Poster Presentation

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An Alternative to Western Blotting Using Capillary Electrophoresis

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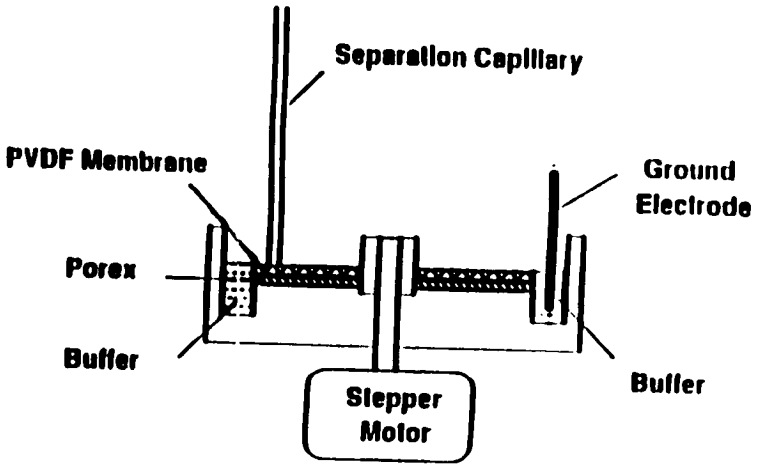
INTRODUCTION

Polyacrylamide gel electrophoresis (PAGE) performed on slab gels is a well established and frequently used technique for the separation of proteins contained in various sample matrices. Following electrophoresis, the macromolecules are often transferred to a membrane solid support (e.g. polyvinylidene difluoride) for post-run staining and analysis.

By comparison use of capillary electrophoresis (CE) continues to increase due in part to the technique's ability to resolve large biopolymers and to the availability of commercial instruments. Although valuable quantitative and comparative information is obtained by on-capillary UV detection, further characterization of protein samples is often desired.

This poster describes two post-separation analytical procedures that rely on a membrane fraction collector to capture proteins eluting from the capillary following separation. Utilizing routine capillary zone electrophoresis (CZE) conditions, proteins collected onto PVDF membranes were analyzed using both non-specific and antigen-specific staining techniques. In a separate series of experiments, protein amino acid compositions were determined with picomole quantities of samples isolated from membranes. Hydrolysates were derivatized with a recently developed fluorescent reagent, 6-aminoquinolyl-N-hydroxysuccinimdylcarbamate (AQC) followed by reversed phase HPLC.

Figure 1



MEMBRANE FRACTION COLLECTOR

MATERIALS AND METHODS

CE Separations with a Membrane Fraction Collector:

All capillary zone electrophoretic (CZE) separations were performed on a 75µm x 60cm fused silica capillary at a constant voltage of + 7kV using a **Waters™ Quanta 4000** system with a positive polarity power supply (Millipore, Milford, MA USA). The electrolyte was 100mM sodium phosphate, pH 7.0, containing 1.0 M trimethylammonium propane sulfonate (Waters™AccuPure™ Z1-methyl reagent).

Egg white lysozyme, horse heart cytochrome c, horse heart myoglobin, human transferrin and bovine serum albumin were purchased from Sigma Chemical Co.(St. Louis, MO), dissolved in electrolyte and passed through a Millex-HV 0.45µm filter (Millipore). All samples were loaded hydrostatically onto the capillary with volumes ranging from 18 to 100 nl.

The eluate of the capillary was continuously applied on a PVDF membrane (Immobilon-P™, Millipore) using a rotating platform consisting of stepper motor driven platen capped with a polyethylene disk as shown in Fig. 1. Details of the fraction collector assembly and operation have been previously described (Ref. 1 - 3). The disk was rotated at a rate of 0.033 rpm for this series of experiments.

Protein Staining Techniques:

Twenty second injections of the five protein standard mixture were loaded onto the capillary, separated by CZE and collected onto the rotating Immobilon-P membrane. Protein component concentrations ranged from 250 ng/ml to 250 µg/ml. Total protein component detection was obtained by soaking the membranes for 15 min. at room temperature with AuroDye™ Forte colloidal gold solution (Amersham International, Amersham, UK).

Immunodetection of proteins was carried out as follows: The membranes were first blocked for 1 hr. at room temperature with 10mM potassium phosphate, pH 7.5 containing 150mM sodium chloride, 1% bovine albumin and 0.1% sodium azide. The membranes were then incubated for 1 hr. at room temperature with a 1:2000 dilution of goat anti-human transferrin IgG (Sigma T-2027) in 10mM potassium phosphate, pH 7.5 containing 150mM sodium chloride, 1% bovine albumin, 0.05% Tween-20 and 0.1% sodium azide (i.e., conjugate diluent). Three, 5 min. washes in 10mM potassium phosphate, pH 7.5 containing 150mM sodium chloride and 0.1% sodium azide (i.e., wash buffer) were performed, followed by a 1 hr. incubation with rabbit anti-goat IgG alkaline phosphatase conjugate (Sigma, 1:8000 dilution) in conjugate diluent. Excess conjugate was removed by three washes with buffer followed by a Milli-Q water rinse and chromagenic signal generation was generated by incubation in BCIP / NBT alkaline phosphatase substrate per the manufacturer's directions (Kirkegaard and Perry Laboratories, Inc. Gaithersburg, MD).

Chemiluminescent signals were generated using the protocol above with the following modifications. Membranes were washed for 10 min. in 10mM Tris, pH 9.5 containing 10mM sodium chloride and 1mM magnesium chloride. The membranes were then covered with Lumigen-PPD substrate (Millipore) and allowed to incubate at room temperature for 10 min. Excess Lumigen-PPD reagent was removed and the membranes were sealed in clear plastic bag. In a darkroom, a piece of X-ray film (Kodak XAR) was sandwiched against the sealed membranes and the film was exposed for 60 min. prior to development.

Separation of amino acid derivatives were performed as follows: Samples were separated on a Waters AccQ•Tag™ reverse phase column (3.9 x 150mm) using a ternary eluent system and a multistep gradient. The HPLC system was a Waters AccQ•Tag Amino Acid Analysis System (Millipore) consisting of a 625 LC System equipped with a column heater, a 717 plus Autosampler with heater/chiller accessory, a 470 Scanning Fluorescence Detector and a Millennium™ 2010 Workstation .

Compositional data was calculated according to previous publications (Ref. 6) where % Error = 100 x (experimental - true residue value) / true residue value. The overall compositional accuracy was assessed as the Average % Error where Average % Error = Σ absolute % Error / 16.

Amino Acid Composition Determinations:

One hundred nanoliters of cytochrome c and myoglobin (5mg dry weight /ml each) were injected onto the capillary, separated and collected onto Immobilon-P membranes. The air dried membranes were wet with methanol then rinsed copiously with Milli-Q water. Following re-drying of the membranes, the proteins were visualized with 20% methanol and the bands excised with a clean razor. The strips were placed in a 6 x 50mm glass test tube and hydrolyzed with 6N HCl with 1% phenol using a Waters Pico•Tag® Workstation and a vapor phase procedure previously described (Ref. 4). Following hydrolysis, the amino acids were extracted with 100 µl of 4% aqueous SDS: acetonitrile (1:1, v/v) followed by 50 µL wash with the same solvent, and the combined extracts were vacuum dried. As controls, 2087 ng of cytochrome c and 1708 ng of myoglobin were individually hydrolyzed.

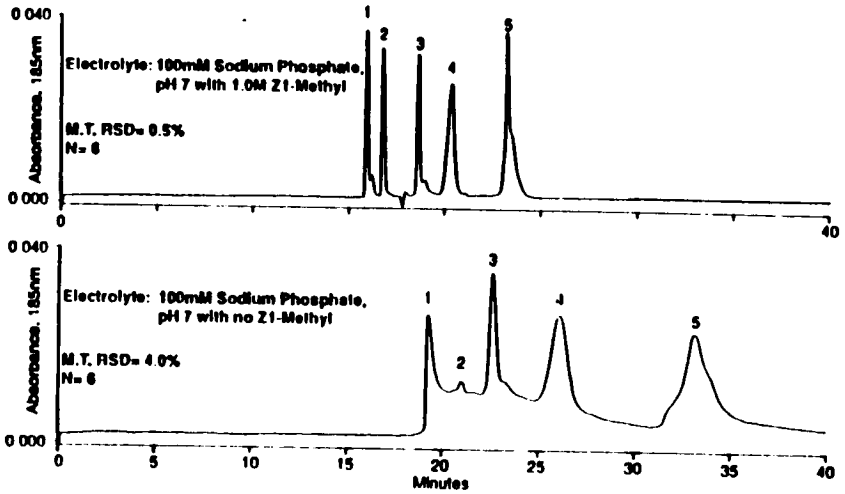
Amino acid derivatization was performed as follows: Standard amino acid mixtures (10 µL, 0.1mM) were buffered with 70 µL of 0.2M sodium borate, pH 8.8 containing 5mM disodium ethylenediamine tetraacetic acid and derivatized with 20 µL of 10mM 6-aminoquinoly-N-hydroxysuccinimidyl carbamate in acetonitrile (AQC, AccQ•Fluor™ Reagent, Waters) according to a previously published procedure (Ref. 5). Protein hydrolysates were reconstituted with 20 µL of 0.1M HCl, buffered with 60 µL of sodium borate and derivatized in a similar fashion.

RESULTS AND DISCUSSION

Reproducibility of CZE Separations:

Previous work has demonstrated that proteins interact with the walls of untreated capillaries and generate irreproducible migration times (Ref. 2). This phenomenon was also observed in the separation of five protein standards under regular CZE separation conditions (Fig. 2: Bottom). The broad tailing peaks suggest protein interaction with the negatively charged capillary wall. Furthermore, not only was cytochrome c (pI = 9.3) poorly recovered from the capillary but the overall migration time reproducibility was less than satisfactory. Under these conditions, the migration time relative standard deviation (M.T. RSD) was found to be 4.0% for six consecutive runs. Protein peak shape and resolution were significantly improved by the addition of 1.0M zwitterion (i.e., trimethylammoniumpropane sulfonate, Z1-Methyl. Millipore) to the phosphate buffer electrolyte (Fig. 2: Top). Furthermore, the M.T. RSD was improved from 4.0% to 0.4%.

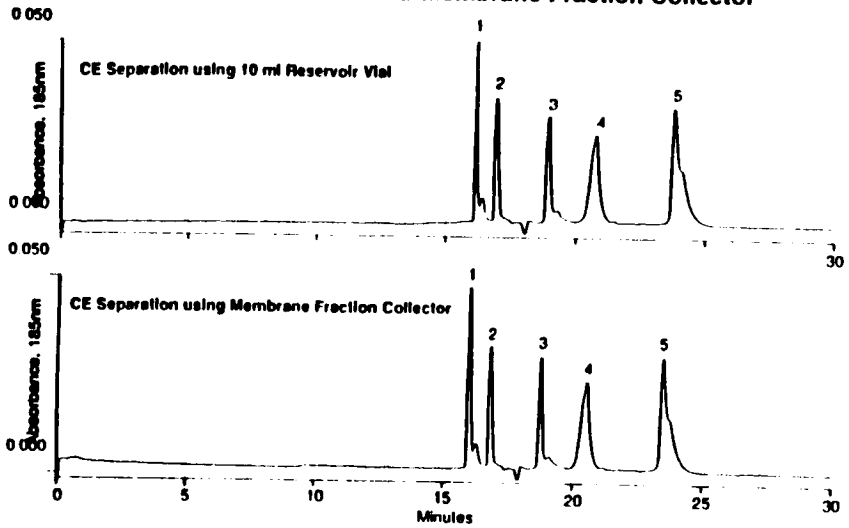
Figure 2: Effect of Electrolyte Additive on Protein Separations



Capillary: Accusep 75µm x 60cm (total)
Sample: 1= Lysozyme, 2= Cytochrome c, 3= Myoglobin, 4= Transferrin, and 5= BSA at 250 µg/ml in 100mM Sodium Phosphate, pH 7

Load: 20 second hydrostatic
Run: + 7 kV

Figure 3: Separation of Protein Mixture With and Without CE Membrane Fraction Collector



Capillary: Accusep 75µm x 60cm (total)
Sample: 1= Lysozyme, 2= Cytochrome c, 3= Myoglobin, 4= Transferrin, and 5= BSA at 250 µg/ml in electrolyte
Electrolyte: 100mM Sodium Phosphate, pH 7.0 with 1.0M Z1-CH3

Load: 20 second hydrostatic
Run: + 7 kV

Effect of Membrane Fraction Collector on CE Separations:

This experiment was performed to determine whether the installation and utilization of the CE membrane fraction collector had a deleterious effect on the CZE separation of the 5 protein standard mixture. Fig. 3 shows the electropherograms for separations performed with (Top) and without (Bottom) the collection device. As indicated, the relative protein peak shapes, migration times and resolution were nearly identical. These data indicate that methods developed for traditional CZE can be transferred directly to a system equipped with the described membrane fraction collector device.

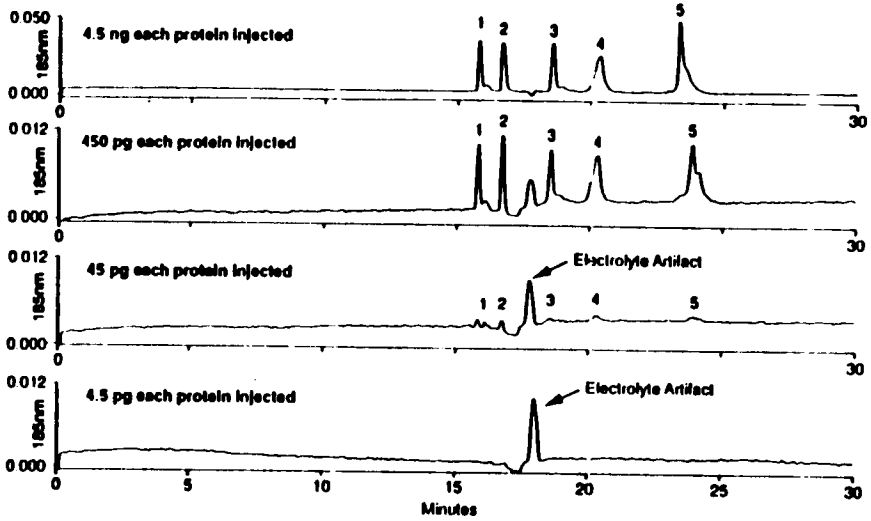
On-Capillary Absorbance Detection and Post-Electrophoresis Staining:

Samples containing the five protein component mixture spanning three orders of magnitude in concentration were prepared and separated by CZE. The amounts of each protein component injected were 4.5 ng, 450 pg, 45 pg, and 4.5 pg. The electropherograms from on-capillary, 185nm absorbance detection are shown in Fig. 4. At the 4.5 ng and 450 pg levels, the five protein components are easily detected. However, at the 45 pg level, the peaks are barely discernible and no proteins are detected at the 4.5 pg amount.

Samples separated by CZE and collected on Immobilon-P membranes were subjected to total protein staining as well as antigen specific, anti-human transferrin immunodetection procedures. Figure 5 shows data from the 4.5 ng analysis at which level total protein band staining with the colloidal gold reagent was clearly visible. Bands were faintly seen at the 450 pg level and not detected at or below the 45 pg level (data not shown).

Immunodetection was performed using antibodies specific to human transferrin, and as such, only the transferrin protein bands were detected on the membranes. Non-specific immunodetection was not seen at any protein concentration tested. Visualization of the antibody bound transferrin with the chromagenic and chemiluminescent substrates was also clearly evident at the 4.5 ng level with minimum level of transferrin detection being 45 pg (data not shown). This level is comparable to that of detection at 185nm.

Figure 4: CE Separations Using Membrane Fraction Collector



Capillary: Accusep 75µm x 60cm (total)
Sample: 1= Lysozyme, 2= Cytochrome c, 3= Myoglobin, 4= Transferrin, and 5= BSA at 250, 25, 2.5 and 0.25 µg/ml in electrolyte
Electrolyte: 100mM Sodium Phosphate, pH 7.0 with 1.0M Z1-CH3
Load: 20 second hydrostatic
Run: + 7 kV

Amino Acid Analysis of Proteins Recovered from PVDF Membranes

The CE / membrane fraction collector separation of 100 nl of horse cytochrome c and myoglobin is shown in Figure 6. These results demonstrate that successful component resolution can be obtained even with relatively large loading volumes.

The amino acid compositional analyses data from these samples are presented in Table 1. Protein recovery from the hydrolyzed and extracted membranes were 88% and 73%. The compositional accuracy for cytochrome c and myoglobin were 9.5% and 11.6% respectively. These values were only slightly higher than that obtained from the analysis of of the cytochrome c and myoglobin controls (i.e., 5.8% and 7.4% respectively) and are significantly better than previous reports of hydrolysate analysis with membrane adsorbed samples with microgram or lower amounts (Ref. 7-9).

As expected, increased CE component resolution was observed using decreased sample load volumes however compromises is amino acid compositional accuracy resulted (data not shown). Techniques such as capillary isoelectric focusing or collection of repetitive low volume injections are currently being explored which may help overcome this apparent tradeoff.

Stained Proteins on PVDF Membrane Obtained From Capillary Electrophoresis Membrane Fraction Collector

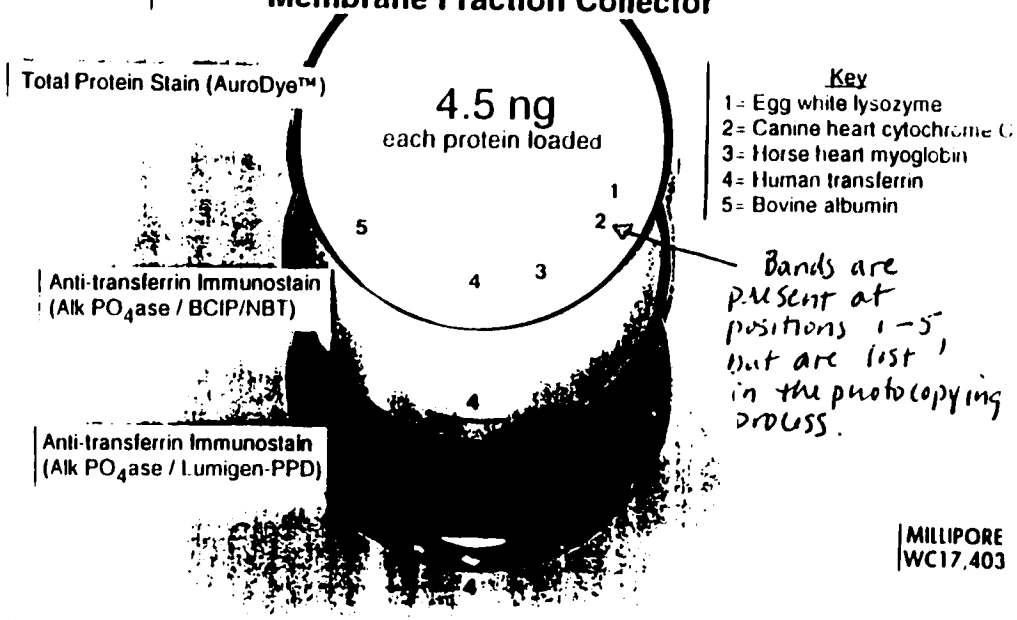
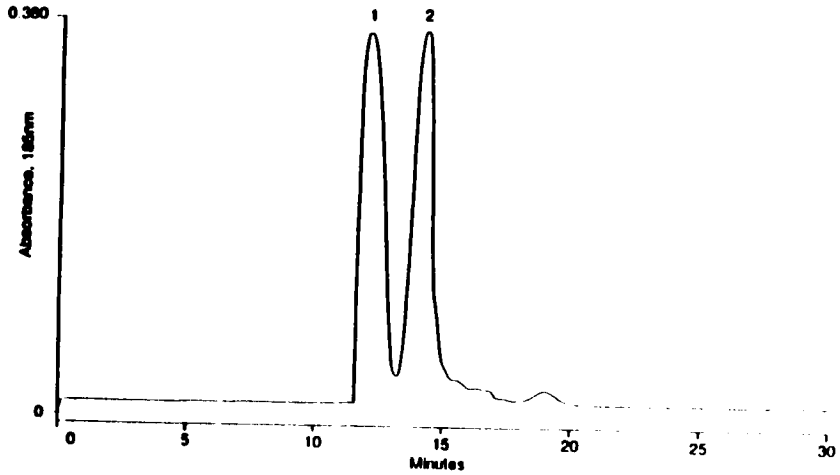


Figure 6: Electropherogram of Cytochrome c and Myoglobin using the CE Membrane Fraction Collector



Capillary: Accusep 75µm x 60cm (total)
Sample: 1= 415 ng of Cytochrome c, 2= 341 ng Myoglobin,
Electrolyte: 100mM Sodium Phosphate, pH 7.0 with 1.0M Z1-CH3
Load: 20 second hydrostatic
Run: + 7 kV

Table 1. Representative Amino Acid Analysis Results for Samples Purified by CE a[illegible] "[illegible]cted on PVDF Memb[illegible]s

	Cytochrome c		Myoglobin	
	Lit. Value	CE Sample	Lit. Value	CE Sample
Asp	8	9.6	10	12.9
Ser	0	n.d.	5	5.6
Glu	12	12.8	19	19.3
Gly	12	12.3	15	16.0
His	3	2.9	11	9.2
Arg	2	2.4	2	2.7
Thr	10	9.1	7	7.4
Ala	6	6.3	15	15.7
Pro	4	4.4	4	4.8
Tyr	4	3.2	2	1.8
Val	3	3.1	7	7.2
Met	2	1.4	2	1.7
Lys	19	17.8	19	18.1
Ile	6	5.8	9	8.4
Leu	6	5.8	17	15.9
Phe	4	3.9	7	6.6
Average Error		9.5%		11.6%
ng Injected on CE		415		341
ng Hydrolyzed		365		249
Recovery		88%		73%

Conclusions:

Post-run analysis of samples separated by capillary electrophoresis has been problematic due to limited sample masses as well as to difficulties encountered collecting discrete fractions from the capillary. The data presented in this study provide evidence of the utility of a membrane fraction collector interface for performing post-run analysis of CZE separated proteins. The interface, since it is based on the continuous transfer of molecules emerging from the end of the capillary onto the moving membrane surface, preserves the spatial resolution of the separation and permits CE to be coupled with other analytical methods including immunodetection and amino acid composition analysis. Significant findings this studies include:

1. Proteins collected with the membrane fraction collector following CZE can be visualized at the low picogram level using antigen-specific immunodetection procedures. This level of sensitivity is comparable to that obtained using non-selective, on-capillary detection at 185nm.

2. Membrane-bound samples are readily amenable to reversed phase HPLC amino acid analysis following vapor-phase hydrolysis and derivatization with AQC.

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