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Short communication

Separation of bovine serum albumin and its monoclonal antibody from their immunocomplexes by sodium dodecyl sulfate–capillary gel electrophoresis and its application in capillary electrophoresis-based immunoassay

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Abstract

A non-competitive immunoassay was performed by sodium dodecyl sulfate–capillary gel electrophoresis with UV detection using bovine serum albumin (BSA) and monoclonal anti-BSA. BSA, anti-BSA and their immunocomplexes were well resolved under non-denaturing conditions. A linear calibration curve was obtained and can be used for the quantification of anti-BSA. The limit of detection of anti-BSA was 0.1 μM under the present conditions. Compared with capillary zone electrophoresis, we believed that this method has the potential to be used as a more general format for performing capillary electrophoresis-based immunoassay of medium- and large-sized analytes.

Keywords: Bovine serum albumin; Monoclonal antibodies

1. Introduction

Capillary electrophoresis (CE) is a powerful technique for the separation of macromolecules, such as peptides, proteins and immunocomplexes [1]. Capillary electrophoresis-based immunoassay (CEIA) utilizes the selectivity of immuno-reactivity in combination with the sensitivity and efficiency of capillary zone electrophoresis (CZE) to improve the performance of immunoassays [2]. Nielsen et al. [3] first reported the use of CZE with UV detection to separate human growth hormone (hGH) from its immunocomplex at the μM range. In 1993, Schultz and Kennedy [2] first demonstrated the concept of performing competitive and non-competitive immunoassays using CZE with laser-induced fluorescence (LIF) detection.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) is a well-established separation tool for the size separation and purity assessment of protein molecule [4]. Traditional SDS–PAGE requires time-consuming gel preparation and visualization techniques with the advantage that a large number of sample can be analyzed at the same time. Cohen and Karger first demonstrated that high-performance SDS–PAGE can be conducted in narrow, fused-silica capillaries for rapid molecular mass

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determination of proteins and polypetides [5]. SDS–capillary gel electrophoresis (SDS–CGE) has been introduced for the size separation of peptides [5], proteins [6–11] and carbohydrate molecules [12,13] based on their molecular size. The advantages of using SDS–CGE for size separation of protein over conventional SDS–PAGE are direct quantification by on-column UV or LIF detection, high resolution, fast single-sample analysis, and use of nanoliter sample volumes. There is, however, no report of CEIA using the CGE format. Theoretically, this method separates molecules according to the sizes of the molecules. There are certainly size differences between the antigen, antibodies and their immunocomplexes [5]. The antibody–antigen interaction has often led to detectable mobility shift of the resulting immunocomplexes in conventional gel electrophoresis, e.g., [14].

In this paper we report a non-competitive immunoassay by SDS–CGE with UV detection using a commercial SDS 14-200 kit. Bovine serum albumin (BSA), monoclonal anti-BSA and their immunocomplexes were well resolved under non-reducing conditions. A good linear calibration curve was obtained and can be used for quantification of anti-BSA.

2. Experimental

2.1. Chemicals

SDS–CGE was performed using an eCAP SDS 14-200 kit (Part No. 477420) from Beckman Instruments (Fullerton, CA, USA). The kit included an SDS-coated capillary, 65 cm×100 μm I.D., SDS 14-200 gel buffer, SDS Orange G reference marker, SDS sample buffer, 0.12 *M* Tris–HCl–1% SDS, pH 6.6, and SDS 14-200 test mixture containing seven proteins with molecular masses ranged from 14 200 to 205 000. A HiTrap Protein G affinity column (1 ml) (catalogue No. 17-0404-01) was obtained from Pharmacia Biotech (Uppsala, Sweden). Glycine (catalogue No. 161-0718) was from Bio-Rad Labs. (Richmond, CA, USA). Tris–HCl (T-3253), BSA (A4503) and monoclonal anti-BSA (mouse ascites) (B-2901 Lot 027H4822) were purchased from Sigma (St. Louis, MO, USA). Phosphate-buffered saline (PBS) (P-3813, Sigma) was composed of 138 m*M* NaCl and 2.7 m*M* KCl in 10 m*M* phosphate buffer (pH 7.4).

2.2. SDS–CGE

A P/ACE 5010 system (Beckman) equipped with a UV detector was used. An IBM PS/2 Model 350-P100 personal computer with Beckman P/ACE system software controlled the CE unit. A coated fused-silica capillary SDS-coated capillary, 47 cm×100 μm I.D. in an UV cartridge was used. The present CGE system used a reversed polarity mode with the cathode on the inlet and the anode on the outlet. Before separation, the capillary was rinsed with 1 *M* HCl for 2 min and rinsed with gel buffer for 5 min. The SDS 14-200 gel buffer was sonicated for 10 s to remove air bubbles before use. Samples were introduced into the capillary by applied low pressure (0.5 p.s.i.) for 30 s (1 p.s.i.=6894.76 Pa). The separation was performed in the polymer buffer solution supplied with the kit (SDS gel buffer). The voltage used was 14.1 kV (300 V/cm). On-line UV absorbance detection was done at 214 nm. The temperature of the gel-filled capillary columns was controlled to 23°C by the liquid cooling system of the P/ACE instrument.

2.3. Test mixture preparation of CGE

The protein test mixture that came with the eCAP 14-200 kit was dissolved in 750 μl of SDS sample buffer (0.12 *M* Tris–HCl–1% SDS, pH 6.6). A 750-μl volume of deionized water was then added and mixed thoroughly. This protein test mixture was aliquoted into 200 μl fractions, each containing 0.45 mg total protein. The aliquots were stored at −20°C until use. The standards for SDS–CGE were prepared by mixing 200 μl of the test mixture, 100 μl sample buffer, 10 μl Orange G reference marker, 5 μl 2-mercaptoethanol and 85 μl deionized water with a vortex mixer for 2 min until the protein was totally dissolved. The mixture was boiled in a water bath at 100°C for 10 min in a closed microcentrifuge vial, and cooled in an ice bath for 3 min before injection into the CE system.

2.4. Purification of monoclonal anti-BSA

The mouse monoclonal anti-BSA ascites contained large amount of proteins, such as albumin. These proteins interfered with the anti-BSA in the capillary gel electrophoresis. Therefore, the anti-BSA ascites was purified with HiTrap Protein G affinity column before CEIA in CGE.

A 0.5-ml sample of monoclonal anti-BSA (mouse ascites) was loaded onto a HiTrap Protein G (1 ml) column that had been equilibrated with 5 ml of start buffer (20 m*M* sodium phosphate, pH 7.0). The column was first washed with 5 ml of start buffer until no protein was detected by a DU 650 spectrophotometer (Beckman) at A_{280} in the effluent. Anti-BSA was eluted by 3 ml of elution buffer (0.1 *M* glycine–HCl, pH 2.7), neutralized by 1 *M* Tris–HCl, pH 9.0, and desalted by a Centricon-30 concentrator (Amicon, Beverly, CA, USA) which had a molecular mass 30 000 cut-off membrane. The concentration of the purified anti-BSA was 2.96 mg/ml (20 μ*M*) as determined by measuring its absorbance in the spectrophotometer.

2.5. Non-competitive immunoassay of anti-BSA

BSA and purified monoclonal anti-BSA antibody were prepared with 10 m*M* PBS (pH 7.4). To perform non-competitive immunoassays, 25 μl of BSA (60 n*M*) was mixed with 25 μl anti-BSA of different concentrations ranging from 0.1 μ*M* to 16 μ*M*. The mixtures were incubated for 10 min before addition of 25 μl of SDS sample buffer, 2.5 μl of orange G reference marker and 22.5 μl deionized water. The mixture was vortexed for 2 min. Air bubbles in the mixture were removed by centrifugation at 5000 *g* for 2 min before injection. In this experiment, the separations were performed under non-denaturing conditions. No 2-mercaptoethanol was added and no boiling of the sample was done as these procedures could cleave the disulfide bridges within the antibody [6]. Intra-assay variation was defined as the variation in the concentration of anti-BSA determined in three consecutive runs performed on the same day. Inter-assay variation was determined by the variation in the anti-BSA concentration determined in three runs performed on separate days.

3. Results and discussion

3.1. CGE of test mixture

A standard protein test mixture containing α-lactalbumin, carbonic anhydrase, ovalbumin, BSA, phosphorylase B, β-galactosidase, myosine and Orange G (reference marker) are well separated under the manufacturer's recommended CGE conditions (SDS 14-200 kit, Beckman). A linear relationship (r^2=0.9989) existed between logarithm of the molecular mass of standard proteins (14 200–205 000) and 1/relative migration time (RMT) of the protein standards (Fig. 1). This calibration curve was used for subsequent molecular mass determination of antibody and its immunocomplexes.

3.2. CGE of BSA, its antibody and complex

The electropherogram of BSA (30 μ*M*) showed two peaks (Fig. 2A). The larger one was BSA with estimated molecular mass of 64 000. The smaller one was probably contaminant. The limit of detection (LOD) (signal-to-noise ratio=3) of BSA in this condition was 500 n*M*. Intra-assay precision was determined by determining a single sample succes-

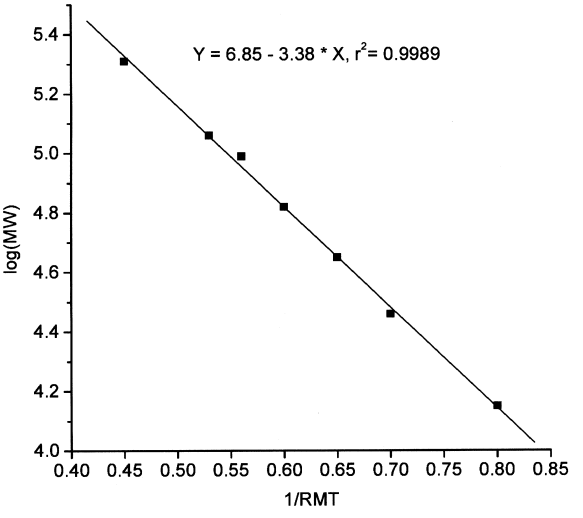

Fig. 1. Molecular mass standard curve of CGE by plotting the logarithm of molecular mass vs. 1/relative migration time (RMT) for protein standards: α-lactalbumin (14 200), carbonic anhydrase (29 000), ovalbumin (45 000), BSA (66 000), phosphorylase B (97 400), β-galactosidase (116 000) and myosine (205 000).

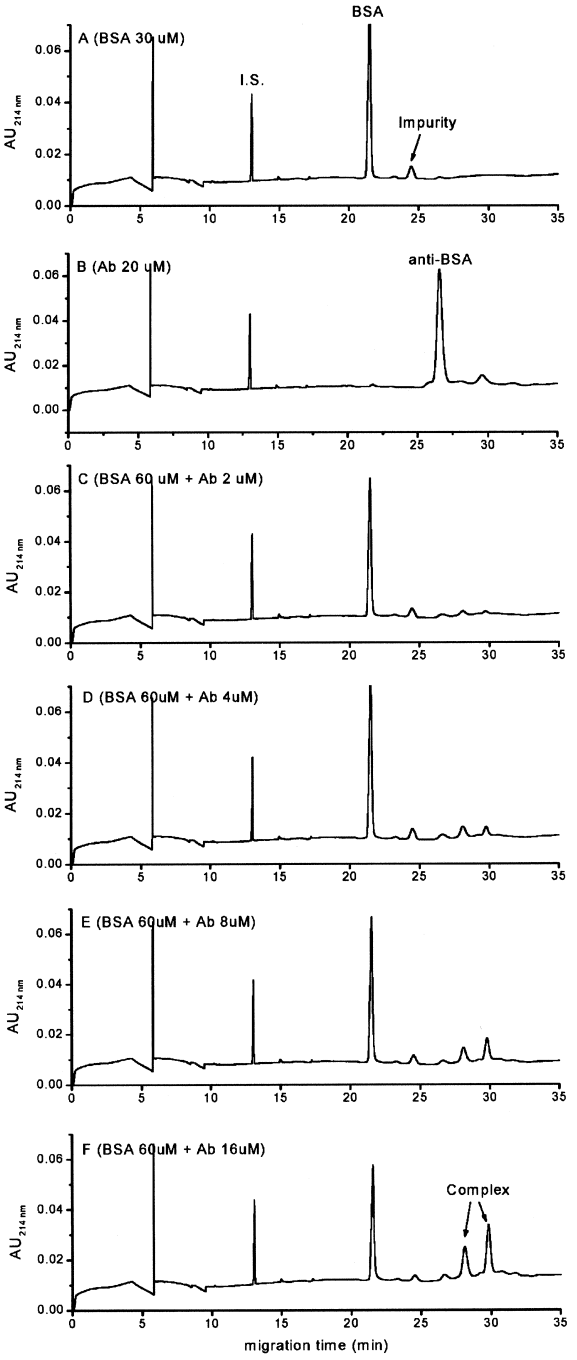

Fig. 2. CGE electropherograms of (A) BSA (30 μ*M*); (B) anti-BSA (20 μ*M*); and the mixtures of BSA (60 μ*M*) with different concentrations of monoclonal anti-BSA (C) 2 μ*M*; (D) 4 μ*M*; (E) 8 μ*M* and (F) 16 μ*M*.

sively for eight times. The mean (±SD) of the intra-day runs of RMT and area ratios of BSA:reference marker were 1.60±0.01 and 2.44±0.01, respectively. The relative standard deviations (RSDs) of the RMT were less than 1% and the RSDs of area ratios of BSA:reference marker were less than 5%. The good reproducibility is probably due to the use of coated capillary and SDS gel buffer, which reduce protein adsorption onto the capillary wall. Fig. 2B shows the purified monoclonal anti-BSA (20 μ*M*) contained two peaks. The dominant one was anti-BSA as its molecular mass was about 147 000. The small one was probably impurities with molecular mass of 231 000.

BSA and anti-BSA were mixed in different molar ratios and the electropherograms are shown in Fig. 2. The two new peaks eluted at the end of the electropherogram with estimated molecular masses of 209 000 and 258 000, respectively, corresponded to the immunocomplexes. As the molecular masses of BSA and anti-BSA as determined by the present CGE system are 64 000 and 147 000, respectively (Fig. 2A and B), the two immunocomplex peaks are likely to represent the binding of one molecule of anti-BSA molecule to one and two BSA molecules.

While denaturing gel electrophoresis is useful in determining the molecular mass of individual subunit in protein molecules, gel electrophoresis in non-denaturing buffer systems allows determination of the molecular mass of proteins in its ''native'' form [15,16]. Denaturation causes separation of the protein subunits and that some of the denatured proteins may have secondary structures different from their native structure. It is known that molecular size and shape affect the mobility of the molecules in sieving gel. In addition, denaturation may also affect the binding of SDS to the protein in the expected 1:1.4 ratio. This results in a change in the charge-to-mass ratios, which in turns causes a change in migration time and therefore the estimated molecular mass [4]. In using CGE for immunoassay, the assay mixture has to be analyzed in a non-denaturing condition. Upon denaturation with 2-mercaptoethanol, antibodies will be broken down into the heavy chain and light chain [7]. However, the factors mentioned above do not seem to have a great effect on the molecular mass determination in the present BSA and anti-BSA system as the molecular masses estimated agree closely to the reported values. This is

probably because that the protein standards used to generate the standard curve are mainly monomeric molecules, whose shape and structure are less likely to be affected in a denatured or non-denatured condition.

In the present system, BSA, anti-BSA and their immunocomplexes were completely baseline resolved by SDS–CGE. This is attributed to the large difference in molecular masses between BSA, anti-BSA and their immunocomplexes. The binding of SDS to proteins in the SDS–CGE condition makes the charge of all protein molecules similar. Thus CGE can separate SDS-bound proteins according to their molecular masses [5,10]. As the concentration of antibody increased, more immunocomplexes were formed, and hence the peak heights and areas of the immunocomplexes increased.

Non-competitive CEIA was performed by adding increasing amount of anti-BSA (0.1–16 μM) to excess amount of BSA (60 μM). The electropherograms are shown in Fig. 2C–F. As the concentration of anti-BSA increased, the peak height and the area of the complexes increased. The areas of the immunocomplexes were proportional to the concentration of anti-BSA as the amount of BSA was in excess. The LOD defined as the concentration of anti-BSA which gave a peak height of immunocomplex three-times the baseline noise ($S/N=3/1$) was 0.1 μM of anti-BSA. The RSDs of assay of area of immunocomplex ranged from 1.88% to 4.60%. A linear calibration curve was obtained by plotting the area of complex versus concentration of anti-BSA ($y=40\ 216x-11\ 559$, $r^2=0.9993$).

Similarly, this CGE method can be used to determine the concentration of the other analytes non-competitively. As CGE can separate antibody, analyte and their immunocomplexes easily using a common separation protocol, this method has the potential to be used as a general format for performing CEIA of medium and large sized analytes. However, those analytes that are not stable in the presence of SDS would not be able to use the present system for CEIA. The CGE format is in great contrast to the CZE format that requires optimization of the separation condition for each analyte. Theoretically, it is also possible to perform competitive CEIA in this format with fluorescent-labeled antigen and LIF detection.

The main drawback of the CGE method is that its sensitivity is low even with fluorescent detection. This is mainly due to the low sensitivity of the detector in case of UV detection or high fluorescent background noise of the CGE gel in case of LIF detection. The other drawback of the method is that the analysis time is much longer than that of the CZE format. The first problem may be solved by using fluorescent dye and laser with excitation wavelength that will not excite the CGE gel or by the use of time-resolved fluorescent detector. The latter increases the signal-to-noise ratio by delaying the determination of the fluorescent signals of the dye and making use of the phenomenon that the autofluorescent signal decays at a faster rate than those emitted by the fluorescent dye. Using a shorter capillary may solve the second drawback. However, this strategy can only be used when the difference in molecular sizes between the antibody and the analyte is large.

4. Conclusion

Non-competitive CEIA of BSA and monoclonal anti-BSA can be performed in SDS–CGE with UV detection. BSA, anti-BSA and immunocomplex can be well separated at the baseline level. Compared with CZE, this method has the potential to be used as a more common format to perform CEIA of medium and large-sized molecules.

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