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Capillary electrophoretic protein separations in polyacrylamide-coated silica capillaries and buffers containing ionic surfactants

Mark A. Strege and Avinash L. Lagu

Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285 (USA)

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ABSTRACT

Capillary electrophoretic protein separations of high efficiency and resolution were obtained using polyacrylamide-coated silica capillaries and buffers containing ionic surfactants. The presence of micellar concentrations of sodium dodecyl sulfate or cetyltrimethylammonium chloride minimized protein-capillary wall interactions, and facilitated concurrent separations of a mixture of both acidic and basic proteins, while the polyacrylamide coating provided increased resolution and migration time reproducibility via a reduction in electroosmotic flow. Attempts to obtain size-based protein separations via sieving through buffers containing the hydrophilic polymers methylcellulose and polyethylene glycol were unsuccessful.

INTRODUCTION

There exists a great demand for rapid, high-resolution bioanalytical techniques to monitor the isolation and purification of proteins. Capillary electrophoresis (CE) is one such method which potentially offers rapid and quantitative protein analyses of high resolution and efficiency.

Although there are several reports of the use of CE for the successful separation of proteins (see ref. 1 for a review), the inherent tendency of these macromolecules to adsorb to the walls of fused-silica capillaries has hindered the achievement of efficient and reproducible separations, and has prevented the adoption of CE as a routine qualitative or quantitative bioanalytical technique [2].

Among the strategies employed to facilitate successful separations of proteins via CE, the use of ionic surfactants may have significant applicability. Although evidence has suggested that ionic surfactants denature proteins [3], for some applications a denaturing medium is acceptable and perhaps beneficial, since analyte solubility may be maximized under these conditions [4–6]. The ability of the anionic surfactant sodium dodecyl sulfate (SDS) to denature and impart a strong negative charge upon proteins has been successfully exploited for the facilitation of size-based separations of these molecules via sieving through cross-linked polyacrylamide in the slab gel format, using a technique known as SDS-polyacrylamide gel electrophoresis (PAGE) [7,8]. Recently, sieving separations of SDS--protein complexes have been demonstrated inside silica capillaries employed in conjunction with both cross-linked [9,10] and non-cross-linked polyacrylamide gel [11]. Non-ionic surfactant-coated octadecylsilane-derivatized capillaries have been reported to facilitate successful separations of native proteins in the presence of reduced electroosmotic flow [12]. Also, a polyacrylamide capillary coating was found to be beneficial in regard to the reduction of both protein-silica adsorption and electroosmotic flow in the presence of a zwitterionic surfactant [13]. Al-

Correspondence to: A. L. Lagu, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285, USA.

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though they offer significant potential for protein analyses, separations of protein-ionic surfactant complexes obtained in free solution inside polyacrylamide-coated capillaries, where the electroosmotic flow is minimal, have not been reported.

In this paper, the applicability of the use of ionic surfactants for the separation of proteins inside polyacrylamide-coated capillaries is demonstrated.

EXPERIMENTAL

Chemicals

The proteins used in this investigation were purchased from Sigma (St. Louis, MO, USA) and were used without further purification. SDS, cetyltrimethylammonium chloride (CTAC) (25% solution in water), and methylcellulose (high viscosity) were obtained from Fluka (Ronkonkoma, NJ, USA). Sodium acetate and mesityl oxide were purchased from Mallinckrodt (Paris, KY, USA) and Aldrich (Milwaukee, WI, USA), respectively. Polyethylene glycol (M_r = 40 000) was obtained from Serva (New York, NY, USA).

Apparatus

Separations were performed in a commercial instrument (P/ACE System 2100, Beckman, Palo Alto, CA, USA) equipped with, unless otherwise noted, 67 cm (60 cm inlet-to-window) 50 μm I.D. capillaries. All capillaries were thermostated at 25°C. Injections of the protein mixtures were carried out hydrodynamically via application of a low positive pressure [0.5 p.s.i. (1 p.s.i. = 6894.76 Pa)] for 5 s, unless otherwise noted.

Procedures

The internal surfaces of the capillaries used in this investigation were coated with polyacrylamide in the manner described by Hjerten [14]. Sodium acetate buffers were adjusted to the desired pH by the addition of glacial acetic acid. Acidic buffers were employed to prolong the polyacrylamide coating lifetime, since silica derivatizations such as that described in ref. 14 are known to be unstable in an alkaline environment [15]. Buffer surfactant concentrations are reported in this study as % (w/v). Protein samples were made up as 1 mg/ml (each protein) solutions. The peaks corresponding to proteins in the mixtures were identified via analyses of injections of the mixture spiked with 10-s injections of samples of the individual proteins. Buffers and samples were made using deionized water, and were filtered through 0.45-μm pore size filters (Millipore. Milford, MA, USA) and degassed prior to use. To substantiate reproducibility, all separations were performed at least in triplicate in three different capillaries. Separation efficiencies were calculated by an HP1000 minicomputer using an empirical equation developed for the characterization of either Gaussian or skewed chromatographic peaks [16].

RESULTS AND DISCUSSION

Permanent modification of silica capillary walls has proven to be an effective method, under certain conditions, for minimizing protein-silanol interactions as well as the electroosmotic flow [1,17]. The use of non-cross-linked polyacrylamide bonded to capillary walls through the use of an organosilane reagent has been reported to reduce protein adsorption and enhance separation efficiencies [14]. However, using a 67-cm capillary coated in this manner, together with a 50 m*M* sodium acetate pH 4.5 buffer, acceptable separation (30 kV, outlet = cathode) of a mixture, solubilized in running buffer, of bovine serum albumin (BSA) (M_r = 68 000, p*I* = 4.8), β-lactoglobulin (M_r = 18 400, p*I* = 5.2), myoglobin (M_r = 17 200, p*I* = 7.0), ribonuclease A (M_r = 13 700, p*I* = 9.3), and lysozyme (M_r = 14 300, p*I* = 11.0) was not obtained (see Fig. 1). The electroosmotic flow inside this capillary was presumed negligible, since an injected neutral marker (0.5% mesityl oxide) dit not pass the detector window during a 90-min separation (outlet = cathode). Under these conditions, where the pH of the buffer is below the p*I* of each of the five proteins, all of the analytes would be expected to migrate toward the cathode. However, β-lactoglobulin and BSA were not recovered, and the peaks corresponding to lysozyme, ribonuclease, and myoglobin were observed to tail significantly. These results indicated that adsorption of the proteins to either the silica surface or the wall-anchored polyacrylamide chains had occurred, probably via electrostatic and/or hydrogen bonding mechanisms [18,191.

A separation (30 kV, outlet = anode) of the same mixture of proteins solubilized in 0.5% SDS in wa-

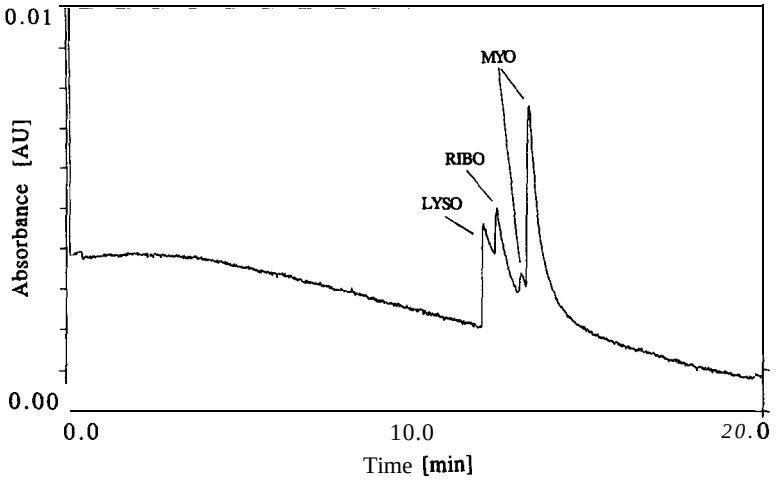

Fig. 1. Separation (outlet = cathode) of a 5-s injection of a mixture of myoglobin (MYO), β-lactoglobulin, lysozyme (LYSO), BSA, and ribonuclease (RIBO) in a polyacrylamide-coated capillary (60 cm inlet-to-window, 67 cm total length) using a 50 m*M* sodium acetate pH 4.5 buffer, 30 kV applied potential, and 214 nm UV detection

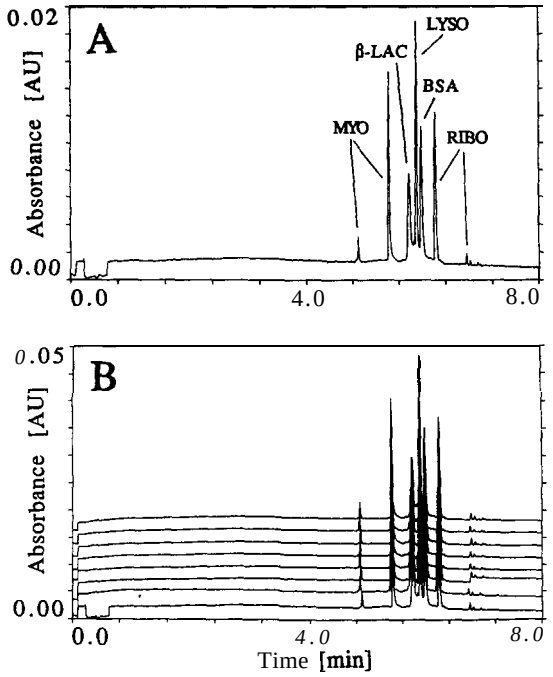

Fig. 2. (A) Separation (outlet = anode) of a 5-s injection of a mixture of myoglobin (MYO), β-lactoglobulin (β-LAC), lysozyme (LYSO), BSA, and ribonuclease (RIBO) in a polyacrylamide-coated capillary (60 cm inlet-to-window, 67 cm total length) using a 50 m*M* sodium acetate pH 4.5, 0.5% SDS buffer, 30 kV applied potential, and 214 nm UV detection; (B) Eight replicate injections obtained using the conditions listed in (A).

ter was obtained in a 67-cm polyacrylamide-coated capillary using 50 m*M* sodium acetate pH 4.5, 0.5% SDS as running buffer, and is displayed in Fig. 2A. The association of the anionic surfactant with the proteins promoted the anodic migration of all the analytes, and also appeared to minimize protein adsorption, since peaks corresponding to all five proteins were observed, and peak tailing was not evident. Under these conditions, all five proteins were well resolved. The reproducibility of this separation is demonstrated in Fig. 2B, where eight replicate electropherograms are overlayed. No buffer replenishment was performed between injections. Migration time and peak area precision data obtained from these separations are listed in Table I. Using myoglobin as a standard, the method was linear over the domain 25 to 6000 μg/ml (correlation coefficient = 0.999) employing peak area for quantitation. The upper limit of the linear response using peak height was 2000 μg/ml. The multiple minor peaks observed in the separations of the model protein mixture were believed to represent contaminants present in the commercially available protein standards.

To determine the effect of surfactant concentration upon the separation of the protein mixture, electropherograms were obtained at 30 kV (outlet =

TABLE I

RELATIVE STANDARD DEVIATIONS (%) OF MIGRATION TIME AND PEAK AREAS OBTAINED FOR PROTEINS SEPARATED IN 50 m*M* SODIUM ACETATE pH 4.5, 0.5% SDS

All data are mean values from eight replicate injections.

Protein	Migration time precision (R.S.D., %)	Peak area precision (R.S.D., %)
Myoglobin	0.3	4.9
β-Lactoglobulin	0.2	4.6
Lysozyme	0.2	4.7
Bovine serum albumin	0.2	4.8
Ribonuclease	0.2	4.6

anode) using a 67-cm polyacrylamide-coated capillary and 50 m*M* sodium acetate pH 4.5 buffer with SDS concentrations of 0.1, 0.2, 0.5, and 1.0%. The results displayed in Fig. 3 revealed significant changes in resolution between the components in the mixture, particularly lysozyme, *β*-lactoglobulin, and BSA, taking place as surfactant concentration was increased. These occurrences may be effects of the differential tendencies of the protein-SDS complexes to associate with SDS micelles (the critical micelle concentration of SDS in water is cu. 0.2% [20,21]), the number of which grow as surfactant concentration increases. The strongly anionic micelles can be expected to migrate at a much faster rate than that of the protein-surfactant monomer complexes.

To investigate the effect of the polyacrylamide capillary coating upon protein separations, an electropherogram (30 kV, outlet = anode) of the protein mix solubilized in 0.5% SDS was obtained in a 67-cm bare silica capillary using 50 m*M* sodium acetate pH 4.5, 0.5% SDS as running buffer. Under

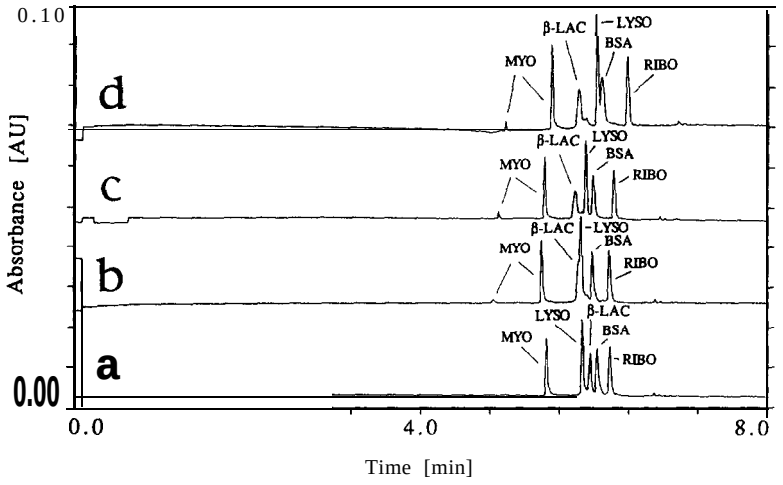

Fig. 3. Separations (outlet = anode) of a 5-s injection of a mixture of myoglobin (MYO), *β*-lactoglobulin (*β*-LAC), lysozyme (LYSO), BSA, and ribonuclease (RIBO) in a polyacrylamide-coated capillary (60 cm inlet-to-window, 67 cm total length) using 30 kV applied potential, 214 nm UV detection, and 50 m*M* sodium acetate pH 4.5 buffers containing (a) 0.1% SDS; (b) 0.2% SDS: (c) 0.5% SDS; (d) 1.0% SDS.

these conditions, the negatively charged protein-surfactant complexes appeared to be able to slowly electrophoretically migrate toward the anode, overcoming the weak cathodic electroosmotic flow. However, due to the slow net migration of the proteins, not all of the individual components of the injected mixture migrated past the detector window during the course of a 100-min separation, and the peaks that were observed during this timeframe were very broad (data not shown). Therefore, it appeared that the presence of a polyacrylamide capillary coating significantly improved protein separations performed in the presence of SDS at pH 4.5 by minimizing the electroosmotic flow inside the capillaries.

An investigation of the polyacrylamide capillary coating lifetime was performed by monitoring the efficiency (the algorithm employed for the determination of efficiency considers peak asymmetry [16]) and migration time of myoglobin in separations of the protein mixture obtained in a single coated capillary over time. Efficiency and sample migration times can be expected to decrease and increase, respectively, in response to degradation of the capillary coating. These changes may occur as the coating degrades due to an increase in both protein-silica interactions and a cathodic silanol-generated electroosmotic flow which opposes the anodic electrophoretic migration of the negatively charged protein-SDS complexes. Separations (30 kV, outlet = anode) of the protein mixture were obtained in a 67-cm polyacrylamide-coated capillary using 50 m*M* sodium acetate pH 4.5, 0.5% SDS buffer at 0, 1, 5, 11, 12, and 16 days following derivatization. The efficiencies and migration times of the major peak corresponding to myoglobin were measured during this time period. In the acidic environment (pH 4.5) employed in this investigation, the polyacrylamide coating appeared to be stable for cu. 11 days before the effects of coating degradation became evident, as demonstrated in the plots of migration time and efficiency vs. capillary coating lifetime in Fig. 4.

Cationic surfactants have also been utilized to minimize protein adsorption during CE separations in bare silica capillaries [22,23]. A separation (30 kV) of the protein mixture obtained in a 67-cm polyacrylamide-coated capillary using 50 m*M* sodium acetate pH 4.5, 0.5% CTAC buffer is displayed in

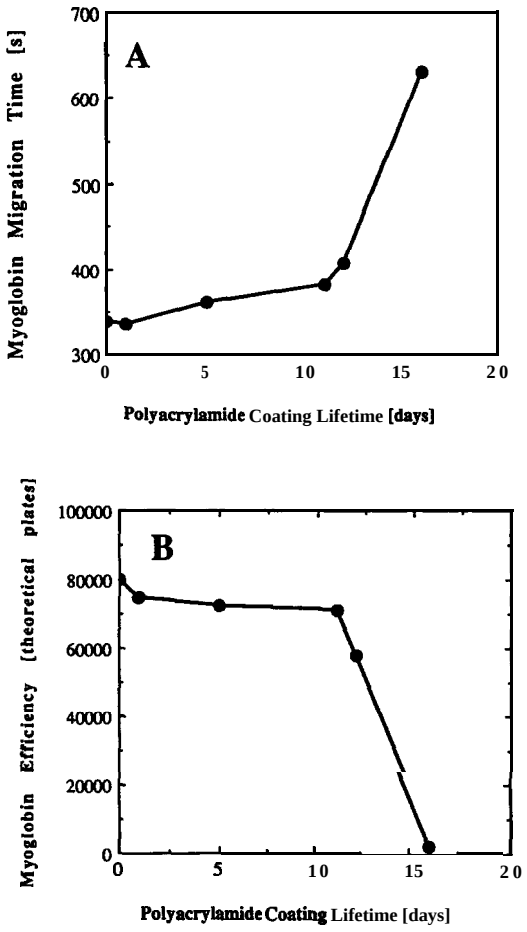

Fig. 4. (A) Plot of myoglobin peak migration time vs. capillary coating lifetime obtained following separations of the protein mixture in a polyacrylamide-coated capillary (60 cm inlet-to-window, 67 cm total length) using a 50 m*M* sodium acetate pH 4.5, 0.5% SDS buffer, 30 kV applied potential, and 214 nm UV detection; (B) Plot of myoglobin peak efficiency vs. capillary coating lifetime obtained from the same separations described in A.

Fig. 5. Since the proteins formed complexes of net positive charge in the presence of the cationic surfactant, the outlet reservoir was made cathodic for these separations. Both analyte resolution and migration order varied significantly from that of the separation obtained in SDS, probably reflecting differences in the magnitudes of the positive and negative zeta potentials of the migrating species in the presence of CTAC and SDS, respectively. Interestingly, while lysozyme generated only one peak

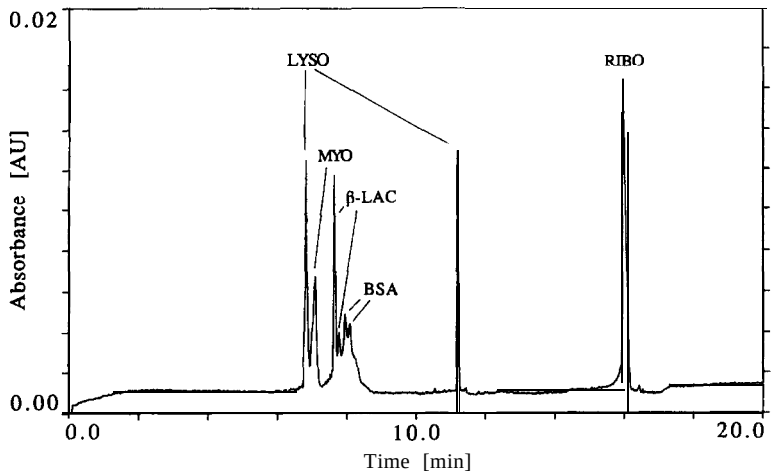

Fig. 5. Separation (outlet = cathode) of a 5-s injection of a mixture of myoglobin (MYO), β-lactoglobulin (β-LAC), lysozyme (LYSO), BSA, and ribonuclease (RIBO) in a polyacrylamide-coated capillary (60 cm inlet-to-window, 67 cm total length) using a 50 m*M* sodium acetate pH 4.5, 0.5% CTAC buffer, 30 kV applied potential, and 214 nm UV detection.

when analysed in SDS butler, two major peaks were found to correspond to the lysozyme separated in CTAC buffer. These peaks may represent different conformations of the protein which appear to be present and separable in micellar solutions of CTAC. Lysozyme is known to unfold reversibly in solution, and has been reported to generate two peaks in reversed-phase liquid chromatographic separations [24,25].

To determine the effects of the presence of a hydrophilic linear polymer network upon protein analyses achieved inside the polyacrylamide-coated capillaries, separations (30 kV, outlet = cathode) of the protein mixture (solubilized in running buffer) obtained in 50 m*M* sodium acetate pH 4.5 and 0.1% methylcellulose or 5.0% polyethylene glycol (PEG) using a 47-cm polyacrylamide-coated capillary were performed. Methylcellulose is a material which has displayed the ability to sieve double-stranded DNA fragments [26,27], and it has been suggested that 5.0% PEG may provide sieving capacity sufficient to separate the monomer, dimer, and trimer of BSA [26]. As mentioned earlier, under these conditions in the absence of ionic surfactant, where the pH is below the isoelectric points of all five proteins, the analytes can be expected to possess a net positive charge, facilitating their migration toward the cathode. In the presence of both methylcellulose and PEG, the proteins appeared to migrate very slowly through the capillary (perhaps due to the high viscosity of the buffers), and only three very broad peaks appeared during the course of a 100-min separation (data not shown).

A more acceptable analysis was achieved by reversing the instrument polarity and separating (30 kV) a l-s high-pressure (20 p.s.i.) injection (from the "outlet end" of the capillary), using the 7-cm short section of the capillary which lies between the detector window and the outlet reservoir for the analysis. The electropherogram obtained using this technique, with a 50 m*M* sodium acetate pH 4.5, 5.0% PEG buffer, is displayed in Fig. 6A. A separation obtained in a buffer containing 0.1% methylcellulose instead of 5.0% PEG was similar (see Fig. 6B). The order of migration correlated with protein p*I*, as the high positive zeta potential of the basic proteins apparently enables them to migrate more rapidly than myoglobin and the acidic proteins. The presence of the hydrophilic polymer also reduced peak tailing relative to that observed to occur in sodium acetate buffer alone (see Fig. 1). In both separations, β-lactoglobulin appeared to split into two components. These may be β-lactoglobulins A and B present in the commercially available

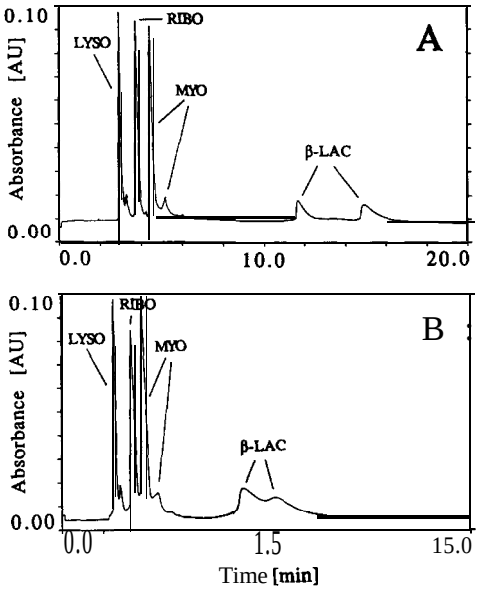

Fig. 6. (A) Separation (outlet = anode) of a l-s high-pressure (20 p.s.i.) injection of a mixture of myoglobin (MYO), β-lactoglobulin (β-LAC), lysozyme (LYSO), BSA, and ribonuclease (RIBO) in a polyacrylamide-coated capillary (7 cm inlet-to-window, 47 cm total length) using 30 kV applied potential, 214 nm UV detection, and 50 m*M* sodium acetate pH 4.5, 0.5% SDS buffer also containing (A) 5.0% PEG; (B) 0.1% methylcellulose.

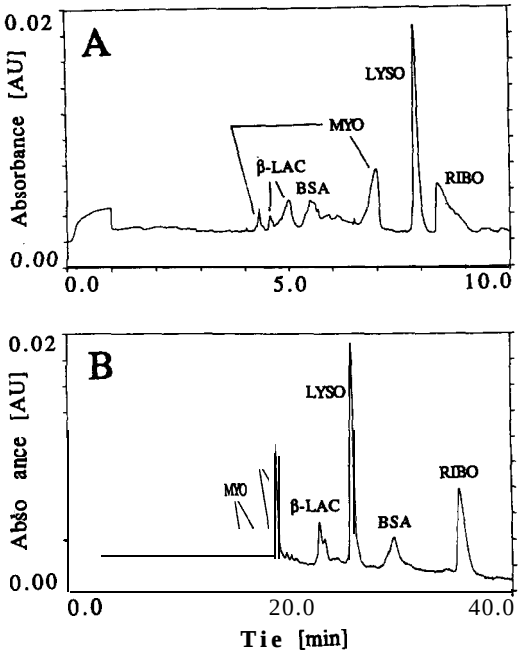

Fig. 7. (A) Separation (outlet = anode) of a 5-s injection of a mixture of myoglobin (MYO), β-lactoglobulin (β-LAC), lysozyme (LYSO), BSA, and tibonuclease (RIBO) in a polyacrylamide-coated capillary (40 cm inlet-to-window, 47 cm total length) using 30 kV applied potential, 214 nm UV detection, and 50 m*M* sodium acetate pH 4.5, 0.5% SDS buffer also containing (A) 5.0% PEG; (B) 0.1% methylcellulose.

standard, an occurrence which has also been reported in other studies [12,28,29]. BSA, the protein with the greatest acidic character, was not identified in these separations, probably due to the very low electrophoretic mobility of the native species at a pH so close to its p*I*.

The incorporation of 0.5% SDS into both the sample of the protein mixture and the running buffer facilitated the separation (30 kV, outlet = anode) displayed in Fig. 7A and B, achieved using a 47-cm polyacrylamide-coated capillary in the normal manner (i.e. using 40-cm capillary section between the inlet and detector window for the separation). The buffers employed for the separations presented in Fig. 7A and B were 50 m*M* sodium acetate pH 4.5 solutions contaning 0.1% methylcellulose and 5.0% PEG, respectively. The separation of the protein mix, including the order of protein elution, obtained in the presence of 5.0% PEG (Fig. 7A) was significantly different from that obtained in its absence (see Fig. 2). However, this phenomenon may be due to differential tendencies among proteins for interaction with the hydrophilic PEG network, and is probably not a result of sieving since BSA, by far the largest protein in the mixture, eluted earlier than three of the smaller proteins in the separation displayed in Fig. 7A. Also, no evidence for the occurrence of sieving was observed when the buffer containing 0.1% methylcellulose was employed to separate the protein mixture in the presence of 0.5% SDS (see Fig. 7B), since the migration order of the proteins observed in this separation was identical to that displayed in Fig. 2.

CONCLUSIONS

The results of this study suggest that protein separations of high efficiency and resolution may be obtained in free solution, in the absence of polymeric additives, through the use of both polyacrylamide-coated capillaries and buffers containing ion-

ic surfactants. Although attempts to obtain size-based protein separations using buffers containing hydrophilic polymer networks were unsuccessful, future work employing linear, non-cross-linked polyacrylamide gels in conjunction with polyacrylamide-coated capillaries may offer significant potential for the separation of proteins by CE. Also, the lifetimes of polyacrylamide capillary coatings may be significantly lengthened through the use of silica derivatizations of greater stability, such as that described by Cobb *et al.* [15].

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REFERENCES

1 M. V. Novotny, K. A. Cobb and J. Liu, *Electrophoresis*, 11 *(1990) 735.*
2 W. D. Pickering, *LC–GC*, 7 (1989) 752.
3 F. W. Putnam, *Adv. Prot. Chem., 4 (1948) 79.*
4 T. C. Furman, J. Epp, H. M. Hsiung, J. Hoskins, G. L. Long, L. G. Mendelsohn, B. E. Schoner, D. P. Smith and M. C. Smith, *Biotechnology, 5 (1987) 1047.*
5 P. T. Wingfield, P. Graber, G. Buell, K. Rose, M. G. Simona and B. D. Burleigh, *Biochem. J., 243 (1987) 1047.*
6 R. J. Kirschner, N. T. Hatzenbuhler, M. W. Moseley and C. C. Tomich, *J. Biotechnol.*, 12 *(1989) 247.*
7 A. L. Shapiro, E. Vinuela and J. V. Maizel, *Biochem. Biophys. Res. Commun., 28 (1967) 815.*
8 U. K. Laemmli, Nature, 227 (1970) 680.
9 S. Hjerten, *J. Chromatogr., 270 (1983)* 1.
10 A. S. Cohen and B. L. Karger, *J. Chromatogr., 397 (1987) 409.*
11 A. Widhalm, C. Schwer, D. Blaas and E. Kendler, *J. Chromatogr., 549 (1991) 446.*
12 J. K. Towns and F. E. Regnier, *Anal.* Chem.. 63 (1991) 1126.
13 M. A. Strege and A. L. Lagu, *J. Liq. Chromatogr., 16 (1993)* in press.
14 S. Hjerten, *J. Chromatogr., 347* (1985) 191.
15 K. A. Cobb, V. Dolnik and M. Novotny, *Anal. Chem., 62* (1990) *2478.*
16 J. P. Foley and J. G. Dorsey, *Anal.* Chem., 55 (1983) 730.
17 J. R. Mazzeo and I. S. Krull, *Biotechniques*, 10 *(1991) 638.*
18 P. Holt and J. Bowcott, *Biochem. J., 57 (1954) 471.*
19 R. A. Messing, *J. Am. Chem. Soc.*, 91:9 *(1969) 2370.*
20 D. W. Armstrong, *Sep. Purif. Methods*, 14 (1985) 213.
21 J. G. Dorsey, *Adv. Chromatogr., 27 (1987) 167.*
22 J. E. Wiktorowicz and J. C. Colburn, *Electrophoresis*, 11 (1990) 769.
23 A. Emmer, M. Jansson and J. Roeraade, *J. Chromatogr., 547 (1991) 544.*
24 K. Benedek, S. Dong and B. L. Karger, *J. Chromatogr., 317 (1984) 227.*
25 *N.* Nimura, H. Itoh, T. Kinoshita, N. Nagae and M. Nomura. *J. Chromatogr., 585 (1991) 207.*
26 M. Zhu, D. L. Hansen, S. Burd and F. Gannon, *J. Chromatogr., 480* (1989) 311.
27 M. A. Strege and A. L. Lagu, *Anal.* Chem., 63 (1991) 1233.
28 H. H. Lauer and D. McManigill, *Anal. Chem., 58 (1986) 166.*
29 J. S. Green and J. W. Jorgenson, *J. Chromatogr.. 478 (1989) 63.*