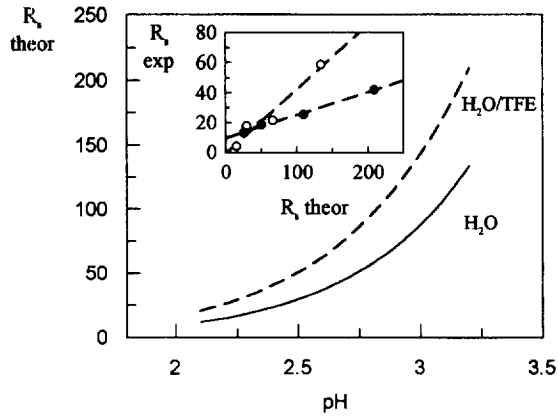

Fig. 6. Theoretical resolution calculated using Eq. 5 for GGXA separation in H_2O and in H_2O–TFE (62.5:37.5, v/v) solution as a function of pH. The inset shows the correlation between the experimental and the theoretical values (○ = H_2O; ● = TFE–H_2O).

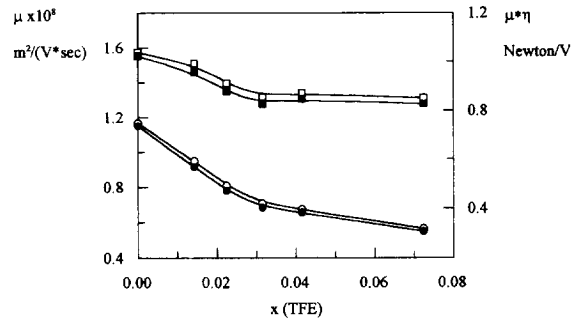

Fig. 8. Mobilities (circles) and values of the product of mobility and viscosity (squares) of leu-enkephalin (open symbols) and met-enkephalin (closed symbols) as a function of TFE molar fraction. pH, 2.2; other CZE conditions as reported under Experimental. The viscosity of TFE–H_2O solutions was calculated according to Ref. [12]. In order to offer a correct comparison with the results obtained with other peptides, the scale amplitudes correspond exactly to those utilized in Fig. 1.

3.2. Met- and leu-enkephalin

The mobility of met- and leu-enkephalin as a function of TFE concentration is presented in Fig. 8. In this case, the values of the product $\mu\eta$ indicate stabilization of the Stokes radius at ca. 0.03 TFE molar fraction. The titration of the enkephalins at a 0.0418 TFE molar fraction [corresponding to a concentration of 25% (v/v)] allowed the apparent pK_a values reported on Table 3 to be obtained. The pK_a values of tyrosines were not determined owing to solubility problems. Since the CZE separations were performed at acidic pH values, an exact knowledge of these constants is useless for the charge determination (at acidic pH values the phenolic group of tyrosine can be considered to be completely associated).

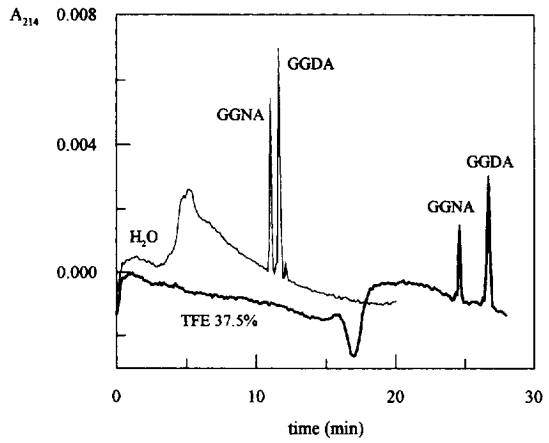

Fig. 7. CZE separation of GGDA and GGNA peptides in H_2O and H_2O–TFE (62.5:37.5, v/v) solution at an apparent pH of 2.8. The CZE conditions are reported under Experimental.

The charge of the enkephalins in water and at 0.0418 TFE molar fraction at acidic pH values is presented in Fig. 9 and the mobility as a function of the peptide charge at this TFE concentration is presented in Fig. 10. From these values, the mean Stokes radius obtained for the enkaphalins was 6.8 ± 0.4 Å in water and 7.0 ± 0.2 Å in TFE–H_2O. The insignificant increase in the Stokes radius is in agreement with the small variation of the $\mu\eta$ product reported in Fig. 8. From these results, the decrease in mobility can be principally attributed to the increase in viscosity.

The number of theoretical plates and the resolution as a function of TFE molar fraction at pH 2.2 are presented in Fig. 11. Also in this case, an increase in N can be observed, again linked to

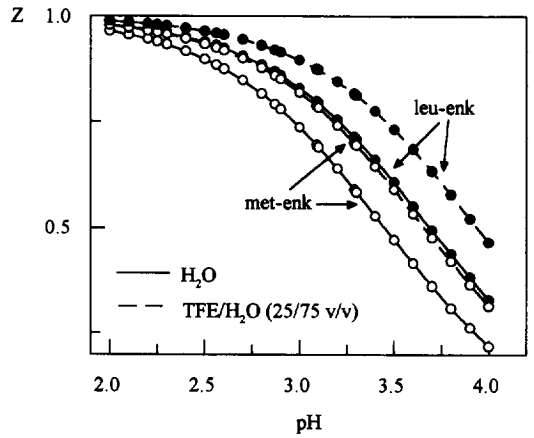

Fig. 9. Theoretical charges (Z) of enkephalins in H_2O and TFE–H_2O (25:75, v/v) as a function of pH calculated from the pK_a values in Table 3.

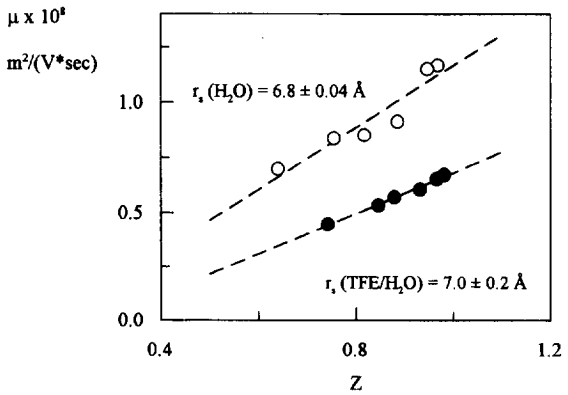

Fig. 10. Mobilities of enkephalins as a function of their charge (Z) in (○) H_2O and (●) TFE–H_2O (25:75, v/v). The Stokes radius was calculated using the slope of the linear regression and Eq. 1.

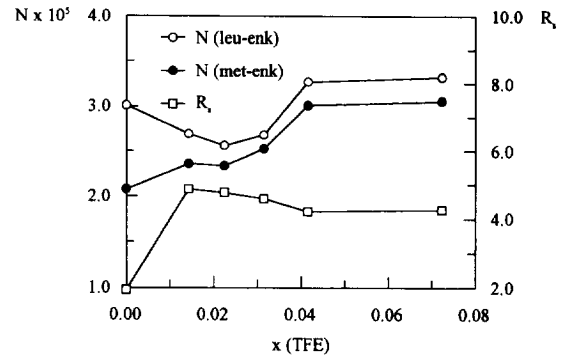

Fig. 11. Number of theoretical plates (N) and spatial resolution (R_s) for CZE separation of met- and leu-enkephalin as a function of TFE concentration.

a better temperature dispersion. The increase in R is not relevant. In fact, the charge difference between the two peptides is not greatly modified by TFE (Table 3). The theoretical resolutions calculated (Eq. 5) as a function of pH at a 0.0418 TFE molar fraction and in water are approximately the same (Fig. 12), until a pH of about 3.5 is reached. Nonetheless, the experimental resolution measured in TFE (Fig. 12, inset) is greater than that in water, principally owing to a decrease in the peak width.

The data obtained from ^{13}C NMR spectra (Table 4) are in agreement with a reduced interaction of enkephalins with TFE. The CD spectra (Fig. 13) indicate that met- and leu-enkephalin also do not assume a well defined conformation owing to the interaction with TFE. Nevertheless, the changes observed suggested that the TFE interaction affects the rotational power as a function of TFE concentration (not reported). Also for enkephalins the TFE interaction promotes a change in the peptide population, excluding some of the possible conformations, towards more structured forms. The reduction of mean conformational flexibility could provide, also in this case, a modification of the electrophoretic mobility and of the peak dispersion.

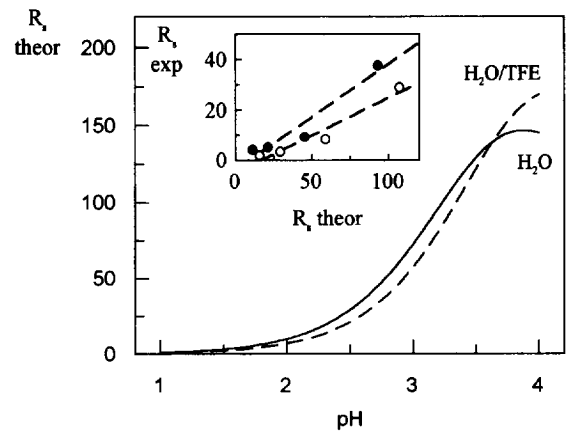

Fig. 12. Theoretical resolution calculated using Eq. 5 for enkephalin separations in H_2O and TFE–H_2O (25:75, v/v) solution as a function of pH. The inset shows the correlation between the experimental and the theoretical values (○ = H_2O; ● = TFE–H_2O).

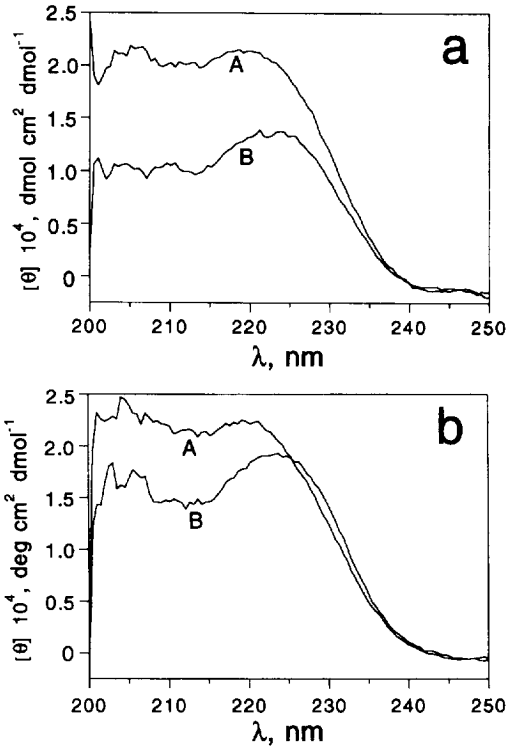

Fig. 13. CD spectra of (a) met-enkephalin and (b) leu-enkephalin in solutions containing (A) 0% and (B) 90% TFE. Apparent pH, 2.2; other experimental conditions as reported under Experimental.

3.3. ACTH fragments

Owing to the low availability and the high cost of ACTH fragments, the titration of these peptides and the measurement of the ^{13}C NMR spectra were not performed. Therefore, a precise determination of their charge was not possible. Nonetheless, since the use of TFE in the CZE separations on GGXA peptides and enkephalins display different results, we decided to perform further experiments. The mobility of ACTH fragments as a function of TFE molar fraction is reported in Fig. 14. It is evident that in this case the peptide behaviours are completely different to those in the previous examples. The biphasic aspect of the $\mu\eta$ curve leads to the assumption of a decrease in the Stokes radius at low TFE concentration and a small increase at higher concentration. Although an accurate charge

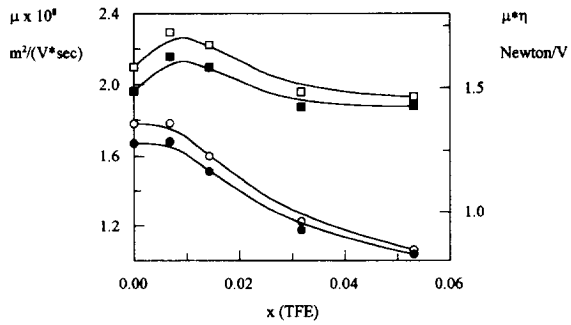

Fig. 14. Mobilities (circles) and values of the product of mobility and viscosity (squares) of ACTH fragment 5–10 (open symbols) and ACTH fragment 4–10 (closed symbols) as a function of TFE molar fraction. pH, 2.2; other CZE conditions as reported under Experimental. The viscosity of TFE–H_2O solutions was calculated according to Ref. [10]. In order to offer a correct comparison with the results obtained with other peptides, the scale amplitudes correspond exactly to those utilized in Fig. 1.

value for these peptides was not obtainable, the large decrease in the resolution as a function of increasing TFE concentration at an apparent pH of 2.2 (Fig. 15), clearly expected from the results in Fig. 14, should derive from a convergence of the pK_a values at high TFE concentrations.

3.4. Tryptic peptides of myoglobin

The use of TFE in the CZE separation of the small polar peptides described previously leads to a general increase in N and it can provide, for

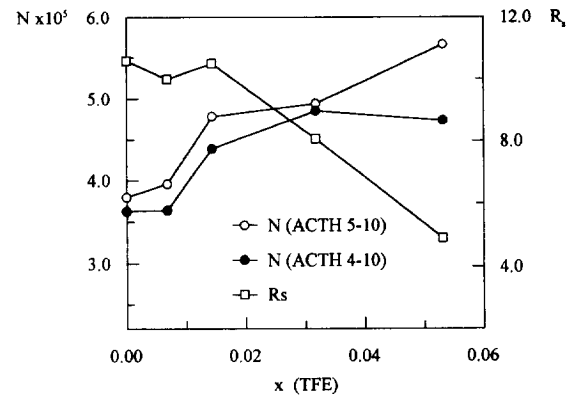

Fig. 15. Number of theoretical plates (N) and spatial resolution (R_s) for CZE separation of ACTH fragments as a function of TFE concentration.

some peptides, a large increase in resolution. As described, these effects are principally attributable to an increase in viscosity and to a modification of the peptide charge, deriving from modification of the dielectric constant of the solution. The structuring properties of TFE do not exert great effects for peptides smaller than about ten amino acids. For this reason, the effect of TFE on the peptides deriving from the tryptic digestion of horse skeletal muscle myoglobin was also studied. In this case the protease digestion provides large peptides which can be easily structured by TFE at high concentration, with a consequent improvement in the performance of the separation. In Fig. 16, the CZE separation of the tryptic digest of myoglobin at 0.0531 TFE molar fraction [corresponding to a concentration of 30% (v/v)] is presented and compared with that obtained, at the same pH values, in aqueous buffer. The CZE separation at 30% (v/v) TFE clearly indicates a strong modification of the selectivity on the zone pertaining to the small polar peptide and a large improvement in the separation performance in the zone pertaining to the large apolar peptides. An accurate NMR study of the effect of TFE on the myoglobin fragment 132–153 was performed by Van Buuren and Berendsen [13]. The structure-forming properties of TFE provide strong stabilization of the fragment conformation, generating two stable α-helical sections which are not observed in aqueous solution. From these results, it can be postulated that the increase in performance observed for the large apolar myoglobin fragments derives primarily from a structure stabilization.

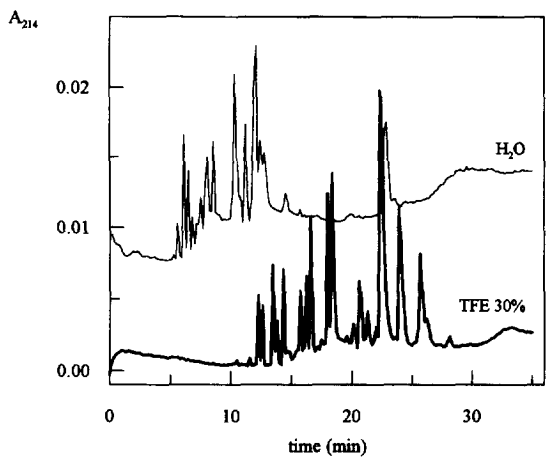

Fig. 16. CZE separations of the tryptic digest of horse skeletal muscle myoglobin in water and TFE–H_2O (30:70, v/v) at pH 2.2. Other conditions are reported under Experimental.

4. Discussion

The results obtained in this study indicate that electrophoretic peptide separations in the presence of TFE as organic cosolvent display several advantages over separations performed in aqueous solution. They can be summarized as it follows:

(i) An increase in solution viscosity is reflected in a decrease in ion mobility with a consequent lowering of the current. The decreased Joule effect permits better temperature control in the capillary during the analysis, resulting in an increased number of theoretical plates.

(ii) The modification of the dielectric constant induces a change in the peptide protonic dissociation constants, usually a decrease in those of the carboxylic groups and an increase in those of the ammonium groups. Therefore, a possible enhancement of the charge differences of similar peptides and thus an increase in the electrophoretic resolution can be obtained. In any case, the charge change provides a good option for modulation of the separation selectivity.

(iii) The interaction of TFE with the peptide can modify its Stokes radius, with a consequent modification of the mobility. The interaction of TFE depends strongly on the peptide sequence, as clearly demonstrated by the different results obtained with different small peptides.

(iv) The structuring properties of TFE contribute to an increase in the separation performance. This behaviour, not very important for small polar peptides, becomes a relevant factor for the improvement of the CZE separation of large apolar peptides.

(v) Finally, an additional important TFE property is its capability to increase the stability of the capillary coating. In fact, after the use of a

TFE solution, the coating remains stable for more than 3 months, even with continuous utilization of the capillary. Normally, with the use of aqueous solutions alone, the coating is not stable for longer than 30–40 runs. This coating stabilization could derive from passivation of the silica wall by a small release of fluorine by TFE.

For the aforementioned properties, when a CZE separation of medium-sized peptides is characterized by insufficient performance, the use of TFE–water mixtures as an electrophoretic CZE medium is highly advisable.

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