The Determination of a Small Amount of Biological Constituent by the Use of Chemiluminescence. XIV. The Flow-Injection Analysis of Protein Using Ultrasonic Chemiluminescence of Luminol

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Synopsis. A flow-injection analysis method for the determination of a small amount of protein has been established by the use of the ultrasonic chemiluminescence of luminol. The present method was based on the phenomenon that the catalytic activity of cobalt(II) for the ultrasonic chemiluminescence of luminol decreased in the presence of protein. The apparatus consisted of only a luminol solution line equipped with an ultrasonic irradiation bath and a cobalt(II) catalyst solution line equipped with a sample inlet. Under optimum conditions, similar calibration curves were obtained for bovine serum albumin, bovine serum γ-globulin, human serum albumin, and human serum γ-globulin. According to the present method, these proteins could be determined in the concentration range 5.0×10^{-6}—1.0×10^{-1} g dm^{-3}, with a detection limit of 200 pg (injected sample volume 40 mm^3, S/N=2) and a coefficient of variation of 2.9% (n=8) at a rate of about 60 samples per hour.

The authors have already reported the flow-injection analysis (FIA) method for the determination of a small amount of protein using a 5-animo-2,3-dihydro-1,4-phthalazinedione (luminol) or a 1,10-phenanthroline (phen)-hydrogen peroxide (H_2O_2)-copper(II) system.[1,2] These methods were about 10^2—10^6 times as sensitive as the conventional methods, such as colorimetry and fluorometry for the determination of protein.[3,4] However, an improvement of the analytical capabilities and a simplification of the flow system have also been desired in these methods.

A selective, sensitive method for the determination of cobalt(II) (Co(II)) was established by Suzuki et al., in which the ultrasonic chemiluminescence (CL) reaction of luminol was utilized.[5] This method was carried out using a simple flow-injection system without a H_2O_2 line. On the other hand, the phenomenon that the catalytic activity of the cobalt(III) complex compound for the CL reaction between luminol and H_2O_2 decreased in the presence of protein was found by the authors in the previous paper.[6]

In the present study, a new FIA method for the determination of protein was established by the use of an ultrasonic CL reaction by which Co(II) at sub-pg levels could be detected and the complex formation between Co(II) and protein. Some improvements were achieved compared with the previous study,[2] regarding the following points: detection limit, coefficient of variation, sampling rate, and simplification of a flow system.

Experimental

Reagents. All the reagents used were of a commercially available special grade. Ion-exchange water was distilled for use. A 5.0×10^{-9} mol dm^{-3} Co(II) catalyst solution was prepared by diluting a Co(II) stock solution containing 1.0×10^{-3} mol dm^{-3} cobalt(II) chloride, 1.0×10^{-3} mol dm^{-3} hexadecyltrimethylammonium bromide (HTAB), and 1.0×10^{-1} mol dm^{-3} hydrochloric acid with a buffer solution (pH 10.2) containing 1.0×10^{-1} mol dm^{-3} boric acid, 1.0×10^{-1} mol dm^{-3} potassium hydroxide, and 1.0×10^{-3} mol dm^{-3} HTAB. A 5.0×10^{-5} mol dm^{-3} luminol solution containing 1.0×10^{-1} mol dm^{-3} sodium hydroxide (NaOH) was prepared for use. All the sample solutions of bovine serum albumin (BAS) from Nakarai Chemical Ltd., human serum albumin (HSA) from Chemical Dynamics Corporation, and bovine serum γ-globulin (BγG) and human serum γ-gulobulin (HγG) from Sigma Chemical Co. were prepared by dissolving and diluting them with the above-mentioned buffer solution.

Apparatus and Procedure. A schematic diagram of the FIA apparatus was shown in Fig. 1. The luminol solution (a) was fed at a flow rate of 1.5 cm^3 min^{-1} by means of a pump (P_1) (PERISTA MINI PUMP SJ-1211) and passed through a cooling bath (k), followed by the exposure to the ultrasonic irradiation in sonication cell (m) (0.7 cm i.d., 1.4 cm^3 volume) made of poly-propylene by mean of an oscillator (Tomy Seiko Co., Handy Sonic Model UR-20P; 28 kHz, max. 20W) equipped with an exponential horn (1). Then, the luminol solution was fed into a flow cell at a flow rate

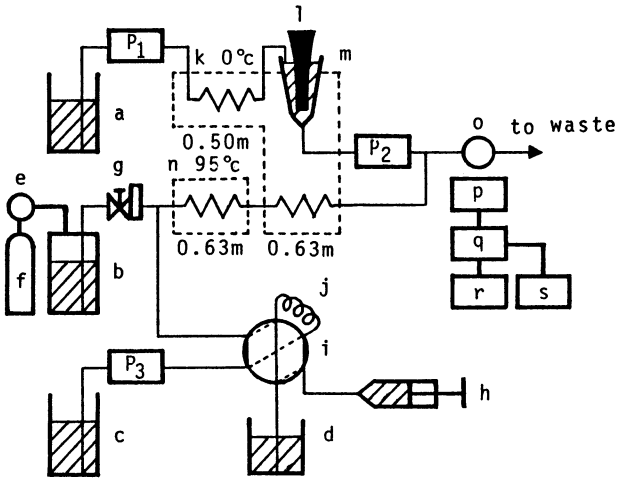

Fig. 1. Schematic diagram of the flow-injection system. a: Luminol solution, b: Co(II) catalyst solution, c: buffer solution, d: sample solution, e: regulator, f: N_2 cylinder, g: flowmeter equipped with a needle valve, h: syringe, i: six-way cock, j: sampling loop, k: cooling bath, l: exponential horn, m: sonication cell, n: heating bath, o: flow cell, p: photomultiplier, q: amplifier, r: recorder, s: integrator, and P_1, P_2, P_3: pump.

of 1.5 cm^3 min^{-1} by means of a pump (P_2) (PERISTA MINI PUMP SJ-1221).

The Co(II) catalyst solution (b) was fed at a flow rate of 1.0 cm^3 min^{-1} by means of an N_2 gas cylinder; the buffer solution (c) was fed at a rate of 1.5 cm^3 min^{-1} by means of a high-pressure pump (P_3) (Nishio kogyo Co., Ltd., SSP DM3M-2044). A definite volume (40 mm^3) of a protein sample solution (d) was injected into the buffer solution line through the sampling loop (j) and the six-way cock (i) (Reodyne). The injected protein sample solution was mixed with the Co(II) catalyst solution, passed through the heating bath (n) and the cooling bath, mixed with the luminol solution, and finally fed to the flow cell.

The CL intensity of the solution containing no sample was recorded as a base line, while the CL intensity of the solution containing protein was record as a negative peak because the catalytic activity of Co(II) decreased due to the formation of a complex compound between Co(II) and protein. The amount of protein was estimated from the peak area.

Results and Discussion

Optimization of Experimental Conditions. Prior to the determination of protein, the optimum concentrations of luminol, NaOH, Co(II), and HTAB were examined. Bovine serum albumin was used as a model protein.

The relationship between the luminol concentration and the peak area as well as the relationship between the NaOH concentration and the peak area were examined. Both the luminol concentration and the NaOH concentration did not affect the noise of the base line. From the experimental results, the optimum concentrations of luminol and NaOH were chosen as the concentrations which gave the maximum peak area. The relationship between the Co(II) concentration and the peak area or detection limit (S/N=2) are shown in Fig. 2. Both the peak area and the noise of the base line increased with increasing Co(II) the concentration; however, the optimum Co(II) concentration was chosen as the Co(II) concentration which gave a minimum detection limit.

In order to examine the catalytic effect of micelle on the CL intensity, HTAB (cationic surfactant), TritonX-100 (nonionic surfactant), and sodium dodecyl sulfate (SDS) (anionic surfactant) were used. The CL intensity in the presence of 1.0×10^{-3} mol dm^{-3} HTAB or 1.0×10^{-3} mol dm^{-3} TritonX-100 and in the absence of surfactant were 160, 27, and 75 mV respectively. Since SDS did not dissolve in the buffer solution (pH 10.2), HTAB was used in the present study. The relationship between the HTAB concentration and the peak area or detection limit (S/N=2) are shown in Fig. 3. Both the peak area and the noise of the base line increased with increasing HTAB concentration; however, the optimum HTAB concentration was chosen as the HTAB concentration which gave a minimum detection limit.

On the basis of these results, the following optimum

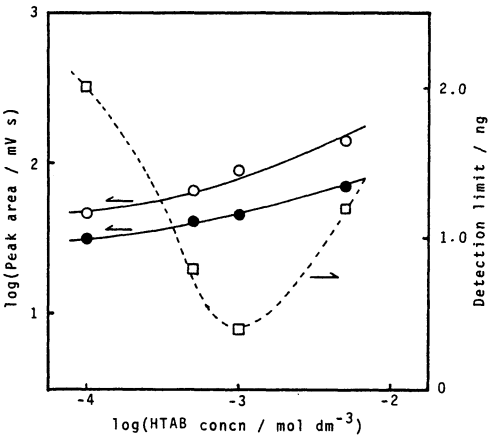

Fig. 3. Relationship between HATB concn and peak area or detection limit. ○: 1.0×10^{-3} g dm^{-3}- and ●: 1.0×10^{-4} g dm^{-3}-BSA. □: Detection limit (S/N=2). Conditions: 5.0×10^{-5} mol dm^{-3} luminol, 1.0×10^{-1} mol dm^{-3} NaOH, and 5.0×10^{-9} mol dm^{-3} Co(II).

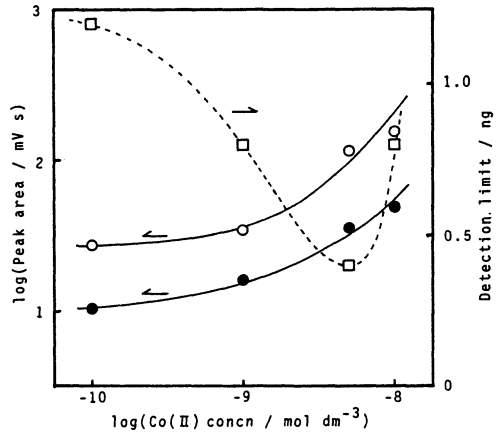

Fig. 2. Relationship between Co(II) concn and peak area or detection limit. ○: 1.0×10^{-3} g dm^{-3}- and ●: 1.0×10^{-4} g dm^{-3}-BSA. □: Detection limit (S/N=2). Conditions: 5.0×10^{-5} mol dm^{-3} luminol, 1.0×10^{-1} mol dm^{-3} NaOH, and 1.0×10^{-3} mol dm^{-3} HTAB.

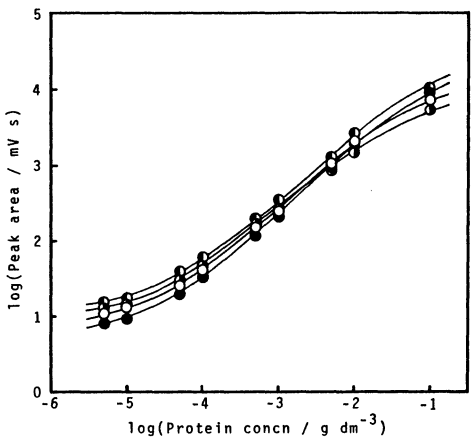

Fig. 4. Calibration curves of various proteins. ○: BSA, ●: BγG, ◑: HSA, and ◐: HγG. Conditions: 5.0×10^{-5} mol dm^{-3} luminol, 1.0×10^{-1} mol dm^{-3} NaOH, 5.0×10^{-9} mol dm^{-3} Co(II), and 1.0×10^{-3} mol dm^{-3} HTAB.

Table 1. Comparison of the Present Method with the Other FIA Methods

Method	Detection limit[a]	Determination range	Sampling volume	C.V.[b]	Sampling rate
	ng	g dm^{-3}	mm^3	%	sample h^{-1}
Present	0.2	5.0×10^{-6}—1.0×10^{-1}	40	2.9	60
Dyestuff-binding[7]	40	2.0×10^{-3}—5.0×10^{-1}	20	0.2	60
Electrode[8]	120	1.0×10^{-3}—2.0×10^{-1}	40	5	
Spectrophotometry[9]	1000	1.0×10^{-1}—1.0×10^{-1}	10	<1	60
Lowry[10]	1000	5.0×10^{-3}—2.0×10^{-2}	100	<2	90

a) As BSA. b) Coefficient of variation.

concentrations of various reagents were used in the study thereafter: 5.0×10^{-5} mol dm^{-3} luminol, 1.0×10^{-1} mol dm^{-3} NaOH, 5.0×10^{-9} mol dm^{-3} Co(II), and 1.0×10^{-3} mol dm^{-3} HTAB.

Determination of Various Proteins. The calibration curves shown in Fig. 4 were obtained under the optimized conditions for a determination of various proteins. The responses for BSA, BγG, HSA, and HγG were almost similar. These proteins could be determined in the concentration range of 5.0×10^{-6}—1.0×10^{-1} g dm^{-3} with a detection limit 200 pg (injected sample volume 40 mm^3, S/N=2).

Since H_2O_2 was unnecessary in the present method, the FIA apparatus used in the present study consisted only of the luminol solution line equipped with an ultrasonic irradiation bath and the Co(II) catalyst solution line equipped with a sample inlet. The peak shape obtained by the present method was much sharper than that obtained by the previous method.[2] This seemed to be due to a reduction in the dilution degree of the sample solution in the simplified FIA system, which had two lines different from the previous system having three lines. The detection limit (S/N=2), coefficient of variation, and sampling rate are as follows: 1 ng (injected sample volume 50 mm^3), 4.9% (1.0×10^{-3}g dm^{-3} BSA, n=10), and about 20 samples per hour (1.0×10^{-2} g dm^{-3} BSA) in the previous method; 200 pg (injected sample volume 40 mm^3), 2.9% (1.0×10^{-3} g dm^{-3} BSA, n=8), and about 60 samples per hour (1.0×10^{-2} g dm^{-3} BSA) in the present one. Protein up to 250 pg could be detected using the previous method; however, it can be obtained only by the use of the amplifying effect of an amino acid. As can be seen from the above-mentioned results, the present method was superior to the previous one regarding analytical capabilities and a simplification of the flow system.

Comparison of the Present Method with the Other FIA Methods. Several FIA methods for a determination of protein have recently been reported. As can be seen from Table 1, the present method was superior regarding the detection limit and the concentration range for a determination of protein to the other FIA methods.

References

1) T. Hara, M. Toriyama, and K. Tsukagoshi, *Bull. Chem. Soc. Jpn.*, **57**, 1551 (1984).
2) T. Hara, T. Ebuchi, A. Arai, and M. Imaki, *Bull. Chem. Soc. Jpn.*, **59**, 1833 (1986).
3) K. Sano, "Bunseki-library 3 Rinsho Kagaku Bunseki II," ed by the Japan Society for Analytical Chemistry, Tokyo Kagaku Dojin, Tokyo (1979).
4) K. Sugawara and M. Soejima, "Seibutsu Kagaku Jikken-ho 7 Tanpakushitsu No Teiryo-ho," Japan Scientific Societies Press, Tokyo (1981).
5) T. Komatsu, M. Ohira, M. Yamada, and S. Suzuki, *Bull. Chem. Soc. Jpn.*, **59**, 1849 (1986).
6) T. Hara, M. Toriyama, and K. Tsukagoshi, *Bull. Chem. Soc. Jpn.*, **57**, 289 (1984).
7) T. Shuto, M. Koga, I. Tanaka, T. Akiyama, and H. Igisu, *Bunseki Kagaku*, **36**, 256 (1987).
8) C. J. Yaun and C. O. Huber, *Anal. Chem.*, **57**, 180 (1985).
9) L. C. Davis and G. A. Radke, *Anal. Biochem.*, **161**, 152 (1987).
10) R. A. Salerno, C. Odell, N. Cyanovich, B. P. Bubnis, W. Morges, and A. Gray, *Anal. Biochem.*, **151**, 309 (1985).