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Adsorption and Desorption of Serum Proteins Using Cellulosic Affinity Membrane Modified with *N*-Acetyl-L-phenylalanine

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ABSTRACT

A cellulosic affinity membrane modified with *N*-acetyl-L-phenylalanine (*N*-Ac-L-Phe) was prepared intending to separate and purify the serum proteins. A porous cellulose membrane was first reacted with acrylonitrile to afford a cyanoethyl cellulose membrane (CEC membrane). After reduction of the cyano groups of the CEC membrane to obtain aminopropyl cellulose membrane (APC membrane), *N*-Ac-L-Phe was bound to the APC membrane through amide linkage (APC-Phe membrane). The pH dependence of the adsorption of serum proteins on the APC-Phe membrane was investigated in a dead-end flow mode, using bovine serum albumin (BSA) and γ-globulin (BγG) as model proteins. The adsorption behavior of BSA and BγG on the APC-Phe membrane

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was rather independent of pH, and the amount of BSA adsorbed on the membrane was greater than that of BγG. The BSA adsorbed on the APC-Phe membrane was recovered with hydroxycarboxylic acid solutions; whereas, it was not effectively eluted with NaCl solution.

Key Words: Affinity membrane; Porous cellulose membrane; Phenylalanine; Albumin; Globulin.

INTRODUCTION

Albumin and γ-globulin are the major components of serum proteins. They play important roles in the human body. For example, serum albumin has many important physiological functions that contribute significantly to osmotic blood pressure and aid in the transport, distribution, and metabolism of many endogenous and exogenous substances, including bile acids, bilirubin, long-chain fatty acids, amino acids, steroids, metal ions, chloride ion, and numerous pharmaceuticals.[1] It is well known that the loss of albumin in renal disease causes serious problems. On the other hand, γ-globulin is a mixture of, mainly, IgG and IgM, and the former can be a useful medicine. A large amount of human serum albumin and other therapeutically important proteins, such as IgG, coagulation factor VIII, and protease inhibitors, are isolated from plasma.[2] The increasing diversity of therapeutic proteins derived from plasma relies on adequate techniques to obtain highly purified and physiologically active proteins. The differential precipitation with ethanol by Cohn's process has 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 the separation and purification of proteins.

Separation methods based on the affinity interactions between biological materials are often regarded as among the most effective techniques in protein purification. There are many specific affinity systems that can distinguish biomolecules. For example, biospecific affinity, such as an interaction between antigen and antibody, shows the highest specificity. On the other hand, group-specific physicochemical affinity that attracts a series of molecules having the same functional groups or structural moieties is also important. These affinities include electrostatic interaction, hydrophobic interaction, and/or hydrogen bonding. Recently, affinity separation, such as affinity chromatography, has been extensively developed.[3–5] Furthermore, since Brandt et al.[6] proposed a membrane-based affinity separation system, many investigators have reported affinity membranes.[7–12] In principle,

membrane adsorbents avoid the major diffusional limitation to the transport of solute molecules to their binding sites in chromatographic beads, which becomes the rate-limiting factor during large-scale purification, and binding capacities of affinity membranes are independent of flow rate, on account of convective rather than diffusive mass transport.[6] However, synthetic polymers, such as nylon and polyethylene (PE), are often used for affinity matrix. There are few reports about affinity membranes using natural macromolecular materials for affinity matrix.[13,14] We have studied affinity membranes with matrices that are naturally occurring chitosan[15,16] and cellulose.[17–19]

In this study, a porous cellulose membrane was used as affinity matrix, and *N*-acetyl-L-phenylalanine (*N*-Ac-L-Phe) was adopted as a ligand for serum proteins. Naturally occurring cellulose is rather biocompatible, and phenylalanine is one of the hydrophobic amino acids. Affinity chromatography and membrane based on hydrophobic interaction have wide applications for protein purification.[8,20] Bovine serum albumin (BSA) and γ-globulin (BγG) were chosen as model serum proteins. This article deals with the adsorption and desorption of BSA and BγG using the porous cellulose membrane modified with *N*-Ac-L-Phe. Our modification procedure for the cellulose membrane with *N*-Ac-L-Phe is an alternative route to the conventional method.[21]

EXPERIMENTAL

Materials

Microfiltration membrane made of cellulose acetate, 90 mm in diameter, 125 μm in thickness, 0.2 μm in average pore size, and 66% in porosity, was generously supplied by Advantec, Tokyo, Japan. Acrylonitrile was purchased from Wako Pure Chemical Industries, Osaka, Japan. BH_3 in tetrahydrofuran (1 mol/dm^3 BH_3–THF complex) and *N*-Ac-L-Phe were obtained from Aldrich Chemical Co., Milwaukee, WI. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was purchased from Dojin Lab., Kumamoto, Japan. BSA was of monomer grade from Calbiochem, San Diego, CA. 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, THF, and *N*,*N*-dimethylformamide (DMF) were distilled just before use, and other chemicals were used as received.

Modification of Porous Cellulose Membrane

To obtain a porous 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, and washed with methanol then with deionized water. The morphology of the cellulose membrane was observed with a Hitachi S-4500 scanning electron microscope (SEM) after freeze drying and coating with gold. The modification of the cellulose membrane was carried out according to the synthetic route outlined in Fig. 1.

The porous cellulose membrane (0.23 g) was immersed in 50 cm^3 of 1 mol/dm^3 NaOH solution overnight, and 5 cm^3 of acrylonitrile was added. The solution was stirred for 5 h at room temperature, and the membrane was washed with deionized water and lyophilized. The resulting cyanoethyl cellulose membrane (CEC membrane) was immersed in 50 cm^3 of THF and 25 cm^3 of 1 mol/dm^3 BH_3–THF was added dropwise with a syringe at 0°C. The CEC membrane was reduced for 1 h at 0°C, for 1 h at room temperature, then for 5 h at 60°C, with bubbling dry nitrogen through the solution. After this, 6 mol/dm^3 HCl was carefully added until there was no further liberation of gaseous hydrogen. The membrane was washed with alkaline solution then

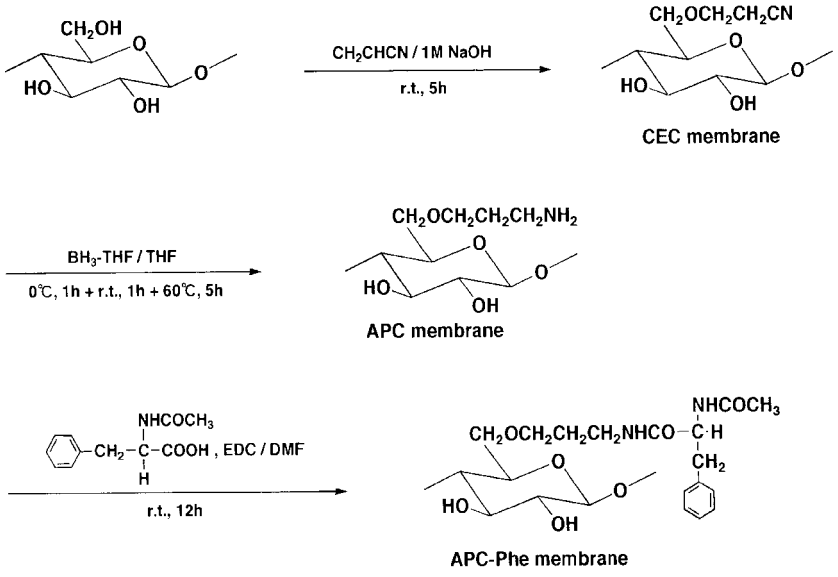

Figure 1. Synthetic route of cellulosic affinity membrane modified with *N*-Ac-L-Phe.

with deionized water. The obtained aminopropyl cellulose membrane (APC membrane) was dehydrated with acetone then with DMF. The APC membrane was finally immersed in 50 cm^3 of DMF in which 0.414 g of *N*-Ac-L-Phe and 0.383 g of EDC were dissolved. The reaction mixture was gently stirred overnight at room temperature under a nitrogen atmosphere. After the reaction, the membrane was thoroughly washed with acetone, methanol, then deionized water. The membrane was further washed with the eluent, which is used in the desorption experiment and then extensively washed with deionized water.

IR spectra of the membranes were recorded on a Hitachi 270-50 IR spectrophotometer by the KBr pellet method. The degree of substitution with *N*-Ac-L-Phe was estimated from the difference in weight between the APC and APC-Phe membranes.

Measurement of Zeta Potential of BSA and BγG

BSA was dissolved in 0.1 mol/dm^3 citrate-0.2 mol/dm^3 Na_2HPO_4 buffer solutions (pH 3–5) and 0.067 mol/dm^3 phosphate buffer solutions (pH 5–8) to afford 10 g/dm^3 solutions. After being degassed and filtered through a 0.45-μm filter, zeta potential of BSA at each pH was measured by laser Doppler anemometry using a Coulter DELSA 440SX. For BγG, 0.01 mol/dm^3 citrate-0.02 mol/dm^3 Na_2HPO_4 buffer solutions (pH 5–8) were used.

Adsorption and Desorption of BSA and BγG

The adsorption and desorption of serum proteins using the APC-Phe membrane were investigated employing an Advantec TSU-90A filtration apparatus illustrated in Fig. 2. An effective membrane area was 59 cm^2. The membrane mounted was previously washed with 100 cm^3 of 0.067 mol/dm^3 phosphate buffer whose pH was adjusted to that of the protein solution, and 200 cm^3 of ca. 0.15 g/dm^3 BSA (or BγG) solution, which was prepared using the phosphate buffer (pH 5.5–7.4), 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 effluent was fractionated by a fraction collector. The membrane was then washed with 50 cm^3 of the same phosphate buffer, 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.

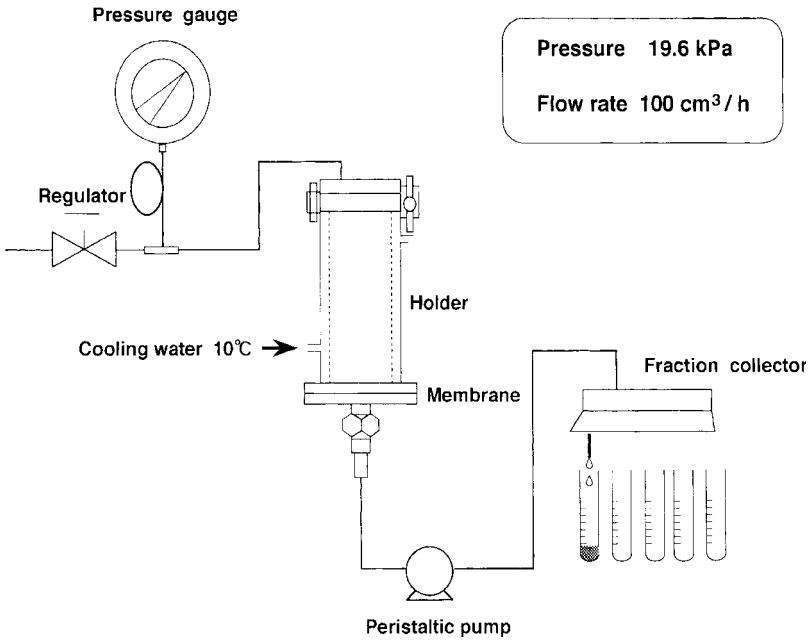

Figure 2. Experimental apparatus for adsorption and desorption of serum proteins in the dead-end flow mode.

RESULTS AND DISCUSSION

Figure 3 displays SEM picture of the porous cellulose membrane. The microporous structure of the membrane is well maintained. This cellulose membrane has the same porosity and mechanical strength as the original cellulose acetate membrane. Generally, cellulose is modified with amino acids through the cyanogen bromide, oxirane, or triazine methods.[21] In this article, we propose an alternative route for the modification of the porous cellulose membrane with *N*-Ac-L-Phe, which is outlined in Fig. 1. Figure 4 shows IR spectra of the CEC, APC, and APC-Phe membranes. As can be seen, the CEC membrane has the absorption peak at 2250 cm^{-1} assigned to the aliphatic cyano group, the APC membrane has the absorption peak at 1570 cm^{-1} assigned to the amino group, and the APC-Phe membrane has the absorption peaks at 1650 and 1560 cm^{-1} assigned to the amide I and II, respectively, and 750 and 700 cm^{-1} assigned to the phenyl group. Apparently, the APC-Phe membrane has *N*-Ac-L-Phe residue via aminopropyl spacer as shown in Fig. 1.

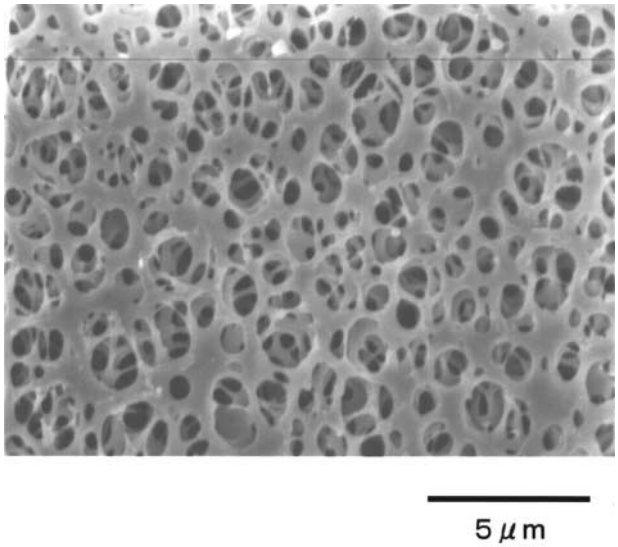

Figure 3. Scanning electron micrograph of porous cellulose membrane.

Using the APC-Phe membrane thus obtained, the experiments of the adsorption and desorption of serum proteins were carried out in a dead-end flow mode. Figure 5 depicts a typical process of the adsorption and desorption of BSA. This process includes three steps, that is, adsorption, washing, and elution. The breakthrough capacities and binding capacities of affinity membranes can be improved by stacking the membranes.[13] The amount of BSA adsorbed on the APC-Phe membrane was calculated from the difference in concentration between the feed solution and effluent at the adsorption step. The APC-Phe membrane was also used for the experiments of the adsorption and desorption of BγG. An adsorption–washing–elution profile similar to that for BSA was obtained. According to Kassab et al.,[1] interactions between immobilized ligands and protein molecules may result from the ionization state of them and from the conformational state of protein molecules. Thus, the pH dependence of the amounts of BSA and BγG adsorbed on the APC-Phe membrane was investigated. Figure 6 shows that the adsorption of BSA and BγG on the APC-Phe membrane is rather independent of pH, similar to the cellulosic affinity membrane modified with tannic acid through its carboxyl group.[19] As reported, such pH dependence is probably due to the fact that the membrane has no dissociable group.[19] On the other hand, the isoelectric points of BSA and BγG are p$I \approx 4.6$ and p$I \approx 6$, respectively, as shown in

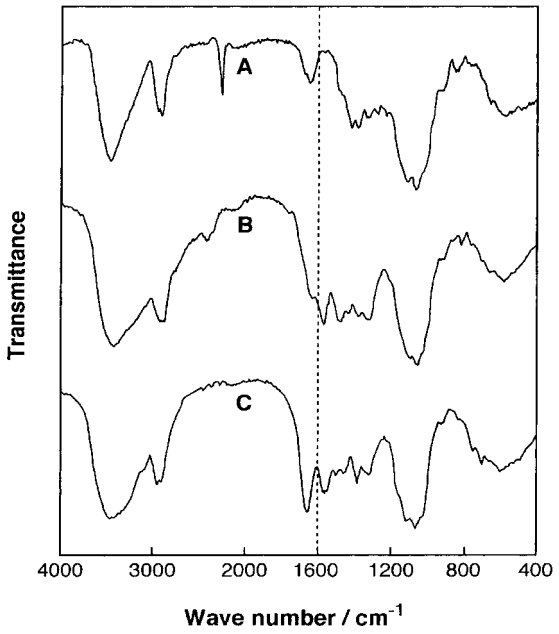

Figure 4. IR spectra of the modified cellulose membranes. A, CEC membrane; B, APC membrane; C, APC-Phe membrane. The substitution degree of the APC-Phe membrane was 0.35.

Fig. 7. BSA and BγG molecules are expected to become compact around these isoelectric points because the intramolecular electrostatic repulsion is small. Considering the result that the amounts of BSA and BγG adsorbed on the APC-Phe membrane did not decrease even at higher pH, the hydrophobic interaction and/or hydrogen bonding between *N*-acetylphenylalanyl or (*N*-acetylphenylalanyl)aminopropyl groups of the immobilized ligands and protein molecules are conceivable.

Since the amount of BSA adsorbed on the APC-Phe membrane is higher than that of BγG likely due to the hydrophobic pocket of BSA, the adsorption and recovery of BSA using the APC-Phe membrane was investigated in detail. The adsorption pH was fixed at pH 7.4, which is the physiologically significant pH. Figure 8 shows the dependence of the amount of adsorbed BSA on the substitution degree of the APC-Phe membrane. The degree of substitution could be varied from 0 to 0.4 by changing the amount of *N*-Ac-L-Phe in the reaction mixture. The substitution degree of 0 means the APC membrane, and the APC membrane also adsorbed only 1.16 mg of BSA. Of course, the original

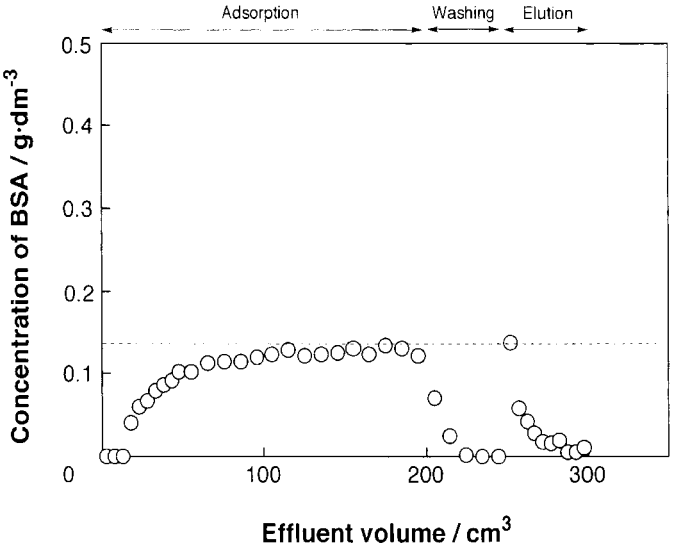

Figure 5. Typical process of adsorption and desorption of BSA in the dead-end flow mode. Broken line represents feed concentration of BSA.

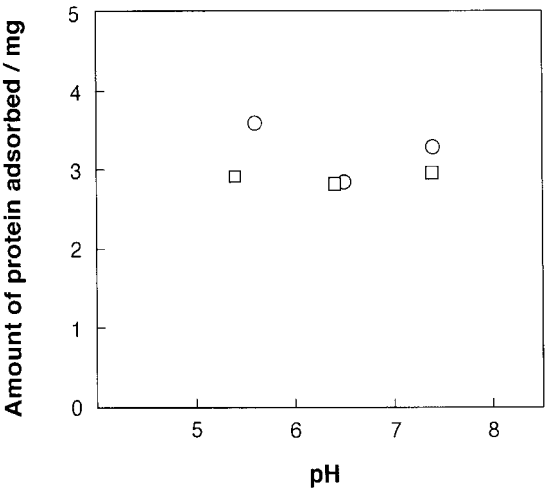

Figure 6. pH dependence of amounts of BSA and BγG adsorbed on the APC-Phe membrane. ○, BSA; □, BγG. Experimental data are an average of two measurements.

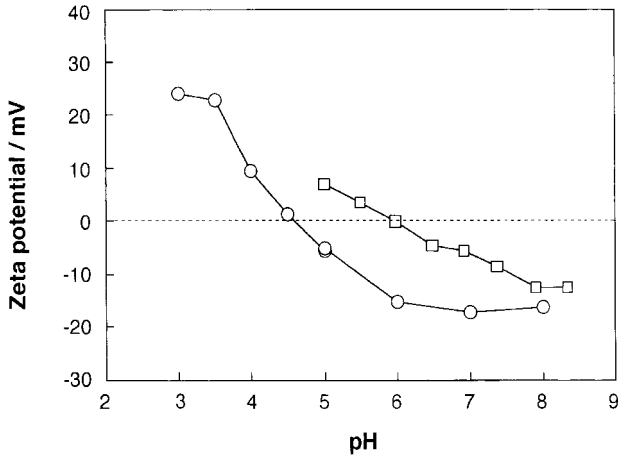

Figure 7. pH dependence of zeta potentials of BSA and BγG. ○, BSA; □, BγG.

cellulose membrane adsorbed no detectable amount of BSA, that is, the cellulose matrix does not show nonspecific adsorption of BSA. As compared with the parent APC membrane, a larger amount of BSA was obviously adsorbed on the APC-Phe membrane; the higher the substitution degree,

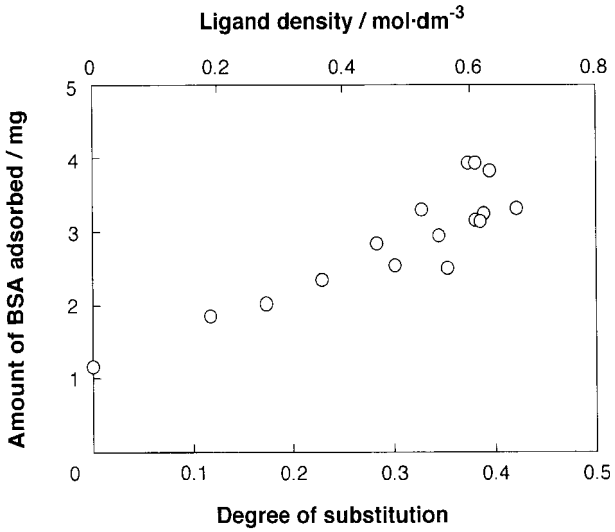

Figure 8. Dependence of adsorption amount of BSA on the substitution degree.

the greater the adsorption amount of BSA. Nevertheless, the value measured for the APC membrane cannot be considered as a background for the APC-Phe membrane, because the original binding sites of the APC membrane could be masked by *N*-Ac-L-Phe ligands.

The recoveries and concentration factors, C_{max}/C_0, (C_{max}, maximum concentration in effluent; C_0, feed concentration) of BSA adsorbed on the APC-Phe membrane using different eluents are summarized in Table 1. With 1 mol/dm^3 NaCl solution, BSA adsorbed on the APC-Phe membrane was not effectively recovered. In addition, the recovery of BSA with 0.02 mol/dm^3 citrate buffer solutions was independent of pH, probably because the APC-Phe membrane has no dissociable group as described previously. The concentrated 0.2 mol/dm^3 citrate buffer, however, improved the recovery of BSA. On the APC-Phe membrane, electrostatic interaction seems not to contribute to the adsorption and desorption of BSA. It is known that hydrophilic organic solvents with a low dielectric constant break the structure of water and reduce the hydrophobic interaction. Accordingly, aqueous solution of such a hydrophilic organic solvent was also used as eluent. Twenty percent ethylene glycol, however, could not increase the recovery of BSA at all. Introduction of a hydrophobic group at a high density onto agarose-based bead induces a lower elution percentage of the protein after adsorption and elution cycle. It was reported that the elution percentage of BSA from the PE-based membranes decreased with increasing hydrophobic ligand density.[12] Therefore, the density of the hydrophobic group is a very important factor

Table 1. Recovery of BSA adsorbed on the APC-Phe membrane.

Eluent	$\frac{C_{max}}{C_0}$	Recovery (%)
1 mol/dm^3 NaCl	0.93	32.9
3 mol/dm^3 NaCl	0.32	16.2
0.02 mol/dm^3 Citrate buffer (pH 5.6)	0.66	49.8
0.02 mol/dm^3 Citrate buffer (pH 2.3)	1.30	54.2
0.2 mol/dm^3 Citrate buffer (pH 2.3)	1.71	71.4
0.2 mol/dm^3 Tartaric acid (pH 1.72)	2.50	86.8
0.2 mol/dm^3 Malic acid (pH 2.08)	2.69	96.2
0.2 mol/dm^3 Succinic acid (pH 2.33)	2.11	84.1
0.2 mol/dm^3 Glycolic acid (pH 2.16)	2.03	83.0
20% Ethylene glycol	0.10	14.0
50% Ethylene glycol containing 1 mol/dm^3 NaCl	0.67	73.0

Adsorption pH was 7.4 and eluent volume was 50 cm^3.
C_{max} is the maximum concentration in effluent. C_0 is the feed concentration.

for achieving a higher elution percentage of the proteins. These results suggest that hydrophobic interaction is the greater contribution to the adsorption of BSA on the APC-Phe membrane. BSA strongly interacts with the *N*-Ac-L-Phe ligands, requiring strong elution conditions for dissociation of BSA from the APC-Phe membrane. Several possibilities exist for improving the desorption of proteins: optimization of the buffer composition or addition of the particular substance or cofactor. The recovery of BSA with aqueous ethylene glycol was increased by the addition of NaCl.[8] NaCl is thought to affect the conformation of BSA. In concentrated salt solutions, electrostatic interactions between proteins are screened and the protein–protein pair potential does not depend on the net charge of the protein. Among the eluents used in this part, aqueous solution of hydroxycarboxylic acids, such as tartaric acid, malic acid, succinic acid, and glycolic acid, are quite effective for recovery of BSA. Malic acid especially recovered the greatest amount of BSA and attained the highest concentration factor. It appears that hydroxyl and carboxyl groups of hydroxycarboxylic acids take part in the elution of BSA adsorbed on the APC-Phe membrane. Further investigation should be made into this point.

CONCLUSION

The porous cellulosic affinity membrane modified with *N*-Ac-L-Phe, the APC-Phe membrane, could be prepared. The amount of BSA and BγG adsorbed on the APC-Phe membrane was rather independent of pH. It seems, therefore, that these serum proteins were hydrophobically adsorbed on the APC-Phe membrane through *N*-acetylphenylalanyl or (*N*-acetylphenylalanyl)-aminopropyl groups of the immobilized ligands. Moreover, the amount of BSA adsorbed on the APC-Phe membrane was greater than that of BγG. The adsorbed BSA could be eluted effectively with hydroxycarboxylic acid solutions. From these, it can be expected that the cellulosic affinity membrane modified with *N*-Ac-L-Phe is applicable for the separation and purification or, at least, preceding separation and purification, of serum proteins using group-specific physicochemical affinity.

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