Published in final edited form as:

Talanta. 2007 March 30; 71(5): 1981–1985.

CSE-MECC two-dimensional capillary electrophoresis analysis of proteins in the mouse tumor cell (AtT-20) homogenate

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Abstract

Two-dimensional capillary electrophoresis was used for the separation of proteins and biogenic amines from the mouse AtT-20 cell line. The first-dimension capillary contained a TRIS-CHES-SDS-dextran buffer to perform capillary sieving electrophoresis, which is based on molecular weight of proteins. The second-dimension capillary contained a TRIS-CHES-SDS buffer for micellar electrokinetic capillary chromatography. After a 61 seconds preliminary separation, fractions from the first-dimension capillary were successively transferred to the second-dimension capillary, where they further separated by MECC. The two-dimensional separation required 60 minutes.

Introduction

Capillary electrophoresis is an interesting separation technique for the characterization of proteins. However, the small sample volume and small optical path length used in capillary electrophoresis results in poor concentration detection limits, which prohibits the use of CE for separation and determination of trace-level proteins in many biological samples. Recently, a few techniques have been developed to improve concentration detection limits for CE, such as isotachopheresis [1], field amplification [2], and on-capillary concentration using a small pore plug [3].

Alternatively, improved detection limits are produced by use of laser induced fluorescence detection of native and derivatized proteins [4-10]. Native fluorescence is usually based on tryptophan, which is a rare amino acid in most organisms, has modest molar absorptivity and fluorescence quantum yield, and requires excitation in the ultraviolet with expensive and temperamental lasers. In contrast, derivatization can target the relatively abundant lysine residues to produce strongly fluorescent product. However, derivatization is not straightforward for analysis of trace proteins because excess derivatizing reagent and the presence of fluorescent impurities can interfere with the separation. Also, incomplete labeling of reaction sites results in complex electropherograms generated by multiple reaction products [11-12]. Fluorogenic reagents have proven useful for protein analysis [13]. These reagents are not fluorescent until they react with a primary amine; they generate low background signals compared to traditional fluorescent derivatizing reagents. We have exploited 3-(2-furoyl) quinoline-2-carboxaldehyde (FQ) to generate highly fluorescent proteins that are separated efficiently when used a buffer system that contains an anionic surfactant, without noticeable bandbroadening due to multiple labeling [9].

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While providing rapid analysis of the protein in biological samples, one-dimensional separations are inherently limited in their resolution of complex mixtures. Fortunately, that resolution can be increased by combining two or more separation methods. As Giddings pointed out, the spot capacity of a multidimensional separation is given by the product of the peak capacity for the one-dimensional separations, assuming that the separations are not correlated [14]. A number of two-dimensional capillary electrophoresis methods have been developed for separation of complex protein mixtures [15-17].

AtT-20 is a mouse tumor cell line. These cells secrete β -endorphin and ACTH and have served as a useful model for studying neuropeptide synthesis, transport, and release [18-20]. Strong evidence has been presented that these cells have many enzymatic and structural features common to neuropeptide secreting neurons [21]. Mackie *et al.* reported the transfection of the AtT-20 cell line with the rat cannabinoid (CB1) receptor [22]. This transfected cell line (AtT-20, CB1) was used as a model for studying Δ^9 -tetrahydrocannabinol (THC) induced changes in protein profile in neurons.

We have employed capillary electrophoresis to investigate the sub-cellular localization of components in this cell line (23). Our first generation separation of components from the AtT20 cell line employed relatively low electric fields, and the separation required over 3 hours. In this paper, we now report an improved separation for this cell line that requires 60 minutes for completion.

2 Materials and Methods

2.1 Reagents

Unless otherwise stated, all chemicals were obtained from Sigma (St Louis, MO). 3-(2-furoyl)-quinoline-2-carboxaldehyde (FQ) and potassium cyanide (KCN) were purchased from Molecular Probes (Eugene, OR, USA). UltraTrol dynamic pre-coatings was purchased from Target Discovery, Inc. (Pal Aito, CA, USA). FQ solution was prepared with HPLC grade methanol (Fischer Sci., Houston, TX, USA). Stock solutions of 2-(N-cyclohexylamino) ethanesulphonic acid (CHES), Tris [hydroxymethyl] aminomethane (TRIS), sodium dodecyl sulfate (SDS) were made by dissolving appropriate amounts of reagents in nanopure (Barnstead, Dubuque, WI) distilled water. All chemicals were of analytical reagent grade and were used as received. All solution and buffer were prepared in nanopure distilled water.

Separation buffers were made by mixing aliquots of stock solutions (0.2-0.5 M) and nanopure distilled water. pH was adjusted by adding 0.5 M NaOH or 0.5 M HCl. The buffer solutions were then filtered through a 0.22 μ m membrane filter (Millipore Corporation, MA) to remove particulates. Finally the buffer solution was degassed before use by sonication for 30 minutes. Sieving matrices for capillary sieving electrophoresis experiments were prepared by dissolving dextran (513 kDa or 100-200 kDa) in the CSE buffer. Any insoluble particles were removed by spinning the CSE buffer using a centrifuge (15000 rpm, 20 minutes).

2.2 Two-dimensional CE-LIF instrument and data processing

Our two-dimensional capillary electrophoresis instrument employs a post-column sheath flow laser induced fluorescence detector as described elsewhere [15,17,23-25]. A 29.8 mW, 473 nm solid-state laser (Lasermate Corporation, Pomona, CA, USA) was used for excitation of FQ labeled proteins. Fluorescence signal was collected at a right angle with a 60x, 0.7 NA microscope objective, filtered by a 580 nm long-pass filter from Omega Optical (Brattleboro, VA, USA), and single photons were counted using an avalanche photodiode unit (Perkin-Elmer, Fremont, CA, USA). Electrophoresis was performed by applying voltage using two high-voltage (0-30 kV) power supplies (CZE 1000R, Spellman, Hauppauge, NY, USA). Both

first-dimension and second-dimension capillaries were uncoated fused-silica capillary. A laboratory-built multipurpose injection assembly was used to clean and condition the capillary and to inject sample [25].

The injection end of the first dimension capillary was placed in the injection reservoir and the other end was carefully aligned with the second dimension capillary using an interface. The interface was made of a square piece of Plexiglas with four channels in the shape of a cross. Two capillary sleeves (large outer diameter fused silica capillary) were used to guide the separation capillaries. The interface was connected to the interface reservoir containing the second capillary buffer. The detection end of the second dimension capillary was inserted into a sheath flow cuvette.

Data were collected with a PC using software written in LabView. The two-dimensional capillary electrophoresis data were treated using software written in MATLAB 7.0.

2.3 Dynamic pre-coating and conditioning of capillaries

Before each experiment, the capillaries were cleaned, conditioned, and coated with UltraTrol, a commercial dynamic coating. First, the capillaries were purged with 1.0 M NaOH for 5 minutes followed by water for another 5 minutes again. Next, the capillaries were coated with UltraTrol for 5 minutes. Then the capillaries were flushed with MECC buffer for 5 minutes to eliminate excess coating. Finally, the first-dimension capillary was flushed with CSE buffer to replace MECC buffer in it for another 5 minutes. High voltage was applied and let run for 20 minutes to achieve a stable baseline.

2.4 Two-dimensional CE experiments

In the two-dimensional capillary electrophoresis system, two 20-cm long, 31- μ m inner diameter capillaries were used as the first dimension capillary for CSE and second dimension for MECC, respectively. The CSE running buffer contained 50 mM TRIS, 50 mM CHES, 5 mM SDS (pH 8.4) and 5% dextran; dextran acts as a sieving matrix. The MECC running buffer contained 50 mM TRIS, 50 mM CHES, 15 mM SDS (pH 8.4).

The voltage program for the two dimensional capillary electrophoresis is shown in Fig. 1. Samples were injected into the first capillary by applying -5 kV for 2 seconds on the first capillary electrode. The injection reservoir was filled with the sieving matrix and a preliminary separation was achieved by applying 1100 V/cm for 61 seconds. The pre-run time was determined experimentally so that at the end of the pre-run, the fastest migrating proteins approached close to the interface. A series of transfer and MECC separation cycles then began. A fraction of partially separated sample from the first capillary was transferred into the second capillary by applying -15 kV on the second capillary and -25 kV on the first capillary for 1 second. Once the fraction was transferred the second dimension separation was performed by applying -18 kV at both electrodes, which drove separation in the second capillary while holding analyte stationary in the first. These cycles of fraction transfer and second capillary separation were repeated under computer control until the entire sample was transferred and separated in the second capillary.

2.5 AtT-20 cell culture and pre-column labeling of cell homogenate

AtT-20 (CB1) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated horse serum, 100 UI/mL streptomycin, 100 UI/mL penicillin at 37 °C in a 5% CO₂ atmosphere. Culture medium was changed after every three days. At ~70% confluence level cells were harvested by treating with 1X trypsin-EDTA and were washed three times with PBS to remove traces of culture medium. Cells were lysed with 1% SDS on ice for 20 minutes and then stored at -80 °C.

To denature proteins, 5 μ L of 2% SDS was added to 5 μ L of protein sample and heated for 4 minutes at 90 °C. The sample was transferred into a 500 μ L microcentrifuge tube which contained 100 nmol of previously dried FQ. 2.5 μ L of 10 mM KCN and 2.5 μ L water were added to the microcentrifuge tube and then heated for another 5 minutes at 65 °C. After completion of the derivatization reaction the sample was diluted to 100 μ L with 50 mM TRES-50 mM CHES-5 mM SDS (pH 8.4).

3 Results and discussions

3.1 The effect of molecular weight and concentration of dextran in CSE buffer on analysis of proteins in AtT-20 cell homogenate

Polymer concentration and molecular weight are two important characteristics in sieving electrophoresis. In order to obtain high separation efficiency, the concentration of dextran (100-200 kDa) was investigated from 5% to 7% in 100 mM TRIS-100 mM CHES- 3.5 mM SDS buffer (pH 8.4). The results indicated that the separation efficiency improved slightly at 7% compared with 5%. However, separation time and viscosity also increased with polymer concentration. A higher molecular weight dextran (513 kDa) was also investigated. Higher separation efficiency was obtained with the higher molecular weight polymer at the same concentration. However, CSE buffers with higher than 5% dextran (513 kDa) were very viscous and required unacceptably high pressure to fill the capillary. We therefore chose to employ 5% dextran (513 kDa) for further studies.

3.2 The effect of CHES-TRIS-SDS concentration in CSE buffer on analysis of proteins in AtT-20 cell homogenate

The concentrations of CHES, TRIS and SDS in CSE buffer have a significant effect on the separation of proteins through their influence on ionic strength and Joule heating, viscosity of electrolyte, and adsorption on the capillary wall. The effect of the concentrations of CHES, TRIS and SDS on separation of proteins in AtT-20 cell homogenate was investigated. The results indicated that the separation of proteins was better in the 50 mM CHES-50 mM TRIS-5 mM SDS buffer than that in 100 mM CHES- 100 mM TRIS-3.5 mM SDS buffer, presumably because of excessive Joule heating at higher ionic strength, and the 50 mM CHES-TRIS-3.5 mM SDS buffer was used in subsequent studies.

3.3 The effect of CHES-TRIS concentration in MECC buffer on analysis of proteins in AtT-20 cell homogenate

SDS concentration was held constant at 15 mM for MECC separations, and the concentrations of CHES and TRIS were varied over the range 50- 100 mM. The results showed that varying the concentrations did not result in changes in the migration order of proteins but had a significant effect on the resolutions. As expected, the current roughly doubled from 0.28 μ A to 0.42 μ A with the increased CHES and TRES concentrations, and the separation degraded. This phenomenon can be attributed to the excessive Joule heating. In order to obtain a higher efficiency, while avoiding the generation of excessive Joule heating, 50 mM CHES-TRIS 15-mM SDS was selected as for the MECC buffer.

3.4 Analysis of proteins in AtT-20 cell homogenate

3.4.1 The relationship of logarithm of molecular weight standard proteins (log MW) and migration time in CSE—A calibration curve was constructed between migration time and molecular weight by analysis of a set of standard proteins, Fig. 2A. Migration time increased with the logarithm of molecular weight, Fig 2B. The least-squares regression equation for this line was $y=0.5034x-0.7762$ with a correlation coefficient $r = 0.993$. Detection

limit was 300 zmol for carbonic anhydrase injected onto the capillary, which is rather high for this protein.

3.4.2 Analysis of proteins in AtT-20 cell homogenate by two-dimensional CE—

The proteins in AtT-20 cell homogenate were analyzed by two-dimensional capillary electrophoresis. After the 61 seconds preliminary separation, fractions from the first-dimension capillary were successively transferred to the second-dimension capillary, where they further separated by MECC. The second-dimension separation was completed in 60 minutes. The typical two-dimensional gel stained image and landscape image from AtT-20 cell homogenate are shown in Fig. 3.

A non-linear least-squares regression method was used to fit a Gaussian surface to the 50 most intense components in this separation. The median spot width, expressed as the standard deviation of the Gaussian surface, was 3.6 transfers in the CSE dimension and 0.68 s in the MECC dimension. A plot of the spot position in the CSE versus the MECC dimension was uncorrelated ($r = 0.22$). We can estimate the spot capacity as the product of the peak capacity in the two dimensions. In this case, there were 209 fractions transferred from the CSE capillary, which gives a peak capacity in that dimension of 14.5. The MECC dimension was 19 seconds in duration, which gives a peak capacity in that dimension of 7. The total spot capacity of the separation was 100.

4 Conclusions

Based on peak capacities of one-dimensional CSE and one-dimensional MECC separations (data not shown) the spot capacity of CSE×MECC was estimated to be over a thousand. In reality, about 60 spots were resolved. Band broadening due to molecular diffusion was the main reason for such a huge loss. One-dimensional separations were completed within a few minutes whereas two-dimensional CE took about 1 hour. Proteins remain in the first dimension capillary for a relative long time before separation in the second capillary. Molecular diffusion during the long separation results in loss of resolution. Shorter experimental time by using smaller capillaries and higher field strengths would be a potentially effective approach to improve the overall the two-dimensional CE spot resolution.

ACKNOWLEDGMENT

This work was funded by the National Institutes of Health grant R01GM071666.

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Fig 1.
The voltage program for the two-dimensional capillary electrophoresis.

Fig 2.

(A) Electropherograms of 1- μ M solution of standard proteins. Capillary: 25 cm x 147 μ m O.D. x 31 μ m I.D., Coated by UltraTrol; Buffer: 100 mM CHES-100 mM TRIS-3.5 mM SDS-5% Dextran (513 kDa), pH 8.4; Injection: -4 kV, 5 s; Separation: -20 kV. (B) The relationship of log MW (kDa) and migration time of standard proteins.

Fig 3. Protein gel stained image (A), landscape image (B), and landscape image magnified by 10X (C) from AtT-20 cell homogenate. The 50 components used to estimate run-to-run variation in migration time are marked with a $\oplus$.