ELSEVIER

Journal of Chromatography B, 757 (2001) 79–88

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Improved capillary electrophoresis conditions for the separation of kinase substrates by the laser micropipet system

Huaina Li, Hayley Y. Wu, Yan Wang, Christopher E. Sims, Nancy L. Allbritton*

Department of Physiology & Biophysics and Center for Biomedical Engineering, University of California, Irvine, CA, 92697-4560, USA

Received 18 September 2000; received in revised form 23 January 2001; accepted 26 January 2001

Abstract

Phosphorylated and nonphosphorylated forms of peptide substrates for protein kinase C (PKC) and calcium-calmodulin activated kinase II (CamKII) were separated by capillary zone electrophoresis. Electrophoresis of the peptide substrates and products in biologic buffer solutions in uncoated capillaries yielded asymmetric analyte peaks with substantial peak tailing. Some of the peptides also exhibited broad peaks with unstable migration times. To improve the electrophoretic separation of the peptides, several strategies were implemented: extensive washing of the capillary with a base, adding betaine to the electrophoretic buffer, and coating the capillaries with polydimethylacrylamide (PDMA). Prolonged rinsing of the capillaries with a base substantially improved the migration time reproducibility and decreased peak tailing. Addition of betaine to the electrophoretic buffer enhanced both the migration time stability as well as the theoretical plate numbers of the peaks. Finally PDMA-coated capillaries brought about significant improvements in the resolving power of the separations. These modifications all utilized an electrophoretic buffer that was compatible with a living biologic cell. Consequently they should be adaptable for the new capillary electrophoresis-based methods to measure kinase activation in single cells.

Keywords: Peptides; Kinases; Betaine

1. Introduction

Recently a new strategy (the laser-micropipet) was developed to measure the activation of enzymes in small, single mammalian cells [1,2]. For this method a cell pre-loaded with fluorescent enzyme substrates, was rapidly lysed by a shock wave generated from a single laser pulse. The cellular contents were collected into an uncoated capillary, separated by electrophoresis, and then detected by laser-induced fluorescence. The total time needed for cell lysis, collection of the cellular contents, and initiation of electrophoresis was under 33 ms. The fast speed with which these events occurred gave the method a subsecond temporal resolution (with respect to the measurement of enzyme activation). Since enzymatic activity in cells can be modulated on subsecond timescales and since many enzymes are activated in response to cellular damage, the fast temporal resolution was required to accurately reflect the level of enzyme activation in the cell just prior to lysis [3,4]. In part the laser-micropipet method attained the fast

*Corresponding author. Tel.: +1-949-824-6493; fax: +1-949-824-8540.

E-mail address: nlallbri@uci.edu (N.L. Allbritton).

0378-4347/01/$ – see front matter
PII: S0378-4347(01)00080-9

rate of cell sampling by loading the cellular contents with the surrounding biologic medium directly into the capillary. The electrophoretic separations were also performed in the same solution. This buffer was high in salt (130 m*M* NaCl) and near neutral in pH (pH 7.4) and sometimes yielded asymmetric analyte peaks, peak tailing, loss of resolution, and unstable migration times for the fluorescent substrates and products. For these reasons the laser-micropipet system would benefit from an improvement in the separation conditions for the enzymatic substrates and products.

A strength of the laser-micropipet technique is its potential to perform measurements of multiple enzymes simultaneously within the same cell. However to achieve this potential, large numbers of substrates and products must be successfully separated and identified. For one subset of enzymes, kinases and phosphatases, the substrates are peptides typically 10–20 amino acids in length and frequently possess a high net positive charge (often greater than +4) [5–7]. The combination of the highly charged peptides with the biologic-based electrophoretic buffer in an uncoated capillary can further degrade separations. Since the substrates/products and uncoated capillary walls are generally opposite in charge, it is likely that interactions between the substrates/products and the capillary wall are partly responsible for the suboptimal separation characteristics. Kinases and phosphatases are key information transducers in cells; therefore, conditions which improve the separation of the substrates and products of these enzymes would greatly enhance the utility of the new laser-micropipet technique.

A very large number of strategies have been employed to overcome capillary wall interactions with positively charged peptides including manipulation of the buffer composition and the use of capillary coatings. An enormous body of literature exists on this topic and the reader is referred to recent reviews for detailed discussions [8–12]. A frequently employed tactic is the use of buffers at either pH extreme and/or the addition of surfactants to diminish peptide–wall binding. Dawson et al. established a capillary electrophoresis-based assay for kinases and phosphatases using peptide substrates in a phosphate buffer (pH 2.0) [13]. Westerlund and co-workers have also separated enkephalin-related peptides and protein kinase A peptide substrates in a low pH (pH 2–3), phosphate buffered solution [14,15]. The acidic pH reduces the charge on the capillary wall diminishing peptide–wall interactions and improving the efficiency of the separations. Another strategy to minimize the adsorption of the peptides onto the capillary wall has been the use of buffer solutions with a pH greater than the isoelectric point of the peptide [16]. Wu and Tsai [17] and Adamson et al. [18] have developed sensitive methods to separate protein kinase substrates and their phosphorylated products in borate-buffered solutions at basic pH values. Covalently attached or adsorbed coatings on the capillary wall can also reduce wall interactions with cationic peptides and proteins. The acrylamide-coating method of Hjertén is widely used through out the field of capillary electrophoresis to increase the separation efficiency of many different types of analytes [19]. Gamble et al. utilized capillaries coated with [(acryloylamino)propyl]-trimethylammonium to separate phosphopeptides in an acidic buffer solution [20]. An alternative method to minimize peptide–wall interactions is the use of buffer additives which adhere to the capillary wall reversing or diminishing the surface charge, for example polyethyleneimine or poly(vinyl alcohol) [20–22]. While many of these methods result in excellent separation properties for positively charged peptides, a feature common to many of the above techniques is that the electrophoresis buffers are not compatible with biological cells. Placement of a cell in a nonphysiologic separation buffer prior to the measurement (and lysis) will result in cellular damage activating cellular repair processes some of which utilize kinases and other enzymes [3,4]. Thus these improvements in electrophoretic conditions cannot readily be combined with the laser-micropipet technique to improve the separation of enzyme substrates.

In the present paper, we sought to develop strategies to improve the separation of cationic peptides using the laser-micropipet methodology. To minimize the time required for cellular lysis, collection of the cellular contents, and initiation of electrophoresis, a key consideration was that the electrophoretic buffer remain compatible with a living biologic cell. The cellular contents could then be collected in the

biologically compatible electrophoretic buffer and separation initiated immediately. Initially fused-silica capillaries that were extensively washed in a basic solution were compared to capillaries that were rinsed for much shorter times. Betaine, a biologically compatible buffer additive (at low concentrations) was also used to reduce adsorptive interactions between the peptides and inner wall of the bare fused-silica capillaries. Finally polydimethylacrylamide (PDMA)-coated capillaries were also evaluated for their ability to improve the separation characteristics of the peptide substrates and products. Specifically these modifications were tested for their ability to decrease peak tailing, enhance separation efficiency, and improve migration time reproducibility.

2. Experimental

2.1. Reagents

All fluorescent reagents were obtained from Molecular Probes (Eugene, OR, USA). All peptides including phosphopeptides were synthesized by the Beckman Peptide and Nucleic Acid Facility at Stanford University. All peptides were amidated on the carboxyl terminus. N-terminally labeled fluorescent peptides was prepared as previously described [23]. Peptides were labeled with fluorescein by incubation with fluorescein (–5′ and –6′) succinimydyl ester unless stated otherwise. The PKC peptide substrate F-PKC (RFARKGSLRQKNV) was derived from the pseudosubstrate region of PKC [5]. Another PKC substrate F-NG (AAKIQASFRGHMARKK) was derived from the PKC phosphorylation site on the neurogranin protein [6,7]. The CamKII peptide substrate F-CamKII (KKALHRQETVDAL) was derived form the threonine 286 autophosphorylation site of CamKII [24]. The underlined residues are the phosphorylation sites. The peptides adsorbed readily to glass and polypropylene, and so were stored in concentrated aliquots in siliconized tubes. The concentration of the fluorescein-labeled peptides was estimated by comparison of their fluorescence (excitation 480 nm, emission 520 nm) to that of standards of hydrolyzed fluorescein succinimydyl ester.

γ-Methacryloxypropyltrimethoxysilane (MAPS), *N,N,N′,N′*-tetramethylethylenediamine (TEMED), and ammonium persulfate (APS) were obtained from Sigma. *N,N*-Dimethylacrylamide (DMA) and trimethylglycine hydroxide inner salt (betaine) were purchased from Aldrich. Fused-silica capillary tubing (I.D. 50 μm×365 μm O.D.) was obtained from Polymicro Technologies. All other reagents and materials were purchased from Fisher Scientific (Pittsburgh, PA, USA). ECB, a physiologic extracellular buffer, was composed of 135 m*M* NaCl, 5 m*M* KCl, 10 m*M* 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 2 m*M* $MgCl_2$, and 2 m*M* $CaCl_2$ and adjusted to pH 7.4 with NaOH. TBE buffer was composed of 44.5 m*M* Tris base, 44.5 m*M* boric acid (pH 8.3) and 10 m*M* EDTA.

2.2. Pretreatment of the inner surface of fused-silica capillaries

The fused-silica capillary tube was first washed with water and flushed with 0.1 *M* NaOH for varying periods of time as described in the text. The capillary was then flushed sequentially with water, 0.1 *M* HCl, and water for varying times.

2.3. Capillary electrophoresis

Capillary electrophoresis and fluorescence detection was performed as described previously with the following exceptions [2]. The capillary length was 65 cm with an optical window created 54 cm from the inlet unless stated otherwise. The outlet reservoir was held at a negative potential of 12 kV and the inlet reservoir was placed at ground potential unless stated otherwise. Under these conditions with ECB as the electrophoretic buffer, the current was ~70 μA. Samples were loaded into the capillary by gravitational fluid flow, and the volume loaded was calculated from Poiseulle's equation, spontaneous fluid displacement, and diffusion (for 30 μm I.D. capillaries only) [25–28]. Typically, buffer solutions were loaded into the capillary by placing the capillary inlet into the sample solution and elevating the level of the inlet solution 2 cm above the level of outlet solution for 7 s. Analytes were detected by laser-induced fluorescence as described previously [2]. Current versus voltage curves were performed for each new buffer system or capillary coating as

described previously [25]. The voltages utilized were always well within the linear range of the curve to prevent artifacts due to Joule heating.

2.4. Linear polydimethylacrylamide coating of capillaries

Pretreatment and activation of the inner surface of the capillaries with a methacryl group were performed according to the procedure described by Hjertén and Kubo [29]. Coupling of linear polyacrylamide to the activated inner surface was carried out by the Hjertén method, with some modifications [19,29]. Capillaries were first flushed sequentially with 0.1 *M* NaOH (12 h), water (30 min), 0.1 *M* HCl (4 h), and water (30 min). The capillaries were then flushed with 50% methanol (2 h) followed by 100% methanol (2 h). Finally, the capillaries were filled with 5% MAPS in methanol and allowed to stand overnight at room temperature. After washing the capillaries with 100% methanol (1 h), 50% methanol (1 h), and water (1 h), drying in air (30 min), the capillaries were immediately filled with 3% DMA, 0.5 mg/ml APS, and 0.2% TEMED in TBE buffer which was previously degassed with nitrogen for 10 min. The capillaries were incubated overnight at room temperature to completely couple the polymer to the activated inner surface of the capillaries. Uncoupled DMA was removed by flushing the capillaries with water at room temperature.

2.5. Measurement of electroosmotic fluid flow (EOF)

EOF was measured using the neutral marker, BODIPY FL propanol (0.1 μ*M*), as described previously [30]. New capillaries (65 cm×50 μm I.D.× 365 μm O.D.) were rinsed with ECB for 10 min prior to measurement of EOF. The electroosmotic mobility (μ_{eo}) was calculated from the equation: $\mu_{eo}=L_d/(Et_m)$ for L_d (the capillary length to the detector), t_m (the migration time), and E (the field strength) [25].

2.6. Calculation of the theoretical plates

The theoretical plates (N) were calculated from the equation: $N=2\pi\cdot(t_m\cdot h/A)^2$ with the variables defined as the peak area (A), peak height (h) and migration time (t_m) [31].

3. Results and discussion

3.1. Electrophoresis of kinase substrates and products after prolonged washing with base

Typically, fused-silica capillaries were prepared for use by flushing with deionized water (15 min), 0.1 *M* NaOH (2 h or 30 min), deionized water (15 min), 0.1 *M* HCl (30 min), and deionized water (15 min) using a vacuum-based flow system [17,32]. Prior to use the capillaries were flushed with ECB. Capillaries were always washed between electrophoretic runs [~2 min (~18 μl) of each 0.1 *M* NaOH, water, 0.1 *M* HCl, water, and ECB]. Many (although not all) peptide substrates exhibited asymmetric peaks with tailing, inconsistent migration times, and variable peak areas (Fig. 1A and B). Of the four peptides shown in Fig. 1A and B, the phosphorylated form of F-CamKII (PF-CamKII) possessed the most variability in peak area and the most variable migration time with an RSD of 7.1% ($n=6$).

Cifuentes and co-workers have demonstrated that improved separations can be obtained by increasing the concentration of base used to rinse the fused-silica tubing [32,33]. The higher quality separation is likely due to many factors one of which may be the removal of surface contaminates. Other factors such as a more uniform display of silanols may also contribute to more consistent surface properties. For these reasons, the fused-silica capillary was first washed with water (15 min) and then flushed with NaOH (0.1–0.5 *M*, 2–24 h). The capillary was then washed sequentially with water (15 min), 0.1 *M* HCl (2 h), and water (15 min). These wash steps were only performed prior to the first electrophoretic run. Between electrophoretic runs the capillaries were washed briefly (~2 min of each 0.1 *M* NaOH, water, 0.1 *M* HCl, water, and ECB). Longer initial wash times with base yielded greater migration time reproducibility possibly due to a more consistent EOF (Fig. 1C and D). The RSD of the migration time for PF-CamKII was less than 3.5% ($n=6$). The increased wash time also decreased peak tailing for

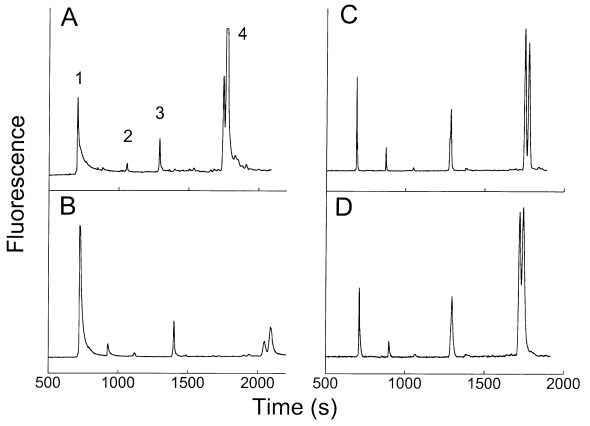

Fig. 1. Electropherogram of the four peptides: F-PKC, phosphorylated F-PKC (PF-PKC), F-CamKII, and phosphorylated F-CamKII (PF-CamKII). A mixture of the peptides [0.6 nl of F-PKC (4 n*M*), PF-PKC (0.5 n*M*), F-CamKII (3 n*M*), and PF-CamKII (12 n*M*)] was loaded into a capillary (65 cm×50 μm I.D.×360 μm O.D.) and separated by application of −12 kV to the outlet reservoir while the inlet was held at ground potential. The detection window was placed 54 cm from the inlet end of the capillary and the electrophoretic buffer was ECB. Prior to electrophoresis, capillaries received the standard pretreatment (A, B) or extensive washing (24 h) with base (C, D). Electropherograms of the first (A, C) and fifth (B, D) electrophoretic runs are displayed. The peak identities determined by electrophoresing single species were F-PKC (peak 1), PF-PKC (peak 2), F-CamKII (peak 3), and PF-CamKII (peak 4). The split peak for PF-CamKII is due to the partial separation of the fluorescent peptide isomers (see Section 3.2).

all four peptides and most significantly for F-PKC, the peptide with the greatest positive charge (Fig. 1C and D). Increasing the concentration of base did not further improve the separation characteristics. While the EOF in capillaries varied considerably for different lot numbers of capillary tubing, with in a lot the capillaries washed with 0.1 *M* NaOH for 30 min possessed a greater EOF than those washed for 12 h or longer (Table 3). Traditionally the peak tailing seen with basic peptides has been attributed to interactions of the peptides with the ionized silanol groups on the capillary walls [8]. However, the prolonged washing in base is extremely unlikely to have decreased the number of silanol groups. Consequently the diminished peak tailing after prolonged washing in base is most likely attributable to alterations in peptide interactions with groups other than the ionized silanols. Prolonged washing in base may remove surface contaminants that interact with the peptides. All subsequent experiments utilized capillaries that were washed for over 12 h in 0.1 *M* NaOH.

3.2. Betaine as a buffer additive for the separation of kinase substrate peptides

While prolonged rinsing of the capillary with base improved the separation characteristics for most substrate peptides, it was not sufficient for every peptide. A peptide substrate of PKC derived from the neurogranin protein (F-NG) consistently migrated as a very broad bifurcated peak (Fig. 2A). To improve the migration properties a variety of buffer additives that were biologically compatible were considered.

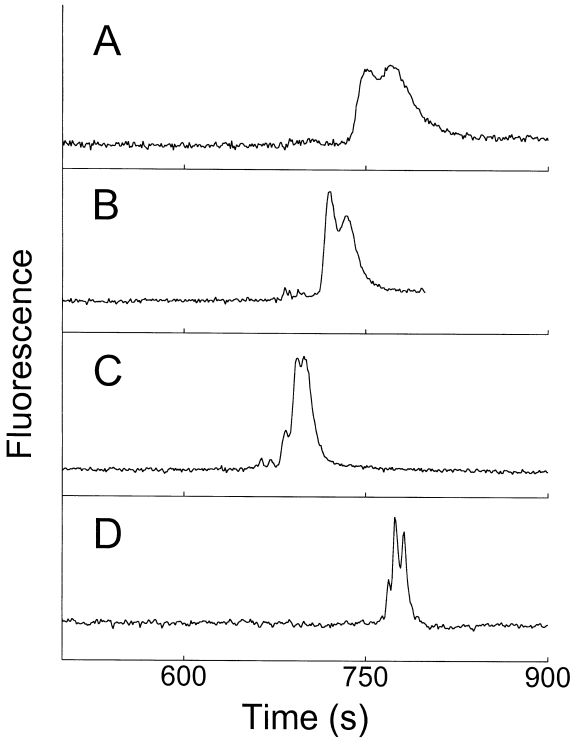

Fig. 2. Electropherogram of the peptide F-NG with and without betaine. F-NG (0.6 nl of 16 n*M*) was loaded into a capillary (65 cm×50 μm I.D.×360 μm O.D.) and separated by application of −12 kV to the outlet reservoir while the inlet was held at ground potential. The detection window was placed 54 cm from the inlet end of the capillary and the electrophoretic buffer was ECB with varying concentrations (A, 0 m*M*; B, 10 m*M*; C, 100 m*M*; D, 1 *M*) of betaine.

Zwitterions were particularly attractive since they do not contribute to the conductivity of the electrophoretic buffer, but can still associate with the negatively charged capillary surface or with the positively charged peptides to minimize peptide–wall interactions. An additional advantage is that electroosmotic pumping can frequently be maintained with these buffer additives. Several additional factors were considered in the selection of the zwitterion for this study. The compound must be zwitterionic at neutral pH, have a high solubility in biologic buffers, and not traverse the plasma membrane of cells, i.e., it must be hydrophilic. Betaine was used as buffer additive since it possesses all of the desired features. Bushey and Jorgenson have used betaine as buffer additive to separate lysozyme and α-chymotrypsinogen A [34]. F-NG was electrophoresed in ECB with different concentrations of betaine (Fig. 2). As the concentration of betaine was increased the peaks sharpened dramatically, most likely due to a decreased binding of the peptide to the wall of the capillary (Table 1). The multiple peaks apparent on the F-NG electrophoretic trace at high betaine concentrations are due to contaminants in the F-NG sample as well as the two isomers of fluorescein used to label the peptide. The contaminants are most likely the result of errors during peptide synthesis or incomplete removal of protecting groups on the amino acid side chains during cleavage of the peptide from the synthetic resin. Peak sharpening was also observed for other substrate peptides electrophoresed in the presence of betaine (Fig. 3). As little as 1 m*M* betaine substantially increased the number of theoretical plates (*N*)

Table 1
Theoretical plate numbers achieved with betaine

[Betaine] (m*M*)	F-PKC	PF-PKC	F-CamKII	PF-CamKII
0	53 100	227 600	72 100	130 600
1	176 700	261 400	109 400	132 800
10	148 800	245 400	95 800	174 900
100	151 900	266 000	97 300	187 600

For the bifurcated peak (F-PKC, peak 1, Fig. 3) and the peak with a shoulder (F-CamKII, peak 3, Fig. 3), the height of the major peak, and the area of the combined peaks was utilized to calculate an apparent *N*. For PF CamKII the first of the two peaks (peak 4, Fig. 3) was utilized to calculate *N*.

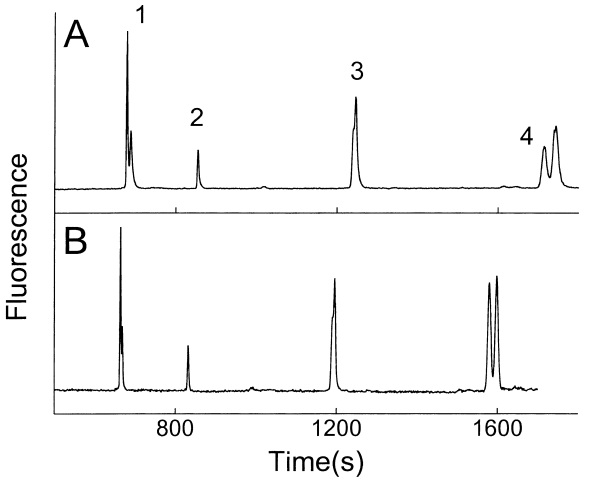

Fig. 3. Electropherogram of the peptides F-PKC, PF-PKC, F-CamKII, and PF-CamKII in the absence and presence of betaine. A mixture of the peptides [0.6 nl of F-PKC (4 n*M*), PF-PKC (0.5 n*M*), F-CamKII (3 n*M*), and PF-CamKII (12 n*M*)] was loaded into a capillary (65 cm×50 μm I.D.×360 μm O.D.) and separated by application of −12 kV to the outlet reservoir while the inlet was held at ground potential. The detection window was placed 54 cm from the inlet end of the capillary and the electrophoretic buffer was ECB without (A, 0 m*M*) or with (B, 10 m*M*) betaine. The peak labels are identical to those of Fig. 1.

obtained for many of the substrates (Table 1). Betaine also improved the reproducibility of the migration time of the peptide substrates. Without betaine the RSD of the migration time of F-NG, F-PKC, PF-PKC, F-CaMKII and PF-CaMKII was between 2 and 4% (Table 2). When betaine (10 m*M*) was added to the buffer the RSD was decreased to 1.7% or less. The improvement in the number of theoretical plates and the migration time stability, was most likely due to a decrease in the interactions of the peptides with the capillary wall. At a concentration of 1 *M* betaine the EOF was decreased compared to that at 100 m*M* betaine or without betaine (Table 3). The modest decrement in EOF at very high betaine concentrations was also observed by Bushey and Jorgenson [34]. The slightly lengthened migration time at high betaine concentration was most likely due to the slower EOF. At intermediate betaine concentrations (10, and 100 m*M*) the migration times of the components of the F-NG sample were shortened (Fig. 2). This result was reproducible. The reason for the faster migration

Table 2
Peptide migration time reproducibility (RSD) (%, $n=6$)

[Betaine] (m*M*)	F-PKC	PF-PKC	F-CamKII	PF-CamKII	F-NG
0	2.2%	3.5%	2.7%	3.4%	3.7%
10	0.7%	0.89%	1.7%	1.6%	1.7% ($n=4$)

Conditions were as described in Fig. 3. RSD is the relative standard deviation. When a group of poorly resolved peaks were present on the electropherogram, the average migration time of the peaks was utilized.

Table 3
Electroosmotic fluid flow

Capillary pretreatment with 0.1 *M* NaOH	[Betaine] in electrophoretic buffer	Capillary coating	Lot 1[a] [$\cdot 10^{-4}$ cm^2/(V s)]	Lot 2[a] [$\cdot 10^{-4}$ cm^2/(V s)]
1 h	0	–	2.8±0.2	2.9±0.02
12 h	0	–	2.4±0.1	2.6±0.08
12 h	100 m*M*	–	2.3±0.05	2.6±0.01
12 h	1 *M*	–	2.1±0.01	2.3±0.03
			Coating 1[a]	Coating 2[a]
12 h	0	PDMA	0.79±0.01	0.37±0.01

[a] The EOF was measured for capillary tubing from two different lots or for two different PDMA-coated capillaries.

velocities at intermediate betaine concentrations is not understood. Since the EOF (at intermediate betaine concentrations) was similar to that without betaine (Table 3), the faster velocities were mostly likely the result of betaine interacting with the peptide rather than the capillary wall.

In many of the electropherograms, the peptide peaks actually appear as a bifurcated peak, i.e., two poorly resolved peaks (Figs. 1–3). This is most noticeable in Fig. 3B for PF-CaMKII. In this instance the two peaks are nearly resolved to baseline. Since the peptides were labeled with two isomers of fluorescein succinimydyl ester (fluorescein-5 succinimydyl ester and fluorescein-6 succinimydyl ester), we suspected that these two poorly resolved peaks represented the two isomers of the fluorescein-labeled peptide. To determine whether this was indeed the case, peptides were also labeled with a single isomer (fluorescein-5 succinimydyl ester). Peptides labeled with the single isomer of fluorescein succinimydyl ester always migrated as a single peak with a migration time coincident with one of the peaks of the mixed isomer peptide (Fig. 4). These results suggested that the two poorly resolved peaks on the electropherograms represent the two isomers of fluorescein-labeled peptide.

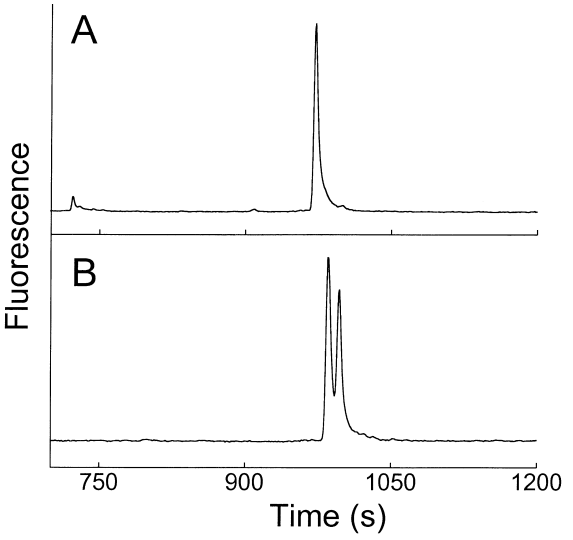

Fig. 4. Electropherogram of PF-CamKII labeled with single (5′, panel A) or mixed (5′ and 6′, panel B) isomers of fluorescein succinimydyl ester. PF-CamKII [100 pl of single isomer (9 n*M*) or mixed isomer (12 n*M*)] was loaded into a capillary and separated by electrophoresis in ECB containing 500 m*M* betaine. Electrophoresis was initiated by application of −18 kV to the outlet end the capillary (60 cm×30 μm I.D.×360 μm O.D.) while the inlet end was held at ground potential. The distance from the inlet end to the detection window was 49 cm.

3.3. Linear PDMA-coated capillary for peptide separation

Deactivation of silanol groups on the capillary surface by covalent attachment of a neutral polymer chain can result in a significant reduction in analyte adsorption onto the wall and consequent improvements in resolution. Dovichi introduced a series of N-substituted acrylamide coatings for separation of alkaline proteins [35]. For example, covalent linkage of dimethylacrylamide (DMA) produces a highly stable and hydrophobic coating, polydimethylacrylamide (PDMA), resulting in substantially less protein adsorption onto the capillary wall. Capillaries coated with PDMA were tested for their ability to separate peptide substrates for protein kinase C (PKC). F-PKC was electrophoresed in uncoated or PDMA-coated capillaries. Electropherograms obtained with the bare capillaries possessed two poorly resolved peaks as described previously; however, electropherograms from the PDMA-coated capillaries displayed two well separated peaks (Fig. 5). The peptide isomers were resolved to baseline in the coated capillaries. Electrophoresis of F-NG in the PDMA-coated capillaries also resulted in separations with a much higher resolution compared to that of bare capillaries (Fig. 6A). Five peaks were now present on the electropherogram of F-NG, suggesting that the sample actually contained multiple different impurities. For comparison F-NG migrated as three peaks in uncoated capillaries with 1 *M* betaine in the electrophoretic buffer. Thus the separation efficiency of the PDMA-coated capillaries was also superior to that of uncoated capillaries combined with betaine (compare Fig. 3D to Fig. 6A). However, a significant drawback of electrophoresis in the PDMA-coated capillaries was the very long migration times – 5000 s versus ~780 s in the uncoated capillaries. Since μ_{eo} was much lower than in the coated capillaries compared to the bare capillaries (Table 3), the longer migration time was due to the loss of EOF. Another consequence of the greatly diminished EOF in PDMA-coated capillaries was that analytes of opposite charge did not migrate in a common direction unless additional forces were applied. When F-CamKII (net positive charge) and PF-CamKII (net negative charge) were loaded into a capillary and electrophoresed, only one peak was present on the electropherogram. When a hydrodynamic force was applied to the capillary during electrophoresis (by raising the fluid level surrounding the inlet end 8 cm over that surrounding the outlet end), two peaks were identifiable (Fig. 6B). *N* was 8100 (F-CamKII) and 8500 (PF-CamKII). While the addition of hydrodynamic fluid flow forced both peptides to migrate in the same direction, the parabolic pattern of fluid flow diminished the efficiency of separation.

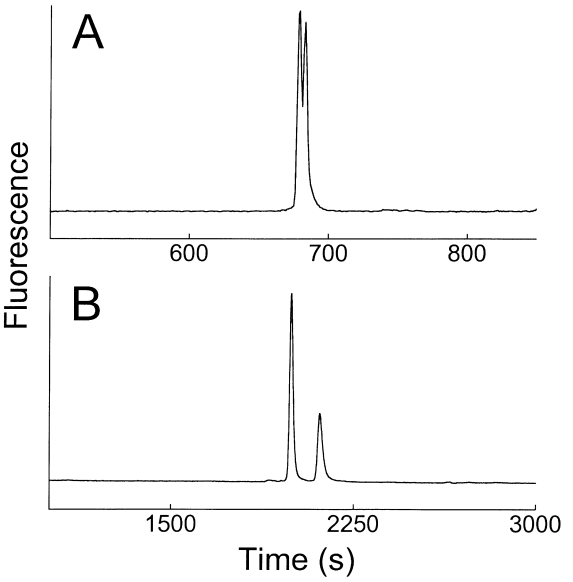

Fig. 5. Electropherogram of F-PKC in an uncoated (A) or PDMA-coated (B) capillary. F-PKC (0.6 nl of 4 n*M*) was loaded into a capillary (65 cm×50 μm I.D.×360 μm O.D.) and separated by application of −12 kV to the outlet reservoir while the inlet was held at ground potential. The detection window was placed 54 cm from the inlet end of the capillary and the electrophoretic buffer was ECB.

4. Conclusion

We have demonstrated that several simple modifications to the electrophoretic conditions used with the laser-micropipet technique can greatly enhance the RSD of substrate/product migration times, increase the theoretical plate number of the peptide

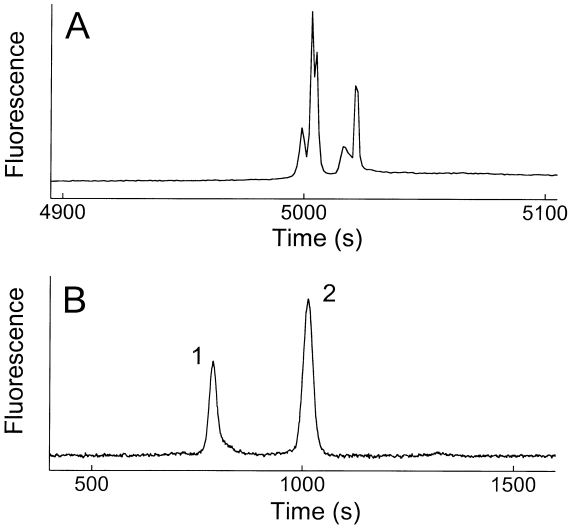

Fig. 6. Electropherogram of F-NG or F-CamKII and PF-CamKII in a capillary coated with PDMA. (A) F-NG (0.6 nl of 16 n*M*) was loaded into a capillary (65 cm×50 μm I.D.×360 μm O.D.) and separated by application of −12 kV to the outlet reservoir while the inlet was held at ground potential. The detection window was placed 54 cm from the inlet end of the capillary and the electrophoretic buffer was ECB. (B) F-CamKII and PF-CamKII (3 nl of 2 n*M* each) was loaded into a capillary (43 cm×50 μm I.D.×360 μm O.D.) and separated by application of +6.7 kV to the outlet reservoir while the inlet was held at ground potential. The detection window was placed 25.5 cm from the inlet end of the capillary and the electrophoretic buffer was ECB. Both peptides were labeled with a single isomer of fluorescein. The fluid level surrounding the inlet of the capillary was 8 cm higher than that around the outlet during electrophoresis. The peak identities determined by electrophoresing single species were PF-CamKII (peak 1) and F-CamKII (peak 2).

peaks, and improve the resolving power of the method. These alterations include more extensive pretreatment of the capillary with base which may act by decreasing the presence of surface contaminants. Adding betaine to the electrophoretic buffer or PDMA-coating of the capillaries enhanced the separations by diminishing undesirable interactions between the substrates/products and the capillary walls. In addition all of these modifications are compatible with a living biologic cell, and consequently should be easily combined with the laser-micropipet method broadening the range and numbers of enzyme substrates that can be utilized with the technique.

Acknowledgements

We thank Joseph S. Soughayer for helpful suggestions. This work was supported by the NIH (CA78858 and NS/MH39310 to N.L.A.) and AI01513 to C.E.S.

References

[1] G.D. Meredith, C.E. Sims, J.S. Soughayer, N.L. Allbritton, Nat. Biotechnol. 18 (2000) 309.
[2] C.E. Sims, G.D. Meredith, T.B. Krasieva, M.W. Berns, B.J. Tromberg, N.L. Allbritton, Anal. Chem. 70 (1998) 4570.
[3] T. Togo, J.M. Alderton, G.Q. Bi, R.A. Steinhardt, J. Cell Sci. 112 (1999) 719.
[4] P.L. McNeil, R.A. Steinhardt, J. Cell Biol. 137 (1997) 1.
[5] C. House, B.E. Kemp, Science 238 (1987) 1726.
[6] E. Klann, S.J. Chen, J.D. Sweatt, Proc. Natl. Acad. Sci. USA 90 (1993) 8337.
[7] S.J. Chen, E. Klann, M.C. Gower, C.M. Powell, J.S. Sessoms, J.D. Sweatt, Biochemistry 32 (1993) 1032.
[8] S.F.Y. Li, I. Rodriguez, Anal. Chim. Acta 383 (1999) 1.
[9] T.A. van de Goor, Pharm. Biotechnol. 7 (1995) 301.
[10] E.C. Rickard, J.K. Towns, Methods Enzymol. 271 (1996) 237.
[11] I. Messana, D.V. Rossetti, L. Cassiano, F. Misiti, B. Giardina, M. Castagnola, J. Chromatogr. B 699 (1997) 149.
[12] C.K. Larive, S.M. Lunte, M. Zhong, M.D. Perkins, G.S. Wilson, G. Gokulrangan, T. Williams, F. Afroz, C. Schoneich, T.S. Derrick, C.R. Middaugh, S. Bogdanowich-Knipp, Anal. Chem. 71 (1999) 389R.
[13] J.F. Dawson, M.P. Boland, F.B. Holmes Charles, Anal. Biochem. 200 (1994) 149.
[14] M. Thorsteinsdottir, I. Beijersten, D. Westerlund, Electrophoresis 16 (1995) 564.
[15] I. Beijersten, D. Westerlund, Electrophoresis 17 (1996) 161.
[16] P.D. Grossman, K.J. Wilson, G. Petrie, H.H. Lauer, Anal. Biochem. 173 (1988) 265.
[17] W.S. Wu, J.L. Tsai, Anal. Biochem. 269 (1999) 423.
[18] N. Adamson, P.F. Riley, E.C. Reynolds, J. Chromatogr. 646 (1993) 391.
[19] S. Hjertén, J. Chromatogr. 347 (1985) 191.
[20] T.N. Gamble, C. Ramachandran, K.P. Bateman, Anal. Chem. 71 (1999) 3469.
[21] A. Cifuentes, H. Poppe, J.C. Kraak, F.B. Erim, J. Chromatogr. B 681 (1996) 21.
[22] H.G. Lee, D.M. Desiderio, J. Chromatogr. B 691 (1997) 67.
[23] C.L. Lee, J. Linton, J.S. Soughayer, C.E. Sims, N.L. Allbritton, Nat. Biotechnol. 17 (1999) 759.
[24] P.I. Hanson, M.S. Kapiloff, L.L. Lou, M.G. Rosenfeld, H. Shulman, Neuron 3 (1989) 59.
[25] R. Weinberger, Practical Capillary Electrophoresis, Academic Press, San Diego, CA, 1993.

[26] H.A. Fishman, R.H. Scheller, R.N. Zare, J. Chromatogr. A 680 (1994) 99.
[27] H.A. Fishman, N.M. Amudi, T.T. Lee, R.H. Scheller, R.N. Zare, Anal. Chem. 66 (1994) 2318.
[28] J. Crank, in: The Mathematics of Diffusion, Clarendon Press, Oxford, 1989, p. 2.
[29] S. Hjertén, K. Kubo, Electrophoresis 14 (1993) 390.
[30] H. Tian, L.C. Brody, D. Mao, J.P. Landers, Anal. Chem. 72 (2000) 5483.
[31] S. Terabe, K. Otsuka, T. Ando, Anal. Chem. 61 (1989) 251.
[32] A. Cifuentes, P. Canalejas, A. Ortega, J.C. Diez-Masa, J. Chromatogr. A 823 (1998) 561.
[33] A. Cifuentes, P. Canalejas, J.C. Diez-Masa, J. Chromatogr. A 830 (1999) 423.
[34] M.M. Bushey, J.W. Jorgenson, J. Chromatogr. A 480 (1989) 301.
[35] C. Gelfi, M. Curcio, M.G. Righetti, R. Sebastiano, A. Citterio, H. Ahmadzadeh, N.J. Dovichi, Electrophoresis 19 (1998) 1677.