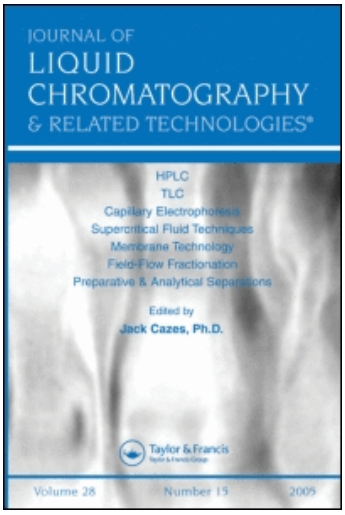

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STUDIES OF MIGRATION TIME REPRODUCIBILITY OF CAPILLARY ELECTROPHORETIC PROTEIN SEPARATIONS

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ABSTRACT

Studies of the migration time reproducibility of trypsinogen were performed using a capillary electrophoresis system designed for high resolution protein separations, where analyte adsorption and electroosmotic flow were minimized through the use of poly-(acrylamide)-derivatized capillaries coated with 3-(dimethyldodecylammonio)-propanesulfonate, a zwitterionic surfactant. The results of this investigation suggested that oxidation/reduction-induced pH drifts occurring at the electrodes had a significant impact upon protein migration. Acceptable migration time reproducibility (<2% RSD for a series of nine injections) was found to be dependent upon buffer replenishment at both electrodes prior to each injection. These results indicate that instruments which can provide pre-injection buffer replenishment may be necessary for the qualitative or quantitative analysis of proteins using poly-(acrylamide) coated capillaries.

INTRODUCTION

The potential of capillary electrophoresis (CE) to separate large biomolecules such as proteins has attracted the attention of biotechnology researchers and manufacturers from the fields of both liquid chromatography and electrophoresis. CE is a technique offering advantages when compared to both classical gel electrophoresis and high performance liquid chromatography (HPLC). CE separations require very small sample volumes (<10 µl), and can be highly efficient, rapid, and quantitative. In addition, the electrophoretic

separation mechanism of CE is very different from the analyte-stationary phase interactions employed by HPLC, making analyses by the two methods complementary.

Although these advantages may make CE appear very attractive as an analytical tool, many issues must be resolved before it can be considered a routine quantitative technique. Of prime importance among these is separation reproducibility. In comparison to HPLC, which employs motor-driven pumps to effect analyte elution, capillary electrophoretic analyte migration is entirely dependent upon chemical processes, and it seems evident that superior migration reproducibility should be readily achievable if the environment within the capillary is controlled. However, achievement of a reproducible inner capillary chemical environment, along with that of other important parameters affecting quantitation such as sample injection and sensitive detection capability, has proven to be a challenge. A 1989 survey of several researchers in the biotechnology industry revealed that, despite its potential, a multitude of variables and unknowns had prevented CE from being utilized as a quantitative technique (1). Also, a recent study of the analysis of insulin formulations by CE and HPLC, conducted by the US Food and Drug Administration, found conventional (non-micellar, unmodified silica) CE to be unsuitable for regulatory purposes (2). Variations in sample injection, separation temperature, the internal surface of the capillary, and buffer composition can exert a powerful influence upon a CE separation, and changes in any of these variables from run-to-run can have deleterious effects upon reproducibility. A review of considerations for the use of CE in industrial applications estimated that environmental variations inside a capillary can easily decrease separation reproducibility to levels such that coefficients of variation of 10 to 20% are observed (3). These reports, along with many others, have emphasized the importance of the chemical state of the internal capillary surface. In addition, very thorough reports of the effects of temperature have been published (4-7), and a variety of injection techniques have been studied (8-10). The effects of a variety of buffer cations and anions upon the CE separations of dansylated amino acids have also been reported (11, 12). Although buffer replenishment has been found to improve migration time reproducibility (8), no systematic study of migration time reproducibility has been carried out specifically as it relates to buffer replenishment and the internal chemical environment of the capillary..

Of the multitude of analytes to which CE separation technology can be applied, proteins in particular have presented unique challenges to the analyst. Their inherent tendency to adsorb to the inner walls of fused silica capillaries results in considerable peak broadening and asymmetry, making it difficult to attain the high separation efficiencies predicted by theory. In addition, the silica surface can be significantly altered by the presence of adsorbed material, in effect changing the electroosmotic flow upon which subsequent separations in bare silica capillaries depend. Efforts to achieve acceptable CE

protein migration time reproducibility have included the use of dilute alkaline rinses (13-16), a neutrally charged electroosmotic flow marker facilitating the measurement of analyte electrophoretic mobility (17), low pH (18), derivatized capillaries (18-20), or the use of ethylene glycol as a buffer additive (15). Several reports have stressed the importance of buffer replenishment for obtaining reproducible protein separations in non-coated capillaries (15, 21, 22). HPLC methods developed for the qualitative analysis of peptides (peptide mapping) have been shown to provide retention time reproducibilities varying by less than 2% RSD (23, 24). Since proteins possess significantly greater potential for secondary interactions than peptides do, this level of precision may also be considered both an estimate of the capabilities of HPLC protein separations and a target for the reproducibility of analyses of proteins by CE. An investigation of the changes occurring in the environment inside a capillary during a series of CE separations could facilitate both an understanding and an optimization of protein analysis reproducibility, and a major restraint preventing the use of CE as a qualitative and quantitative technique for the analysis of proteins could be overcome.

In this study, we report the changes in protein migration time and buffer environment observed during series of replicate protein analyses by CE. The system employed was one in which temperature variation, analyte adsorption, and electroosmotic flow were minimized.

MATERIALS AND METHODS

Capillary electrophoresis was performed with an automated P/ACE 2000 instrument (Beckman Instruments, Inc., Palo Alto, CA) controlled by an IBM PS/2 Model 80 386 computer fitted with P/ACE software (Beckman) running in a WINDOWS (Microsoft, Redmond, WA) environment. Analog data (10 Hz sampling rate) were collected directly from the on-column absorbance detector (214 nm) on an in-house centralized chromatography computer system based on the Hewlett-Packard Model 1000 minicomputer. The capillary cassette was fitted with a 50-μm-i.d. fused silica capillary (Polymicro Technologies, Phoenix, AZ) 97 cm in length (90 cm to the detector). The internal surfaces of the capillaries used in this investigation were coated with poly(acrylamide) in the manner described by Hjerten (25). Injection of the sample was achieved using low pressure (0.5 psi), and the temperature was controlled at 25 ± 0.1 °C. Glass vials (4 ml capacity) were employed as sample and inlet and outlet buffer reservoirs.

Trypsinogen, ribonuclease A, lysozyme, α-chymotrypsinogen, and cytochrome C were purchased from Sigma Chemical Co. (St. Louis, MO). 3-(dimethyldodecylammonio)-propanesulfonate (DDAPS) was obtained from Fluka

(Ronkonkoma, NJ). Acrylamide, ammonium persulfate, and N, N, N', N'-tetramethylenediamine (TEMED) were purchased from Bio-Rad (Richmond, CA).

Phosphate buffers were prepared using monobasic sodium phosphate, adjusted to the specific pH by adding 1 M sodium hydroxide or dilute phosphoric acid as needed. Analyses were performed using an applied potential of 25 kV in automated sequences consisting of nine consecutive 60 min separations, unless otherwise noted. For the verification of the reproducibility of the experimental results, all sequences were performed at least in triplicate. Samples were made up as 1 mg/ml solutions in running buffer. All buffers and samples were filtered through a 0.45-μm pore size filter (Millipore) and degassed prior to use. CE separations were performed employing a cathodic outlet (detector end) electrode. Efficiencies were calculated by the HP-1000 minicomputer using an empirical equation developed for the characterization of either Gaussian or skewed chromatographic peaks (26). Injection volumes and plug lengths were estimated using Poiseuille's equation (27), calculated assuming a solution viscosity equal to that of water at 30 °C. Buffer conductivity was measured using a Model 604 conductivity meter (Amber Science, Eugene, OR).

RESULTS AND DISCUSSION

To investigate the effects of changes in buffer composition upon migration reproducibility for a series of replicate protein separations and to achieve optimum reproducibility, it was deemed necessary to initially minimize the variation in parameters which may govern analyte migration; capillary temperature, analyte-wall interactions, and electroosmotic flow.

Acceptable reproducibilities may be difficult to achieve without accurate control of the desired capillary temperature during CE separations, since electrophoretic mobilities have been found to change 2% / °C (27). Joule heating occurring during electrophoresis influences capillary temperature, which in turn affects variables such as buffer viscosity, chemical equilibria, and pH, upon which migration time for a given species is dependent. The instrument employed in this investigation utilized a Peltier thermoelectric device to control capillary temperature. A system of this design has been shown to provide optimum capillary temperature control when compared to natural convection or forced air cooling (4). Ohm's law plots of current vs. applied voltage (29) obtained using 25, 50, and 100 mM sodium phosphate, 5 mM DDAPS buffers displayed linearities over potentials ranging up to 30 kV (the instrumental limit), indicating that the capillary temperature was maintained consistently throughout these studies, and suggesting that temperature

variations did not play a significant role in the determination of protein migration reproducibility.

Silanol groups present on the surface of the inner capillary wall can cause a major impediment which may prevent the achievement of acceptable protein migration reproducibility. Since proteins can adsorb on the silica surface and change the electroosmotic flow, migration time can change from injection to injection (19). Permanent modification of the capillary wall with an uncharged coating is perhaps the most attractive method for controlling protein-wall interactions (30, 31). Using derivatized capillaries, researchers have reported significant improvements in protein separation efficiencies (18, 19, 32-34).

To reduce protein adsorption and minimize electroosmotic flow, the inner walls of the capillaries used in this investigation were derivatized with poly-(acrylamide) (25), a technique also successfully utilized for the separation of DNA restriction fragments (35). Separations of 2 sec injections of trypsinogen in 25 mM sodium phosphate pH 4.3, obtained in non-coated and coated capillaries (see Figures 1a and 1b), revealed a measurable reduction of peak tailing in the coated capillary, although adsorption is clearly evident in both systems. The increased baseline noise present in Figure 1b may be due to a background absorbance generated by the polyacrylamide coating. Asymmetry factors (36, 37) corresponding to the peaks in Figures 1a and 1b were estimated to equal 37 and 10, respectively (the peak in Figure 1a tails badly and returns to the baseline asymptotically). Incorporation of a zwitterionic surfactant, 3-(dimethyldodecylammonio)-propanesulfonate (DDAPS), into the separation buffer at a 5 mM concentration provided a significant enhancement of peak shape (peak asymmetry $\approx$ 1.00), as displayed in Figure 1c. Conditioning the coated capillary with a 15 min buffer rinse prior to the first separation was found to be sufficient for saturation of the poly-(acrylamide) wall coating with a surfactant layer. A similar approach has been employed by Towns and Regnier, who deposited a layer of non-ionic surfactant upon octadecylsilane-derivatized silica capillaries to inhibit protein adsorption (34). Like non-ionic surfactants, zwitterionic surfactants are non-conducting, non-denaturing solublizing agents (38). A 5 sec injection (plug length $\approx$ 1.5 mm, volume $\approx$ 3 nl) of a mixture of five basic proteins separated using these techniques is displayed in Figure 2. During this separation, the electroosmotic flow was virtually eliminated (ie. $< 7.54 \times 10^{-10}$ m^2/V s, calculated using an equation presented by Jorgenson and Lukacs (39)), as determined by the fact that a neutral marker (1% mesityl oxide) did not appear at the detector after a 60 min separation (anode at the injection end) following injection of the sample into the short end of the capillary 7 cm from the detector window. Efficiencies ranged from 384,000 for cytochrome C to 684,000 theoretical plates for trypsinogen. By reducing the injection time, injected volume, and plug length to the

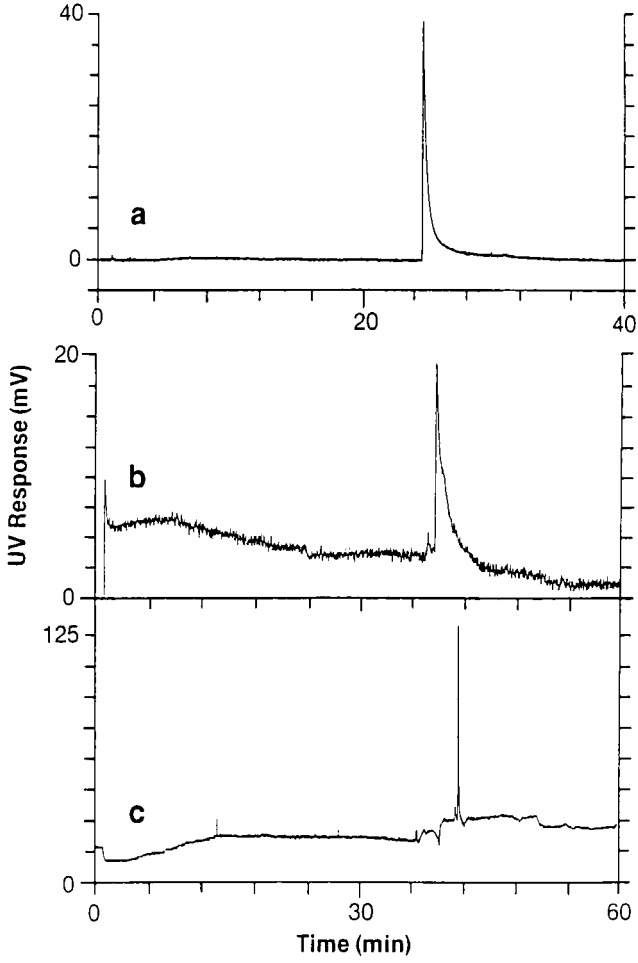

Figure 1. Electropherograms of 2 sec injections of 1 mg/ml trypsinogen separated at 25 kV in (a) 25 mM sodium phosphate pH 4.3, non-coated capillary (peak asymmetry ≈ 37); (b) 25 mM sodium phosphate pH 4.3, poly-(acrylamide)-coated capillary (peak asymmetry ≈ 10); (c) 25 mM sodium phosphate, 5 mM DDAPS, poly-(acrylamide)-coated capillary (peak asymmetry ≈ 1.00).

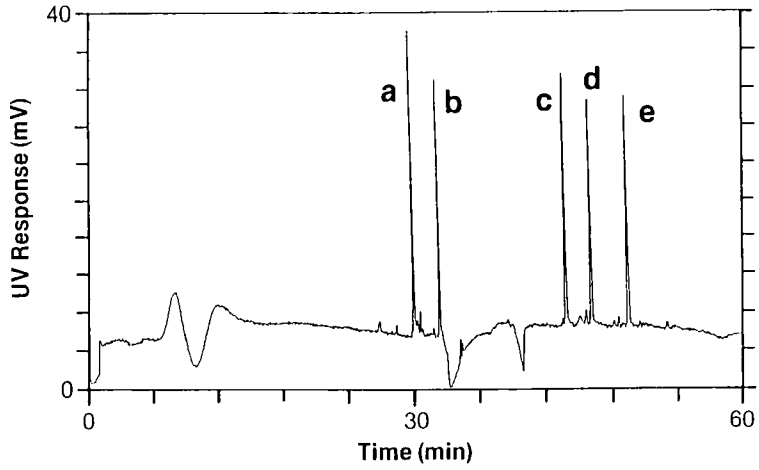

Figure 2. Electropherogram of a 5 sec inj of a mixture of 5 model proteins (1 mg/ml each) in 25 mM sodium phosphate, 5 mM DDAPS, separated in a poly-(acrylamide)-coated capillary at 25 kV. Proteins and respective efficiencies were as follows; (a) cytochrome c, N=384,000; (b) lysozyme, N=490,000; (c) ribonuclease a, N=435,000; (d) trypsinogen, N=685,000; (e) α-chymotrypsinogen, N=610,000.

approximate instrumental limits of 1 sec, 0.6 nl, and 0.3 mm, respectively, trypsinogen efficiency increased to a value of 1.35×10^6 theoretical plates (data not shown). This result suggests that the efficiency of trypsinogen in this system may be limited only by diffusion and the limits of the instrument to inject and detect narrow sample plug lengths. The theoretical relationship of plug length to efficiency has been detailed in several reports (40-42), and this phenomena was also observed experimentally by Tsuda et al., who performed separations of pyridine, a non-adsorbing analyte (43). With such high efficiencies achieved, it appeared that the adsorption of trypsinogen to the capillary walls was minimal under the conditions employed.

Since the electrophoretic migration of an analyte is dependent upon its zeta potential and the magnitude of the applied electric field (44), investigators generally have employed a constant potential for obtaining separations by CE. The results of nine replicate 1 hour separations (25 kV applied potential) of 5 sec injections of trypsinogen obtained using 25, 50, and 100 mM sodium phosphate pH 4.3, 5 mM DDAPS (sodium phosphate is a buffer recommended for the separation of proteins by CE at low pH (18)) and 25 mM sodium

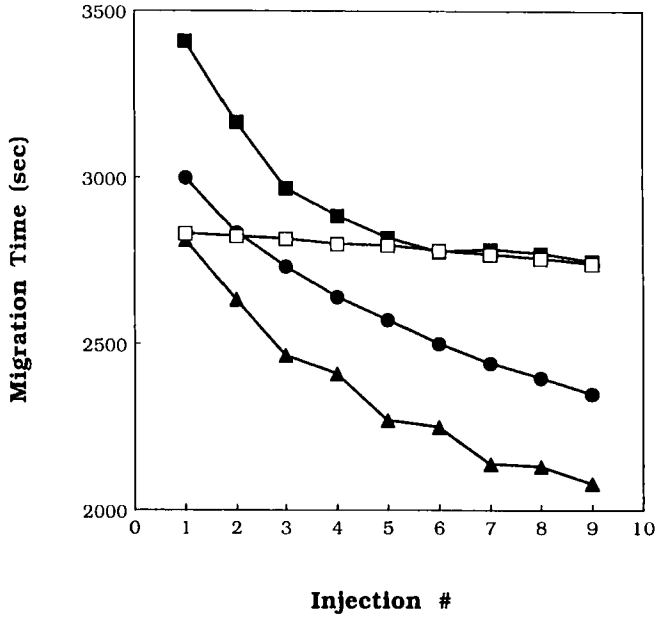

Figure 3. Trypsinogen (1 mg/ml, 5 sec injection) retention time plotted vs. separation replicate for a series of nine replicate separations obtained at 25 kV using the same buffer vials for each separation with no replenishment or rinse between injections, in (▲) 25 mM sodium phosphate pH 4.3, 5 mM DDAPS; (●) 50 mM sodium phosphate pH 4.3, 5 mM DDAPS; (■) 100 mM sodium phosphate pH 4.3, 5 mM DDAPS; (□) 25 mM sodium acetate pH 4.3, 5 mM DDAPS.

acetate pH 4.3, 5 mM DDAPS buffers, with no capillary rinse between runs, are plotted as trypsinogen migration time vs. injection # in Figure 3, and a set of electropherograms corresponding to the separations obtained in 50 mM sodium phosphate, 5 mM DDAPS is displayed in Figure 4. In addition to a relative dependence of migration time upon ionic strength, the data plotted in Figure 3 revealed that protein migration time in the phosphate buffers decreased with each injection, changing by approximately 25% between the first and ninth replicate, independent of buffer concentration. The reproducibility of the observed drift is displayed in Figure 5, where the results of five sets of nine separations are overlaid, each set obtained using fresh 25 mM sodium phosphate pH 4.3, 5 mM DDAPS buffer. The injection-to-injection trypsinogen peak area reproducibility displayed in these five series of nine replicate trypsinogen injections was found to correspond to a 11.4%

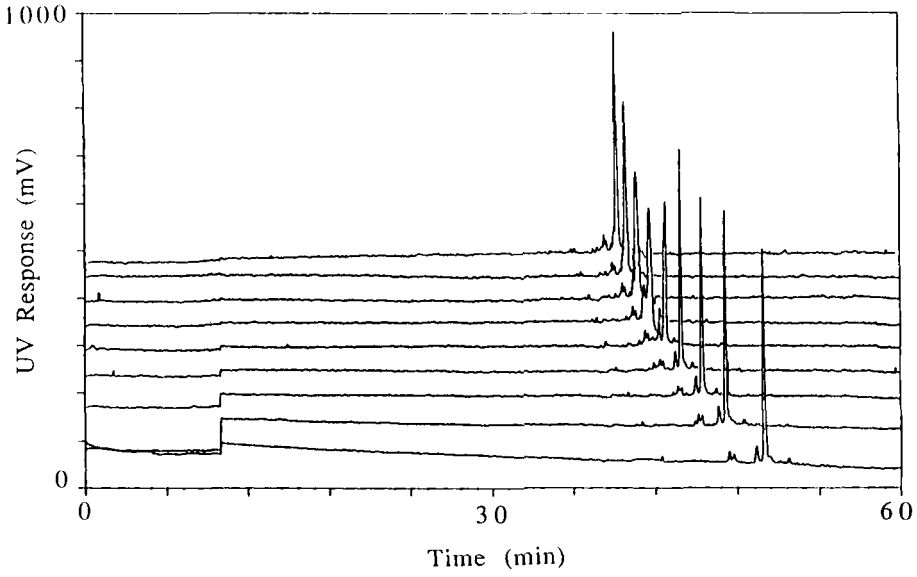

Figure 4. Electropherograms corresponding to nine replicate 5 sec injections of 1 mg/ml trypsinogen obtained at 25 kV in 50 mM sodium phosphate pH 4.3, 5 mM DDAPS, using the same buffer vials for each separation with no replenishment or rinse between injections.

RSD. Sample solvent evaporation, which can be anticipated to have a significant concentrating effect upon samples stored inside the autosampler in 50 µl microvials for extended periods of time, was minimized through the utilization of full 4 ml vials (fresh sample was used for each sequence of nine replicates) as sample reservoirs. Factors affecting CE sample injection volume reproducibility in instruments similar to the one used in this study have been analyzed thoroughly in other reports (2, 8-10, 45, 46), and therefore no further investigations of this issue were attempted in this study.

For all replicate sets between the first and ninth separations, the generated currents were observed to vary by less than 5%, and the conductivities of the buffers in the inlet and outlet reservoirs at the conclusion of nine separations were not found to have changed significantly from that of the fresh buffer. These observations suggested that ion depletion was not responsible for the observed decrease in run-to-run migration time. Electroosmotic flow measurements, obtained using 0.5% mesityl oxide as a neutral marker, revealed that the bulk flow inside the capillary had not increased during the course of the nine

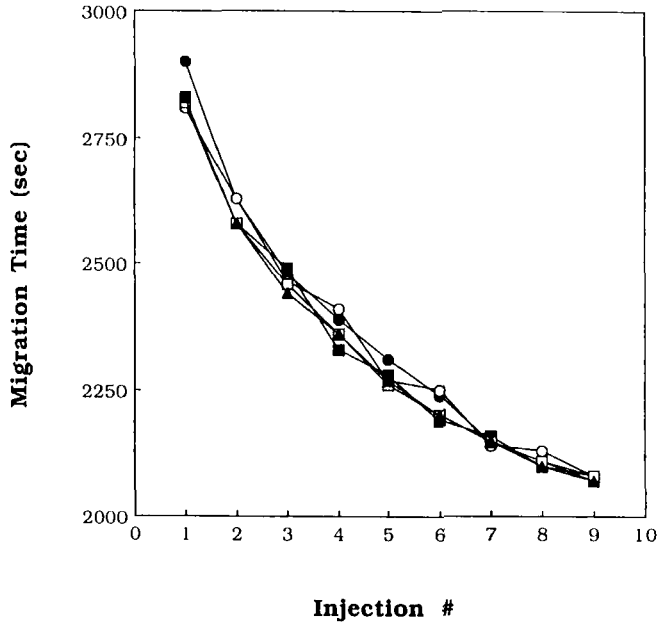

Figure 5. The reproducibility of the trypsinogen migration time drift observed when using the same buffer vials for each separation with no replenishment or rinse between injections, displayed as overlaid plots of data from five sets of nine replicate 5 sec injections separated in 25 mM sodium phosphate pH 4.3, 5 mM DDAPS at 25 kV; (●) Set 1; (■) Set 2; (□) Set 3; (○) Set 4; (▲) Set 5.

separations. However, after 9 hours at 25 kV, the pH values of the inlet and outlet buffers were found to have decreased to pH 3.41 and increased to pH 5.45, respectively, from that of the fresh buffer. These pH variations, along with those observed in the 25 mM sodium acetate buffer and a 25 mM sodium chloride solution after similar treatment, are displayed in Figure 6. To determine the magnitude of the pH changes generated by the elevated temperatures inside the autosampler compartment, a buffer vial not exposed to electrophoresis was incubated for nine hours inside the instrument as a control. The pH of the buffer inside this vial was found to have increased only 0.09 pH units, suggesting that temperature-induced pH changes were insignificant. Instead, the pH drifts observed during this study appeared to have occurred as a result of oxidation and reduction processes taking place at the anode and cathode, respectively, creating pH variations which could not

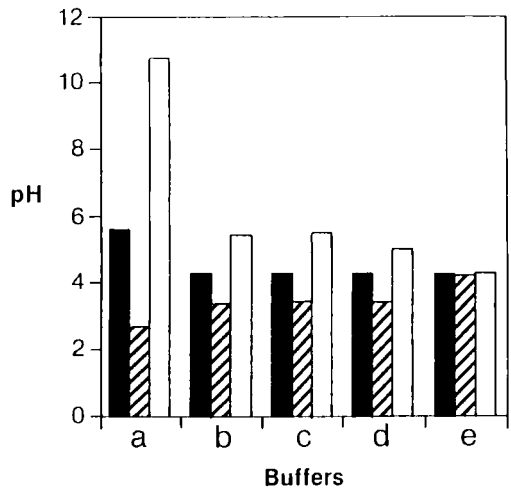

Figure 6. The pH of salt solutions before (■) and after nine hours at an applied potential of 25 kV (inlet =▨, outlet =❑). (a) 25 mM NaCl; (b) 25 mM sodium phosphate, 5 mM DDAPS; (c) 50 mM sodium phosphate, 5 mM DDAPS; (d) 100 mM sodium phosphate, 5 mM DDAPS; (e) 25 mM sodium acetate, 5 mM DDAPS.

be completely neutralized by the phosphate buffers employed in this study. A similar observation was also reported by Svensson, who, while performing isoelectric focussing experiments, recognized that at the electrodes there is a continuous production of hydrogen and hydroxyl ions, causing the anode and cathode to naturally become more acidic and alkaline, respectively, with the passage of an electric current (47).

The drifts toward shorter migration times observed with consecutive injections (see Figures 3 and 4) suggest that the acidic environment which developed in the inlet reservoir may have increased the positive zeta potential of trypsinogen and dominated the migration of the protein over the higher pH in the outlet reservoir. This is probably due to the fact that the detector window is 7 cm from the outlet reservoir, thereby significantly diminishing the effect of the higher pH environment on the protein. The relatively smaller trypsinogen migration time drift (3.2% from the first to the ninth replicate) observed while employing the sodium acetate buffer (see Figure 3) correlated with its superior ability, relative to sodium phosphate, to buffer pH changes near pH 4.3 (the pK_a of acetic acid is at pH 4.75, while phosphoric acid displays pK_as at pH 2.12 and pH 7.21) (see Figure 6). To

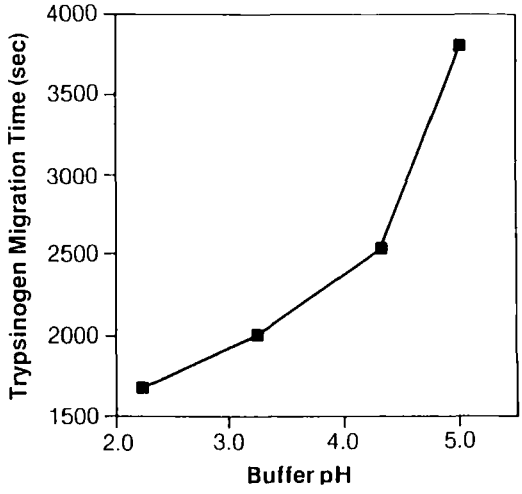

Figure 7. Trypsinogen migration time plotted as a function of buffer pH. Separations of 5 sec inj of 1 mg/ml protein were performed in 25 mM sodium phosphate, 5 mM DDAPS at 25 kV.

determine if the pH changes in the buffer reservoirs were responsible for the observed trypsinogen migration time decrease, an experiment was performed wherein the buffer pHs were deliberately varied. The capillary was filled with pH 4.3 phosphate buffer, and buffers of pH 3.4 and 5.4 were employed at the inlet and outlet, respectively, during a 25 kV separation. As expected, trypsinogen eluted at a time (2066 sec) corresponding to a ca. 25% decrease relative to a separation obtained using a consistent pH 4.3 environment. Thus, this result appeared to confirm the dependence of protein migration time upon the pH environments in the buffer reservoirs. A plot of trypsinogen migration time vs. buffer pH demonstrating the dependence of protein electrophoretic mobility upon pH is displayed in Figure 7. The increase in trypsinogen retention time which occurred as pH was increased from pH 2 to pH 5 probably took place in response to the ionization of multiple aspartic acid residues ($pK_a \approx 4.5$) present in the protein (48) which induced a decrease in its net positive charge.

Several reports of protein separations by CE advocate a rinse with buffer, from inlet to outlet, of at least 1 column volume between separations (15, 21, 22). To determine the effects of a between-run inlet buffer rinse upon trypsinogen migration time reproducibility in 25 mM sodium phosphate pH 4.3, 5 mM DDAPS at 25 kV, a 3 min (ca. 2 column

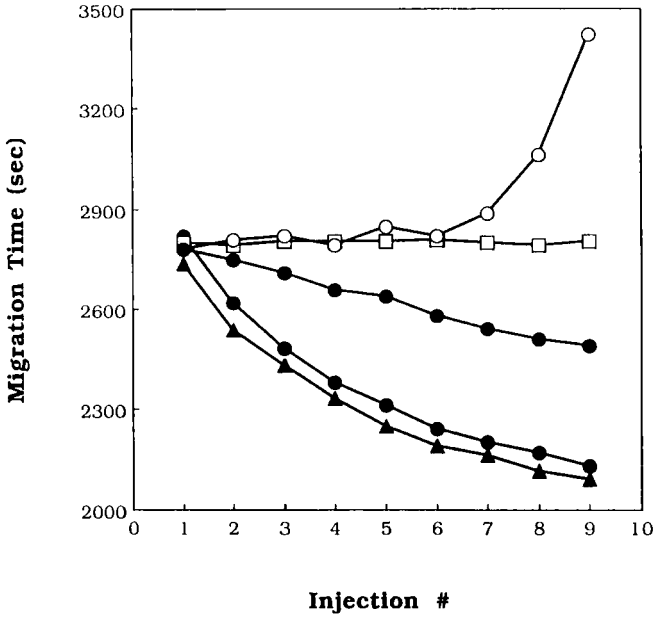

Figure 8. Trypsinogen (1 mg/ml, 5 sec inj) migration time plotted vs. separation (25 kV) replicate for a series of nine replicate separations in 25 mM sodium phosphate, employing the following prior to each separation; (■) 2 column volume inlet rinse; (○) 2 column volume rinse from a fresh inlet buffer, followed by separation using the fresh inlet buffer, outlet buffer not changed; (●) 2 column volume inlet rinse, separations performed using 12.5 μamps constant current; (▲) 2 column volume inlet rinse, separations performed using 0.338 W constant power; (□) complete buffer replenishment, ie. fresh inlet and outlet buffers and a 2 column volume inlet buffer rinse.

volume) rinse from the inlet reservoir to an empty waste vial was inserted between nine replicate trypsinogen (5 sec injection) separations. Migration times were found to decrease by ca. 10% upon completion of the ninth replicate (see Figure 8). This variation was less than that observed when no between-run rinse was employed. Apparently, elimination of the pH gradient inside the capillary by the pre-injection inlet rinse served to reduce the run-to-run trypsinogen migration drift, relative to that observed when no rinse was employed. Theoretical work has predicted that the presence of a pH gradient inside a capillary can have a significant effect upon the migration of analytes (49), and this phenomenon has been

exploited by Foret et al., who optimized a separation of five model proteins by dynamically controlling the pH of the separation buffer (50).

Several commercial instruments currently available possess an autosampler tray which can coordinate with the capillary inlet. With this type of configuration, where the inlet can be inserted into a variety of samples and buffers, an analyst may program a run of multiple samples, each of which can also be separated using an inlet vial of fresh buffer, following a rinse from this vial. This technique may be crucial when electroosmotic flow and non-coated capillaries are employed, since studies have suggested that the state of the cathode (outlet) buffer has little or no effect upon separations in these systems (51). To investigate the effects of fresh inlet buffer upon migration time reproducibility in a poly-(acrylamide)-coated capillary, nine replicate separations of trypsinogen (5 sec injection) were obtained at 25 kV in 25 mM sodium phosphate pH 4.3, 5 mM DDAPS following a 3 min. rinse with fresh buffer from the inlet to a waste vial, employing an inlet vial containing fresh buffer for each separation. The results, plotted in Figure 8, demonstrate an increase in migration time of approximately 20% by the ninth replicate, and suggest that the increased pH (pH 5.50) which developed in the outlet buffer during this series of separations may have significantly reduced the mobility of trypsinogen by lowering its net charge as the protein approached the outlet reservoir. Thus, it appeared that the employment of fresh inlet buffer between protein separations in a series of replicate analyses, in a system without significant electroosmotic flow, was not sufficient to achieve reproducible migration times.

Although analyte electrophoretic migration is directly dependent upon the applied potential, operation at constant current or constant power has been found to offer some advantages. If a cooling system is inadequate or not available, constant current operation can result in a somewhat self-compensating effect of decreasing voltage with increasing column temperature, in effect reducing migration time variations due to temperature changes (28, 52), as had been suggested for slab gel electrophoresis (53). Power supplies delivering a constant power output, independent of changes in the electrical resistance, can ensure that heat generation is constant throughout a separation (44, 54). However, as detailed earlier, temperature variations are presumed to have minimal effect upon the migration time reproducibility obtained in the system utilized for this investigation. Since pH changes in the inlet and outlet buffer reservoirs were observed to be responsible for the migration time variations obtained using constant potential, it was not expected that the use of either constant current or constant power could improve trypsinogen migration time reproducibility. Indeed, replicate separations of 5 sec injections of trypsinogen (buffer vials not changed, no inlet rinse between injections) obtained in 25 mM sodium phosphate pH 4.3, 5 mM DDAPS using both constant current (15.5 μamps) and constant power

(0.388 W) (see Figure 8) displayed approximately 25% reductions in trypsinogen migration time following completion of the ninth replicate, trends identical to that observed when constant potential was employed in the absence of any between-injection rinse or replenishment (see Figure 3).

Since the oxidation / reduction phenomena taking place in both the inlet and outlet reservoirs caused significant pH changes, it appeared that the only way to achieve a consistent capillary environment prior to each injection in a series of multiple analyses was to completely replenish the buffer. This conclusion was also reached by Schwartz, et al., who achieved a <2% RSD in migration time precision for a neutral marker (uracil) using a vacuum-driven buffer replenishment system and non-coated capillaries (8). The instrument used in the present study was equipped with autosampler trays for both the capillary inlet and outlet positions, permitting the analyst to change both reservoirs to vials of fresh buffer for each separation in a series of up to 10 replicate separations. Utilizing the instrument in this capacity, nine replicate trypsinogen injections were obtained at 25 kV in 25 mM sodium phosphate pH 4.3, 5 mM DDAPS. A 3 min inlet rinse with fresh buffer was also employed prior to each injection to ensure consistency of the pH environment inside the capillary. The results, plotted in Figure 8, display migration time reproducibilities superior to that obtained by the other techniques investigated in this study. The trypsinogen migration time RSD of 0.7% obtained from these measurements is competitive with those achievable in HPLC systems employed for peptide mapping (23, 24). Using fresh buffers for all separations, day-to-day and capillary-to-capillary migration time reproducibilities of 0.9% (n=5) and 1.5% (n=5), respectively, were obtained. Capillaries treated in the manner described in this report were stable for a period of at least two weeks before the effects of coating degradation, as evidenced by a significant decrease in efficiency, became evident.

CONCLUSIONS

The employment of acidic buffers containing zwitterionic surfactants in silica capillaries derivatized with poly-(acrylamide) facilitated the achievement of very efficient separations of basic proteins, while virtually eliminating the electroosmotic flow. Because most CE separations are limited, due to joule heating effects, to buffer salt concentrations of 100 mM or less, the results from a series of replicate protein separations revealed the importance of utilizing a buffer salt with optimum buffer capacity at the desired pH, such as sodium acetate at pH 4.3, and suggested that complete buffer replenishment between injections may be necessary to achieve reproducible migration times. It can be postulated that, in addition to proteins, such buffer replenishment may be necessary for obtaining

reproducible migration times for a variety of solutes possessing ionizable groups. pH drift occurring at the inlet electrode may also affect migration time reproducibilities inside non-coated capillaries, where the electroosmotic flow is responsible for the dependence of the environment inside the capillary upon the inlet (anode) reservoir buffer. Thus, for qualitative and quantitative CE analysis employing capillary coatings which minimize electroosmotic flow, the use of instruments that can allow an analyst to perform complete buffer replenishment appears to be essential for the achievement of reproducible migration times.

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