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Observation of the complex formation between Cu(II) and protein by capillary electrophoretic system incorporating an UV/CL dual detector

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Abstract

We investigated the complex formation between Cu(II) and human serum albumin (HSA) through a biuret reaction by use of capillary electrophoretic system incorporating an ultra-violet absorption (UV) and chemiluminescence (CL) dual detector. Cu(II)–tartrate complex and Cu(II)–human serum albumin complex were detected by UV detection (282 nm) with on-capillary, followed by CL detection (luminol–hydrogen peroxide CL reaction) with end-capillary. We examined the effects of the reaction time and temperature on the UV and CL responses. On the basis of the obtained results we considered the Cu(II)–human serum albumin complex formation processes and its catalytic activity for the CL reaction. The system easily, rapidly, and simultaneously produced useful information concerning the complex formation of Cu(II) and human serum albumin due to the presence of the both detectors.

Keywords: Capillary electrophoresis; Chemiluminescence; Biuret reaction; Dual detector; Cu(II)–human serum protein complex

1. Introduction

The capillary electrophoretic (CE) system has received much attention as a powerful separation instrument in the fields of not only analytical chemistry but also pharmaceutical chemistry and medicine [1–4]. Absorption and fluorescence phenomena are the most basic principles of the detection technique in instrumental analysis, and they have been commonly adopted as a detection technique in CE systems. While chemiluminescence (CL), which has a profound relationship to the above detection principles, has also been found to be a useful detection technique in FIA, HPLC, and CE systems [5–9].

We developed a capillary electrophoretic system incorporating an ultra-violet absorption (UV)/CL dual detector, taking advantage of the CL reaction of luminol–hydrogen peroxide and the batch-type CL detection cell [10]. UV detection was carried out using the on-capillary method while CL detection was performed using the end-capillary method. In the previous study examination of isoluminol isothiocyanate (ILITC) as a model sample revealed two main peaks with UV detection and one main peak with CL detection. The first peak in the UV detection data corresponded to the main peak in the CL detection data. We then determined that the ILITC sample included natural ILITC as well as an impurity that had absorption behavior but did not have CL property and labeling ability. The system quickly and precisely provided information concerning the impurity via the UV/CL dual detector.

On the other hand, the biuret reaction is well known as an analytical method for determining protein through the complex formation between Cu(II) and protein in an alkaline solution [11–13]. The biuret reagent is prepared by dissolving copper sulfate (II), potassium sodium tartrate (masking reagent), and sodium hydroxide into water in this order. Potassium sodium tartrate works as a masking reagent to avoid polymerization of Cu(II) hydroxide through hydrolysis reaction in an alkaline solution. The biuret reaction is generally carried out by mixing protein (analyte) and biuret reagent at 37 °C for ca. 1 h.

The UV/CL dual detector that we have developed in CE not only can adapt to large amount of analytes possessing absorption or affecting CL in a CL reaction, but also can provide multi-

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information for the same sample in CE. In this study, in order to demonstrate the unique performance of the CE with UV/CL dual detector, we tried to examine the complex formation processes in the biuret reaction, i.e., the complex formation between Cu(II) and protein, with the CE system.

2. Experimental

2.1. Reagents

All reagents used were of commercially available and analytical grade. Ion-exchanged water was distilled for use. Luminol was purchased from Nacalai Tesque. Human serum albumin (HSA) was purchased from Sigma Co. Hydrogen peroxide solution (30 wt.%) was purchased from Wako Pure Chemical Industries, Ltd.

2.2. Biuret reaction

Biuret reagent (1.0×10^{-3} M Cu(II), 2.0×10^{-2} M potassium sodium tartrate as a masking reagent, and 1.0×10^{-2} M sodium hydroxide) was prepared according to the conventional preparation procedure. An aliquot amount of HSA was dissolved with the biuret reagent in order to start the biuret reaction, which was allowed to proceed for different durations and at different temperatures. After the reaction the solution was subjected to the CE system with the dual detector.

2.3. CL detection cell

A batch-type CL detection cell [10] was used in the CE system incorporating the UV/CL dual detector. The detection cell was made of quartz and the inner volume was ca. 0.8 ml. The CL detection cell was enclosed in a small light-tight box together with a photomultiplier tube to produce a compact CL detector. A fused-silica capillary and a platinum wire as a grounding electrode were fixed to the detection cell. In other words, the cell also worked as an outlet reservoir including an electrolyte solution. As analytes emerged from the capillary, they reacted with reagents to produce visible light. The CL light was detected by the photomultiplier tube located at the bottom of the cell.

2.4. Analytical procedure

A 10 mM phosphate buffer (pH 10.8) was prepared as the running buffer. A 10 mM phosphate buffer (pH 10.8) containing 2×10^{-2} M hydrogen peroxide and 5×10^{-3} M luminol was left for 18 h at 4 °C, and then used as the cell buffer, because in the preliminary experiment it was found that a base-line CL became more stable after the mixture was left in the way.

A high voltage (10 kV) was applied to electrodes using a dc power supplier (Model HCZE-30PNO. 25, Matsusada Precision Devices Co. Ltd.). A fused-silica capillary of 75 μm i.d. and 75 cm length was used; 50 cm length for UV detection (282 nm) and 75 cm length for CL detection. Sample injections were performed by gravity for 15 s at a height of 25 cm. The sample migrated into the running buffer toward the CL detection cell and mixed with the reagents. The absorption detection was carried out on-capillary by a modified SPD-6A spectrophotometric detector (Shimadzu Co.), followed by end-capillary CL detection by the CL detector. The outputs from the detectors were fed to an integrator (Chromatopac C-R8A, Shimadzu Co.) to produce electropherograms.

3. Results and discussion

3.1. Electropherograms of a mixture of Cu(II) and HSA after the biuret reaction

As described in the Section 2, a sample of a mixed alkaline solution of Cu(II) and HSA, which was prepared by mixing biuret reagent and HSA, was injected. Free Cu(II) (strictly speaking, Cu(II)–tartrate complex) and Cu(II)–HSA complex were detected by both UV and CL detection. Fig. 1 shows the electropherograms obtained for the Cu(II) and HSA mixtures, which were prepared with different reaction times of 0, 60, 120, and 240 min at 20 °C. In UV detection, Cu(II)–HSA complex and free Cu(II) were detected based on the absorption of the wave length 282 nm at ca. 13 and 30 min, respectively, and in CL detection they were detected based on the catalytic activity of the complexes for luminol–hydrogen peroxide CL reaction at ca. 20 and 45 min, respectively. As shown here, CL detection was more sensitive than UV detection.

3.2. Effect of reaction time on UV response

In UV detection, as the sensitivity was poor, we were unable to measure the peak area of free Cu(II) (Cu(II)–tartrate complex). We had not expected the detection of Cu(II)–tartrate complex with UV detection, because of its very low molar absorption coefficient. However, the peaks were observed on the elctropherograms as shown in Fig. 1, although without quantitativeness. The peaks were identified to Cu(II)–tartrate complex by considering the retention times obtained with UV and CL detections.

HSA possesses a molar absorption coefficient of ca. 35,000 at 280 nm [14]. So that, the sensitivity of absorption detection for HSA is not sufficient in CE using a capillary less than 100 μm i.d., at most 10^{-5}–10^{-6} M [15], although it much depends on each instrument. In this study the peak area of the Cu(II)–HSA complex was detected and quantitatively plotted against the reaction times (0–300 min) (Fig. 2), although they were small. The peak area was almost constant above 120 min, indicating that the complex formation between Cu(II) and HSA in the biuret reaction almost reaches equilibrium.

3.3. Effect of reaction time on CL response

In Fig. 3 the peak areas of free Cu(II) (Cu(II)–tartrate complex) and Cu(II)–HSA complex in the CL detection are plotted against the reaction times (0–300 min). The peak area of free Cu(II) decreased up to ca. 120 min and remained almost constant thereafter. Thus, the complex formation between Cu(II) and HSA in a biuret reaction almost reaches equilibrium. However,

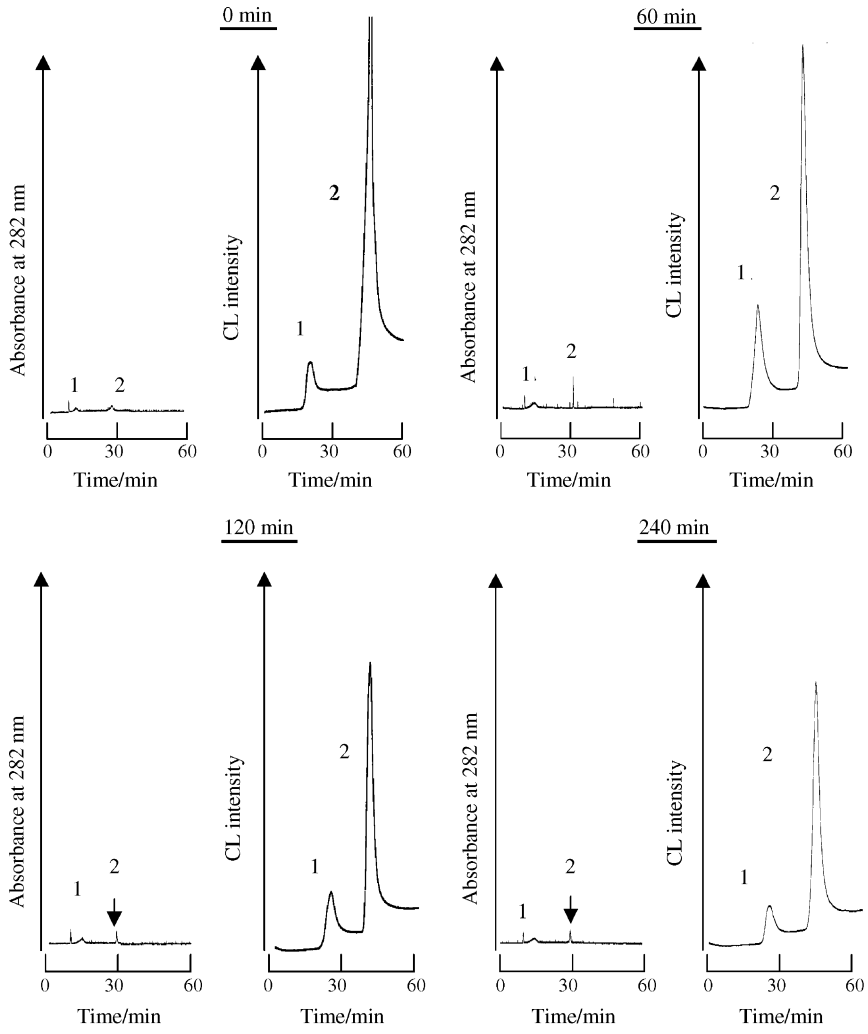

Fig. 1. Electropherograms of a mixture of Cu(II) and HSA after the biuret reaction obtained using the CE system incorporating a UV/CL dual detector. Peak identifications: (1) Cu(II)–HSA complex and (2) free Cu(II) (Cu(II)–tartrate complex). Conditions: Fused silica capillary of 75 μm i.d., effective length of 50 cm for UV detection and effective length of 75 cm for CL detection, applied voltage; 10 kV, reagent; 10 mM phosphate buffer (pH 10.8) as a migration buffer and 10 mM phosphate buffer (pH 10.8) containing 2.0×10^{-2} M hydrogen peroxide and 5×10^{-3} M luminol in the outlet reservoir, and sample; mixture of 4.0×10^{-5} M HSA and 1.0×10^{-3} M Cu(II) at 20 °C.

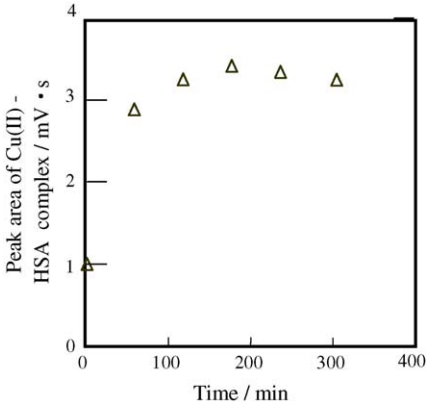

Fig. 2. Relationships between the reaction time and UV response. Conditions: fused silica capillary of 75 μm i.d., effective length of 50 cm for UV detection and effective length of 75 cm for CL detection, applied voltage; 10 kV, reagent; 10 mM phosphate buffer (pH 10.8) as a migration buffer and 10 mM phosphate buffer (pH 10.8) containing 2.0×10^{-2} M hydrogen peroxide and 5×10^{-3} M luminol in the outlet reservoir, and sample; mixture of 4.0×10^{-5} M HSA and 1.0×10^{-3} M Cu(II) at 20 °C.

the peak area of the Cu(II)–HSA complex indicated a maximum CL peak area at around 60 min. Hence, the catalytic activity of the Cu(II)–HSA complex for luminol and hydrogen peroxide CL reaction must decrease when the reaction time is greater than ca. 100 min. We previously reported the decreasing of the catalytic activity of Cu(II) complex in the CL reaction in FIA for protein analysis [16], in which the four coordinating sites of Cu(II) were occupied by protein as a biopolymer [17,18].

Furthermore, we tried to calculate the binding mole ratio of Cu(II) to HSA in the following way. By using the calibration curve of free Cu(II) in the CL detection data and the peak area of free Cu(II) in Fig. 1 (120 min), the amount of the bound Cu(II) to HSA surface was estimated. And then, a mole ratio of 12:1 (Cu(II):HSA) was obtained under the present conditions.

3.4. Effect of reaction temparature on UV and CL responses

We also examined the effect of the reaction temperature on the electropherogram of the mixture of Cu(II) and HSA after the

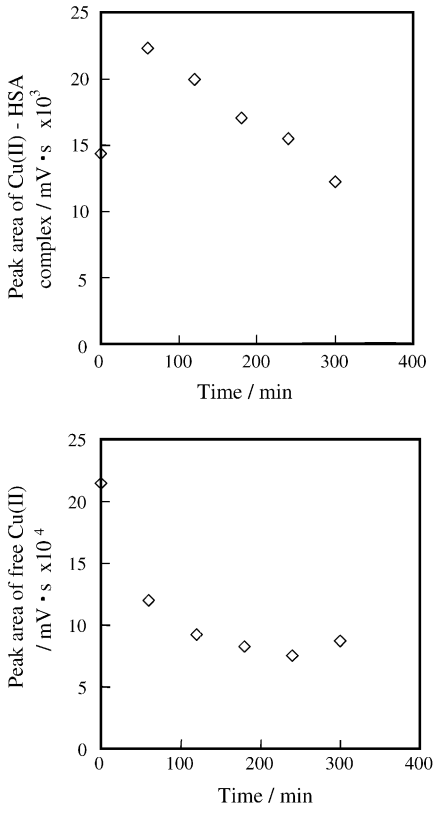

Fig. 3. Relationships between the reaction time and CL response. Conditions: fused silica capillary of 75 μm i.d., effective length of 50 cm for UV detection and effective length of 75 cm for CL detection, applied voltage; 10 kV, reagent; 10 mM phosphate buffer (pH 10.8) as a migration buffer and 10 mM phosphate buffer (pH 10.8) containing 2.0×10^{-2} M hydrogen peroxide and 5×10^{-3} M luminol in the outlet reservoir, and sample; mixture of 4.0×10^{-5} M HSA and 1.0×10^{-3} M Cu(II) at 20 °C.

biuret reaction (30, 50, 70 and 90 °C for 1 h). In UV detection, the peak area of free Cu(II) decreased while that of Cu(II)–HSA complex increased with increasing reaction temperature. Thus, the complex formation proceeded more effectively at higher temperature.

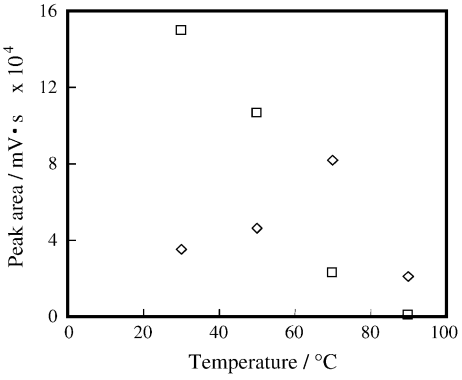

Fig. 4. Relationships between the reaction temperature and CL response. (◊); Cu(II)–HSA complex and (□); free Cu(II). Conditions: fused silica capillary of 75 μm i.d., effective length of 50 cm for UV detection and effective length of 75 cm for CL detection, applied voltage; 10 kV, reagent; 10 mM phosphate buffer (pH 10.8) as a migration buffer and 10 mM phosphate buffer (pH 10.8) containing 2.0×10^{-2} M hydrogen peroxide and 5×10^{-3} M luminol in the outlet reservoir, and sample; mixture of 4.0×10^{-5} M HSA and 1.0×10^{-3} M Cu(II) for 1 h.

The resultant peak areas in CL detection are plotted against the reaction temperatures in Fig. 4. The peak areas of free Cu(II) decreased with increasing reaction temperature, also indicating that complex formation proceeded more effectively at higher temperature. On the other hand, a maximum peak area of Cu(II)–HSA complex was achieved at a temperature of about 70 °C. Although the concentration of Cu(II)–HSA complex increased with increasing reaction temperature, the catalytic activity of the complex decreased as the complex formation became more tight at the higher reaction temperatures, which led to four coordinating sites-occupied Cu(II) complex. The above two phenomena must bring about the maximum CL peak area due to Cu(II)–HSA complex shown in Fig. 4.

4. Conclusion

Conditioning of the capillary inner wall is one of the most important steps in the CE system. The difficulty in controlling the state of the inner wall often leads to poor reproducibility in CE analysis, and thus comparing data obtained using different CE systems is problematic. However, in the present system, the same sample is subjected to one capillary electrophoretic procedure, and then detected by both UV and CL detectors. The UV and CL data are simultaneously obtained for the same sample, and easily and accurately compared. We successfully demonstrated that the CE system, incorporating a UV/CL dual detector, provided useful and interesting information regarding Cu(II)–protein complex formation through biuret reaction. The UV/CL dual detector not only can adapt to large amount of analytes possessing absorption or affecting CL in a CL reaction, but also can provide multi-information for the same sample in CE.

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