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Separation of proteins by sodium dodecylsulfate capillary electrophoresis in hydroxypropylcellulose sieving matrix with laser-induced fluorescence detection

Shen Hu, Zheru Zhang, Lillian M. Cook, Eric J. Carpenter, Norman J. Dovichi*

Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada

Abstract

Sodium dodecyl sulfate capillary electrophoresis by using hydroxypropylcellulose as the sieving matrix was developed for separation of proteins. 3-(2-furoyl)quinoline-2-carboxaldehyde, a fluorogenic dye, was used as the pre-column reagent to label proteins, which allows the use of laser-induced fluorescence to improve the detection sensitivity. Five standard proteins within the molecular mass range of 14 000–97 000 were used to test this method and a calibration curve was obtained between the molecular mass of these proteins and their peak migration times. This method was also applied to the separation of proteins from HT29 human colon adenocarcinoma cell extracts, and, typically, nearly 30 protein components could be resolved in a 20-min separation. Similar separation patterns were observed for the cell extract proteins when three running buffer systems were employed, indicating that buffer composition did not have much influence on the separation based on HPC sieving.

Keywords: Sodium dodecylsulfate capillary electrophoresis; Capillary electrophoresis; Proteins; Hydroxypropylcellulose; Sodium dodecylsulfate

1. Introduction

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) has been used for the separation and molecular mass determination of proteins for over 30 years [1,2]. This method provides high resolution for protein separation, especially when combined with isoelectric focusing to analyze complex protein mixtures in a two-dimensional (2D) mode. Another advantage of this method lies in its feasibility for preparing and purifying proteins. This is important for biochemists to study the structure and function of proteins. Although SDS–PAGE is a dominant method for protein separation, it has some limitations. Firstly, it is time-consuming to prepare the gel and perform the separation and detection. Secondly, the detection for SDS–PAGE is realized by staining in most cases. This detection mode lacks accurate quantitation and full automation, resulting in difficulty in handling and storing the data. Hence, developing a high-resolution capillary electrophoresis (CE) method for separation of SDS-proteins is of great interest since CE has advantages such as on-line detection, high rapid separation and full automation. Hjerten first reported the use of polyacrylamide gel for capillary zone electrophoresis (CZE) separation of membrane proteins [3]. Later, Cohen et al. reported a cross-linked polyacrylamide capillary gel electrophoresis method for the separation of SDS-proteins [4].

*Corresponding author: Tel.: +1-780-492-2845; fax: +1-780-492-8231.

E-mail address: norm.dovichi@ualberta.ca (N.J. Dovichi).

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Recently, various polymer solutions have been employed as the replaceable sieving matrix for CE separation of proteins instead of cross-linked polyacrylamide. This approach simplifies the SDS-CE since the polymer sieving solutions can easily be prepared and put into the capillary. The polymer solution is also replaceable after each run, which adds flexibility for SDS-CE method. For example, different injection modes, gels, and buffers can be applied. Linear polyacrylamide (LPA) is a commonly used sieving matrix for SDS-CE [5,6]. However, LPA has significant UV absorbance, affecting the linear dynamic range and detection limit of UV detectors [7]. Polyethylene oxide is another important linear sieving polymer in SDS-CE. This sieving matrix is UV-transparent, which can improve UV detection sensitivity. The molecular mass of polyethylene oxide employed for separation of SDS-proteins is often around 100 000 [7,8,10]. Other reported sieving polymers include dextran and pullulan, which are both slightly branched. Dextran within a very broad molecular mass range from 1270 to 2 000 000 has been reported for separation of SDS-proteins [7,9,10], this polymer is also UV-transparent. In terms of UV detection sensitivity, pullulan is also superior to LPA since its UV absorbance, especially in the low UV wavelength range (below 230 nm), is much lower than LPA. The molecular size of pullulan used for separation of SDS-proteins is 50 000–100 000 [11,12]. From the existing publications, a polymer size around 100 000 is reasonable for separation of SDS-proteins considering the separation performance and ease of preparation.

A UV detector is often used for CE separation of SDS-proteins. Unfortunately, this detector is not particularly sensitive. Another problem involved is that some sieving polymers used for SDS-CE have high UV absorbance [13]. Compared with UV detection, laser-induced fluorescence (LIF) offers low background and extraordinarily high detection sensitivity. So, it is important to develop LIF detection methods for SDS-CE. To date, there have been only a few publications on developing LIF detection for SDS-CE separation of proteins [14–19].

In this work, a new SDS-CE method was developed for the separation of standard proteins and HT29 cancer cell proteins using hydroxypropylcellulose (HPC) as a replaceable matrix. The proteins were combined with SDS, labeled with the fluorogenic reagent 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ), separated in HPC sieving matrix and then detected by laser-induced fluorescence. This work indicates that HPC might be a useful sieving matrix for the separation of SDS-proteins.

2. Experimental

2.1. Apparatus

The CE instrument with a sheath-flow cuvette and LIF detector was built in the laboratory [19]. The high voltage was provided by a 0–30 kV d.c. power supply (CZE 1000, Spellman, Plainview, NY, USA). The excitation for LIF detection was provided by an argon ion laser (Model 2211-15SL, Uniphase, San Jose, CA, USA) operated at 12 mW. The 488 nm laser line was focused at ~30 μm from the tip of the capillary by using a 6.3× objective (Melles Griot, Nepean, Canada). Fluorescence signal collected passed through a 630DF30 bandpass filter (Omega Optical, Brattleboro, VT, USA) to a photomultiplier tube (R1477, Hamamatsu, Middlesex, NJ, USA), which was biased at 900 V. Data sampling was accomplished by a 16-bit data acquisition board (NB-MIO16XH-18, National Instruments, Austin, TX, USA) connected to a Macintosh computer.

2.2. Reagents

Acrylamide and ammonium persulfate (APS) were purchased from Life Technologies (Burlington, Canada). γ-methacryloxypropyltrimethoxysilane (MAPS) and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad (Richmond, CA, USA). SDS was obtained from BDH (Toronto, Canada). Enzyme-grade 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was obtained from Fisher Scientific (Fair Lawn, NJ, USA). HPC (M_r 100 000) was obtained from Aldrich (Milwaukee, WI, USA).

Tris, tricine and standard proteins including α-lactoalbumin (M_r 14 200), carbonic anhydrase (M_r 29 000), ovalbumin (M_r 45 000), bovine serum

albumin (BSA, M_r 66 000) and phosphorylase *b* (M_r 97 400) were purchased from Sigma (Oakville, Canada). FQ and KCN were obtained from Molecular Probes (Eugene, OR, USA). Stock solution of 25 m*M* KCN was prepared with water. Stock solution of 100 m*M* FQ was prepared with methanol. 10 μl FQ solution was placed into a 500 μl microcentrifuge tube and the solvent was vacuumed with a Speed Vac (Savant, Farmingdale, NY, USA). The dried FQ aliquots (100 nmol) were stored at −20°C. These precautions were used to prevent FQ degradation in solutions.

2.3. Capillary coating

Capillaries were coated as described by Hjertén [20], and Schmalzing et al. [21]. In brief, fused-silica capillaries of 150 μm O.D.×50 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA) were flushed sequentially with 0.1 *M* sodium hydroxide, water and methanol. Then the inner surface of the capillaries was derivatized with MAPS in a 1:1 methanol solution and left at room temperature overnight. The capillary was then filled with a degassed 4% acrylamide solution containing 0.1% APS and 0.1% TEMED overnight to form a linear polyacrylamide on the capillary wall. Next, the capillary was flushed with water to push excess polyacrylamide (not attached) out of the capillary. Finally, the capillary was filled with 37% formaldehyde (pH 10) for cross-linking the polyacrylamide. After 12 h, the capillary was flushed with water and both ends were dipped into water for storage.

2.4. Sieving buffer

HPC sieving buffer solution was prepared by dissolving appropriate polymers in the running buffer. Three running buffers, including HEPES, tricine and Tris–tricine, were used to prepare corresponding sieving buffer solutions. Before use, the polymer solution was degassed for 30 min and centrifuged at 8000 rpm for 5 min. A laboratory-made pressurizing device was used to fill the capillary with the polymer solution. The polymer solution was preconditioned at the running voltage for 20 min before each run.

2.5. Sample preparation

2.5.1. Standard proteins

To denature the protein, 5 μl protein solution was mixed with 5 μl 5% SDS solution and then heated at 90°C for 5 min. Thiols, such as 2-mercaptoethanol or dithreitol, were not included because they may react with FQ and affect the protein labeling. Then 5 μl of the denatured protein solution was derivatized by mixing with 5 μl of 25 m*M* KCN solution in a 500-μl microcentrifuge tube containing 100 nmol of previously dried FQ. The mixture was incubated for 5 min at 65°C and then diluted with 40 μl running buffer.

2.5.2. Cell extract

Roughly 10^6 HT29 human colon adenocarcinoma cells were washed five times with phosphate-buffered saline (PBS) and resuspended in 40 μl water. Then the cells were lysed by sonication and the suspension was centrifuged at 1000 rpm for 10 min.

Typically, 5 μl of the cell extract supernatant was mixed with 5 μl of 5% SDS and heated at 90°C for 5 min. Then 5 μl of this solution was added into a 500-μl microcentrifuge tube with 5 μl of 25 m*M* KCN and 100 nmol of previously dried FQ. The derivatization reaction was then performed at 65°C for 5 min and diluted with 40 μl running buffer.

3. Results and discussion

Fluorescent labeling is often used to improve the sensitivity of protein analysis by CE. However, pre-column labeling often produces heterogeneous reaction products due to multiple labeling. In CZE, this will lead to a set of distinct peaks or a broad envelope [22], resulting in poor electrophoretic resolution. Compared with CZE, this multi-labeling problem might not be as serious in SDS-CE. Most proteins bind SDS at a constant mass ratio of 1.4 g SDS/g protein. Since proteins have high molecular masses, individual protein molecules will combine with a large number of SDS molecules to become highly charged. This charge prevents further heterogeneity in the charge-to-size ratio of individual protein population caused by derivatization. In addition, SDS-CE is a size-based method for protein

separation. Attaching labels to the large protein molecule should not dramatically change the size of the protein molecule and therefore its migration velocity [14,17]. Moreover, the separation medium used in SDS-CE always contains submicellar SDS, which is similar to that in SDS–PAGE. The submicellar SDS tends to make multiple labeled product peaks collapse, which improves the high-efficiency separation [19,23].

In this work, we use FQ to label SDS-proteins. FQ is a fluorogenic dye, so it can be in high excess without causing a high background signal. This is very important when labeling proteins at low concentration and improves the detection sensitivity [19]. A large excess of FQ (100 nmol) and elevated temperatures (65°C) were utilized to improve the reaction efficiency [23].

Cellulose is an important separation medium for separation of biopolymers. For instance, hydroxyethylcellulose (HEC) and hydroxypropylmethyl cellulose (HPMC) are important sieving matrixes for CGE separation of DNA [24]. Cellulose derivatives such as cellulose acetate or nitrocellulose are often used in classic electrophoresis methods for separation of proteins [25,26]. Recently, different types of cellulose including methylcellulose, HEC, HPMC, and HPC were also used as additives in CZE to improve the separation of proteins [27–31].

In this work, HPC with a molecular mass of 100 000 was tested as the sieving polymer for separation of SDS-proteins. The electropherogram for five standard proteins obtained in Tris–tricine–HPC sieving buffer is presented in Fig. 1. These standard proteins have molecular masses from 14 000 to 97 000 and a linear calibration curve ($r=0.983$) is obtained between the molecular mass of these proteins and their migration times (Fig. 2). The average theoretical plate number for these five proteins is estimated to be 18 000. Multiple labeling might have caused the low separation efficiency, although a similar separation efficiency was reported earlier [14–17]. Another possible reason is that some proteins consist of several glycoforms, which may co-elute and result in low efficiency. The detection limits ($S/N=3$) for these standard proteins are 30 n*M* for lactoalbumin, 7 n*M* for carbonic anhydrase, 8 n*M* for ovalbumin, 4 n*M* for BSA, and 5 n*M* for phosphorylase. Compared with SDS-CE–UV [7], this method provides much better sensitivity.

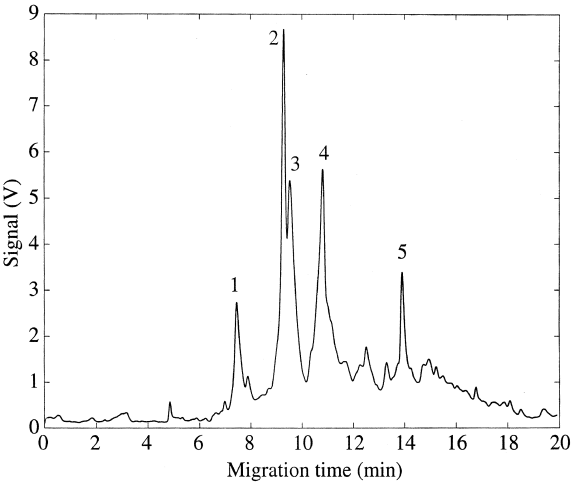

Fig. 1. Electropherogram of standard proteins using 2% HPC sieving polymer and LIF detection. Conditions: capillary length, 20 cm; running voltage, −300 V/cm; injection, −300 V/cm for 5 s; running buffer, 20 m*M* Tris–20 m*M* tricine with 0.1% SDS (pH 8.0); polymer, 2% HPC. Protein peaks: 1, α-lactoalbumin ($1.2\cdot10^{-6}$ *M*); 2, carbonic anhydrase ($1.0\cdot10^{-6}$ *M*); 3, ovalbumin ($5.5\cdot10^{-7}$ *M*); 4, BSA ($3.1\cdot10^{-7}$ *M*); 5, phosphorylase ($2.1\cdot10^{-7}$ *M*).

The SDS-CE method described above was used to separate the proteins in HT29 cell extract. The cells were lysed by sonication, and SDS was added to denature the proteins. Finally, the denatured protein sample was mixed with FQ and KCN for fluorescent labeling. Fig. 3A shows a typical separation pattern for HT29 cell protein extract obtained in Tris–tricine

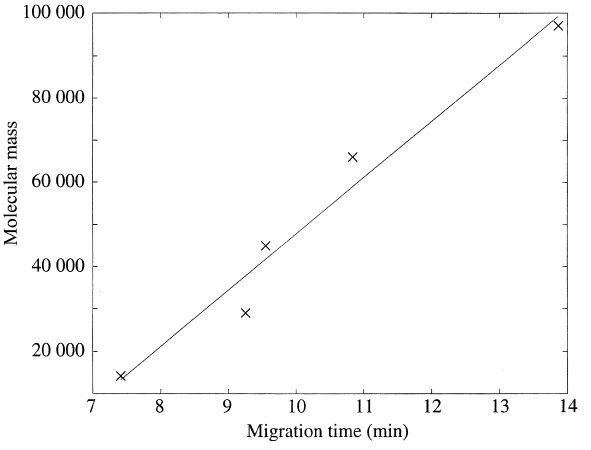

Fig. 2. Plot of molecular mass vs. migration time for the separation shown in Fig. 1.

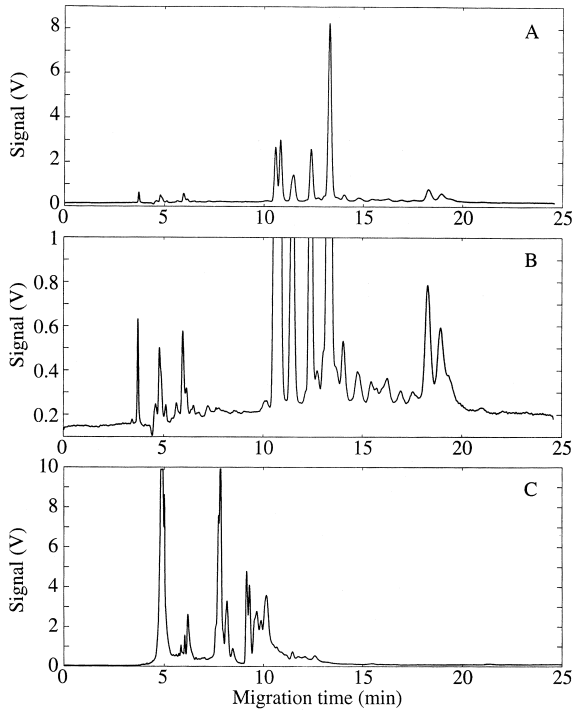

Fig. 3. Separation of proteins from HT29 cell extract in 20 m*M* Tris–20 m*M* tricine–0.1% SDS buffer (pH 8.0). (A) with 2% HPC; (B) with 2% HPC, expanded scale of (A); (C) with no HPC. Other conditions as in Fig. 1.

buffer with sieving polymer HPC. Comparing the results obtained for standard proteins (Fig. 1), the major component has a molecular mass of ~100 000, which was noted before in this cell line [32] Fig. 3B presents the same electropherogram as Fig. 3A but with an expanded fluorescence scale. Clearly, a typical run allows between 25 and 30 peaks to be separated in 20 min, which demonstrates the potential of this method to rapidly analyze complex protein samples. The electropherogram for the HT29 cell extract proteins obtained in the same Tris–tricine buffer but without HPC is shown in Fig. 3C. It can be seen that the pattern for the cell extract proteins in this case is very different from that obtained in Tris–tricine buffer containing HPC. The SDS-protein complexes migrated very fast under free solution CE mode since they did not encounter any resistance from the HPC polymer. The separation window under this condition (Fig. 3C) is only about half of that under SDS-CE mode with HPC (Fig. 3A). Further, lack of resistance from HPC led to more sample injection volume for free solution mode than that for SDS-CE with HPC, and a corresponding improved detection limit [19].

The effect of the buffer composition on the separation of cell extract proteins was also examined. Two other running buffers, 25 m*M* HEPES or 40 m*M* tricine, were used in sieving buffers with the same concentrations of HPC and SDS for HT29 cell extract proteins, Fig. 4A and B. Compared with the electropherogram shown in Fig. 3A obtained in Tris–tricine–HPC sieving buffer, the three sieving buffers present similar patterns for cell extract proteins. This result is reasonable because all three sieving buffers contain the same polymer. It was observed that Tris–tricine–HPC sieving buffer provided a more stable current in the capillary than HEPES–HPC or tricine–HPC. Hence, practically, Tris–tricine is a better buffer system for SDS-CE-HPC method.

We did not use thiols to reduce disulfide bonds; the thiols interfered with the derivatization chemistry. The thiols help control the secondary structure of the protein, and the unreduced proteins are likely to take a slightly more compact structure and may migrate slightly faster than the fully reduced form.

In conclusion, this is the first study of using SDS-CE–LIF with HPC sieving matrix for separation of proteins. This method provides better

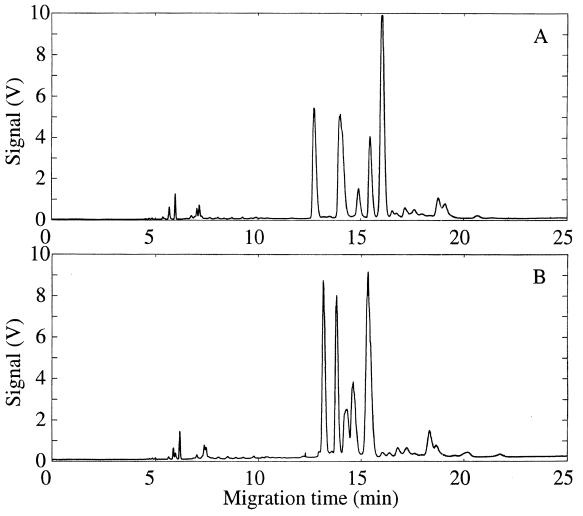

Fig. 4. Effect of buffer composition on the separation of proteins from HT29 cell extract. Polymer: 2% HPC; Buffer: (A) 25 m*M* HEPES with 0.1% SDS (pH 8.0); (B) 40 m*M* tricine with 0.1% SDS (pH 8.0). Other conditions as in Fig. 1.

sensitivity than SDS-CE–UV. Moreover, its analysis speed is fast with separation under 20 min. This method was applied to analysis of proteins from HT29 cancer cells. More than 20 peaks are observed for a typical protein extract in a 20-min separation, indicating that this method is promising for separation of complex protein samples.

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