Chitosan-based membrane chromatography for protein adsorption and separation

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**A B S T R A C T**

A chitosan-based membrane chromatography was set up by using natural chitosan/carboxymethylchitosan (CS/CMCS) blend membrane as the matrix. The dynamic adsorption property for protein (lysozyme as model protein) was detailed discussed with the change in pore size of the membrane, the flow rate and the initial concentration of the feed solution, and the layer of membrane in membrane stack. The best dynamic adsorption capacity of lysozyme on the CS/CMCS membrane chromatography was found to be 15.3 mg/mL under the optimal flow conditions. Moreover, the CS/CMCS membrane chromatography exhibited good repeatability and reusability with the desorption efficiency of ~90%. As an application, lysozyme and ovalbumin were successfully separated from their binary mixture through the CS/CMCS membrane chromatography. This implies that such a natural chitosan-based membrane chromatography may have great potential on the bioseparation field in the future.

1. Introduction

With the development of biotechnology, the bioseparation techniques with adsorptive membranes become more and more important due to their efficiency in overcoming mass transfer limitation [1]. The advantage of membrane chromatography lies in the predominance of convective material transport [2] because the membrane adsorber acts as a short and wide chromatographic column [3]. Membrane chromatography is widely employed for purification and recovery of biomolecules such as polypeptides, proteins, nucleic acids, polynucleotides, and so on [4–6].

Ion exchange membrane, a widely used membrane adsorber, has attracted many interests because of its broad applicability, high resolution, and large adsorption capacity in large-scale protein purification processes [7]. It can separate the proteins with similar molecular weights but different charge conditions easily. For instance, Zeng and Ruckenstein [8] used macroporous anion-exchange chitosan membranes with controlled pore sizes to separate protein mixtures. They selected five proteins and the results showed that all these five proteins were recovered efficiently (91–98%). Lin et al. [9] employed both cellulose phosphate cation-exchange membrane and diethylaminoethyl cellulose anion-exchange membrane to separate lysozyme, bovine serum albumin, and γ-globulin. Avramescu et al. [10] also successfully separated bovine serum albumin and bovine hemoglobin through a novel ion-exchange membrane, which was prepared by the incorporation of various types of Lewatit ion-exchange resins into an ethylene–vinyl alcohol copolymer porous matrix.

The natural polymer-based ion-exchange membranes are obvious benefit for bioseparation because of their good biocompatibility. However, most of the ion-exchange membranes reported are from either synthetic polymers or synthetic/natural polymer blends/composites [11–14], and only few are from natural polymers, as mentioned above [9,15].

Chitosan (CS), derived from chitin that is the main structural component of the invertebrate exoskeleton and the fungal cell wall, is an abundant natural polymeric resource [16]. It is considered as an attractive material that can be potentially used in biomedical fields because of its favorable physicochemical and biological properties, such as good biocompatibility, non-toxicity, and antibacterial property [17,18]. In our previous research, we developed two natural polymer ion-exchange membranes, i.e., chitosan/carboxymethyl cellulose (CS/CMC) and chitosan/carboxymethyl chitosan (CS/CMCS) blend membrane [15,19–23]. We have performed the characterizations of those two materials by FTIR spectroscopy and X-ray diffraction, and also tested their mechanical properties as well as swelling ratios in different pH buffer solutions [22,23]. In the meantime, we have fully studied the adsorption properties of two model protein, lysozyme and ovalbumin on both blend membranes and successfully separated each protein from their binary mixture in static mode [15,19]. In this article, we reported our trials to use CS/CMCS membrane as a matrix to compose membrane chromatography and discussed the dynamic adsorption and separation properties of such a CS/CMCS membrane chromatography.
2. Experimental

2.1. Materials

CS flake (deacetylation degree = 72%, molecular weight = 850,000) and CS powder (deacetylation degree = 99%, molecular weight = 40,000) were purchased from Jinan Haidebei Marine Biological Product Co., Ltd. (Jinan, China). Sample of lysozyme and ovalbumin (Grade V, minimum purity of 98%) was obtained from Sigma-Aldrich. CS with 72% deacetylation degree was further deacetylated according to the literature [24]. The final deacetylation degree was 92% as determined by titration [25]. CMCS was prepared by the established procedure reported previously [26]. The substitution degree of carboxymethyl groups on CMCS was determined by $^1$H NMR [26,27]. The total carboxymethyl substitution degree was 1.18, where the O-substitution degree was 1.00 and the N-substitution degree was 0.18, indicating most of the amino groups on the original CS molecular chains were preserved [15].

2.2. Preparation of the macroporous CS/CMCS blend membranes

The macroporous CS/CMCS blend membranes were prepared following the method in our previous work [15,19]. In brief, 2 wt% CMCS aqueous solution was added dropwise into 2 wt% CS acetic acid solution under stirring, and then the porogen silica particles and crosslinking agent glutaraldehyde were added. The CMCS content in the final CS/CMCS blend membrane was set to 40 wt% and the crosslinking degree is 3%. In this paper, we used three different sizes of silica particle, thus obtained three different macroporous membranes. After 3 h of stirring, the solution was poured into a poly(ethylene terephthalate) box and allowed to dry. Then, the dried membranes were immersed into 5 wt% NaOH aqueous solution to dissolve the silica and generated the macroporous membranes. Finally, the membranes were further crosslinked under mild alkaline conditions using epichlorohydrin [28]. The pore size and the thickness of the membranes were determined from their SEM photographs taken with TS-5136MM scanning electron microscopy at 20 kV, using the software provided with the equipment. The average pore size was obtained by measuring at least 20 pores. The thickness of the membrane obtained here was used to calculate the volume of the membrane, and further calculate the dynamic adsorption capacity according to Eq. (1) (see below).

2.3. Dynamic adsorption of CS/CMCS membrane chromatography for lysozyme

We utilized the experimental setup similar to Chen and Juang’s [29], but improved the membrane stack holder according to the literature of Ghosh and Wong [1] (Fig. 1). The CS/CMCS blend membranes were cut into 5 cm diameter slice and put into 0.01 mol/L borate buffer solution (pH = 9.2) for 4 h to reach the swelling equilibrium. Then three to five membranes were packed into the stack holder to form membrane stack. The diameter of effective membrane area in the stack holder was 4 cm. Lysozyme solutions with concentration from 0.27–0.75 mg/mL in 0.01 mol/L borate buffer solution were loaded through the stack holder with the flow rate of 2–4 mL/min controlled by a peristaltic pump (DDB-320, Shanghai Zhisun Instrument Co., Ltd.). After ~160 min of lysozyme loading, the membrane stack was washed with fresh 0.01 mol/L borate buffer solution to release the unbounded lysozyme, and then eluted with 0.01 mol/L borate buffer solution containing 1.5 mol/L NaCl to desorb lysozyme [3]. At last, the membrane stack was regenerated with 2 mol/L NaCl solution. The permeate in the adsorption-washing-elution process was collected with a fraction collector and the lysozyme concentration in permeate was measured with a Hitachi UV 2910 UV–vis spectrometer at 280 nm. We chose 0.01 mol/L borate buffer solution in this work because the CS/CMCS blend membrane showed maximum lysozyme adsorption capacity at this pH value (pH = 9.2) in our previous work in batch system [15].

The dynamic binding capacity at 10% breakthrough (defined as when the protein concentration in the permeate is equal to 10% of the feed concentration) of lysozyme was calculated according to the following equation [30]:

$$Q_{{\text{B10}}} = C_p \left( V_p - V_b \right) / V_b$$

(1)

where $Q_{{\text{B10}}}$ is the dynamic binding capacity (mg/mL) at 10% breakthrough, $C_p$ is the concentration of lysozyme loaded (mg/mL), $V_p$ is the volume of lysozyme solution loaded at 10% breakthrough (mL), $V_b$ is the holdup volume of the apparatus (mL), and $V_b$ is the volume of membranes (mL).

The desorption efficiency of lysozyme from the CS/CMCS membrane was calculated according to the following equation:

$$DS = q_e / (q_i - q_e)$$

(2)

where $q_i$ is the lysozyme amount in elution, $q_f$ is the initial lysozyme amount in feed, and $q_e$ is the lysozyme amount that removes from the membrane during the washing process.

2.4. Separation of lysozyme–ovalbumin binary mixture through CS/CMCS membrane chromatography

Three layers of CS/CMCS blend membrane with average pore size of 55 μm were packed as membrane stack. 20 mL feed solution composed of 0.52 mg/mL of lysozyme and 0.52 mg/mL ovalbumin in 0.01 mol/L borate buffer solution was loaded through the membrane stack with the flow rate of 2 mL/min. Then, the membrane stack was washed with fresh 0.01 mol/L borate buffer solution, and eluted with 0.01 mol/L borate buffer solution containing 1.5 mol/L NaCl. HPLC (Waters 600E with BioSuite™ 250 SEC column) was used to examine the composition and concentration of the permeate from the whole separation process.

3. Results and discussion

3.1. Adsorption of lysozyme on the CS/CMCS membrane chromatography

Fig. 2 shows the typical adsorption–washing–elution curve of lysozyme on the CS/CMCS membrane chromatography. At first few
minutes, the lysozyme was totally adsorbed on the membrane, so there was almost no lysozyme found in the permeate. Afterwards, the lysozyme concentration in the permeate increased gradually indicating less and less lysozyme was adsorbed on the membrane during the adsorption process. During the washing process, some nonspecific adsorbed lysozyme was easy to be washed out. At final elution process, the adsorbed lysozyme was quickly desorbed from the membrane.

Dynamic adsorption capacity, which is defined as the amount of protein absorbed in per unit volume of membrane, is always used to evaluate the adsorption property of the membrane chromatography [31]. In this article, the dynamic adsorption capacity at 10% breakthrough was selected for discussion as it seems to be most frequently used by researchers [1].

3.2 Influence factors on the dynamic adsorption behavior of lysozyme on the CS/CMCS membrane chromatography

3.2.1 Effect of the pore size of the membrane
The porogen method was applied to prepare macroporous CS/CMCS blend membrane, so the pore size of membrane was controlled by the silica particle size we used. In this study, three kinds of macroporous CS/CMCS blend membrane were successfully prepared with different pore size by varying silica particle size. The average pore size of three different blend membranes was 45, 55, and 68 μm, respectively.

From the breakthrough curve of lysozyme adsorption, it was found that the medium pore size membrane (55 μm) had the best performance (Fig. 3). The dynamic adsorption capacity of such a membrane chromatography was 8.4 mg/mL, which was higher than either the one with small pore size (45 μm, 7.0 mg/mL) or the one with large pore size (68 μm, 6.6 mg/mL). This is probably because the medium pore size membrane had the suitable effective adsorptive area and contact time to the feed solution.

3.2.2 Effect of the flow rate
The breakthrough curves at three different flow rates, i.e., 2, 4, and 6 mL/min were obtained, in which the obvious difference was found (Fig. 4). Flow distribution is a major concern in chromatography as in most types of separation process [2]. In membrane chromatography, the radial dimension of the adsorptive media is far greater than its axial dimension, so the flow rate plays an important role in adsorption process. It was found that the increase in flow rate caused a dramatic decrease of dynamic adsorption capacity on the CS/CMCS membrane chromatography, namely from 8.4 to 1.2 mg/mL when the flow rate increased from 2 to 6 mL/min. Obviously, increase of the flow rate shortens the contact time between the protein and the membrane, so in our case (large pore size and good convection), the slow flow rate is favorable to achieve a high dynamic adsorption capacity.

3.2.3 Effect of feed concentration
The initiate lysozyme concentration also found to play an important role in the dynamic adsorption on the CS/CMCS membrane chromatography. An increase in initial concentration resulted in the increase of protein adsorption capacity (Fig. 5).
of dynamic adsorption capacity. The high lysozyme concentration in the feed provided more protein that flowed through the membranes if the flow rate was the same. Thus from the breakthrough curves in Fig. 5, the dynamic adsorption capacity of 8.4, 9.0, and 13.4 mg/mL was achieved corresponding to the initiate lysozyme concentration of 0.27, 0.52, and 0.75 mg/mL, respectively.

3.3. Desorption efficiency of lysozyme on the CS/CMCS membrane chromatography

Here 20 mL of 0.52 mg/mL lysozyme solution was used to study the desorption efficiency of the CS/CMCS membrane chromatography. The amount of lysozyme in the feed solution was ensured to be entirely adsorbed according to the previous studies (Fig. 5). From Fig. 7 it was found that almost all of the lysozyme in the feed did adsorb in the membrane as the lysozyme concentration in the permeate was almost zero. In the meantime, the adsorbed lysozyme in the membrane was quite stable that was not washed out during the washing process. The lysozyme was found to be quickly desorbed from the membrane in the elution process. After elution, most of the lysozyme was desorbed and the desorption efficiency was almost 90% in the second and third circle (Table 1). The relative low desorption in the first circle may because the membranes had not reach the equilibrium state entirely. Moreover, the data shown in Table 1 indicated the good repeatability and reusability of such a membrane chromatography.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Adsorption amount of lysozyme (mg)</th>
<th>Desorption amount of lysozyme (mg)</th>
<th>Desorption efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>10.1</td>
<td>8.65</td>
<td>86</td>
</tr>
<tr>
<td>Second</td>
<td>10.0</td>
<td>9.01</td>
<td>90</td>
</tr>
<tr>
<td>Third</td>
<td>10.1</td>
<td>8.95</td>
<td>89</td>
</tr>
</tbody>
</table>

*Lysozyme concentration: 0.52 mg/mL; flow rate: 2 mL/min; pore size: 55 μm; membrane layer: 3.

3.4. Separation of lysozyme-ovalbumin binary mixture through CS/CMCS membrane chromatography

Protein bioseparation is one of the major applications on membrane chromatography, in which the lysozyme and ovalbumin are often selected as model proteins [1]. In this research, lysozyme-ovalbumin binary solution was also prepared as feed solution to test the separation property of the CS/CMCS membrane chromatography. In our previous static study, it has been confirmed that the ovalbumin did not adsorb on CS/CMCS blend membrane while pH = 9.2. Fig. 8 shows the fraction curves of lysozyme and ovalbumin during the separation process. It was found that the ovalbumin passed through the membranes directly except some nonspecific adsorption that was removed easily during the washing process. On the contrary, lysozyme was adsorbed on the membrane tightly from the binary protein solution as from its pure solution (Fig. 7). In the elution process, only lysozyme was found in the permeate. Based on the fact that there was no lysozyme found in the permeate during the adsorption and washing process, and no ovalbumin found in the elution process, we may conclude that the lysozyme and ovalbumin were successfully separated from their binary solution through the CS/CMCS membrane chromatography.

4. Conclusion

Macroporous CS/CMCS blend membrane was successfully used as the matrix to set up a membrane chromatography for protein
adsorption and separation. Lysozyme was selected as the model protein and its dynamic adsorption property on the CS/CMCS membrane chromatography was extensively investigated by varying the pore size of the membrane, the flow rate and the initial concentration of feed solution as well as the layer of membrane in membrane stack. The results showed that the suitable pore size (55 μm), low flow rate (2 mL/min) and high initial lysozyme concentration (0.75 mg/mL) were favorable to achieve a high dynamic adsorption capacity. Although the increase of layer of the membrane also helped to increase the dynamic adsorption capacity, it was not obvious as expected that may be due to the imperfect design of the apparatus. The CS/CMCS membrane chromatography showed good repeatability and reusability with the desorption efficiency of ~90%, and it separated lysozyme and ovalbumin from their binary mixture successfully. All these imply that such a natural chitosan-based membrane chromatography may have great potential on the bioseparation field in the future, for instance separating lysozyme from the egg white.

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References