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Carbohydrate Polymers 40 (1999) 107–113

Carbohydrate Polymers

Recovery of serum proteins using cellulosic affinity membranes modified with tannic acid

N. Kubota[a,*], N. Tatsumoto[a], T. Sano[b]

[a]*General Education Chemistry, Oita Medical University, Hasama-machi, Oita 879-5593, Japan*
[b]*General Education Physics, Oita Medical University, Hasama-machi, Oita 879-5593, Japan*

Received 8 October 1998; received in revised form 15 February 1999; accepted 22 February 1999

Abstract

Two kinds of cellulosic affinity membranes modified with tannic acid were prepared for separation and purification of serum proteins. A porous cellulose membrane was reacted with 1,6-diaminohexane in advance to afford the aminohexyl cellulose membrane (AHC membrane). Then, the tannic acid was bound to the AHC membrane through its hydroxyl group (AHC-COOH membrane) and its carboxyl group (AHC-OH membrane). The pH dependence of the adsorption of serum proteins on the AHC-COOH and the AHC-OH membranes was investigated in a dead-end flow mode, using bovine serum albumin (BSA) and γ-globulin (BγG) as model serum proteins. The adsorption of BSA on the AHC-COOH membrane was dependent on pH, whereas that on the AHC-OH membrane was not. By contrast, the amount of BγG adsorbed on the AHC-OH membrane had a maximum at pH 6.5. The separation of a mixture of BSA and BγG was also conducted using the AHC-OH membrane. The ratio of the amount of BSA to that of BγG adsorbed on the AHC-OH membrane was about unity. The BγG adsorbed on the AHC-OH membrane was effectively eluted with NaCl solution, while it was difficult to recover the BSA adsorbed on that membrane.

Keywords: Bovine serum albumin; γ-Globulin; Porous cellulose membrane; Affinity

1. Introduction

Separation and purification of biomaterials can be grouped into three methods: (i) separation based on the specific precipitation such as ethanol precipitation and salt-fractionation, (ii) separation based on the partition equilibrium such as distillation and extraction, and (iii) separation based on the difference of the mass transfer rate such as chromatography. The ethanol-precipitation method has always been one of the most important industrial fractionations of serum proteins. In laboratories, electrophoresis, gel filtration, and ion-exchange column chromatography are also available for separation and purification of proteins.

There are specific affinities, which can distinguish between biomaterials. Affinity can be classified into two types: (i) biospecific affinity that is the highest specificity such as interaction between antigen and antibody, and (ii) group-specific physicochemical affinity that attracts a series of materials having the same functional groups or structural moieties. These affinities include electrostatic interaction, hydrophobic interaction, and/or hydrogen bonding. Recently, affinity separation such as affinity chromatography (Porath & Hansen, 1991; Lowe, Burton, Burton, Alderton, Pitts, & Thomas, 1992; Zhixin & Kongchang, 1993), affinity cross-flow filtration (Weiner, Sara, Dasgupta, & Sleytr, 1994), and affinity precipitation has been extensively developed. As Brandt, Goffe, Kessler, O'Connor, and Zale (1988) proposed a membrane-based affinity separation system, many investigators have reported affinity membranes (Champluvier & Kula, 1991; Kugel, Moseley, Harding, & Klein, 1992; Suen & Etzel, 1992; Kim, Saito, Furusaki, & Sugo, 1993; Serafica, Pimley, & Belfort, 1994). We have also studied affinity membranes whose matrices are naturally occurring chitosan (Kubota, Kai, & Eguchi, 1994; 1996) and cellulose (Eguchi & Kubota, 1995; Kubota, Nakagawa, & Eguchi, 1996).

In this article, cellulose was used as an affinity membrane matrix, and tannic acid was adopted as a ligand for serum proteins. Naturally occurring cellulose is rather biocompatible, and tannic acid is cheap and has been used as a protein-precipitating agent for a long time (Oh & Hoff, 1979). On the other hand, albumin and γ-globulin are the major components of serum proteins, and they play important roles in the human body. It is well known that the loss of albumin in renal disease causes serious problems. Also, γ-globulin can be a useful medicine. Bovine serum

* Corresponding author.

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PII: S0144-8617(99)00047-8

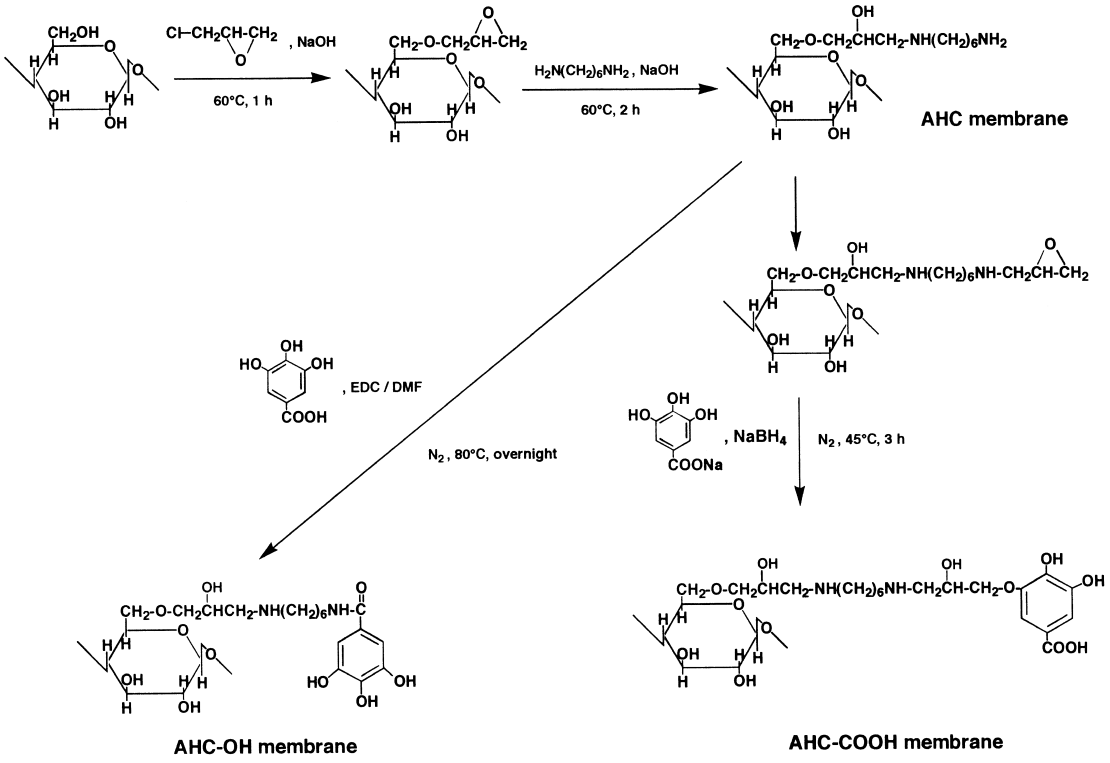

Fig. 1. Synthetic route of cellulosic affinity membranes modified with tannic acid.

albumin (BSA) and γ-globulin (BγG) were chosen as model serum proteins to be separated. The adsorption of BSA in the single-protein system using the porous cellulose membranes modified with tannic acid was previously reported (Eguchi & Kubota, 1995); this paper further deals with the adsorption and desorption of BSA and BγG in a mixed-protein system.

2. Experimental

2.1. Materials

Microfiltration membrane, which is made of cellulose acetate, 90 mm in diameter, 125 μm in thickness, and 0.2 μm in average pore size, was generously supplied by Advantec, Tokyo, Japan. Epichlorohydrin, 1,6-diaminohexane, and tannic acid were purchased from Wako Pure Chemical Industries, Osaka, Japan. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was purchased from Dojin Lab., Kumamoto, Japan. BSA was of monomer grade from Sigma Chemical, St. Louis, USA. BγG was of fraction II grade from Tokyo Kasei Kogyo, Tokyo, Japan. Folin-Ciocalter Reagent from Wako Pure Chemical Industries was used to determine serum proteins. Methanol, acetone, and *N*,*N*-dimethylformamide (DMF) were distilled just before use, and other chemicals were used as received.

2.2. Modification of porous cellulose membrane

To obtain a cellulose membrane, a microfiltration membrane (0.37 g) was immersed in 100 cm^3 of 0.2 mol/dm^3 methanolic KOH, gently stirred for 6 h at room temperature, washed with methanol and then with deionized water. The preparation of the affinity membranes (AHC-COOH and AHC-OH membranes) was carried out according to the synthetic route outlined in Fig. 1.

The AHC-COOH membrane was prepared by a modification of the method by Chibata, Tosa, Mori, Watanabe and Sakata (1986) as follows: The porous cellulose membrane (0.23 g) was immersed in 200 cm^3 of ice-cold 6 mol/dm^3 NaOH solution, left for 30 min at 2°C, and stirred for 30 min at 60°C. After the alkaline solution was exchanged with 100 cm^3 of 1 mol/dm^3 NaOH solution, 25 cm^3 of epichlorohydrin was added, the solution was stirred for 1 h at 60°C, and the membrane was washed with acetone, and then with deionized water. The activated membrane was stirred in 200 cm^3 of 0.1 mol/dm^3 1,6-diaminohexane aqueous solution for 2 h at 60°C and washed with acetone and deionized water. The resulting aminohexyl cellulose membrane (AHC membrane) was again stirred in the mixture of 100 cm^3 of 1 mol/dm^3 NaOH and 25 cm^3 of epichlorohydrin and washed with acetone, and then with deionized water. The activated AHC membrane was immersed in 200 cm^3 of 3% tannic acid aqueous solution whose pH was adjusted to 7.0 with NaOH solution, and then 0.048 g of $NaBH_4$ was added. Nitrogen gas was bubbled

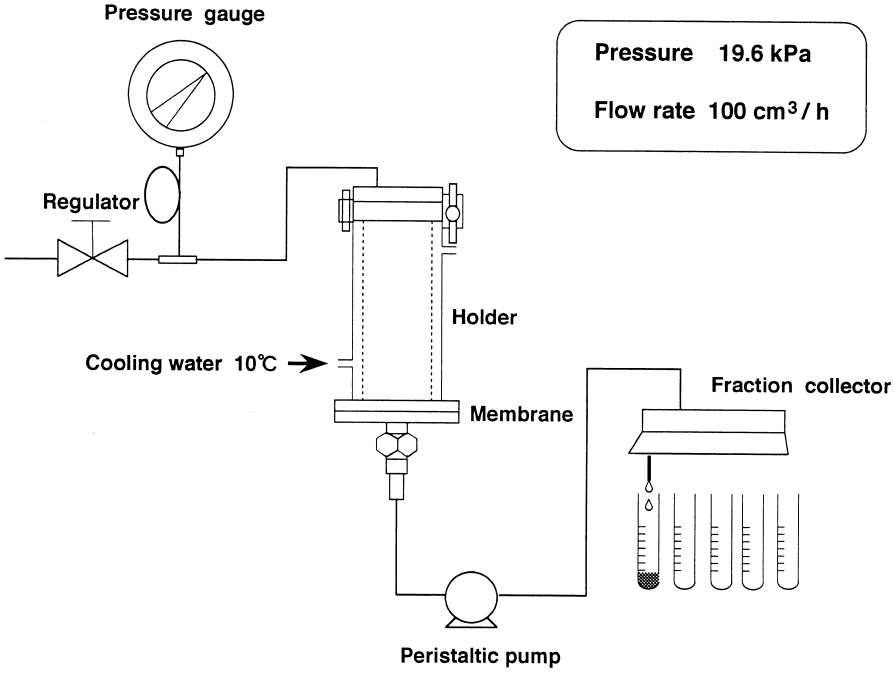

Fig. 2. Experimental apparatus for adsorption and desorption of serum proteins in a dead-end flow mode.

through the solution with stirring for 3 h at 45°C. After the reaction, the membrane was washed with 30% aqueous acetone adjusted to pH 2.0 with HCl solution and then with deionized water. The membrane was further washed with 20% aqueous ethanol adjusted to pH 2.5 with HCl solution, with the eluent which is used in the desorption experiment, and then with deionized water.

However, the AHC-OH membrane was prepared according to the following method: the AHC membrane was immersed in acetone and washed with DMF. The AHC membrane was finally immersed in 100 cm^3 of DMF in which 1.2 g of tannic acid and 1.5 g of EDC were dissolved, and the reaction mixture was gently stirred overnight at 80°C under nitrogen atmosphere. After the reaction, the membrane was thoroughly washed using the same procedure as used for the AHC-COOH membrane.

IR spectra of the membranes were recorded on a Hitachi 270-50 IR spectrophotometer by the KBr pellet method.

2.3. Adsorption and desorption of serum proteins in single-protein system

The adsorption of serum proteins on the AHC-COOH and the AHC-OH membranes was investigated employing an Advantec TSU-90A filtration apparatus (Fig. 2). The effective membrane area was 59 cm^2. The mounted membrane was previously washed with 50 cm^3 of phosphate buffer solution whose pH was adjusted to that of the protein solution, and 200 cm^3 of 0.15 g/dm^3 BSA (or BγG) solution which was prepared using the phosphate buffer (pH 4.8–8.0) was passed through the membrane at a flow rate of 100 cm^3/h. The flow rate was controlled by a peristaltic pump, and the effluents were fractionated by a fraction collector. The membrane was then washed with 50 cm^3 of the same phosphate buffer solution, and the adsorbed protein was eluted with 50 cm^3 of eluent. The concentration of BSA (or BγG) in each fraction was determined with a Hitachi U-2000 spectrophotometer by the Cu-Folin method.

2.4. Adsorption and desorption of serum proteins in mixed-protein system

In a manner similar to the single-protein system, 200 cm^3 of the mixture solution of BSA and BγG (0.15 g/dm^3 each) which was prepared using the phosphate buffer (pH 4.8–8.0) was passed through the AHC-OH membrane, the membrane was washed with 50 cm^3 of the same phosphate buffer solution, and 50 cm^3 of eluent was passed through the membrane. The concentrations of serum proteins in the effluents were determined with a gel permeation chromatographic system, which was composed of an automatic sample injector (Tosoh AS-8020), degasser (Tosoh SD-8022), pump (Hitachi L-6000), column (Tosoh TSK_{gel} $G3000SW_{XL}$), differential refractometer (Tosoh RI-8000), and chromato-integrator (Hitachi D-2500). The eluent was 0.05 mol/dm^3 sodium phosphate solution containing 0.1 mol/dm^3 Na_2SO_4, the flow rate was 1.0 cm^3/min, and the column temperature was 40°C.

3. Results and discussion

According to the procedure shown in Fig. 1, the porous cellulose membrane was modified with tannic acid. As the AHC-COOH membrane was prepared by connecting the

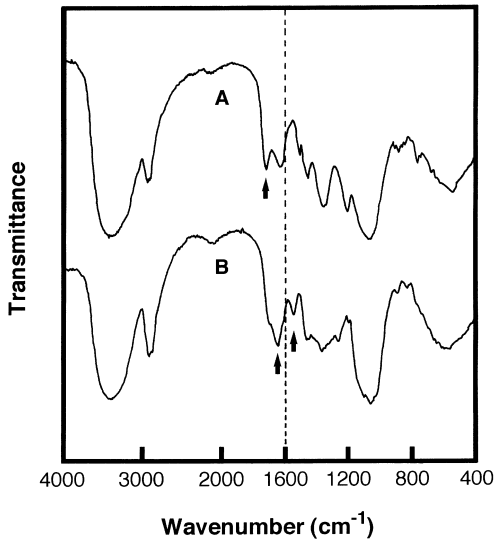

Fig. 3. IR spectra of the membranes modified with tannic acid: (A) AHC-COOH membrane; and (B) AHC-OH membrane.

tannic acid through its hydroxyl group, this membrane has arylic -COOH groups. On the contrary, the AHC-OH membrane was obtained by connecting the tannic acid through its carboxyl group, and the membrane has phenolic -OH groups rather than carboxyl groups. As can be seen in Fig. 3, which shows the IR spectra of the AHC-COOH and the AHC-OH membranes, the AHC-COOH membrane has absorption peak at 1720 cm^{-1} assigned to the carboxyl group, and the AHC-OH membrane has the absorption peaks at 1650 and 1550 cm^{-1} assigned to amide I and II, respectively. The degrees of modification with tannic acid were determined from the difference in dry weight before and after the modification. As a result, the average degrees of modification of the AHC-COOH and the AHC-OH membranes were estimated as 35.4 and 31.4 mol.%-anhydroglucose unit, respectively.

Using the AHC-COOH and the AHC-OH membranes thus obtained, the experiments of the adsorption and desorption of BSA were first carried out. Fig. 4 depicts a typical process of the adsorption and desorption of BSA in the

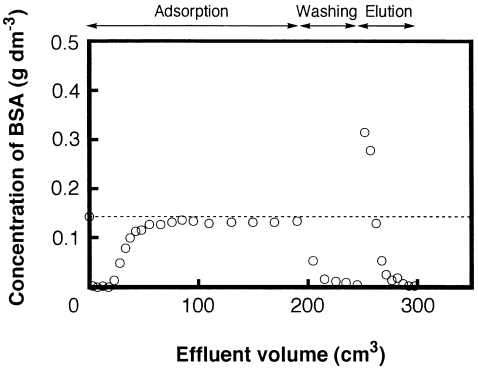

Fig. 4. Typical process of adsorption and desorption of BSA in a dead-end flow mode. Broken line represents feed concentration.

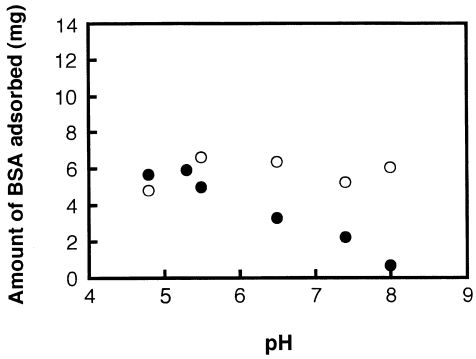

Fig. 5. pH dependence of the amount of BSA adsorbed on the membranes modified with tannic acid: ●, AHC-COOH membrane; and ○, AHC-OH membrane.

dead-end flow mode. This process includes three steps, i.e. adsorption, washing, and elution. The amount of adsorbed BSA was calculated from the difference in concentration between the feed solution and effluent. Similarly, the pH dependence of the amount of BSA adsorbed on the AHC-COOH and the AHC-OH membranes was investigated. Fig. 5 shows that the amount of BSA adsorbed on the AHC-COOH membrane is dependent on the pH of the solution and has a maximum at pH 5.3. In contrast, the amount of BSA adsorbed on the AHC-OH membrane does not depend on pH and is almost the same as the maximum amount of BSA adsorbed on the AHC-COOH membrane. Such pH dependence is probably due to the fact that the dissociation characters of the carboxyl and hydroxyl groups are different and the isoelectric point of BSA is p$I \approx 4.8$. This suggests that the carboxyl groups on the AHC-COOH membrane may be related to the low adsorption of BSA, i.e. the electrostatic repulsion between carboxylate anions on the AHC-COOH membrane and the anionic groups on BSA may reduce the adsorption of BSA on the AHC-COOH membrane at higher pH. Kawamoto, Nakatsubo, and

Table 1
Recovery of BSA adsorbed on the AHC-COOH membrane (adsorption pH was 5.3 and eluent volume was 50 cm^3)

Eluent	C_{max}/C_0[a]	Recovery (%)
1 mol/dm^3 NaCl	1.19	42.1
2 mol/dm^3 NaCl	2.54	78.3
2 mol/dm^3 NaCl (pH 2.0)[b]	0.05	1.7
2 mol/dm^3 NaCl (pH 8.0)[c]	1.08	48.9
0.02 mol/dm^3 Citrate buffer (pH 2.0)	1.19	44.8
0.2 mol/dm^3 Citrate buffer (pH 2.0)	3.06	83.9
0.2 mol/dm^3 Citrate buffer (pH 5.5)	1.10	36.0
0.2 mol/dm^3 Citrate buffer (pH 7.4)	0.56	15.5

[a] C_{max}, maximum concentration in effluent; C_0, feed concentration.
[b] pH was adjusted with 3 mol/dm^3 HCl.
[c] NaCl was dissolved in phosphate buffer.

Table 2
Recovery of BSA adsorbed on the AHC-OH membrane (adsorption pH was 7.4 and eluent volume was 50 cm^3)

Eluent	C_{max}/C_0 [a]	Recovery (%)
2 mol/dm^3 NaCl	1.72	71.4
0.2 mol/dm^3 Citrate buffer (pH 2.0)	1.63	65.4
0.2 mol/dm^3 Citrate buffer (pH 5.5)	1.67	57.8
0.2 mol/dm^3 Citrate buffer (pH 7.4)	2.97	59.6
0.2 mol/dm^3 Malic acid	3.23	80.1
50% Ethylene glycol	0.22	29.2
50% Ethylene glycol containing 1 mol/dm^3 NaCl	2.60	68.3
2 mol/dm^3 Urea	–	–
1 mol/dm^3 Sucrose	–	–
10% Glycerol	–	–

[a] C_{max}, maximum concentration in effluent; C_0, feed concentration.

Murakami (1990) reported that the hydroxyl groups of tannin derivatives may synergistically interact with proteins. Their finding is consistent with our result that the amount of BSA adsorbed on the AHC-OH membrane was greater than that on the AHC-COOH membrane which has fewer hydroxyl groups.

As in the case of the AHC-COOH membrane, the largest amount of BSA was adsorbed at pH 5.3, the adsorption pH was fixed at this pH and the adsorbed BSA was recovered under various elution conditions. Table 1 lists the recoveries and concentration factors of BSA for the AHC-COOH membrane, C_{max}/C_0, where C_{max} is the maximum concentration in the effluent and C_0 is the feed concentration. The adsorbed BSA was effectively recovered with 2 mol/dm^3 NaCl solution. Considering that BSA was scarcely adsorbed at pH 8.0, 2 mol/dm^3 NaCl solution adjusted to pH 8.0 was also used as an eluent. However, the recovery was not improved. With 0.2 mol/dm^3 citrate buffer solution of pH 2.0, the recovery and concentration factor are greater than those at pH 5.5 and 7.4. Considering that all three carboxyl groups of the citric acid are not dissociated at pH 2.0 ($pK_1 = 3.1$, $pK_2 = 4.7$, $pK_3 = 5.4$), the carboxyl groups of citric acid seem to participate in the elution of BSA adsorbed on the AHC-COOH membrane.

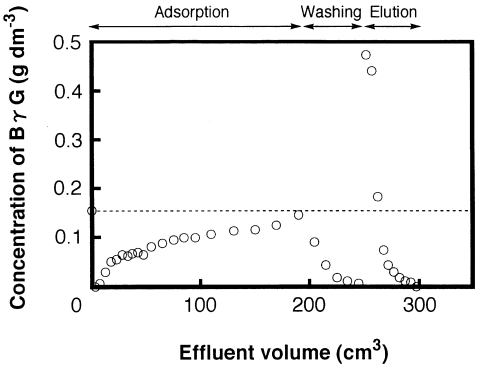

Fig. 6. Typical process of adsorption and desorption of BγG in a dead-end flow mode. Broken line represents feed concentration.

As the adsorption of BSA on the AHC-OH membrane was independent of pH, the adsorption pH was fixed at pH 7.4, which is the physiologically significant pH. The recoveries and concentration factors of BSA adsorbed on the AHC-OH membrane with several eluents are summarized in Table 2. With 2 mol/dm^3 NaCl solution, the recovery of BSA from the AHC-OH membrane is slightly lower than that from the AHC-COOH membrane. Also, the recoveries of BSA with citrate buffers are not so different at all pHs, probably because the AHC-OH membrane has no carboxyl group. On the AHC-OH membrane, ionic interaction does not seem to contribute to the adsorption and desorption of BSA, and hydrophobic interaction and/or hydrogen bonding are conceivable instead. It is known that a hydrophilic organic solvent with low dielectric constant breaks the structure of water and reduces the hydrophobic interaction. Accordingly, a solution of such hydrophilic organic solvent was also used as eluent. Ethylene glycol alone, however, could not increase the recovery of BSA so much, and the recovery became greater by adding NaCl. NaCl is thought to affect the conformation of BSA. Furthermore, urea, sucrose and glycerol, which are known to weaken hydrogen bonding, were not able to recover BSA adsorbed on the AHC-OH membrane. These results suggest that hydrophobic interaction is the greater contribution to the adsorption of BSA on the AHC-OH membrane, and BSA strongly interacts with the AHC-OH membrane. Among the eluents used in this part, malic acid solution recovered the greatest amount of BSA. It appears that hydroxyl and carboxyl groups of malic acid take part in the elution of BSA adsorbed on the AHC-OH membrane.

The AHC-OH membrane was next used for the experiments of adsorption and desorption of BγG, because the amount of BSA adsorbed on the AHC-OH membrane is greater than that adsorbed on the AHC-COOH membrane. A typical process of the adsorption and desorption of BγG is shown in Fig. 6. Compared with Fig. 4, the adsorption rate of BγG is slower than that of BSA. Moreover, the pH dependence of the adsorption of BγG was studied in the same manner as that for the adsorption of BSA. Fig. 7 indicates that the amount of adsorbed BγG is dependent on the pH of the solution and has a maximum at pH 6.5. The BγG molecule is expected to become compact at a pH near the isoelectric point ($pI \approx 7$), because the intramolecular electrostatic repulsion is small. As a result, a large amount of BγG seems to be adsorbed on the AHC-OH membrane at pH 6.5. Considering the result that the amount of BγG adsorbed on the membrane was not negligible even at the highest pH, hydrophobic interaction and/or hydrogen bonding are conceivable. Further investigation should be addressed to this point.

As previously mentioned, in the single-protein system, the amount of BγG adsorbed on the AHC-OH membrane was twice as great as that of BSA on the same membrane at pH 6.5. Consequently, we tried to separate the mixture of BγG and BSA by the AHC-OH membrane. Fig. 8 shows an

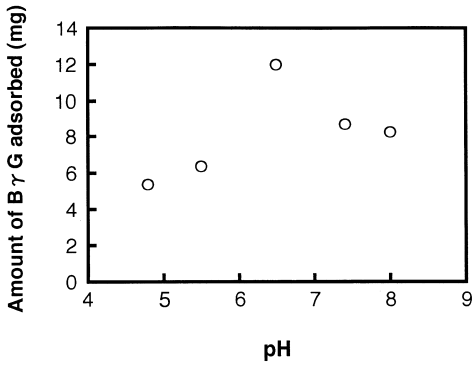

Fig. 7. pH dependence of the amount of BγG adsorbed on the AHC-OH membrane.

example of the separation process for the mixture of BSA and BγG, in which BSA and BγG were in the ratio of 1:1. BSA reached a saturation of the adsorption earlier than BγG, and this phenomenon coincides with the results in the single-protein system. Fig. 9 shows the pH dependence of the adsorption of serum proteins on the AHC-OH membrane in the mixed-protein system. Unlike the single-protein system in which the adsorption of BSA does not depend on pH, both the BSA and the BγG slightly show the pH-dependent adsorption on the AHC-OH membrane, and the total amount of adsorbed proteins is a maximum at pH 6.5. This might be due to the interaction between anionic BSA and cationic BγG. The ratio of the amount of adsorbed BγG to that of adsorbed BSA is about 1 at pH 5.5 and 6.5 and about 0.7 at pH 4.8 and 8.0. Adsorption specificity for BγG, which was observed in the single-protein system, was not apparent in the mixed-protein system. It seems that as BSA was adsorbed prior to BγG, the adsorption site left for BγG is limited and the amount of adsorbed BγG is less. Table 3 compiles the results of the recovery in the mixed-protein system with 2 mol/dm^3 NaCl solutions as eluents. It shows that BγG could be efficiently recovered with NaCl solution, whereas only a small amount of BSA was eluted. Again, this suggests that BSA may tightly interact with the AHC-OH membrane.

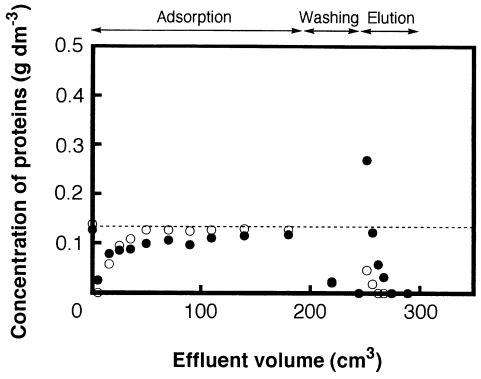

Fig. 8. Typical process of adsorption and desorption of serum proteins in the mixed-protein system. Broken line represents feed concentration: ○, BSA; and ●, BγG.

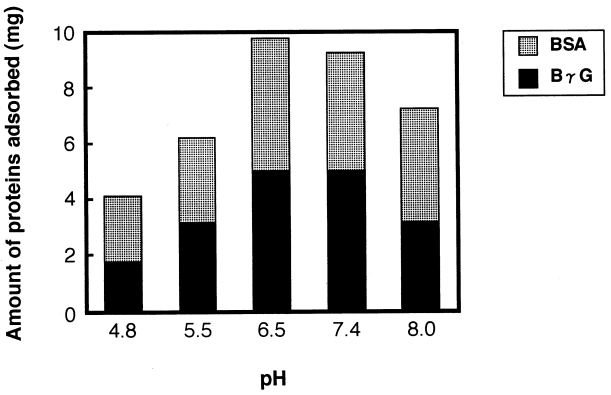

Fig. 9. pH dependence of the amount of serum proteins adsorbed on the AHC-OH membrane in the mixed-protein system.

In conclusion, two kinds of porous cellulosic affinity membranes modified with tannic acid, the AHC-COOH and the AHC-OH membranes, could be prepared. The amount of BSA adsorbed on the AHC-COOH membrane decreased with pH, while that on the AHC-OH membrane was constant. It seems, therefore, that BSA was adsorbed on the AHC-OH membrane hydrophobically, and ionic interaction as well as hydrophobic interaction was important for the AHC-COOH membrane. On the contrary, the amount of BγG adsorbed on the AHC-OH membrane was greater than that of BSA adsorbed on the same membrane, and at pH 6.5, the amount of BγG adsorbed on the AHC-OH membrane had a maximum because the electrostatic repulsion might be reduced at this pH. In the mixed-protein system, moreover, the total amount of BSA and BγG adsorbed on the AHC-OH membrane had a maximum at pH 6.5, and adsorption specificity for BγG was not apparent, unlike in the single-protein system. This is probably due to the slower adsorption rate of BγG. However, BγG could be eluted specifically, because BSA was tightly adsorbed on the AHC-OH membrane. From these as well as previous results (Eguchi & Kubota, 1995), it can be expected that these cellulosic affinity membranes modified with tannic acid are applicable for

Table 3
Recovery of serum proteins adsorbed on the AHC-OH membrane in mixed-protein system using 2 mol/dm^3 NaCl solution (eluent volume was 50 cm^3)

Adsorption pH	Protein	C_{max}/C_0 [a]	Recovery (%)
8.0	BγG	1.00	52.1
	BSA	0.18	6.4
7.4	BγG	1.92	64.9
	BSA	0.28	11.6
6.5	BγG	2.11	58.2
	BSA	0.33	8.1
5.5	BγG	1.21	60.9
	BSA	0.32	11.2
4.8	BγG	0.34	69.9
	BSA	0.33	12.6

[a] C_{max}, maximum concentration in effluent; C_0, feed concentration.

the separation of serum proteins, by further investigation on the eluent which can recover each protein specifically.

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