The Determination of a Small Amount of a Biological Constituent by the Use of Chemiluminescence. XV. A Zeolite Column-Chemiluminescence Detection System for the Separation and the Determination of a Protein Mixture

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To make use of zeolite as the packing material for the separation of proteins by means of adsorption chromatography, the adsorption behavior of protein onto the Na-X type of zeolite (Molecular Sieve 13X) in an aqueous solution or onto a column (3 mm i.d.×50 mm) packed with the same zeolite as above was examined using a ultraviolet absorption detector and model proteins, such as human-serum albumin and human-serum γ-globulin. The following results were obtained: 1) Human-serum γ-globulin was much more adsorbed onto the zeolite than was human-serum albumin and 2) in general, more protein was adsorbed in an acidic solution than in an alkaline solution. Moreover, a chemiluminescence detector using a 1,10-phenanthroline–hydrogen peroxide–copper(II) system, which has been developed by the present authors, was modified to improve its sensitivity for the determination of protein. Both the carrier solution and the eluting solution which were used in combination with a zeolite column with a chemiluminescence detector were optimized so as to give a smooth baseline on a recorder. A microbore zeolite column (1 mm i.d.×250 mm)-chemiluminescence detection system suitable for the separation and the determination of a small amount of a protein mixture was established on the basis of these experimental results. Since human-serum albumin passed through a microbore zeolite column, while human-serum γ-globulin was retained on the column, when a model sample containing human-serum albumin and human-serum γ-globulin was injected into the column, human-serum albumin was first determined, followed by the determination of human-serum γ-globulin after its desorption. Both human-serum albumin and human-serum γ-globulin in the concentration range of 2×10^{-5}—5×10^{-3} g dm^{-3} could be determined economically and conveniently, with a detection limit of about 1 ng (injected sample volume: 50 mm^3; S/N=2). The present method was, therefore, applicable to the separation and the determination of albumin and globulin in a serum sample.

It has been desired to develop a new method for the determination of a trace amount of a protein, such as a tumor marker in serum. The present authors have already reported a flow-injection analysis (FIA) method for the determination of a small amount of a protein, in which method a chemiluminescence (CL) detector using the CL reaction of a 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol)–hydrogen peroxide (H_2O_2)–copper(II) (Cu(II)) system or a 1,10-phenanthroline (phen)–H_2O_2–Cu(II) system is used.[1,2] The determination of a protein was based on the measurement of the decreasing catalytic activity of Cu(II) for the CL reaction in the presence of the protein. These methods were about 10^2—10^6 times as sensitive as the conventional methods,[3-7] such as colorimetry and fluorometry.

Immunoaffinity chromatography and metal-chelate-affinity chromatography, in which conventional affinity columns were combined with the CL detector for the separation of protein, have also previously been established by the present authors for the analysis of a model sample containing albumin and globulin.[8,9] These affinity columns were confirmed to have some excellent characteristics for the separation of proteins, but they were, in general, expensive, difficult to operate, and time-consuming, and they required a complicated operation for their pretreatment and regeneration. Furthermore, some serious problems, such as an abnormally large blank value and a lowering of the sensitivity for the detection of protein, appeared since protein was eluted from the column with an acidic solution, and so the resulting acidic eluate was subjected to the measurement of the CL intensity in an alkaline solution.

In addition to the packing materials used in the above affinity columns, there are several commercially available ones. However, these packing materials are ordinarily difficult to handle and expensive because most of them necessitate a specific regand or functional group. On the other hand, some metal-oxide beads, such as silica and alumina, have been used as the packing material for the separation column of a protein[10,11] because of their ease of handling and their inexpensiveness. Since zeolite is a crystalline aluminosilicate and is stable enough in an alkaline solution, unlike silica, it seems a possible packing material which is inexpensive, easy to handle, and durable enough for use in an alkaline solution. And zeolite shows a cation-exchange phenomenon and has a specific and definite diameter of its cavity. Moreover, zeolite has often been utilized as a packing material for gas chromatography, but only rarely for liquid chromatography,[12] and its adsorption behavior against protein has not yet been examined. The present authors were, therefore, interested in the application of zeolite to protein-separation.

On the basis of the results obtained in the preliminary experiment, the present paper with deal with zeolite as a packing material for the separation of proteins by adsorption chromatography. First, the adsorption behavior of protein onto the Na-X type of zeolite will be examined using a ultraviolet absorption (UV) detector. Moreover, a CL detector using a phen–H_2O_2–Cu(II) system will be modified to improve the sensitivity for the determination of protein. Then, both the carrier solution and the eluting solution used in the combination of a zeolite column with a CL detector will be optimised. A microbore zeolite column-CL detection system suitable for the separation and the determination of a small amount of a protein mixture will be established on the basis of these experimental results.

Experimental

All of the reagents used were of a commercially available special grade. Deionized water was distilled for use. A sodium dihydrogenphosphate (NaH_2PO_4)–disodium hydrogenphosphate (Na_2HPO_4) buffer solution (pH 5.8—8.3) and a sodium hydroxide (NaOH)–sodium chloride (NaCl) buffer solution (pH 12.0—13.0) were mainly used. HSA (Chemical Dynamics Co.) and HγG (SIGMA), which comprise most of the serum protein and the determination of which is regarded as significant in a clinical laboratory, were used as the model proteins. Control serum (Q-PAK-Chemistry Control Serum I, Human) from Hyland Diagnostics was also used. A Molecular Sieve 13X (Nishio Kogyo, Ltd.) (chemical formula; $Na_{86}[(AlO_2)_{86}(SiO_2)_{106}]\cdot 276H_2O$; particle size 100—120 mesh; and cavity diameter 10 Å) was used as the Na-X type of zeolite. Cation-exchanged zeolites, such as Mg-, Ca-, and Zn-X types of zeolites, were prepared from the Molecular Sieve 13X in accordance with the procedure described in the literature.[13–15]

The zeolite was packed in an ordinary (3 mm i.d. ×50 mm) or a microbore column (1 mm i.d. ×250 mm) which has been manufactured by the present authors using a Teflon tube (2 mm o.d. ×1 mm i.d.) and a Teflon line-filter (Nishio Kogyo, Ltd.) (Fig. 1).

The adsorption behavior of protein onto the zeolite in an aqueous solution was examined by means of the depletion method (indirect-determination method)[16,17] (Scheme 1) using a UV detector (measured at 280 nm). The adsorption behavior of protein onto a zeolite column (3 mm i.d. ×50 mm) was also examined by means of the determination of the protein in an eluate by means of a UV detector (measured at 280 nm) connected to a column.

A schematic flow diagram of a zeolite column-CL detection system is shown in Fig. 2. A CL detector using a phen–H_2O_2–Cu(II) system was used, in which the concentrations of phen, H_2O_2, and Cu(II) in the individual solutions were the same as in a previous paper.[2] A carrier solution (d) and an eluting solution (e) were fed is at the flow rate of 1.0 ml min^{-1} by means of a pump (Pharmacia Fine Chemicals, High-precision Pump, P-500) (j). A 50-mm^3 portion of the sample solution was injected into the carrier solution line through a sampling loop (m) and a six-way cock (l), and then passed through a zeolite column (o).

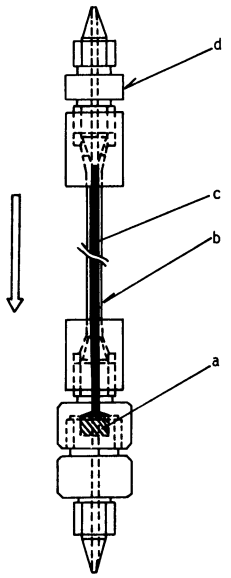

Fig. 1. Setup of a microbore column. a) 10 μm Teflon filter, b) 2 mm o.d.×1 mm i.d. Teflon tube, c) zeolite, and d) joint (0.6 mm i.d.).

(Zeolite + Protein soln)

↓

Degassing

↓

Shaking

(3 h, 4 °C)

↓

Centrifugation

(20 min, 10000 rpm)

↓

Diluting the supernatant with

a buffer soln

↓

Detection using a UV detector

Scheme 1. Outline of a depletion method.

Globulin was adsorbed selectively onto the zeolite column, but albumin was not adsorbed. Then the eluting solution was fed into the column by operating a four-way cock (k) to elute the globulin. The protein in the eluate was detected by the CL detector.

The CL intensity of the solution containing no protein was recorded as a baseline on a recorder, while the CL intensity of the solution containing protein was recorded as a negative peak because the catalytic activity of Cu(II) decreased due to the formation of a complex compound between Cu(II) and the protein. The amount of protein was

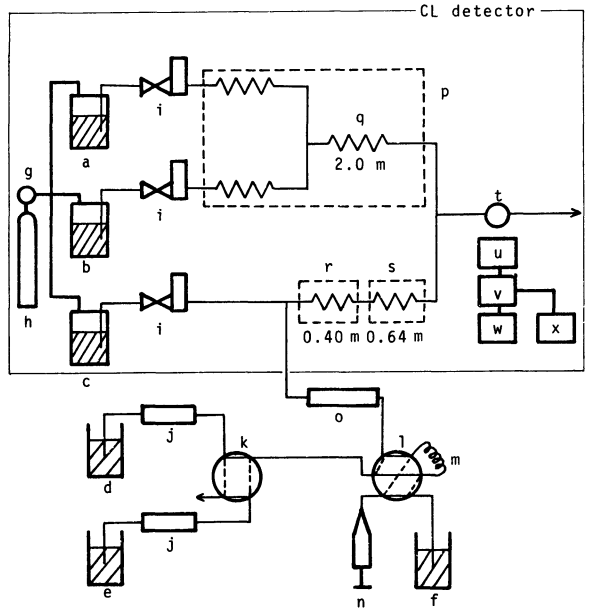

Fig. 2. Schematic flow diagram of the zeolite column-CL detection system. a) Phen soln, b) H_2O_2 soln, c) Cu(II) catalyst soln, d) carrier soln, e) eluting soln, f) sample soln, g) regulator, h) N_2 cylinder, i) flowmeter equipped with a needle valve, j) pump, k) four-way cock, l) six-way cock, m) sampling loop, n) syringe, o) microbore zeolite column, p) thermostat, q) mixing coil, r) reaction tube, s) cooling tube, t) flow cell, u) photomultiplier, v) amplifier, x) recorder, and w) integrator.

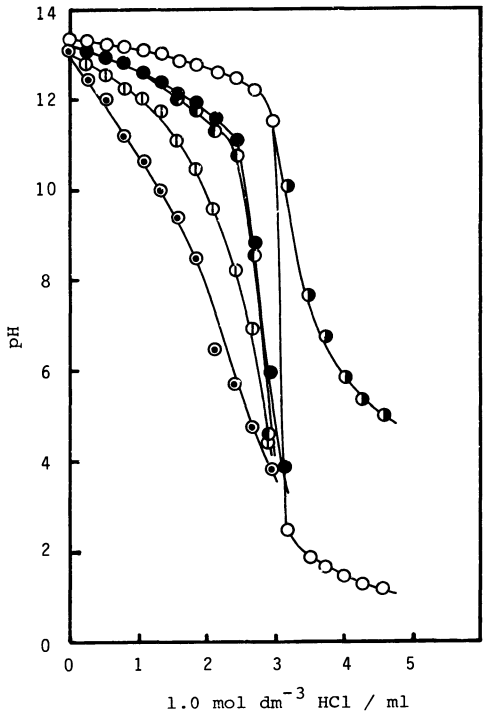

Fig. 3. Titration curves. ○: Zeolite-free, ●: commercially available zeolite (A), ◑: zeolite treated with an alkaline solution (B), ◐: zeolite treated with a neutral solution (C), ⓓ: zeolite treated with an acidic solution (D), and ⊙: proton-type of zeolite (E). Condition: 0.1 mol dm^{-3} NaOH solution (30 ml) containing 0.20 g zeolite was titrated with 1.0 mol dm^{-3} HCl solution.

estimated from the peak area.

Results and Discussion

Preliminary Experiment. To make use of zeolite as a packing material for each mode of chromatography, such as chromatofocusing, metal-chelate-affinity, molecular exclusion, and adsorption chromatographies, preliminary experiments were carried out.

The pH buffering action, which is indispensable for a material used as packing in chromatofocusing,[18,19] was examined by titrating a sodium hydroxide solution containing a definite amount of zeolite with a standard hydrochloric acid solution. The following Na-X-type zeolites were used: A) Commercially available zeolite (untreated zeolite); B) zeolite treated with an alkaline solution (1.32×10^{-1} mol dm^{-3} NaOH–5.00×10^{-2} mol dm^{-3} NaCl; pH 13.0); C) zeolite treated with a neutral solution (3.90×10^{-2} mol dm^{-3} NaH_2PO_4–6.10×10^{-2} mol dm^{-3} Na_2HPO_4; pH 7.0); D) zeolite treated with an acidic solution (7.35×10^{-2} mol dm^{-3} NaH_2PO_4–2.65×10^{-2} mol dm^{-3} Na_2HPO_4; pH 6.4), and E) a proton-type zeolite prepared from the Na-X type of zeolite.[20] As can be seen from Fig. 3, zeolite B, with less proton, showed a buffer capacity in an acidic region, whereas zeolite A≃C, D, and E showed a buffer capacity in an alkaline region, in that order.

In the mode of metal-chelate-affinity chromatography, different affinities of proteins against cation-exchanged zeolite are utilized for the separation of proteins. The amounts of cation on each Mg-, Ca-, and Zn-X-type zeolite, as estimated by chelatometric titration, were 1.0, 1.3, and 1.2 mequiv g^{-1} respectively. Each cation on the cation-exchanged zeolite was held in the cavity of the zeolite crystal; no cation was found in a solution, even after standing for 3 h in a phosphate buffer solution (pH 7.0). Then, the amount of protein adsorbed onto the cation-exchanged zeolite was determined by means of a depletion method after 3 hours' shaking with the phosphate buffer solution (pH 7.0) (Table 1). As is shown in Table 1, the amounts of HSA adsorbed onto the four types of zeolites were almost definite, whereas the amount of HγG adsorbed onto the Zn-X type zeolite was about 6% larger than the amounts of HγG adsorbed onto the other types of zeolites.

Since zeolite, having a definite diameter of its cavity, is quite different from the packing material, which has a pore-size distribution for molecular exclusion chromatography, the molecular-sieve effect on peptide and protein of a column packed with the Na-X type of zeolite was examined. The retention times obtained in

Table 1. Amount of Protein Adsorbed onto Each Cation-Exchanged Zeolite

Zeolite	Relative peak area	
	HSA	HγG
None	1.00	1.00
Molecular sieve 13X (Na-X type)	0.95	0.14
Mg-X type	0.96	0.17
Ca-X type	0.96	0.16
Zn-X type	0.95	0.10

a) Conditions: Zeolite, 1.5 g; soln volume, 3.0 ml; protein concn, 1.0×10^{-1} g dm^{-3}; shaking time, 3 h, and pH 7.0 (Na_2HPO_4–NaH_2PO_2).

Table 2. Retention Times for Polypeptides and Proteins

Sample	Retention time/s
Glycylglycine	321
Glycylglycylglycine	276
Glycylglycylglycylglycine	252
HSA	245
HγG	244

Conditions: Column, 3 mm i.d.×50 mm; polypeptide concn, 1.0×10^{-3} mol dm^{-3}; protein concn, 1.0×10^{-1} g dm^{-3}; injection volume, 50 mm^3; flow rate, 0.1 ml min^{-1}, and measurement wavelength, 220 nm.

the zeolite column for several kinds of polypeptides, HSA, and HγG are shown (Table 2). Here, a phosphate buffer solution containing 1.0 mol dm^{-3} of NaCl was used as the carrier solution because several kinds of polypeptides, HSA, and HγG were not adsorbed onto the zeolite column in a 1.0 mol dm^{-3} NaCl solution. No difference was observed between the retention times of HSA and HγG, but some differences were observed between the retention times of protein and polypeptides or among the retention times of polypeptides. These differences seemed to be based on the molecular-sieve effect, since the retention time decreased with an increase in the molecular weight of the polypeptides and proteins.

To investigate if zeolite could be used as a packing material for adsorption chromatography, much like silica beads,[10] the effect of the pH on the amount of protein adsorbed onto the Na-X type of zeolite in an aqueous solution was examined by means of a depletion method. The experiment was carried out in a solution of pH 5.8—8.3, in which the crystal structure of zeolite was stable and the protein was not denatured. The following results were obtained: 1) much more HγG was adsorbed onto the zeolite than HSA, and 2) more protein was adsorbed in an acidic solution than in an alkaline solution.

On the basis of the results thus obtained, a column packed with the Na-X type of zeolite was used in the present paper for the separation of proteins by means

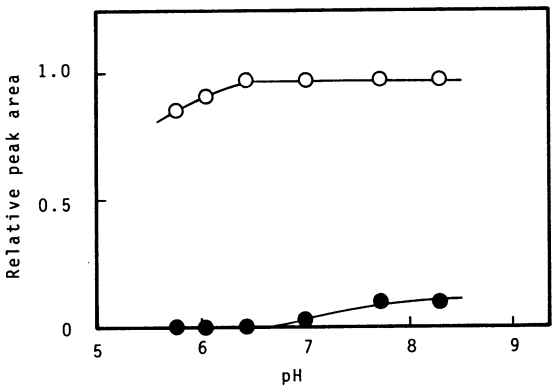

Fig. 4. Adsorption of protein onto column packed with zeolite. ○: HSA and ●: HγG. Conditions: Column size 3 mm i.d.×50 mm, protein concn 1.0×10^{-1} g dm^{-3}, injection volume 50 mm^3, and flow rate 0.1 ml min^{-1}.

of adsorption chromatography. The application of a zeolite column to the other chromatography will be described later.

Adsorption Behavior of Protein onto Zeolite. The adsorption behavior of protein onto the Na-X type of zeolite was further examined.

The adsorption equilibrium of protein on the zeolite in an aqueous solution was attained in about 1 h; thereafter, the amount of adsorbed protein did not change, even an standing for 5 h. In the present study, the amount of protein adsorbed at equilibrium was determined after 3 hours' standing.

It was found that the adsorption behavior of protein onto the zeolite in an aqueous solution was shown by Langmuir's adsorption isotherm.[16,17] The amount of HSA or HγG adsorbed onto the zeolite was constant above protein concentrations of about 4 g dm^{-3} or 6 g dm^{-3} respectively.

The effect of the pH on the amount of protein adsorbed onto the zeolite column was also shown (Fig. 4). Here, a series of experiments were carried out at an individual, definite pH value as follows: A 50-mm^3 protein solution was placed in the column, and then the corresponding buffer solution was fed, followed by the measurement of the protein eluted from the column by means of a UV detector connected to the column. The relative peak area in Fig. 4 was referred to as the ratio of the peak area of the protein passing through the column to the peak area of the protein obtained without passing through the column. The results obtained using the zeolite column showed a trend similar to that obtained in a preliminary experiment using the zeolite in an aqueous solution.

The adsorption of protein onto a silica bead is known to be attributable to the negative charge due to a hydroxyl group on its surface in an aqueous solution. Since the adsorption of protein onto the zeolite was thought to be similar, Results 1) and 2)

described in the preliminary experiment were explained as follows: 1) was explained by considering that globulin, having a much more positive charge than albumin, showed a larger affinity than albumin against the negative charge due to a hydroxyl group on the zeolite surface, while 2) was explained by considering that protein, as an amphoteric electrolyte, had a much more positive charge in an acidic solution than in an alkaline solution.

As is shown in Fig. 4, HγG was completely adsorbed onto the zeolite column from the phosphate buffer solution (pH 6.4), while HSA was not adsorbed from the same buffer solution. The effect of the coexisting substance on the adsorption of HγG onto the zeolite column was examined by the use of the phosphate buffer solution (about pH 6.4) containing: 1) NaCl as a moderator of the ionic strength, 2) urea as a denaturing agent, 3) glycine as a competitive inhibitor, and 4) ethylene glycol or 1,4-dioxane as a representative of a polar-to-nonpolar solvent (Table 3). The amount of HγG adsorbed onto the zeolite column tended to decrease with the use of all these solutions except the glycine solution. Neither HSA nor HγG was adsorbed onto the column at all by the use of a phosphate buffer solution (pH 6.4) containing 1.0 mol dm^{-3} of NaCl.

On the basis of these results, albumin and globulin in a control serum were separated and determined using a zeolite column-UV detection system. A control serum sample diluted 100 times (50 mm^3) was placed in the zeolite column, after which a phosphate buffer solution (pH 6.4) was fed in. Here, globulin was adsorbed onto the column, while albumin passed through the column. The globulin on the column was eluted by a phosphate buffer solution (pH 6.4) containing 1.0 mol dm^{-3} of NaCl. As can be seen from Fig. 5, the practical peak for globulin is given by II′, which corresponds to the difference between the true peak II for globulin and the negative peak II″ for the interference fringe appearing in the mixing area of two kinds of solutions. The recovery of 50 mm^3 of the 1.0×10^{-1} g dm^{-3} HγG solution was 80% by the present method.

Table 3. Effects of the Coexisting Substance on Adsorption of Protein onto Zeolite Column

Solution	Relative peak area	
	HSA	HγG
Phosphate buffer soln (pH 6.4)	0.97	0.00
Phosphate buffer soln (pH 8.3)	0.97	0.10
Phosphate buffer soln -1.0 mol dm^{-3} sodium chloride (pH 6.4)	0.96	0.97
Phosphate buffer soln -1.0 mol dm^{-3} Urea (pH 6.4)	0.97	0.07
Phosphate buffer soln -1.0 mol dm^{-3} glycine (pH 6.4)	0.96	0.00
Phosphate buffer soln (pH 6.4) -ethylene glycol (9:1)	0.97	0.15
Phosphate buffer soln (pH 6.4) -1,4-dioxane (9:1)	0.97	0.10

Conditions: Column, 3 mm i.d.×50 mm; protein concn, 1.0×10^{-1} g dm^{-3}; injection volume, 50 mm^3, and flow rate, 0.1 ml min^{-1}.

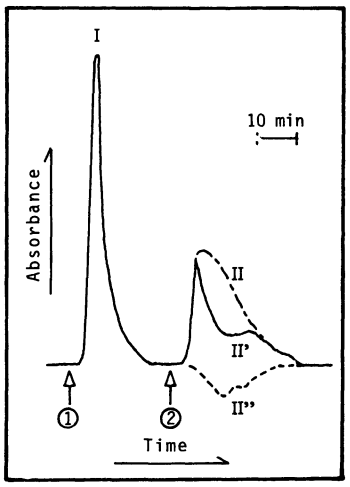

Fig. 5. Chromatogram of control serum using a zeolite column-UV detection system. ①: Sample was injected, ②: eluent was fed. I: Peak for albumin, II: true peak for globulin, II′: practical peak for globulin, and II″: negative peak for interference fringe.

Improvement of the Sensitivity of a CL Detector and Optimization of a Carrier Solution and an Eluting Solution. In the previous papers,[1,2] the reaction between Cu(II) and protein in the CL detector was done at pH 10.2 using a borate buffer solution. On the other hand, the biuret reaction for the determination of protein based on the formation of a complex compound between Cu(II) and protein is generally carried out in a solution above pH 12. This suggests that the effect of the pH on the reaction between Cu(II) and protein should be reinvestigated in order to improve the sensitivity of a CL detector.

Then, the pH (pH ①) of the solution being passed through a reaction tube (r in Fig. 2), the pH (pH ②) of the solution being passed through a flow cell (t in Fig. 2), the CL intensity of a base-line (CL_{BL}), and the percentage of the decrease in the rate of CL intensity (CL_{DR}) ($|$(the lowering of the CL intensity in the presence of protein)/(CL_{BL})$|\times100$) were measured using various pH values of phen, the Cu(II) catalyst, and the carrier solutions. Based on the results obtained, the relationship between pH ① and CL_{DR}, as well as that between pH ② and CL_{BL}, were established (cf. Figs. 6 and 7). From Figs. 6 and 7, the optimum values may be supposed to be about pH 13 for pH ① and about pH 10.5 for pH ②.

In order to separate and determine a small amount

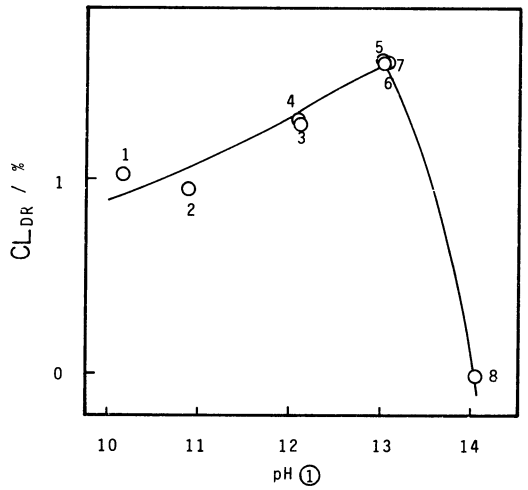

Fig. 6. Relationship between pH ① and CL_{DR}.

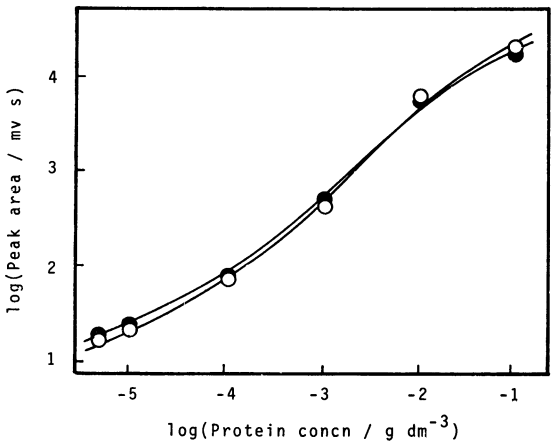

Fig. 8. Calibration curves of proteins using a FIA method. ○: HSA and ●: HγG.

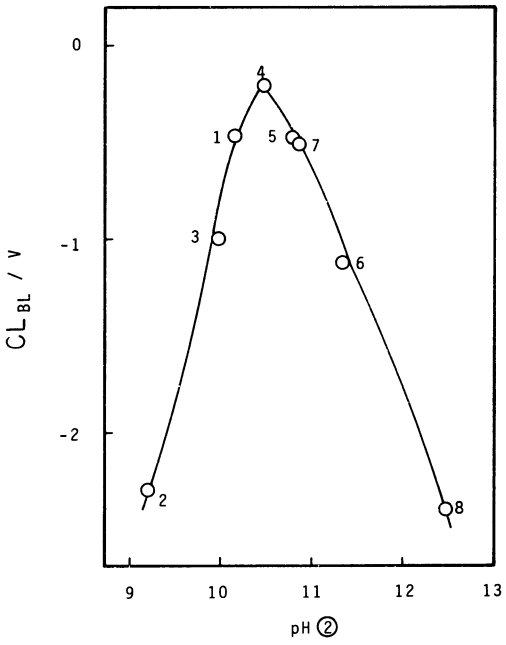

Fig. 7. Relationship between pH ② and CL_{BL}.

of a protein mixture using a zeolite column-CL detection system, the following conditions are required: 1) pH ① and pH ② were satisfied with the optimum pH values, as above, and 2) the change in CL_{BL} with the change in from a carrier solution to an eluting solution was negligible. The buffer capacities of the Cu(II) catalyst, carrier, and eluting solutions, which significantly influenced the pH ①, pH ②, and the change in CL_{BL}, were changed, and then the change in CL_{BL} was measured. Here, the phosphate buffer solutions (pH 6.4—6.5) were used as carrier solutions because these buffer solutions gave the most favorable results for the separation of HSA and HγG on the zeolite column. The NaOH-NaCl buffer solutions (pH 12.0—13.0) were also used as eluting solutions, because all the proteins adsorbed onto the zeolite column were expected to be eluted. As a result, the conditions using a Cu(II) catalyst solution (3.96×10^{-1} mol dm^{-3} NaOH–1.50×10^{-1} mol dm^{-3} NaCl; pH 13.2), a carrier solution (7.35×10^{-3} mol dm^{-3} NaH_2PO_4–2.65×10^{-3} mol dm^{-3} Na_2HPO_4; pH 6.5), and an eluting solution (1.32×10^{-2} mol dm^{-3} NaOH–5.00×10^{-3} mol dm^{-3} NaCl; pH 12.0) were recommended for the following experiments, because the change in CL_{BL} with the change from a carrier solution to an eluting solution was negligibly small and because the pH ① and pH ② were near to the optimum values of about 13 and about 10.5 under these conditions.

The calibration curves for HSA and HγG under the above conditions were obtained by means of an FIA method without using a zeolite column (Fig. 8). Both proteins could be determined in the concentration range of 5×10^{-6}—1×10^{-1} g dm^{-3}, with the detection limit of 250 pg (injected sample volume 50 mm^3, S/N=2). The present FIA method was about 4 times as sensitive as the previous method,[2] in which up to 250 pg of protein could be detected only by the use of the amplifying effect of an amino acid. Though several FIA methods for the determination of protein have recently been reported,[21-24] the present FIA method is much superior to these FIA mehods in termes of the detection limit and the concentration range.

Development of a Zeolite Column-CL Detection System. A zeolite column-CL detection system was investigated in an attempt to develop a method for the separation and determination of a small amount of a protein mixture. To minimize the dilution of a sample solution in a columm, a microbore column was used in the present study. An ordinary zeolite column (3 mm i.d.×50 mm) or a microbore zeolite column (1 mm i.d.×250 mm), both packed with the Na-X type of zeolite, was combined with the CL

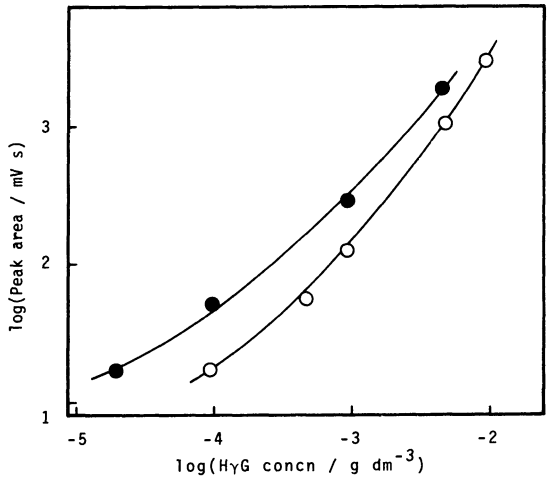

Fig. 9. Comparison of HγG calibration curves obtained on a microbore and an ordinary columns. ○: Ordinary column (3 mm i.d.×50 mm) and ●: microbore column (1 mm i.d.×250 mm).

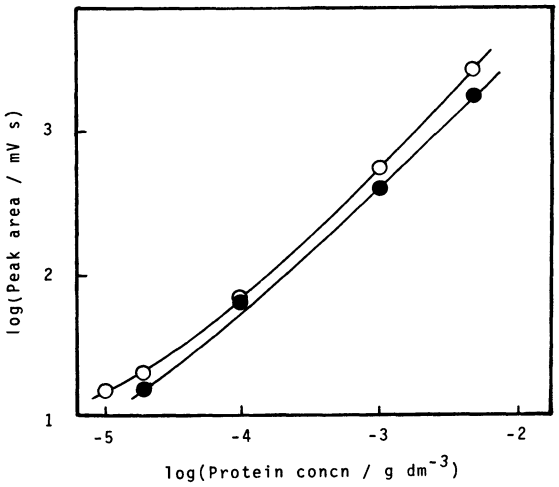

Fig. 10. Calibration curves of proteins using a microbore zeolite column-CL detection system. ○: HSA and ●: HγG.

detector; they were then used under the conditions specified above. 50-mm^3 HγG sample solutions in various concentrations were injected and adsorbed on the column, followed by elution and the measurement of the HγG. The calibration curves of HγG eluted from an ordinary zeolite column and a microbore zeolite column are shown in Fig. 9. As can be seen from Fig. 9, HγG could be determined by the use of a microbore column less well than by the use of an ordinary column.

It was first confirmed that 5.0×10^{-3} g dm^{-3} HSA was not adsorbed onto the microbore zeolite column, while the same concentration of HγG was completely adsorbed by the carrier solution. The model sample containing HSA and HγG was separated on the microbore zeolite column, and then the HSA passing through the column was first determined, followed by the determination of the HγG after its desorption. According to the present method using the microbore zeolite column, both HSA and HγG in the concentration range of 2×10^{-5}—5×10^{-3} g dm^{-3} could be determined with a detection limit of about 1 ng (injected sample volume, 50 mm^3; S/N=2) (Fig. 10). Here, the recovery of 1.0×10^{-3} g dm^{-3} of HγG was 84%, the coefficients of the variation of HSA and HγG were 5.4% and 8.3% (1.0×10^{-3} g dm^{-3} protein; n=5), and the time necessary for an analysis was about 16 min, including adsorption, elution, and regeneration. The present method may, therefore, be characterized as follows: 1) High sensitivity and a wide dynamic range for the determination of protein; 2) a consecutive operation for the separation and the determination of protein by means of high-performance liquid chromatography, and 3) the advantage of a zeolite column, that is, its inexpensiveness, ease of operation, and convenience for pretreatment and regeneration.

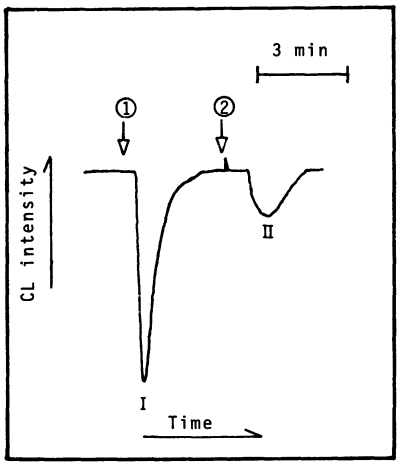

Fig. 11. Chromatogram of control serum using a microbore zeolite column-CL detection system. ①: Sample was injected, ②: eluent was fed, I: peak for albumin, and II: peak for globulin.

The immunoaffinity chromatography and metal-chelate-affinity chromatography using the CL detector previously developed by the present authors could determine up to 15 ng of HSA or 5 ng of bovine serum γ-globulin.[8,9] However, the present method could determine up to 1 ng of HγG. The high sensitivity of the present method may be supposed to be due to: 1) Improvement in the sensitivity in the CL detector, 2) utilization of a microbore column, and 3) the combination of the zeolite column with the CL detector using an alkaline-eluting method. The separation and the determination of several kinds of proteins using a pH-gradient method or a step-by-step eluting method are now in progress.

Finally, the present method was applied to a control serum sample. A control serum sample was diluted 10^4 times, and then an aliquot (50 mm^3) of it was analyzed

by the present method (Fig. 11). As is shown in Fig. 11, there were two peaks, I and II; Peak I corresponded to albumin, and Peak II, to globulin.

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