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The Determination of a Small Amount of Biological Constituent by the Use of Chemiluminescence. XIII. High Sensitive Metal Chelate Affinity Chromatography

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A chemiluminescence detector using a 1,10-phenanthroline-hydrogen peroxide-copper(II) system was successfully combined with a metal chelate affinity column. A mixture comprising bovine serum albumin, lysozyme, and bovine serum γ-globulin as model proteins was completely separated by the pH-gradient elution method on a TSK gel Chelate-5PW adsorbed with zinc ions, also, each protein could be quantitatively determined. According to the present method, using a 0.4 cm^3 column of bed volume, 1) the determinable concentration range for bovine serum γ-globulin was formed to be 1.0×10^{-4}—1.0×10^{-1} g dm^{-3} with a detection limit of 5 ng for a 50 mm^3 of injection volume, 2) the recovery of 1.0×10^{-3} g dm^{-3} bovine serum γ-globulin was 85% with 7.9% of the coefficient of variation (n=5), and 3) the time necessary for an analysis was about 50 min. The present method was about 200 times more sensitive than that using an ultraviolet detector and was also applicable to a human serumn sample.

In order to separate and determine each small amount of protein in the mixture by high-performance liquid chromatography (HPLC), highly sensitive HPLC was attempted using a chemiluminescence (CL) detector. In the present study, metal chelate affinity chromatography (MCAC) was used since the authors were interested in the affinity between metal ions and proteins;[1] MCAC using the affinity is a relatively new method for the separation of a mixture of various proteins.

The concept of metal chelate affinity chromatography was first described by Porath and co-worker.[2] Its sensitivity when using a conventional ultraviolet (UV) detector was still unsatisfactory for the determination of a small amount of biological constituent.

In order to improve the sensitivity of MCAC, an attempt to combine it with CL detection using a 1,10-phenanthroline (phen)-hydrogen peroxide (H_2O_2)-copper(II) (Cu(II)) catalyst system was carried out in the present study. The determination of a protein is based on a measurement of the decreasing catalytic activity of Cu(II) for a CL reaction in the presence of the protein.[3] In this case, the stable Cu(II)-protein complex formed as a result of heating caused a lowering of the free Cu(II) concentration.[1] Chemiluminescence detection, developed by the authors, is characterized by the following: 1) high sensitivity, 2) application to HPLC (including the determination of a small amount of protein without labeling), 3) a wide dynamic range, and 4) a relatively simple apparatus.[1,3,4]

By using a CL detector, which was developed by the authors for the determination of various proteins, they could be determined over a concentration range 5.0×10^{-6}—1.0×10^{-1} g dm^{-3}, with a detection limit of 250 pg (for a 50 mm^3 of injection volume).[3] Though the CL detector was shown by the authors to be applicable to immunoaffinity chromatography in which a specific single protein in a serum could be analyzed by an immune reaction,[4] no investigation has been made with regard to the applicability of the CL detector to the determination of a small amount of protein separated by HPLC.

The CL detector was shown to be highly sensitive and its response was influenced by the pH, salt content, solvent, flow-rate, and temperature.[3] Therefore, suitable conditions for elution using the CL detector were investigated, and the experimental conditions necessary for the acquisition of a well-defined chromatogram were optimized. A metal chelate affinity column was successfully connected to the CL detector (developed by the authors), and a mixed sample containing bovine serum albumin, lysozyme, and bovine serum γ-globulin as a model proteins was subjected to pH-gradient elution, followed by CL detection. The sensitivity of the present method was about 200 times as high as that of the conventional UV method.

Experimental

Reagents. All of the reagents used were of a commercially available special grade. Deionized water was distilled for use. A phen solution, a H_2O_2 solution, and a Cu(II) catalyst solution were prepared as described in a previous paper,[2] except for the use of a borate buffer of pH 12.19 for the preparation of a Cu(II) catalyst solution. The borate buffer solution (Buff-A; pH 8.04) contained 5.0×10^{-3} mol dm^{-3} boric acid (H_3BO_3), 5.0×10^{-4} mol dm^{-3} potassium hydroxide (KOH), and 2.0×10^{-1} mol dm^{-3} sodium chloride (NaCl). The borate buffer solution (Buff-B; pH 12.19) used for the preparation of a Cu(II) catalyst solution contained 2.0×10^{-1} mol dm^{-3} H_3BO_3 and 2.0×10^{-1} mol dm^{-3} KOH. An acetic acid solution for pH-gradient elution was prepared by dissolving 2.5×10^{-2} mol acetic acid in 1 dm^3 Buff-A. The sample solution of bovine serum albumin (BSA) (Sigma Chemical Co.), human serum albumin (HSA) (Sigma Chemical Co.), bovine serum α-globulin (BαG) (Sigma Chemical Co.), bovine serum γ-globulin (BγG) (Sigma Chemical Co.), lysozyme (from chicken egg white) (Sigma Chemical Co.), α_2-macroglobulin (from human) (Sigma Chemical Co.),

apoferritin (from human) (ICN Pharmaceuticals, Inc.), α-fetoprotein (AFP) (Midorijuji Co.), and control serum (Q-PAC-Chemistry Control Serum I) (HYLAND) were prepared by dissolving them in Buff-A and by diluting them with Buff-A.

Preparation of an MCAC Column. The following columns were used for MCAC: TSK gel Chelate-5PW (7.5 mm i.d.×75 mm) (Toyo Soda, Tokyo), and HR-5/5 (5 mm i.d.×50 mm) (Pharmacia) charged with Chelating Sepharose 6B (Pharmacia). Their bed volumes were 3.3 cm^3, 0.4 cm^3, in the above-mentioned order. All of the charged gel, which has an iminodiacetic acid group at the end of a spacer, showed a chelate-forming ability against such metal ions as Cu(II), zinc(II) (Zn(II)), and cobalt(II); each 1 cm^3 of the charged gel was capable of adsorbing at least a 20 μmol of the metal ion.

Zinc(II) was selected as an added metal ion in the present study because Zn(II) could not be detected in an eluate using a CL detector and several proteins were selectively adsorbed and eluted on the Zn(II) containing gel.

An MCAC column was prepared as follows: A definite volume of 0.03 mol dm^{-3} Zn(II) solution (pH 4), prepared by dissolving zinc chloride in a dilute acetic acid solution, was delivered into a column through a sampling loop. In practice, the column was loaded with Zn(II) calculated on the basis of its adsorbing capacity, only to 67% of its length in order to avoid any contamination of the eluate with Zn(II). Thereafter, the column was preconditioned by feeding Buff-A (equivalent to 15 times of a bed volume) at a flow rate of 1.0 cm^3 min^{-1}. All of the fundamental experiments for the elution of proteins from a column were carried out using a Zn(II) loaded TSK gel Chelate-5PW; HR-5/5 was in order to improve of the sensitivity, based upon a reduction of the bed volume.

Apparatus and Procedure. The MCAC method was carried out using the HPLC system (Fig. 1) equipped with a CL detector and an apparatus for the pH-gradient. The CL detector used was the same one as described in previous papers.[3,4] A 50 mm^3 portion of a protein sample solution was injected into the eluent solution line, that is, an MCAC column (j) through the sampling loop (i) and the six-way cock (g). Then, the protein was adsorbed on an MCAC column.

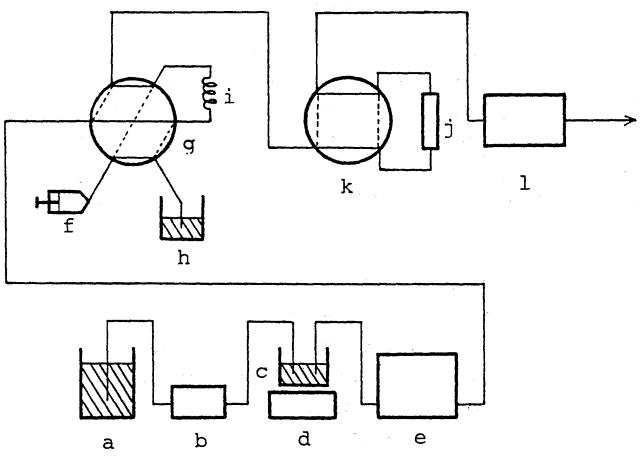

Fig. 1. Schematic diagram of the HPLC–CL system. a: Acetic acid solution, b: peristaltic pump, c: gradient-mixer, d: magnetic stirrer, e: constant flow pump, f: syringe, g: six-way cock, h : sample solution, i: sampling loop, j: MCAC column, k: four-way cock, and l: CL detector.

The pH-gradient elution was carried out as follows: The sample adsorbed on the MCAC column was eluted by a pH-gradient elution method. An acetic acid solution (a) for pH-gradient elution was fed into a gradient-mixer (c), which was charged with 50 cm^3 Buff-A at a flow rate of 0.5 cm^3 min^{-1} using a peristaltic pump (b). The solution in a gradient-mixer was well-stirred by a magnetic stirrer (d) and was fed into the MCAC column at a flow rate of 1.0 cm^3 min^{-1} using a constant flow pump (e). The protein eluted from the MCAC column was detected by a CL detector (l) as described in previous papers.[3,4]

The FIA method using direct-injection was carried out by injecting a sample solution (h) into the eluent line and by operating a four-way cock (k) and a six-way cock (g) equipped with a sampling loop (i).

Results and Discussion

Connection of the CL Detector with an MCAC Column. In a previous paper,[3] the optimum pH value of the solution during the CL reaction of a phen-H_2O_2-Cu(II) system was reported to be about 10.2 for the determination of a protein. Since the adsorption of a protein onto an MCAC column and the elution of a protein from it were usually carried out in the pH range of 8.0—4.0,[2,5–7] the pH value and buffer capacity of the solution during the CL reaction had to be adjusted to about pH 10.2 with a Cu(II) catalyst solution. Judging from the experimental result, this was accomplished by an adding a Cu(II) solution containing a borate buffer solution of pH 12.19 (Buff-B). A phen solution and a H_2O_2 solution were prepared as described in a previous paper.[2] Under these conditions, calibration curves for BSA, lysozyme, and BγG were obtained by an FIA method (Fig. 2). These proteins in a concentration range of 2.0×10^{-5}—1.0×10^{-1} g dm^{-3} could be determined by injecting each 50-mm^3 sample into an FIA system, with a detection limit of 1 ng (S/N=2) and a coefficient of variation (CV) of 5.9% (n=6) at 1.0×10^{-3} g dm^{-3} protein. Hereafter, the detection limit is based on S/N=2.

The different calibration curves of BSA, lysozyme, and BγG seemed to be due to differences between in their molecular structures. Relatively linear calibration curves were obtained in a previous study,[3] while the calibration curves shown in Fig. 2 are sigmoid. Since the concentration of NaCl was different in the experiment for the preparation of both calibration curves, the effect of NaCl on the form of the calibration curve was examined and a linear calibration curve was found to assume a sigmoid form with an increasing amount of NaCl. Though there might be some other causes for the sigmoid form, a calibration curve for protein by CL detection was mostly obtained as a sigmoid form.[8–10]

pH-Gradient. A protein adsorbed on an MCAC column can be eluted by such methods as 1) lowering

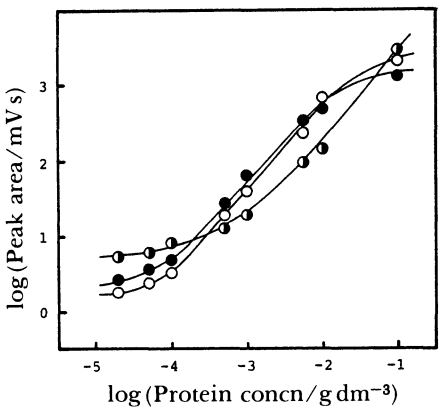

Fig. 2. Calibration curves for proteins by FIA. ○: BSA, ●: BγG, and ◑: lysozyme.

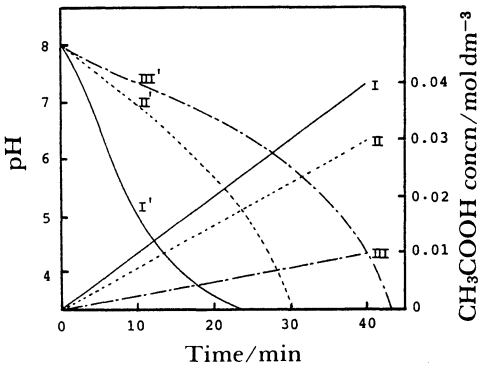

Fig. 3. Relationship among pH, time, and concentration of an acetic acid. Line-I, -II, and -III: relationship between C_A and t and curve-I', -II', and -III': relationship between pH and t.

of the pH, 2) adding a competitor,[7] and 3) adding ethylenediaminetetraacetic acid. Among these methods, methods 2) and 3) were unsuitalbe for the present purpose because they did not give smooth base-lines on the CL detector, whereas method 1) was shown to be useful. Since any protein adsorbed on a Zn(II) column was eluted at an individual pH-band and all the proteins used in this study were completely eluted near pH 4, a pH-gradient method in which the pH of the eluent changed continuously from 8.0 to 4.0 was proposed. During the course of an investigation regarding the preparation of the pH-gradient, it was found that 1) the base-line of the CL detector was most stable as a result of adding a definite concentration of acetic acid to Buff-A and by lowering the pH of the eluent, and 2) that the stability of the CL detector was further improved by adding salt (NaCl) to Buff-A. The pH-gradient of the eluent was prepared by using the gradient apparatus based on principle of a single stage of a continuously stirred tank reactor; the optimum conditions could be obtained. The pH change of the eluate from the Zn(II) column against a linear increase in the concentration of acetic acid (C_A) in the eluent is shown in Fig. 3. Line-I,-II, and -III show the relationship between C_A and the feed time (t), while curve-I', -II', and -III' show the relationship between pH and t. Line-III and curveIII' were best for the separation of proteins since the base-line stability of the CL detector was excellent and an approximately linear pH-gradient could be applied.

Effect of NaCl. A definite amount of salt was usually added to the eluent in MCAC in order to avoid any undesirable ion exchange and any association between proteins. According to the preliminary experiment, the retention time of BSA, lysozyme, and BγG varied according to the concentration of NaCl. However, the sensitivity of the CL detector became lower upon increasing the amount of NaCl. Therefore, their relations were examined in detail. So far, it has been experimentally accepted that the base-line separation of a binary mixture is feasible if the peak resolution

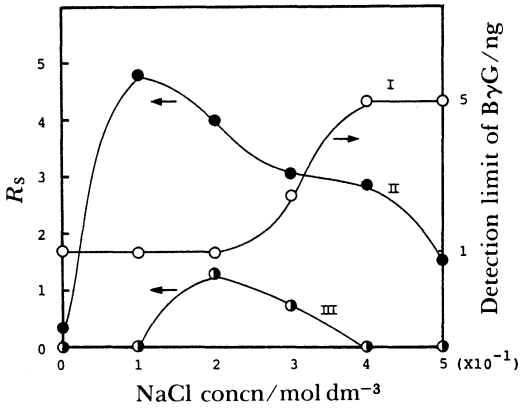

Fig. 4. Effect of NaCl on detection limit and R_S. ○: detection limit of BγG, ●: R_S between BSA and BγG, and ◑: R_S between BSA and lysozyme.

(R_S) exceeds 1.25 in a chromatogram.

The effect of the NaCl concentration on the detection limit of BγG and on the R_S between BSA and BγG as well as between BSA and lysozyme are shown in Fig. 4. As can be seen from curve-I in Fig. 4, the detection limit of BγG increases with increasing the concentration of NaCl. From curve-II in Fig. 4, BSA and BγG could also be completely separated when the eluent contained more NaCl than 0.1 mol dm^{-3} (R_S exceeded 1.25). From curve-III in Fig. 4, BSA and lysozyme could also be separated when the eluent contained 0.2 mol dm^{-3} NaCl (where R_S exceeded 1.25). On the basis of these results, an eluent containing 0.2 mol dm^{-3} NaCl was shown to be most suitable for a sensitive detection of BSA, lysozyme, and BγG as well as for the base-line separation of a BSA and BγG mixture or a BSA and lysozyme mixture. Therefore, an eluent containing 0.2 mol dm^{-3} NaCl was used for the separation of BSA, lysozyme, and BγG in the present experiment. Though the retention times of these proteins varied according to the concentration of NaCl in the eluent, the reason for such a phenomenon still remains unsolved.

Affinity against a Zn(II) Column. The affinity of various proteins against a Zn(II) column was examined under the above-mentioned conditions and the results are shown in Table 1. Judging from the retention time, 1) both BSA and HSA showed no affinity, 2) lysozyme showed moderate affinity, and 3) BαG, BγG, α_2-macroglobulin, and HγG showed strong affinity. α-Fetoprotein, a cancer marker, also showed no affinity.

Separation and Determination of Proteins by MCAC. As can be seen from the above results, BSA, lysozyme, and BγG, all the retention times that were mutually different, were selected as model proteins. The separation of a mixture consisting of the above-mentioned three proteins and the determination of individual proteins were carried out by MCAC. A protein mixture (50 mm^3) dissolved in Buff-A was injected onto a Zn(II) column, and pH-gradient elution was started 7 min after the injection of a sample solution. A chromatogram is shown in Fig. 5. As can be seen from Fig. 5, BSA, lysozyme, and BγG could be almost completely separated. Calibration curves of BSA, lysozyme, and BγG were obtained by MCAC using a Zn(II) column (Fig. 6). Both BSA and BγG in 50 mm^3 were determinable in the concentration range of 5.0×10^{-4}—1.0×10^{-1} g dm^{-3}, with a detection limit of each 25 ng. Lysozyme was also determinable in a concentration range of 8.0×10^{-4}—1.0×10^{-1} g dm^{-3}, with detection limit of 40 ng. Bovine serum γ-globulin of 1.0×10^{-3} g dm^{-3} was determined with an average recovery of 78% and a CV of 10.9% (n=6). The time necessary for an analysis was about 150 min. The detection limit of FIA method was 2.0×10^{-5} g dm^{-3} (1 ng) BγG but that of the MCAC method was 5.0×10^{-4} g dm^{-3} (25 ng) BγG. These differences seem to be due to the fact that the MCAC method is accompanied by an the extreme band broadening during separation, while the FIA method is not. The poor results in the above-mentioned average recovery and CV could be explained on the basis of the extreme band broadening. It was, therefore, concluded that the bed volume of a TSK gel Chelate-5PW was too large for the successful analysis of a low concentration of protein mixture using a CL detector.

Reduction of Bed Volume. In order to improve the detectability of a CL detector by reducing bed volume of a Zn(II) column, HR-5/5 having a bed volume 0.4 cm^3 was prepared and applied to a mixture of BSA, lysozyme, and BγG. Each calibration curve was obtained and is shown in Fig. 7. Both BSA and BγG

Table 1. Affinity Against a Zn(II) Column

Protein	Sample load/ng	Retention time/s
BSA	50	210
HSA	50	227
Apoferritin	50	227
AFP	38.8	287
Lysozyme	50	563
BαG	50	1487
BγG	50	1496
α_2-Macroglobulin	50	1559
HγG	50	1498

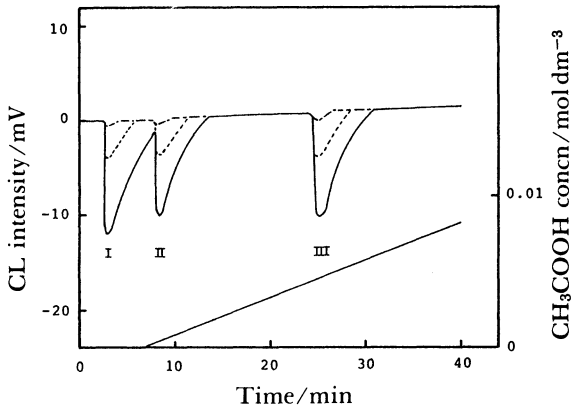

Fig. 5. A chromatogram of a BSA, lysozyme, and BγG mixture. I: BSA, II: lysozyme, and III: BγG, solid line: 1.0×10^{-1} g dm^{-3}, dotted line: 1.0×10^{-2} g dm^{-3}, broken line: 1.0×10^{-3} g dm^{-3}, and straight line: acetic acid concentration.

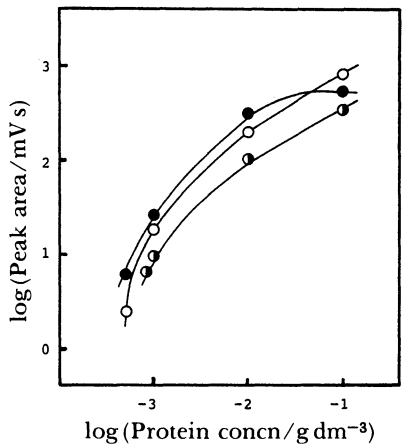

Fig. 6. Calibration curves for BSA, lysozyme, and BγG by MCAC. ○: BSA, ●: BγG, and ◑: lysozyme.

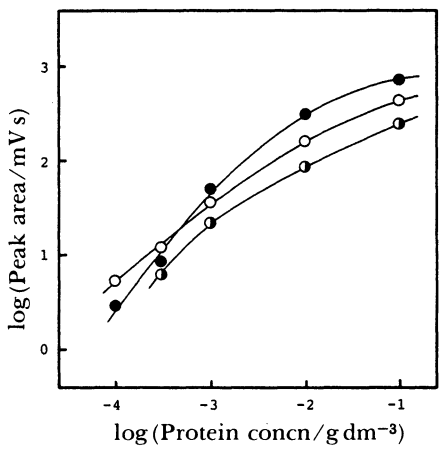

Fig. 7. Calibration curves for proteins by MCAC using a HR-5/5. ○: BSA, ●: BγG, and ◑: lysozyme.

in a 50 mm^3 sample solution were determinable in a concentration range of 1.0×10^{-4}—1.0×10^{-1} g dm^{-3}, with a detection limit of 5 ng each. Lysozyme was also determinable in the concentration range 3.0×10^{-4}—1.0×10^{-1} g dm^{-3}, with a detection limit of 15 ng. Bovine serum γ-globulin in a concentration of 1.0×10^{-3} g dm^{-3} could be determined with an average recovery of 85%, and a CV of 7.8% (n=5). The detection limit of BγG against HR-5/5 (0.4 cm^3 bed volume) was 5 ng whereas that of a TSK gel Chelate-5PW (3.3 cm^3 bed volume) was 25 ng under the same conditions. The time necessary for an analysis was about 60 min. Moreover, HR-5/5 could be confirmed from Fig. 7 to be satisfactorily usable for analyzing of a mixture consisting of a low concentration of proteins.

The lowest detectable concentration of BγG using a UV detector and a CL detector were compared under the same conditions using a TSK gel Chelate-5PW: 1.0×10^{-1} g dm^{-3} BγG for a UV detector, and 5.0×10^{-4} g dm^{-3} BγG for a CL detector. The sensitivity of a CL detector was about 200 times as high as that of a UV detector.

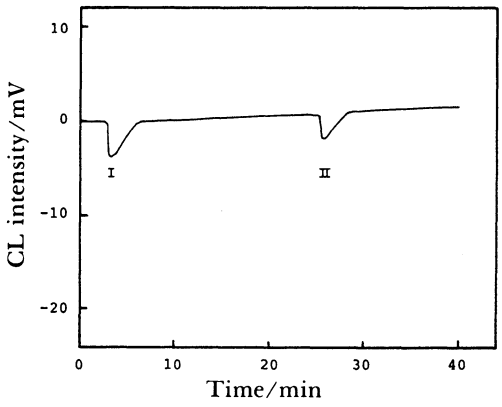

Fig. 8. A chromatogram of human serum. I: HSA and II: human serum globulins.

Finally, a control serum was diluted 5000 times with Buff-A and an aliquot (50 mm^3) was analyzed by the present method using a TSK gel Chelate-5PW (Fig. 8). As shown in the figure, there are two peaks: peak I corresponds to HSA and peak II to human serum globulins.

It has been concluded from the results that by the use of a CL detector and an MCAC column having a small bed volume, sensitive MCAC suitable for the separation and determination of low concentrations of proteins could be established. The present study suggests the possibility of combining a CL detector with a wide range of columns, including an MCAC column.

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