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Short communication

Immunoassay for human serum albumin using capillary electrophoresis–semiconductor laser-induced fluorometry

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Abstract

Capillary electrophoresis combined with semiconductor laser-induced fluorometry was applied to an immunoassay of human serum albumin. Human serum albumin was labeled with a fluorescent molecule (Cy5), which has an absorption maximum at 649 nm. The labeled albumin was purified by ultrafiltration in order to reduce signals, which are unreacted labeling reagent, product, and fragment products derived therefrom. After the purification, no signal for unreacted labeling reagent and fragment products was detectable in the electropherogram of the labeled albumin. The labeled albumin was then reacted with anti-albumin to form an immunocomplex, which was separated from the excess free albumin. The competitive immunoassay was used in the determination of human serum albumin in a controlled serum sample, using the labeled albumin. The obtained value was found to be 0.21 ± 0.02 mg/ml, which is in good agreement with other known values.

Keywords: Semi-conductor laser-induced fluorometry; Human serum albumin

1. Introduction

High-resolution capillary electrophoresis (CE) is suitable for the determination of biological materials, such as peptides [1], proteins [2], and DNA [3,4], and has recently been shown to be a powerful technique for immunoassay [5–15] because its use involves a short analysis time, small amount of sample, high detection sensitivity, and the capability for simultaneous analysis of multi-components. Capillary electrophoresis immunoassay (CEIA) has been applied to the separation of small molecules, e.g., insulin [5,6], methamphetamine [7], cyclosporin [8], salicylic acid [9], vancomycin [10] and morphine [11]. This method also represents a potentially useful technique for the selective determination of large molecules. However, it is difficult to determine macromolecules, such as proteins by CEIA, since the immunocomplex has nearly the same electrophoretic mobility as the protein. Some studies have been reported on the determination of large proteins, i.e., human immunoglobulin G (IgG) [12,13], bovine serum albumin (BSA) [14], lactoferrin [15] using CEIA. In these reports, the separation efficiency for the immunocomplex was poor because it was adsorbed onto the capillary wall.

On the other hand, laser fluorometry represents an ultra-sensitive detection technique in CE, but practical applications are limited because of the high cost

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of the laser and difficulties associated with its maintenance and operation. A semiconductor laser can be used to solve these problems. Compared with other lasers, diode lasers have the advantages of lower cost and smaller size [16]. Therefore, the semiconductor laser can be used for the determination of amino acids [17], proteins [18], DNA [19], etc., as a conventional detection method with high sensitivity. Cyanine derivatives, which have absorption maxima at red region and produce fluorescence, are often employed as labeling reagents in laser-induced fluorescence detection using a semiconductor laser. For example, Cy5, which has an absorption maximum at 649 nm, is a labeling reagent suitable for excitation by a diode laser emitting at 635 nm [17] and helium–neon laser emitting at 632.8 nm [11]. Wang et al. reported a CEIA method to detect anti-bovine serum albumin in mouse serum using Cy5 [13]. A problem in laser fluorometry using a labeling reagent is that an excess amount of the labeling reagent must be added in order to obtain high labeling efficiency, typically resulting in a considerable number of unfavorable peaks. The sample separation is often unsuccessful because large peaks, which are assigned to the signals of unreacted labeling reagent, and fragment products derived therefrom, are always found in the electropherogram, especially when both the labeling reagent and the sample molecules have similar electrophoretic mobilities. Therefore, the labeled sample molecules must be purified by using a procedure recommended by the manufacturer (gel electrophoresis and/or dialysis) or liquid chromatography [11,13].

In this study, semiconductor laser-induced fluorometry (SLIF) was applied to CEIA. Ultrafiltration was employed to remove the unreacted labeling reagent, which results in some large peaks and makes the detection of trace amounts of the analyte difficult. In the case of the protein used in this study, a large difference in hydrodynamic volume exists between the labeling reagent and protein, so that ultrafiltration can be used as an effective method to exclude the labeling reagent. After purification by ultrafiltration, a competitive immunoassay was examined using the labeled protein. The separation was optimized by pH adjustment and the addition of a surfactant, sodium dodecyl sulfate (SDS), which suppressed the adsorption of the immunocomplex to the capillary wall.

2. Experimental

2.1. Apparatus

The experimental apparatus used in this study is reported elsewhere [20]. A semiconductor laser, emitting at 635 nm (LDA1035, Life Laser-Innovation, Switzerland) with 1.5 mW of output power, was used as an excitation light source for SLIF detection. The laser beam was focused with a objective lens (Nikon 22405, Tokyo, Japan) to the capillary. Fluorescence was collected with a lens (Olympus 101247, Tokyo, Japan) and detected by a photomultiplier tube (R3896, Hamamatsu Photonics) after passing through a pinhole, bandpass filter (CWL: 672 nm, FWHM: 5 nm) and longpass filter (cut-off: $<$640 nm). A high-voltage power supply (HCZE-30P, Matsusada Precision Devices) was used for applying the high voltage. Fused-silica capillaries (50 μm I.D.$\times$375 μm O.D.) were purchased from G.L Science (Tokyo, Japan). The total and effective lengths of the capillary were 60 and 45 cm, respectively. Fluorescence signals were recorded using a personal computer (Power Macintosh 6200/75, Apple Computer) and an interface (compare module, Rainin Instrument). An absorption spectrophotometer, UV-2400PC (Shimadzu, Kyoto, Japan) was employed for the determination of the concentration of labeled human serum albumin.

2.2. Chemicals

Human serum albumin and sodium hydrogenphosphate were purchased from Wako (Osaka, Japan). Monoclonal anti-human albumin (mouse ascites fluid) was obtained from Cedarlane Labs. (Ontario, Canada). Sodium dihydrogenphosphate was purchased from Kishida (Osaka, Japan). CAPS (*N*-cyclohexyl-3-aminopropanesulfonic acid) was obtained from Dojindo (Kumamoto, Japan). Cy5 was obtained from Amersham Life Science (Tokyo, Japan). Normal human serum was purchased from Scantibodies Lab. (CA, USA). All the solutions were

prepared by using deionized water prepared by means of a WG-25 purification system (Yamato Kagaku, Tokyo, Japan).

2.3. Preparation of Cy5-labeled albumin

A Cy5 solution was prepared by dissolving 0.2 mg of Cy5 in 500 μl of dried dimethylformamide. Human albumin was labeled by stirring Cy5 with a solution of 1 mg/ml albumin in a volume ratio of 4:1 for 2 h. After the reaction had been completed, ultrafiltration (15 min, 6000 *g*) was carried out to remove the unreacted labeling reagent with an ultrafree-MC (Millipore: M_r cut-off 10 000). The labeled albumin on the membrane filter was recovered by adding 400 μl of phosphate buffer solution (pH 7.4) in a filter cup of the ultrafree-MC and then mixing by a vortex mixer (Automatic Labmixer HM-10H, Iuchi, Japan) for 1 min. The concentration of the labeled albumin was determined from absorbance and extinction coefficient (250 000 M^{-1} cm^{-1} [21]) at 649 nm. The labeled albumin was stored in a dark room at a temperature of 4°C.

2.4. Competitive immunoassay

Anti-albumin and labeled albumin were dissolved in 100 m*M* phosphate buffer, pH 7.4. Anti-albumin and labeled albumin solutions were diluted to 0.20 and 0.1 mg/ml, respectively, and equal volumes of these solutions were mixed. An aliquot of the mixed solution (10 μl) was placed in a micro-tube, and 0.01~1 mg/ml unlabeled albumin was added. These solutions were incubated for 10 min and then, injected into a capillary for 10 s by means of a siphon method (the inlet was raised 6 cm above the outlet). Serum samples used in this study were prepared by diluting normal human serum obtained from Scantibodies Lab.

3. Results and discussion

A competitive immunoassay using laser-induced fluorometry is a promising technique for the trace analysis of human albumin. However, the signals of unreacted labeling reagents and fragment products derived therefrom in the electropherogram degrade the detection sensitivity of the protein and can obscure the desired signal altogether if their migration behavior is similar. A possible strategy to solve this problem is to optimize the ratio of the protein to the labeling reagent in the preparation. In this approach, optimization of concentration of labeling reagent is required to minimize the effect of the signals derived from unreacted labeling reagents. A low concentration of the labeling reagent might be preferable, in terms of decreasing the signals. However, labeling efficiency would be poor as the concentration of the labeling reagent is decreased. The labeling reagent used in the experiments, Cy5, has a high labeling efficiency from 4 to 10 of the labeling reagents/protein molar ratio. Thus, labeling efficiency must be sacrificed in order to decrease the signals derived from unreacted labeling reagents. Ultrafiltration is useful for sample purification in that it is capable of removing the excess labeling reagents and fragments without decreasing labeling efficiency. Fig. 1 shows the electropherogram of albumin before the ultrafiltration procedure (Fig. 1A) and after (Fig. 1B). The concentrations of albumin in Fig. 1A and B were 50 and 500 μg/ml, respectively. In Fig. 1A, the signal corresponding to the unreacted labeling reaagent appears as the largest peak in the electropherogram. However, after ultrafiltration, no signal derived from unreacted labeling reagents was detectable in the electropherogram. Therefore, ultrafiltration is effective in removing the labeling reagent with an absorption band in the deep red region. However, it should be noted that a loss of sample always occurred, due to adsorption on the membrane filter (<10%). Such adsorption is explained by hydrophobic and/or electrostatic interactions between cellulose membrane and labeled albumin as follows. The isoelectric point (p*I*) value of native albumin is around 4.5 so that albumin is negatively charged at pH 7.4, which is the pH value of the buffer solution used for recovering the labeled albumin from the membrane filter. However, p*I* of the labeled albumin is changed since amino groups have reacted with Cy5. Furthermore, several amino groups are unreacted with Cy5, so that albumin molecule can have protonated amino groups at side chains. Therefore, adsorption may be the result of

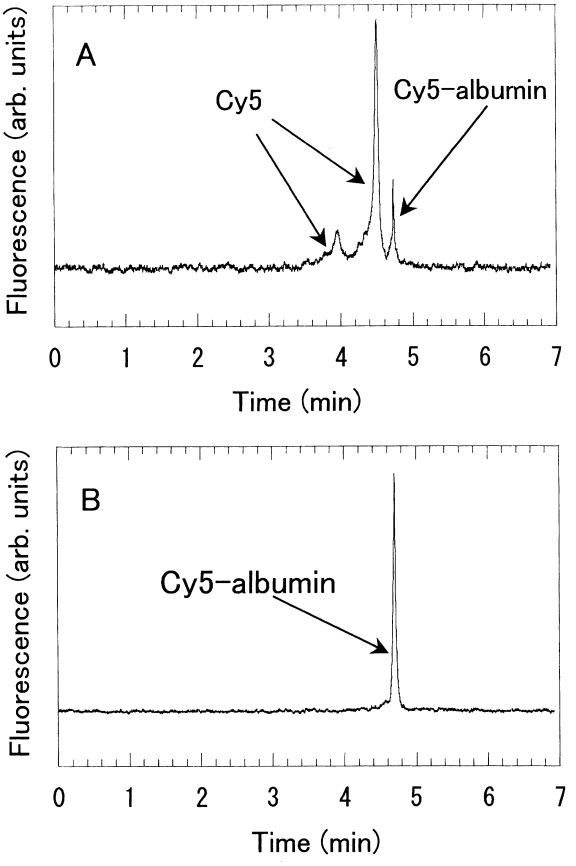

Fig. 1. Electropherogram of labeled albumin. (A) Unfiltered sample; the concentration of albumin, 50 μg/ml, (B) filtered sample; the concentration of albumin, 500 μg/ml. CE buffer; 100 m*M* CAPS buffer (pH 9.5), LIF detection at 635 nm, applied voltage of 17 kV (5 μA), the uncoated capillary: 60 cm (effective length 45 cm)×50 μm I.D. Other conditions as described in the text.

hydrophobic and/or electrostatic interactions between the negatively charged cellulose membrane and the labeled protein, which has a partially positive charge.

We initially attempted a non-competitive immunoassay for the determination of albumin by using labeled anti-albumin. However, in this case the immunocomplex could not be separated from labeled anti-albumin because the molecular mass of the immunocomplex (M_r 217 000) is close to that of the anti-albumin (M_r 150 000) [11] and there is no difference in the electrophoretic mobilities between the anti-albumin and immunocomplex. Therefore, competitive immunoassay was examined. The labeled albumin could be separated from its complex due to the large difference in molecular mass. Furthermore, several buffer solutions (pH 7.0~10.0, buffer concentration 50~200 m*M*, SDS or no SDS) were examined, in an attempt to obtain good resolution (the value calculated from the electropherogram was 1.7). A 100 m*M* CAPS buffer (pH 9.5) with no SDS was found to give the highest resolution. Fig. 2 illustrates the electropherograms obtained by varying the concentration of the anti-albumin. The sample solutions contain a specific amount of labeled albumin (0.20 mg/ml) and various amounts of anti-albumin (0.03~0.04 mg/ml). The two peaks were assigned to free albumin and the albumin–anti-albumin complex, although peak broadening was observed for the immunocomplex. The reason for this peak broadening is adsorption of the complex to the capillary wall due to the hydrophobicity of the anti-albumin. Anti-albumin is relatively more hydrophobic than albumin, resulting in its adsorption to the wall. We also observed a tailing peak, which usually indicates adsorption of an analyte to the capillary wall, when the labeled anti-albumin was used as a sample (data not shown). As concentration of anti-albumin is increased, the peak intensity of the immunocomplex becomes higher. Based on these results, a competitive immunoassay was performed to determine albumin under separation conditions of 100 m*M* CAPS buffer (pH 9.5)

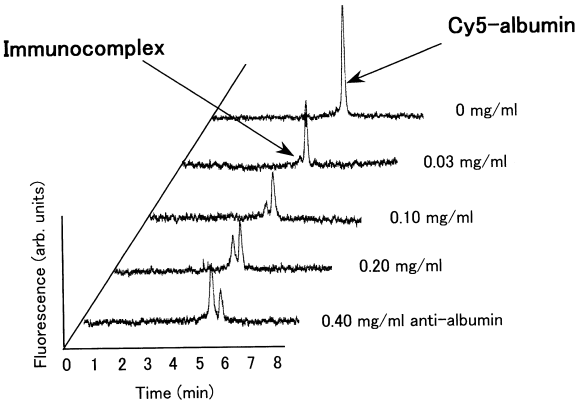

Fig. 2. Electropherogram showing the separation of the immunocomplex (labeled albumin–anti-albumin) from the labeled albumin by using CE–SILF. Each sample contains 0.20 mg/ml of labeled albumin, and 0.03, 0.10, 0.20, or 0.40 mg/ml of anti-albumin.

and 17 kV of applied voltage (total length of capillary: 60 cm, effective length: 45 cm, electric field: 280 V/cm). The concentrations of labeled albumin and anti-albumin were 0.1 and 0.2 mg/ml, respectively. Fig. 3 shows the electropherograms obtained by adding native albumin to a solution which contained labeled albumin and anti-albumin. Fig. 4 shows the relation between the ratio of the peak area of the labeled albumin to that of the complex vs. the logarithm of the concentration of native albumin. Each data point represents an average value obtained from four runs. The detection limit, which was defined as the concentration when a ratio of the change in the peak height of free labeled albumin to noise is equal to 3, was 0.02 mg/ml, and an albumin concentration of 0.02~1.0 mg/ml can be reliably determined by the present assay. The concentration limit of detection is not as good as those reported by the other methods [22,23], However, mass sensitivity is much higher because of the small injection volume. Furthermore, the present method can offer several advantages in addition to high speed, low cost, and simplicity in the operation which requires no wash step in the reaction. These results indicate that the present method can clearly be applied to the quantitative analysis of albumin.

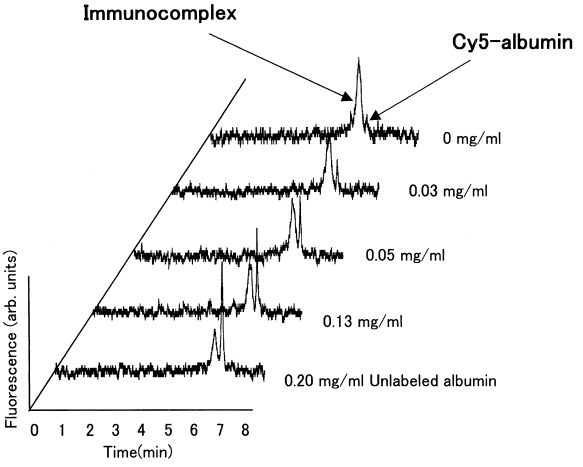

Fig. 3. Electropherogram showing the separation of immunocomplex from labeled albumin in a competitive immunoassay using CE–SILF. The sample solutions contain 0.20 mg/ml of anti-albumin, 0.10 mg/ml of labeled albumin, and 0.03, 0.05, 0.13, or 0.20 mg/ml of unlabeled albumin. For conditions see Fig. 1.

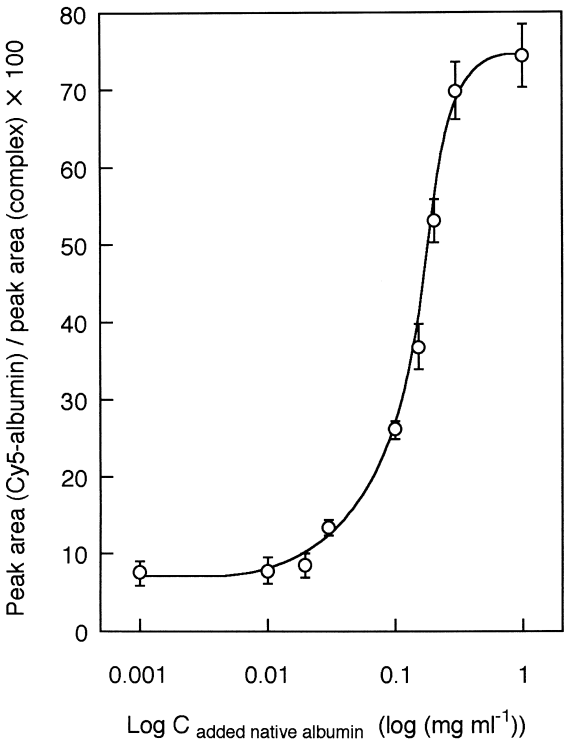

Fig. 4. Calibration curve for the competitive immunoassay in CE–SILF as the increase in the ratio of labeled albumin after the addition of different amounts of unlabeled albumin. Error bars in the figure show one standard deviation for the ratio of labeled albumin determined.

Albumin in a standard human serum was determined by using the calibration curve shown in Fig. 4 in order to confirm whether this method is applicable to the determination of albumin in the solution containing various materials in the living body. Using sample solutions of 0.2 and 0.15 mg/ml albumin, excellent values were obtained for four runs (0.21 ± 0.02 mg/ml and 0.15 ± 0.03 mg/ml). Therefore the present technique provides reliable values in the determination of albumin in human serum.

4. Conclusion

A competitive immunoassay using CE–SLIF easily separated the free antigen and immunocomplex and was used to accurately determine albumin levels. Furthermore, this method could be applied to analy-

sis of human serum sample and gave excellent quantitative values. Ultrafiltration was found to be an effective and necessary pretreatment in removing unreacted labeling reagent and fragments derived therefrom, from the labeled antigen solution, which was used as a probe. The use of immunoassay for the determination of large macromolecules, such as albumin, has considerable potential for use in areas of clinical chemistry since this method can be remarkably miniaturized. In the future, we plan to apply this method to the diagnosis of various diseases and to further extend this method for use in microfluidic device format.

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