Highly Sensitive Detection of Proteins Separated by Capillary Zone Electrophoresis Using On-Line Chemiluminescence Detection

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Keywords Chemiluminescence detection, on-line, protein, capillary electrophoresis, stacking

In a previous study¹, Rhodamine B isothiocyanate (RITC) was used as a dyestuff for labeling proteins so as to improve the sensitivity of on-line chemiluminescence detection (CLD) of proteins separated by capillary zone electrophoresis (CZE) in a phosphate buffer solution (pH 3.5). Using this method bovine serum albumin (BSA) in the concentration range of 7×10^{-8} – 7×10^{-6} M ($1 \text{ M} = 1 \text{ mol dm}^{-3}$) could be determined with a lower detection limit of 5×10^{-8} M (absolute amounts, 1 fmol). Since RITC was not examined in an alkaline solution, RITC was used at pH 9.0 in the present study. Tetramethylrhodamine isothiocyanate isomer R (TRITC), which is expected to be similarly sensitive as RITC, has also been examined at pH 9.0. TRITC gave a better result than did RITC. The result obtained was as follows: *ca.* 1×10^{-6} M of BSA could be determined using TRITC in about 20 min with a detection limit of 1×10^{-8} M, corresponding to 200 amol of BSA. Using the method of stacking^{2,3} for the sample components across the concentration boundary between the sample and background electrolyte, *ca.* 1×10^{-7} M of BSA could be further determined using TRITC in about 20 min with a detection limit of 6×10^{-9} M.

Experimental

Reagents

All of the reagents used were commercially available and of analytical-reagent grade. Deionized water was distilled before use. Each 1×10^{-4} M solution of RITC and TRITC was prepared by dissolving them in a carbonate buffer solution (pH 9.0). BSA, bovine serum γ globulin (B γ G), ovalbumin (Ova), α -lactalbumin (α -lacta) (molecular weights of 66296, 150000, 42699 and 14200, respectively) dissolved in a 25 mM carbonate buffer solution were used, except for the stacking experiments. 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 solution.

Apparatus

The experiment was carried out using the same apparatus as that reported regarding a previous experiment.^{4,5}

Procedures

A freshly available capillary tube was treated with 1 M hydrochloric acid for 10 min, and then washed with distilled water before use. The carrier solution was filtered through a Millipore (Millex-GSSLG0250S) 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 an imidazole buffer solution alone at a rate of $5 \mu\text{l min}^{-1}$ using a syringe pump. Then, the CL reagent solution containing H_2O_2 and TCPO was fed at a rate of $30 \mu\text{l min}^{-1}$ by another pump until a steady electrolytic current was obtained. A sample solution, which was left standing for 4 h after mixing of a dye-stuff and a protein, was introduced into the capillary tube by siphoning after the high voltage had been switched off. A high voltage ranging from 0 to 20 kV was gradually applied over a period of twenty seconds while operating the pumps. The CL measurement was started as soon as the voltage reached 20 kV. In accordance with a method of Burgi^{2,3}, stacking a TRITC-BSA complex was also attempted.

Results and Discussion

Both the spectrophotometric and CZE properties of proteins, dye-stuffs, and complexes were examined, and the following results were obtained. The absorption spectra of TRITC and its BSA complex in the wavelength region between 400 and 800 nm were almost the same, and showed maximum absorption at 548 nm. A mixed solution (pH 9.0) of TRITC and BSA was subjected to CZE; the two electropherograms shown in Fig. 1 (A) and (B) were at 548 and 210 nm, respectively. The third electropherogram (C) was obtained at 548 nm for TRITC alone (Fig. 1 (C)). As can be seen from Fig. 1, TRITC

Fig. 1 Electropherograms of TRITC and the TRITC-BSA complex with UV-VIS detection. Capillary, 60 cm, 50 μ m i.d., fused silica; separation distance, 35 cm; applied voltage, 20 kV; buffer, 25 mM carbonate (pH 9.0). Sample solutions were prepared by dissolving TRITC (and BSA) in an electrophoretic buffer. (A) BSA and TRITC, detected at 548 nm. (B) BSA and TRITC, detected at 210 nm. (C) TRITC, detected at 548 nm. The BSA and TRITC concentrations were 1×10^{-5} M and 2×10^{-4} M, respectively. Peaks: (I)=TRITC, (II)=TRITC-BSA complex.

Fig. 2 Electropherograms of TRITC-protein complexes detected by CL. Capillary, 80 cm, 50 μ m i.d., fused silica; applied voltage, 20 kV; buffer, 25 mM carbonate (pH 9.0). TCPO-CL reaction conditions: (1) 1 mM TCPO and 100 mM hydrogen peroxide in acetonitrile and (2) 200 mM imidazole buffer (pH 6.15). All sample solutions were prepared by dissolving protein(s) and RITC or TRITC in the electrophoretic buffer. (A) BSA (8×10^{-7} M) and TRITC (4×10^{-5} M), (B) Ova (1×10^{-6} M), α -lacta (1×10^{-6} M) and RITC (5×10^{-5} M), (C) B γ G (8×10^{-7} M) BSA (8×10^{-7} M), α -lacta (8×10^{-7} M) and TRITC (4×10^{-5} M). Peaks: (a) and (f)=TRITC, (b)=TRITC, (c)=TRITC-BSA, (d)=RITC-Ova, (e)=RITC- α -lacta, (g)=TRITC-B γ G, (h)=TRITC-BSA, (i)=TRITC- α -lacta.

was found to co-migrate with BSA in CZE, since peaks (I) and (II) in (A) corresponded to peak (I) in (C) and peak (II) in (B), respectively. A similar result to this was also obtained for a RITC-BSA complex. Figure 2 shows electropherograms of RITC-protein complexes and TRITC-protein complexes detected by CL. On the

Fig. 3 Relationship between sample buffer concentration and CL intensity. Capillary, 80 cm, 50 μ m i.d., fused silica; applied voltage, 20 kV; running buffer, 100 mM carbonate (pH 9.0); sample buffer, carbonate (pH 9.0); sample, 4×10^{-7} M BSA solution containing 2×10^{-5} M TRITC. TCPO-CL reaction conditions as in Fig. 2.

basis of the migration times known for the individual RITC- and TRITC-protein complexes, peaks (a), (c), and (f), as well as peaks (b), (d), (e), (g), (h), and (i) in Fig. 2 were identified as being due to TRITC, RITC, and TRITC, and as TRITC-BSA, RITC-Ova, RITC- α -lacta, TRITC-B γ G, TRITC-BSA, and TRITC- α -lacta complexes, respectively.

Samples containing various amounts of BSA and a definite amount of each dye-stuff were analyzed by the present method, and a linear calibration curve for the CL intensity of BSA was obtained for both dyes. Using the calibration curve, the BSA in the concentration range of up to 2×10^{-6} M could be determined by RITC with a detection limit of 2×10^{-8} M; the BSA in the concentration range of up to 1×10^{-6} M could be determined by TRITC with a detection limit of 1×10^{-8} M. Since approximately 20 nl (2×10^{-8} dm³) of the sample was taken by siphoning for 15 s at a height difference of 15 cm, the absolute amount of detectable BSA was 400 amol for RITC and 200 amol for TRITC, respectively.

An optimization for stacking of a TRITC-BSA complex was carried out as follows: (1) Mixed sample solutions of each definite amount of TRITC and BSA were prepared as carbonate buffer solutions of 100, 75, 50, and 25 mM (pH 9.0); the resulting sample solutions were subjected to a CZE-CLD system where a 100 mM carbonate buffer (pH 9.0) was used as a running buffer. The relationship between the sample buffer concentration and the CL intensity is shown in Fig. 3, including the result obtained for a carbonate buffer-free sample. Considering the result shown in Fig. 3 and the expected rise in the temperature inside a capillary tube due to a decrease of the buffer concentration, a 25 mM carbonate buffer was chosen in the following experiment. (2) The relationship between the injection time and the CL intensity was examined in order to determine the stacking

Fig. 4 Relationship between injection time and CL intensity. Capillary, 80 cm, 50 μ m i.d., fused silica; applied voltage, 20 kV; running buffer, 100 mM carbonate (pH 9.0); sample buffer, 25 mM carbonate (pH 9.0); sample, 4×10^{-7} M BSA solution containing 2×10^{-5} M TRITC. TCPO-CL reaction conditions as in Fig. 2.

effect; the results are shown in Fig. 4. An injection time of 4 min was selected, since the CL intensity increased with increasing injection times. However, the separation of the TRITC-BSA complex from TRITC deteriorated.

Various BSA samples containing a definite amount of TRITC were analyzed by the present method, and a linear calibration curve was obtained for the CL intensity against the BSA amount. Using the calibration curve, the BSA in the concentration range of up to 1×10^{-7} M could be determined with a detection limit of 6×10^{-9} M.

In summary, (1) TRITC was more sensitive than RITC for the detection of proteins, and (2) by the proposed method using the stacking effect, BSA could be estimated with a detection limit of 6×10^{-9} M.

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(Received May 2, 1994)

(Accepted July 27, 1994)