Operation Modes Can Affect the Activity of Immobilized Enzyme onto Silk Fibroin Nanofibrous Membrane

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Abstract

In the present study, we report that the selection of operation mode is important to take the full advantage of nanofibrous membrane in enzyme immobilization. Silk fibroin nanofibrous membrane has been prepared by electrospinning, and α-chymotrypsin was immobilized as a model enzyme. When the immobilized enzyme was operated in the membrane reactor mode, the Michaelis constant, $K_m$, was lower and the $V_{max}$ was higher compared to the batch reactor mode. No concentration gradient was observed in the membrane reactor mode and the immobilized enzyme was stable even after 7 times of re-use. Our results suggests that the enzyme immobilized nanofibrous membrane should be operated in the membrane reactor mode rather than in the bath reactor mode.

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Introduction

Enzymes have advantages over chemical catalysts because of regio- and site-specificity (Koeller et al., 2001). However, the cost of enzymes was always the major barrier for the industrial applications of enzymes. Enzyme immobilization is one of the techniques to solve the problem. Once the enzyme is immobilized onto an insoluble support, it can be re-used multiple times, which compensates the cost of enzyme. In addition, the immobilized enzyme has increased stability, which enables more flexible operation conditions. However, the inherent catalytic activity of enzyme will be decreased upon immobilization. This could be overcome by increasing the amount of enzyme that immobilized onto the support. Therefore, highly porous support is favored in enzyme immobilization due to the high specific surface area (Zhou et al., 2013; Garcia-Galan et al., 2011).

A nanofibrous membrane prepared by electrospinning has been used in various fields due to its high specific surface area, and it could be an excellent candidate for the enzyme immobilization. There are many studies on the enzyme immobilization onto nanofibrous membrane (Wang et al., 2009). Various materials were utilized as a support including synthetic and natural polymers. Silk protein has been also used as a support of enzyme immobilization. We have previously immobilized α-chymotrypsin (CT) onto electrospun silk fibroin (SF) nanofibrous membrane. The immobilized enzyme had good stability against denaturation (Lee et al., 2005a; 2005b).

In the present study, we immobilized CT onto electropspun SF
Immobilization of CT on SF nanofibrous membrane

In order to immobilize CT on the SF, the amine groups of SF were activated with glutaraldehyde (GA). To one piece of SF nanofibrous membrane, 10% (v/v) GA in 0.2 M sodium carbonate buffer (pH 9.2) was added to activate the SF. The reaction was continued for 1 h at 25°C. The activated SF was washed 2 times with distilled water and 3 times with 0.1 M sodium phosphate buffers (pH 7.4). The activated SF was mounted on the membrane holder and an ice-cold buffer solution of CT (1 mg/mL) in 0.1 M sodium phosphate buffer (pH 7.4) was circulated overnight at 4°C. The unbound CT was washed out 20 mL of ice-cold 0.5 M of NaCl in 0.1 M sodium phosphate buffer (pH 7.4). Further it was washed 30 mL of the same buffer but without NaCl.

Quantification and Activity Test of CT

The bound CT was measured by bicinchoninic acid assay methods using bovine serum albumin as standard. The amount of bound CT was calculated by subtracting the amount of remaining CT from the initial amount of CT. N-benzoyl-DL-tyrosine-p-nitroanilide hydrochloride (BTPNA) was used as substrate of CT. For general purpose, the substrate solution were prepared by mixing 5 mM BTPNA in DMSO, distilled water and 0.1 M sodium phosphate buffer (pH 7.4) in a ratio of 10:15:3, respectively. In order to measure the activity of the immobilized CT, the increase of absorbance at 400 nm was measured using UV spectrometer (UVICON 923, Kontron Instruments, USA). If the value of absorbance exceeded 2 then it was diluted until it reached below 1.5. The activity of immobilized CT was defined as μmol BTPNA hydrolyzed in 1 min by 1 mg of immobilized CT applying an absorbance coefficient of \( \varepsilon_{400} = 9520 \text{ M}^{-1} \text{cm}^{-1} \). The Michaelis-Menten parameters was calculated by the Lineweaver–Burk plot after reacting enzyme immobilized SF nanofibrous membrane with the substrate solution having different BTPNA concentration. In this case, the final concentration of BTPNA was 0.0625, 0.125, 0.25, 0.5, 1.0 and 2.0 mM.

Operation modes of immobilized CT

The substrate solution was reacted with enzyme immobilized SF nanofibrous membrane in two modes. For the batch reactor mode, the enzyme immobilized SF nanofibrous membrane...
was immersed in 10 mL of substrate solution and incubated for 30 min at 25°C. For the membrane reactor mode, the enzyme immobilized SF nanofibrous membrane was mounted on the membrane holder, and the substrate solution was passed through the membrane using a peristaltic pump just once at 25°C. The flow rate of the substrate solution was changed in order to optimize the reaction condition for membrane reactor mode. For the recycling of immobilized CT, 10 mL of substrate solution was passed through the membrane in each cycle, and the membrane was washed with 30 mL of 0.1 M sodium phosphate buffer (pH 7.4) during the intervals.

### Results and Discussion

A high surface area and low diffusional limitation is the fundamental requirements in the selection of morphology of support for enzyme immobilization. High surface area enables high enzyme loading which can compensate the loss of activity after immobilization. Low diffusional limitation is required in order to maximize the enzyme activity. Porous materials are favored due to the high surface area but the diffusional limitation is usually accompanied. Non-porous materials can be free from the diffusional limitation but low surface area would be more problematic. From this point of view, an electrospun nanofibrous membrane has a high surface area due to its fine diameter, and each fiber is considered as non-porous material. The spaces between the fibers could be considered as pores but they are far greater than that could be found in a porous material. In this study, we prepared SF nanofibrous membrane by electrospinning. The mean diameter of SF nanofiber was 135 ± 25 nm and the thickness of the SF nanofibrous membrane was 111.3 ± 17.5 µm (Table 1). The amount of CT that immobilized onto 1 g of SF was 106.8 ± 9.6 mg. Since the flow rate of substrate solution through the membrane defines the retention time of enzyme in the membrane, we first checked the effect of flow rate on the activity of immobilized CT. As shown in Fig. 1, the activity of immobilized CT decreased with the increase of flow rate. It is obvious that the activity of immobilized CT will be increased with prolonged retention time. Based on the results of Fig. 1, the flow rate was fixed to 30 mL/h for further experiments. In our previous reports, the enzyme immobilized SF nanofibrous membrane was operated like a batch type reactor (Lee et al., 2005a; 2005b). In that case, the nanofibrous membrane was simply immersed in the substrate solution. However, nanofibrous membrane consists of multiple layers of nanofibers. Based on the fiber diameter and the thickness of SF membrane, it would have almost 1,000 layers of nanofibers. Therefore, we supposed that there might be some diffusional limitation of the substrate, which is frequently observed in the highly porous supports. In order to verify this assumption, we compared the Michaelis-Menten parameters of the batch reactor mode and the membrane reactor mode (Table 1). In the batch reactor mode, no additional force was applied to assist the penetration of substrate into the membrane. On the other hand, in the membrane reactor mode, the substrate was forced to penetrate into the membrane by peristaltic pump. The $K_m$ indicates the affinity between the substrate and the enzyme. The membrane reactor mode had much lower $K_m$ value indicating higher affinity than the batch reactor mode. The membrane reactor mode had also

<table>
<thead>
<tr>
<th>Operation Modes</th>
<th>Fiber Diameter (nm)</th>
<th>Thickness (µm)</th>
<th>Bound CT (mg/g)</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (µM/sec/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane</td>
<td>135 ± 25</td>
<td>111.3 ± 17.5</td>
<td>106.8 ± 46.0</td>
<td>0.150 ± 0.020</td>
<td>25.342 ± 1.445</td>
</tr>
<tr>
<td>Batch</td>
<td>0.265 ± 0.082</td>
<td>0.123 ± 0.015</td>
<td>106.8 ± 46.0</td>
<td>0.265 ± 0.082</td>
<td>0.123 ± 0.015</td>
</tr>
</tbody>
</table>

**Fig. 1.** Effect of substrate flow rate on the activity of CT immobilized onto SF nanofibrous membrane in the membrane reactor mode.
Fig. 2. Relative absorbance of substrate solution that elutes from the membrane reactor.

Fig. 3. Operational stability of CT immobilized onto SF nanofibrous membrane in the membrane reactor mode.

higher $V_{\text{max}}$ than the batch reactor mode. These parameters indicate that the membrane reactor mode is more effective than the batch reactor mode. The enhanced efficiency of membrane reactor mode can be explained by the enhanced diffusion of the substrate. In the case of batch reactor mode, the substrate might be difficult to penetrate into the nanofibrous membrane without external forces. Therefore, the substrate might not be able to reach deeply inside the membrane. On the other hand, in the case of membrane reactor mode, the substrate passed through all the nanofiber layers, which results in increased chances to react with the immobilized enzyme even located deeply inside the membrane. This result shows that the enzyme immobilized nanofibrous membrane should be operated in a membrane reactor mode in order to take the full advantage of nanofibrous membrane.

Fig. 2 shows the relative absorbance of each volume fraction that eluted from the reactor. The absorbance is maintained throughout the volume fraction indicating that there is no void volume at the beginning. In the case of packed-bed reactor, there is concentration gradient at the beginning of reaction and needs to be stabilized (Balcão et al., 1996). However, due to the small thickness of the membrane, the data indicates that there is no concentration gradient throughout the reaction.

Fig. 3 shows the operational stability of the immobilized CT. Even after 7 times of re-use, the activity of the immobilized CT was maintained. The operational stability of an immobilized enzyme is the most important factor that determines the potential of the system for industrialization. In the membrane operating mode, high pressure was applied in order to pass the substrate solution through the membrane. Our concern was whether such high pressure can denature the immobilized enzyme. However, our result indicates that the CT immobilized onto the SF nanofibrous membrane did not affected by the pressure and have high potential for industrialization.

Reference


