Determination of a Small Amount of a Biological Constituent by the Use of Chemiluminescence. XII. Highly Sensitive Immunoaffinity Chromatography

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Synopsis. Highly sensitive immunoaffinity chromatography was established by combining a micro-bore affinity column with a chemiluminescence detector for protein monitored by a flow-injection method using a 1,10-phenanthroline–hydrogen peroxide–copper(II) system. After having optimized the bed volume of a column, the flow rate of a buffer solution which was used for an immune reaction, and the time necessary for an immune reaction in a column, human serum albumin in the range of 3×10^{-4}—1×10^{-1} g dm^{-3} could be determined, with the detection limit of 15 ng as the injected amount. Human serum γ-globulin and bovine serum γ-globulin showed somewhat nonspecific adsorption onto an affinity column with values of 0.16 and 0.14 based on the specific adsorption 1.00 of human serum albumin onto a column, whereas bovine serum albumin, bovine serum α-globulin, and ovalbumin showed no nonspecific adsorption.

Cyclic immunoaffinity chromatography was previously established by the authors by combining an affinity column with a chemiluminescence (CL) detector for protein using the chemiluminescent reaction of a 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol)–hydrogen peroxide (H_2O_2)–copper(II) (Cu(II)) system.[1,2] According to this method, a small amount of protein could be determined continuously and selectively without using a labeling procedure. However the sensitivity of this method was not yet satisfactory for the determination of a trace amount of α-fetoprotein (AFP) as a tumor marker. Furthermore, some problems as to both nonspecific adsorption onto an immunoaffinity column and the cycling of a sample solution still remained undissolved.

On the other hand, a highly sensitive CL detector for protein monitored by a flow-injection method using a 1,10-phenanthroline (phen)–H_2O_2–Cu(II) system was established by the authors in the previous paper[3]. This method was 40 times superior to the previous one using a luminol–H_2O_2–Cu(II) system in their detection limits of protein.

In the present study, the above mentioned CL detector using a phen–H_2O_2–Cu(II) system was used for the determination of protein together with a micro-bore immunoaffinity column which required no cycling of the sample solution. Experimental conditions such as the bed volume of an affinity column, the flow rate of a buffer solution which was used for an immune reaction, and the time necessary for an immune reaction in a column were optimized, followed by the determination of human serum albumin. The result showed that marked improvement could be achieved compared with the previous work in the following points: The detection limit of protein, the nonspecific adsorption onto a column, the time necessary for a measurement, and the durability of a column.

Experimental

All reagents were of commercially available special grade. Ion exchanged water was distilled for use. A phen solution, a H_2O_2 solution, and a Cu(II) catalyst solution containing L-arginine hydrochloride were prepared as in the previous paper.[3] Human serum albumin (HSA) (Miles Laboratories, Inc.), bovine serum albumin (BSA) (Nakarai Chemicals, Ltd.), human serum γ-globulin (HγG) (Sigma Chemical Co.), bovine serum γ-globulin (BγG) (Sigma Chemical Co.), bovine serum α-globulin (BαG) (ICN Pharmaceuticals, Inc.), ovalbumin (Ova) (Sigma Chemical Co.), and human control serum (Q-PAK-Chemistry Control Serum I; Hyland) were diluted with a 8.34×10^{-3} mol dm^{-3} phosphate buffer solution (Buff-A, pH 7.3) consisting of a 2.78×10^{-3} mol dm^{-3} potassium dihydrogenphosphate solution and a 5.56×10^{-3} mol dm^{-3} disodium hydrogenphosphate solution.

Rabbit anti-HSA (DAKO) was immobilized onto CNBr-Activated Sepharose 4B (Pharmacia Fine Chemicals) in accordance with the instruction paper. About 5 mg of antibody was immobilized onto 1 cm^3 of support. This value was obtained by the measurement of the remaining antibody by the UV monitor (Hitachi, Ltd., wavelength-

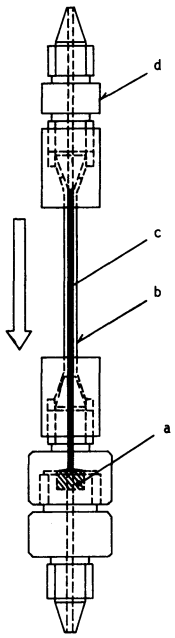

Fig. 1. Structure of a micro-bore column.
a: 10 μm Teflon filter, b: 2 mm o.d.×1 mm i.d. Teflon tube, c: immunosorbent, and d: joint (0.6 mm i.d.).

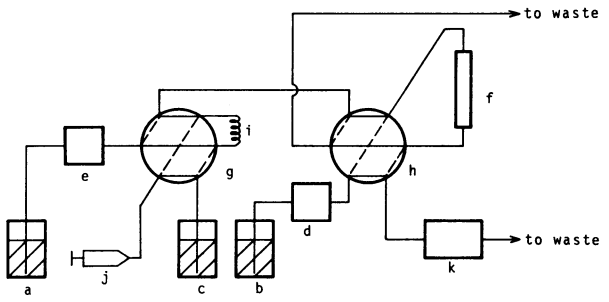

Fig. 2. Schematic flow diagram of the immunoaffinity chromatography system equipped with a CL detector.
a: Buffer solution (Buff-A), b: eluent (Buff-B), c: sample solution, d and e: pump, f: column, g and h: six-way cock, i: sampling loop, j: syringe, and k: CL detector.

tunable effluent monitor, 634A). This immunosorbent was packed in a micro-bore column (1 mm i.d. ×50 mm) which was manufactured using a Teflon tube (2 mm o.d. ×1 mm i.d.) and a Teflon line-filter (Nishio Kogyo, Ltd.) (Fig. 1).

A flow diagram of the immunoaffinity chromatography system equipped with the CL detector devised by the authors is shown in Fig. 2. A buffer solution which was used for an immune reaction (Buff-A) (a) was fed at the flow rate of 0.2 cm^3 min^{-1} by the pump (e) (Atto, SJ1211), and an eluent (Buff-B, pH 2.2) (b) consisting of a 8.0×10^{-3} mol dm^{-3} hydrochloric acid solution and a 2.5×10^{-2} mol dm^{-3} potassium chloride solution was fed at the flow rate of 1.0 cm^3 min^{-1} by the pump (d) (Pharmacia Fine Chemicals, High-precision Pump, P-500). A 50 mm^3 portion of HSA solution was injected into the Buff-A line through a sampling loop (i) and a six-way cock (g), and passed through an affinity column (f). The immune reaction was performed by feeding Buff-A to the column for 10 min after the injection of a sample solution. Then Buff-B was fed to the column to liberate HSA by operating a six-way cock (h). HSA in the eluate from the column was detected by the CL detector (k). Subsequently Buff-A was fed again to the column for 2 min to regenerate it by operating a six-way cock (h).

Results and Discussion

The optimum pH value of an eluent was determined so as to liberate sufficient HSA from an affinity column (Fig. 3). The gel immobilized with antibody was packed in a column (3 mm i.d. ×50 mm, Omnifit), and to it, 200 mm^3 of 1.0×10^{-1} g dm^{-3} HSA solution was loaded. Then HSA was liberated by feeding the eluent at various concentrations of hydrochloric acid (the concentration of potassium chloride was constant: 2.5×10^{-2} mol dm^{-3}). HSA in the eluate from the column was detected by means of the UV monitor, and the peak area was measured by use of an integrator (Shimadzu Chromatopac E1A). Judging from Fig. 3, 8.0×10^{-3} mol dm^{-3} hydrochloric acid–2.5×10^{-2} mol dm^{-3} potassium chloride buffer solution (pH 2.2) was employed as an eluent.

In the previous work, the equipment and the procedure for analysis were complicated since it required the cycling of a sample solution for an

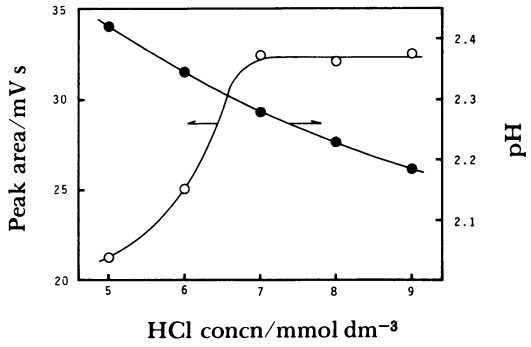

Fig. 3. Effect of HCl concn on liberation.
○: peak area and ●: pH of eluent. Conditions: 2.5×10^{-2} mol dm^{-3} KCl and 1.0×10^{-1} g dm^{-3} HSA.

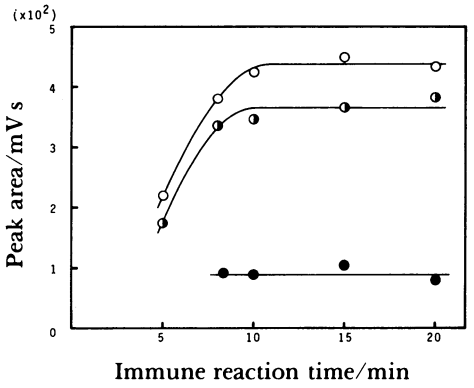

Fig. 4. Relationship between the immune reaction time and the peak area of liberated protein.
○: 1 mm i.d.×50 mm, ◑: 1 mm i.d.×30 mm, ●: 1 mm i.d.×15 mm column. Condition: 4.0×10^{-3} g dm^{-3} HSA.

immune reaction. Moreover, the cycling of a sample solution was thought to badly influence the time necessary for a measurement, the durability of the column, the coefficient of variation, and nonspecific adsorption onto the column. Therefore, a micro-bore immunoaffinity column in the present study was manufactured using a Teflon tube (1 mm i.d.) and a Teflon line-filter (pore size=10 μm) which required no cycling of a sample solution (Fig. 1). The flow rate of Buff-A was selected to be 0.2 cm^3 min^{-1}, the minimum flow rate achieved by the pump used, so as to perform the immune reaction sufficiently on a column during a single passage of a sample solution. Using this flow rate, the bed volume of a column and the reaction time (the time of feeding Buff-A) required were investigated using a 4.0×10^{-3} g dm^{-3} HSA solution. The relationship between the immune reaction time and the peak area of the liberated protein is shown in Fig. 4 using several columns of various bed volume (1 mm i.d. ×15, 30, and 50 mm). In case of a 1 mm i.d. ×70 mm column, the blank value was so large that the measurement was disturbed. Therefore a 1 mm i.d. ×50 mm column was employed so as to reduce the blank value and to obtain the maximum peak area at the same time. The immune reaction time was

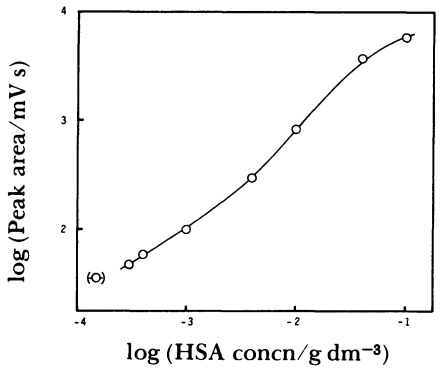

Fig. 5. Calibration curve of HSA.
(—O—): Blank value.

Table 1. Selectivity of an Affinity Column

Protein	Selectivity
HSA	1.00
HγG	0.16
BSA	0.00
BγG	0.14
BαG	0.00
Ova	0.00

Protein concn = 1.0×10^{-2} g dm^{-3}.

selected to be 10 min. A 1.0×10^{-1} g dm^{-3} BSA solution was analyzed under the above mentioned conditions, but no protein peak was observed. Liberation of HSA was performed by feeding Buff-B at the flow rate of 1.0 cm^3 min^{-1}. HSA less than 1.0×10^{-2} g dm^{-3} could be completely eluted within 5 min. Then the column was regenerated for 2 min at the flow rate of 1.0 cm^3 min^{-1} by feeding Buff-A corresponding to the volume of 50 times as large as the bed volume. The time necessary for an overall measurement (immune reaction-liberation-regeneration) was 17 min.

Under the condition mentioned above, the calibration curve of HSA obtained by the present method is shown in Fig. 5. As can be seen from Fig. 5, HSA in the range of 3×10^{-4}—1×10^{-1} g dm^{-3} could be determined with the lower limit of 15 ng. The coefficient of variation for six analyses of 4.0×10^{-3} g dm^{-3} HSA was 4.0%, and the affinity column was durable for about 40 replicate analysis.

The selectivity of the affinity column is indicated on the basis of the analytical value of HSA (Table 1). Each 1.0×10^{-2} g dm^{-3} of protein was used throughout this experiment. HγG and BγG showed somewhat nonspecific adsorption with values of 0.16 and 0.14 based on the specific adsorption 1.00 of HSA, whereas BSA, BαG, and Ova showed no nonspecific adsorption. The determination of HSA in a serum sample was next examined. Various amounts of HSA were estimated in either the presence or absence of a definite amount of control serum (containing about 6×10^{-3} g dm^{-3} HSA, diluted to a volume of 5000 times with a buffer solution), and the relationship between HSA concentration and the analytical value was obtained by the use of the calibration curve shown in Fig. 5

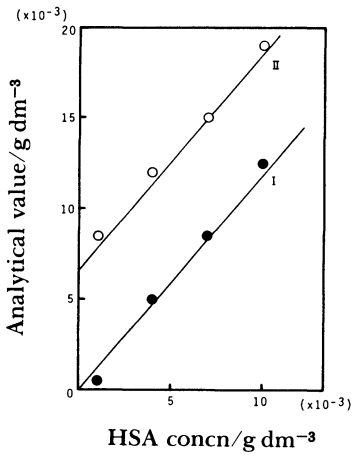

Fig. 6. Relationship between HSA concn and analytical value.
●: HSA and O: HSA+control serum.

(Fig. 6). The difference between straight lines I and II in the graph was approximately equal to the amount of HSA in the added control serum. This means that the present method was applicable to the selective determination of protein in a serum sample.

According to the previous cyclic immunoaffinity chromatography using a luminol-H_2O_2-Cu(II) chemiluminescence system, the detection limit of protein, nonspecific adsorption onto a column, the time necessary for a measurement, durability of a column, and the coefficient of variation were 100 ng, 0.00—0.63 (5 kinds of protein, on the basis of the analytical value of HSA), 34 min, about 20 times, and 5.7% (n=5, 1.0×10^{-2} g dm^{-3} HSA) respectively. In the present study, they were as follows; 15 ng, 0.00—0.16, 17 min, about 40 times, and 4.0% (n=6, 4.0×10^{-3} g dm^{-3} HSA) respectively.

Recently a small amount of protein such as AFP has attracted public attention as a tumor marker. As for AFP, Single Radial Immunodiffusion method and Reverse Passive Haemagglutination method have been generally used for simple screening. Their detection limits are 5000 and 200 ng cm^{-3} respectively, and the latter method is only qualitative.[4] Though HSA was analyzed in the present study by immobilizing anti-HSA on agarose gel, a trace amount of protein such as AFP might be analyzed continuously and selectively with the sensitivity required for practical use if suitable antibody should be immobilized.

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