Influence of preparation conditions on properties of chemical modified nylon affinity membrane (used for γ-globulin adsorption)

GAN, Hong-Yu*(甘宏宇) SHANG, Zhen-Hua(商振华) WANG, Jun-De(王俊德)
LIU, Xue-Liang(刘学良)
Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, Liaoning 116012, China

Microporous polyamide membrane (Nylon 6, PA) was modified by acid hydrolysis and then coupled with hydroxyethyl cellulose (HEC) to amplify reactive groups and reduce nonspecific interaction with proteins. 1,6-Diaminohexane (DAH) as space arm and phenylalanine (Phe) as ligand were immobilized onto nylon membrane through the activation of s-triazine trichloride ($CyCl_3$). The affinity membrane obtained can be used to adsorb γ-globulin specifically. The optimum conditions of preparing affinity membrane and adsorbing bovine γ-globulin were studied.

Keywords Preparation, property, affinity chromatography, nylon membrane, γ-globulin

Introduction

Membrane affinity separation has the advantages of large surface area, short diffusion path and low pressure drop and low ratio of denaturation of biomolecule in separation process.[1] Ideally, membrane matrix used for protein separation must fulfill the following conditions: high hydrophilicity and low nonspecific protein adsorption; a narrow pore size distribution; chemical and mechanical resistance as well as enough reactive groups.[2] Nylon membrane was used in this paper for its good mechanical rigidity. However, nylon membrane has low concentration of primary amino group leading to low ligand density, and the nonspecific adsorption of protein.[3]

In this work nylon membrane was modified by being hydrolyzed firstly to amplify primary amino groups and coated with hydroethyl cellulose (HEC) secondly to increase the active sites and reduce nonspecific adsorption. In the process of preparing affinity membrane s-triazine trichloride ($CyCl_3$) was the activator, 1, 6-diaminohexane (DAH) was the space arm and phenylalanine (Phe) was the ligand. The optimum conditions of binding of HEC, DAH and Phe and adsorption of bovine γ-globulin (BGG) were studied in this work. The affinity membrane prepared according to the optimum conditions was set in a membrane separator designed for preparative purification of γ-globulin.[4]

Experimental

Optimizing the conditions of synthesis of affinity membrane, Fig. 1 shows the procedure of preparing affinity membrane.

Membrane was hydrolyzed in 1 mol/L HCl at room temperature for 24—72 h. The density of terminal amino groups was assayed by ninhydrin method.[5] After hydrolysis nylon membrane was activated with $CyCl_3$ according to a procedure described by Smith III *et al.* with some modification.[6] To determine the optimum condition of binding HEC, activated membranes was shaken in 2% (*W*%) HEC at 19—55℃ (pH 6—9 adjusted by Na_2CO_3). The amount of HEC bound was assayed by phenol-sulfuric acid method and glucose was used as the standard.[7] To determine the optimum conditions of binding DAH and Phe, only activated nylon membrane (not HEC-bound membrane) was used. The activated nylon

* Received October 20, 1999; accepted March 2, 2000.
Project (No. 29675026) supported by the National Natural Science Foundation of China.

membrane was shaken in 1—3% (*W*%) DAH solution at 15—55℃ for 2—16 h. The amount of DAH bound was assayed by ninhydrin method. To determine the optimum condition of binding Phe the activated nylon membrane was shaken in Phe solution with different concentrations at 45℃ for at least 10 h. Ligand density was determined by measuring the difference of the amount of Phe in solution before and after reaction through ninhydrin method. According to the optimum conditions, affinity membrane was prepared. At the end the affinity membrane was shaken in 1 mol/L 2-mercaptoethanol (pH 6—7 adjusted by Na_2CO_3) for 10 h at 45℃ to block residual chloride atoms. Then the membrane was washed with sufficient amount of water and dried *in vacuo*.

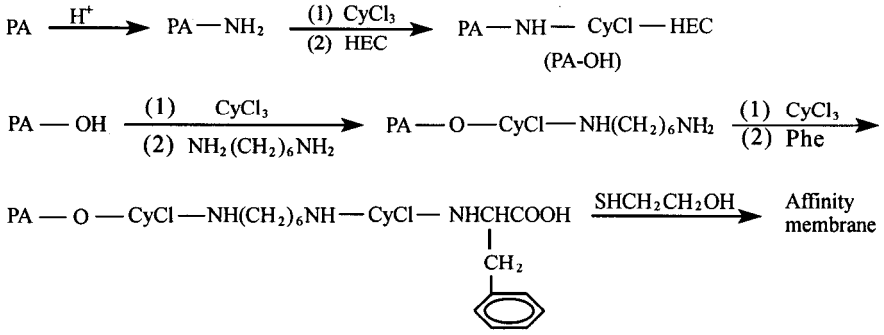

Fig. 1 Procedure for preparing nylon affinity membrane.

Optimizing the conditions of affinity adsorption of BGG

The amount of 0.04 g HEC-binding or affinity membrane was equilibrated with phosphate buffer of different pHs or ionic strength for 10 min. Then the membrane was shaken in 4 mL of 0.2—0.3 mg/mL BGG dissolved in the equilibration buffer for 24 h at room temperature. The amount of protein adsorbed on membrane was determined by A_{280}.

Dynamic adsorption of BGG

Two affinity membrane sheets (47 mm, diameter) were set into a membrane separator. The procedure of affinity purification is equilibration, adsorption, washing, elution and regeneration. The stack was equilibrated with phosphate buffer (pH 7.4, $I = 0.18$) firstly. Then BGG solution was fed. After adsorption the stack was washed with equilibration buffer till A_{280} is less than 0.02. The protein adsorbed was eluted with a mixture of 1 mol/L sodium chloride solution and ethylene glycol (1 :1, *V*: *V*). Then the stack was washed with bidistilled water and equilibrated with buffer again. All solutions were made with bidistilled water and filtered.

Results and discussion

Acid hydrolysis

Increase of terminal amino group on nylon membrane with reaction time was shown in Table 1. The number of terminal amino group on the membrane increased from 10.5 to 24.4 μmol/g after hydrolyzing for 72 h. When the amount of amino group exceeded 27 μmol/g, mechanical integrity of the membrane would be destroyed according to our observation.

Table 1 Effect of reaction time on the amount of terminal amino groups on nylon membrane

Reaction time (h)	Terminal amino groups (μmol/g)
0	10.5
24	12.5
48	18.2
72	24.4

Optimum condition of binding HEC

HEC was bound to nylon membrane after activation by $CyCl_3$. Effects of pH, reaction time and temperature on the amount of HEC bound were shown in Table 2.

Table 2 Binding HEC to activated nylon membrane under various conditions

pH	Amount of binding (mg glucose/g membrane)	Reaction time (h)	Amount of binding (mg glucose/g membrane)	Temperature (℃)	Amount of binding (mg glucose/g membrane)
5.0	5.71	2	6.09	25	4.45
6.7	5.79	4	6.09	35	5.17
7.3	5.63	6	5.31	45	6.11
8.1	5.65	10	4.56	55	6.08

* Experimental condition of binding HEC: effect of pH: 2% HEC, 25—35℃, 4 h; effect of reaction time: 2% HEC, 45℃, pH 7.9; effect of temperature: 2% HEC, pH 6.7, 7 h.

Table 2 shows that pH has little effect on the amount of HEC-binding between pH 5 and pH 8. The amount of HEC bound on the membrane decreased with the increase of reaction time and the reason is unknown. It can also be seen from Table 2 also that increase in temperature is good to the HEC-binding. Generally substituting of the second chloride atom of $CyCl_3$ needs temperature of about 45—60℃. Considering the mechanical strength of nylon membrane, binding of HEC at 45℃ is suitable. The optimum condition of binding HEC is pH 6—7, 45℃, 2—4 h and 2% HEC. And at this condition the amount of HEC bound on the membrane is 8 mg glucose/g.

Optimum condition of binding DAH

Because Phe is a small molecule, while γ-globulin is a large biomelocule, space arm is needed to reduce resistance and increase adsorption capacity. DAH was used as space arm in this work. Effects of concentration of DAH, reaction time and temperature on amount of DAH bound on the activated nylon membrane were shown in Table 3.

Table 3 Binding DAH to activated nylon membrane under various conditions

Concentration of DAH (W%)	Terminal amino group (μmol/g)	Temperature (℃)	Terminal amino group (μmol/g)	Reaction time (h)	Terminal amino group (μmol/g)
1	20.2	15	23.9	2	13.7
2	13.4	45	25.8	4	21.5
3	16.5	55	23.4	16	23.5

* Experimental conditions of binding DAH: effect of concentration of DAH: 25℃, 2 h; effect of temperature: 2% DAH, 3.5 h; effect of reaction time: 2% DAH, 15℃.

It can be seen from Table 3 that the amount of DAH bound on membrane decreased with the increase of concentration of DAH, because DAH is basic and the possibility of hydrating of chloride atoms of $CyCl_3$ increased with the increase of DAH concentration. According to the amount of DAH bound it can be known that nearly all reactive chloride atoms of $-CyCl_2$ on the membrane can react with DAH when the concentration of DAH is 1%. Table 3 shows that the suitable temperature is 45℃ and the reaction time should be over 4 h. The optimum condition of binding DAH is in 1% DAH solution at 45℃, for at least 4 h.

Optimum condition of binding Phe

Phe as a hydrophobic amino acid can be used to purify γ-globulin specifically. Immobilizing Phe on activated nylon membrane is affected by many factors, such as concentration of Phe and buffer, reaction time and pH of buffer. The results were shown in Table 4.

It can be seen from Table 4 that the ligand density increased with the increase of concentration of Phe. Thus > 3.6 mg/mL Phe can be used according to experimental need. Table 4 shows that the optimum pH of buffer is 5.8 (the isoelectric point of Phe). When pH

was lower than 5.8 – NH_2 on Phe would be NH_3^+, which was not good to nucleophilic substitution. It can also be seen from Table 4 that ligand density increased with the decrease of phosphate concentration in the buffer. A reasonable explanation is that increase of concentration of buffer inhibited cleavage of carboxyl group and the charge density on nitrogen decreased, which was not good to nucleophilic substitution too. But for certain buffer capacity buffer with concentration lower than 0.03 mol/L is not suitable. The optimum procedure of binding Phe is dissolving Phe in 0.03—0.05 mol/L pH 5.8 phosphate buffer and reacting at 45℃. And for complete reaction overnight can be chosen to bind ligand. Affinity membrane was prepared according to the optimum condition. The ligand density of 102 μmol/g on membrane can be achieved when concentration of Phe was 6 mg/mL.

Table 4 Binding Phe to activated nylon membranes under various conditions

Concentration of Phe (mg/mL)	Ligand density (μmol/g)	pH	Ligand density (μmol/g)	Concentration of buffer (mol/L)	Ligand density (μmol/g)
0.2	13.5	5.8	81.1	0.03	83.0
0.54	20.4	6.5	66.6	0.1	65.5
0.93	29.1	7.0	73.8	0.15	25.8
3.65	75.3	7.5	48.2	0.2	40.8
		8.0	28.1		

* Experimental conditions of binding Phe: effect of concentration of Phe: 0.04 mol/L pH 5.8 phosphate buffer, 45℃, 7 h; effect of pH of buffer: 1 mg/mL Phe, 0.05 mol/L phosphate buffer, 45℃, 7 h; effect of concentration of buffer: 1 mg/mL Phe, pH 5.8 phosphate buffer, 45℃, 7 h.

Effect of ionic strength on adsorption

Fig. 2 shows the effects of ionic strength on BGG adsorption on affinity membrane and HEC-bound membrane. It can be seen from Fig. 2 that the adsorption capacity of affinity membrane increased with the decrease of ionic strength, but at the same time the adsorption on matrix increased also. The optimum ionic strength is 0.18, at this point the affinity adsorption is maximum and the nonspecific adsorption caused by matrix is undetermined. That adsorption increased with the decrease of ionic strength shows that ionic bond plays an important role in adsorption of BGG.

Effect of pH *of buffer on affinity adsorption*

It can be seen from Fig. 3 that the optimum pH is 7.2, at this point the affinity adsorption is maximum and the nonspecific adsorption caused by matrix is undetermined. Adsorption increased with the decrease of pH shows that ionic bond plays an important role in adsorption of BGG. The effects of ionic strength and pH on adsorbing BGG show that the optimum condition of adsorbing BGG is pH 7.2 with 0.18 of ionic strength. At this condition, the amount of adsorption is maximum and the nonspecific adsorption caused by modified matrix is undetermined. While at the same condition, nonspecific

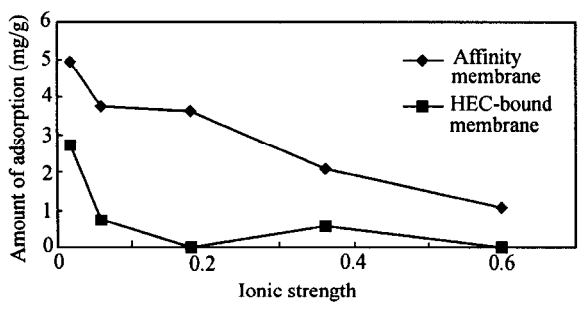

Fig. 2 Effect of ionic strength on affinity adsorption.

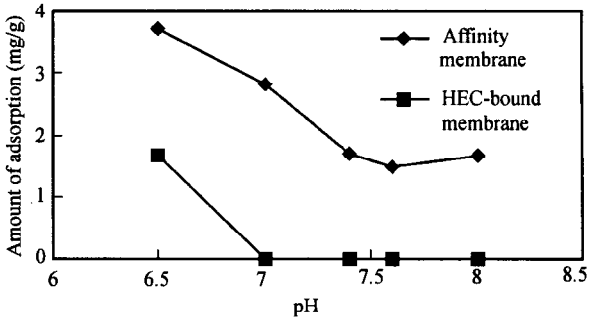

Fig. 3 Effect of pH of buffer on affinity adsorption.

adsorption of original membrane was 5.5 mg/g. It indicates that binding HEC can decrease nonspecific adsorption of nylon membrane effectively. On the other hand that 50% ethylene glycol must be used in eluant shows that there is hydrophobic interaction between Phe and BGG. So both ionic bond and hydrophobic interaction play important role in affinity adsorption of BGG.

Adsorption of BGG *on membrane stack*

Affinity membrane was set into a membrane separator to make a stack. After equilibration of stack with phosphate buffer 0.2 mg/mL BGG was loaded onto the stack and then the stack was washed, eluted. The peak of BGG is thin and hybrid was separated from BGG. A typical chromatography was shown in Fig. 4. The affinity membrane stack can be used for a long time if only the membrane was not fouled.

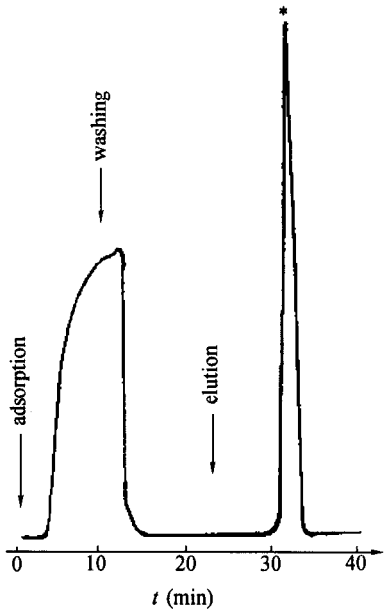

Fig. 4 Affinity chromatography of γ-globulin. * peak of γ-globulin; Stack: 2 × Φ47 mm; Concentration of γ-globulin: 0.18 mg/mL; Rate of adsorption: 2 mL/min; Rate of washing: 10 mL/min; Rate of elution: 1 mL/min; Chart speed: 2 mm/min.

References

1. Charcosset, C., *J. Chem. Technol.*, **71**, 95(1998).
2. Suen, S.Y.; Etzel, M.R., *Chem. Eng. Sci.*, **47**, 1355 (1992).
3. Turkova, J., *J. Chromatog. Library* (*Affinity Chromatography*), Elsevier Science Publishers B. V., Netherlands, 1993, p.55.
4. Guo, W.; Shang, Z. H.; Yu, Y. N.; Zhou, L. M., *Biomed. Chromatog.*, **11**, 164(1997).
5. Kugel, K.; Moseley, A.; Harding, G. B.; Klein, E., *J. Membr. Sci.*, **74**, 115(1992).
6. Smith III, N. L.; Lenhoff, H. M., *Anal. Biochem.*, **61**, 392(1994).
7. Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F., *Anal. Chem.*, **28**, 350(1956).

(E9910139 SONG, J.P.; LING, J.)