PREPARATION OF FREEZE-DRIED POROUS MEDIA IN A MICROCHANNEL: A New Platform for Enzymatic Reactions

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ABSTRACT
Lipase from candida rugosa was immobilized in a freeze-dried polyvinyl alcohol (PVA) matrix for investigating the enzymatic reactivity. This matrix was prepared in a microreactor to achieve continuous enzymatic reactions. The performances of the lipase immobilized microreactors were compared in terms of the micro channel type and the reaction conditions. The freeze-dried matrix made in the microchannel possessed porous microstructure with 2–20 μm of well-aligned pores. These pore structures were obtained as a replica of the ice crystals formed during the freezing stage. Experiments suggested that the enzymatic reaction with the immobilized lipase was a diffusion controlled system. It was found that the present microreactors exhibited approximately 10 times higher reaction performance than the large scaled plug flow reactor with the freeze-dried specimens. The freeze-dried microstructure fabricated in a microspace had a significant effect of improving enzymatic reactions. A solution flow in the microchannel is under an influence of both the porous microstructure and the channel structure. When the channel size was smaller than 1000 μm, the flow regime would mainly be controlled by the freeze-dried microstructure.

KEYWORDS
Enzyme, reaction, microchannel, freeze-drying, microstructure, porous structure.

INTRODUCTION
Biological reactions catalyzed by enzymes are widely recognized as a useful tool for tailoring chemical products. Micro process engineering is an emerging field for chemical engineers for the last decades, expecting specific effects on reaction in a microspace, such as improvement of reaction yield, enhancement of the reaction selectivity etc. Enzymes are catalytic proteins that accelerate chemical reactions. Enzymes are usually selective for their substrates and catalyze limited numbers of reactions from among large numbers of reactions. It is a useful feature if it can be introduced in a μTAS. A challenge is to immobilize a biological catalyst in a microchannel. Our research group has been developed a new technique for the enzyme immobilization [1]. Our approach is to produce a polymeric porous media directly in a microchannel by applying a freeze-drying technique [2], and the porous matrix supports enzymes. As shown in Figure 1, freeze-drying a colloidal suspension produces porous material, where the pore structures are replicas of the ice crystals formed during freezing [3-5]. First, a colloidal polymeric suspension with enzyme are introduced in a micro channel. The micro device with the solution is then frozen and subsequently dried under vacuum condition. This process produces freeze-dried matrix in the channel. The formation of the porous freeze-dried matrix could be confirmed as the example shown in Figure 2. A chitosan solution was set in microchannels (channel diameter 40 μm) fabricated by UV lithography on a 500 μm thickness of PMMA film, and then freeze-dried. Porous network structures made by chitosan were fairly well formed in the channels. Enzymes can be stabilized in these network bones made from polymers.
In this study, *lipase from candida rugosa* was immobilized in a freeze-dried polyvinyl alcohol (PVA) matrix for investigating the enzymatic reactivity. This matrix was prepared in a microreactor to achieve continuous enzymatic reactions. The performances of the lipase immobilized microreactors were compared in terms of the micro channel type and the reaction conditions.

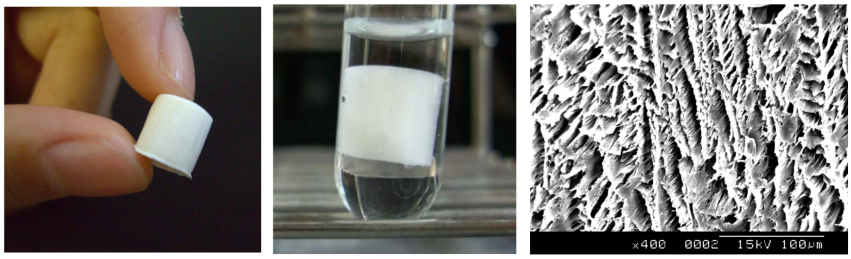

Figure 1: Freeze-dried porous media made from polymeric colloidal suspensions and the internal microstructure.

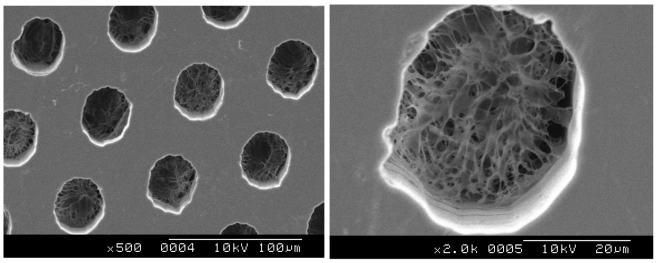

Figure 2: Freeze-dried porous media prepared in a microchannel (PMMA).

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EXPERIMENT

A 5 wt% polyvinyl alcohol (PVA) suspension was prepared by dissolving PVA powder into distilled water. Large sized freeze-dried specimens for analyzing the bulk reactivity were prepared by using a freezing system equipped with a plate heat exchanger. In the setup, temperature of the heat exchanger was controlled by internally circulated coolant. A sample holder with a cylindrical hole (made from PTFE, diameter D=10 mm, Height H=10 mm) was set on the cooling plate, and the sample solution was set in the space. 30 µL of lipase solution (1, 2, 5, 10 mg/ml) was carefully mixed with this suspension. The sample solution in the sample holder was cooled at selected cooling rate (-1.0 °C/min) and completely frozen at -40°C. The obtained frozen samples were subsequently freeze-dried for 48 hours in a vacuum chamber. Enzymatic activity of the immobilized lipase in the freeze-dried specimen was tested by hydrolysis reaction of p-nitrophenylacetate in a batch system. A freeze-dried specimen was put in a test tube with a 2 mL of the substrate solution (12 mg/mL of p-nitrophenylacetate). A 30 µL of solution was continuously sampled to detect nitro phenol production using an ultraviolet-visible (UV-Vis) spectrophotometer (Nanodrop 2000C, Thermo Fisher Scientific Inc., USA). A 30 µL of crude lipase solution was added to the substrate solution for taking a control data.

Microreactors were fabricated on PDMS by SU-8 mold prepared by UV-LIGA. Three different reactors were used in this study. The differences were in the total volume and width of the channel as the detail appeared in the following section. The microreactor was set in the housing plates tightly fixed with screw threads. A PVA suspension containing 10 mg/mL of lipase was slowly introduced in the reactor with a syringe. After the microchannel was fully filled with the solution, the reactor set was placed on the freeze-drying system to freeze and dry following the protocol addressed above. The performance of the reactor was evaluated by the reaction yield of the p-nitrophenylacetate under a plug flow condition. A 12 mg/mL of p-nitrophenylacetate solution was supplied with a syringe pump to the reactor with flow rates of 10, 15, 25 and 40 µL/min. The nitro phenol concentration in the external solution was analyzed to evaluate the reaction yield. The obtained yield values were plotted as a function of the space time, τ [s] (the reactor volume divided by the flow rate of the substrate solution) for the comparison of the reactor performance. A packed column reactor was separately prepared with the freeze-dried specimens, where two piece of the specimens were packed in a vinyl tube (inner diameter 8 mm, inner volume 769 µL). The substrate solution was similarly supplied to this reactor, and the reaction yields were compared with those obtained from the microreactors.

RESULTS

A batch test of the enzymatic reaction was conducted with the freeze-dried specimen. As seen in the results depicted in Figure 3, the hydrolysis reaction catalyzed by the immobilized lipase was the Michaelis-Menten type as the reaction rate increased as increasing the amount of the enzyme. The reaction rate observed in the immobilized system was however lower than that for the crude system. Freeze-drying gives porous microstructure in the resultant dried matrix. So a substrate must diffuse on to the pore surface with immobilized enzymes for yielding the product, and the product must come out from the pores. The results suggested that the present system was clearly a diffusion controlled system.

Lipase immobilized microreactor was prepared by the freeze-drying method to investigate the performance of the reactor (Figure 4). The freeze-dried matrix made in the microchannel possessed, as seen in the SEM images, porous microstructure with 2–20 µm of well-aligned pores. As noted above, these pore structures were the replica of the ice crystals formed during the freezing stage. Anyway, this structured material was perfectly packed in the microchannel. A worry was the pressure drop caused by this packing material when loading a substrate solution. When the channel size was around 200 µm in diameter and 5 cm in length, the microreactor made by PDMS was deformed by the pressure given by the loading solution at 40 µL/min. This caused a serious leakage and performance loss. We thus selected microreactors that are available to apply the present immobilization technique. After the freeze-dried foam meet a substrate solution, the polymeric matrices were rehydrated and swelled. The spongy freeze-dried matrix turned into hydrogel. Due to the swelling, the pore size in the gel matrix was not identical to that in the dried matrix. The size, however, was not evaluated yet.

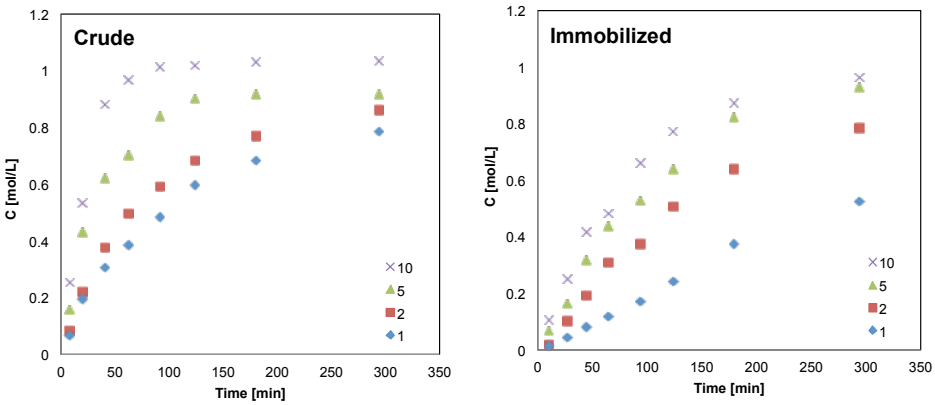

Figure 3: Batch hydrolysis test of immobilized lipase (at 25°C).

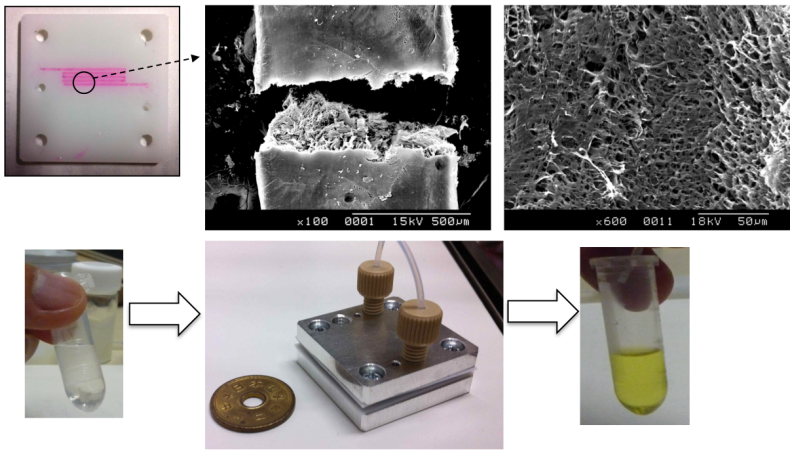

Figure 4: Freeze-dried microstructure prepared in a microreactor.

The performances of the prepared reactors were compared in Figure 5. It is noteworthy that all the microreactors exhibited higher performance than the packed column reactor. At the space time of 45 sec, the microreactor A and B showed approximately 10 times higher reaction performance than the packed column reactor. The results support that the present immobilization method can be effectively applied in a micro system. The volume of the reactor B and C were equal to 7.5 µL. They were different in the channel width (the path length). The channel width for the reactor B (500 µm) was greatly smaller than that for the reactor C. The reaction performance for these two reactors were equivalent in the space time range between 10 to 30 s. However, the performance for the reactor C was inferior to that for the reactor B at the higher space time range. It means that the efficacy of the enzymes in the reactor C could not be brought out at slower flow rate. The volume of the reactor A was double than that for the reactor B. The performances of these reactors were almost equivalent. A scale-down from the reactor A to B (channel width 1000 µm to 500 µm) did not significantly improve the reaction. The present method is to produce a secondary microstructure in a microspace. A solution flow in the microchannel is under an influence of both the porous microstructure and the channel structure. When the channel size was smaller than 1000 µm, the flow regime would mainly be controlled by the secondary microstructure. In any cases, the freeze-dried microstructure fabricated in a microspace had a significant effect of improving enzymatic reactions. In the related studies of our research group, other enzymes, such as amylase, protease etc., were also applied to confirm considerable enzymatic activities. This approach would be useful not only for chemical material processing but also for analytical system based on biological activity, for example, for antigen-antibody reaction.

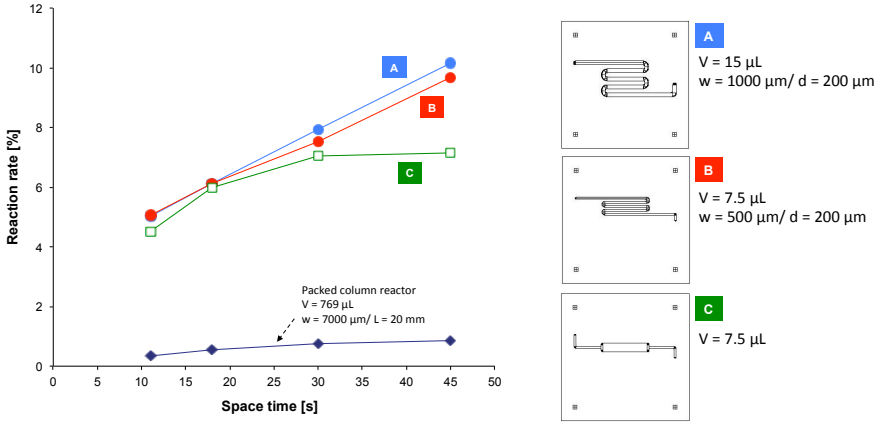

Figure 5: Reaction rate as a function of space time (reactor volume/flow rate).

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