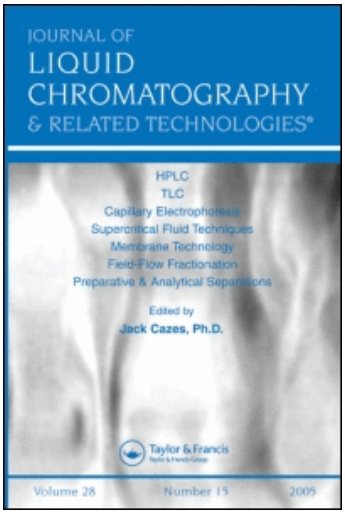

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USE OF HIGH IONIC STRENGTH BUFFERS FOR THE SEPARATION OF PROTEINS AND PEPTIDES WITH CAPILLARY ELECTROPHORESIS

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ABSTRACT

The use of high ionic strength buffers with capillary electrophoresis in untreated fused silica capillaries is demonstrated for the separation of proteins and peptides. Short, small i.d. (20-25 μm) capillaries are useful as a quick screening tool for protein analysis. Electropherograms of standard proteins run from pH 5.0 to 10.0 in 0.5 M sodium phosphate buffers suggest that at these high ionic strengths, the proteins (relative to the electroosmotic flow markers) are not always predictable functions of their pI's. Consecutive runs of standard proteins yield excellent migration time and peak area reproducibility. Milk proteins, particularly the caseins, could be separated with the addition of 4 M urea to the buffer. Alternatively, the addition of a zwitterion to a phosphate-based buffer provide different selectivities for the protein standards. The accompanying reduction in ionic strength allows for

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the use of wider diameter (50 μm) and longer (57 cm) capillaries. Finally, an improved micropreparative separation of peptides from a tryptic digest is demonstrated with a high ionic strength borate buffer. Using a 200 μm i.d. capillary and an automated fraction-collection protocol, individual peaks are isolated and sequenced from a single run.

INTRODUCTION

Capillary electrophoresis (CE) has made significant advances in recent years and is establishing itself as a reliable technique for the analysis of a wide variety of substances. While the applicability of CE to protein separations was recognized early (1,2), difficulties remain up to date.

The challenge of protein separation by CE results not only from differences in analyte stability but also from interactions with the silica surface. Several approaches have been proposed for resolving these issues which tend to focus on the charge density on the silica surface. This charge density has been reported to be ca. 13,000 charges/μm^2 in 50 mM buffer (3). A variety of techniques have been developed to reduce the magnitude of the surface charge: 1) by operating at low pH; 2) by covalent modification of the capillary wall; 3) by shielding the surface with polymers; 4) by the addition of competing charged species; 5) by modifying the analyte charge; and 6) by coating the surface dynamically. (For recent reviews, see references (4,5,6) Although effective in some cases, none of these of methods has broad-based applicability.

Often, methods which have been designed to prevent adsorption may introduce new problems for analysts: 1) coatings and additives can modify the electroosmotic flow so that extremes in pH are needed to control migration direction; 2) protein binding to the capillary surface may be driven by a combination of electrostatic and hydrophobic forces; 3) additives can alter results by interacting with the analyte; and 4) multiple techniques may be required to handle the diversity of protein applications.

This paper will focus on the use of high ionic strength buffer systems for the separation of proteins and peptides. Earlier work by Lauer and McManigill (2) and Jorgenson et al. (1,7,8) provided a basis for the use of these conditions for protein and peptide separations. Recently, Swedberg (9) employed a high phosphate/small i.d. capillary combination in their work

with coated capillaries. Chen used small i.d. (25 μm) untreated fused silica capillaries for rapid, reproducible separations of serum proteins (10). In these papers, it was recognized that the combination of an effective cooling system and a small i.d. capillary provides efficient removal of Joule heat. This work extends these observations and provides a basis for the use of a high ionic strength buffer system as a tool for protein analysis. In addition, other configurations which use wider i.d. capillaries and/or zwitterion additives are also investigated. Finally, we demonstrate the advantages of high ionic strength buffers in the analytical and micropreparative separations of tryptic peptides. In the latter application a 200 μm i.d. capillary is employed.

MATERIALS AND METHODS

A P/ACE System 2000 (Beckman Instruments, Inc., Palo Alto, CA) was used with P/ACE system software controlled by an IBM PS/2 model 50Z. 25 μm i.d. capillaries (Polymicro Technologies, Phoenix, AZ) were used at a total capillary length of 21-23 cm by removing the mandrel from the cartridge. For micropreparative work a 107 cm x 200 μm or 57 cm x 75 μm uncoated fused silica capillary was used.

Phosphate buffers with a pH of between 5 and 10 were prepared by mixing appropriate amounts of 0.5 M mono-, di- and/or trisodium phosphate. For the milk protein analysis, the 0.5 M sodium phosphate buffer pH 7.0 contained 4 M urea. Buffer containing 280 mM sodium borate and 30 % ethylene glycol was prepared by diluting 400 mM sodium borate, pH 8.55, with ethylene glycol; the final pH was measured at 7.34. In some cases, a proprietary zwitterionic additive, ZB-4, was incorporated in the buffer. All buffers were filtered through a 0.45 μm filter before use.

Protein standards, obtained from Sigma Biochemicals (St. Louis, MO) and Serva Biochemicals (Westbury, NY) included bovine trypsin inhibitor, cytochrome c, carbonic anhydrase, soybean trypsin inhibitor, myoglobin, conalbumin, β-lactoglobulin B and β-lactoglobulin A. Each protein concentration was between 0.3 to 1.0 mg/mL. In some samples, 0.01% v/v of dimethylformamide (DMF) was added to the sample diluent as a neutral electroosmotic flow marker.

Pasteurized, low-fat, nonfat and powdered milk and β-lactoglobulin were obtained from local suppliers.

On-line detection with the P/ACE system was set at 200 or 214 nm with a 50x200 μm aperture in the P/ACE cartridge. Temperature of the capillary during electrophoresis was set and maintained at the ambient temperature, usually 23°C or 25°C. Samples were introduced by pressure injection and electrophoresed at the voltage and current stated in each electropherogram.

For the separations on the small i.d. (20-25 μm) capillaries, between runs, the capillary was sequentially rinsed with 2 column volumes of 1.0 M NaOH and water (0.3 min high pressure each), followed by reconditioning with 5-10 volumes of run buffer (1.5-2.5 min high pressure). In the case of the zwitterion separations, a rinse with 5-10 volumes of buffer without additive was inserted between the sodium hydroxide and run buffer. Capillaries used for the separation of tryptic digests were conditioned using two column volumes of 280 mM sodium borate-30% glycerol rinsed under high pressure, followed by a column volume rinsed at low pressure.

Sample Preparation

Protein standards were dissolved in sample diluent buffer containing 75 mM NaCl, 20 mM potassium phosphate, 0.01% sodium azide, pH 7.0 (PBS). For ionic strength studies, protein standards were dissolved in 25% PBS at 1.7 mg/mL each and pressure-injected for 3 sec. Powdered nonfat milk was dissolved in water. All milk samples were diluted 1:5 in PBS containing 4 M urea. For fraction collection experiments, bovine Beta-lactoglobulin A was digested to completion with trypsin (Sigma). The time course of the reaction was followed by removing aliquots as samples for analysis by a capillary electrophoresis method similar to the described work (data not shown). The reaction was terminated by adding trifluoroacetic acid to a final concentration of 5%. The resultant final concentration of β-lactoglobulin used for the fraction collection experiments was 8.6 mg/mL based upon the monomeric molecular weight of 18,367.

Gold-based Method Translator for Fraction Collection

The fraction collection techniques are driven by an in-house DOS-based program called a 'method translator' (not commercially available). This software program converts a Beckman System Gold P/ACE analytical method into one which collects selected peaks. With the exception of voltage parameters and electrolyte/collection vials, all components and parameters of

the CE system were identical for the two types of runs. A Gold data file from the analytical run containing peak start/stop times and peak names are matched against entries in a user made "wish list". This "wish list" contains peak names, peak apex times, vial numbers and voltages for peaks to be collected. After retrieving information from the 'wish list', the program integrates the voltage vs. time profile in the method to derive volt-min. parameters defining the predicted start/stop periods when each of the selected peaks elutes from the capillary outlet. The program then exports these parameters to create a System Gold method containing voltage values and vial changes for the fraction collection run.

The fractions were collected in 10 µL of 100 mM sodium borate, pH 8.4 at 5 kV. Between peak collection periods, the capillary outlet returned to the starting outlet buffer vial and voltage. Collection periods were longer than the peak widths in the analytical run due to the reduced electric field used during these periods.

RESULTS AND DISCUSSIONS

Utility of short, small i.d. capillaries

The separation of standard proteins at high ionic strengths at a variety of pH's are shown in Figures 1 and 2. For these separations, short, small i.d. (20-25 µm), untreated fused-silica capillaries were employed. An electroosmotic flow marker (DMF) was added to the sample. The isoelectric points of the proteins are listed in Table 1 along with their molecular weights. Small i.d. capillaries are advantageous with respect to minimizing Joule heat generation (10). Hence, high ionic strength buffers can be used with these capillaries.

In Figure 1, first note that proteins with a ΔpI of 0.2 (i.e. ß-lactoglobulin A and B) can be separated with the short, untreated capillary, indicating the potential utility of the high molarity phosphate buffer system. Similar results have also been obtained using a coated capillary, but with longer analysis times (11). However, the stability of coated capillaries operated under neutral or high pH conditions is a concern. We have found excellent long-term stability of the untreated capillaries used in Figure 1.

Second, run time reproducibility for the standard protein set was evaluated with and without column regeneration (see Table 2). Migration

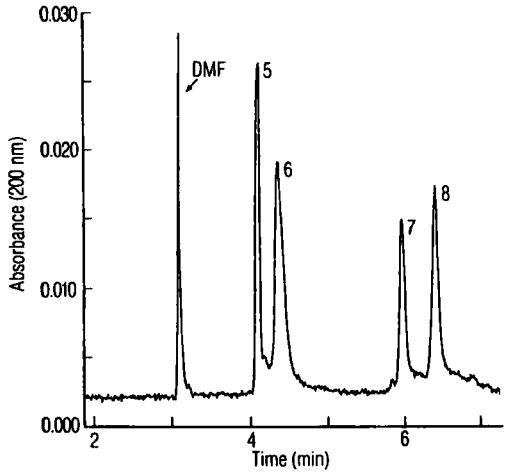

Figure 1. Separation of protein-standard mixture in 500 mM phosphate buffer. Peak identification: (5) myoglobin, (6) conalbumin, (7) ß-lactoglobulin B, and (8) ß-lactoglobulin A. DMF was added as an electroosmotic flow marker. Buffer: 0.5 M sodium phosphate, pH 7.0. A 22 cm length x 20 um i.d. capillary was used. The field strength was 410 V/cm.

time and peak area precision are given for 9 consecutive runs shown in Figure 1. In this case, the capillary was washed with base and water, as described in Material and Methods. It can be seen that the precision in migration time is less than 1% relative standard deviation (RSD), while peak area precision is better than 2.3% RSD.

In the case where the capillary was not regenerated with sodium hydroxide and buffer between runs, the electroosmotic flow rate appears to increase initially and reaches a constant flow rate after approximately an hour. Protein adsorption was not observed with the 500 mM phosphate buffer so that this phenomenon must be related to surface equilibration. The increased electroosmotic flow strongly suggests a time-dependent and buffer concentration-dependent interaction between the phosphate buffer and silica surface (12,13). In addition, the relatively high ionic strength allows for a greater variation in sample matrix conditions yet still promotes conditions favorable for focussing or sample stacking (14). Short capillaries are

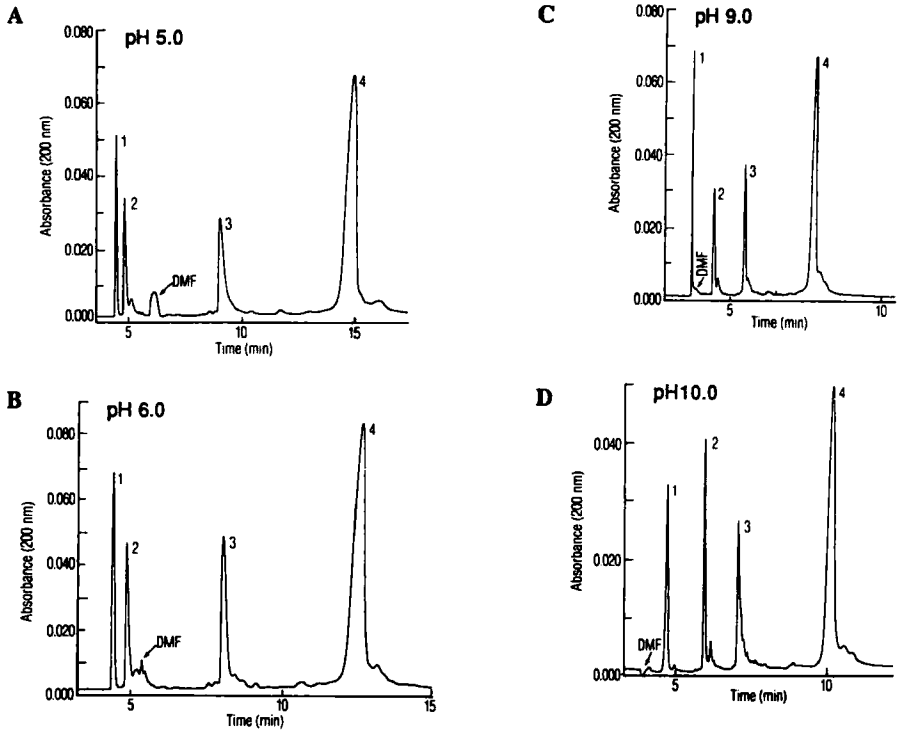

Figure 2. Influence of pH on resolution in high phosphate buffers. Peak identification: (1) bovine trypsin inhibitor, (2) cytochrome c, (3) carbonic anhydrase, and (4) soybean trypsin inhibitor. A 23 cm length x 25 μm i.d. capillary was used in conjunction with a 0.5 M sodium phosphate buffer. DMF was used as an electroosmotic marker. Detection was at 200 nm and the field strength was 350 V/cm. Capillary temperature was set at 23°C. Sample injection time was 20-40 s. The pH's of these separations were 5.0 (Figure 1A); 6.0 (Figure 1B); 9.0 (Figure 1C); or 10.0 (Figure 1D), respectively.

TABLE 1.

Proteins Separated

#	Protein	pI	Mol. Weight
1.	Bovine Lung Trypsin Inhibitor	10.5	500
2.	Cytochrome c	10.7	12,500
3.	Carbonic Anhydrase	5.9	29,000
4.	Soybean Trypsin Inhibitor	4.5	21,000
5.	Myoglobin	7.0	17,500
6.	Conalbumin	6.6	77,000
7.	ß-Lactoglobulin B	5.4	36,000
8.	ß-Lactoglobulin A	5.2	36,000
9.	lysozyme	11.0	14,000

TABLE 2.

Reproducibility of Migration Time and Area for 9 Consecutive Runs *

Protein	Time (min.)	RSD (%)	Area	RSD (%)
DMF (marker)	4.66	0.35	---	---
myoglobin	6.53	0.92	2.03	1.81
conalbumin	6.92	0.53	1.86	2.33
ß-lactoglobulin B	9.46	0.39	2.30	0.93
ß-lactoglobulin A	10.22	0.37	2.69	0.68

** Proteins were run in 0.5 M sodium phosphate buffer, pH 8.0 on a 21 cm x 25 μm capillary at 350 V/cm.

advantageous for protein separations as their residence times are generally short, leading to a decreased chance of adsorption.

A second set of standard proteins was used to evaluate the effect of pH on mobility (pI's=4.5-10.5). Results with 0.5 M sodium phosphate buffer are shown in Figure 2. Panels A-D show the separation of the electroosmotic flow marker DMF, bovine lung trypsin inhibitor, cytochrome c, carbonic anhydrase and soybean trypsin inhibitor. At a buffer pH of 7.0 or below, bovine trypsin inhibitor and cytochrome c migrate earlier than the electroosmotic flow marker, DMF, as anticipated. Carbonic anhydrase, with a pI of 5.9, however, migrates substantially later than DMF at a buffer pH of 5.0 (Figure 2A). At pH 6.0 (Figure 2B), carbonic anhydrase occurs much later than DMF despite the buffer pH and the pI of the protein being almost the same. Furthermore, at buffer pH 9.0 (Figure 2C), cytochrome c elutes unexpectedly after DMF. Similarly, at buffer pH 10.0 (Figure 2D), both cytochrome c and bovine trypsin inhibitor migrate after DMF. The five proteins run in Figure 2 are also separated well at pH 7.0 (data not shown).

The above results suggest that under these high ionic strength conditions – two phosphate pKa's are titrated – the effective charge on the protein is altered. Hence, expected migration relative to an EOF marker at a given pH may not be observed. Furthermore, the change in resolution with pH seemed to be selective. At least two of the proteins showed no change in resolution between pH's 6 and pH 9. This may be attributed to the relatively low or similar histidine content of these proteins.

Turning to some examples with samples in a biological matrix, electropherograms of pasteurized, low-fat, nonfat and powdered milk are shown in Figure 3. In addition to a variety of proteins, of which casein is the major component, milk contains vitamins, carbohydrate and variable amounts of lipid. Milk proteins traditionally are separated with gel electrophoresis as well as ion exchange and reversed phase HPLC. CE may offer advantages in terms of resolution and speed compared to the latter techniques. With a pH 7 run buffer containing 4 M urea, alpha and beta-casein are unequivocally separated. In the absence of urea, however, the two species exist as an aggregate and co-migrate as an unresolved broad peak (data not shown).

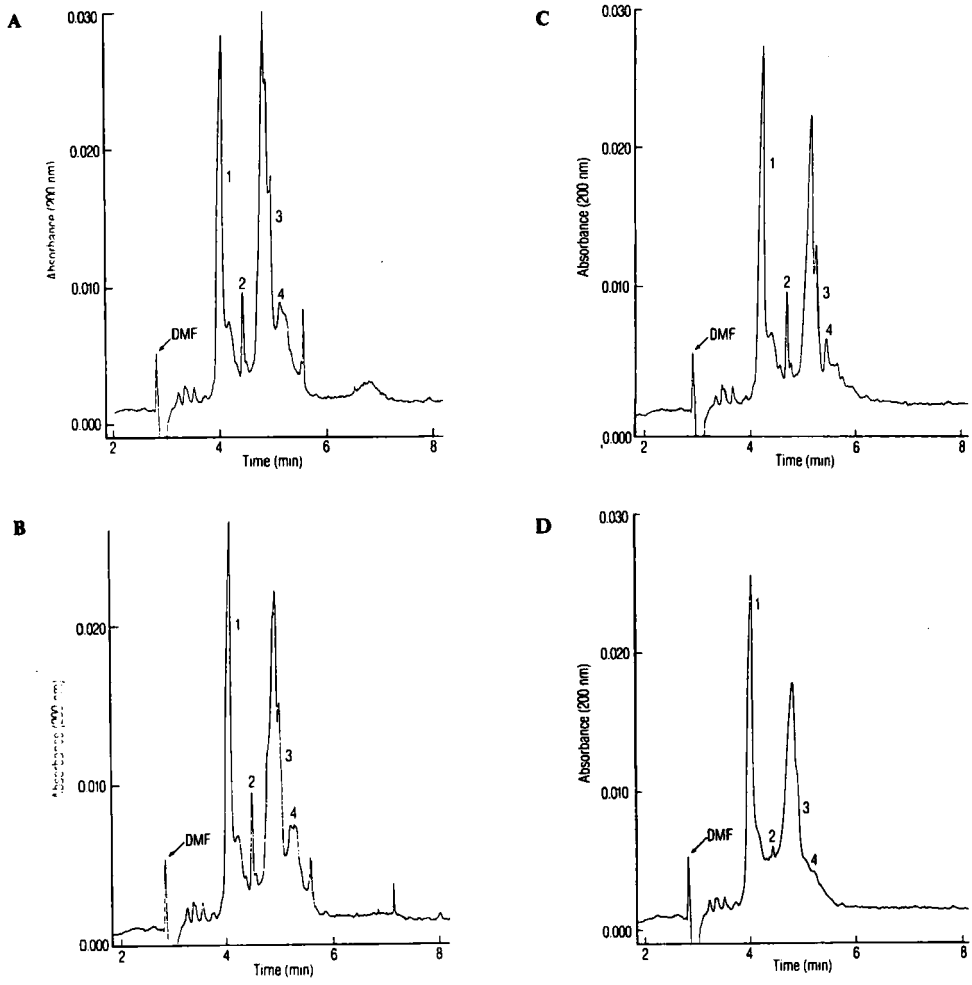

Figure 3. Electropherograms of pasteurized (A), low-fat (B), nonfat (C) and powdered milk (D). Powdered nonfat milk was dissolved in water. All milk samples were diluted 1:5 in PBS containing 4 M urea and pressure injected for 30 s onto a 23 cm x 21 μm capillary. The run buffer consisted of 500 mM phosphate, 4 M urea at a final pH of 7.0. Run voltage was 10 kV.

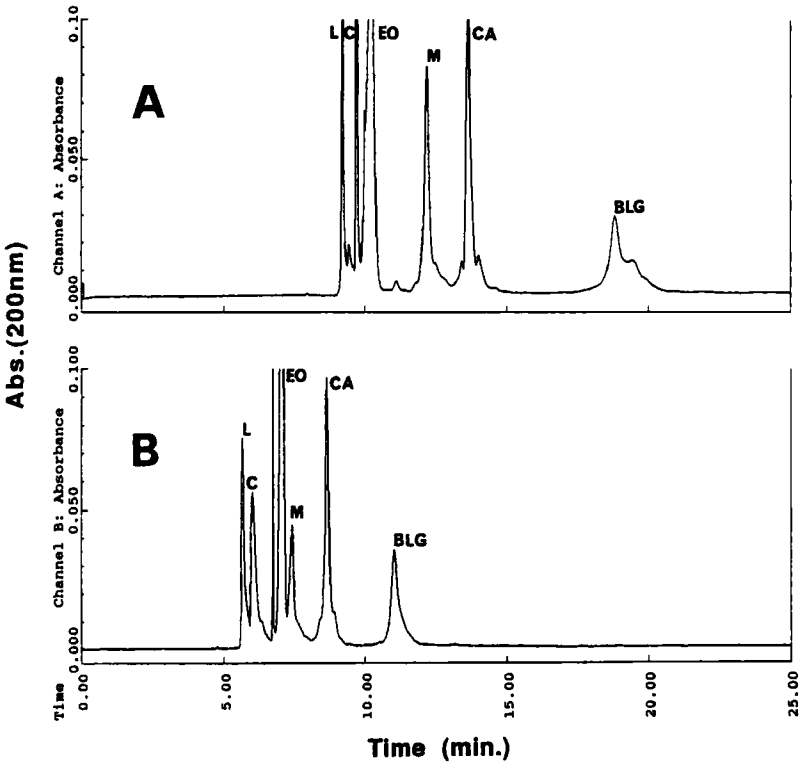

Figure 4. Effect of capillary diameter on resolution in concentrated phosphate buffers. Protein standards: (L) lysozyme, (C) cytochrome c, (M) myoglobin, (CA) carbonic anhydrase, and (BLA) ß-lactoglobulin-A. The proteins were dissolved in 25% PBS at 1.7 mg/mL each and pressure-injected for 3 sec onto a 50 μm i.d. X 27 cm capillary. Note that the run voltage and buffer were 3.75 kV and 400 mM potassium phosphate, respectively (Figure 3A), or 5 kV and 250 mM potassium phosphate, respectively (Figure 3B). The buffer pH was 6.5 and the capillary temperature was 25 °C.

Effect of capillary diameter and ionic strength

The influence of capillary diameter on resolution in concentrated phosphate buffers is shown in Figure 4. In contrast to Figures 1-3, a 50 μm i.d. capillary was used. With this larger i.d. capillary, lysozyme, cytochrome c, myoglobin, carbonic anhydrase and ß-lactoglobulin-A could be separated with 400 mM potassium phosphate (Figure 4A) or 250 mM potassium phosphate (Figure 4B). Elution times are observed to increase as the ionic strength is increased, as would be expected when electroosmotic flow is reduced (data not shown). Note that in spite of the lower field in (A) compared to (B), the wattage is higher (the current is higher in this case) and the ß-lactoglobulin-A peak is broadened. Apparently, under these high salt conditions excessive Joule heating causes this dimeric protein to undergo conformational changes or to unfold (15). This problem is circumvented by lowering the phosphate concentration to 250 mM (Figure 4B); here, a symmetrical peak shape was obtained.

Effect of capillary length / addition of zwitterions

According to theory, resolution is generally increased as the capillary length is increased (16). However, could these theoretical expectations be realized with protein analytes in high ionic-strength buffers? Additional peak broadening might be expected to occur from increased exposure to the silica surface, from prolonged time at elevated temperatures, or from extended interactions with buffer components. Results from a 50 um i.d. and 57 cm long capillary are shown in Figure 5A. Compared to Figure 4B (27 cm length), all protein peaks appear to be more efficiently separated-approximately twice the plate count was obtained for the 57 cm length. Increased time at run conditions did not result in additional peak broadening for this sample set. This would suggest that increased efficiency for protein separations might also be obtained with longer capillary lengths.

An alternative to the high phosphate buffer was obtained with the zwitterion additive. The effect of the proprietary additive ZB-4 on the separation is shown in Figure 5B. This compound functions in an analogous fashion to that shown by Bushey and Jorgenson (8), i.e. protein adsorption is suppressed while conductivity is not substantially increased. Note that in Figure 5B, the phosphate was reduced to 100 mM and only 250 mM zwitterion was added-conditions were not optimized. In some cases, resolution is better

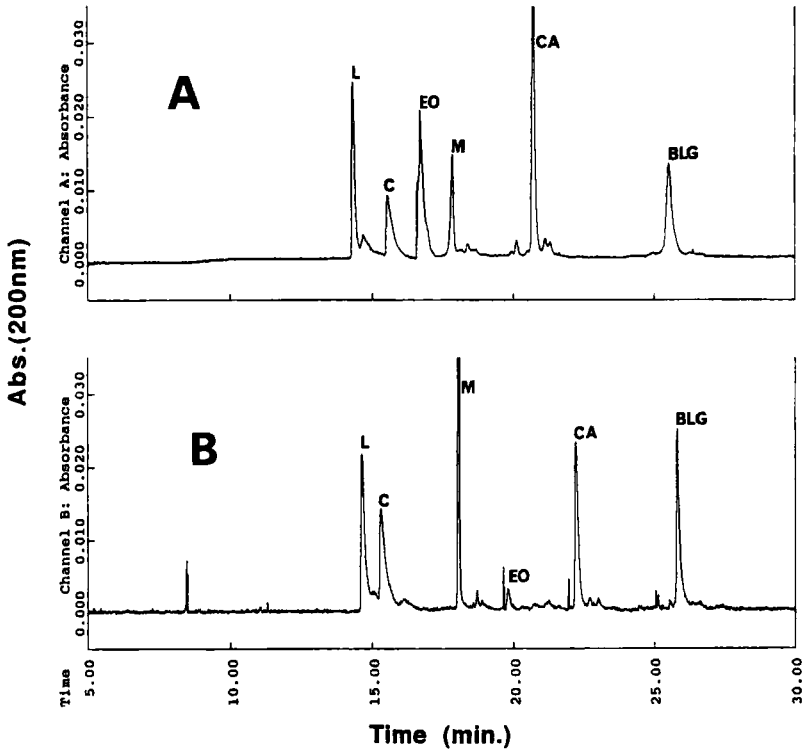

Figure 5. The effect of column length and zwitterion additive on separation efficiency. Protein standards at 0.5 mg/ml were prepared as in Figure 4. Peak identification: (L) lysozyme, (C) cytochrome c, (M) myoglobin, (CA) carbonic anhydrase, and (BLA) ß-lactoglobulin A . Samples were injected for 6 s on to a 50 um i.d. X 57 cm capillary. Separation was in a 250 mM phosphate, pH 6.0 buffer (Figure 4A) , or in 100 mM phosphate + zwitterion at pH 5.6 (Figure 4B). The run voltage was 10.5 kV and the capillary temperature was set at 25 °C. The signal-to-noise ratio increased significantly for myoglobin and ß-lactoglobulin-A with the addition of zwitterion.

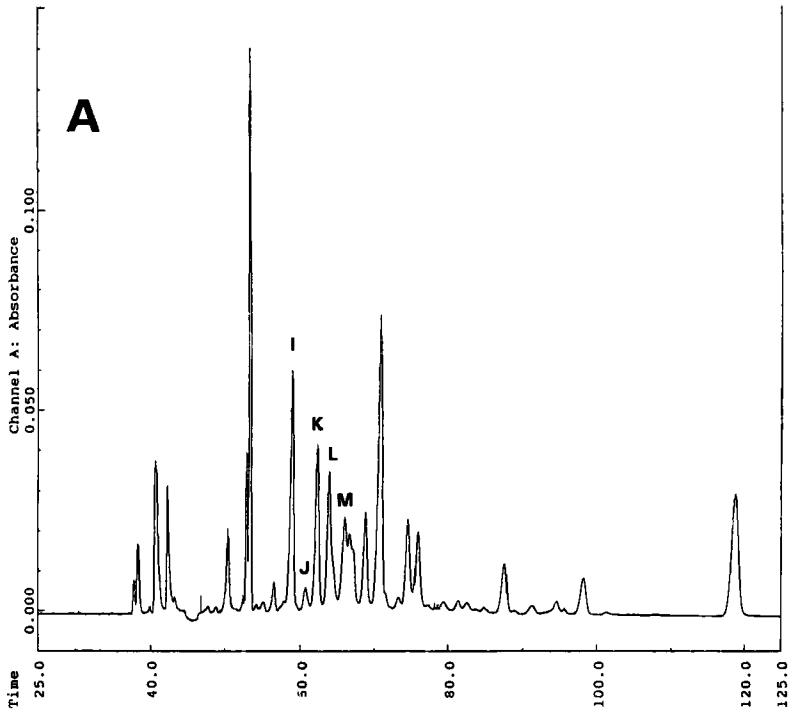

Figure 6. Micropreparative collection of tryptic peptides. Figure 6A shows the electropherogram of a tryptic digest of ß-lactoglobulin A. The concentration of the digest was 8.6 mg/mL in water. The sample was run on a 107 cm length x 200 um i.d. capillary in a 280 mM sodium borate, 30% ethylene glycol, pH 8.55 buffer. The run voltage was 20 kV at a current of 160 μA. The temperature was controlled at 25 °C for each run. Detection was at 214 nm. The automated, fraction collection electropherogram for peaks I, K, L and M is shown in Figure 6B. The top panel of the Figure 6B shows the complete tryptic digest injected on a 57 cm length x 75 um i.d. capillary at 30 kV (73 μA). The bottom traces show, from top to bottom, the re-injected collected fractions I, K, L and M, respectively.

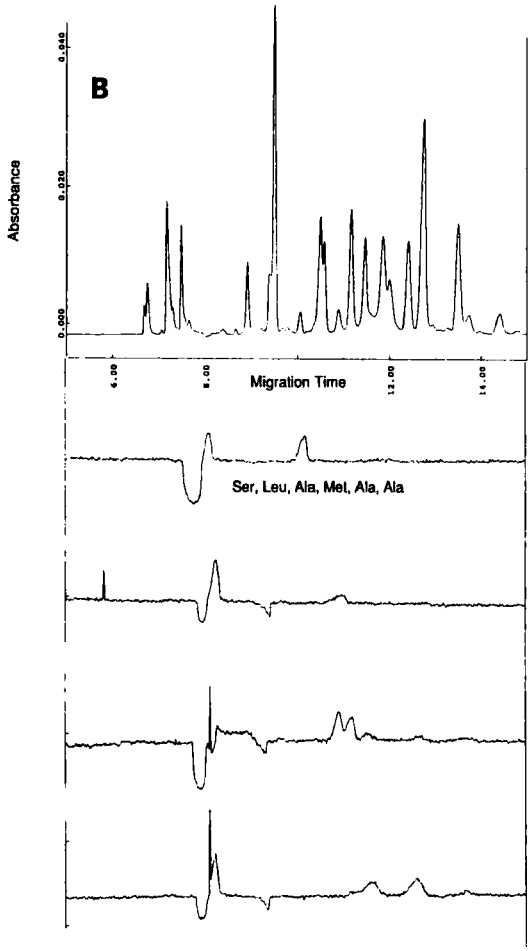

Figure 6. (continued)

than the 250 mM phosphate buffer. Note the increased efficiency for ß-lactoglobulin-A and myoglobin in Figure 5B compared to Figure 5A. The zwitterion may provide alternative ion-pairing capabilities for these proteins.

Micropreparative application

The potential to recover and analyze fractions is a fundamental component of most analytical schemes. Recently this capability

(micropreparative) has been extended to capillary electrophoresis (17-22). However, a major limitation of previous configurations was the need for multiple collection runs due to the requirement for small bore capillaries (20).

Recently, the application of larger bore capillaries (200 μm i.d.) to peptide fraction collection has been demonstrated (21). Compared to an analytical capillary of 75 μm i.d., a seven-fold increase in sample load is potentially obtainable with the larger capillary. However, with the increase in size comes a decrease in performance due to Joule heating. Our objective was to define a buffer system which would be compatible with existing digestion conditions and with subsequent sequence analysis.

Earlier results (data not included) had shown that overall performance increased with buffer ionic strength. At the same time, it was necessary to reduce the voltage gradient to avoid over heating. A comparison of the analytical and micropreparative separations in high ionic strength borate buffer is shown in Figure 6. In both the analytical and the micropreparative run the tryptic digest of β-lactoglobulin A was separated in a buffer consisting of 280 mM sodium borate, 30% ethylene glycol at pH 8.55 (see Figure 6A and 6B). Note that ethylene glycol was added to reduce the electroosmotic flow and to improve resolution. Although the capillary length was increased (57 cm to 107 cm) and the voltage was reduced (30 kV to 20 kV), the results from the micropreparative run (Figure 6A) compare favorably to the analytical run. The high ionic strength buffer promotes sample focusing for the relatively high concentrations of protein and buffer used during the tryptic digestion (14). This effect along with the increase in resolution more than compensates for the longer run times and increased Joule heat experienced with the higher ionic strength.

To illustrate the compatibility of this technique with fraction collection and subsequent protein sequencing, several peaks were collected. Four peaks, I, K, L and M were collected and reinjected on the analytical capillary for verification of collection (see Figure 6B lower panel; runs were aligned with the entire digest for comparison). Note the large refractive index artifact from the water/sample injection plug. The sequence of peak I (Ser-Leu-Ala-Met-Ala-Ala), although not a principal tryptic peptide, does corresponds to a known sequence in the protein. Using pressure injection, the estimated sample load was ca. 200 nl (9.4 pmole of peptide I). This corresponds well with a 7.3 pmole recovery obtained in the first cycle of the sequencer. More work is necessary to elucidate sequences of the fractions K, L, M.

Automation is key to successful fraction collection of multiple peaks by capillary electrophoresis. For this purpose, a preliminary software program was developed in which specified peaks (based on a previous run) are designated for collection. The reproducibility of the method (≤ 0.5 % RSD) permits the use of parameters from a preceding run to be utilized as a good predictor of migration time elution "windows" in a subsequent run. The results presented here with the 200 um i.d. capillary are preliminary and look promising. On-going research in our laboratory continues to explore capillary electrophoresis as a micropreperative tool for a variety of applications.

CONCLUSIONS

The utility of protein analysis by CE in a short, small i.d. untreated fused-silica capillary with a high ionic strength phosphate buffer system has been explored in model protein systems and with a "real" sample. With 0.5 M sodium phosphate, separation of proteins can be performed rapidly and reliably with excellent precision over a wide range of buffer pH's when using these capillaries. The use of a high ionic strength buffer in CE typically would result in high currents, but the heat dissipation of the P/ACE system coupled with the use of a narrow bore capillary, provides efficient removal of Joule heat, making operation under these conditions successful. An additional advantage is that with short capillaries, analysis times are typically fast and consequently, adsorption is minimized. These capillaries should therefore prove useful as a screening tool for protein chemists. However, longer capillaries typically yield better resolution. In some cases, substitution of zwitterion additives for the buffer may enhance resolution. Larger bore capillaries are advantageous with respect to sample detectability as the detector path length is larger while they have a more favorable stray light-to-signal ratio than narrow bore capillaries. Preliminary data from our group and others (21,22) show that unconventionally large i.d. capillaries (100-500 μm) can be utilized for micropreparative work.

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