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Free solution capillary electrophoresis of proteins using untreated fused-silica capillaries

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ABSTRACT

Numerous efforts have been made to separate proteins by capillary zone electrophoresis (CZE). The most common optimization techniques are changing the pH of the running buffer, coating the capillary surface with a hydrophilic polymer, or using additives in the sample solution. Surface coatings and solution additives can reduce the adsorption of the protein onto the capillary surface, but they diminish the separation efficiency and the resolution of CZE. This paper reports the successful separation of proteins in a untreated fused-silica capillary by raising the pH of the running buffer and washing between runs with 1.0 *M* sodium hydroxide. Under these conditions, model proteins and proteins in human serum have been determined by CZE. It is shown that the results from CZE are compatible with those of sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

INTRODUCTION

Small organic molecules, including amino acids, small peptides, bases and water-soluble vitamines, can be separated by capillary zone electrophoresis (CZE) or micellar electrokinetic capillary chromatography in untreated fused-silica capillaries [1–3]. When CZE is applied to the separation of multicharged proteins, however, adsorption becomes a serious problem [1].

Several strategies have been developed to overcome the problems caused by the adsorption of proteins onto the silanol surface of fused-silica capillaries. First, to remove the Coulombic interaction between the charged proteins and the silanol surface, running buffers of various pH values can be used. For fully protonated silanols, pH values lower than three have been used to remove the negative charges from the capillary surface [4]. Although some proteins can be separated, most are aggregated or precipitated under acidic conditions. In addition, separations of proteins without electroosmotic flow are insufficient. The use of higher pH values (above 9), where most of the proteins are negatively charged above their p*I* values is fairly successful in the separation of proteins [5,6]. Second, the use of a polymer-coated tube for CZE was described by Hjertén [7] and Zhu *et al.* [8]. Although capillaries coated with a hydrophilic polymer reduce the adsorption of the protein and the electroosmotic flow, the separations take longer and the peak resolution is decreased. Third, alkali

metal salts were used in the running buffer to minimize the adsorption of proteins by competition between potassium ions and proteins on the silanol surfaces [9]. As an increase in conductivity requires the use of a lower voltage, the separation times are very long (> 30 min). Finally, additives such as ethylene glycol were used in the sample solutions [6]. Combining a higher pH running buffer with additives, gave reproducible protein separations, except for ethylene glycol itself, which showed extra peaks by forming complexes with the borate buffer.

In this study, separations of model proteins of various p*I* values were performed using a high pH running buffer. Conventional protein analysis was performed with sodium dodecyl sulphate–polyacrylamide gel electrophoreis (SDS-PAGE), in which separations are based on the molecular weights of proteins [10]. The separation of proteins by CZE, which is mainly based on the p*I* values of proteins, are compared with the SDS-PAGE separation of modified human serum proteins. These results provide sound correlations between conventional gel electrophoresis and CZE.

EXPERIMENTAL

Apparatus

The CZE system used is similar to that described previously [1]. A high-voltage power supply (0–40 kV, Glassman, Whitehouse Station, NJ, USA; Model PS/EH 40R 2.5CTZR) was used to drive electrophoretic process across the capillary. The platinum wires connected to the anode and cathode of the power supply were immersed in 3 ml of buffer. This system was isolated in a Plexiglass box for operator safety.

A straight length of fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA), 100 cm long (63.5 cm to detector, 360 μm O.D. × 50 μm I.D.) was used as a separation tube. Detection was performed by the on-column measurement of UV absorption at 200 nm with an ISCO CV^4 UV detector (ISCO, Lincoln, NE, USA).

CZE procedure

Prior to each run the capillary was rinsed with 1 *M* sodium hydroxide solution, distilled water and running buffer. The capillary was filled with running buffer, usually 50 m*M* sodium borate buffer, pH 9.5, or various buffers as indicated in the figure legends. A sample was introduced by siphoning at a height of 15 cm. Using this sample injection techniques 5–15 nl of sample solution were carried into the capillary. The analysis was performed by applying a 20–30-kV voltage in the constant voltage mode.

Reagents

Proteins containing isoelectroic point (p*I*) standards (amyloglycosidase from *Aspergillus niger*, trypsin inhibitor from soybean, β-lactoglobulin A from bovine milk, carbonic anhydrase II from bovine erythrocyte, carbonic anhydrase I from human erythrocyte, myoglobin from horse heart and trypsinogen from bovine pancreas), SDS, acrylamide, glycine, N,N,N′,N′-tetramethylethylenediamine (TEMED), bovine serum and protein A-Sepharose were purchased from Sigma (St. Louis, MO, USA). The immunoaffinity matrix ALB-AWAY was from Kendrick Labs. (Madison, WI, USA). Human standard serum, SRM 909, was obtained from the National

Institute of Standards and Technology (NIST) (Gaithersburg, MD, USA). Samples of patient serum were gifts from Chungnam Medical School (Daejon, South Korea).

Sample preparation

Albumin-depleted serum were prepared using a immunoaffinity matrix (ALB-AWAY) as decribed previously [11]. Briefly, 10 μl of serum were incubated with 100 μl of 75 m*M* sodium phosphate buffer, pH 7.4, and 100 μl of pre-washed ALB-AWAY immunoaffinity gel for 2 h at room temperature. The supernanant liquid was separated and analyzed by CZE and SDS-PAGE.

Immunoglobulin G (IgG) was removed from human serum using protein A-Sepharose. Human serum (100 μl) was mixed with 0.15 *M* phosphate buffer (pH 8.0, 300 μl) and the mixture was shaken at room temperature for 4 h. The supernatant was kept for CZE and SDS-PAGE analysis.

IgG-binding protein A-sepharose gel was briefly washed with 0.1 *M* citrate buffer, pH 3.5, to obtain IgG. The supernatant containing IgG was analyzed.

Gel electrophoresis

The protein patterns of serum samples were analyzed by SDS-PAGE under non-reducing conditions. The proteins were separated on a 10% polyacrylamide slab gel by the Laemmli procedure [10]. The gels were stained with Coomassie Brilliant Blue.

RESULTS AND DISCUSSION

Separation of model proteins by CZE

The adsorption of proteins on an uncoated fused-silica capillary at neutral pH has been illustrated previously [1,8]. Although several workers have tried to overcome this problem by deactivating the silanol surface of the capillary using a low pH [4], high salt concentration [9], or by coating the surface with hydrophilic polymers [8], the reduction in electroosmotic flow diminishes the high separation efficiencies and fast separation which are the advantages of CZE.

The separation of proteins in an uncoated fused-silica capillary at alkaline pH using 50 m*M* sodium borate buffer, pH 9.5, is demonstrated in Fig. 1. In this buffer, the electropherogram of these model proteins at 22 kV showed sharp peaks. Glycine contamination in the model proteins gives two broad peaks, which are consistent with pure glycine at this pH.

This procedure is similar to that reported by Lauer and McManigill [5] in which the reproducibility was poor and the peaks deteriorated. In this experiment, the run-to-run reproducibility of migration time and peak height have been improved, as shown in Table I, by washing the capillary between analyses.

The coefficients of variation (C.V.) in retention time are less than 0.5% and those in peak height are less than 4%, except for the small peaks 3 and 7. Differences between these data and those reported previously [5] may be caused by differences in the nature of the chosen proteins. Depending on the nature of the proteins, including the p*I* value, hydrophobicity, molecular weight and structure, the adsorption of proteins during separation by CZE can be changed.

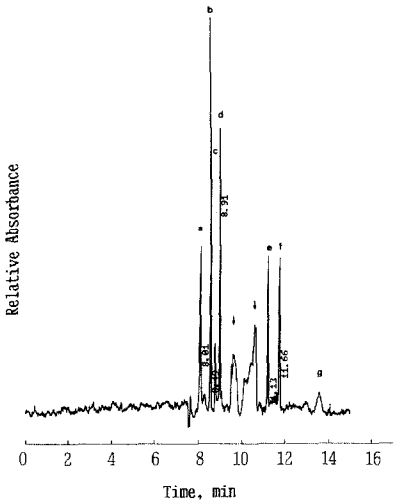

Fig. 1. Zone electrophoretic separation of seven model proteins on a 100 cm × 50 μm I.D. bare fused-silica column. Peaks: a = Trypsinogen, pI = 9.3, mol. mass = $24.5 \cdot 10^3$; b = myoglobin, pI = 6.8, 7.2, mol. mass = $17.5 \cdot 10^3$; c = carbonic anhydrase I, pI = 6.6, mol. mass = $29.7 \cdot 10^3$; d = carbonic anhydrase II, pI = 5.9, mol.mass = $29.7 \cdot 10^3$; e = trypsin inhibitor, pI = 4.6, mol.mass = $7.5 \cdot 10^3$; f = β-lactoglobulin A, pI = 5.1 mol.mass = $17.5 \cdot 10^3$; g = amyloglucosidase, pI = 3.6, mol.mass = $170 \cdot 10^3$. Conditions, buffer, 50 mM sodium borate buffer (pH 9.5); applied voltage, 22 kV; temperature, ambient; detection wavelength, 200 nm.

Protein separation of red blood cell lysate

Proteins in human red blood cell lysate were separated under alkaline conditions (50 mM sodium borate, pH 9.5) in uncoated fused-silica capillaries. A good reproducibiltiy of peak shape (Fig. 2) and migration time was observed (Table II), in which less than 0.7%. The first small sharp peak (a), which is considered to be carbonic anhydrase, and the second unknown peak are completely separated from large hemoglobin peak. These shapes were reproducible in seven consecutive runs, with

TABLE I

REPRODUCIBILITY OF MIGRATION TIME AND PEAK HEIGHT FOR THE ANALYSES OF MODEL PROTEINS WITH CZE

Conditions as in Fig. 1.

Peak	Retention time (n = 5)		Relative peak height (n = 5)	
	Average ± S.D. (min)	C.V. (%)	Average ± S.D.	C.V. (%)
a	7.97 ± 0.029	0.36	5.64 ± 0.08	1.42
b	8.45 ± 0.030	0.36	19.00 ± 0.31	1.63
c	8.63 ± 0.031	0.36	3.06 ± 2.05	67.00
d	8.87 ± 0.030	0.34	11.62 ± 0.29	2.5
e	11.09 ± 0.061	0.55	6.59 ± 0.23	3.49
f	11.62 ± 0.067	0.58	8.49 ± 0.33	3.89
g	13.47 ± 0.092	0.68	1.13 ± 0.14	12.40

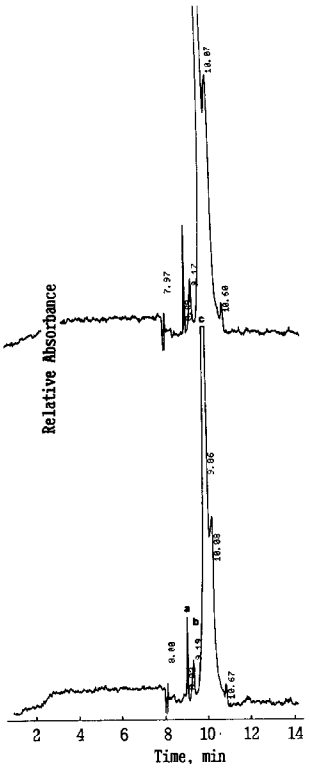

Fig. 2. Separation of proteins in human red blood cell lysate. First and seventh consecutive run. Other conditions as in Fig.1.

washing between runs with 1.0 *M* sodium hydroxide solution. This result is comparable to the data of Zhu *et al.* [8] in which they used a coated capillary for the same sample. With a coated capillary, the small peaks were not well separated from the haemoglobin peak. This indicates the possibility that the reduction in electroosmosis by coating the capillary decreases the separation efficiency of CZE.

TABLE II

REPRODUCIBILITY OF MIGRATION TIME FOR THE ANALYSES OF HUMAN RED BLOOD CELL LYSATE WITH CZE

Conditions as in Fig. 2.

Peak	Migration time ($n=5$)	
	Average ± S.D. (min)	C.V. (%)
a	8.858 ± 0.0620	0.70
b	9.112 ± 0.0453	0.50
c	9.782 ± 0.0655	0.67

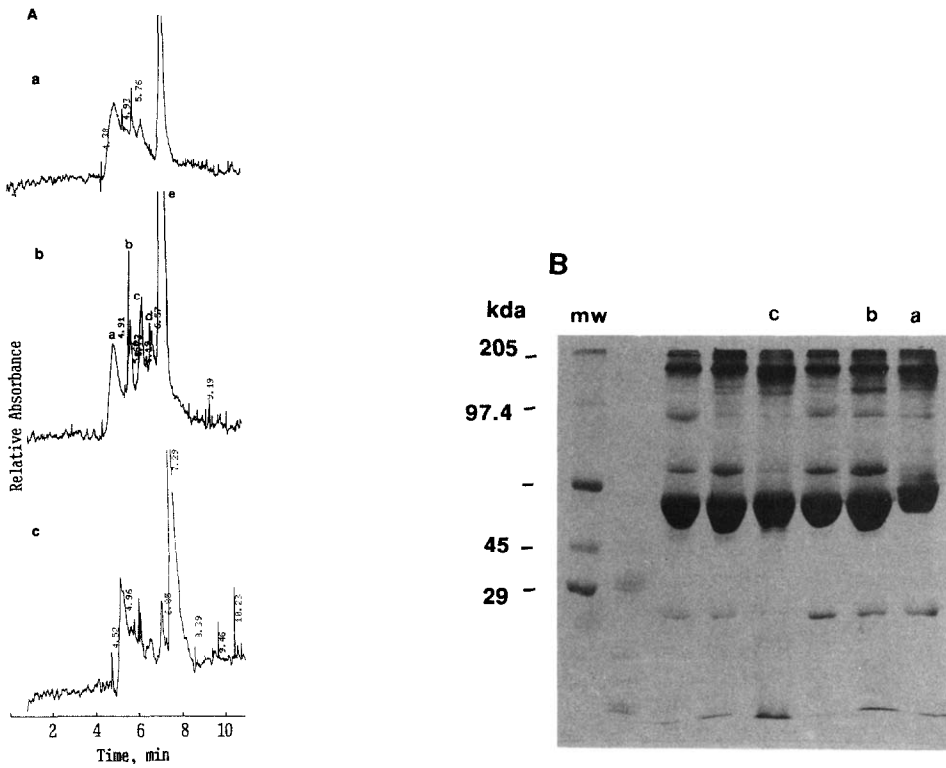

Fig. 3. (A) Separation of proteins with CZE from bovine serum (a), from NIST standard reference material (b) and human serum from a patient (c). Conditions as in Fig. 1, except the applied voltage = 30 kV. (B) Separation of proteins with SDS-PAGE under non-reducing conditions based on molecular mass. mw = Molecular mass; kda = kilodalton.

Comparison between CZE and conventional SDS-PAGE for the separation of proteins in human serum

Protein profiles of human serum in gel electrophoresis have been adopted for the diagnosis of diseases in the clinical laboratory. To estimate the possiblity of using CZE for this purpose, CZE electropherograms of serum proteins are compared with protein profiles of SDS-PAGE under non-reducing conditions in Fig. 3. Fig. 3A shows the CZE electropherograms of bovine serum, human serum from NIST standard reference material and from a hospital patient. The protein profiles in conventional SDS-PAGE in Fig. 3B correspond to those in Fig. 3A.

The reproducibility of migration time under alkaline condition with a 1 *M* sodium hydroxide solution wash in between analyses is shown in Table III. The C.V. value is $< 2\%$. It is obvious that the resolving power of this method with CZE is superior to that with the gel electrophoresis.

Modifications of the protein profiles in human serum have been performed by depleting IgG with protein A-sepharose or serum albumin with an immunoaffinity column (see under Experimental). The depletion of IgG is shown by the disapparance of the 160 kda molecular mass protein band corresponding to IgG in SDS-PAGE

TABLE III

REPRODUCIBILITY OF MIGRATION TIME FOR THE ANALYSES OF HUMAN SERUM WITH CZE

Conditions as in Fig. 3.

Peak	Migration time ($n = 5$)	
	Average ± S.D.	C.V. (%)
a	4.90 ±0.0894	1.825
b	5.372±0.0584	1.087
c	5.916±0.0722	1.220
d	6.312±0.1128	1.787
e	6.908±0.1340	1.940

(Fig. 4B); the IgG peak, which is broad in CZE as a result of heterogeneity, is also missing (Fig. 4A). This indicates that the molecular mass distribution of IgGs is fairly homogeneous, but the p*I* values are varied. Serum albumin has been depleted with anti-human serum albumin antibody bound to the gel. The albumin peak in CZE (Fig. 5A) and the band is SDS-PAGE (Fig. 5B) are also simultaneously reduced. The identification of the peaks in CZE is possible by comparing these two methods. CZE can also be used to check the progress of biological reactions *in situ*.

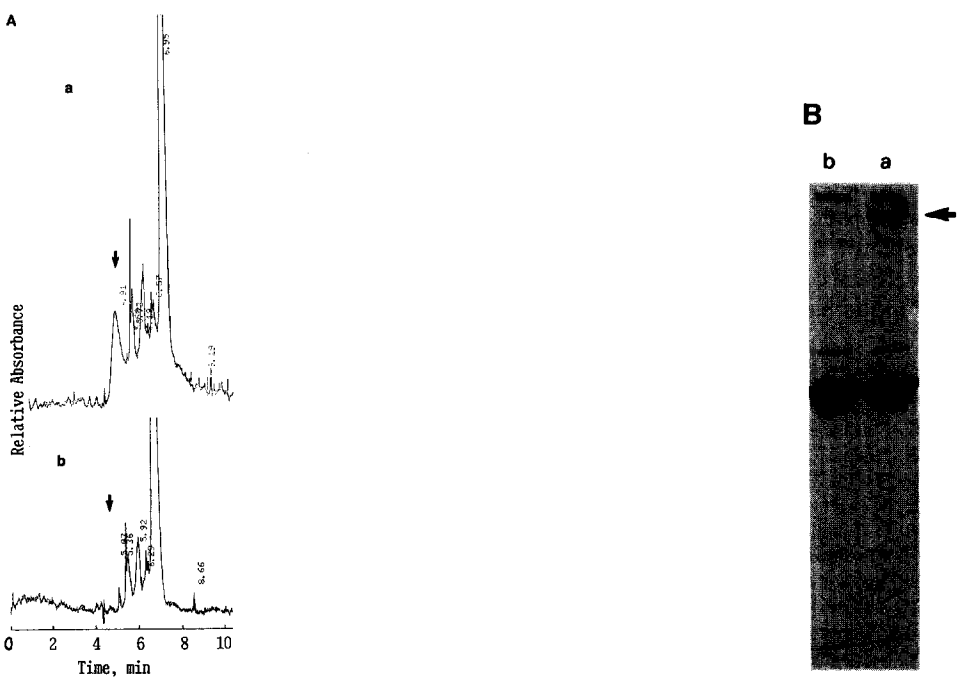

Fig. 4. Comparison between normal human serum (a) and IgG-depleted human serum (b). (A) CZE electropherogram under the same conditions as Fig. 3. (B) Protein profiles in SDS-PAGE. Arrow indicates the peak or band of IgG.

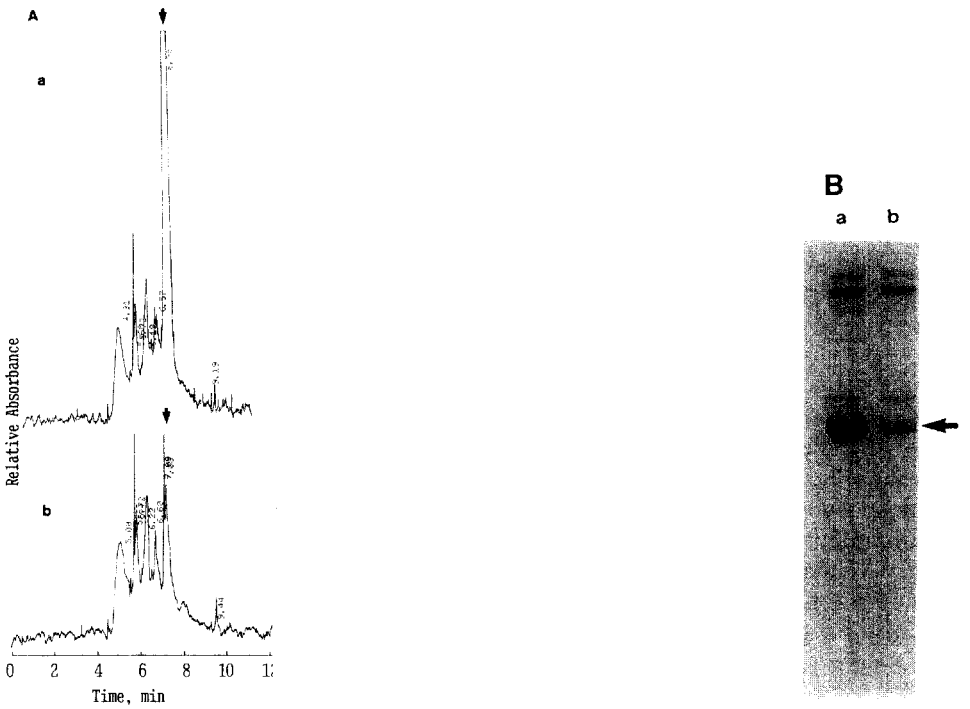

Fig. 5. Comparison between normal human serum (a) and albumin-depleted human serum (b). (A) CZE electropherogram under the same conditions as in Fig. 3. (B) Protein profiles in SDS-PAGE. Arrow indicates the peak and band of serum albumin.

In conclusion, proteins in model systems or in complicated biological samples can be determined in an uncoated fused-silica capillary under alkaline conditions. The reproducibility of the migration time and peak height is good (C.V. < 1%) except for a few proteins. The CZE electropherogram based on the p*I* values of proteins is compatible with the data obtained by SDS-PAGE, which is a separation technique based on molecular mass.

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