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Ultra-fast high-performance capillary sodium dodecyl sulfate gel electrophoresis of proteins

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Abstract

An ultra-fast analysis of proteins, based on sodium dodecyl sulfate (SDS)-mediated gel electrophoresis was developed, in which protein molecular mass standards ranging from M_r 14 200 to 94 700 were separated within 3 min. A 50 μm diameter uncoated fused-silica capillary column and a high field strength are used. The effects of the SDS concentration in the separation gel buffer and in the sample buffer on the resolution of protein test mixture were studied. The influence of the heat treatment of the sample prior analysis is also discussed.

1. Introduction

Capillary electrophoresis (CE) is one of the fastest growing analytical techniques in the biotechnology field. In the last few years new applications have been developed and old, well established methods have been transferred for this low sample and low solvent consuming analytical method.

Since the early development of CE the implementation of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) has been an important goal [1,2]. Traditional SDS-PAGE requires time-consuming gel and sample preparation which are then followed by tedious visualization techniques. An apparent advantage of slab gel electrophoresis is, however, that multiple samples can be analyzed on the same gel. In capillary gel electrophoresis, detection and quantitation are performed on-line [3]. Most of the commercially available instruments are automated and their control and data acquisition are managed by computers.

High-molecular-mass linear acrylamides [4], dextrans [5] and polyethylene oxide (PEO) [6,7] are also appropriate for SDS-mediated protein separations [8,9], when they are used in small-diameter capillaries. Protein molecular mass estimation by SDS-mediated capillary gel electrophoresis can be performed by using a single standard curve or by Ferguson analysis [10,11].

In our study we employed different solutions of M_r 100 000 linear PEO polymer or eCAP200, a commercially available gel for protein analysis [12]. The use of extremely high applied electric field strengths (at the several hundreds V/cm level) and narrow-bore capillary columns allows to attain ultra-fast separation of the test mixture. Effects of the SDS concentration in the separation gel buffer and in the sample buffer were studied on the resolution of the test proteins.

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2. Materials and methods

2.1. Instrumentation

A Beckman P/ACE System 2000 (Beckman Instruments, Fullerton, CA, USA) automated CE instrument, with System Gold instrument control and data evaluation software was used for analysis. The detection wavelength was 214 nm. The temperature of the analysis was set at 20°C unless stated otherwise.

2.2. Materials

The electrophoresis calibration kit for low-molecular-mass proteins (Pharmacia, Piscataway, NJ, USA) contained the following proteins: phosphorylase *b* (PHB, 94 000), bovine serum albumin (BSA, 67 000), ovalbumin (OVAL, 43 000), carbonic anhydrase (CA, 30 000), soybean trypsin inhibitor (STI, 20 100) and α-lactalbumin (ALACT, 14 400). SDS stock solution was from Amres (Solon, OH, USA). The eCAP 200 for SDS-gel electrophoresis was purchased from Beckman Instruments. The M_r 100 000 PEO was purchased from Aldrich (Milwaukee, WI, USA).

2.3. Capillaries

The separations were performed in 100 or 50 μm I.D. and 375 μm O.D. fused-silica capillaries from Polymicro Technologies (Phoenix, AZ, USA). Most of the work was done with capillaries of 20 cm effective and 27 cm total length. The capillaries were first washed with 1 *M* NaOH, HPLC water, 1 *M* HCl and then conditioned with the separation buffer. Between runs the capillary was washed with 1 *M* HCl and water to remove surface adhered material.

2.4. Electrophoresis

For the optimization of the separation buffer 3% (w/v) solutions of the M_r 100 000 PEO were used. The background buffer concentration, pH, except the SDS concentration were kept constant in all experiments and no attempt was made to optimize them. The SDS concentration in the separation buffer system was varied in the experiments between 0.1 and 1.0%. The electrophoresis was performed in uncoated capillaries at various field strengths. The samples were introduced by pressure injection (0.5 p.s.i.; 1 p.s.i. = 6894.76 Pa) using the P/ACE apparatus.

2.5. Samples

The protein samples in general were prepared according to the method described in the Instruction Manual to the eCAP 200 kit [13]. The final sample buffer contained 0.06 *M* Tris–HCl, pH 6.6 with 5% 2-mercaptoethanol and SDS. The SDS concentration was varied between 0.1 and 1.0% and the appropriate concentration is noted in the text. The samples, in general, were boiled for 5 min, than cooled on ice for 3 min, finally the samples were centrifuged, and transferred to the sample vials of the P/ACE system.

3. Results and discussion

3.1. The effect of capillary diameter on the SDS-PEO gel electrophoresis of proteins

The analysis time for SDS-mediated protein separation techniques using CE is usually around 20–30 min. However, when CE is used one of the major claimed advantages is that the separation technique provides on-line quantitative results. The success of the analysis is immediately apparent since the visualization performed by on-line UV detection as opposed to slab gel where the success of the analysis can be checked only after visualization. The documentation and storage of the analysis data is also easier and more comfortable with HPCE.

In HPCE the usual sequence of the analysis is as follows; preparation of the capillary for analysis, introduction of the separation media, injection of sample, analysis of the sample and finally the regeneration of the capillary. Because of the above order the different samples are introduced sequentially, meaning that consecutive samples spend increasing amounts of time in

the autosampler prior to analysis. The incubation of the samples can lead to physical or chemical changes in the samples or in the sample matrix. Such physical change can be the evaporation or polymerization of the sample (matrix) and oxidation or degradation can be mentioned as frequently occurring chemical modifications. The half-lives of the above-mentioned reactions are in the order of magnitude of hours. Consequently if we can shorten the analysis time significantly, some of the problems generated by the long waiting period in the autosampler can be eliminated.

We attempted to shorten the separation time without compromising the resolution provided by the capillary SDS gel electrophoresis. The minimum capillary length in the P/ACE system is 27 cm total length providing 20 cm effective capillary length (from the injection point to the detection window). The separation of standard proteins using a 100 μm I.D. capillary at 300 V/cm is shown in Fig. 1A. Next we repeated the analysis using a 50 μm I.D. capillary assuming that the efficiency would improve because of decreased diffusion and thermal dispersion. The electropherogram obtained under these conditions is displayed in Fig. 1B, showing baseline separation, sharp peaks and good signal-to-noise ratio. It seems that the efficiency improvement is protein dependent and in general the look of the electropherogram remained the same as in Fig. 1A. However, it is important to note that the injection time was increased from 5 to 20 s for comparable peak heights.

An increase of the applied electric field strength in the analysis should increase the rate of migration. This could have two effects; shorter migration times and sharper peaks, i.e. higher efficiency due to the decreased diffusion. However, these effects might be influenced by the Joule heating inside the capillary. Fig. 1C shows the electropherogram at 633 V/cm. The separation is comparable with the previously shown separations but the duration of the analysis is under 5 min.

Table 1 contains some of the data we used for the characterization of the separation such as the separation window which is the difference between the migration times of the last and the first peak, the average peak width at half height, and the peak capacity which we calculated by dividing the separation window with the average peak width at half height. As is apparent, the decrease in the diameter of the capillary does not effect the parameters of Table 1. However, doubling the electric field decreased the separation window and peak width so the peak capacity remained unchanged.

Next we repeated the analysis by doing 10-, 30- and 60-s pressure injections. For these experiments we selected a 633 V/cm field strength. The reproducibility of the injection, measured by the reproducibility of migration times for each standard protein, is listed in Table 2. The relative standard deviations of the migration times for all peaks were under 1%. The signal as a function of the injection time showed good linearity as listed in Table 3.

A further increase of the field strength to 888 V/cm provided good resolution and sharp peaks as shown in Fig. 2, but the effect of Joule heating is apparent on the baseline. All of the six molecular mass standards migrated in less than 3 min. Under these physical conditions it seems that 888 V/cm is the practical limit for the separation, but with better heat exchange an even faster analysis could be performed.

3.2. Effect of SDS concentration in the separation buffer and in the sample buffer

The separations shown above were performed after the sample preparation step has been optimized. It is known from SDS-PAGE that the efficiency of the separations is largely dependent on proper sample treatment. The effects of the SDS concentration in the separation buffer and in the sample were studied on the HPCE separation of a protein test mixture. The quality of the separations were measured by the resolution between the α-lactalbumin and the soybean trypsin inhibitor (peaks 1 and 2). They were selected for the evaluation because these two have the smallest difference in molecular mass, and that is a molecular mass range of considerably interest in general, and for our other applica-

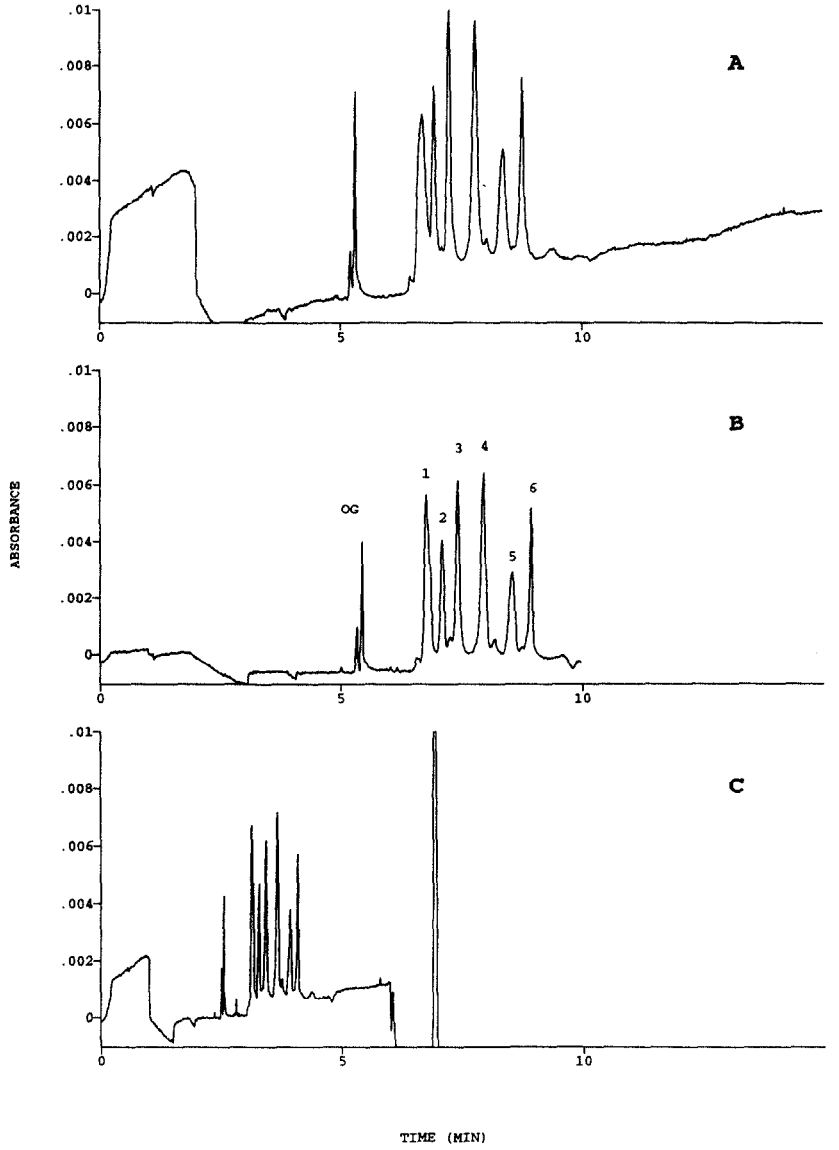

Fig. 1. The effect of capillary diameter on the separation of standard proteins using eCAP SDS 14-200 kit. (A) 27 cm × 100 μm I.D. capillary; 300 V/cm field strength; 5-s pressure injection; (B) 27 cm × 50 μm I.D. capillary; 300 V/cm field strength; 20-s pressure injection; (C) 27 cm × 50 μm I.D. capillary; 633 V/cm field strength; 20-s pressure injection. Peaks: OG = orange G; 1 = ALACT; 2 = STI; 3 = CA; 4 = OVAL; 5 = BSA; 6 = PHB.

tions particularly. The resolution was calculated according to the following formula; $R_s = 2(t_{STI} - t_{ALACT})/(w_{STI} + w_{ALACT})$, where t_{STI} and t_{ALACT} are the migration times for STI and ALACT, while w_{STI} and w_{ALACT} are the peak widths of the STI and ALACT peaks at the baseline. As shown in Fig. 3, the increase of the SDS concentration in the separation buffer from 0.1 to 1% decreases the observed resolution form 2.0 to 1.6. It is important to note that migration time of the peaks did not change significantly when the SDS concentration was increased in the

Table 1
Effect of analysis parameters on the performance of the separation in SDS-PEO gel electrophoresis

Field strength (V/cm)	Capillary diameter (μm)	Separation window (min)	Average half height (min)	Peak capacity
300	100	2.058	0.11	19
300	50	2.167	0.10	22
633	50	0.947	0.05	20

Table 2
Reproducibility of injection for each standard protein

Protein	Migration time (min)	R.S.D. (%) ($n = 3$)
ALACT	3.17	0.83
STI	3.30	0.28
CA	3.44	0.09
OVAL	3.68	0.14
BSA	3.95	0.08
PHB	4.10	0.18

separation buffer. However, the widths of the peaks are changed, consequently this is the reason behind the decrease in the efficiency.

Next we studied the role of SDS in the sample buffer. A 10-fold decrease in SDS concentration in the sample buffer leads to very poor separation of the test proteins (Fig. 3). The observed resolution between α-lactalbumin and soybean trypsin inhibitor peaks drops from 2.2 to 0.94. Again the migration time practically does not change in these experiments and the deteriorated separation corresponds to the amount of SDS present in the sample buffer. Apparently the critical step in terms of the resolution is the formation of the SDS–protein complex. At low concentration of SDS is not sufficient for the formation of homogeneous SDS–protein complexes. At high SDS concentration sufficient SDS is available and good separation could be obtained. Returning to our previous observation on the role of SDS in the separation buffer, it seems reasonable to assume that once the SDS–protein complex is properly formed it remains relatively stable and high concentrations of SDS in the separation buffer are not required for further stabilization. Actually the resolution decreased by 20% when excess SDS was applied.

Table 3
Linear regression data of the peak area vs. injection time plots for different proteins

Protein	Slope	Intercept	r^2
ALACT	1.44	1.37	0.999
STI	0.76	0.62	0.997
CA	1.33	0.75	0.999
OVAL	1.51	0.59	0.999
BSA	0.90	−2.42	0.996
PHB	0.81	−0.62	1.000

3.3. Effect of the sample treatment on the separation

Our data clearly show that the sample treatment is critical to achieve good separation of proteins using SDS-mediated gel electrophoresis. Next we concentrated on the generation of the SDS protein complexes. The heat treatment is a sample preparation step during which the proteins are denatured, unfolded and the SDS–protein complexes are formed. When the protein test mixture was heated up to 80°C for 5 min the peak efficiencies were 27% lower compared to the instance when the same test mixture was immersed in boiling water for 5 min. The magnitude of the efficiency improvement at higher temperatures is protein dependent and probably it is recommended to be optimized for a particular protein of interest. As far as the duration of the heat treatment is concerned, shorter then

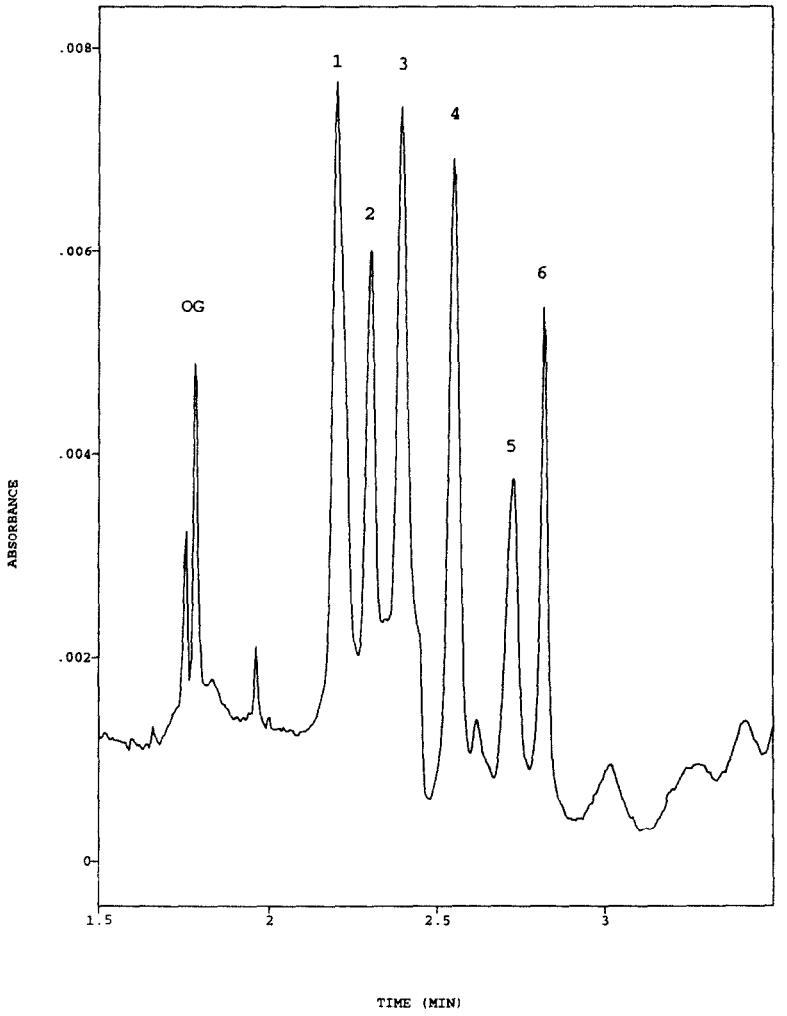

Fig. 2. Separation of standard proteins at high field strength. Capillary dimensions; 27 cm × 50 μm I.D. Electrophoresis conditions: 888 V/cm field strength and 20-s pressure injection. Peaks as in Fig. 1.

5 min boiling time also decreased the separation efficiency.

4. Conclusions

In capillary gel electrophoresis the samples are introduced sequentially. During repetitive analysis this type of sample introduction can lead to unwanted artifacts and/or extra peaks in the electropherogram. The artifacts can be generated by inducing chemical changes in the original sample. These chemical changes could be oxidation or reduction, degradation, association or dissociation or conformational rearrangements of the protein sample. If the half-life of the modification is comparable to the time scale of the series of analysis, the appearance of extra peaks would be expected on consecutive electropherograms. The impact of the modification on the analysis would be reduced by faster separations.

A fast capillary SDS gel analysis method was developed using the eCAP 200-containing separating gel, without compromising efficiency

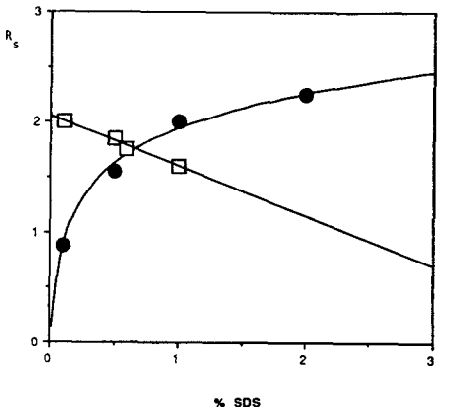

Fig. 3. Effect of the SDS concentration in the separation buffer (□) and in the sample buffer (●) on the resolution between the α-lactalbumin and carbonic anhydrase peaks.

or resolution. The analysis can be performed at 888 V/cm in a 27 cm × 50 μm I.D. capillary in less than 3 min. Based on our results, it is recommended to boil (100°C) the sample proteins with a sample buffer containing 1% SDS and sufficient amounts of reducing agent (5% 2-mercaptoethanol) at least for 5 min. It is important to note that longer boiling times (> 5min) do not cause any significant changes in peak efficiencies and in peak resolution.

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