Recovery of Serum Proteins Using Cellulosic Affinity Membrane Modified by Immobilization of Cu^{2+} Ion

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SYNOPSIS

An affinity membrane was prepared from a porous cellulose membrane, and adsorption and recovery of serum proteins were investigated from the viewpoint that affinity membranes are efficacious against separation and purification of biomaterials. Into the cellulose membrane, iminodiacetate (IDA) group that acts as a ligand to metal ions was introduced (Cell–IDA membrane), and then Cu^{2+} ion was immobilized (Cell–IDA–Cu membrane). Bovine serum albumin (BSA) and γ-globulin (BγG), which are the major proteins in blood, were adopted as model proteins to be separated. The Cell–IDA–Cu membrane had large adsorption capacity for these proteins despite the low degree of modification. The amounts of proteins adsorbed on the Cell–IDA–Cu membrane increased with increasing pH, and BγG was adsorbed more than BSA. High protein recoveries from the Cell–IDA–Cu membrane were obtained. The separation of these proteins was also conducted under the optimum conditions of adsorption and recovery, and BγG was concentrated more than BSA although the initial concentration of BγG was lower than that of BSA. © 1996 John Wiley & Sons, Inc.

INTRODUCTION

A number of studies on the separation and purification of biomaterials, such as proteins and enzymes, have been developed with a growing demand for them in many fields ranging from food to drug. Affinity column chromatography is one of the most powerful techniques to separate and purify them, and different kinds of packings are available. Immobilized metal affinity chromatography (IMAC) was also evolved for the isolation of biomaterials.[1] There have been extensive discussions on the properties as well as preparation of IMAC.[2-6] In IMAC, transition metal ions immobilized on polymer matrices are coordinated by proteins through their electron donor groups, such as histidine, cysteine, and tryptophan residues.[7-12]

In addition to the development of novel adsorbents for affinity columns, improvements in adsorbent geometry have been undertaken to process a large quantity of proteins rapidly. In order to obtain the higher performance on the processing ability of column chromatography, it is necessary to use smaller beads with higher packing density. Under the high operating pressure, however, they bring about a considerable loss of pressure. Consequently, attempts to remove such a defect have been made by use of porous silica or sturdy polymer beads.[10] Recently, Brandt and coworkers[13] proposed the concept of affinity membrane originated from microporous membrane, which is a kind of column having the lowest bed height and the widest bed cross-section.[13] At the same time, they suggested that affinity membranes can avoid the loss of pressure under the high operating pressure and that affinity membranes are of greater advantage than affinity columns, since the distance of mass transfer is short in the micropores of affinity membranes.

Industrial fractionation of serum proteins is largely based on precipitation with ethanol or salt. In laboratories, on the other hand, ion-exchange column chromatography, gel filtration, and electrophoresis are available as well. The definition of affinity is classified into the bio-specific and group-specific affinities.[14] The former uses substrate, inhibitor, or antibody as a ligand, shows high specificity, and is expensive.[15,16] The latter, on the contrary, includes a ligand that interacts with a par-

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ticular functional group or chemical structure, shows rather low specificity, and is not expensive.[17-20] Immobilized metal affinity membrane[17,18,21] is one of the group-specific affinity membranes.

The purpose of this work is to prepare the affinity membrane derived from cellulose and to recover serum proteins efficiently. Naturally occurring cellulose was adopted as an affinity membrane matrix because it is known for its biocompatibility and is used as a hemodialysis membrane. Iminodiacetate (IDA) groups were introduced into hydroxyl groups of the porous cellulose membrane as a ligand (Cell–IDA membrane), and Cu^{2+} ion was immobilized on them (Cell–IDA–Cu membrane). The adsorption and recovery of bovine serum albumin (BSA) and γ-globulin (BγG) using the Cell–IDA–Cu membrane were investigated.

EXPERIMENTAL

Materials

Microfiltration membrane of 90 mm diameter, 0.125 mm thickness, and 0.2 μm average pore size (Advantec, Tokyo, Japan), which is made of cellulose acetate, was employed to obtain porous cellulose membrane. Epichlorohydrin, sodium IDA, copper sulfate hexahydrate, imidazole, and glycylglycine were of reagent grade, from Wako Pure Chemical Industries, Osaka, Japan. Methanol was distilled just before use, and other chemicals were used as received. BSA and BγG were of fraction V and II grades, respectively, from Tokyo Kasei Kogyo, Tokyo, Japan. BSA monomer[22] was also purchased from Sigma Chemical (St. Louis, MO). Folin-Ciocalter Reagent, from Wako Pure Chemical Industries, was used to assay proteins.

Preparation of Cell–IDA–Cu Membrane

All the following reactions were carried out in a 200-cm^3 cylindrical reaction flask.

A piece of cellulose acetate membrane (0.37 g) was immersed in 100 cm^3 of 0.02 mol/dm^3 methanolic KOH, gently stirred for 6 h at room temperature, and washed, first with methanol and then with deionized (DI) water.

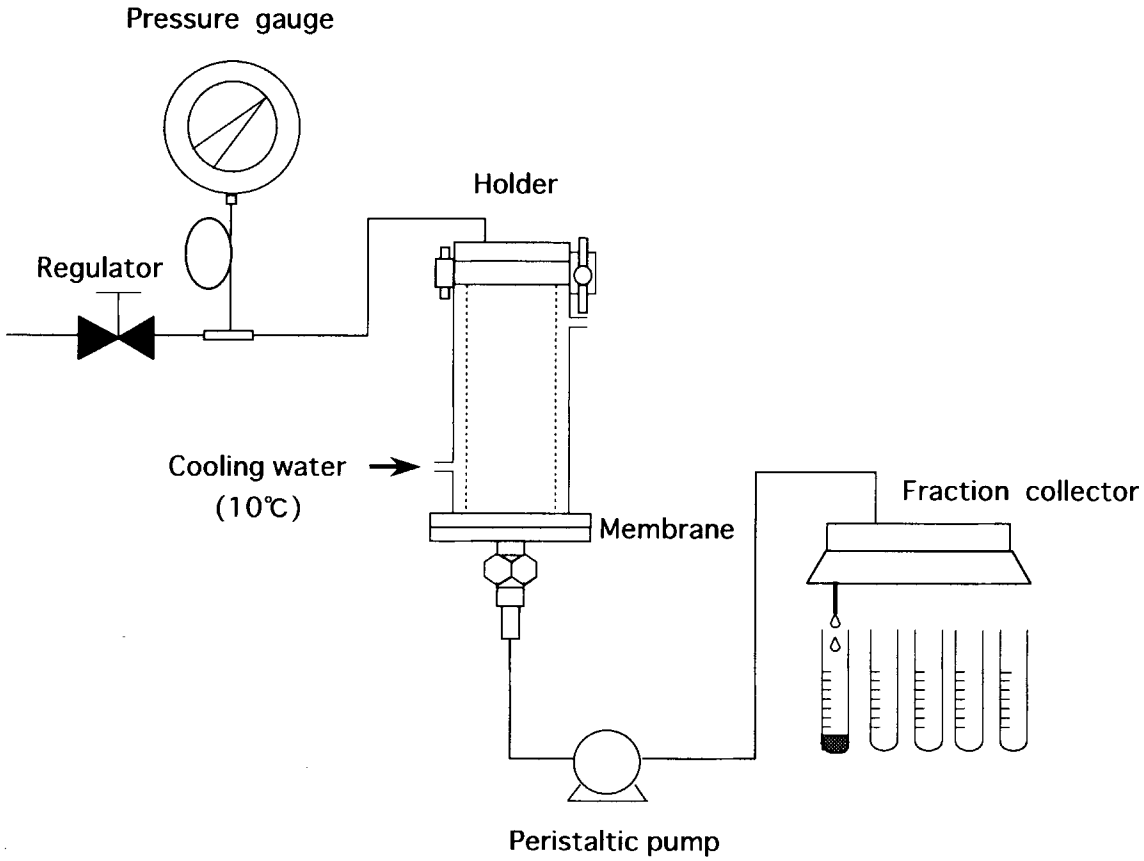

Figure 1 Schematic representation of an experimental apparatus used in protein adsorption and recovery.

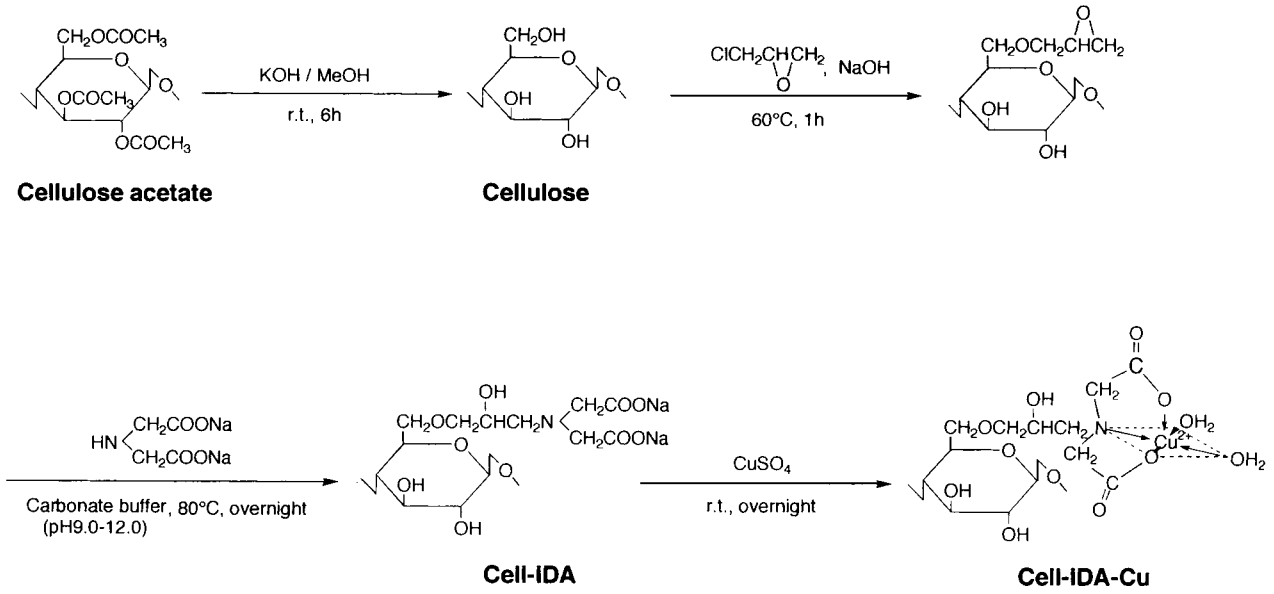

Figure 2 Synthetic route of the Cell–IDA–Cu membrane.

The cellulose membrane obtained (0.23 g) was immersed in 50 cm^3 of ice-cold 6 mol/dm^3 aqueous NaOH, left for 0.5 h at 2°C, and stirred for 0.5 h at 60°C. After the alkaline solution was exchanged with 100 cm^3 of 1 mol/dm^3 aqueous NaOH, 25 cm^3 of epichlorohydrin was added; the solution was stirred for 1 h at 60°C,[23] and the membrane was washed with methanol and then DI water. The membrane was next immersed in 100 cm^3 of 0.2 mol/dm^3 IDA solution whose pH was adjusted to 11.0 with 1 mol/dm^3 carbonate buffer, and gently stirred overnight at 80°C. Infrared (IR) spectra of the cellulose membrane and the Cell–IDA membrane that was treated with aqueous HCl (pH 1.0) were recorded on a Hitachi 270-50 IR spectrophotometer by the KBr pellet method.

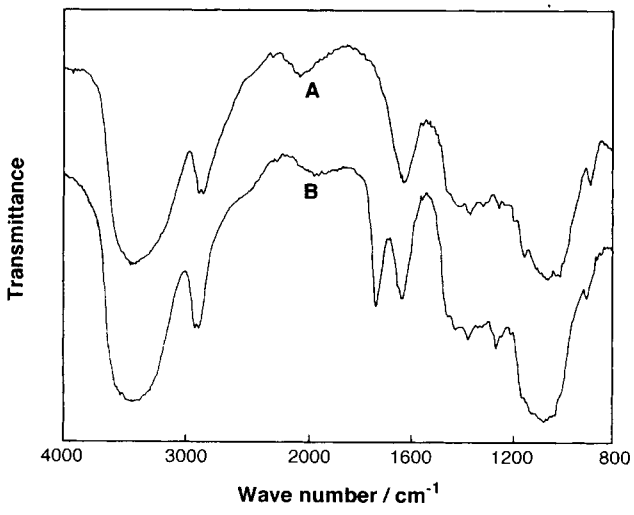

Figure 3 IR spectra of cellulose and Cell–IDA membranes: (A) cellulose membrane, (B) Cell–IDA membrane treated with aqueous HCl of pH 1.0.

The Cell–IDA membrane was immersed in 50 cm^3 of 0.1 mol/dm^3 $CuSO_4$ solution and allowed to stand overnight. After washing with DI water, it was further washed with 0.02 mol/dm^3 citrate buffer (pH 5.5). The morphology of the cellulose and Cell–IDA–Cu membranes were observed with a Hitachi S-2400 scanning electron microscope (SEM) after freeze-drying and coating with gold. The Cell–IDA–Cu membrane was washed repeatedly with 1 mol/dm^3 aqueous HCl, and the washings were collected. The concentration of Cu^{2+} ion in the washings was determined with a Hitachi Z-8100 atomic absorption spectrophotometer, and the degree of modification of the Cell–IDA–Cu membrane was estimated.

Adsorption and Desorption of Serum Proteins

The Cell–IDA–Cu membrane was mounted on an Advantec TSU-90A filtration apparatus equipped with a cooling system, pressure gauge, and fraction collector, as shown in Figure 1. The membrane was previously washed with 50 cm^3 of phosphate buffer whose pH was adjusted to that of protein solution. A solution of 100 cm^3 of 0.15 g/dm^3 BSA (or BγG), which was adjusted to the predetermined pH with one of the various buffer solutions (pH 3.8–5.5, $CH_3COOH/NaOH$; pH 5.5–7.4, KH_2PO_4/Na_2HPO_4; pH 7.4–10.0, $KH_2PO_4/NaOH$), was passed through

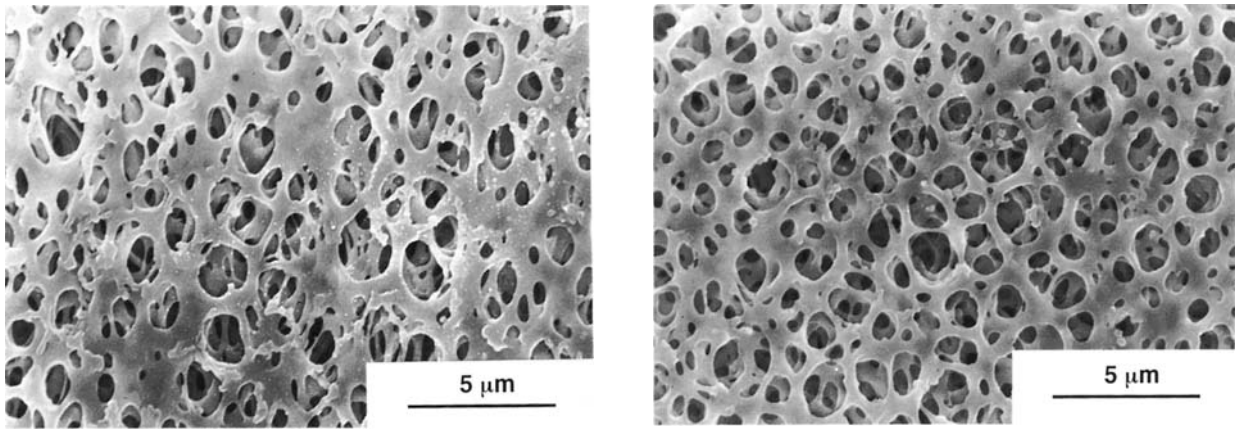

Figure 4 Scanning electron micrographs of freeze-dried cellulose and Cell–IDA–Cu membranes: (A) Cellulose membrane, (B) Cell–IDA–Cu membrane.

the membrane at a flow rate of 100 cm^3/h, and the concentration of BSA (or BγG) in each fraction was determined with a Hitachi U-2000 spectrophotometer using the Cu–Folin method.

Recovery of BSA and BγG in a Single-protein System

A solution of 200 cm^3 of 0.15 g/dm^3 BSA (or BγG), adjusted to pH 7.4, was passed through the Cell–IDA–Cu membrane at a flow rate of 100 cm^3/h, and the membrane was washed with 50 cm^3 of phosphate buffer adjusted to the same pH. Then the adsorbed protein was eluted by 100 cm^3 of eluent. Eluents adopted were 2 mol/dm^3 NaCl solutions at various pHs and imidazole solutions in various concentrations (pH 7.5). The concentrations of protein and Cu^{2+} ion in each fraction were determined by the Cu–Folin method and atomic absorption spectrophotometry, respectively.

Separation of BSA and BγG in a Mixed-protein System

A 200-cm^3 solution of 0.15 g/dm^3 BSA and 0.05 g/dm^3 BγG was passed through the Cell–IDA–Cu membrane at a flow rate of 100 cm^3/h, and the membrane was washed with 50 cm^3 of phosphate buffer whose pH was adjusted to that of the protein mixture. A 50-cm^3 eluent was passed through the membrane. Eluents adopted here were 2 mol/dm^3 NaCl (pH 4.7), 10 mmol/dm^3 imidazole (pH 8.0), and 10

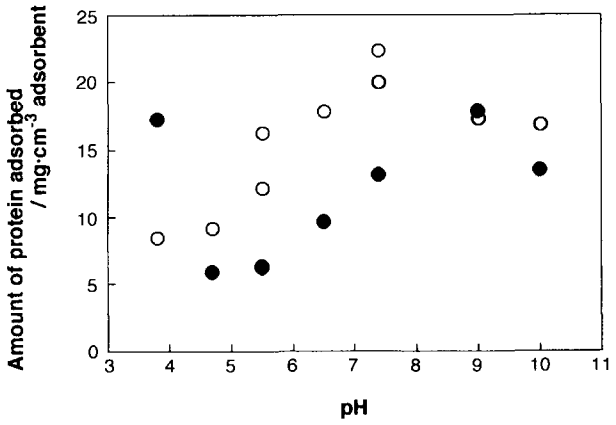

Figure 5 pH dependence of amounts of proteins adsorbed on the Cell–IDA–Cu membrane in the single-protein system: (●) BSA, (○) BγG.

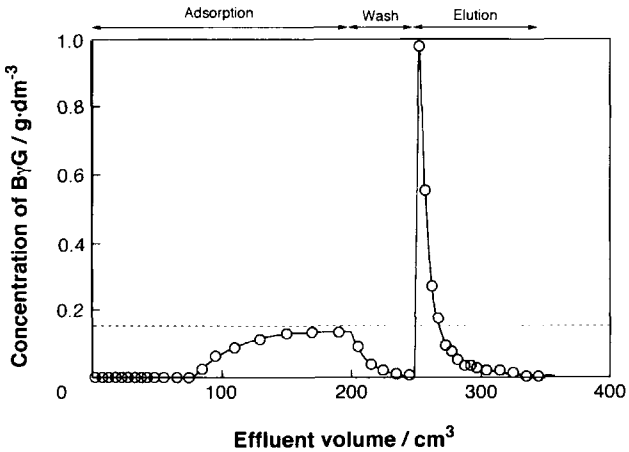

Figure 6 Typical protein recovery process: Broken line represents the concentration of BγG in the feed solution.

Table I pH Dependence of Protein Recovery and Cu^{2+} Ion Leakage in the Case Using 2 mol/dm^3 NaCl Solution as an Eluent[a]

Protein	Elution pH	Maximum Protein Concentration[b] (mg/dm^3)	Protein Recovery[c] (%)	Cu^{2+} Ion Leakage[d] (μg)
BSA	4.23	728.7	84.6	9.89
	4.70	631.2	67.8	5.02
	5.76	456.5	61.2	7.17
	6.73	67.41	22.2	8.55
	7.34	17.45	8.70	6.20
BγG	4.33	916.1	70.5	
	4.75	977.0	74.6	
	5.75	396.8	42.6	
	6.63	73.92	16.7	
	7.73	24.75	3.34	

[a] Adsorption was carried out using the protein solution of 0.15 g/dm^3 (pH 7.4) at a flow rate of 100 cm^3/h.
[b] Determined on each fraction of 5 cm^3.
[c] Estimated by dividing the amount of protein eluted into that adsorbed.
[d] Total amount of Cu^{2+} ion in the solution eluted (100 cm^3).

mmol/dm^3 glycylglycine (pH 8.5) solutions. The concentrations of BSA and BγG in the mixture were determined by a Tosoh gel permeation chromatograph system (pump, CCPS; column, TSK-G3000SW$_{XL}$; column heater, U-620; detector, RI-8000); the eluent was 0.05 mol/dm^3 phosphate buffer (pH 5.0) containing 0.1 mol/dm^3 Na_2SO_4, flow rate was 1.0 cm^3/min, and column temperature was 40°C. Cu^{2+} ion was determined by atomic absorption spectrophotometry.

RESULTS AND DISCUSSION

Cellulose offers its hydroxyl groups to a variety of reagents and thus makes it possible to modify properties. The preparation of the Cell–IDA–Cu membrane was carried out according to the synthetic route depicted in Figure 2. Figure 3 shows IR spectra of the cellulose membrane and the Cell–IDA membrane that was treated with aqueous HCl. As can be seen, the Cell–IDA membrane has the conspicuous absorption peak at 1740 cm^{-1} assigned to carboxylic acid of IDA group. Figure 4 displays SEM pictures of the cellulose and Cell–IDA–Cu membranes. Although the surface of the membranes appears to be somewhat rough due to the damage during chemical reactions, the porous structure is well maintained. The average degree of modification of the Cell–IDA membrane was estimated as 6.5 mol %–anhydroglucose units (AGU) from the difference in weight before and after the modification with IDA. The average modification degree of the Cell–IDA–Cu membrane evaluated by the determination of Cu^{2+} ion was 4.4 mol %-AGU. The modification degree in this system was not so high, due to the solid-phase modification of the swollen cellulose membrane. At the surface of the membrane, however, Cu^{2+} ion seems to be immobilized in a little higher degree than the average modification degree.

The Cell–IDA–Cu membrane thus obtained was used for the experiments of adsorption and desorption of serum proteins. Figure 5 shows the pH dependence of the amounts of BSA and BγG adsorbed on the Cell–IDA–Cu membrane. In both cases, the amount of adsorbed protein increased with increasing pH. This is due to the fact that histidine, cysteine, and tryptophan residues, which play an important role as a ligand,[7-12] can coordinate to the Cu^{2+} ion at higher pH. As pH of the protein solution decreases, the degree of protonation of these amino acid residues increases and their coordination ability decreases. Furthermore, at pH 3.8, a very large amount of BSA was adsorbed. This is presumably a result of the electrostatic interaction between the carboxylate anion of IDA and the BSA cation.

Figure 6 represents a typical protein recovery process for BγG. This process includes three steps, i.e., adsorption, wash, and elution. Adsorption pH was fixed at 7.4 because adequately large amounts of proteins were adsorbed at this pH and it is the physiologically significant pH. In the adsorption step, protein was all adsorbed in the beginning; the adsorption curve passed a breakthrough point and then reached the same concentration as that of the feed solution. Next, in the wash step, the Cell–IDA–Cu membrane was washed until the concentration of protein in the washings reached zero. Finally, in

Table II Influence of Imidazole on Protein Recovery and Cu^{2+} Ion Leakage in the Elution Step (pH 7.5)[a]

Protein	Imidazole Concentration (mmol/dm^3)	Maximum Protein Concentration[b] (mg/dm^3)	Protein Recovery[c] (%)	Cu^{2+} Ion Leakage[d] (μg)
BSA	5	508.4	70.4	6.24
	10	532.6	80.0	7.00
	20	477.6	90.5	9.58
	50	701.3	60.6	7.51
BγG	1	230.3	58.9	9.52
	2	433.2	89.4	3.71
	5	710.2	74.5	7.97
	10	1209	82.1	10.1

[a] Adsorption was carried out using the protein solution of 0.15 g/dm^3 (pH 7.4) at a flow rate of 100 cm^3/h.
[b] Determined on each fraction of 5 cm^3.
[c] Estimated by dividing the amount of protein eluted into that adsorbed.
[d] Total amount of Cu^{2+} ion in the solution eluted (100 cm^3).

the elution step, protein adsorbed on the Cell–IDA–Cu membrane was eluted, mostly in the beginning of the step. NaCl solutions (2 mol/dm^3) at various pHs and imidazole solutions in various concentrations were used as eluents. Table I summarizes the pH dependence of recoveries of BSA and BγG in the case using 2 mol/dm^3 NaCl solution as a eluent. For both proteins, the lower pH was effective to obtain the higher recovery. This find can be explained by the pH dependence of the amounts of proteins adsorbed on the Cell–IDA–Cu membrane, which was discussed above. NaCl solution in high concentration was effective for obtaining high protein recoveries, probably due to the shielding effect on proteins that are polyelectrolytes. Since imidazole can coordinate to the Cu^{2+} ion as a competitive ligand to proteins, it was also adopted as a eluent in this study. Table II compiles the contribution of imidazole to protein

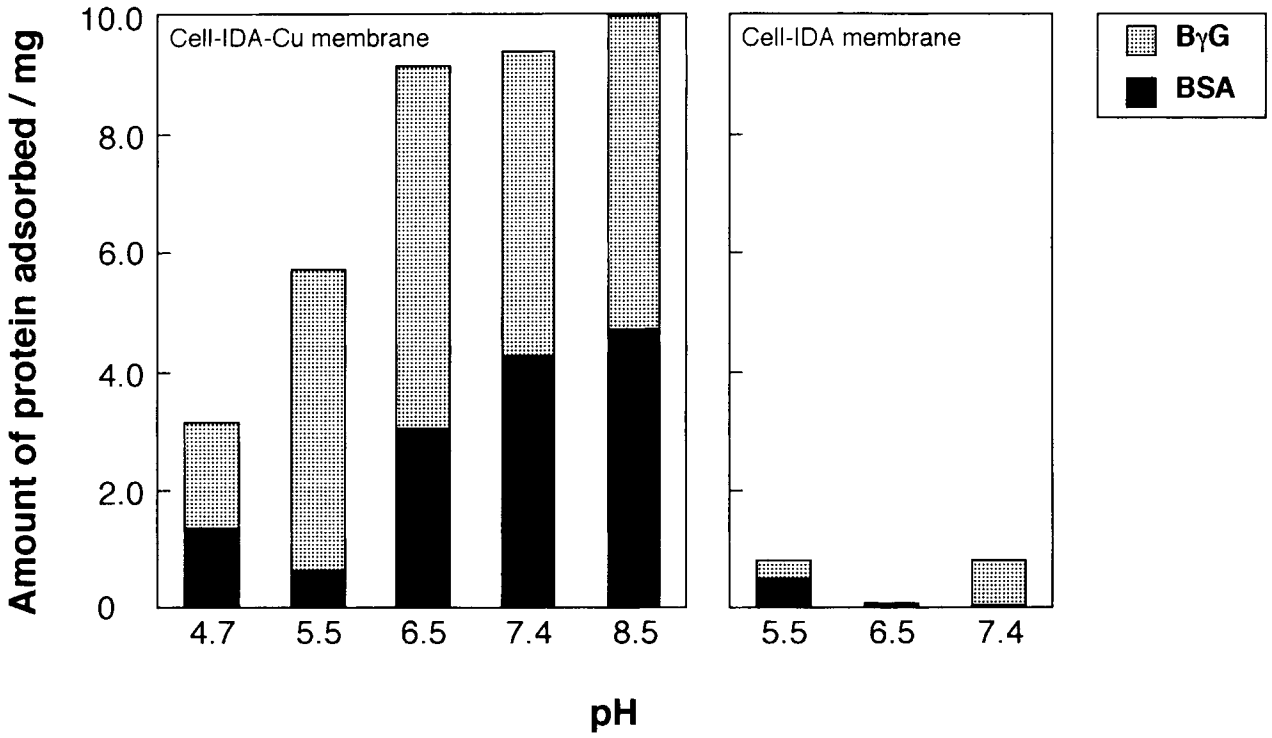

Figure 7 pH dependence of amounts of proteins adsorbed on the Cell–IDA–Cu membrane in the mixed-protein system.

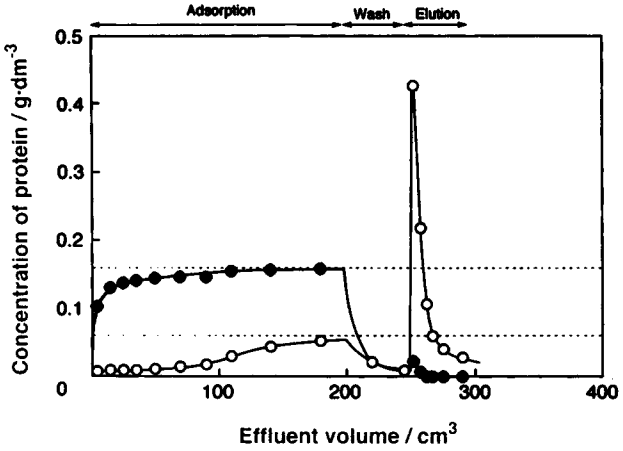

Figure 8 Typical protein recovery process in the mixed-protein system: (○) BγG, (●) BSA. Broken lines represent the concentrations of BSA and BγG in the feed solution.

recoveries. BSA recoveries were almost the same over the imidazole concentration range of 5 to 50 mmol/dm^3. On the other hand, BγG recoveries increased with increasing the concentration of imidazole ranging from 1 to 10 mmol/dm^3. This is attributable to the difference in the histidine residue content between them.

It is possible that Cu^{2+} ion tightly captured by protein leaks from the Cell–IDA–Cu membrane. The leakage of metal ions from immobilized metal affinity membranes may cause the pollution of products and reduce the performance of affinity membranes. Table I lists the leakage of Cu^{2+} ion, along with the protein recoveries. The Cu^{2+} ion leakage was below 10 μg (0.1 ppm in concentration) under all the elution conditions. Each Cu^{2+} ion concentration is lower than that in blood.[24] Table II also lists the Cu^{2+} ion leakage in the case using imidazole solution as a eluent. All concentrations of Cu^{2+} ion were again below 0.1 ppm. Imidazole is a monodentate ligand and its coordination ability seems lower than IDA.

Since the protein adsorption behavior in the mixed protein system sometimes does not show the additive property of each protein, due to the interaction between them,[25] the adsorption of BSA and BγG on the Cell–IDA–Cu membrane in the mixed protein system was further investigated. As shown in Figure 7, BγG was adsorbed more than BSA under every pH condition, although the initial concentration of BγG to BSA was in the ratio 1 : 3, which is essentially the same as that in blood. At pH 5.5, in particular, the ratio in the amount of BγG to BSA adsorbed on the Cell–IDA–Cu membrane was 8 : 1. This is probably due to the fact that globulin has saccharide chains, but this point is still ambiguous. Further investigation on other serum proteins such as fibrinogen and enzymes should be made. Figure 7 shows the results of the adsorption of BSA and BγG on the Cell–IDA membrane, which does not contain Cu^{2+} ion at all, as well as that on the Cell–IDA–Cu membrane. This figure demonstrates that protein adsorption on the Cell–IDA–Cu membrane arises from the interaction between Cu^{2+} ion and proteins, not the interaction between carboxylate groups of IDA and proteins.

Figure 8 represents a typical result of protein recovery in the mixture system of BSA and BγG. Obviously, the breakthrough point of BγG is larger than that of BSA, and the concentration of BγG eluted is higher than that of BSA. Table III is the compilation of the protein recovery and Cu^{2+} ion leakage in the case using various eluents. At all the experiments, pH in the adsorption step was fixed at 5.5 because the concentration efficiency in this step is significantly high at this pH, as mentioned above. By use of 2 mol/dm^3 NaCl solution whose pH was

Table III Effect of Various Eluents on BγG Recovery and Cu^{2+} Ion Leakage[a]

Eluent	Maximum BγG Concentration[b] (mg/dm^3)	BγG Recovery (mg)	BγG/BSA Ratio (by weight)	Cu^{2+} Ion Leakage[c] (μg)
2 mol/dm^3 NaCl (pH 4.7)	427	4.97	11.1	4.71
10 mmol/dm^3 imidazole (pH 7.5)	561	6.22	6.13	3.44
10 mmol/dm^3 glycylglycine (pH 8.5)	101	3.51	9.71	607

[a] Adsorption was carried out using the mixture solution of 0.15 g/dm^3 BSA and 0.05 g/dm^3 BγG (pH 5.5) at a flow rate of 100 cm^3/h.
[b] Determined on each fraction of 5 cm^3.
[c] Total amount of Cu^{2+} ion in the solution eluted (50 cm^3).

adjusted to 4.7, the ratio of BγG to BSA reached 11 : 1. It appears that increase in the ionic strength leads to the great conformational change of BγG because pI of BγG (ca. 7) is apart from the elution pH, while it leads to the small conformational change of BSA because pI of BSA (ca. 4.7) is close to the elution pH. Moreover, imidazole does not show the remarkable effect on the recovery of BγG. Glycylglycine, which is a bidentate ligand, brought about high Cu^{2+} ion leakage: 2 orders higher than that in the case using imidazole solution as eluent.

As previously mentioned, the cellulose membrane modified by immobilization of the Cu^{2+} ion revealed good properties as an affinity membrane. By using higher pH in the adsorption step and lower pH in the elution step with NaCl and imidazole, the serum proteins were concentrated up to 8 times higher than the initial protein solution and the recoveries reached 90%. In the specific recovery from the mixture of BSA and BγG, in which the concentration of BγG to BSA was in the ratio 1 : 3, BγG was concentrated more than BSA under the optimum conditions of adsorption and recovery. The Cu^{2+} ion leakage through the elution step was lower than 0.1 ppm.

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