The Determination of a Small Amount of Biological Constituent by the Use of Chemiluminescence. VII. An Attempt of Cyclic Immunoaffinity Chromatography

Tadashi HARA,* Motohiro TORIYAMA,† Takashi EBUCHI, and Masakatsu IMAKI††
Department of Chemical Engineering, Faculty of Engineering, Doshisha University, Karasuma Imadegawa, Kamigyo-ku, Kyoto 602
†Government Industrial Reserch Instituent, Nagoya, Hirate-machi, Kita-ku, Nagoya 462
††Horiba Ltd., Kisshoin, Minami-ku, Kyoto 601
(Received February 4, 1986)

Synopsis. An immunoaffinity chromatography has been established by combining a chemiluminescence detector developed by the authors with two kinds of immunoaffinity columns connected in series. According to the present method, human serum albumin and ovalbumin in the range of 100 ng to 15 μg in their mixtures could be continuously and selectively determined.

It has been desired to develop an analytical technique for a sensitive, continuous, and selective determination of the desired constituents in serum.

Through immunoassay, used as a labeling reagent, a synthesized metal-complex compound having catalytic activity for a chemiluminescence(CL) reaction, was continuously carried out by the sandwich method mentioned in a previous paper. It required six operating steps and tedious labeling.[1]

So far, there have been several reports[2-5] in which the desired constituent was specially separated on an affinity column based on a biological reaction analyzed by means of a suitable detector. However, the detector used for such a measurment was not always very sensitive.

The method for a flow-injection analysis of protein was established by the authors on the basis of the phenomenon that the catalytic activity of copper(II) against a CL reaction between 5-amino-2,3-dihydro-1,4-phtalazinedione (luminol) and hydrogen peroxide(H_2O_2) is lowered in the presence of protein. According to this method, 2×10^{-4}—1×10^{-1} g dm^{-3} of protein could be sensitively determined with a detection limit of 10 ng.[6,7] A highly sensitive detector for the HPLC of protein was manufactured by making use of the same phenomenon that is described above, and the detector was shown to be also useful for the post-column detection of protein in HPLC.[7]

It was therefore expected that an immunoaffinity chromatography for the sensitive, continuous and selective determination of the desired constituents in a sample without labeling might be established by combining the CL detector with the affinity columns used for an immune reaction.

Experimental

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[8] with a buffer solution (pH 12.8) consisting of a 0.20 mol dm^{-3} boric acid (H_3BO_3) solution and a 0.25 mol dm^{-3} potassium hydroxide (KOH) solution. A 1.0×10^{-4} mol dm^{-3} luminol solution was prepared by use of a buffer solution (pH 10.2)(0.20 mol dm^{-3} H_3BO_3–0.20 mol dm^{-3} KOH buffer solution). A 5.0×10^{-4} mol dm^{-3} H_2O_2 solution was prepared in a manner similar to that described in a previous paper.[7] These reagents were used for the CL detector developed by the authors. Human serum albumin(HSA)(Miles Labolatolies, Inc.), bovine serum albumin(BSA)(Nakarai Chemicals, Ltd.), ovalbumin (Ova)(Sigma Chemical Co.), human serum γ-globuline-(HγG)(Sigma Chemical Co.), bovine serum γ-globulin-(BγG)(Sigma Chemical Co.), bovine serum α-globulin-(BαG)(ICN Pharmaceuticals, Inc.), and 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) and goat anti-Ova(Cappel), respectively, were immobilized onto 2 g of porous glass beads (CPG-3000, 200/400 mesh, 29260 nm mean pore diameter, ELECTRO-NUCLEONICS, INC.) by a periodate method.[1] Each amount of anti-HSA and anti-Ova immobilized onto a gram of support was 18.5 and 3.10 mg, as determined by the CL detector developed by the authors.

A flow diagram of the immunoaffinity chromatography system equipped with the CL detector devised by the authors is shown in Fig. 1, and its operating procedure is shown in Scheme 1. Buff-A(a) was fed at a flow rate of 1.8 cm^3 min^{-1} by the pump(d)(Atto, SJ 1211) and an elluent(b)(pH 2.5; 4.2×10^{-3} mol dm^{-3} hydrochloric acid –2.5×10^{-2} mol dm^{-3} potassium chloride buffer solution) (Buff-B) was fed at a flow rate of 1.0 cm^3 min^{-1} by the pump(e)(Pharmacia High-precision Pump P-500). The porous glass beads immobilized with anti-HSA and anti-Ova were packed in a column 1(Col. 1)(15×1 mm i.d.) and a column 2(Col. 2)(15×1 mm i.d.), respectively.

A 50-mm^3 mixed solution containing HSA and Ova was

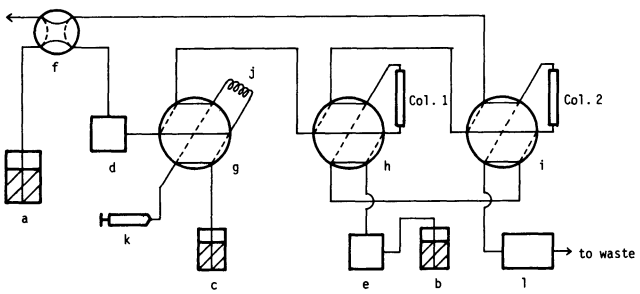
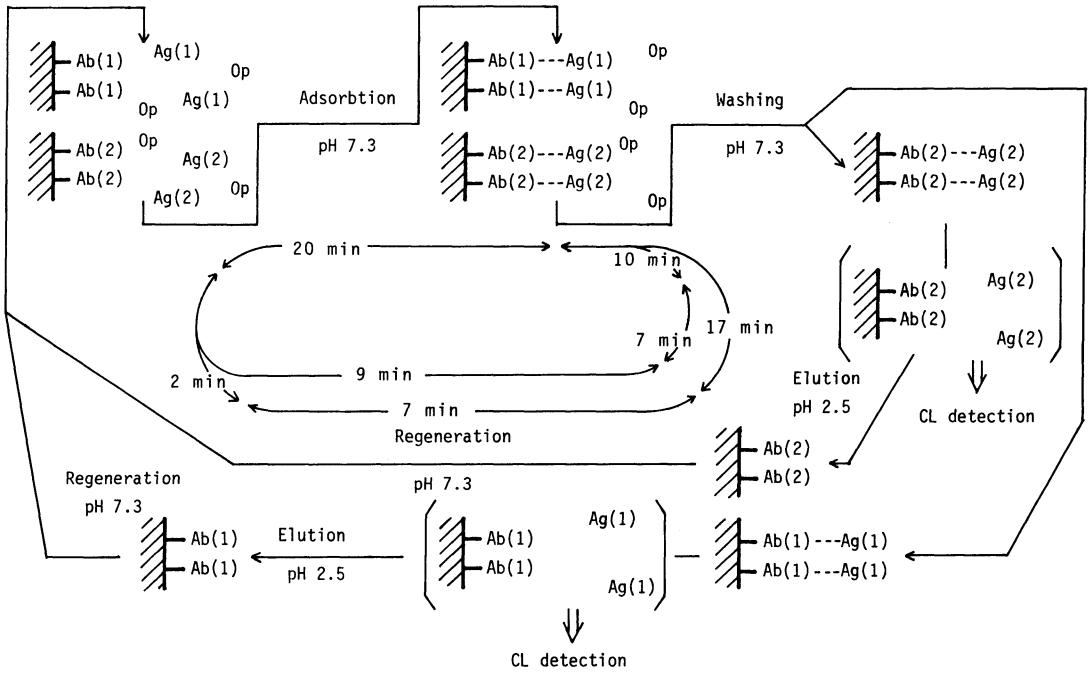


Fig. 1. Schematic flow diagram of the immunoaffinity chromatography system equipped with a CL detector. a: Buffer solution (Buff-A), b: eluent(Buff-B), c: sample solution, d and e: pump, f: four-way cock, g,h, and i: six-way cock, j: sampling loop, k: syringe, and l : CL detector.



Scheme 1. Operating procedure for the immunoaffinity chromatography. Ab: Antibody, Ag: antigen, and Op: other protein.

injected into the Buff-A line through the sampling loop(j) and a six-way cock(g). A sample flaction was repeatedly passed through columns 1 and 2 for 20 min by operating a four-way cock(f). Here, HSA and Ova were collected on columns 1 and 2, respectively, by an immune reaction. Then Buff-A was fed to columns 1 and 2 for 10 min by operating a four-way cock(f) to wash out coexisting constituents. Ova was eluted from column 2 by operating a six-way cock(i); then cock(i) was again operated to feed Buff-A. HSA was eluted from column 1 by operating a six-way cock(h) and then cock(h) was again operated to feed Buff-A. Ova and HSA in each eluate was detected by a CL detector(1) as mentioned in a previous paper.[7] Columns 1 and 2 were regenerated by operating the six-way cocks and by feeding Buff-A.

Results and Discussion

Four steps consisting of adsorption, washing, elution (CL detection), and regeneration were developed for optimum operation as follows: both the flow rate and the feeding time of Buff-A for adsorption and regeneration were chosen so that each analytical value of a mixed sample containing 500 ng HSA and 500 ng Ova might give a definite value; both the flow rate and the feeding time of Buff-A for washing were also chosen so that the analytical value of 500 ng BSA, as a coexisting constituent, might give a minimum value; HSA and Ova, less than 500 ng, could almost be completely eluted in 7 min from affinity columns. The chosen experimental conditions are shown in Scheme 1.

A chromatogram for the mixture consisting of 500 ng of both HSA and Ova is shown in Fig. 2. The selectivities of affinity columns 1 and 2 are indicated on the basis of the analytical values of HSA and Ova, respectively (Table 1). Each 500 ng of protein was

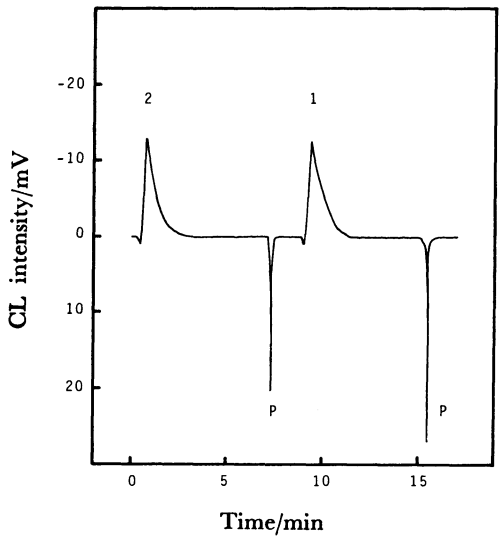


Fig. 2. Chromatogram of the mixture of HSA and Ova. 1: HSA, 2: Ova, and P: shock peak appeared owing to the exchange between the two cylinders in the pump.

Table 1. Selectivity of the Affinity Columns

Proteins (500 ng)	Column 1 (anti-HSA)	Column 2 (ati-Ova)
HSA	1.00	0.00
Ova	0.00	1.00
BSA	0.58	0.00
HγG	0.18	0.07
BγG	0.63	0.23
BαG	0.40	0.15

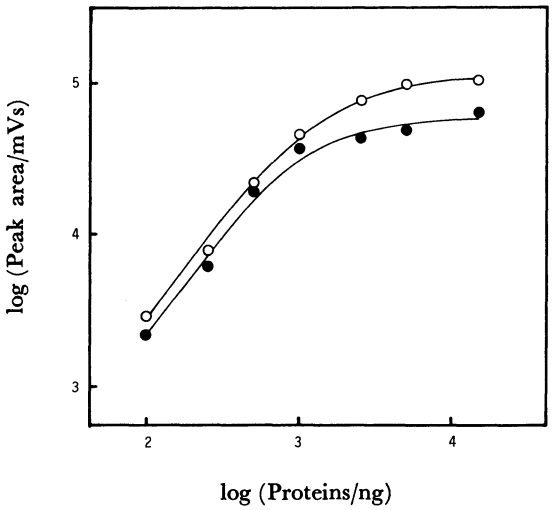


Fig. 3. Analysis of the mixture of HSA and Ova. ○: HSA and ●: Ova.

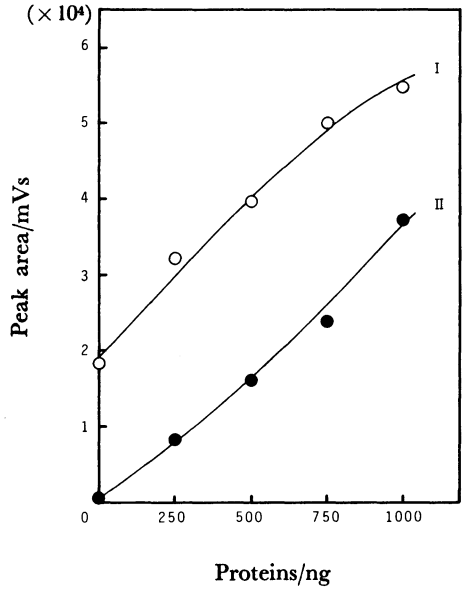


Fig. 4. Relationship between protein concentration and peak area.
○: Analytical value obtained by use of column 1 and ●: analytical value obtained by use of column 2.

used throughout this experiment. As can be seen from Table 1, the selectivity of column 1 (immobilized with anti-Ova) was fairly good. Porous glass beads were subjected to the immobilization treatment described in a previous paper[1] in the absence of antibodies. It was charged in a column to analyze HγG. According to the obtained result, no nonspecific adsorption was obtained. This suggests that nonspecific adsorption of HγG onto an affinity column is based on a cross reaction due to the use of inpure antibodies and HγG.

The present method was applied to a mixture of HSA and Ova, and each in the range of 100 ng—15 μg could be successfully determined (Fig. 3). The difference between two calibration curves seemed to result from a difference between the amounts of immobilized antibodies. The coefficient of variation for five analyses of a mixture containing each 500 ng HSA and Ova were 5.7% for HSA and 9.3% for Ova. The time necessary for an analysis was 46 min and the affinity columns were durable for about 20 replicate analyses. The beads immobilized with antibodies were stable and available for about 3 months after their preparation. By assuming the following (1)—(3): (1) 2 g of beads are immobilized with antibodies at a time, (2) 5.9 mg of beads are charged in a column, and (3) an affinity column is durable for about 20 replicate analyses, therefore, about 6800 analyses are feasible. Thus, the present method is sufficiently economical.

To examine the applicability of the present method to a practical sample, a control serum was diluted 5000 times and to it a definit amount of HSA and Ova was added. This was followed by an analysis of the resulting three mixtures (Fig. 4). Curves I and II in Fig. 4 were obtained from columns 1 and 2, respectively. Curve I showed the sum of albumin and non-specifically adsorbed protein in the control serum and of the added HSA. Curve II passed through an original point. This meant that the added Ova alone could be selectively analyzed without being affected by the serum matrix.

Judging from these results, the present method is sufficiently applicable to a practical sample if a selective column immobilized with antibodies is available.

References

1) T. Hara, M. Toriyama, and M. Imaki, *Bull. Chem. Soc. Jpn.*, **58**, 1299 (1985).
2) S. Ohlson, L. Hansson, P. Larsson, and K. Mosbach, *FEBS Lett.*, **93**, 5 (1978).
3) J. R. Sportsman and G. S. Wilson, *Anal. Chem.*, **52**, 2013 (1980).
4) R. R. Walters, *Anal. Chem.*, **55**, 1395 (1983).
5) S. C. Crowley and R. R. Walters, *J. Chromatogr.*, **266**, 157 (1983).
6) T. Hara, M. Toriyama, and K. Tsukagoshi, *Bull. Chem. Soc. Jpn.*, **57**, 1551 (1984).
7) T. Hara, M. Toriyama, and T. Ebuchi, *Bull. Chem. Soc. Jpn.*, **58**, 109 (1985).
8) T. Hara, M. Toriyama, and K. Tsukagoshi, *Bull. Chem. Soc. Jpn.*, **56**, 1382 (1983).