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Fluorescein thiocarbamyl amino acids as internal standards for migration time correction in capillary sieving electrophoresis

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Abstract

A number of algorithms have been developed to correct for migration time drift in capillary electrophoresis. Those algorithms require identification of common components in each run. However, not all components may be present or resolved in separations of complex samples, which can confound attempts for alignment. This paper reports the use of fluorescein thiocarbamyl derivatives of amino acids as internal standards for alignment of 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ)-labeled proteins in capillary sieving electrophoresis. The fluorescein thiocarbamyl derivative of aspartic acid migrates before FQ-labeled proteins and the fluorescein thiocarbamyl derivative of arginine migrates after the FQ-labeled proteins. These compounds were used as internal standards to correct for variations in migration time over a two-week period in the separation of a cellular homogenate. The experimental conditions were deliberately manipulated by varying electric field and sample preparation conditions. Three components of the homogenate were used to evaluate the alignment efficiency. Before alignment, the average relative standard deviation in migration time for these components was 13.3%. After alignment, the average relative standard deviation in migration time for these components was reduced to 0.5%.

1.0 Introduction

Run-to-run drift in migration time in capillary electrophoresis can arise from changes in a wide range of experimental parameters, including temperature, buffer composition, electro-osmotic mobility, and applied potential. A number of alignment algorithms have been developed to correct for that drift [1–4]. In general, these algorithms require identification of one or more common components in each sample. However, it may be challenging to identify those common components in complex samples.

This paper reports the use of fluorescein thiocarbamyl derivatives of aspartic acid and arginine as internal standards to align the capillary sieving electrophoresis (CSE) separations of a cellular homogenate labeled with 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ). The aspartic acid thiocarbamyl migrates before components in the FQ-labeled homogenate while the arginine thiocarbamyl migrates after most of the FQ-labeled components. The use of these internal standards and a simple correction algorithm improves the relative precision in migration time by over an order of magnitude.

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2.0 Experimental

2.1 Chemicals and Reagents

Unless otherwise stated all chemicals are from Sigma (St. Louis, MO, USA). The fluorogenic reagent FQ was purchased from Molecular Probes (Eugene, OR, USA). The dynamic coating UltraTrol LN was from Target Discovery (Palo Alto, CA, USA). All solutions were made using deionized water from a Barnstead Nanopure Diamond (Boston, MA, USA). All buffers were filtered using 0.22µm Millipore Stericup filters (Billerica, MA, USA).

2.2 MCF-7 samples

The MCF-7 breast cancer cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Conditions for tissue culture were previously reported [5].

Two different cultures of MCF-7 cells were homogenized. The first set of homogenate samples was 70% confluent. The second set was made 2 days later and was 90% confluent. The cells were lifted from their culture flasks using a solution containing 0.25% w/v trypsin and 0.53mM EDTA. The cell suspension was washed 3 times with cold phosphate-buffered saline (PBS). To fix the cells, the cells were suspended in 70% ethanol and kept on ice for 15 min. The cells were then washed 2 times with cold PBS to get rid of any residual ethanol. The cells were finally resuspended in 1% sodium dodecyl sulfate (SDS) and heated for 5 min at 95 °C to lyse the cells. The lysed homogenate samples were spun down and 20 µL aliquots of the supernatant were placed in tubes and put in a -80 °C freezer.

2.3 Fluorescein thiocarbamyl amino acid synthesis

The first separation of fluorescein thiocarbamyl derivatives of amino acids (FTC-amino acids) by capillary electrophoresis was reported in 1988 [6]. We employed a modified version of that protocol for preparation of our derivatives. A 100 µL aliquot of 6 mM fluorescein isothiocyanate isomer 1 (FITC) in acetone was combined with 300 µL of 3 mM L-arginine (99.5%) and L-aspartic acid (99.5%) solution in an amber tube and allowed to react in the dark overnight at room temperature. The next day, the fluorescein thiocarbamyl derivative solutions were diluted to 2 mL with a 40 mM Na₂HPO₄ buffer at pH 7.8.

2.4 HPLC purification of FTC-Amino acids

The fluorescein thiocarbamyls were purified using a Vision Workstation BioCAD Family HPLC with a Zorbax Eclipse-AAA column (Agilent Technologies, Santa Clara CA, USA). The mobile phases consisted of 40 mM Na₂HPO₄ pH 7.8 and ACN:MeOH:water (45:45:10 v/v). The purified thiocarbamyl derivatives were collected and diluted with a 100 mM 2-(cyclohexylamino)ethanesulfonic acid (CHES), 100 mM Tris, and 3.5 mM SDS buffer to a concentration that was appropriate for capillary electrophoresis.

2.5 Labeling of protein homogenate

The MCF7 protein homogenates were labeled with the fluorogenic reagent, FQ [5,7–8]. Frozen homogenate MCF-7 samples in 1% SDS were heated to 95 °C for 5 min to denature the protein. Next, 20 µL of the heated sample was added to a tube containing 5 µL of 5 mM KCN in 10 mM borate in a tube with 100 nmol of lyophilized FQ and heated for 5 min at 65 °C. The labeled sample was diluted with 150–200 µL of a 100 mM CHES, 100 mM Tris, and 3.5 mM SDS buffer and stored on ice. Immediately before analysis, 2–3 µL of purified fluorescein thiocarbamyl-arginine and 2–3 µL of purified fluorescein thiocarbamyl-aspartic acid solutions were added.

2.6 CE instrument

Analytes were detected by laser-induced fluorescence in a post-column sheath-flow cuvette. The sheath-flow cuvette-based capillary electrophoresis instrument has been previously described [6,9–11]. Briefly, the locally constructed instrument used a 6 mW 473 nm solid-state diode laser (Lasermate Group, Pomona, CA, USA) for excitation. Fluorescence was collected with a 60 $\times$, 0.7 NA microscope objective and was filtered by a 580 nm long-pass filter (Omega Optical, Brattleboro, VA, USA). Single photons were counted using an avalanche photodiode (Perkin-Elmer, Fremont, CA, USA) at 80 Hz using a National Instruments Card (PCI-6035E) that was programmed with LabVIEW running on a PC. The electropherograms were processed using Matlab.

Separations were performed with a 38 cm long, 30 μ m inner diameter fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA). Capillaries were treated with the dynamic coating Ultratrol (Target Discovery, Palo Alto, CA, USA) using the manufacturer's protocol. The separation was performed by sieving electrophoresis with a 5% w/v dextran (400–500 kDa), 100 mM CHES, 100 mM Tris, and 3.5 mM SDS separation buffer. The sheath flow buffer contained 100 mM CHES, 100 mM Tris, and 3.5 mM SDS. The samples were electrokinetically injected with -5 kV for 3 s. Separations were performed at a potential of either -15 or -20 kV.

3. Results

3.1 Criteria for internal standards

There are at least two criteria for the choice of internal standards for correction of migration times in capillary electrophoresis with fluorescence detection. As the first criterion, one standard should migrate before the fastest component in the sample and the other standard should migrate after the slowest migrating component. As a second criterion, it is experimentally simpler to employ standards that have similar excitation and emission spectra as the labeled proteins. While we have reported the use of a two-color fluorescence detector to separate the signal from proteins and standards labeled with different dyes [12], the two-color instrument is experimentally complex.

3.2 Fluorescent dyes as internal standards

We first considered the use of Oregon Green and sodium fluorescein as standards. These dyes have sufficient overlap with the excitation and emission spectra as FQ-labeled proteins to be detected with our fluorescence system. Unfortunately, these dyes migrated in the middle of the FQ-labeled homogenate and were not further investigated.

3.3 Fluorescein thiocarbamyl amino acids

We next investigated fluorescein thiocarbamyl derivatives of amino acids as standards to normalize migration time. Our capillary sieving electrophoresis buffer employs SDS as a component. This anionic surfactant complexes with proteins, imparting a negative charge to the proteins. Fluorescein isothiocyanate reacts with the α -amine of amino acids (in addition to the ϵ -amine of lysine) to produce the negatively charged carbamyl. Fluorescein itself is dianionic at basic pH. The least negatively charged fluorescein thiocarbamyl derivative of a common amino acid is arginine, which takes a -2 charge ($+1$ from the guanidinium group of the amino acid, -1 from the carboxylic acid of the amino acid, and -2 from fluorescein) whereas the derivatives of aspartic acid takes a -4 charge (-2 from the two carboxylic acids on the amino acid and -2 from fluorescein). Of course, the guanidinium group of arginine may interact with the negatively charged surfactant, inducing a more negative charge to the derivative in the separation buffer. Nevertheless, the fluorescein thiocarbamyl of aspartic acid migrated

before all components in the FQ-labeled cellular homogenate and the fluorescein thiocarbamyl of arginine migrated after almost all components in the FQ-labeled homogenate.

We used liquid chromatography to purify the labeled amino acids, which removed unreacted labeling reagent and its hydrolysis products and other fluorescent impurities. The purified thiocarbamyl-amino acids do not have a free primary amine and do not react with the FQ-labeling reagent.

3.4 Evaluation of the fluorescein carbamyl derivatives of arginine and aspartic acid as internal standards to correct for migration time differences in capillary sieving electrophoresis

Twenty-one CSE runs were performed over eight days in a two-week period using two different MCF-7 homogenate samples and two different voltages. Figure 1 shows the runs before alignment. The samples were aligned using a two-point migration time normalization method [4]. Figure 2 shows the runs after alignment.

Non-linear regression analysis was used to fit a Gaussian function to three peaks in each run; peak width was estimated as the standard deviation of the Gaussian function. Peak 1, migration time 5.6 min, had a standard deviation in peak position of 1.10 s and an average peak width of 0.54 s. Peak 2, migration time 6.25 min, had a standard deviation in peak position of 2.21 s and an average peak width of 1.29 s. Peak 3, migration time 8.15 min, had a standard deviation in peak position of 3.50 s and an average peak width of 3.02 s. The uncertainty in peak location is comparable to the peak width itself. Before alignment, the average relative standard deviation in migration time for these three peaks was 13%. After alignment, the average standard deviation in migration time was reduced to 0.5%.

These results are somewhat surprising. The low molecular weight amino acid derivatives are far too small to interact with the sieving matrix and presumably are separated under submicellar capillary electrophoresis condition, whereas the proteins are separated by size under CSE conditions. Presumably, peak 3 is generated by a high molecular weight component whose migration time is particularly sensitive to the composition of the sieving buffer, and the larger standard deviation for this component is presumably due to the different separation mechanisms for the standards and sample. Nevertheless, these internal standards work remarkably well to align the complex sample even with different separation mechanisms.

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Figure 1. Twenty-one capillary electrophoresis runs before alignment. Peaks are offset for ease of viewing.

Figure 2. Twenty-one capillary electrophoresis runs after alignment. FTC-Arg and FTC-Asp are the peaks generated by the fluorescein thiocarbamyl derivatives of arginine and aspartic acid. Peaks 1, 2, and 3 were used to evaluate the run-to-run migration time precision. Peaks are offset for ease of viewing.