Capillary Zone Electrophoretic Separation of Protein Labeled with Rhodamine B Isothiocyanate and Its On-Line Chemiluminescence Detection

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Synopsis. To improve the sensitivity of on-line chemiluminescence dectection of proteins separated by capillary zone electrophoresis, rhodamine B isothiocyanate has been used as a dye-stuff for labeling proteins. By the present method, bovine serum albumin (BSA) in the concentration range of 7×10^{-8}—7×10^{-6} M (1 M=1 mol dm^{-3}) could be determined with a lower detection limit of 5×10^{-8} M (absolute amounts: 1 fmol).

During an investigation regarding the separation of proteins by capillary zone electrophoresis (CZE) in a phosphate buffer solution (pH 3.5), Eosine Y (EY) was found for the first time to comigrate with a protein in a capillary tube in a complexed form,[1] followed by chemiluminescence detection (CLD) of EY associated into the complex. The present method was successfully extended using Rose Bengal (RB) in place of EY.[2] The detection limit of BSA was 4×10^{-7} M for EY and 2×10^{-7} M before RB. Since rhodamine B isothiocyanate (RITC) was expected to be more sensitive in the CLD than RB,[3] RITC was used to improve the sensitivity of CLD in this work.

Experimental

All of the reagents used were of a commercially available analytical-reagent grade. Deionized water was distilled before use. Each 1×10^{-4} M solution of EY and RITC was prepared by dissolving them in a phosphate buffer solution (pH 3.5). BSA, human serum albumin (HSA), human serum γ globulin (HγG) and α-lactalbumin (α-lacta) (molecular weights: 66296, 66241, 156000, and 14200, respectively) dissolved in a 25 mM phosphate buffer solution (pH 3.5) were used. An acetonitrile solution containing hydrogen peroxide (30% H_2O_2) and bis (2,4,6-trichlorophenyl) oxalate (TCPO) was used as a chemiluminescence (CL) reagent. A surfactant (FC-135) offered by Sumitomo-3M Co. was added to a carrier solution to achieve excellent resolution.

The experiment was carried out using the same apparatus and procedure as that reported in a previous paper, except for using (1) an imidazole buffer solution (pH 6.0) instead of a phosphate buffer solution and (2) a DC power supplier delivering 0—−20 kV.

A freshly available capillary tube was treated with 1 M hydrochloric acid for 10 min and washed with distilled water before use. The carrier solution containing a definite amount of FC-135 was filtered through a MILLIPORE Millex-GSSLGS0250S filter and degassed before use. The capillary tube was filled with a carrier solution by means of a 100 μl microsyringe before use. First of all, a high voltage was applied while feeding the imidazole buffer solution alone at a rate of 5 μl min^{-1} using a pump. Then, the CL reagent containing H_2O_2 and TCPO was fed at a rate of 15 μl min^{-1} by the other pump until the stationary electrolytic current was obtained. A sample solution was introduced into the capillary tube by siphoning after a high voltage had been made off. A high voltage ranging from 0 to −20 kV was gradually applied over a period of twenty seconds under the operation of pumps. The CL measurement was started as soon as the voltage reached −20 kV.

Result and Discussion

Both spectrophotometric and CZE properties of proteins, dye-stuffs, and complexes were between those examined, and the following results were obtained. The absorption spectra of RITC and its BSA complex in the wavelength region between 400 and 800 nm were almost the same, and showed their maximum absorptions at 560 nm. A mixed solution (pH 3.5) of RITC and BSA was subjected to CZE; the two electropherograms shown in Figs. 1 (A) and (B) were at 560 and 210 nm, respectively. Another electopherogram (C) was also obtained at 560 nm for RITC alone (Fig. 1 (C)). In our previous paper,[1] EY showed a bathochromic shift (from 519 to 531 nm) in the presence of BSA, and was found to comigrate with BSA in CZE. From the results shown in Fig. 1, RITC was confirmed, similarly to EY, to comigrate with BSA in CZE, though RITC showed no bathochromic shift in the presence of BSA under conditions similar to those used for EY. Though RITC has two geometrical isomers, based on the linked position of an isothiocyanate group, and gave two peaks corresponding to the individual isomers in the electropherogram (Fig. 1 (C)) detected at 560 nm, only one peak due to a RITC-BSA complex in the presence of BSA was observed in the electropherogram (Fig. 1 (A)). This means that the complexes between BSA and the RITC isomers could not be separated under the CZE conditions used, because the molecular mass of BSA was much larger than that of RITC and the mobility in CZE depended mainly on size and charge of the complex ion. A result similar to this was also obtained from the CL data, as shown below.

Each complex between RITC and BSA or HSA or HγG was subjected to CZE, and their respective electropherograms were obtained using CL detection. The CL intensity was examined against the reaction time after mixing at pH 3.5. The result showed that the reaction between RITC and BSA (or HSA) went to completion within 1 h, while the reaction between RITC

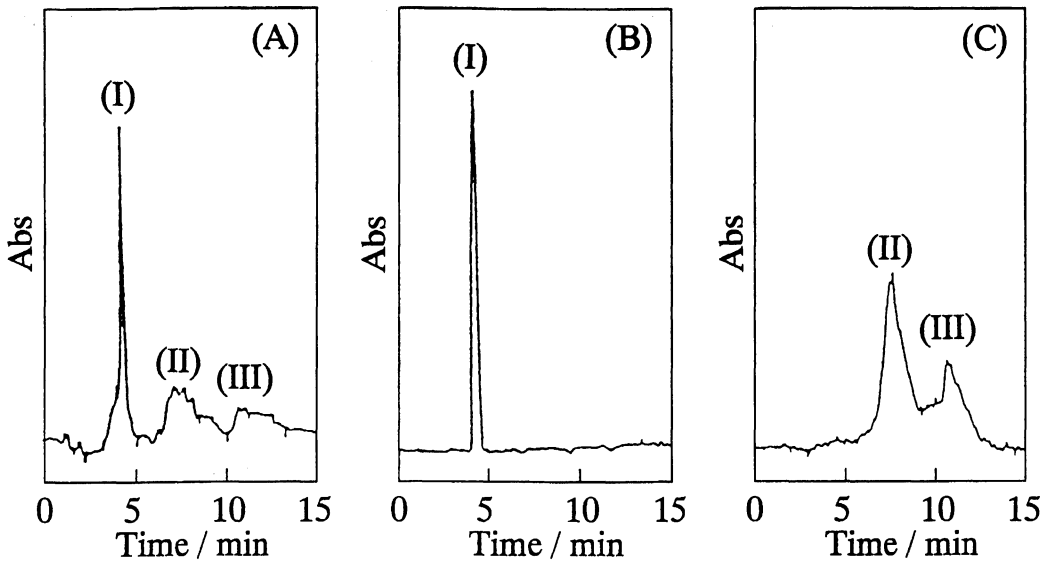

Fig. 1. Electropherograms of RITC and RITC–BSA complex with UV-vis detection. Capillary, 60 cm of 50 μm i.d. fused silica, 35 cm separation distance; Applied voltage, −20 kV; Buffer, 25 mM phosphate containing 50 ppm FC-135 (pH 3.5). (A) Sample: 1×10^{-5} M BSA solution containing 2×10^{-4} M RITC, Detection λ=560 nm. (I) RITC–BSA complex; (II,III) isomers of RITC. (B) Sample: 1×10^{-5} M BSA solution containing 2×10^{-4} M RITC, Detection λ=210 nm. (I) RITC–BSA complex. (C) Sample: 2×10^{-4} M RITC, Detection λ=560 nm. (II,III) isomers of RITC.

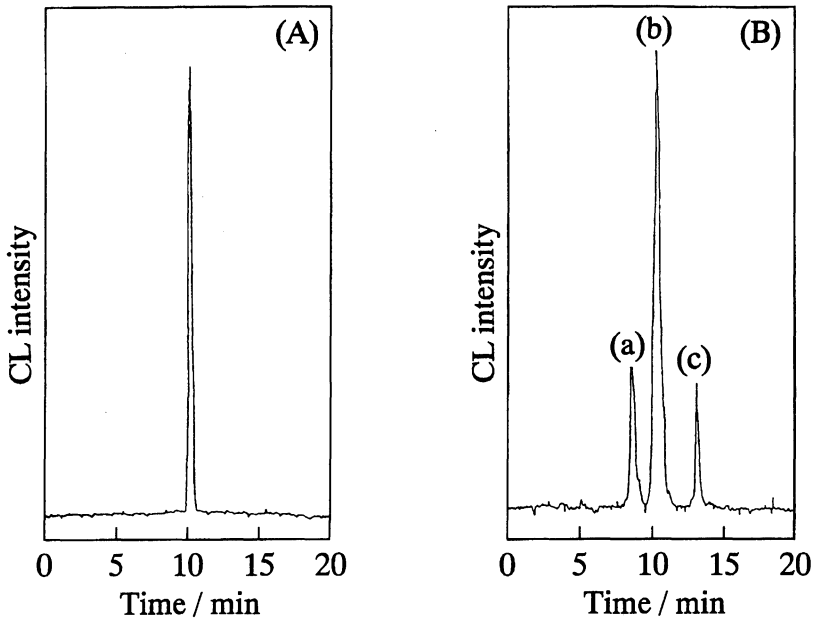

Fig. 2. Electropherograms of RITC–BSA complex and RITC–protein complexes with CL detection. Capillary, 70 cm of 50 μm i.d. fused silica; Applied voltage. −20 kV; Buffer, 25 mM phosphate containing 50 ppm FC-135 (pH 3.5). TCPO-CL reaction conditions were as follows: (1) 1 mM TCPO and 100 mM hydrogen peroxide in acetonitrile and (2) 200 mM imidazole buffer (pH 6.0). (A) Sample: 1×10^{-6} M BSA solution containing 5×10^{-5} M RITC. (B) Sample: 1×10^{-6} M protein solution containing 5×10^{-5} M RITC. (a) RITC–HγG complex. (b) RITC–HSA complex. (c) RITC–α-lacta complex.

and HγG necessitated about 4 h up to completion. In the reaction HSA behaved similarly to BSA.

Various BSA samples containing a definite amount of RITC were analyzed by the present method, and a linear calibration curve for the determination of BSA was obtained from the CL results. Using the calibration curve, the BSA in the concentration range of 7×10^{-8}—7×10^{-6} M could be determined with a detection limit of 5×10^{-8} M lower than 2×10^{-7} M given in a previous paper.[2] Since approximately 20 nl (2×10^{-8} dm^3) of sample was taken by siphoning for 15 s at a height difference of 15 cm, the present method was fea-

Table 1. Relative CL Intensity for Several Proteins

Proteins	Relative CL intensity[a)]	
1×10^{-6} M	EY	RITC
BSA	100	227
HSA	89.6	218
HγG	82.3	65.4

a) Relative CL intensity was based on that of EY–BSA complex.

sible for detecting 1 fmol (1×10^{-15} mol) of BSA, and was fairly sensitive compared to the conventional UV-vis detection limit of 1×10^{-5} M. The coefficient of variation in 6 measurements was 2.0%; this value was within the permissible range in a routine analysis. The sensitivity of the present method is expected to be enhanced by improving the apparatus as well as optimization of the procedure.

The relative CL intensity for several proteins, based on that (=100) of an EY–BSA complex, are given in Table 1. The data in Table 1 were obtained within 1 h after mixing a dye-stuff and protein. As shown in Table 1, the relative CL intensities of the protein complexes of RITC were more than 2-times those of the corresponding complexes of EY, respectively, except for its HγG complex. As mentioned before, the relative CL intensity of the RITC–HγG complex increased with increasing reaction time; therefore, the data given in Table 1 are acceptable.

Electropherograms of a RITC–BSA complex and of a mixture containing RITC–HSA, –HγG, and –α-lacta complexes were obtained by the present method, and are shown in Fig. 2 (A) and (B), respectively. Using the migration times obtained for the individual RITC–protein complexes, peaks (a), (b), and (c) in Fig. 2 (B) were identified as being peaks due to RITC–HγG, RITC–HSA, and RITC–α-lacta complexes, respectively. As can be seen from these electropherograms, several RITC–protein complexes could be separated and CL detected by the present method using RITC.

It is supposed that the present method using RITC will be applicable to many fields, including immunoassay, in the future.

References

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