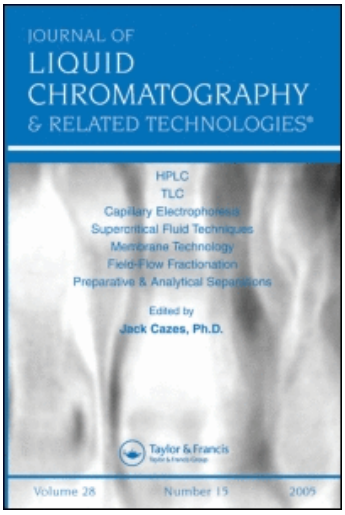

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PERFORMANCE OF UNCOATED AND COATED CAPILLARIES IN FREE ZONE ELECTROPHORESIS AND ISOELECTRIC FOCUSING OF PROTEINS

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ABSTRACT

Separation of standard proteins was compared in uncoated fused silica capillaries and in capillaries coated with a linear polymer covalently attached to the capillary wall. Performance of coated versus uncoated capillaries was assessed for free zone separations and capillary isoelectric focusing. In free zone separations performed under acidic conditions, separation profiles and run-to-run reproducibility were comparable, although resolution was poor in both cases. In free zone separations performed under basic conditions, separation profiles and reproducibility were clearly improved using coated capillaries. Use of coated capillaries was essential for obtaining useful results in isoelectric focusing.

INTRODUCTION

Capillary electrophoresis has been used with wide success for separation of peptides employing uncoated fused silica capillaries and

acidic buffers (1). Although there is great interest in applying the technique to separation of proteins, considerable difficulties have been encountered using these conditions. Many proteins are unstable at low pH, and may aggregate and precipitate. Separation of some standard proteins at low pH has been described (2-4), but this cannot be a general strategy for free zone protein separations because of stability considerations and insufficient differences in mass to charge ratios. Since the vast majority of proteins have isoelectric points between 4 and 9, optimal separations should be achieved at pH values across this range. Also, proteins are generally most stable at physiological pH values. Unfortunately, the surface characteristics of fused silica capillaries and of most proteins impose two obstacles to achieving satisfactory results: electroendosmosis and solute adsorption. At pH values above the pK of the silanol group, the inner wall of the capillary will carry a negative charge, generating a high electroendosmotic flow and active sites for protein adsorption. Protein adsorption will reduce detector signal, compromising quantitative accuracy. More importantly, adsorption will change the surface characteristics of the capillary wall, causing variations in electrososmotic flow, thereby compromising quantitative precision.

Recently we have reported the combination of three strategies to improve protein separations under neutral and alkaline conditions (5). These are the use of coated capillaries, the incorporation of zwitterion additives to the analysis buffer, and the use of capillary wash solutions between analyses. Hjertén (6) has shown that a linear hydrophilic polymer covalently attached to the capillary wall greatly reduces electroendosmosis, and we observed significant reduction in protein adsorption using capillaries coated with a modification of Hjertén's procedure. Addition of zwitterions to the electrophoresis buffer further reduces the amount of protein adsorption, while purging the capillary after analysis with an acidic wash strips any residual adsorbed protein, improving run-to-run repeatability. This communication, a continuation of our previous study, describes free zone electrophoresis results with a wider variety of proteins and compares the performance of coated and uncoated capillaries in isoelectric focusing.

MATERIALS AND METHODS

β-lactoglobulin A, β-lactoglobulin B and α-lactalbumin were obtained from Sigma Chemical Co. (St. Louis, MO.). Hemoglobins A, F, S, and C were obtained from Isolab (Akron, HO). Phycocyanin was obtained from Bio-Rad Laboratories (Richmond, CA). All capillary electrophoresis separations were performed using the Microsampler 100 capillary electrophoresis system (Bio-Rad Laboratories, Richmond, CA). The capillaries were enclosed in cartridges, and, where indicated, were coated internally with a covalently bonded linear polymer after the method of Hjertén (6). For free zone electrophoresis separations, proteins were prepared in 1:10 aqueous dilutions of the analysis buffer at concentrations of 25 - 100 μg/ml. Capillary dimensions were 25 μm i.d. x 20 cm length. Samples were loaded into the capillary electrophoretically at 8 kV constant voltage for 8 seconds, and separations were obtained at 8 kV constant voltage. Detection was at 200 nm. All free zone analysis buffers contained a proprietary mixture of zwitterionic additives. For isoelectric focusing separations, samples were prepared in a 2% final concentration of Bio-Lyte® 3-10 ampholytes (Bio-Rad Laboratories, Richmond, CA) and loaded into the capillary by pressure. Capillary dimensions were 25 μm i.d. x 14 cm length. The anolyte was 10 mM phosphoric acid and the catholyte was 20 mM sodium hydroxide. Samples were focused at 6 kV under constant voltage for approximately 2 minutes. Mobilization of focused zones was performed after the method of Hjertén (7) by replacing the catholyte with 20 mM sodium hydroxide + 80 mM sodium chloride. Mobilization was carried out at 7 kV constant voltage. Detection was at 280 nm.

RESULTS AND DISCUSSION

Free Zone Capillary Electrophoresis

A mixture of six standard proteins was selected for comparision. These range in pI value from 4.2 to 7.4 and in size from 18,000 - 64,000

TABLE 1.
Characteristics of Standard Proteins

Protein	Isoelectric Point	M_r
α-lactalbumin	4.2-4.5	16,000
phycocyanin	4.65	19,000
β-lactoglobulin A	5.13	18,400
β-lactoglobulin B	5.23	18,400
hemoglobin A	6.95	64,500
hemoglobin F	6.90	64,500
hemoglobin S	7.25	64,500
hemoglobin C	7.40	64,500

M_r (Table 1). When separated under acidic conditions using 0.1M sodium phosphate (pH 2.5) as the analysis buffer with positive-to-negative polarity, the electropherograms obtained with coated and uncoated capillaries are very similar (Figures 1 and 2). Under these conditions, the silanol groups on the surface of the uncoated capillary are weakly ionized if at all, and there will be few active sites for protein adsorption and negligible electroendosmotic flow. The separation pattern is reproducible from run-to-run on both the coated and uncoated capillaries, and the coated capillary appears to offer little advantage under these conditions aside from a slightly better resolution of α-lactalbumin from hemoglobin A and hemoglobin S from phycocyanin. However, it is clear that the resolution obtained with either a coated or uncoated capillary under acidic conditions is poor.

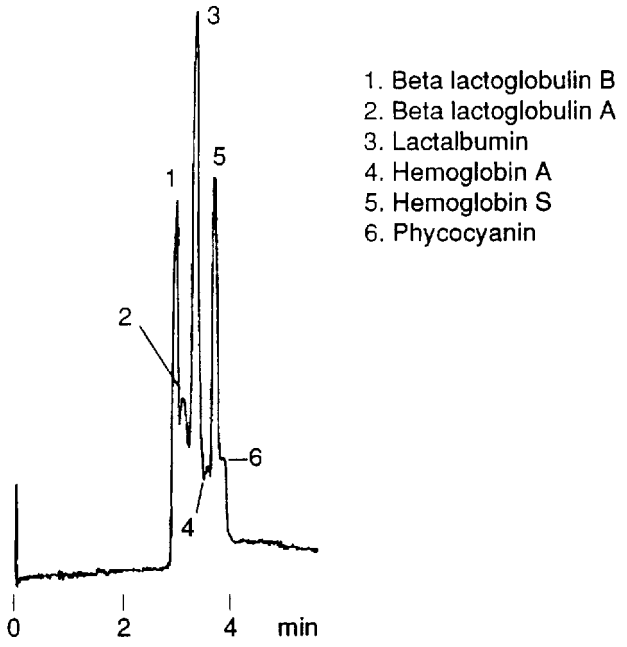

FIGURE 1. Free zone separation of standard proteins at pH 2.5 in a coated capillary.

When the protein mixture was run under alkaline conditions using a 0.3M sodium borate buffer (pH 8.5) with zwitterionic additives, all six components were completely resolved within 10 minutes using a coated capillary (Figure 3). Since all proteins in the mixture have pI values below 8.5, this separation was performed with negative-to-positive polarity. Run-to-run reproducibility is satisfactory, suggesting that adsorption is minimal under these conditions. The capillary was purged for 30 seconds with 0.1M sodium phosphate (pH 2.5) followed by deionized water between analyses.

When the separation of the six-component mixture was attempted using an uncoated capillary with the same buffer and positive-to-negative polarity, no peaks were observed (Figure 4). Measurement of electroendosmosis using urea as an uncharged marker indicated that the

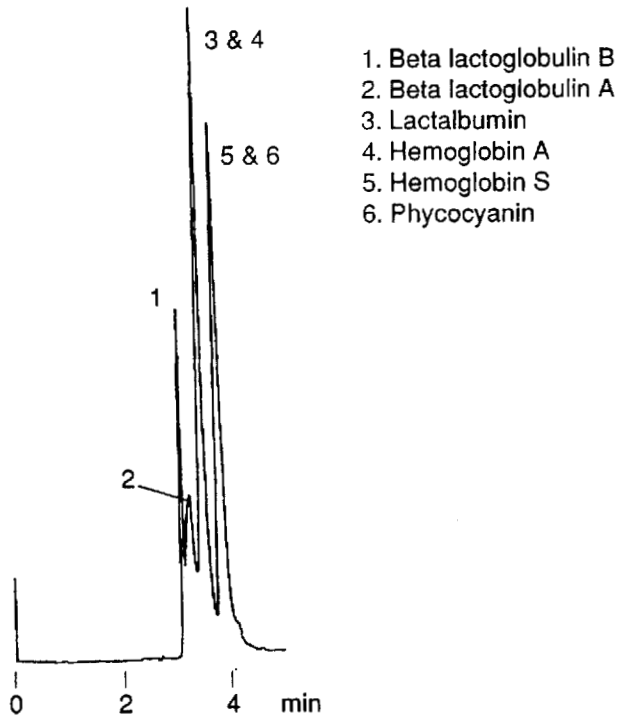

FIGURE 2. Free zone separation of standard proteins at pH 2.5 in an uncoated capillary.

electroendosmotic flow coefficient at this ionic strength was 9.4 x 10^{-5} cm^2 $sec^{-1}V^{-1}$. The electrophoretic mobility of the fastest migrating peak, β-lactoglobulin A, was 18.6 x 10^{-5} cm^2 $sec^{-1}V^{-1}$ (determined using a coated capillary). Therefore, since the direction of electrophoretic migration was opposite in direction and of similar magnitude to electroendosmotic flow, the net rate of electromigration of these proteins during loading and analysis was quite low. When this experiment was repeated using identical conditions but with negative-to-positive polarity, peaks were observed (Figure 5). The migration order was the same as that observed in Figure 3, but with much longer migration times. Again, this reflects the contribution of electroendosmosis to electromigration in the uncoated capillary.

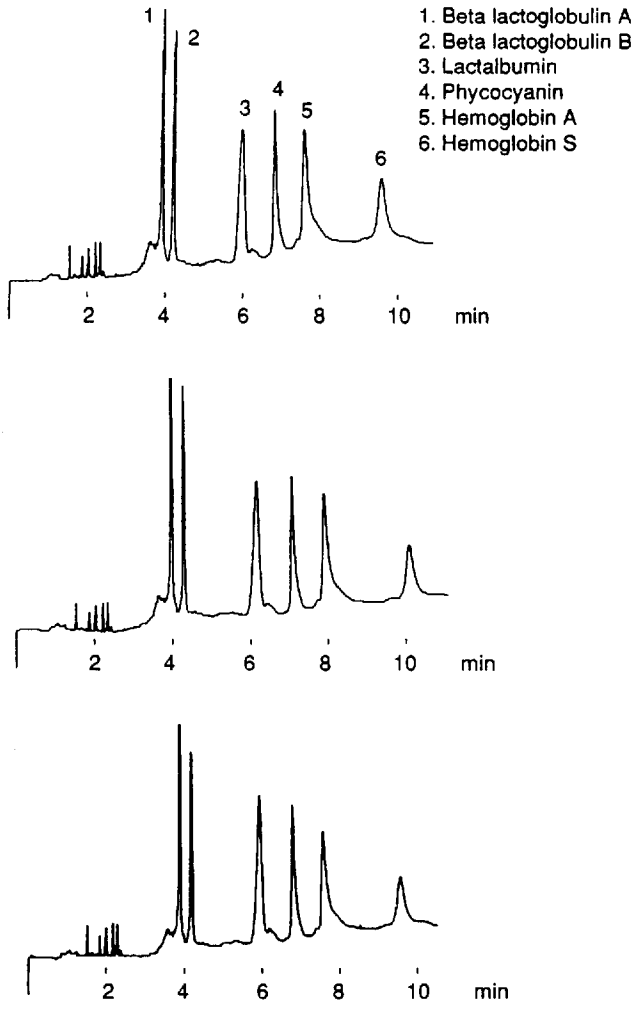

FIGURE 3. Free zone separation of standard proteins at pH 8.5 in a coated capillary, three successive runs.

FIGURE 4. Free zone separation of standard proteins at pH 8.5 in an uncoated capillary with positive-to-negative polarity.

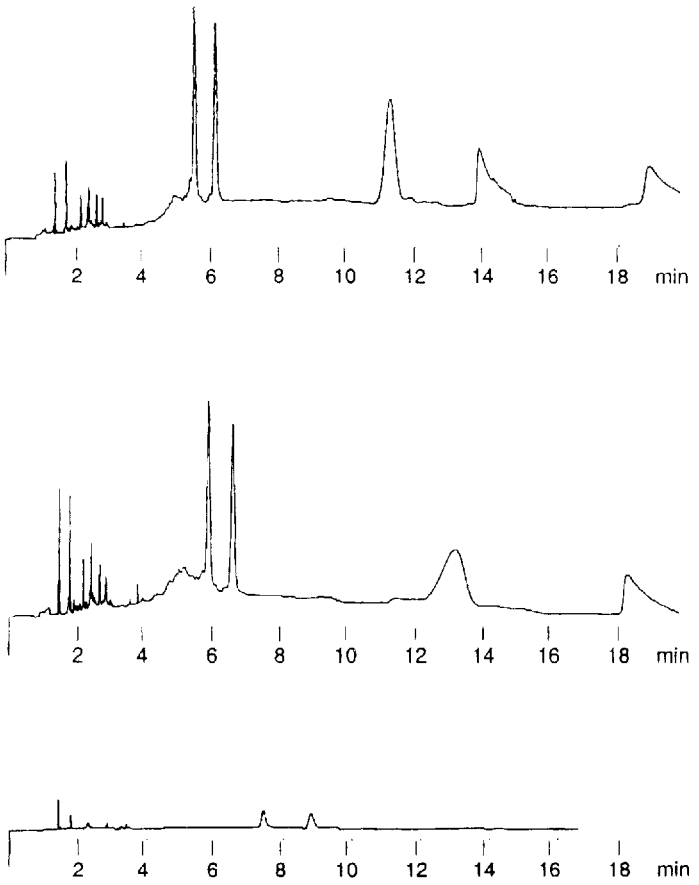

FIGURE 5. Free zone separation of standard proteins at pH 8.5 in an uncoated capillary with negative-to-positive polarity, three successive runs.

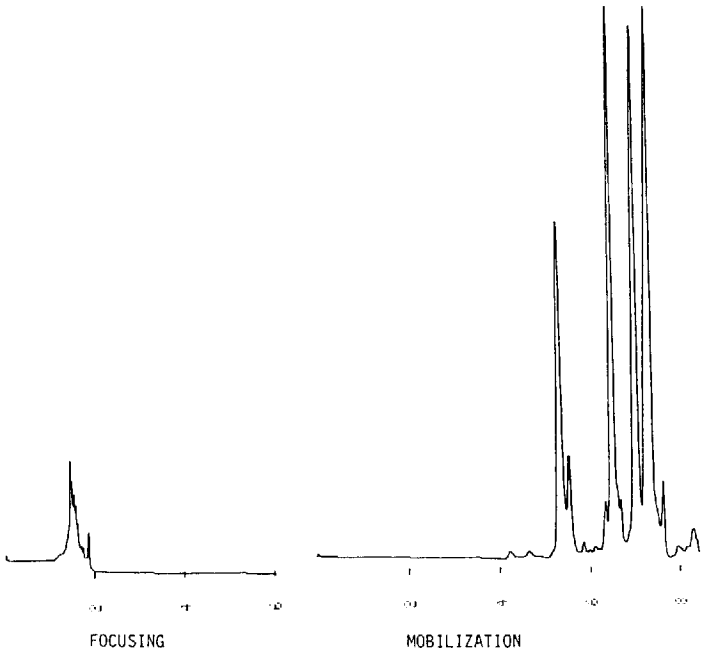

FIGURE 6. Isoelectric focusing of hemoglobin variants in a coated capillary.

A comparison of successive runs on the uncoated capillary (Figure 5) indicates that, in contrast to the coated capillary, protein adsorption to the unprotected silica surface results in diminishing detector response and increasing migration time. It should be noted that the capillary was purged between analyses as described above.

Capillary Isoelectric Focusing

Isoelectric focusing is widely used for high resolution of proteins based on differences in isoelectric points. The resolving power of IEF depends upon formation of a stationary pH gradient and high field strength to maintain stable focused zones. As in conventional gel isoelectric focusing, capillary IEF is performed by generating a pH

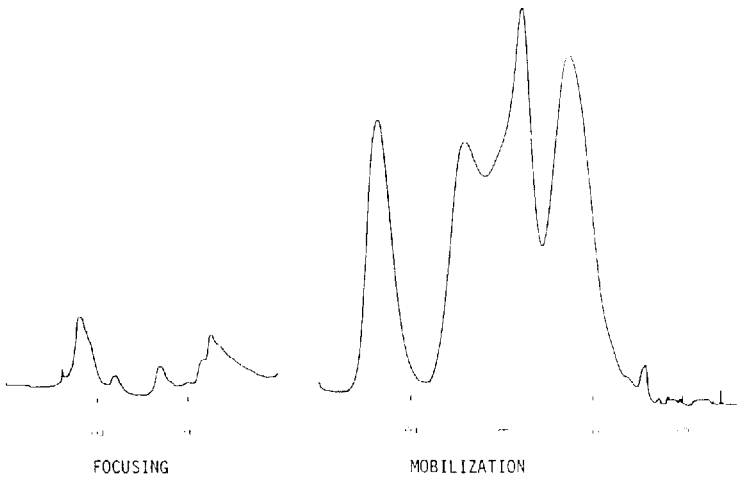

FIGURE 7. Isoelectric focusing of hemoglobin variants in an uncoated capillary.

gradient between an acidic anolyte and a basic catholyte. However, detection of focused zones in capillary IEF is achieved by mobilizing the zones past a fixed monitor point after the focusing step. This is usually accomplished by modifying the anolyte or catholyte solution (7). We typically use cathodic mobilization by addition of salt to the catholyte (8). Isoelectric focusing of a mixture of hemoglobin variants in a coated capillary with cathodic mobilization is shown in Figure 6. Note that proteins migrating past the detector during focusing generated a characteristic focusing electropherogram.

A significant level of electroendosmosis interfered with both the focusing and mobilization processes (Figure 7). Electroendosmosis during focusing caused displacement towards the cathode, and could be detected by delayed migration in the focusing electropherogram. Electroendosmosis during mobilization caused reduced migration times in the mobilization electropherogram. In both steps, electroendosmosis resulted in zone broadening. In most cases, use of uncoated capillaries

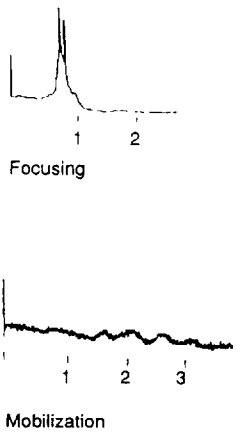

FIGURE 8. Isoelectric focusing of hemoglobin variants in an uncoated capillary.

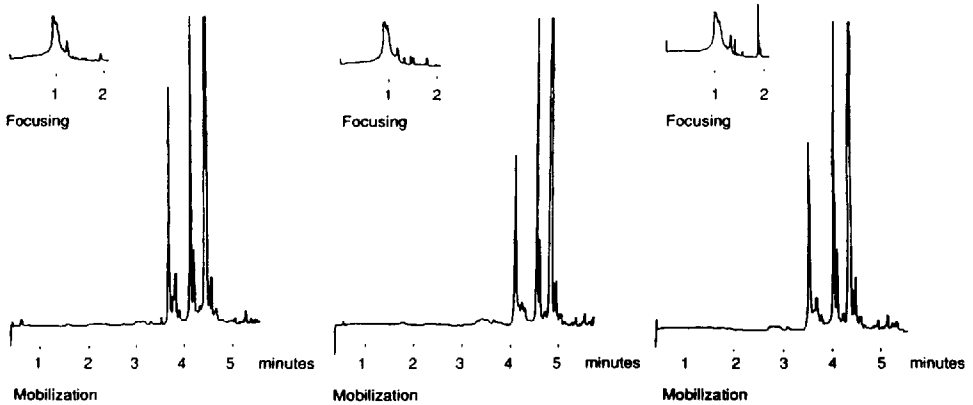

FIGURE 9. Isoelectric focusing of hemoglobin variants in a coated capillary, three successive runs.

resulted in no detectable peaks during mobilization (Figure 8). Using a coated capillary, reproducible mobilization electropherograms were obtained in successive IEF separations (Figure 9). We conclude that use of coated capillaries is essential for consistent and high resolution separations in capillary isoelectric focusing.

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