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Short communication

Membrane affinity chromatography of alkaline phosphatase

Wei Guo, Zhenhua Shang, Yinian Yu, Liangmo Zhou*

Dalian Institute of Chemical Physics, Academia Sinica, P.O. Box 100, Dalian 116012, China

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Abstract

A method for the preparation of a microporous cellulose membrane is described. The triazine dyes Cibacron Blue F3GA and Active Red K2BP were immobilized as affinity ligands. Up to 90 mg of Active Red K2BP can be coupled to 1 g of membrane matrix. A membrane cartridge containing red affinity membranes was also prepared. The flux of the cartridge containing 80 sheets of membrane can reach 5.0 ml/min with a pressure drop of 0.1 MPa. On this cartridge, the chromatography of alkaline phosphatase was performed with a 60% recovery of activity and a 40-fold purification.

1. Introduction

Affinity chromatography is a unique method in separation technology as it is the only technique that permits the purification of biomolecules based on biological functions rather than individual physical or chemical properties. The high specificity of affinity chromatography is due to the strong interaction between the ligand and biomolecule of interest. On the other hand, because of the strong interaction, elution of a biomolecule may be very difficult and time consuming, which may seriously lower the activity recovery of samples. To solve this problem, membrane affinity chromatography (MAC) was introduced several years ago [1,2].

Membrane separation allows the processing of a large amount of sample in a relatively short time owing to its structure, which provides a system with rapid reaction kinetics [3]. The integration of membrane and affinity chromatography provides a number of advantages over normal affinity chromatography using columns, especially with regard to time and activity recovery [4,5].

There are two technical problems that have hampered further increases in the use of MAC, the distribution of the sample or mobile phase and the sealing of the bundling system. The former problem was solved by Josic et al. [6] using simple distribution plates but the latter was not so easy, especially because when using many individual membranes as the stationary phase the bundling of thin membranes often leads to leakages in the system, which in turn causes the mobile phase and the sample to flow beyond the edges and therefore past the membranes. One solution to this problem is to add a sealing ring between every two individual membranes, as done by Millipore (Tokyo, Japan) in their commercial membrane cartridge product.

In this work, an affinity membrane medium was prepared from chemically cross-linked cellulose film. The membrane obtained was more

* Corresponding author.

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chemically and mechanically stable than the original cellulose microfiltration membrane, and had a large pore size and high porosity. The triazine dyes Cibacron Blue F3GA and Active Red K2BP were immobilized as affinity ligands. Using a special sealing technique, 80 sheets of cellulose membrane were bonded together. With the addition of two distribution plates, an affinity membrane cartridge was also prepared. The flux of the membrane cartridge can reach 5.0 ml/min with a pressure drop of 0.1 MPa. The membrane cartridge with red dye affinity ligand was used for the purification of alkaline phosphatase.

2. Experimental

2.1. Materials

Quantitative filter-paper Xinhua No. 1 was purchased from Hangzhou Xinhua Paper Manufactory (Hangzhou, China), Sepharose 4B from Pharmacia (Uppsala, Sweden), epoxy propane chloride of analytical-reagent grade from Tianjin Chemical Plant (Tianjin, China), Cibacron Blue F3GA and Active Red K2BP from Serva (Heidelberg, Germany) and Shanghai Eighth Chemical Dyestuff Factory (Shanghai, China), respectively, and alkaline phosphatase and its substrate, *p*-nitrophenyl phosphate, from Dongfeng Biochemical (Shanghai, China).

2.2. Apparatus

The experiments were performed on a Bio-Rad (Richmond, CA, USA) automated Econo System which consists of a Model EP-1 Econo Pump, a Model EM-1 Econo UV monitor with portable optics module equipped with two interchangeable filters (254 and 280 nm), a Model 2110 fraction collector and a Model ES-1 Econo system controller. Chromatograms were recorded and processed on a Hewlett-Packard Model 3394A integrator. The loading of sample on the affinity cartridge was carried out with a Gilson (Middleton, WI, USA) Minipuls 3 peristaltic pump in a refrigerator (3–7°C). The C, H and N contents of the dye-immobilized affinity membrane were determined on a Carla Erla (Milan, Italy) Model 1106 elemental analyser.

2.3. Preparation of cellulose film

An 80-g filter-paper was dispersed in 1000 ml of aqueous solution containing 50 g of NaOH and 5 g of $NaBH_4$ and the mixture was heated and kept boiling until a uniform pulp was achieved. Then the pulp was cast on a glass plate and frozen at −30°C in a freezer for 45 min. After thawing at room temperature, the plate was immersed in 10% HCl for 1 h. The cellulose film was then removed and rinsed with water until neutral. After dipping in acetone for 2.5 h, the cellulose film was dried in air.

2.4. Preparation of affinity membrane

A 12-g amount of NaOH was dissolved in 50 ml of water and 100 ml of dimethyl sulphoxide, to which 20 ml of epoxy propane chloride and 0.5 g of $NaBH_4$ were added. At room temperature, 40 sheets of cellulose films with a diameter of 40 mm were soaked in the above solution individually, then gently shaken for 20 min. The films were then removed, 4 g of NaOH and 10 ml of epoxypropane chloride were added to the remaining solution and the films were soaked individually again in the solution. The solution was heated to 50°C and stirred every 10 min for 3 h, then the cross-linked membranes were washed with hot distilled water until neutral. The membranes were dispersed in 10% ammonia solution for 24 h at room temperature, washed thoroughly to remove residual ammonia and finally soaked in a solution of 2.0 g of Active Red K2BP, 380 ml of water and 20 ml of 15% Na_2CO_3. The temperature was held at 50°C for 20 h with stirring every 10 min. Finally, the red membranes were washed with distilled water until the absorbance at 520 nm of the eluate was zero.

2.5. Methods

The activity of the enzyme was determined with a *p*-nitrophenyl phosphate substrate as described by Schlesinger and Barret [7] and the protein content was determined by the Coomassie Blue method described by Gogstad and Grossberg [8]. The pore size of the prepared cellulose membrane was determined by the method Yasuda and Tsai [9].

3. Results and discussion

One of the most important factors in affinity chromatography is the development of solid supports. In membrane affinity chromatography, the selection of the membrane material and the preparation of the membrane are dominant factors affecting chromatographic performance. We chose cellulose as the best material as it has long been used in membrane preparation and it is a good matrix for affinity coupling. However, the commercially available cellulose derivatives membranes, which usually are produced by a solution casting method, have a relatively low porosity and are not suitable for chromatography owing to the high pressure drop through the bundling of membranes. Therefore, in this work, a new type cellulose microfiltration membrane prepared in our laboratory was used as a membrane matrix. This membrane, consisting of coarse fibres, has a higher porosity and larger pore size (1–2 μm) than ordinary cellulose microfiltration membranes, but is less uniform in structure. To enhance the mechanical and chemical stabilities, cross-linking by epoxypropane chloride was carried out prior to immobilization of triazine dyes.

Sample distribution is one of the main problems, especially when wide but thin membrane cartridges are used in separation. Josic et al. [6] overcame this problem by installing distribution plates before and behind the membrane. They compared over twenty patterns of plates. In this work, distribution plates were prepared according to the optimum conditions described by Josic.

The other main problem in the preparation of membrane cartridges, the sealing of the system, which becomes more serious when the bundling of many single membranes is adopted, was solved with greater effort. In this work, a special bonding method was performed. The circumference with a width of 1 mm on the edge of each individual membrane was saturated with an elastic glue. After removing the excess glue, up to 80 sheets of membrane can be packed and bonded together with mild pressure. The structure of the cartridges prepared using this method was better with regard to both sealing and separation efficiency and, because that the membrane consisted of coarse fibres and had a large pore size and high porosity, the pressure across the cartridge during chromatography can be relatively low. The construction of the membrane cartridge used in the experiment is shown in Fig. 1. The flux through a membrane cartridge containing 80 sheets of membrane is shown in Fig. 2.

For cellulose membrane cross-linking, 2.4 *M* NaOH was used. This concentration exceeded that necessary for the cross-linking only. A high concentration alkali in the cross-linking increased the amount and activity of the hydroxyl groups in the cellulose membrane obtained. In this experiment, two kinds of cellulose membranes was prepared using different cross-linking conditions: one (alkali-treated cellulose mem-

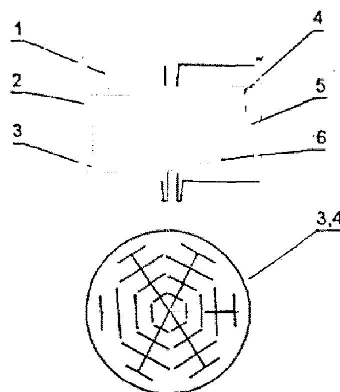

Fig. 1. Construction of the membrane cartridge for membrane affinity chromatography. The diameter of the membranes used was 40 mm. 1 = Rubber cover; 2 = glass holder; 3, 4 = distribution plates; 5 = bonding part; 6 = affinity membranes.

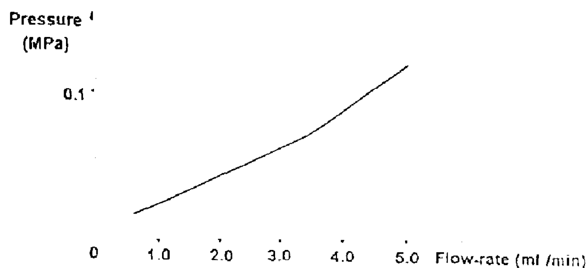

Fig. 2. Flux of membrane cartridge (18 × 40 mm I.D., containing 80 sheets of cellulose membrane).

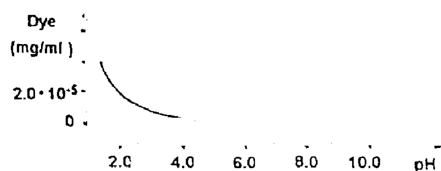

Fig. 3. Ligand leakage of membrane affinity media. 18 × 40 mm I.D. cartridge, containing 80 sheets of Active Red K2BP-immobilized cellulose membrane. Mobile phase, 0.5 *M* NaCl; flow-rate, 0.5 ml/min.

brane) was cross-linked in 2.4 *M* NaOH and the other (initial cellulose membrane) was cross-linked in 0.4 *M* NaOH. Based on elemental analysis, the capacities of these two cellulose membranes for the immobilization of triazine dyes were compared (Table 1).

A major advantage of the triazine dyes as affinity ligands is their stability. Triazine linkages are less prone to ligand leakage than cyanogen bromide-activated polysaccharides [10]. The membrane cartridge prepared in this experiment with Active Red K2BP as ligand was eluted with a pH gradient from pH 1 to 12, and the absorbance of the eluate was determined (Fig. 3). The results showed that when the pH of the eluent was higher than 3.5, no dye released from the stationary phase was observed in the eluate.

Triazine dyes can serve as analogues for nucleotides so that a protein will bind them at nucleotide-binding sites. They have been used successfully in the purification of many enzymes, especially kinases and dehydrogenases. The

Table 1
Effect of alkali treatment of the cellulose membrane on its capacity for the immobilization of triazine dyes

Dye	Capacity (mg/g)	
	Original cellulose membrane	Alkali-treated cellulose membrane
Cibacron Blue F3GA	20	100
Active Red K2BP	39	90

phosphatase can be purified on a dye-immobilized medium. In this work, commercial alkaline phosphatase from calf intestine was purified on an affinity membrane cartridge containing 80 sheets of red cellulose membrane (Fig. 4). The phosphatase was eluted with 1 *M* NaCl, and an unknown component with a stronger affinity to the ligand was eluted with 60% ethylene glycol. The results of recovery experiments are given in Table 2. The recovery of alkaline phosphatase

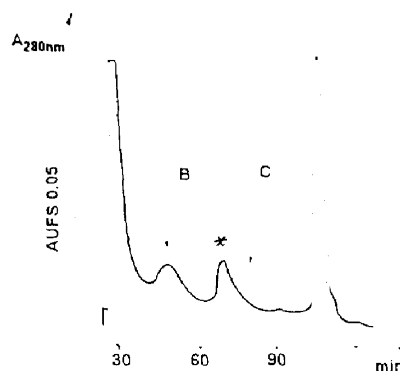

Fig. 4. Membrane affinity chromatography of alkaline phosphatase on Active Red K2BP-immobilized cellulose membranes. A 10.3-mg amount of alkaline phosphatase was dissolved in 2.0 ml of 0.1 *M* NaCl–0.1 *M* NaOAc–HCl buffer (pH 7.5) and was applied to a 18 × 40 mm I.D. cartridge, containing 80 sheets of membrane, at a flow rate of 0.1 ml/min at 4°C. The cartridge, at room temperature, was first equilibrated with 1.0 ml of 0.1 *M* NaCl–0.1 *M* NaOAc–HCl buffer (pH 7.2) (solution A), then the alkaline phosphatase and other impurities were eluted with 1.0 *M* NaCl–0.1 *M* NaOAc–HCl buffer (pH 7.0) (solution B) and 1.0 *M* NaCl–0.1 *M* NaOAc–HCl buffer (pH 7.0) containing 60% ethylene glycol (solution C), respectively. The flow-rate of mobile phase was 0.8 ml/min. The asterisk indicates alkaline phosphatase.

Table 2
Purification of alkaline phosphatase by membrane affinity chromatography

Enzyme	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Enrichment (-fold)	Yield (%)
Initial sample	2.0	10.3	10 800	1050		
Product of MAC	8.0	0.15	6480	$4.2 \cdot 10^4$	40	60

activity was 60% and a 40-fold purification was achieved.

Acknowledgements

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