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On-line chemiluminescence detection of proteins separated by capillary zone electrophoresis

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ABSTRACT

In a capillary zone electrophoretic (CZE) experiment in phosphate buffer (pH 3.5), Eosin Y was found to migrate together with protein in a capillary tube as a supramolecular complex. This finding gave the possibilities not only of overcoming the problem of protein determination but also of measuring the Eosin Y comigrating with protein by a chemiluminescence (CL) method with high sensitivity. Labelling of protein with dyestuff was achieved simply by mixing the protein and a dyestuff. On-line CL detection of the protein separated by CZE was feasible by measuring the CL intensity of a bis(2,4,6-trichlorophenyl) oxalate (TCPO)–H_2O_2–dyestuff system by means of an interface between CZE and CL detection. Several xanthene dyestuffs including Eosin Y were examined and Rose Bengal was found to be more sensitive than Eosin Y used in previous work. Using the present method, $5 \cdot 10^{-7}$–$1 \cdot 10^{-4}$ mol dm^{-3} of bovine serum albumin (BSA) could be determined using Rose Bengal in about 20 min with a detection limit of $2 \cdot 10^{-7}$ mol dm^{-3} (signal-to-noise ratio = 3), corresponding to 4 fmol of BSA. The results for fifteen kinds of proteins including BSA are reported.

INTRODUCTION

The separation and detection of a protein as a supramolecular complex using capillary electrophoresis were reported in a previous paper [1]. The method was based on the fact that Eosin Y migrated together with protein through a capillary tube as a supramolecular complex, where the protein was detected by measuring Eosin Y. Eosin Y accompanied by protein could be determined by measuring the chemiluminescence intensity of a bis(2,4,6-trichlorophenyl) oxalate (TCPO)–H_2O_2–Eosin Y system by means of an interface between capillary zone electrophoresis (CZE) and chemiluminescence (CL) detection. This was the first report of on-line CL detection of proteins separated by CZE. However, the method was not very sensitive for the detection of proteins, and several problems remained unresolved. So far, two successful studies involving luminol CL detection [2] and acridinium CL detection [3] have been reported on the application of CL detection to CZE. Wide-bore CZE with CL detection [4] has also been reported for the determination of dansylated compounds.

As Eosin Y is a xanthene dyestuffs, other xanthene dyestuffs such as Rose Bengal, Erythrosin B and Phloxin B were examined in this study, and Rose Bengal was found to be more sensitive than Eosin Y. The present system of coupling CZE and CL detection was successfully applied to the separation and detection of various proteins, including albumin and globulin. The interaction between a protein and a dyestuff and the behaviour of the supramolecular com-

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plex formed were examined by spectrophotometry and CZE. The present system should be also applicable to other experiments involving CL detection.

EXPERIMENTAL

Apparatus

The apparatus shown in Fig. 1 was used. A high-voltage supply delivering 0–30 kV (Model HepLL-30PO, 08-L1; Matsusada Precision Device, Shiga, Japan) was used for the application of high voltages. Separation was performed in a fused-silica capillary tube (50 mm I.D.) (Polymicro Technologies, Phoenix, AZ, USA). CL detection was carried out by means of the CL detector shown in Fig. 1b. The CL reagent solution, obtained by dissolving TCPO and H_2O_2 in acetonitrile, and the buffer solution were fed into a four-way joint by use of two pumps [LC-9A (Shimadzu, Kyoto, Japan) and CCPD (Tosoh, Tokyo, Japan)], where the protein–dyestuff complex that passed through a capillary tube from the upper part to the lower part was mixed with the above-mentioned feed solution consisting of the CL reagent solution and the buffer solution. All the Teflon tubes were of 500 μm I.D. The resulting CL was detected by a photomultiplier (R-464; Hamamatu Photonics, Shizuoka, Japan) and measured by a photon counter (C1230; Hamamatu Photonics). A Shimadzu SPD-6AV UV–Vis spectrophotometric detector was used for the measurement of absorption in the UV–Vis region in CZE. A Shimadzu UV-1200 spectrophotometer was used for examining the optical properties of proteins, dyestuffs and their complexes.

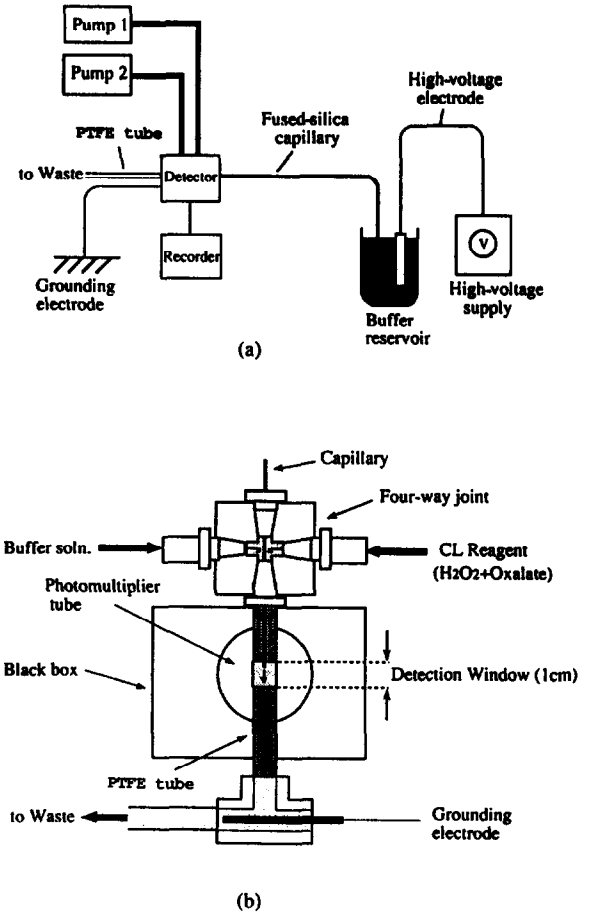

Fig. 1. Schematic diagrams of (a) the apparatus and (b) the CL detector.

Reagents

All of the reagents used were of commercially available special grade. Ion-exchanged water was distilled prior to use. A 0.01 *M* aqueous solution prepared by dissolving a definite amount of a dyestuff, *i.e.*, Eosin Y, Rose Bengal, Erythrosin B, Uranine (special grade from Nacalai Tesque, Kyoto, Japan), Phloxin B (special grade from Tokyo Kasei Kogyo, Tokyo, Japan), 3,4,5,6-tetrachlorofluorescein (TCFl), 2′,7′-dichlorofluorescein (2′,7′-DCFl) or 4′,5′-dichlorofluorescein (4′,5′-DCFl) (donated by Professor Y. Nishikawa, Kinki University, Osaka, Japan), in water was diluted with buffer solution prior to use. Bovine serum albumin (BSA), human serum albumin (HSA), rabbit serum albumin (RSA), bovine serum γ-globulin (BγG), human serum γ-globulin (HγG), ovalbumin (Ova) (from chicken egg, grade V), conalbumin (Cona) (from chicken egg), α-lactalbumin (α-lacta) (from bovine milk), β-lactglobulin (β-lact) (from bovine milk), haemoglobin (from bovine blood), myoglobin (from horse skeletal muscle, type I), avidin (from chicken egg), α-chymotrypsinogen (bovine), ribonuclease A (bovine) and β-casein (from bovine milk) were all pur-

chased from Sigma (St. Louis, MO, USA) and were used after dissolution in buffer solution (pH 3.5) consisting of 50 m*M* phosphoric acid and sodium hydroxide solutions. Commercially available hydrogen peroxide (30%) and TCPO from Nacalai Tesque were used as CL reagents, and actonitrile was used to dissolve these reagents. Surfactant FC-135 (provided by Sumitomo–3M, Tokyo, Japan) was also used to improve CZE separations.

Procedure

A fused-silica capillary tube was filled with phosphate buffer solution by using a 100-μl microsyringe. CL reagent solution (H_2O_2 + oxalate) was fed at a rate of 15 μl min^{-1} from pump 1 and buffer solution was fed at a rate of 5 μl min^{-1} from pump 2. The introduction of sample solution into the capillary tube was achieved by siphoning (injection time = 15 s) under the operation of pumps 1 and 2. CZE separation was carried out by applying a voltage of up to 20 kV between a high-voltage electrode and a ground electrode. The protein–dyestuff complex leaving the end of the capillary tube was immediately mixed with a mixture of CL reagent solution and buffer solution, followed by measurement of the CL intensity.

RESULTS AND DISCUSSION

The absorption spectra of xanthene dyestuffs and their complexes with BSA are shown in Fig. 2. As can be seen the bathochromic shift, in which the wavelength of maximum absorption of the xanthene dyestuffs Eosin Y, Phloxin B, Rose Bengal and Erythrosin B shifted to higher values, was observed in the presence of BDA. In a previous study [5] the authors examined the interaction between Eosin Y and BSA in phosphate buffer (pH 3.1) and the following data were obtained by the Klotz's method [6]: Eosin Y:BSA = 10.1:1 (mole ratio in combination), and association constant = $3.97 \cdot 10^5$ mol^{-1} dm^3 [5]. These values seemed to be reasonable, but they did not guarantee that Eosin Y would migrate with proteins in a capillary. Eosin Y showed its λ_{max} at 522 nm in phosphate buffer (pH 3.5), and

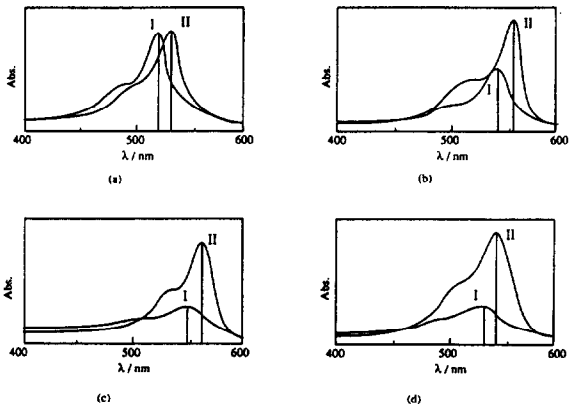

Fig. 2. Absorption spectra of xanthene dyestuffs and their complexes with BSA. Peaks: I = BSA absent and II = BSA present. (a) Eosin Y; (b) Phloxin B; (c) Rose Bengal; (d) Erythrosin B.

λ_{max} shifted to 535 nm in the presence of BSA. These bathochromic shifts are shown in Table I together with the migration times in CZE. As can be seen, no dyestuff gave a migration peak in the visible region except Uranine, showing that the dyestuff itself did not migrate at all under the experimental conditions. The migration time of BSA alone in CZE was obtained by detection at 210 nm and it was shown in Table I to agree with those of the dye–BSA complexes except for Uranine.

Electropherograms of the Eosin Y–BSA system are shown in Fig. 3. The peak in Fig. 3B is attributed to the Eosin Y–BSA complex as Eosin Y alone did not give any peak in the visible region, as mentioned above.

It was concluded from these results that the xanthene dyestuffs such as Eosin Y, Phloxin B, Rose Bengal and Erythrosin B and TCFl, 2′,7′-DCFl and 4′,5′-DCFl migrated together with BSA as their complexes.

The optimization of the interface between CZE separation and CL detection was carried out with regard to the following: (1) relationship between the pH of buffer solution and CL intensity; (2) relationship between TCPO concentration and CL intensity or signal-to-noise ratio; and (3) relationship between H_2O_2 concentration and CL intensity or signal-to-noise ratio. From a CL intensity–pH plot in the pH range 4–7, the optimum pH of the buffer solu-

TABLE I

COMPARISON OF DIFFERENT FLUORESCENT DYES

Sample	λ_{max} (nm)		Migration time in CZE (min)[a]	
	BSA absent	BSA present	BSA absent	BSA present[b]
Eosin Y	519	531	No peak	3.5
Rose Bengal	551	562	No peak	3.5
Erythrosin B	529	541	No peak	3.5
Phloxin B	540	553	No peak	3.5
TCFl	–	470	No peak	3.5
2',7'-DCFl	–	503	No peak	3.5
4',5'-DCFl	–	495	No peak	3.5
Uranine	–	430[c]	20.4	20.4[c]
BSA	–	–	–	3.5[d]

[a] Conditions as in Fig. 3.
[b] Detected at λ_{max} in the presence of BSA.
[c] No interaction with BSA was observed.
[d] Detected at 210 nm.

tion was adjusted at 6.0, where the CL intensity of the TCPO–H_2O_2 system was maximum. Although the CL intensity or signal-to-noise ratio of the TCPO–H_2O_2 system increased linearly with increase in TCPO concentration up to $1.0 \cdot 10^{-3}$ mol dm^{-3}, a $1.0 \cdot 10^{-3}$ mol dm^{-3} TCPO concentration was selected because the TCPO solubility under the experimental conditions was limited. The CL intensity of the TCPO–H_2O_2 system increased with increase in H_2O_2 concentration up to 0.12 mol dm^{-3}, but the signal-to-noise ratio gave a maximum value against H_2O_2 concentration, and 0.10 mol dm^{-3} H_2O_2 was chosen.

As the surfactant FC-135 had been reported to prevent the adsorption of proteins on the inner wall of a capillary and to give reproducible results [7], it was also used in the present study. The effects of FC-135 concentration on the peak sharpness (Fig. 4) and the CL intensity (Fig. 5) of the Eosin Y–BSA system were examined in

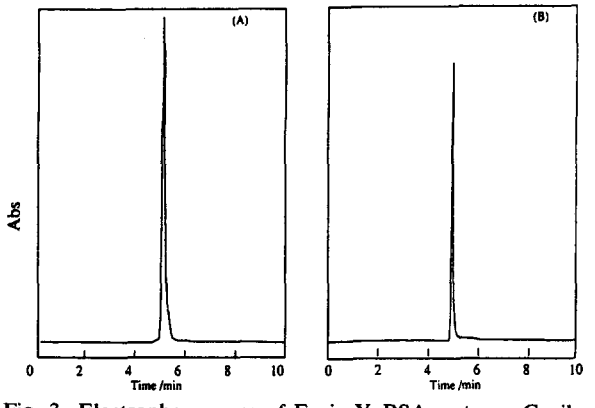

Fig. 3. Electropherograms of Eosin Y–BSA systems. Capillary, 59 cm of 50 μm I.D. fused-silica tube; applied voltage, 15 kV; buffer, 25 m*M* phosphate solution (pH 3.5) containing FC-135; sample, 5.0 g l^{-1} BSA solution containing $3 \cdot 10^{-4}$ *M* Eosin Y. Detection at (A) 210 and (B) 532 nm.

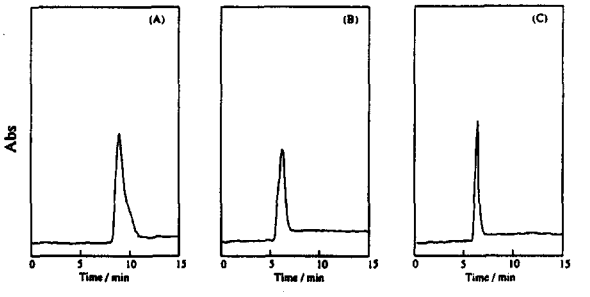

Fig. 4. Effect of FC-135 concentration on the peak shape of Eosin Y–BSA in phosphate buffer (pH 3.5). Capillary, 59 cm of 50 μm I.D. fused-silica tube; applied voltage, 15 kV; sample, 6.6 g l^{-1} BSA solution containing $3 \cdot 10^{-4}$ *M* Eosin Y; detection, 532 nm (corresponding to Eosin Y–BSA). Concentration of FC-135: (A) 25; (B) 50; (C) 100 mg dm^{-3}.

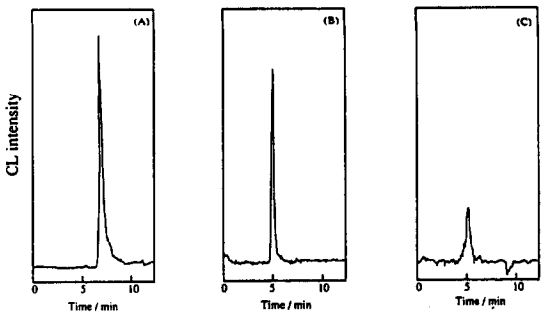

Fig. 5. Effect of FC-135 concentration on the CL intensity of Eosin Y–BSA in phosphate buffer (pH 3.5). Capillary, 85 cm of 50 μm I.D. fused-silica tube; applied voltage, 20 kV; sample, 1.0 g l^{-1} HSA solution containing $5 \cdot 10^{-5}$ *M* Eosin Y. Concentration of FC-135: (A) 25; (B) 50; (C) 100 mg dm^{-3}.

phosphate buffer (pH 3.5). The peak of Eosin Y–BSA became sharper with increase in FC-135 concentration (Fig. 4), but the CL intensity decreased (Fig. 5). The final concentration of FC-135 in the buffer solution used in the capillary tube was adjusted to 50 μg dm^{-3} in subsequent experiments.

Various samples containing 1 g l^{-1} of BSA and $3 \cdot 10^{-5}$ mol dm^{-3} each of Eosin Y, Rose Bengal, Erythrosin B, Phloxin B, TCFl, 2′,7′-DCFl and 4′,5′-DCFl were subjected to CZE separation and CL detection. The results obtained are given in Table II, where the CL intensity in the batch reaction, the peak height at each λ_{max} and the Cl intensity in CZE separation are shown relative to those of Eosin Y (= 100). The values for Rose Bengal were higher and the values for the other dyestuffs were lower than those for Eosin Y.

In the presence of Rose Bengal, the calibration graph for BSA was linear in the concentration range $5 \cdot 10^{-7}$–$1 \cdot 10^{-4}$ mol dm^{-3} with a detection limit of $2 \cdot 10^{-7}$ mol dm^{-3} (signal-to-noise ratio = 3) and a correlation coefficient of 0.998. In the presence of Eosin Y, the calibration graph was linear in the concentration range $1 \cdot 10^{-6}$–$1 \cdot 10^{-4}$ mol dm^{-3} BSA with detection limit 5×10^{-7} mol dm^{-3} (signal-to-noise ratio = 3) and a correlation coefficient of 0.998. According to the present method, BSA in the above-mentioned concentration range could be determined with a relative standard deviation of 4.9% and its detection limit was satisfactory in comparison with the usual detection limits (*ca.* 10^{-5} mol dm^{-3}) with UV detection in CZE.

The relative CL intensities for various proteins with respect to the BSA–Eosin Y complex (= 100) are given in Table III. For all the proteins tested, the Rose Bengal complexes gave larger relative CL intensities than those for Eosin Y complexes. CL was observed for all protein–Rose Bengal complexes. The relative CL intensities for the complexes between Rose Bengal and BSA, HSA, RSA, BγG, HγG, Ova and Cona were relatively large whereas those for the complexes between Rose Bengal and the other proteins were much smaller. Similar results were obtained for various protein–Eosin Y complexes, but no CL was observed for Eosin Y

TABLE II

RELATIVE SIGNAL INTENSITIES[a]

Sample[b]	Batch reaction: CL intensity	CZE	
		Peak height at λ_{max}	CL intensity
Eosin Y	100	100	100
Rose Bengal	102	112	138
Erythrosin B	84.0	90.8	78.1
Phloxin B	97.4	80.8	90.6
TCFl	95.0	30.5	–
2′,7′-DCFl	102	29.9	–
4′,5′-DCFl	88.4	28.0	–

[a] Relative signal intensity with respect to the value for Eosin Y (= 100).
[b] Sample containing 1 g l^{-1} of BSA and $3 \cdot 10^{-5}$ *M* of each dyestuff.

TABLE III

RELATIVE CL INTENSITIES FOR VARIOUS PROTEINS

Proteins ($1 \cdot 10^{-4}$ *M*)	Relative CL intensity[a]	
	Eosin Y	Rose Bengal
Serum albumin (bovine)	100	138
Serum albumin (human)	93.9	123
Serum albumin (rabbit)	114	130
γ-Globulin (bovine)	89.4	136
γ-Globulin (human)	94.5	130
Ovalbumin (chicken egg)	42.2	66.1
Conalbumin (chicken egg)	26.1	86.1
α-Lactalbumin (bovine milk)	20.0	28.3
β-Lactglobulin (bovine milk)	–	12.2
Haemoglobin (bovine)	12.0	16.6
Myoglobin (horse)	10.0	14.4
Avidin (chicken egg)	7.88	10.6
α-Chymotrypsinogen (bovine)	3.33	7.07
Ribonuclease A (bovine)	–	1.63
β-Casein (bovine milk)	–	1.01

[a] Relative CL intensity with respect to that of the BSA–Eosin Y complex (= 100).

complexes of β-lact, ribonuclease A and β-casein.

Electropherograms of (A) protein–Eosin Y complexes and (B) protein–Rose Bengal complexes with CL detection are shown in Fig. 6. In Fig. 6A HSA and RSA were selected whereas in Fig. 6B HγG, HSA and α-lacta were selected as model proteins. As can be seen, each protein in the protein–dyestuff complexes produced a sharp CL peak. The theoretical plate number for peak (b) in Fig. 6B is 42 000 according to the equation of Karger *et al.* [8].

Further efforts are being made to improve the sensitivity of the method.

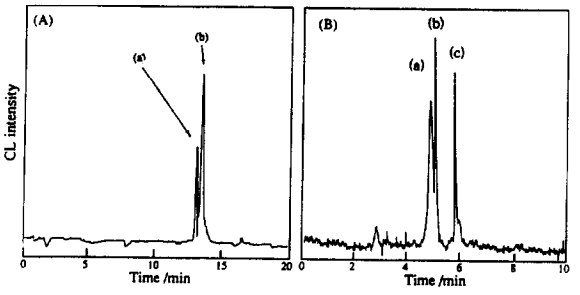

Fig. 6. Electropherograms of (A) protein–Eosin Y and (B) protein–Rose Bengal complexes with CL detection. Capillary, 85 cm fused-silica tube; applied voltage, 20 kV; buffer, 25 m*M* phosphate (pH 3.5) containing 50 mg/l of CFC-135. (A) Sample solution containing 3.3 g l^{-1} of protein and $3 \cdot 10^{-4}$ *M* Eosin Y; (B) sample solution containing 0.5 g l^{-1} of protein and $3 \cdot 10^{-4}$ *M* Rose Bengal. TCPO–CL reaction conditions were as follows: (1) 1 m*M* TCPO and 100 m*M* hydrogen peroxide in acetonitrile (15 μl min^{-1}); (2) 25 m*M* phosphate buffer (pH 6.0) (5 μl min^{-1}). (A) (a) = HSA, (b) = RSA; (B) (a) = HγG, (b) = HSA and (c) = α-lacta.

CONCLUSIONS

On the basis of the above results, the following conclusions were drawn: (1) some kinds of dyestuffs, especially xanthene dyestuffs, migrated together with certain proteins in a capillary tube as their complexes; (2) the dyestuff in a protein–dyestuff complex separated by CZE could be detected in place of protein by measuring its CL intensity; (3) of the dyestuffs tested, Rose Bengal was most sensitive and gave the highest CL intensity; (4) some protein–dyestuff complexes were obtained simply by mixing them and then labelling of the protein was easily carried out; (5) on-line CL detection of the proteins separated by CZE was feasible by measuring the CL intensity of the TCPO–H_2O_2–dyestuff system by means of an interface between CZE and CL detection; (6) with the present method, $5 \cdot 10^{-7}$–10^{-4} mol dm^{-3} of BSA was determined using Rose Bengal in about 20 min with a detection limit of $2 \cdot 10^{-7}$ mol dm^{-3} of BSA (signal-to-noise ratio = 3); (7) BSA, HSA, RSA, BγG, HγG, Ova and Cona, among fifteen proteins tested, could be sensitively determined by the present method.

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