The Determination of a Small Amount of Biological Constituent by the Use of Chemiluminescence. IV. A Highly Sensitive Detector for the HPLC of Protein

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A highly sensitive detector for the HPLC of protein has been manufactured by making use of the phenomenon that the catalytic activity of copper(II) against the chemiluminescence between luminol and hydrogen peroxide is lowered in the presence of protein. According to the present detector, 2×10^{-4}—1×10^{-1} g/dm^3 protein could be determined, with the detection limit of 10 ng. By combining the present detector with a TSK-GEL G3000SW column, 2×10^{-3}—1×10^{-1} g/dm^3 ovalbumin could be determined, with the detection limit of 50 ng. The ovalbumin in 2×10^{-2} g/dm^3 could be determined by the internal-standard method with a coefficient of variation of 8.65% (n=5). Care had to be taken in the detection of the protein acting as a chemiluminescent catalyst.

As detectors for the high-performance liquid chromatography(HPLC) of protein, the following apparatus are now being used: a ultraviolet spectrophotometer(UV), a differential refractometer(RI), a light-scattering spectrophotometer, and so on. The detection limit of RI is inferior to that of UV.[1] A precise determination of protein by the use of a light-scattering spectrophotometer alone is difficult because the signal obtained with it is given by the product of the molecular weight of protein and its concentration.[2] UV is also unsuitable for application to an unknown sample because a measured value varies depending on the kind of protein, and the detection limit of 20 μg/cm^3 protein is still unsatisfactory for the determination of a small amount of protein in a clinical laboratory. To improve the detector sensitivity, a fluorometer has been applied to the determination of peptides after separation by means of HPLC.[3] On the other hand, a method for the flow-injection analysis of protein has been established by the present authors on the basis of the fact that the catalytic activity of copper(II) against the chemiluminescence (CL) between 5-amino-2,3-dihydro-1,4-phthalazinedione(luminol) and hydrogen peroxide(H_2O_2) is lowered in the presence of protein.[4] This method is applicable to the detection of 2×10^{-4}—1×10^{-1} g/dm^3 protein and is more sensitive than any others thus far reported. Moreover, this method shows a similar sensitivity for various kinds of proteins and makes a continuous determination of protein possible. Since this method was expected to be a promising post-column detector for the protein separated in an aqueous HPLC system without losing the inherent property of protein, the analytical conditions, detection limit, and so on were investigated.

Experimental

Reagents. All the reagents used were of a commercially available special grade. A 2.0×10^{-6} mol/dm^3 copper(II) solution was prepared by diluting a 2.0×10^{-2} mol/dm^3 copper(II) stock solution with a buffer solution(Buff-I)(pH 10.2) consisting of 0.1 mol/dm^3 boric acid and 0.1 mol/dm^3 potassium hydroxide. A 1.0×10^{-4} mol/dm^3 luminol solution was prepared by the use of the same buffer solution as above. A 5.0×10^{-4} mol/dm^3 H_2O_2 solution was prepared by diluting a 0.3 wt% H_2O_2 solution with distilled water. A 3.33×10^{-3} mol/dm^3 phosphate buffer solution(Buff-II; pH 6.19), consisting of a 2.67×10^{-3} mol/dm^3 potassium dihydrogenphosphate(KH_2PO_4) solution, a 6.67×10^{-4} mol/dm^3 disodium hydrogenphosphate(Na_2HPO_4) solution, and a 0.15 mol/dm^3 potassium chloride(KCl) solution, was prepared; it was also used as an eluent later on. The sample solutions of bovine serum albumin(BSA) (Sigma Chemical Co.), lysozyme (from chicken egg white) (Sigma Chemical Co.), ovalbumin (Sigma Chemical Co.), and the Gel Filtration Standard(Bio. Rad) were prepared by dissolving them in Buff-II and by diluting them with Buff-II.

Apparatus and Procedure. A HPLC system equipped with a CL detector was set up for the separation and determination of protein (Fig. 1). All the tubes (0.5 mm i.d.) and connectors(n) used were made of Teflon. Each solution of luminol(a) and H_2O_2(b), held at 25 °C by means of a thermostat(q) (Netsudenshi kogyo Co., Ltd.), and of copper(II)(c), held at room temperature, was fed by means of nitrogen gas of 3.3×10^5 Pa pressure gauge, the flow rate was adjusted to 1.0 cm^3/min by the use of a flow-meter equipped with a needle valve(h)(GILMONT). An eluent(buffer solution)(d) was fed at the flow rate of 1.0 cm^3/min by means of a pump(i) (Pharmacia High-precision Pump P-500). A 50-mm^3 portion of a mixed protein solution was injected into the eluent line through the sampling loop(k_1) and the seven-way cock(j_1) (Pharmacia Fine Chemicals, Valve-7), after which the protein was separated on the column(m) (TSK-GEL G3000SW column: 600×7.5 mm i.d.+TSK-GEL G3000SW guard column: 75×7.5 mm i.d.). The protein in an eluate was first mixed with a copper(II) solution, and then the resulting solution was further mixed with a H_2O_2 solution at the position W and with a luminol solution at the position X after having passed through a reaction tube(o) (0.64 m), held at 95 °C and a cooling tube(p) (0.40 m), held at 0 °C, in that order. Finally, the mixed solution was introduced into the same flow cell(r) as in the previous paper. The CL intensity

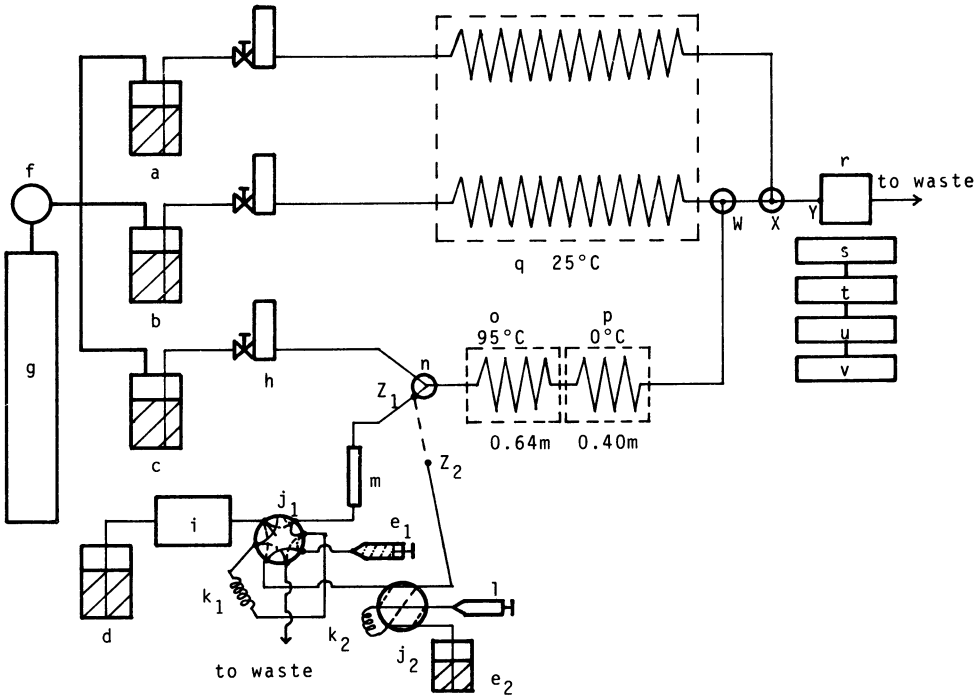

Fig. 1. Schematic diagram of the system equipped with CL detector.
a: Luminol solution, b: H_2O_2 solution, c: copper (II) catalyst solution, d: eluent (buffer solution), e_1 and e_2: sample solution, f: regulator, g: N_2 cylinder, h: flow-meter equipped with a needle valve, i: pump, j_1: seven-way cock, j_2: six-way cock, k_1 and k_2: sampling loop, l: syringe, m: cloumn, n: connector, o: reaction tube, p: cooling tube, q: thermostat, r: flow cell, s: photomultiplier, t: amplifier, u: recorder, and v: integrator.

was measured by means of a photomultiplier(s) (Hamamatsu TV Co., Ltd., R928), amplified with an amplifier(t) (Horiba Ltd., OPE-402), and recorded on a recorder(u) (Yokogawa Electric Works, Ltd., 3046), followed by the measurement of the peak area by the use of an integrator(v) (Shimadzu Chromatopac C-R2A). The distances between X and W or Y were 30 cm and 10 cm respectively.

The flow-injection analysis of protein by a direct-injection method was carried out by injecting a sample solution into the eluent line by operating a seven-way cock (j_1) and a six-way cock(j_2) equipped with a sampling loop(k_2) after connecting Z_1 with Z_2. Though a ghost peak appeared periodically owing to the exchange between the two cylinders in the pump(i), it did not interfere with the determination of the protein.

Results and Discussion

Characteristics of the Detector. The stability of a base line is generally indispensable for the exact determination of protein by means of HPLC. Though the CL intensity was significantly influenced by the temperature, no care was taken about the temperature except for a copper(II) solution in the previous paper. Therefore, no stable base line was obtained over a long period. In the present study, both the luminol solution and the H_2O_2 solution were held at 25 °C so as to give a stable base line. Since the flow rate of eluent in HPLC was generally less than 1.0 cm^3/min, a BSA sample solution was directly estimated by flow-injection analysis under the same conditions as were used in the previous paper except for the use of 1.0 cm^3/min instead of 10 cm^3/min for the flow rate of the buffer solution(Buff-I) (Fig. 1(d)). The detection limit of BSA was 40 ng in the previous paper, while it was 400 ng in this case. This was assumed to be due to the dilution of a sample solution, for the flow rate of each solution of copper(II), luminol, and H_2O_2 was 10 cm^3/min in the previous paper, while that of the buffer solution containing BSA in the present study was 1.0 cm^3/min. Therefore, the flow rate of each solution was selected as 1.0 cm^3/min. The diameter of the tubing was also changed from 1 mm to 0.5 mm so as to reduce the diffusion of a sample. Furthermore, the length of the reaction tube and the cooling tube were changed from 1.0 m to 0.64 m and from 1.6 m to 0.40 m respectively so as to provide the same residence time as in the previous paper. Under these conditions, the maximum diminution in the CL intensity based on the injection of protein was observed at a 1.0×10^{-4} mol/dm^3 luminol, a 5.0×10^{-4} mol/dm^3 H_2O_2, and a 2.0×10^{-6} mol/dm^3 copper(II) solution.

The calibration curve obtained by the flow-injection analysis of BSA, using the direct-injection technique at the above-mentioned flow rate and reagent concentra-

tion, is shown in Fig. 2, together with that obtained in the previous paper. According to the present detector, 2×10^{-4}—1×10^{-1} g/dm^3 BSA could be determined; its detection limit(S/N=2) was the same as in the previous paper. The coefficients of variation for ten measurements of 1×10^{-3} and 1×10^{-2} g/dm^3 BSA were 11.2% and 6.9% respectively. As can be seen from Fig. 2, the slope of the present calibration curve was about one, while that in the previous paper was about one-half. The difference between these two slopes may be supposed to be based on (1) the difference in the reaction conditions for the chelate formation between copper(II) and protein and/or (2) the difference between the residence times in the flow cell in the previous paper(about 0.3 s) and in the present paper(about 3.4 s).

Conditions for the Separation of Protein. A combination of the present detector and gel-permeation chromatography, in which proteins could be separated by the use of an aqueous eluent without changing the higher-order structure of protein, was devised for the separation of proteins. For this combination, care had to be taken in the use of an eluent with a pH value of around 7 against a TSK-GEL G3000SW column and of the alkaline solution for the chelate formation between copper(II) and protein and in the CL reaction.

The effects of the concentration of a phosphate buffer solution and a salt solution on the present detector in the gel-permeation chromatography of ovalbumin and lysozyme were also examined. The effect of the phosphate solution on the chelate formation between copper(II) and protein and the subsequent CL reaction is shown in Fig. 3. The results in Fig. 3 can be understood from the following experimental data.

The pH of the solution in a flow cell was inferred by measuring the pH value of the mixed solution consisting of distilled water, a Buff-I solution, and a phosphate buffer solution (3.33×10^{-2}, 3.33×10^{-3}, 3.33×10^{-4} mol/dm^3) containing a KH_2PO_4 solution and a Na_2HPO_4 solution in a volume ratio of 1:2:1; pH values of 9.8, 10.0, and 10.0 were found, in that order. This shows that the pH value of the solution in a flow cell is around the pH value desired for the measurements of the CL intensity.

On the other hand, the pH value of the mixed solution consisting of a Buff-I solution and a phosphate buffer solution (3.33×10^{-2}, 3.33×10^{-3}, 3.33×10^{-4} mol/dm^3), containing a KH_2PO_4 solution and a Na_2HPO_4 solution in a volume ratio of 1:1, was measured to ascertain the pH value in a reaction tube containing copper(II) and protein; the pH values 9.4, 9.9, and 9.9 were thus obtained.

In other words, the difference in the results of Fig. 3 may be concluded to be primarily due to the difference in the chelate formation by pH. Since the detection limits for A and B in Fig. 3 did not differ much from

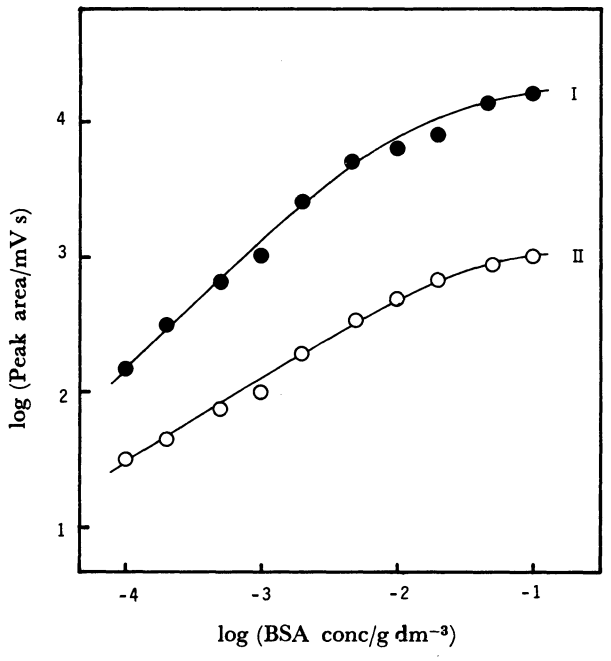

Fig. 2. Calibration curves of BSA by the present method and the previous method.
I: present paper and II: previous paper.
Conditions: previous paper; 5.0×10^{-4} mol/dm^3 luminol, 5.0×10^{-4} mol/dm^3 H_2O_2, 2.0×10^{-6} mol/dm^3 copper(II), and Buff-I(pH 10.15) as a buffer solution and present paper; 1.0×10^{-4} mol/dm^3 luminol, 5.0×10^{-4} mol/dm^3 H_2O_2, 2.0×10^{-6} mol/dm^3 copper(II), and Buff-II(pH 6.19) as a buffer solution.

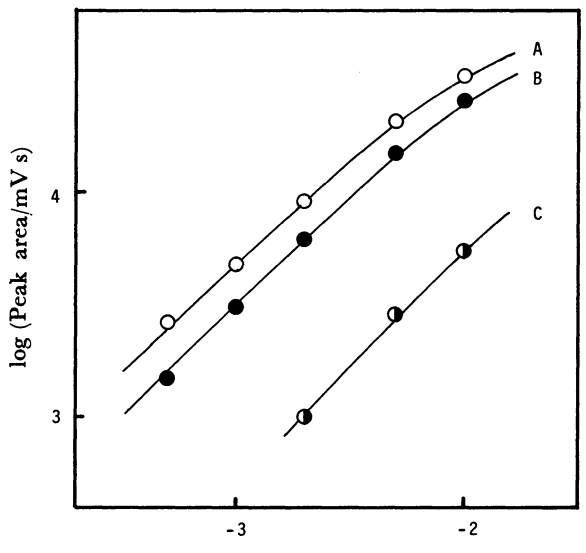

Fig. 3. Effect of concentration of phosphate buffer solution on the present detector.
A: 3.33×10^{-4} mol/dm^3-, B: 3.33×10^{-3} mol/dm^3-, and C: 3.33×10^{-2} mol/dm^3-phosphate buffer solution consisting of KH_2PO_4 solution and Na_2HPO_4 solution.
Conditions: 1.0×10^{-4} mol/dm^3 luminol, 5.0×10^{-4} mol/dm^3 H_2O_2, 2.0×10^{-6} mol/dm^3 copper (II), and ovalbumin as sample.

each other, and since a larger buffer capacity was desired for an eluent, a 3.33×10^{-3} mol/dm^3 phosphate buffer solution was chosen as the eluent for the HPLC.

The ionic strength of an eluent was enhanced by the addition of a salt to suppress the ionic adsorption of protein on the column. The effect of the added salt(KCl) on the present detector is shown in Fig. 4. The base line was lowered with an increase in the concentration of KCl, and then the CL intensity was influenced by the addition of KCl. The peak area in Fig. 4 is shown against the base line in a KCl-free ovalbumin solution. As can be seen from Fig. 4, the peak area was almost definite independent of the concentration of KCl, and the addition of KCl did not affect the reaction between copper(II) and ovalbumin. Since more than 0.2 mol/dm^3 of KCl reduced the CL intensity, and since the detection limits in the 0, 0.05, 0.10, and 0.15 mol/dm^3 KCl solutions were all approximately the same, a 0.15 mol/dm^3 KCl solution was selected as the optimum concentration.

Separation by Means of a Column. The number of theoretical plates was calculated from the peak width and the elution time recorded on recorder chart paper by injecting 1 μg of ovalbumin; the value of 1840 was thus obtained. According to the UV method, the number of theoretical plates 2488 was reported[5] to be obtained under the following conditions: a 0.1 mol/dm^3 phosphate buffer solution as an eluent, a flow rate of 0.875 cm^3/min, 100 μg of ovalbumin, and a 30.5-cm TSK-GEL G3000SW column. This value was calculated to be 5890 at the flow rate and the column length used in the present study.

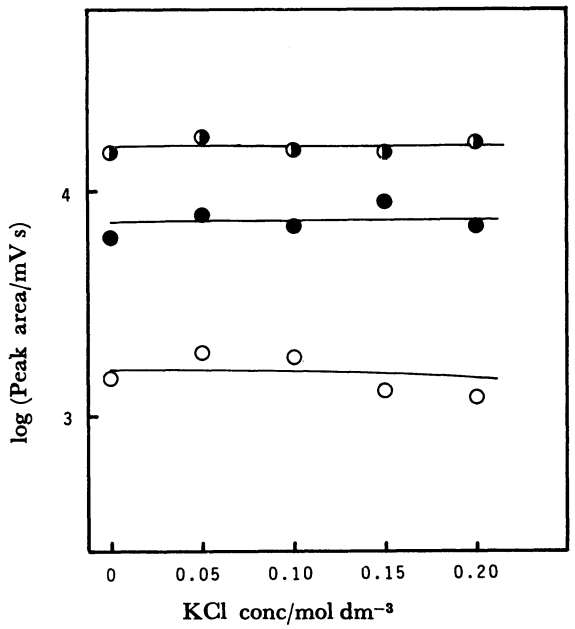

Fig. 4. Effect of added KCl on the present detector.
○: 5.0×10^{-4} g/dm^3-, ●: 2.0×10^{-3} g/dm^3-, and ◑: 5.0×10^{-3} g/dm^3-ovalbumin.
Conditions: 1.0×10^{-4} mol/dm^3 luminol, 5.0×10^{-4} mol/dm^3 H_2O_2, 2.0×10^{-6} mol/dm^3 copper (II), and 3.33×10^{-3} mol/dm^3 phosphate buffer solution consisting of 2.67×10^{-3} mol/dm^3 KH_2PO_4 solution and 6.67×10^{-4} mol/dm^3 Na_2HPO_4 solution.

It has usually been accepted in flow-injection analysis that the band broadening of a sample based on the diffusion in a tube can be neglected at a tube diameter of less than 0.5 mm. Since a tube 0.5 mm i.d. was used in the present study, the diffusion of a sample in the eluate seems not to lower the number of theoretical plates. Therefore, the lowering of the number of theoretical plates may be supposed to be attributed to the residence time in the flow cell, the amount of the sample, and so on.

A 18-mg sample(Gel Filtration Standard) of a mixture consisting of protein aggregates, thyroglobulin-(bovine), γ-globulin(bovine), ovalbumin(chicken), myoglobin(horse), and vitamin B-12 was dissolved in a 0.5-cm^3 Buff-II solution, and from the solution(Sample I) a 50-mm^3 fraction was taken, after which analysis was done by means of HPLC using a UV detector(Hitachi, Ltd., wavelength-tunable effluent monitor, 634). On the other hand, Sample I was diluted to 1:667 with Buff-II, and from the solution(Sample II) a 50-mm^3 fraction was taken, followed by analysis by means of HPLC using the present detector. The chromatograms thus obtained are shown in Fig. 5.

As can be seen from both chromatograms in Fig. 5, the D and E peaks in the chromatogram obtained by the present detector were relatively small in comparison with the A, B, and C. Furthermore, the D and E peaks appeared on the side opposite those in II of Fig. 5 when Sample I was diluted to 1 : 200 and a 50-mm^3 fraction was similarly analysed.

Hemoglobin, catalase, and peroxidase, which are all hemoproteins containing an iron-porphine complex structure, have been well known to act as catalysts in the CL reaction. Since myoglobin is also a hemoprotein, it was expected to act more or less as a catalyst in the CL reaction.

The peak D of myoglobin in II of Fig. 5 was obtained by subtracting the inverse response based on the catalytic activity of myoglobin as a catalyst against the CL reaction from the response based on myoglobin as a protein. Vitamin B-12, containing a cobalt(II)-porphine complex structure in its central position, was experimentally confirmed by the authors to act as a weak catalyst against the CL reaction; then, the E peak was obtained by reasoning similar to that in case of the D peak.

To sum up, the present detector was shown to be a useful one for the sensitive detection of protein in an eluate from the HPLC column, though care had to be taken in the detection of protein acting as a catalyst against the CL reaction.

Determination by Means of a Column. Two calibration curves were obtained as follows: (I) each 50-

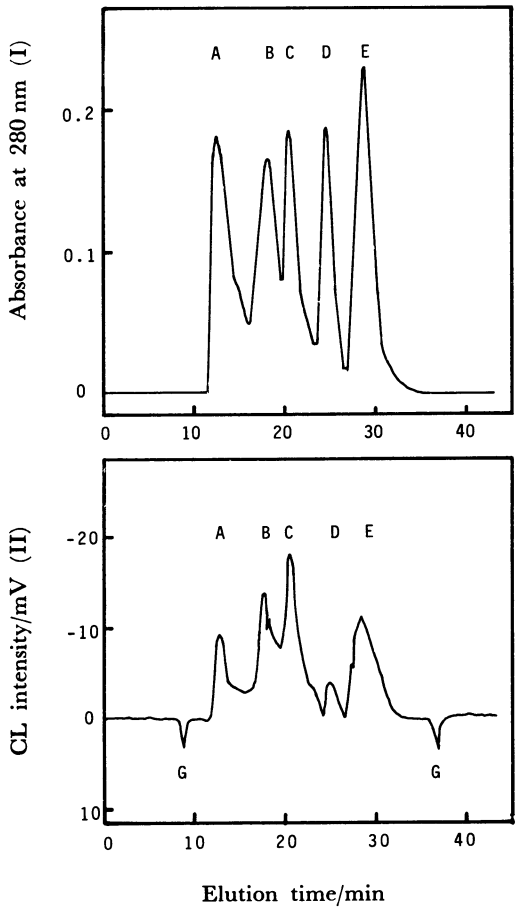

Fig. 5. Chromatograms of Gel Filtration Standard obtained by the present detector and the UV detector.
A: protein aggregates+thyroglobulin(bovine), B: γ-globulin(bovine), C: ovalbumin(chicken), D: myoglobin, E: vitamin B-12, and G: ghost peak.
Conditions: 3.33×10^{-3} mol/dm^3 phosphate buffer solution(Buff-II)(pH 6.19) as eluent, load volume 50 mm^3, I(UV detector); Range 1.28 and Sample-I: Gel Filtration Standard (18 mg mixture sample)/0.5 cm^3 Buff-II, and II(present detector); Sample-II: Sample-I diluted to 1 : 667 with Buff-II.

mm^3 portion in the concentration range of 2×10^{-3}—1×10^{-1} g/dm^3 ovalbumin was injected into a separation column, and the ovalbumin in the eluate was monitored by means of the present CL detector, and (II) each 50-mm^3 portion in the concentration range of 2×10^{-4}—1×10^{-1} g/dm^3 ovalbumin was directly subjected to flow-injection analysis without using a separation column. The results obtained are shown in Fig. 6.

The coefficient of variation for five measurements of 2×10^{-2} g/dm^3 ovalbumin in the former (I) was 12.7%. The deviation of the calibration curve II from a straight line can be understood in terms of the shortage of copper(II) in the reaction with ovalbumin; the shift of the calibration curve I to the higher side of ovalbumin concentration can also be understood by the dilution of the sample in a column. Here, the average recovery of 2×10^{-3}—2×10^{-2} g/dm^3 ovalbumin

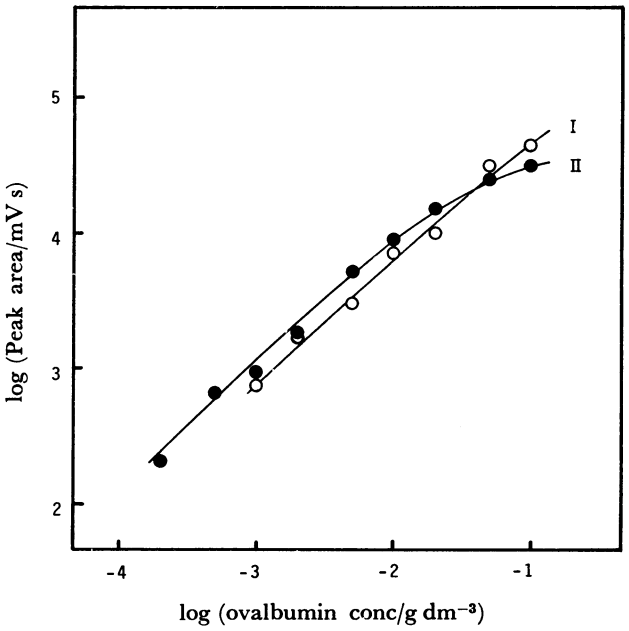

Fig. 6. Calibration curves of ovalbumin obtained by HPLC and flow-injection analysis.
I: through a column and II: column-free.
Conditions: injection volume 50 mm^3, 1.0×10^{-4} mol/dm^3 luminol, 5.0×10^{-4} mol/dm^3 H_2O_2, 2.0×10^{-6} mol/dm^3 copper(II), and Buff-II (pH 6.19) as buffer solution.

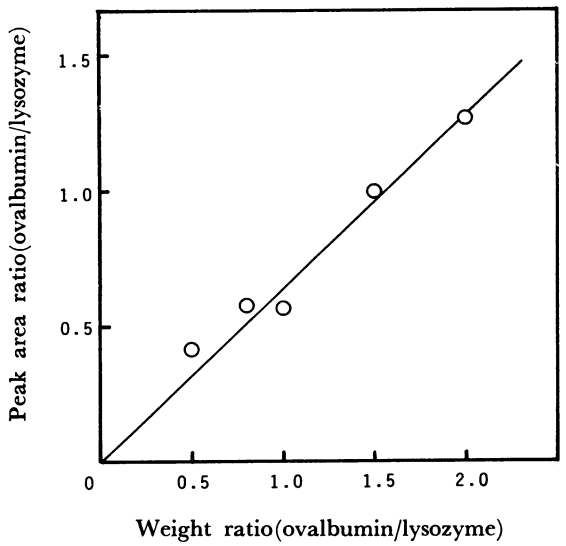

Fig. 7. Calibration curve of ovalbumin by use of lysozyme as internal standard.
Conditions: load volume 50 mm^3, Buff-II (pH 6.19) as eluent, and 2.0×10^{-2} g/dm^3 lysozyme as internal standard.

was 77.3%. Thus, the detection limit of the flow-injection analysis was 10 ng, while that of the column procedure was 50 ng.

Ovalbumin solutions of 1.0×10^{-2}, 1.6×10^{-2}, 2.0×10^{-2}, 3.0×10^{-2}, and 4.0×10^{-2} g/dm^3 were determined by the use of a 2.0×10^{-2} g/dm^3 lysozyme solution as the internal standard (Fig. 7). A linear relationship

was obtained between the weight ratio of ovalbumin to lysozyme and the ratio of the corresponding peak area. The coefficient of variation for five measurements of 2.0×10^{-2} g/dm^3 ovalbumin was 8.65%, and the internal standard method was shown to be superior to the absolute calibration method using the calibration curve I in Fig. 6.

To sum up, it has been found by the present authors that the catalytic activity of copper(II) against the CL between luminol and H_2O_2 is lowered in the presence of protein, and a sensitive and new detector for the HPLC of protein has been developed, 2×10^{-3}—1×10^{-1} g/dm^3 ovalbumin being determined within the detection limit of 50 ng. Since, however, the detection limit of the present detector was damaged by dilution and the low recovery which occurred when the present detector and HPLC were combined, some improvements overcoming these problems have to be done in future studies.

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