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Generation of tryptic maps of α- and β-globin chains by capillary electrophoresis in isoelectric buffers

Laura Capelli[a], Alexander V. Stoyanov[b], Henri Wajcman[c], Pier Giorgio Righetti[a,*]

[a]*Department of Agricultural and Industrial Biotechnologies, University of Verona, Strada Le Grazie, Cà Vignal, 37134 Verona, Italy*
[b]*Institute of Gene Biology, Russian Academy of Sciences, Vavilova Street, 34/5, 117334 Moscow B-334, Russia*
[c]*INSERM U91 and Laboratoire des Hemoglobines, Service de Biochimie, Hopital Henri Mondor, 94010 Creteil, France*

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Abstract

A novel method for generating peptide maps, following tryptic digests of proteins, is reported here: capillary zone electrophoresis in the presence of isoelectric buffers as the sole buffering species. A typical buffer composition comprises 50 m*M* aspartic acid (pH=p*I*=2.77), 0.5% hydroxyethyl cellulose (added as a dynamic coating agent for preventing peptide adsorption to weakly ionized silanols), 5% trifluoroethanol and 1% zwitterionic detergent (CHAPS). With this buffer composition, a high-voltage gradient can be applied (typically 600 V/cm in 75 μm I.D. and 900 V/cm in 50 μm I.D. capillaries), thus drastically reducing the analysis times. The method is applied to the generation of peptide maps of α- and β-globin chains from human adult hemoglobin. In the case of β-peptides, at an operative pH of 2.77, which represents a cross-over point in the titration curve of peptides T2 and T9, the two analytes merge into a single peak. However it is shown that it is possible to change the pH of the zwitterionic buffer by adjusting its concentration in solution. In 30 m*M* Asp (pH 3.0) or 20 m*M* Asp (pH 3.1) resolution of these two peptides is fully restored. Isoelectric, amphoteric buffers thus seem to represent a novel, powerful buffer system able to offer high resolution and high selectivity. © 1997 Elsevier Science B.V.

Keywords: Isoelectric buffers; Buffer composition; Conductivity; Buffering power; Peptides; Globin peptides

1. Introduction

Peptide mapping has been used for many years for the characterization of the primary structure of proteins, ever since Ingram first reported, in a now classical series of papers [1–3], the technique of fingerprinting and discovered that the difference between normal human adult and sickle cell hemoglobins was the replacement of a glutamic acid with a valine residue. Later, he thus spoke of his large-size filter papers used for developing the two-dimensional technique: ''I remember that our first fingerprints looked like a modern watercolour left out in the rain!'' [4] (the recipe for the watercolour: steam the paper for 10 min, dip in 0.2% ninhydrin in acetone and develop at room temperature). In modern times, this cumbersome technique has been replaced by reversed-phase, high-performance liquid chromatography (RP-HPLC), in which selectivity is elicited in a single dimension via hydrophobic interaction of the peptide side chains with the stationary phase. Peptides are usually separated in

*Corresponding author. Address for correspondence: L.I.T.A., Room 3.16, Via Fratelli Cervi No. 93, Segrate 20090 (Milano), Italy.

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trifluoroacetic acid (TFA)–water–acetonitrile mobile phases with ion suppression of acidic residues at pH 2 and ion pairing between TFA and basic residues [5,6]. More recently, capillary zone electrophoresis (CZE) has been adopted as a method complementary to RP-HPLC since, in free solution, the peptide mobilities are proportional to the charge/mass ratio, thus introducing a selectivity parameter not based on a hydrophobicity scale. By and large, the experimental parameters adopted for developing peptide maps by CZE have been based on the use of acidic buffers (typically phosphate, formate, glycinate) at pH values ranging from 1.95 up to 2.8, in concentrations ranging from 25 to 100 m*M* [7–18]. A recent review summarizes all the various mathematical models underlying the mechanism of peptide separation [19].

Use of acidic separation buffers for peptide mapping by CZE possesses some distinct advantages. First of all, at pH values below the p*K* of the free silanols on the fused-silica wall (assessed as pK=6.3, with a wall neutralization at pH 2.3) [20], their dissociation will be significantly suppressed and, in principle, the negative charge on the silica surface abolished. The electroosmotic flow (EOF) should thus be negligible and the peak capacity greatly increased. Additionally, the low pH value ensures that the vast majority of peptides will bear a net positive charge, thus allowing their migration at the cathodic end of the capillary, past the detector window. As a third bonus, the negligible negative charge on the capillary wall will also mean that the coulombic interaction between the fused-silica surface and the peptides will be markedly diminished, thereby removing the most important reason for peak broadening and skewing [21,22] (although quite a few authors report adsorption of peptides to the wall even at pH 2, thus advocating the use of polyacrylamide-coated capillaries) [7,11,18].

However, satisfactory separations are not always obtained using acidic buffer conditions. As the charge of the peptides plays a pivotal role in their separation by CZE, other pH values might be necessary in order to avoid co-migration of peptides having cross-over points at a given pH value in their titration (pH/mobility) curves. Additionally, at low-enough pH values effectively minimizing peptide-wall interaction (i.e., below pH 2), the buffer and bulk water conductivities are so high that only low voltage gradients can be adopted (typically not higher than 200 V/cm), thus greatly lengthening the analysis times (in many reports, up to 50–60 min are required for fully developing complex peptide maps). During such long runs, peptide zones considerably broaden and peak resolution worsens. Recently, we introduced isoelectric buffers (notably His, but also Lys) for separation of oligonucleotides and found we could reduce the analysis times from ca. 30 min down to as low as 4–5 min, at voltage gradients as high as 1000 V/cm, with greatly increased resolution and plate numbers [23,24]. In search for a similar method applicable to peptide maps generation, Righetti and Nembri [25] proposed the use of isoelectric aspartic acid as a background electrolyte, operating at pH=pI=2.77 (at 25°C). These authors could produce peptide maps of casein in only 10–12 min (as opposed to 80 min in standard phosphate buffer, pH 2.0) at voltage gradients as high as 800 V/cm, with much increased resolution. Adsorption of some larger peptides to the wall was completely eliminated by adding to the background, isoelectric Asp buffer, 0.5% hydroxyethyl cellulose (HEC) and 5% trifluoroethanol (TFE).

In the present report, we apply this novel methodology to the generation of tryptic maps of α- and β-globin chains and compare the resolution and peak elution profile to the standard RP-HPLC maps. We show additionally that, while still working with isoelectric Asp, it is possible to modulate the pH of this background electrolyte, moving along by as much as a 0.3 pH unit interval on the pH scale, thus avoiding cross-over points in the pH/mobility curves of some peptides and thus eliciting resolution of co-migrating zones.

2. Experimental

2.1. Materials

Free aspartic acid and TFE were purchased from Fluka, Buchs, Switzerland. Trypsin (TPCK-treated, type XIII) was obtained from Sigma (St. Louis, MO, USA). Fused-silica capillaries (75 or 50 μm I.D., 375 μm O.D.) were purchased from Polymicro Technologies (Phoenix, AZ, USA). HEC (M_n 24 000–27 000) was from Polysciences (Warrington,

PA, USA). Human adult hemoglobin (HbA) was from healthy donors.

2.2. Purification of HbA

Prior to digestion, Hb was purified by small-scale preparative isoelectric focusing (IEF) in flat beds [26]. The gel was made by a suspension of 0.9 g Ultrodex (Pharmacia, Uppsala, Sweden) in 24 ml of a 1.7% solution of Ampholine, pH 6–8. This mixture was poured into a 26×2.5×0.4 cm cassette and submitted to air stream for reducing the gel weight by 30%. The hemolysate (0.5 ml) was applied to the gel and IEF performed over-night at 5°C and 1300 V (2 W). At the end of the IEF procedure, the various Hb components were collected with a spatula and eluted from the gel with water in small columns.

2.3. Globin preparation

Globins were obtained by Hb A precipitation with cold acetone/HCl, diethyl ether washing and drying under a mild stream of oxygen [27]. Prior to digestion, the globin pellet was dissolved in 400 μl of a 0.2% solution of trifluoroacetic acid (TFA) in water and submitted to chain separation by RP-HPLC in an Aquapore RP-300 column (25×0.46 cm) (Brownlee Labs., Santa Clara, CA, USA). Elution was effected with a gradient of two solvents. Solvent A: 20% isopropanol and 80% of a solution of 0.2% TFA in water. Solvent B: 60% isopropanol and 40% of the same TFA solution. The gradient was: 0–20% B from 0 to 2 min; 20–40% B from 2 to 35 min; 40–60% B from 35 to 40 min; isocratic at 60% B for 10 min. The flow-rate was 0.8 ml/min with 280 nm monitoring. Globin chains were collected in small Pyrex tubes and dried under vacuum.

2.4. Aminoethylation and tryptic digestion

For aminoethylation, globin chains were dissolved in 1.5 ml of 6 *M* urea adjusted at pH 8.6 with Tris–HCl. The protein was incubated for 3 h, under nitrogen, with 20 μl of β-mercapthoethanol. At the end of the reaction, reagents in excess and salts were removed by RP-HPLC using a C_8 guard column eluted with a 15-min gradient of acetonitrile, from 0 to 60%, in 0.1% TFA in water. Globin chains were digested overnight, at room temperature, with TPCK-treated trypsin in 25 m*M* ammonium bicarbonate, pH 8.8, using an enzyme-to-substrate ratio of 3–100 (w/w).

2.5. Peptide analysis by RP-HPLC

The tryptic peptides were separated by RP-HPLC at a flow-rate of 1 ml/min on an Aquapore RP-300 column (25×0.46 cm). Elution was effected in 120 min by developing a linear gradient from 0 to 30% acetonitrile in 0.5% TFA. The chromatogram was monitored at 214 nm and the peptides were manually collected in Eppendorf tubes and dried under vacuum [28].

2.6. CZE analysis

CZE of tryptic digests was carried out with a Waters Quanta 4000 instrument. Thirty-seven-cm long (30 cm to detector), for the 75 μm I.D., or 30-cm long (23 cm to detector), for the 50 μm I.D., uncoated capillaries were used. The following background electrolytes were used: (a) 50 m*M* isoelectric aspartic acid (pH=p*I*=2.77 at 25°C) added with 0.5% HEC (M_n=27 000), 5% TFE and 1% CHAPS; (b) 30 m*M* Aps, 0.5% HEC, 10% TFE and 50 m*M* non-detergent sulphobetaine 195 (NDSB) [29], pH 3.0; (c) 20 m*M* Asp, 0.5% HEC, 50 m*M* NDSB-195, pH 3.1. Two types of capillaries were used: either 75 or 50 μm I.D. In the first case, separations were performed at 600 V/cm; in the second case, at 900 V/cm (ca. 18 μA current). The samples were typically injected for 30 s by hydrostatic pressure.

2.7. Calculation of peptide mobility vs. pH

The electric charge on pH relationship for each peptide was calculated as reported earlier [37], by utilizing the dissociation constant values of free amino acids [38]. The peptide mobility (μ) was supposed to be inversely proportional to the cubic root of molecular size (according to Stoke's law), as follows:

$$\mu(\mathrm{pH}) \sim Q(\mathrm{pH})\, M^{-1/3}$$

where Q is the peptide net charge at a given pH. By taking into account the influence of frictional forces, it was possible to obtain different mobility curves for peptides having the same theoretical electric charge but with different molecular mass. Although this is an approximation, it seems to reflect well the behaviour of our peptides in CZE. A number of other formulas for peptide mobilities have been proposed, as reviewed by Castagnola et al. [19]. Nevertheless, it should be remembered that in all cases these approaches also represent approximations.

3. Results

Fig. 1 shows a representative CZE run of four different β-globin peptides, isolated by RP-HPLC and run in buffer (a) (pH 2.77) at 600 V/cm. This Fig. is an overlay of four separate runs, performed with single, isolated peptides, in order to recognize the different fragments as eluted by RP-HPLC. By adding them one by one and sequentially running the enriched mixture, we could identify the 13 major

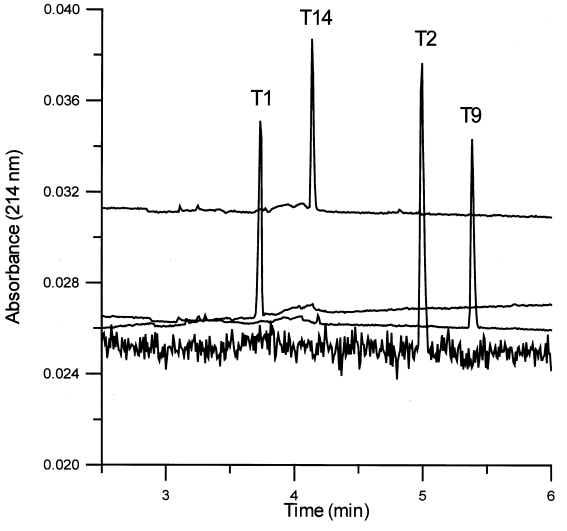

Fig. 1. Separation of selected, RP-HPLC purified fragments of the β-chain by CZE. Run conditions: 37-cm long (30 cm to detector) capillary, run at 22.2 kV (600 V/cm) at ca. 25 μA. Buffer: 50 m*M* Asp, pH 2.77, added with 0.5% HEC, 5% TFE and 1% CHAPS. Sample injected for 15 s by hydrostatic pressure. The results shown represent the superposition of four different runs. Detection at 214 nm.

β-globin peptides. Fig. 2 gives the CZE profiling of all the peptides separated, with the nomenclature typically given in RP-HPLC. It can be noted, however, that at the prevailing pH in buffer (a) (pH 2.77) two peaks (labelled T2 and T9) co-elute into a single zone. We have thus simulated the theoretical pH/mobility curves of the 13 peptides in the pH 2–4 interval: as shown in Fig. 3, indeed at this pH value there is a cross-over point in the titration curves of these two peptides, which could only be eliminated by working at slightly different pH values, such as pH 3.0 or 3.1. However, if one were to work at this pH value with a conventional, non-amphoteric buffer, one would automatically lose the benefit of adopting high voltages (600 V/cm in Figs. 1 and 2) and thus considerably shorten the separation time while gaining in resolving power. It should be noted, however, that, as anticipated by Righetti and Nembri [25] (see their Fig. 5), the pI of 2.77 is only a theoretical value, reached at high enough concentration of Asp (in this case 50 m*M*). By progressive dilution of the buffering ion, one can span a small pH interval, up to at least 0.3 pH units. Thus, it is possible, while still working under isoelectric conditions, to modulate the pH of the background electrolyte simply by varying its concentration. In buffer (b), at 30 m*M* Asp concentration (still ensuring adequate buffering power), the final pH of the background electrolyte is 3.0. When adopting this last buffer (see Fig. 4a), it was possible to separate all the 13 β-globin peptides. By enlarging the 3–6-min separation window (see Fig. 4b) one can indeed appreciate that now the T2 and T9 β-peptides, which co-migrated into a single zone (cf. Fig. 2) are baseline resolved. However, as the pH increased to 3.0, there was a higher risk of peptide adsorption to the naked silica wall (only uncoated capillaries are used throughout) and addition of a zwitterion (NDSB-195) was necessary in order to quench such interaction.

Fig. 5 gives the corresponding RP-HPLC profile of the 13 major β-globin peptides: it is seen that, in order to achieve the same resolution, a total elution time of 60 min is necessary. Thus, our method seems to compare favourably with previously well-established and routinely adopted methods in protein chemistry laboratories.

Fig. 6 gives the theoretical titration curves of nine

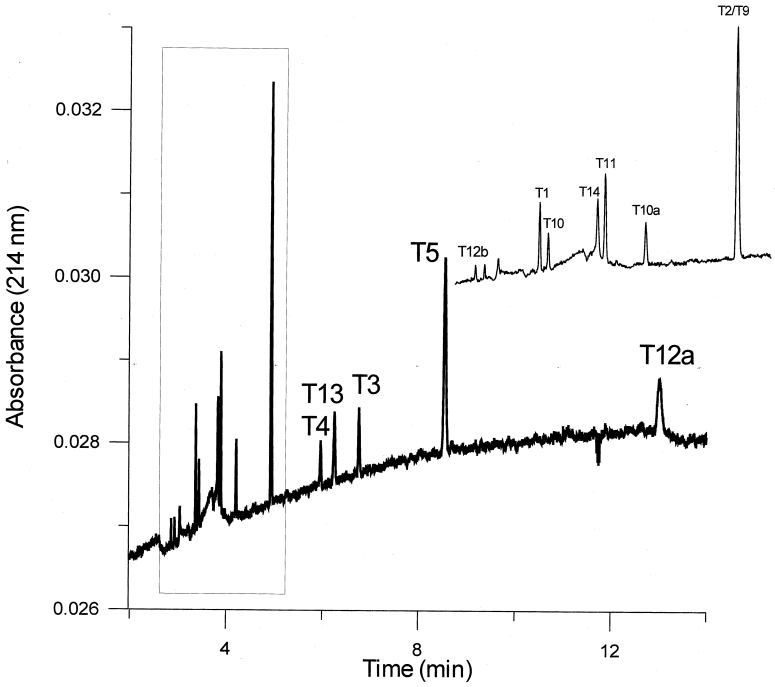

Fig. 2. CZE separation of a peptide digest of β-globin chains. All conditions as in Fig. 1. Note that, at the operative pH (2.77), the two fragments T2 and T9 merge into a single peak. The inset on the right shows an enlargement of the boxed area on the left side (transit times from 2.5 to 5.3 min) with proper peak identification.

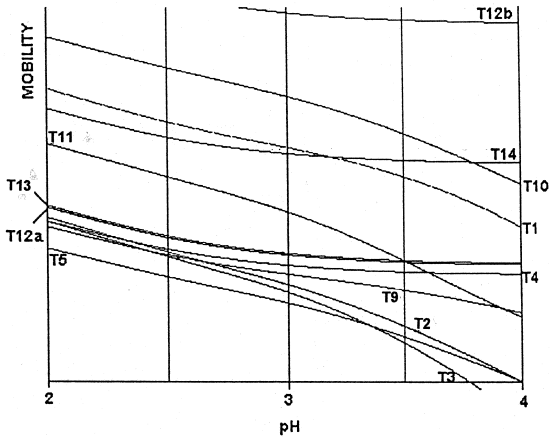

Fig. 3. Theoretical titration (pH/mobility) curves of the major fragments derived from a tryptic digest of β-globin chains. Mobility has been calculated as proportional to the fragment charge devided by the cubic root of molecular mass, as described in Section 2.5. Note the cross-over point at pH 2.77 of the T2 and T9 peptides.

α-globin peptides. It is seen here that a pH of 3.1 is just adequate for resolving these peptides. Fig. 7 gives the corresponding CZE separation. The nine peptides are fully resolved, although the time window available for separation was extremely short (only 3 min) since these peptides have a high net charge at this pH value. All peptides could be identified by adding one by one single, purified peptides isolated from RP-HPLC until reconstituting the full mixture. Fig. 8 displays the corresponding RP-HPLC profile: it is seen that, for developing the full tryptic map, up to 60 min are needed, which renders the CZE technique highly competitive.

In order to summarize the above data, we present in Table 1 the nomenclature, sequence (one-letter code), and theoretically predicted and experimentally found mobility data (in relative units) for all peptides studied in the present work. It should be noted that not all possible peptides generated by tryptic digest of α- and β-chains are visible in the CZE tracings. Since these maps represent a reconstituted profile taken from single, isolated HPLC peaks, the few

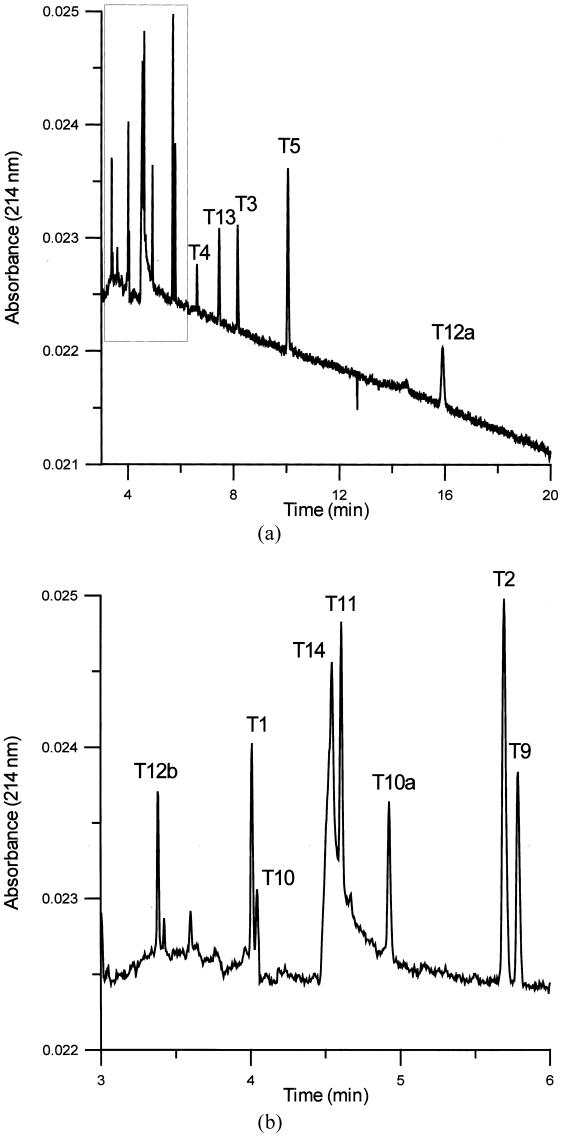

Fig. 4. CZE separation of a peptide digest of β-globin chains. Run conditions as in Fig. 1, except that the buffer was: 30 m*M* Asp, pH 3.0, added with 0.5% HEC, 10% TFE and 50 m*M* NDSB-195. Sample injected for 15 s by hydrostatic pressure. Note in panel b (an enlargment of the boxed area in panel a) the full splitting of peaks T2 and T9, unresolved in the buffer of Fig. 1.

missing bands are those of peptides present in such minute amounts in the original hydrolysate as to go undetected in the second CZE run (allow for some losses in the transfer from the first to the second dimension). It should additionally be noted that, with

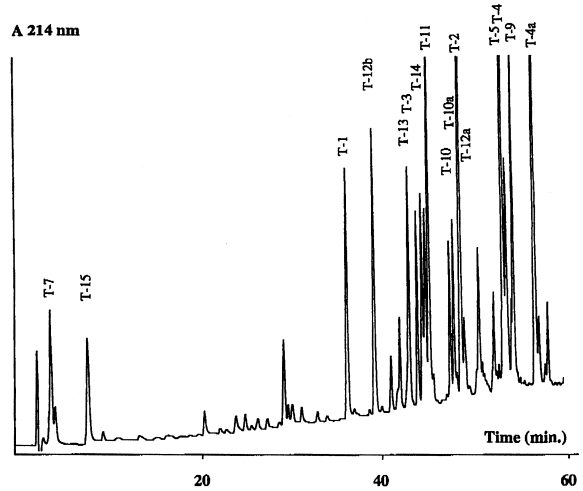

Fig. 5. RP-HPLC separation of the tryptic digest of β-globin chains. Chromatographic conditions as described in Section 2.

a few exceptions, there is a reasonable agreement between experimental and theoretical mobility data.

4. Discussion

4.1. On alternative constructions of peptide maps

We have seen in the introduction that, by far, the most commonly adopted procedure for peptide map generation in CZE is the use of very acidic buffer systems (pH 2.0–2.5) in uncoated capillaries. The method here presented represents a novel alternative,

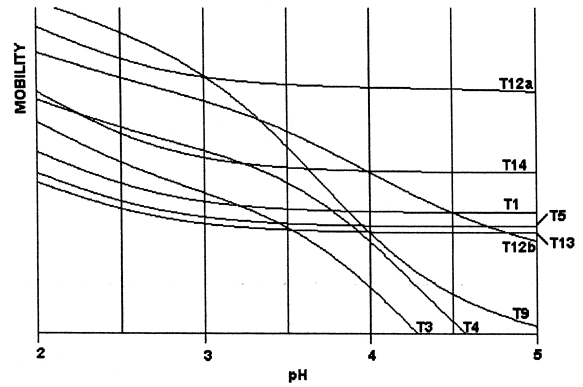

Fig. 6. Theoretical titration (pH/mobility) curves of the major fragments derived from a tryptic digest of α-globin chains.

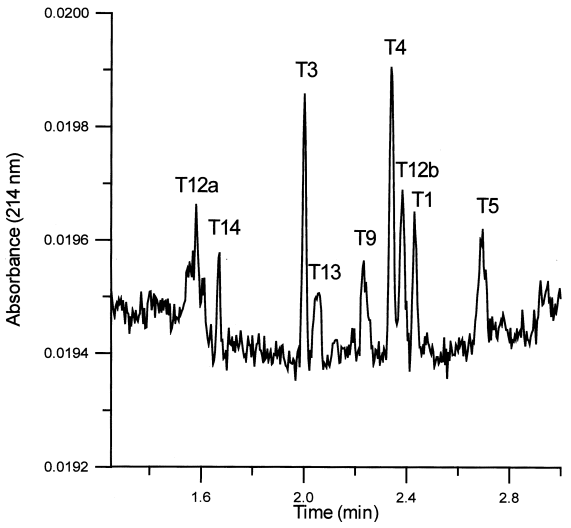

Fig. 7. CZE separation of a peptide digest of α-globin chains. Run conditions as in Fig. 1, except that the buffer was: 20 m*M* Asp, pH 3.1, added with 0.5% HEC and 50 m*M* NDSB-195. Sample injected for 30 s by hydrostatic pressure. Run at 27 kV (900 V/cm). Capillary: 30 cm long (23 cm to detector), 50 μm I.D. Note the splitting of peaks T12a and T1, which in RP-HPLC coelute.

i.e. separations under acidic conditions (pH 2.8–3.1) but in presence of isoelectric buffers, ensuring much lower conductivities and thus compatible with high field strengths and rapid separations. Uncoated capillaries could still be adopted, but under conditions

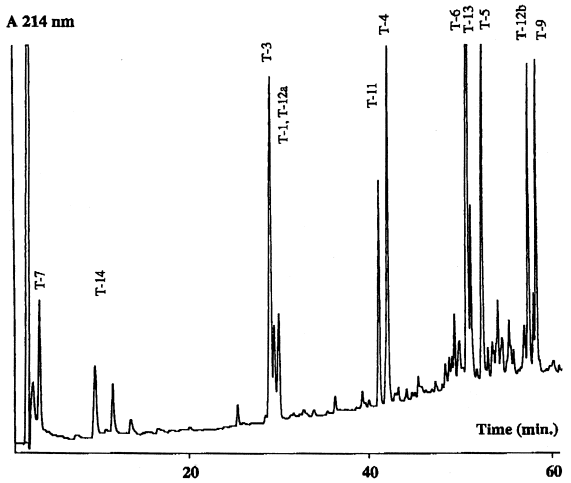

Fig. 8. RP-HPLC separation of the tryptic digest of α-globin chains. Chromatographic conditions as described in Section 2.

ensuring dynamic coating of the inner surface via adsorption of short-chain HEC and possibly ion pairing with zwitterionic substances. However, a number of alternative protocols have been developed, which will be briefly reviewed here.

4.1.1. Separations under alkaline conditions

A number of authors have reported separations at basic pH values, ranging from 8.1 up to 11.8. Rickard's group [30–32] has adopted 100 m*M* tricine, 20 m*M* morpholine buffer, pH 8.15, and obtained good separation of human growth hormone digests. Morpholine was added to minimize interaction of peptides with the silica wall. Cobb and Novotny [33] have developed a buffer system comprising 50 m*M* CAPS, pH 9.5, admixed with 10% methanol for modulating peptide mobility. As an alternate system, a micellar electrolyte was utilized, composed of 50 m*M* CAPS, pH 9.5, in presence of 10% acetonitrile and 60 m*M* SDS. Good resolution of peptide maps of trypsinogen was reported. In another approach, Deyl et al. [34] proposed 2.5 m*M* borate buffer, pH 10.5, for analysis of seven cyanogen bromide cleavage fragments from collagen. Ong et al. [35] reported separation of globin chains at pH values as high as 11.8 in 25 m*M* phosphate buffer. Mildly alkaline pH values (90 m*M* phosphate buffer, pH 7.4) were proposed by Amankwa and Kuhr [36] for separating β-casein digests. In order to ensure migration of peptides towards the detector, the capillary was dynamically coated by adsorption of cationic polymer (polybrene), thus reversing the EOF flow.

4.1.2. Use of ion-pairing agents and other modulators

Kornfelt et al. [39] proposed CZE at neutral pH values of very basic peptides in presence of phytic acid (2–5 m*M*). The enhanced resolution seems to be due to ion-pair formation between the strongly negative oligo-anionic phytic acid and positively charged amino acids, such as Lys and Arg. In another approach, Nashabeh and El Rassi [40] suggest the addition of 50 m*M* tetramethyl ammonium bromide as a selector providing both ion-pair formation and/or hydrophobic interaction with peptides. On a similar line of thinking, Rush et al. [41] adopted the ion-pairing agent heptanesulphonic

Table 1
Amino acid composition (one-letter symbols) of tryptic digest of α- and β-globin chains

Chain	Nomenclature	Sequence	Mobility[a]	
			Experimental	Theoretical
α-Globin	T1	V-L-S-P-A-D-K-	0.65	0.55
	T2	T-N-V-K-		0.50
	T3	A-A-W-G-K-	0.79	0.60
	T4	V-G-A-H-A-G-E-Y-G-A-E-A-L-E-R-	0.68	0.58
	T5	M-F-L-S-F-P-T-T-K-	0.59	0.49
	T6	T-Y-F-P-H-F-D-L-S-H-G-S-A-Q-V-K-		0.83
	T7	G-H-G-K-		1
	T8	K-		0.75
	T9	V-A-D-A-L-T-N-A-V-A-H-V-D-D-M-P-N-A-L-S-A-L-S-D-L-H-A-H-K-	0.71	0.80
	T10	L-R-		0.60
	T11	V-D-P-V-N-F-K-		0.33
	T12a	L-L-S-H-C-	1	0.85
	T12b	L-L-V-T-L-A-A-H-L-P-A-E-F-T-P-A-V-H-A-S-L-D-K-	0.67	0.74
	T13	F-L-A-S-V-S-T-V-L-T-S-K-	0.78	0.50
	T14	Y-R-	0.95	0.76
β-Globin	T1	V-H-L-T-P-E-E-K-	0.83	0.64
	T2	S-A-V-T-A-L-W-G-K-	0.60	0.48
	T3	V-N-V-D-E-V-G-G-E-A-L-G-R-	0.40	0.24
	T4	L-L-V-V-Y-P-W-T-Q-R-	0.50	0.32
	T5	F-F-E-S-F-G-D-L-S-T-P-D-A-V-M-G-N-P-K-	0.33	0.21
	T6	V-K-		0.56
	T7	A-H-G-K-		0.88
	T8	K-		0.68
	T9	V-L-G-A-F-S-D-G-L-A-H-L-D-N-L-K-	0.57	0.46
	T10	G-T-F-A-T-L-S-E-L-H-C-D-K-	0.83	0.51
	T11	L-H-V-D-P-E-N-F-R-	0.70	0.56
	T12a	L-L-G-N-V-L-V-C-	0.21	0.29
	T12b	V-L-A-H-H-F-G-K-	1	1
	T13	E-F-T-P-T-V-Q-A-A-Y-Q-K-	0.43	0.33
	T14	V-V-A-G-V-A-N-A-L-A-H-K-	0.73	0.62
	T15	Y-H-		0.51

[a]The mobility values are given in relative units. To obtain the relative mobility values for each chain, both theoretical and experimental results were normalised to maximal peptide mobility.

acid (100 m*M*) in 40 m*M* phosphate buffer, pH 2.5, for developing the peptide map of erythropoietin digested with endoproteinase. Triethyl ammonium phosphate buffer, pH 2.25, was suggested by Idei et al. [42] for fast analysis of somatostatin analogue peptides. Even heavy water was found to be superior to H_2O, especially at a pH of 7.93, for analysing tryptic fragments of calcitonin and elcatonin [43,44]. Trifluoroethanol (TFE, especially at 37.5%, v/v, in water) was found to induce a stable secondary conformation in peptides and to contribute to a transition from random to well-defined peptide conformations [45]. Markedly improved peptide maps could be obtained in presence of TFE co-solvent. Finally, it should be remembered that also metal ion-supplemented buffers can remarkably enhance resolution of peptides in CZE, especially those containing His residues [46].

4.2. On the use of isoelectric buffers

As stated in Section 1, isoelectric buffers, as originally reported, for CZE, by Hjertèn et al. [47], have much to offer in CZE technology, since they couple a very low electrical conductivity with a satisfactory buffering capacity, conditions which

permit delivering very high voltages on the separation chamber without noxious effects due to excessive Joule heating. In fact, in Ref. [47], separations at voltages as high as 2000 V/cm have been reported, with analyte transit times of the order of 30–120 s (in 15-cm long capillaries). In our hands, too, very good results have been obtained both in analysis of oligonucleotides [23,24] and of peptides [25]. Nevertheless, there are some problems connected with the use of such zwitterionic buffers: (a) the paucity of these species (possessing a high buffering capacity at the p*I* value) along the pH scale; (b) the inability of changing the pH value so as to modulate the analyte charge and mobility, for improved separations. However, there are remedies for that. In the first case, Hjertèn et al. [47] have reported the possibility of fractionating carrier ampholytes into very narrow zones, so that such narrow pH cuts can be used as quasi-isoelectric buffers at any value of the pH scale. To this purpose, Bossi and Righetti [48] have described a simple method for producing such narrow cuts of carrier ampholytes, exploiting multicompartment electrolyzers with isoelectric membranes. In the second case, the remedy has been proposed in the present report. It should be borne in mind that the p*I* of an ampholyte is not an absolute value, but it is a limit value between two extremes, which will be reached asymptotically depending on the concentration of the ampholyte in solution. At extreme dilutions, the pH of the solution will approach the pH of pure water (pH 7.0). At high enough concentrations (which depend on the ΔpK value) the pH of the solution will approach the theoretical p*I* of the ampholyte [49]. We have exploited this subtle concept in order to change the real pH in solution and to affect resolution (at a pH of 3.0–3.1) of two peptides which, at pH 2.77 (approximating the true p*I* of Asp) exhibited coincident mobilities. Thus, simply by diluting the ampholyte (while maintaining adequate buffering power, though!) one can explore, with a single amphoteric buffer, a pH interval of ca. 0.3 pH units, often adequate for moving along the titration curve of difficult separands.

It should be noted that such an operation is not performed at a severe expense of the buffering power (β) of the buffering ion. As noted by Righetti and Nembri [25] (see also their Table 1) Lys and Asp are among the best buffering ions available. At 50 m*M* concentrations Lys (with a p*I*–p*K* value of 0.79) has $\beta = 27.4$ mequiv. l^{-1} pH^{-1} and Asp (with a p*I*–p*K* value of 0.89) has $\beta = 26.4$ mequiv. l^{-1} pH^{-1}. This is an extraordinary buffering power, rarely met by any other ordinary buffer. Even at 30 m*M* concentration, Asp would still maintain a β of 15.8 mequiv. l^{-1} pH^{-1} (and at 20 *M* it would be 10.5 mequiv.), more than adequate for properly titrating and buffering any peptide zone. According to Hjertèn et al. [47], good separations can still be obtained in only 5 m*M* Lys, which would offer a β value of only 2.7 mequiv. l^{-1} pH^{-1}. Why, then, should the pH change quite substantially in our case, upon a modest dilution of Asp (from 50 to 20 m*M*)? Because the p*I* of Asp is situated at such an acidic pH region that, at the prevailing pH in the p*I* zone, bulk water would exhibit appreciable buffering power. The effect of the buffering power and conductivity of water on the shape of pH gradients has been amply covered in the literature describing immobilized pH gradients (see, e.g., Fig. 2.17 in Righetti's book) [50].

As a concluding remark, we note that also Castagnola et al. [51] have exploited CZE for generating peptide maps of whole digests of both α- and β-globin chains. However, since their conditions differ substantially from the ones reported here (in one case, they use 80 m*M* phosphate buffer, pH 2.5; in another they use a micellar system comprising 40 m*M* phosphate, 10 m*M* tetraborate, 33 m*M* SDS, pH 7.1 buffer, supplemented with 10% acetonitrile) and their peaks are unlabelled, it is quite difficult to compare their own with our maps.

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References

[1] V.M. Ingram, Biochim. Biophys. Acta 28 (1958) 539–545.
[2] J.A. Hunt, V.M. Ingram, Biochim. Biophys. Acta 28 (1958) 546–549.
[3] V.M. Ingram, Biochim. Biophys. Acta 36 (1959) 402–411.
[4] V.M. Ingram, Biochim. Biophys. Acta 1000 (1989) 147–150.
[5] W.S. Hancock, C.A. Bishop, R.L. Prestidge, D.R.K. Harding, M.T.W. Hearn, Science 200 (1978) 1168–1170.
[6] H.P.J. Bennett, C.A. Browne, S. Solomon, J. Liq. Chromatogr. 3 (1980) 1353–1365.
[7] F. Nyberg, M.D. Zhu, J.L. Liao, S. Hjertèn, in: C. Schafer-Nielsen (Ed.), Electrophoresis '88, VCH, Weinheim, 1988, pp. 141–150.
[8] P.D. Grossman, K.J. Wilson, G. Petrie, H.H. Lauer, Anal. Biochem. 173 (1988) 265–270.
[9] J.R. Florance, Z.D. Konteatis, M.J. Macielag, R.A. Lessor, A. Galdes, J. Chromatogr. 559 (1991) 391–399.
[10] A. Pessi, E. Bianchi, L. Chiappinelli, A. Nardi, S. Fanali, J. Chromatogr. 557 (1991) 307–313.
[11] M. Castagnola, L. Cassiano, R. Rabino, D.V. Rossetti, J. Chromatogr. 572 (1991) 51–58.
[12] T.A.M. Van De Goor, P.S.L. Janssen, J.W. Van Nispen, M.J.M. Van Zeeland, J. Chromatogr. 545 (1991) 379–389.
[13] C.T. Wu, T. Lopes, B. Patel, C.S. Lee, Anal. Chem. 64 (1992) 886–891.
[14] M.G. Quaglia, S. Fanali, A. Nardi, C. Rossi, M. Ricci, J. Chromatogr. 593 (1992) 259–263.
[15] V.J. Hilser Jr., G.D. Worosila, S.E. Rudnick, J. Chromatogr. 630 (1993) 329–336.
[16] H.G. Lee, D.M. Desiderio, J. Chromatogr. A 666 (1994) 271–283.
[17] B.C. Lim, M.K. Sim, J. Chromatogr. B 655 (1994) 127–131.
[18] M. Castagnola, L. Cassiano, I. Messana, G. Nocca, R. Rabino, D.V. Rossetti, B. Giardina, J. Chromatogr. A 656 (1994) 87–97.
[19] M. Castagnola, I. Messana, D.V. Rossetti, in: P.G. Righetti (Ed.), Capillary Electrophoresis in Analytical Biotechnology, CRC Press, Boca Raton, 1996, pp. 241–275.
[20] M.S. Bello, L. Capelli, P.G. Righetti, J. Chromatogr. A 684 (1994) 311–320.
[21] M.S. Bello, M.Y. Zhukov, P.G. Righetti, J. Chromatogr. A 693 (1995) 113–130.
[22] S. Ermakov, M.Y. Zhukov, L. Capelli, P.G. Righetti, J. Chromatogr. A 699 (1995) 297–313.
[23] C. Gelfi, M. Perego, P.G. Righetti, Electrophoresis 17 (1996) 1470–1475.
[24] A.V. Stoyanov, C. Gelfi, P.G. Righetti, Electrophoresis 18 (1997) 717–723.
[25] P.G. Righetti, F. Nembri, J. Chromatogr. A 772 (1997) 203–211.
[26] B.J. Radiola, Biochim. Biophys. Acta 295 (1973) 412–428.
[27] J.B. Clegg, M.A. Naughton, D.J. Weatherall, J. Mol. Biol. 19 (1966) 91–108.
[28] H. Wajcman, J. Bardakdjian, R. Ducrocq, Ann. Biol. Clin. 50 (1993) 867–870.
[29] L. Vuillard, N. Marret, T. Rabilloud, Electrophoresis 16 (1996) 295–297.
[30] R.G. Nielsen, E.C. Rickard, J. Chromatogr. 516 (1990) 99–114.
[31] E.C. Rickard, M.M. Strohl, R.G. Nielsen, Anal. Biochem. 197 (1991) 197–207.
[32] R.G. Nielsen, R.M. Riggin, E.C. Rickard, J. Chromatogr. 480 (1989) 393–401.
[33] K.A. Cobb, M.V. Novotny, Anal. Chem. 64 (1992) 879–886.
[34] Z. Deyl, V. Rojlicek, M. Adam, J. Chromatogr. 480 (1989) 371–378.
[35] C.N. Ong, L.S. Liau, H.Y. Ong, J. Chromatogr. 576 (1992) 346–350.
[36] L.N. Amankwa, W.G. Kuhr, Anal. Chem. 65 (1993) 2693–2697.
[37] A.V. Stoyanov, Russ. J. Phys. Chem. 68 (1994) 1718–1721.
[38] R.C. Weast (Ed.), Handbook of Chemistry and Physics, 67th ed., CRC Press, Boca Raton, FL, 1987, pp. C-699–C-702.
[39] T. Kornfelt, A. Vinther, G.N. Okafo, P. Camilleri, J. Chromatogr. A 726 (1996) 223–228.
[40] W. Nashabeh, Z. El Rassi, J. Chromatogr. 536 (1991) 31–42.
[41] R.S. Rush, P.L. Derby, T.W. Strickland, M.F. Rohde, Anal. Chem. 65 (1993) 1834–1842.
[42] M. Idei, I. Mezo, Z. Vadasz, A. Horvath, I. Teplan, G. Keri, J. Chromatogr. 648 (1993) 251–256.
[43] P. Camilleri, G.N. Okafo, C. Southan, R. Brown, Anal. Biochem. 198 (1991) 36–41.
[44] G.N. Okafo, P. Camilleri, J. Chromatogr. 547 (1991) 551–553.
[45] M. Castagnola, L. Cassiano, I. Messana, M. Paci, D.V. Rossetti, B. Giardina, J. Chromatogr. A 735 (1996) 271–281.
[46] R.A. Mosher, Electrophoresis 11 (1990) 765–769.
[47] S. Hjertèn, L. Valtcheva, K. Elenbring, J.L. Liao, Electrophoresis 16 (1995) 584–594.
[48] A. Bossi, P.G. Righetti, Electrophoresis 16 (1995) 1930–1934.
[49] H. Rilbe, pH and Buffer Theory: a New Approach, Wiley, Chichester, 1996, pp. 47–49.
[50] P.G. Righetti, Immobilized pH Gradients: Theory and Methodology. Elsevier, Amsterdam, 1990, pp. 103–108.
[51] M. Castagnola, I. Messana, L. Cassiano, R. Rabino, D.V. Rossetti, B. Giardina, Electrophoresis 16 (1995) 1492–1498.