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Protein profiling employing capillary electrophoresis with dendrimers as pseudostationary phase media

Costas Stathakis, Edgar A. Arriaga, Norman J. Dovichi*

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

Abstract

The effects of anionic and cationic dendrimers as buffer additives on electropherograms of chicken sarcoplasmic proteins were examined. Starburst poly(amidoamine) dendrimers of generation G0 (cationic), 0.5, 1.5, 2.5 and 3.5 (anionic) were used in the concentration range of 10^{-5}% to 1% g/ml. Anionic dendrimers, over the concentration range 10^{-5}% to 0.1% g/ml, had a small effect on separation currents, which allows for tuning of selectivity and resolution over a wide range of dendrimer concentration with minimal contribution to buffer conductance and thus Joule heating. The resolution of protein electropherograms improved as a function of anionic dendrimer concentration and generation with a small effect on analysis times. A maximum of 10 components were identified in <10 min in the presence of 10^{-4}% g/ml G2.5 dendrimer. Improved resolution was also observed in the presence of 0.001% g/ml cationic G0 as compared to buffers with no dendrimeric additives. Analysis times however were in excess of 40 min. Although improved protein profiles were obtained in the presence of dendrimers separation efficiencies however were quite low (13 000–76 000 theoretical plates).

Keywords: Buffer composition; Proteins; Dendrimers

1. Introduction

Capillary electrophoresis (CE) has been applied to biomolecular, and in particular to protein separations, since the emergence of the technique. Early reports stressed the promise of efficient and high-resolution protein separations, due to the small diffusion coefficients of the macromolecules [1]. It was soon realized that the predicted efficiencies ($>10^6$ theoretical plates) could not be generated, primarily because of mass-transfer limiting protein–wall interactions. This observation led to the development of covalent [2–6] and dynamic [7–9] coatings to produce a less interactive capillary surface, which was either neutral and hydrophilic or generating the same charge as the proteins at the operating pH. Although a number of these coatings led to substantial increases in separation efficiencies (>300 000 theoretical plates), problems were encountered such as coating deterioration and slow separations due to a low electroosmotic flow (EOF). In the case of dynamic coatings, frequent capillary conditioning was required for acceptable migration time reproducibility. Also, the operating pH range was limited by the requirement that analytes and surface functionalities possess the same charge so as to minimize interactions.

Although there has been much effort devoted to the generation of high efficiency separation of proteins, there has been less effort devoted to improved resolution of complex protein mixtures. Significant effort is required to optimize protein separations because of the range of charged functionalities that a

*Corresponding author.

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protein may acquire. Although proteins possess a range of non-polar groups that render these promising candidates for interactions with such additives as sodium dodecyl sulphate (SDS) micelles and cyclodextrins (CDs), their bulkiness results in poor partitioning ratios [10]. These non-polar groups however may interact with a variety of surfactants [11] or CDs [12] and such interactions may facilitate protein CE separation. Other separation conditions have been explored to control selectivity and resolution in protein separations, including sulphonate-based additives [13], phosphate buffers [3] and pH [14]. Sulphonate additives tend to ion-pair with amino groups in proteins, modifying the analyte charge-to-mass ratio. Although such additives have worked effectively for a number of protein separations, it is often necessary to employ them at high concentrations (>50 mmol/l) resulting in increased buffer conductance and Joule heating. Similarly, use of phosphate buffers has been reported to improve resolution and peak shapes by ion-pairing and wall modification effects. While pH adjustment is commonly used to manipulate protein separations, the pH range is normally restricted to pH>p*I* (isoelectric point) to produce protein–wall charge repulsion for high separation efficiencies.

The use of Starburst dendrimers has been reported in the CE separation of aromatic hydrocarbons [15,16] and dansylated amino acids [17]. A recent review article has examined their use in CE together with a number of other novel surfactants [18]. Dendrimers are also known as unimolecular micelles. Unlike conventional micelles, dendrimers are static structures. They are chemically synthesized starting from a diamine core and the extent of the reaction can be controlled so as to yield dendrimers having different extent of branching. Dendrimers have been applied to drug delivery [19], cell transfection [20], and as immobilizing media for combinatorial synthesis [21]. Their mode of interaction with analyte species has been proposed to involve ion-exchange interactions and hydrophobic partitioning [22].

We report the use of the commercially available Starburst poly(amidoamine) (PAMAM) dendrimers on the separation of chicken sarcoplasmic proteins at pH 7 and in uncoated capillaries. Dendrimers of different generations (0, 0.5, 1.5, 2.5 and 3.5) and in the concentration range 10^{-5}–1% g/ml were used in 20 mmol/l phosphate buffer at pH 7.2. Dendrimer effects on analysis times, separation current, separation selectivity, resolution and efficiency were examined.

2. Experimental

2.1. Instrumentation

The CE instrument was a Quanta 4000 (Waters, Milford, MA, USA) equipped with a pen-ray lamp detector cell assembly and forced air cooling. Detection was at 214 nm (Zn lamp). Data acquisition was done via a Macintosh IIsi computer. A LabVIEW program was written to acquire data. Separation was at an electric field strength of 259 V/cm. Samples were hydrostatically injected for 5 s from 10 cm. A Bruker (Billerica, MA, USA) Proflex matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometer was used to identify proteins in the sample. The mass spectrometer was equipped with a N_2 laser that generated 3 ns duration laser pulses.

2.2. Capillary preparation

Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 75 μm I.D.×365 μm O.D. were used. Unless otherwise noted, capillary end-to-end length was 60 cm and end-to-detection window was 52 cm. Prior to the first run, the capillary was conditioned with the separation medium for periods ranging from 15–30 min. Between runs, the capillary was rinsed with 100 mmol/l KOH for 2 min followed by distilled deionized water for about 2 min using a vacuum (15 mmHg; 1 mmHg=133.322 Pa). When separation electrolytes were changed, the capillary was purged with deionized–distilled water, 100 mmol/l KOH for 5 min followed by a purge with doubly distilled water. For studies where the polymers were used as rinsing agents (0.01–0.5% g/ml), purging of the capillary (15 mmHg) was carried out for 10 min followed by purging with the

separation buffer for 5–10 min. Overnight conditioning of capillaries was done by rinsing with doubly distilled water using gravity-flow. Sample injection was hydrostatic from a height of 10 cm for 5 s unless otherwise noted.

2.3. Reagents

Starburst (PAMAM) dendrimers having generations of 0.5, 1.5, 2.5 and 3.5 were purchased from Aldrich (Milwaukee, WI, USA) and used without further purification. These dendrimers have 8, 16, 32 and 64 surface carboxyl groups, respectively and their molecular masses range from 1 269–12 419 g/mol. Dendrimer solutions were prepared in a phosphate buffer obtained from Waters (Milford, MA, USA) and used in the separation electrolytes at a final concentration of 20 mmol/l and at pH 7.2. The MALDI matrix used was sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid; 0.02690 g) dissolved in 500 μl 0.1% trifluoroacetic acid in water–acetonitrile (2:1) and centrifuged at 10 000 rpm for 1 min. Matrix and 0.1% (v/v) protein sample were mixed at an approximate ratio of 5000:1 and 0.5 μl of the resulting solution was deposited on the MALDI probe and allowed to dry at room temperature. Proteins were purchased from Sigma and used as received.

2.4. Analyte

Frozen chicken tissue was thawed for 1 h. A 30-g sample was homogenized in 30 ml of ice-cold water by grinding the sample at full speed for 15 s, followed by cooling on ice for 45 s. The sample was spun at 4°C for 30 min on an ultracentrifuge. Samples were aliquoted into 500-μl vials and frozen at −20°C. The tissue extract was thawed before use. MALDI-TOF-MS revealed a series of peaks at M_r 20 000, 32 000 and 44 000 corresponding to the proteins myosin (p*I* 6.0), tropomyosin, (p*I* 4.5) and actin (p*I* 4.8), which are known to be abundant in animal muscle tissue [23]. A number of broad peaks were also observed in the M_r $5–10\cdot10^3$ as well as in the M_r $35–45\cdot10^3$ range but could not be assigned with any degree of confidence.

3. Results and discussion

3.1. Dendrimer effects on separation current

The addition of 0.1% dendrimer to the separation buffer resulted in a rise in current of less than 10%; the dendrimeric pseudostationary phase makes a negligible contribution to buffer conductance and Joule heating. This small contribution is probably due to low dendrimer electrophoretic mobility, which is a result of their relative bulkiness and low charge, the latter due to ion-pairing with buffer counterions [22]. In addition, an increase in buffer viscosity with dendrimer concentration will decrease current due to the electroviscous effect [24]. The negligible effects of dendrimers on separation currents allows for tuning of separation selectivity and resolution over a wide range of dendrimer concentration without compromising separation efficiency and migration time reproducibility.

3.2. Anionic dendrimer effects on separation resolution and total analysis times

Fig. 1 presents the separation of the sarcoplasmic sample in a 20 m*M* phosphate buffer in the absence of dendrimers. There are a set of four relatively sharp, early migrating peaks, followed by a poorly resolved, rolling baseline. The first three peaks were identified as actin, tropomyosin, and myosin by co-

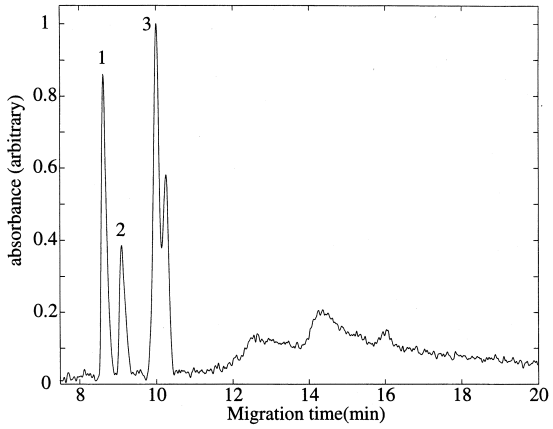

Fig. 1. Electropherogram of chicken protein profile in the absence of dendrimers. Peaks 1–3 co-migrate with actin, tropomyosin and myosin, respectively.

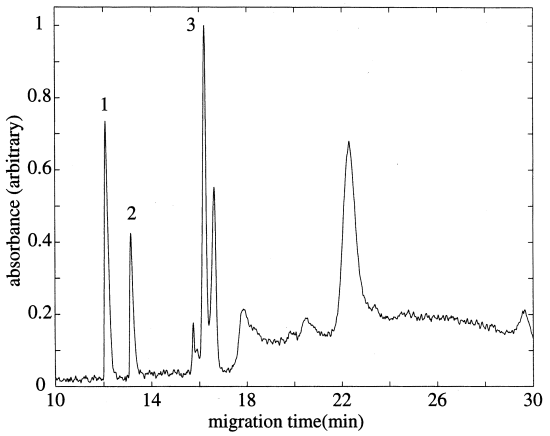

Fig. 2. Electropherogram of chicken protein profile in the presence of 0.001% g/ml G0.5 anionic dendrimer. Peak numbering as in Fig. 1.

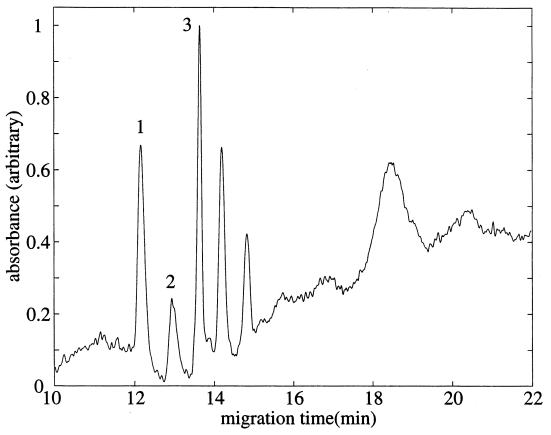

Fig. 3. Electropherogram of chicken protein profile in the presence of 0.1% g/ml G1.5 anionic dendrimer. Peak numbering as in Fig. 1.

migration with standards. There was a dramatic improvement in the separation upon addition of 10^{-3}% g/ml dendrimer G0.5, Fig. 2. A tenfold increase in the concentration of this generation of dendrimer had relatively little effect on the separation.

The addition of 10^{-3}% dendrimer G1.5 produced an electropherogram that was similar to that observed for the same concentration of dendrimer G0.5. However, addition of a relatively high concentration of dendrimer G1.5 produced a much higher resolution separation of the early migrating components, Fig. 3. Finally, a minute amount of the high generation dendrimer G2.5 produced a remarkable effect on the separation, Fig. 4, which generated a noticeable sharpening of the later migrating components.

Ion-exchange protein separations are widely performed in chromatography [25]. Improved resolution in ion-exchange separations often involves increasing ion-exchange capacity, which in this case is equivalent to using higher dendrimer concentration having a nominal or higher dendrimer generation. Ion-exchange interactions are reported to be rapid and reversible in the CE separation of small ions resulting in high efficiencies and good peak shapes [26]. It is likely that the higher generation dendrimers interact primarily with proteins through hydrophobic partitioning [27]. Fig. 4 illustrates improved resolution and similar peak shapes for the early peaks compared to lower generation dendrimers, which indicates the utility of hydrophobic interactions for the CE analysis of larger analytes than the ones conventional micellar electrokinetic chromatography is applied.

3.3. Cationic dendrimer effects on separation resolution and total analysis times

A brief study was conducted to examine the effects the cationic dendrimers (0th generation, 0.001–0.01% g/ml) on protein profiling in 20 mmol/

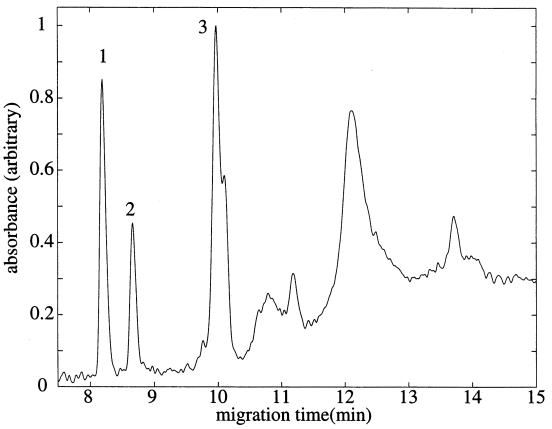

Fig. 4. Electropherogram of chicken protein profile in the presence of 0.0001% g/ml G2.5 anionic dendrimer. Peak numbering as in Fig. 1.

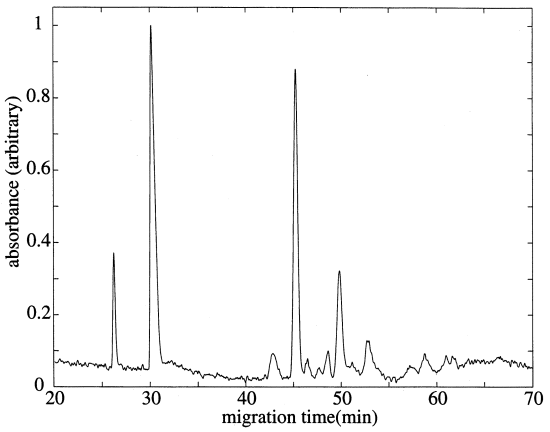

Fig. 5. Electropherogram of chicken protein profile in the presence of 0.001% g/ml G0 cationic dendrimer.

l phosphate buffer at pH 7. The results indicate that although a number of components are resolved at 0.001% g/ml G0, analysis times are quite long (>50 min), Fig. 5. Increasing G0 concentration to 0.01% g/ml results in poor resolution and run times in excess of 90 min, Fig. 6. The increased run times are most likely due to dendrimer–surface silanol interactions [28] which result in decreased electroosmotic flow-rates and thus prolonged migration times. Efficiencies were quite low (16 000–52 000 theoretical plates), which is either due to the inherent heterogeneity of the protein mixture or due to mass transfer limiting protein–wall and/or protein–dendrimer interactions.

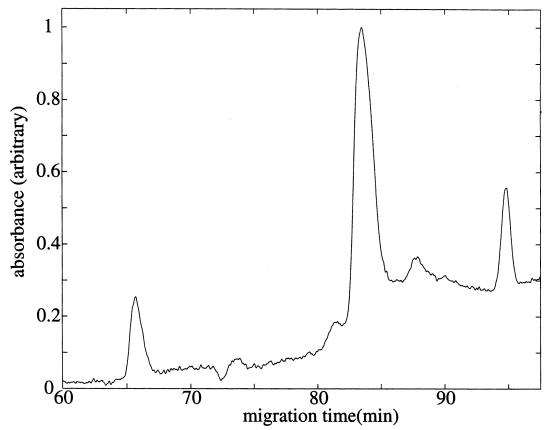

Fig. 6. Electropherogram of chicken protein profile in the presence of 0.01% g/ml G0 cationic dendrimer.

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