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Preparation of α-amylase-immobilized freeze-dried poly(vinyl alcohol) foam and its application to microfluidic enzymatic reactor

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ABSTRACT

Freeze-drying aqueous poly(vinyl alcohol) (PVA) solutions affords porous foam materials that show potential as an interesting entrapping matrix for enzyme immobilization. When an enzyme–PVA solution is freeze-dried, the enzymes are automatically entrapped in the architecture of the PVA matrix. The obtained PVA foams barely disperse in aqueous solution because of physically cross-linked PVA cryogel formation. This method is an in situ immobilization technique such that supporting materials can be produced in any space where the precursor solutions can be loaded. In this study, an attempt was made to prepare freeze-dried PVA foams in a microchannel to prepare an α-amylase–immobilized microreactor. Freeze-dried foams for amylase immobilization were demonstrated to be successfully prepared in microreactors with a ca. 18-μL microchannel volume, and were evaluated by conducting continuous starch hydrolysis reactions over 8 d. The macroscopic pore structures seemed to be only minimally affected by the apparent activities of the immobilized enzymes; however, the cryogel structures, affected by the freezing protocol, and the microchannel materials employed were suggested as the key to determining reaction performances.

1. Introduction

Biological reactions catalyzed by enzymes are widely recognized as a powerful tool for tailoring chemical products. Utilizing the excellent performances of bioactive substances such as enzymes that catalyze complex and specific chemical reactions by converting them into easy-to-handle forms is a considerable challenge for material scientists and engineers. Immobilization techniques are of particular importance for sustaining enzyme stability, providing large catalytic surfaces, and allowing reuse of expensive enzymes [22,25] furthermore, immobilization allows for improvements in enzymatic catalytic activities [15]. Techniques for enzyme immobilization can be classified in various ways. Brady and Jordaan [1] have classified the techniques into entrapment, encapsulation, solid support-based immobilization, and self-immobilization methods. Among these, the solid support-based immobilization method has the potential to improve catalytic performances by modifying the microstructures of the supports. An interesting technique is the use of monolithic porous materials for supporting enzymes. The background and features of monolith materials have been well reviewed by Svec and Huber [23]. A monolith is typically prepared by in situ polymerization [2,5,26] a significant advantage of this technique is that it can be done directly at the site of use, such as in a column for use in chromatographic methods or a reactor channel. Monolith formation could be particularly essential for immobilizing enzymes in microfluidic devices; the microchannel is filled with a mixture of monomers, and polymerization is subsequently induced by a change in temperature or other stimuli. This in situ polymerization technique affords a porous monolithic material with a variety of surface chemical characteristics that can bind enzymes by subsequent immobilization steps [6,8,13,14].

The immobilization technique reported in the present paper involves an entrapment method using poly(vinyl alcohol) (PVA). Aqueous PVA solutions are known to form cryogels, i.e., hydrogels that are formed upon freezing. The research group of Lozinsky has published a number of related contributions in this area, showing that PVA cryogels could be successfully applied to the immobilization and/or encapsulation of biomaterials [9–11]. Cryogelation (also denoted as cryotropic gelation or freeze gelation) involves hydrogel formation induced by concentration enhancement caused by water removal from the system because of ice formation during freezing. One of the unique features of cryogelation is the use of low processing temperatures, which is advantageous for

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entrapping heat-sensitive materials such as proteins. An albumin/chitosan-based cryogel was previously reported to have successfully entrapped and immobilized an enzyme without significant loss of its activity [4]. Furthermore, because cryogels are a physical gel, cryogelation does not always require a cross-linking agent; as a result, the unfavorable denaturation of enzymes could be avoided. A PVA cryogel can be simply obtained by freezing and thawing an aqueous PVA solution at a certain concentration. Once frozen, the PVA solution can be freeze-dried to obtain porous foam-like materials, where the pore structures replicate those of the ice crystals; therefore, control over the pore structure is possible by controlling ice crystal formation, as extensively detailed in previous works [16,18]. When freeze-dried PVA foam is prepared from a PVA solution containing dissolved enzymes, the enzymes are automatically entrapped in the architecture of the PVA matrix. Interestingly, the obtained freeze-dried PVA foams are hardly dispersed in the aqueous solution, permitting their use as catalyst supports for aqueous reactions. The authors would like to emphasize one further interesting feature, which is the major focus of this paper, that is, freeze-dried PVA foams for use in enzyme immobilization in a microreactor, namely an in situ immobilization technique; a monolithic material can be produced in any spaces where precursor solutions can be loaded, and enzymes are simultaneously entrapped during formation of the monolith. This simplicity in processing is a big advantage of the present method. All the processes are carried out under sub-zero temperature, thus this method can reduce denaturation of enzymes.

In the present study, an attempt was made to prepare freeze-dried PVA foams in a microchannel in order to prepare an enzyme-immobilized microreactor. The preliminary attempts were reported by the authors' research group [20]. They demonstrated the concept of freeze-drying immobilization for development of microdevices by stabilizing lipase in a freeze-dried PVA monolith. The influence of microchannel patterns on the resultant enzymatic reaction performance was recently studied with proteolytic microreactors prepared by the freeze-drying immobilization technique [21]. The present study focused much more on processing aspects, that is, the freeze-drying process and the resultant performances of the reactors. As noted above, this process involves cryogelation of PVA and is therefore, closely linked to the kinetics of freezing. Cooling and selection of the reactor material are parameters that directly and indirectly determine the thermal history of the PVA solution in the reactor; consequently, these factors may influence the reactor performance.

In this study, α-amylase from *Aspergillus oryzae* was immobilized in freeze-dried PVA foams. α-Amylase, which hydrolysis starch into low molecular sugar species, is an enzyme that has a wide number of industrial applications. First, the enzymatic activities of immobilized amylase were studied with bulk freeze-dried foam materials. Second, an amylase-immobilized microreactor was prepared by freeze-drying a PVA–amylase solution loaded in the reactor microchannels. Microreactor performances were characterized using continuous hydrolysis reactions of starch, and the influences of the preparation conditions were investigated.

2. Materials and methods

2.1. Materials

Poly(vinyl alcohol) (M_w = 31,000–50,000, 85,000–104,000, and 146,000–186,000, denoted PVA#1, #2, and #3, respectively, in the following sections) were purchased from Sigma–Aldrich (Sigma Chemical Co., Japan). α-Amylase from *A. oryzae* (powder, activity in the lot 37.2 unit/mg) was also purchased from Sigma–Aldrich (Sigma Chemical Co., Japan). Starch (soluble GR for analysis) was purchased from Merck Ltd., Japan. All other chemicals used in this work were of analytical grade and used as received.

2.2. Freeze-dried foam preparation and characterization

Poly(vinyl alcohol) (4% (w/w); PVA#1, #2, and #3) powder was dissolved in distilled water at approximately its boiling temperature. After completion of the dissolution, the solution was weighed and the amount of water loss by evaporation was added. An amylase solution (30 mg-amylase/mL) was mixed with the PVA solution in a ratio of 1:9 (v/v). A 0.6 mL portion of this solution was placed in a cylindrical sample case (made from PTFE or copper, diameter D = 10 mm, height H = 10 mm) and freeze-dried. Freeze-drying was carried out using a plate heat exchanger, and a circulated coolant was used to control the temperature. The sample solution in the sample holder was cooled at a constant cooling rate (−1.0 °C/min), or the supercooled heat exchanger held at −100 °C was controlled with liquid nitrogen and a heating device. The resultant frozen samples were subsequently dried under vacuum (at ca. 5 Pa) at −10 °C for 48 h. The weight of one specimen was approximately 22 mg, containing 1.8 mg of amylase: namely, ca. 0.09 mg-enzyme/piece. The identities (IDs) of the prepared specimens are listed in Table 1.

The apparent enzymatic activities of the immobilized amylase in these freeze-dried foams were evaluated by the hydrolysis reaction of starch as follows: starch (0.2% w/w) was mixed with distilled water, and the solution was heated at 90 °C for 5 min to gelatinize the starches. One piece of freeze-dried foam was placed in 2 mL of the starch solution in a test tube with continuous stirring. In order to analyze the progress of the hydrolysis reaction, a 0.05 mL aliquot of the solution was withdrawn and sampled at selected times. The sampled solutions were reacted with 1 mL of iodine–KI reagent (0.1 g of iodine and 0.2 g of KI were dissolved in 300 mL of distilled water), and the amount of undigested starch was detected using UV–vis spectrophotometry by measuring the absorption at a wavelength of 530 nm.

The microstructures of the freeze-dried samples were studied using scanning electron microscopy (SEM) (S-2400; Hitachi, Japan); both horizontal and vertical cross sections were observed.

2.3. Microreactor preparation and characterization

Microreactors were fabricated using a microchannel plate (made of PTFE or stainless steel plates of 0.5 mm thickness), packing sheet (made of PTFE of 0.5 mm thickness), and housing plates (made of an Al plate of 5 mm thickness). Channel patterns were made on the microchannel plates by end-milling machining, the geometries of which are illustrated in Fig. 1. A microchannel plate was put between the housing plates and held with screws at the corners (Fig. 1). Tubing parts were connected to this assembled microreactor and used in the following process. A poly(vinyl alcohol) (4% w/w, only PVA#3 was used) solution was prepared as

Table 1
Specimen IDs for freeze-dried foams.

Specimen ID	Type of PVA	Sample holder	Freezing condition
F1	PVA#1	PTFE	Constant cooling at −1 °C/min
F2	PVA#2	PTFE	Constant cooling at −1 °C/min
F3	PVA#3	PTFE	Constant cooling at −1 °C/min
F4	PVA#3	Copper	Constant cooling at −1 °C/min
F5	PVA#3	PTFE	Cooled in precooled bath at −40 °C
F6	PVA#3	Copper	Cooled in precooled bath at −40 °C