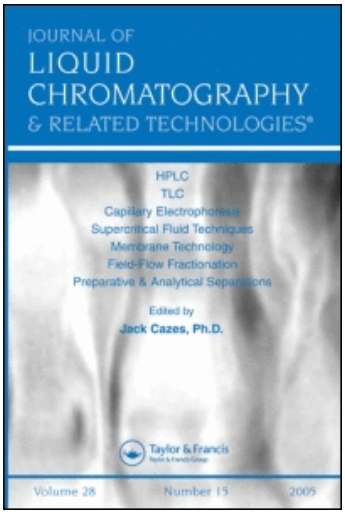

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Application of Capillary Zone Electrophoresis for the Analysis of Proteins, Protein-Small Molecules, and Protein-DNA Interactions

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APPLICATION OF CAPILLARY ZONE ELECTROPHORESIS FOR THE ANALYSIS OF PROTEINS, PROTEIN-SMALL MOLECULES, AND PROTEIN-DNA INTERACTIONS

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ABSTRACT

Capillary electrophoresis with a polyacrylamide-coated capillary was used for the separation of proteins, and the study of protein-small molecule and protein-DNA interactions. The coating (10% T polyacrylamide) is of sufficient thickness and hydrophilicity to reduce protein adsorption and eliminate electroosmotic flow. Data on the effect of sample solvent constituents on the peak shape and position of protein solutes was investigated. The results show that CE is a relevant and fast technique for the study of protein-DNA and protein-drug interaction. Also, the addition of TRIS-buffer to the sample solution resulted in the CE focusing of some basic proteins, while others were not affected.

INTRODUCTION

Capillary Zone Electrophoresis (CZE) is being increasingly recognized as a viable substitute for conventional electrophoresis protocols in molecular biology (1). This is mainly due to its high resolution small sample requirements and automated instrumentation. Recently, several reports appeared that describe CZE studies of protein-small molecule (2-4) and protein -DNA interactions (5,6). An area of unique challenge in the CZE analysis of proteins has been the control and

elimination of adsorption on the capillary inner surface. Adsorption often gives rise to distorted peaks, poor resolution and non-reproducible migration times.

Several strategies, that involve work at extreme pH and the use of buffer additives, were explored to minimize the adsorption of proteins on untreated columns (7-10). A different strategy, that is less restrictive in allowing the separation conditions to be adapted to the requirements of the analytical problem, involves the chemical modification of the fused-silica column surface by a permanent non-adsorptive coating (11-13).

Another area of concern in the CZE analysis of proteins is the effect of sample constituents, other than the protein of interest on peak shape and position. It has been demonstrated by Karger and co-workers (14,15) and others (16,17) that if the sample contains co-ions of the ion of interest with high electrophoretic mobility and if at the same time the mobility of the co-ion of the background electrolyte is lower than the mobility of the ion of interest, a transient isotachophoretic (ITP) migration of the sample ion takes place at the beginning of the migration. This gradually changes to a zone electrophoretic mode. How far does ITP migration proceed before shifting to zone migration depends on the relative mobility and concentration of the sample ion and its co-ions. The transient ITP phenomenon has advantages as well as disadvantages. We have previously shown that the presence of chloride in biological fluids is beneficial for the analysis of nitrate (18). The ITP step created when chloride acts as a leading ion focuses the nitrate peak (over one million theoretical plates) and improves the detection limit. On the negative side, solute migration time and peak shape are largely dependent on the relative concentration of the solute ion and its counter ions, and when the counter ion concentration is too high solute zones migrate close together and eventually co-migrate (18).

We report here on the utility of a polyacrylamide-coated capillary for the analysis of proteins, protein-small molecule and protein-DNA interactions. The coating (10% T polyacrylamide) is of sufficient thickness and hydrophilicity to reduce protein adsorption and eliminate electroosmotic flow.

Data on the effect of sample solvent constituents on the peak shape and position of protein solutes will be presented and discussed.

EXPERIMENTAL

Reagents

Protein samples were purchased from Sigma Chemical Company (St. Louis, MO) as well as Ellman's reagent, EDTA, dithiothreitol and vinyl pyridine. All buffer reagents were purchased from Fisher Scientific (Fair Lawn, NJ). Human immunodeficiency virus type 1 nucleocapsid protein with (NCp7) and without (apo) two coordinated zine ions and reagents attacking the Cysteine thiolates in the metal ion coordination complex were provided by the Viral Protein Laboratory (SAIC AVP, NCI-FCRDC, Frederick, MD). ETS1-P42 (19) and a specific oligonucleotide DNA were provided by the Laboratory of Cellular Biochemistry (SAIC Frederick, NCI-FCRDC, Frederick, MD).

Buffers were prepared using water purified by a Barnstead NANOpure Ultrapure Water System (Dubuque, IA).

Apparatus and method

A CZE P/ACE System 5500 equipped with a P/ACE diode array detector (DAD), an automatic injector, a fluid-cooled column cartridge, and a System Gold data station (all from Beckman Instruments, Inc., Fullerton, CA) was used in this study. All runs were carried out at 25 ° C. The buffers were passed through 0.2-μm nylon filters and degassed prior to use. The capillary inlet and outlet vials were replenished after every tenth run. Injections were made using the pressure mode. The DAD was scanned in the wavelength range of 190-380 nm, and the electropherograms were monitored at 214 nm and 260 nm. Specific experimental parameters are given in the figure captions.

Columns were coated with a thick layer of linear polyacrylamide. The details of the column preparation procedure are given elsewhere (18). Columns coated according to this procedure are

routinely used in our laboratory for the analysis of nitrate and nitrite in biological fluids and in the analysis of proteins and DNA samples. The columns are stable at and below pH 9.0, and could be used over a period of several weeks without any signs of deterioration. Electroosmotic flow (EOF) is negligible, although not completely eliminated. The electroosmotic mobility, μ_{eo} is estimated by measuring the migration time of NO_3^- (18). μ_{eo} values for several columns prepared in our laboratories ranged from 0.03-0.5 x $10^{-4}cm^2v^{-1}sec^{-1}$. μ_{eo} of CZE columns typically ranges from 0-10 x $10^{-4}cm^2v^{-1}sec^{-1}$ depending on column surface chemistry, pH, and buffer additives (18).

RESULTS AND DISCUSSION

Separation of Proteins

The separation of proteins with bare-silica columns is possible only under: (a) very low buffer pH; (b) very high buffer pH; (c) using relatively high salt concentration in the running buffer; and (d) using special additives in the running buffer that associate with either the analyte or the bare-silica surface. All these strategies are inappropriate for the study of protein-DNA complexes, because of the potential interference of extreme pH and additives with the binding activity. The study of protein-DNA complexes would ultimately require more inert non-adsorptive and hydrophilic surfaces than bare-silica in order to accommodate the polyionic nature of these biopolymers. The linear polyacrylamide coated surface used in this study is particularly suited for the study of protein-DNA interaction because: (a) it is hydrophilic; (b) it effectively shields the biopolymers from direct contact with surface silanol groups; (c) it does not interact with the biopolymers; (d) it is chemically stable and resists hydrolytic degradation when in contact with aqueous solutions customarily used in CZE; and (e) the coating procedure yields reproducible capillaries with consistent run-to-run and day-to-day migration times.

Basic proteins are customarily used as probes of the quality of the capillary surface coating because their positive net charge tend to adsorb on the negative charge of the wall on uncoated or poorly shielded surfaces (11-13). The separation of a test mixture of four basic proteins at pH 3 is

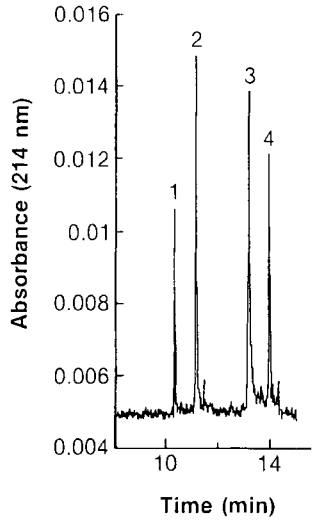

Figure 1. Electropherogram showing the separation of four basic proteins: (1) cytochrome C, (2) lysozyme, (3) ribonuclease A, and (4) α-chymotrypsinogen A. Buffer: 10 mM sodium phosphate, containing 0.1% BRIJ 35; pH: 3.1; Voltage: 15 kV; Instrument: Beckman model P/ACE System 5500; Detection: 214 nm; Injection: 5 seconds at 0.5 psi; Column: 10% T. polyacrylamide-coated fused-silica; Column dimensions: L_{total} = 57 cm, $L_{detector}$ = 50 cm; i.d. = 75 μm; Solute concentration: 0.2-0.5 mg/mL in 10 mM phosphate buffer.

shown in Figure 1. High efficiencies were obtained indicating optimal coverage of the surface active sites. The protein samples were routinely run over time to confirm the stability of the coating. No loss of column efficiency was observed over several weeks of operation. The proteins were dissolved in the running buffer in order to avoid possible complication from transient on-column ITP. When TRIS was added to the sample solvent it acted as a leading ion and resulted in ITP focusing. Cytochrome C and lysozyme were extremely focused, i.e. gave sharper peak than those in fig. 1, while ribonuclease A and α-chymotrypsinogen A were hardly affected (data not shown). Lysozyme was chosen as an example to illustrate the ITP effect. A 50 μl sample of 250 μg/mL lysozyme in 10 mM phosphate buffer was divided equally into two vials. One vial was diluted to 125 μg/mL with water and the other vial was similarly diluted with 20 mM TRIS. The addition of TRIS to the sample

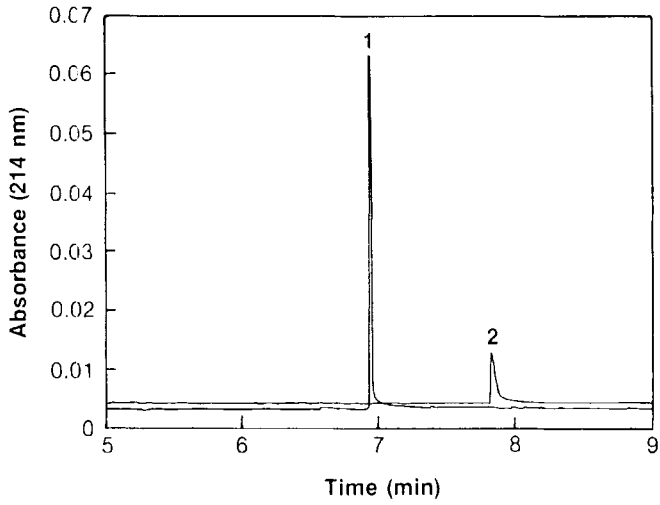

Figure 2. Demonstration of transient on-column ITP focusing. Peak shape of lysozyme with (trace 2) and without (trace 1) TRIS in the sample buffer. Experimental conditions: As in Figure 1, except for column length of 47 cm.

resulted in extreme focusing of the lysozyme peak as illustrated in Figure 2. The number of theoretical plates per meter increased from about 200,000 (Trace 2) to over two million (Trace 1). While this improvement in column efficiency is impressive, it must be cautioned that when the protein peak is focused to such an extent it coelutes with other proteins that are of closely-related charge-to-size ratio (14). Moreover, other compli-cations may arise depending on the relative concentration of the focusing ion (TRIS in this example) and protein. For example, two peaks (one focused and one broad) may appear for a single protein that gives a single peak with no TRIS in the sample solvent (data not shown). The proportions of the areas of the two peaks vary depending on the relative concentration of TRIS in the sample solvent. It may be concluded from the above that the CZE separation of proteins is complicated by the presence of other ions in the sample matrix. Caution must be exercised in interpretation of the electropherogram as a single focused peak might not necessarily indicate a single component and a double peak does not necessarily indicate sample decomposition.

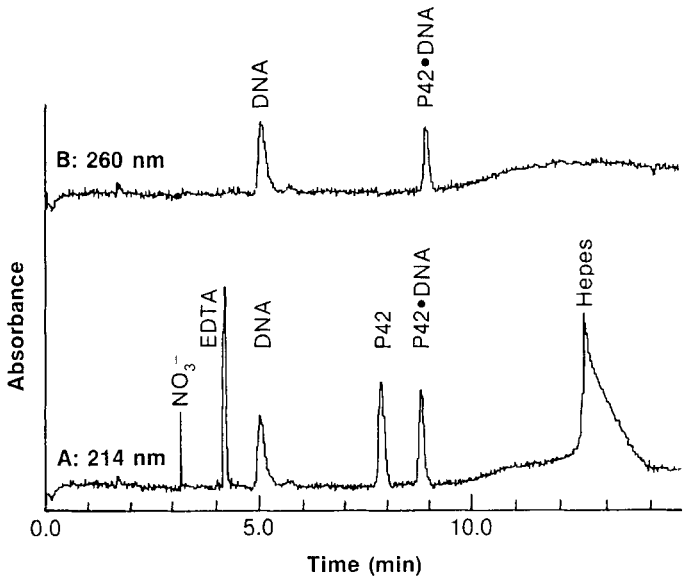

Figure 3. Demonstration of the utility of CZE with coated columns for the study of protein-DNA complexes. Applied voltages: -15 kV; Current: 28 μA; Buffer: 10 mM phosphate; pH 8.8; Other Experimental Conditions: As in Figure 1.

Study of protein-DNA interactions

The linear polyacrylamide coated column used in this study is particularly suited for the study of protein-DNA interactions because it provides a hydrophilic, non-adsorptive surface that does not interfere with the binding activity. Figure 3 gives an electropherogram that was run about 15 minutes after the initial mixing of 30 μl of 0.6 pmole/μl of P42 (Mol. Wt. = 40,780) with 3 μl of 20.8 pmole/μl of a specific oligonucleotide DNA (Mol. Wt. = 12,000). The electropherogram was run at pH 8.8 were all species of interest, P42, DNA and the P42.DNA complex are negatively charged. In a negligible electroosmotic flow environment, and with the detector at the anode end of the column, only negatively-charged components of the injected material will migrate through the length of the column and get detected. The figure demonstrates the unique advantage of CZE over other analytical techniques, instrumental or otherwise. Partial selectivity is obtained when positively-

charged and neutral solutes are separated from the negatively charged solutes of interest. More importantly, only CZE could give peaks for small ions (NO_3^-), complexing agents (EDTA), proteins, DNA and protein-DNA complex in a single run, with real-time on-column detection. Selectivity is also achieved by judicious selection of detector wavelength. For example, Trace B of fig. 3, which was monitored at 260 nm, only shows the peaks of DNA containing species.

Screening of the effect of drugs on proteins

CZE could conveniently be used for the screening of drug-protein interactions. The nucleocapsid protein (NCp7) of human immunodeficiency virus type 1 (HIV-1) (20) was selected to illustrate this application. The protein contains two zinc finger domains which play an important role in the viral replication cycle (21,22). It has been suggested that viral replication depends on the integrity of the Zn finger domains which are preserved in all studied mutant forms of the protein. It is hoped that drugs which disrupt the Zn finger domain may interfere with the virus replication cycle. The protein is positively charged at neutral pH, but migrates very slow towards the negative electrode. In order to shorten the analysis time, the CZE analysis of NCp7 was attempted at pH 3. Figure 4 gives sufficient evidence that shows that the Zn fingers in NCp7 are not disrupted by the change of pH from pH 7 in which the sample is dissolved to pH 3 during migration inside the column. Trace A shows the electropherogram of NCp7 and Trace B shows the electropherogram of the apo form of NCp7. The samples were dissolved in phosphate buffer. TRIS, which is commonly used as a protein buffer constituent, was avoided because the same ITP mechanism described earlier is also operative here. Trace C is an electropherogram of the same sample used to generate Trace A, but with the addition of EDTA which is a strong chelating agent for zinc, and dithiothreitol which is added to obstruct the formation of sulfur bridges after the sulfhydryl residues are freed. The trace clearly shows the gradual conversion of NCp7 to its apo form. Trace D is an electropherogram of the same sample used to generate Trace C but at a later time. In order to test the effect of drugs on NCp7, a 250 μg/mL solution of the protein was prepared in phosphate buffer and 25 μL was

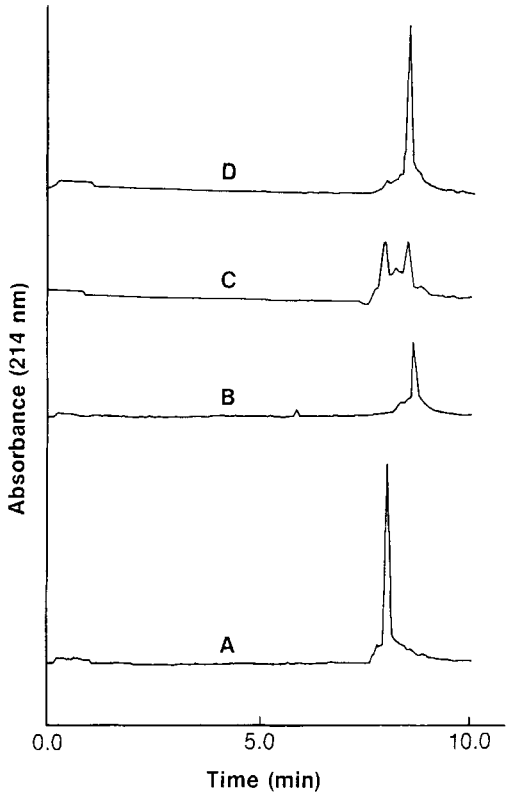

Figure 4. CZE evidence for the transformation of NCp7 to its apo form by the addition of EDTA and dithiothreitol. Trace A: NCp7; B: cpo NCp7; C: NCp7+EDTA+dithiothreitol; and D: Same as C, but at a later time. Experimental Conditions: As in Figure 1.

aliquoted for each determination. The drugs were dissolved in dimethylsulfoxide (DMSO) and less than 1 µl of each drug solution was added to the protein vial to make up a drug-to-protein molar ratio of 6:1. This represents another unique advantage of CZE when only about 6 µg of protein and a few µg of each drug are needed for each screen. Representative examples of NCp7-drug interactions are shown in Figure 5. Trace A shows that NCp7 (second peak) was not disrupted by the addition of vinylpyridine. The reagent modifies free sulfhydryls but does not attack sulfhydryls in the zinc coordination complex of NCp7. Free vinylpyridine is positively charged at pH 3 and is

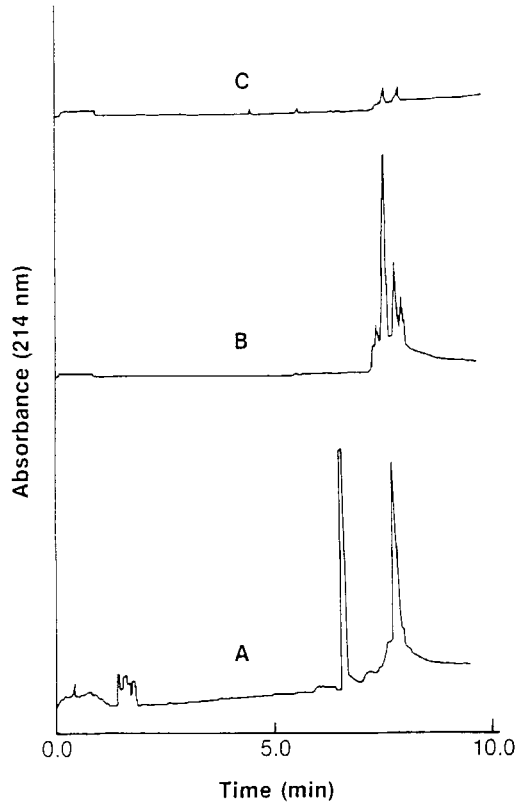

Figure 5. Effect of drugs on NCp7 protein. A: Effect of vinylpyridine; B: Effect of a proprietary drug; and C: Effect of Ellman's reagent. Experimental Conditions: As in Figure 1.

also detected (first peak). Trace B shows an example of a drug that disrupts the Zn fingers, modifies free the sulfhydryl residues and forms several NCp7 derivatives, and trace C gives an example of a drug that disrupts the Zn fingers and causes disulfide cross-links in the protein.

CONCLUSION

The results presented here show that using a polyacrylamide coated capillary can result in excellent separation of basic proteins. Also, the study of protein-drug, protein-DNA interactions by CE is a feasible and efficient method which requires microgram samples.

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