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Immobilization of catalase onto hydrophilic mesoporous poly(ethylene-*co*-vinyl alcohol) monoliths

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ABSTRACT: Poly(ethylene-*co*-vinyl alcohol) monoliths have been fabricated by a thermally induced phase separation method. Catalase was immobilized onto the monolith surfaces after activating the hydroxyl groups of the monolith in the presence of carbonyldiimidazole. The immobilized catalase exhibited the same optimum pH and temperature values to those of the free catalase. In addition, the immobilized catalase showed better thermal stability and high reusability. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42556.

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INTRODUCTION

Monolith is a kind of functional material with a three-dimensional continuous porous structure. Owing to its high permeability, fast mass-transfer performance, large surface area, and ease of chemical modification, monolith can be used for various applications such as separation media,[1,2] enzyme immobilization,[3–6] chromatography,[7,8] and catalysis.[9,10] Recently, our group has developed an easy and template-free technique to prepare polymer-based monolith using a thermally induced phase separation (TIPS) method.[11–14]

Poly(ethylene-*co*-vinyl alcohol) (EVOH) is a crystalline hydrophilic copolymer containing vinyl alcohol and ethylene segments.[15] Because of its hydrophilicity, biocompatibility, thermal stability, and chemical resistance, EVOH has great potential to be used as a unique biomedical material.[16,17] Recently, we have fabricated an EVOH-based monolith by using TIPS technique for the first time.[13,18,19] The EVOH monolith possessing hydrophilic hydroxyl groups has many superior properties over other hydrophobic polymers for enzyme immobilization.[20]

Catalase is widely used in industry, including removal of hydrogen peroxide from milk or fabrics.[21–23] It is also employed for the production of gluconic acid when associated with glucose oxidase.[24,25] Moreover, it can be used for hydrogen peroxide biosensor[26–28] or catalase test.[29,30] Until now, numerous matrixes have been used for the immobilization of catalase.[31–36] However, the immobilization of catalase onto polymeric monolithic materials has never been reported. The advantage of monolithic materials for enzyme immobilization is that they have a continuous pores structure, allowing flow-through of reaction solution.[8] Moreover, they can be fabricated into different shapes by cutting or cast for designing various kinds of reactors.[8]

In this study, we report the immobilization of catalase onto an EVOH-based monolithic material. The effects of pH and temperature on the activity of free and immobilized catalase were investigated by measuring the catalysis efficiency of the catalase for the decomposition of hydrogen peroxide. Furthermore, the thermal stability and reusability of the immobilized catalase were discussed.

MATERIALS AND METHODS

Materials

EVOH with ethylene contents of 27 mol %, catalase, and QuantiPro™ BCA Assay Kit were supplied by Sigma. Carbonyldiimidazole (CDI), isopropanol (IPA), hydrogen peroxide, acetone, and acetonitrile (ACN) were purchased from Wako. All reagents were used as received without further purification.

Instruments

HITACHI S-3500 was used to observe the scanning electron microscope (SEM) images of the monoliths at 15 kV. Before observation, the monoliths were sputtered with pure gold *in vacuo*. UV was measured by a HITACHI U2810 spectrometer.

Preparation of EVOH Monoliths

EVOH monoliths were prepared according to our reported method.[13] The typical procedure to prepare EVOH monoliths is as follows: A mixed solvent of IPA (0.65 mL) and water (0.35 mL) was used to dissolve 150 mg of EVOH pellet at 75°C. Then the solutions were cooled at 20°C to induce phase

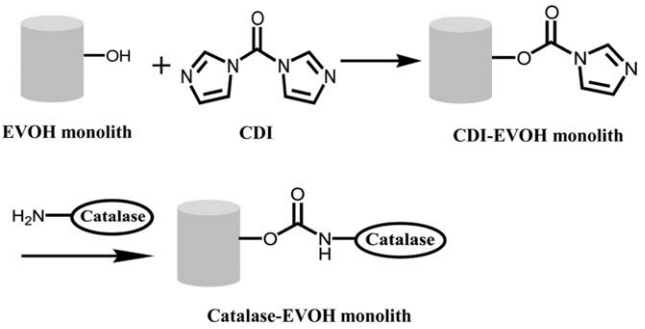

Figure 1. Modification of EVOH monolith and immobilization of catalase.

separation. Subsequently, the monoliths were immersed into acetone for solvent replacement and dried under vacuum.

Immobilization of Catalase

EVOH monolith (0.10 g) was immersed in ACN solution (5.0 mL) in the presence of CDI (40 mg/mL). After reaction for 4 h at 40°C, the monolith was washed with extra amount of acetone and dried under vacuum. The CDI modified EVOH monolith (CDI-EVOH monolith) was stored at 4°C for further uses.

Catalase immobilization process is as follows: CDI-EVOH monolith (50 mg) was added to 10 mL of catalase solution (0.50 mg/mL) in a phosphate buffer (0.10*M*, pH 6.8). The immobilization experiment was conducted at 4°C for 12 h. After immobilization, the catalase immobilized EVOH monolith (catalase-EVOH monolith) was removed from the reaction solution and washed with phosphate buffer (0.10*M*, pH 6.8) for at least three times. Concentration of catalase in the mixture was determined by bicinchoninic acid (BCA) method[37] using a calibration curve prepared with catalase solution of known concentrations. Figure 1 shows a schematic representation for the activation of EVOH monolith and the immobilization of catalase.

Activity Assays of Free and Immobilized Catalase

Catalase is efficient in the decomposition of hydrogen peroxide. To determine the activity of free and immobilized catalase, the decrease in the absorbance of hydrogen peroxide at 240 nm was measured.[32,33] Hydrogen peroxide solutions with the concentration of 2.5–20 m*M* were used to determine enzyme activity. The activity assays of free and immobilized catalase are as follows: 5.0 mL of hydrogen peroxide solution was equilibrated at 25°C for 10 min. Fifty microliter of catalase solution (25 μg/mL) or 20 mg of catalase-EVOH monolith was added to the hydrogen peroxide solution. After 5 min, the reaction was terminated by adding 0.40 mL of HCl solution (1.0*M*) or by removal of catalase-EVOH monolith from the reaction solution for free and immobilized enzyme activity assay, respectively. One unit of activity is defined as the decomposition of 1.0 μmol of hydrogen peroxide per minute at 25°C and pH 6.8. Relative activity was calculated by comparing the specific activity value with the highest value of each set which was assigned as 100% activity.

To determine the pH and temperature profiles of catalase, the activity assays were performed over the pH range of 5.0–9.0 and temperature range of 0–65°C. The effects of pH on the activity of catalase were determined with 20 m*M* of hydrogen peroxide solution with different pH at 25°C. Temperature profiles of catalase were determined with 20 m*M* of hydrogen peroxide solution at pH 6.8.

Reusability and Thermal Stability Studies

The reusability and thermal stability of catalase were also determined by the decomposition of hydrogen peroxide. To determine the reusability of immobilized catalase, a piece of catalase-EVOH monolith (20 mg) was added to 5.0 mL of hydrogen peroxide solution (20 m*M*). After reaction for 5 min, the reaction was terminated by removal of the monolith from the reaction solution. After that, the monolith was washed thoroughly with phosphate buffer (0.10*M*, pH 6.8). Activity test and washing cycle was repeated for eleven times. The decrease in the absorbance of hydrogen peroxide at 240 nm due to its decomposition by immobilized catalase was determined spectrophotometrically.[32,33] Residual activity at each reuse circle was calculated by comparing the activity at each reuse circle with the activity at the first circle which was assigned as 100% activity.

Thermal stability of free and immobilized catalase was tested by measuring the residual enzymatic activity at 50°C in a phosphate buffer (0.10*M*, pH 6.8) for 150 min. Every 30-min time interval, a sample was assayed for its enzymatic activity.

RESULTS AND DISCUSSION

Characterizations of EVOH Monolith and Immobilization of Catalase

The SEM images of the EVOH monolith are shown in Figure 2. Interconnected network of macropores with diameter of micron scale were clearly observed from the SEM images. The average pore sizes was measured as 1.4 μm. This kind of structure allows reaction solution to flow through the monolith easily. Our previous study illustrated that the monolith also had uniform mesopores, which was large enough for enzyme immobilization.[13] The combination of macropores and mesopores afforded the EVOH monolith an ideal matrix for enzyme immobilization. Before enzyme immobilization, the hydroxyl groups of the EVOH monolith were activated with CDI in anhydrous ACN solution. The activated EVOH monolith contained imidazolyl carbamate groups which could react with free amino groups in the enzyme.[19] Catalase was immobilized on the activated hydrophilic EVOH monolith. The amount of catalase immobilized onto the EVOH monolith was determined as 6.0 mg catalase/g monolith.

Effects of pH and Temperature on Activity

The effects of pH on the activity of free and immobilized catalase were performed in a phosphate buffer (0.10*M*, pH 6.8) in the pH range of 5.0–9.0 and the results were shown in Figure 3. It could be observed that the pH profile of the immobilized catalase was similar to the free enzyme when pH is higher than 6.8, but much broader when pH is lower than 6.8, probably due to the secondary interaction between the enzyme and the monolith when pH is lower than 6.8.[38,39] The maximum activity was observed at pH 6.8 for both free and immobilized catalase. The optimum pH value of immobilized enzyme compared

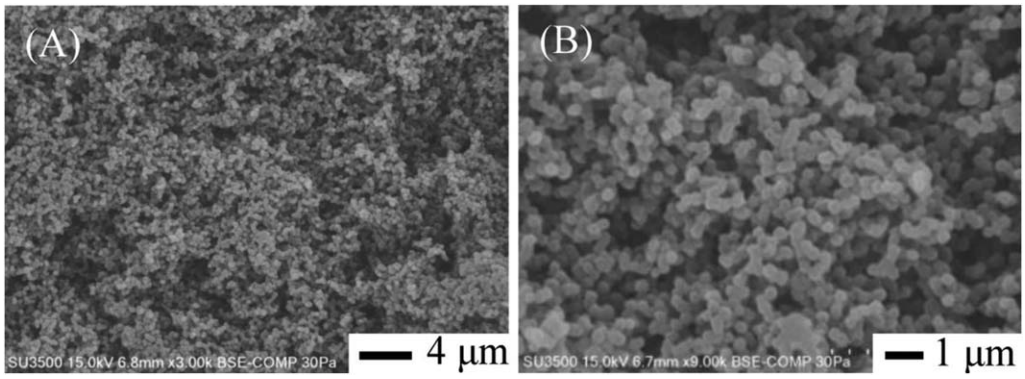

Figure 2. SEM image of EVOH monolith (A). A magnified view of the monolith (B).

with that of free enzyme is determined by the surface and residual charges on the immobilization matrix.[40]

To determine the temperature profile, the activity of free and immobilized catalase was investigated in a phosphate buffer (0.10*M*, pH 6.8) at various temperatures (0–65°C). As it can be seen in Figure 4, the free and immobilized catalase exhibited similar temperature profile. The optimum temperature values for the free and immobilized catalase were found as 30°C. The activity of both the free and immobilized catalase decreased rapidly at temperature higher or lower than 30°C. Generally, effects of temperature on the rates of enzyme-catalyzed reactions do not give much information on the mechanism of enzyme catalysis. But, these effects could indicate the structural changes in enzyme.[41] The similar temperature profile of the free and immobilized catalase indicated that the catalase could retain its structure after immobilization.

Thermal Stability Measurements for Free and Immobilized Catalase

Thermal stability of free and immobilized catalase was determined by measuring the residual enzymatic activity at 50°C in a phosphate buffer (0.10*M*, pH 6.8) for 150 min. Both the residual enzymatic activity of free and immobilized catalase decreased with increasing of incubation time. This may be because that high temperature induced conformational changes of the catalase, and subsequently, inactivation of it. After incubation for 150 min at 50°C, residual enzymatic activities for the

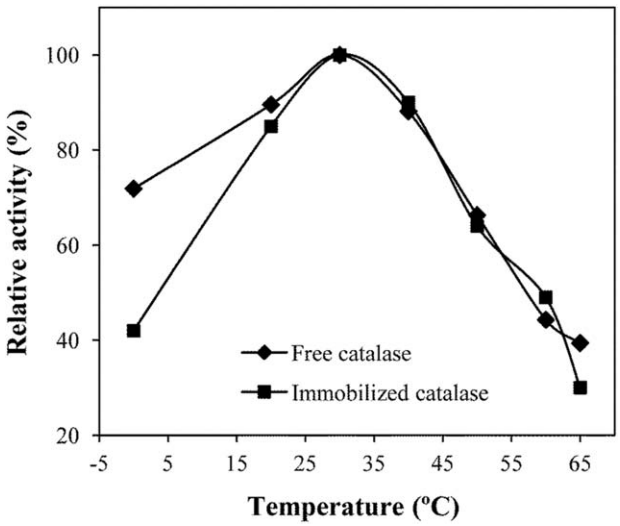

Figure 4. Relative enzyme activity as a function of temperature for free (◆) and immobilized (■) catalase determined by the decomposition of hydrogen peroxide.

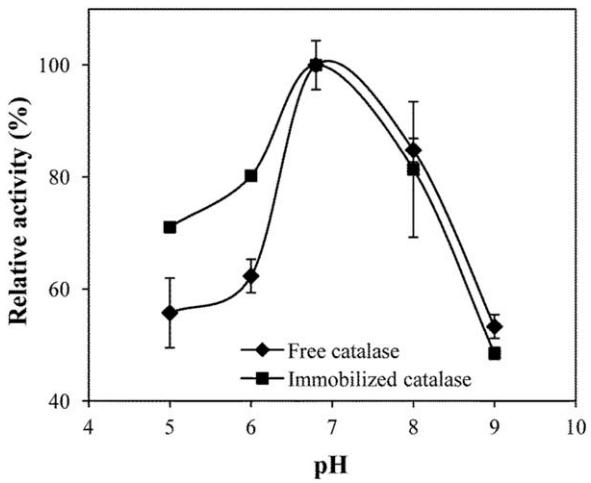

Figure 3. Relative enzyme activity as a function of pH for free (◆) and immobilized (■) catalase determined by the decomposition of hydrogen peroxide.

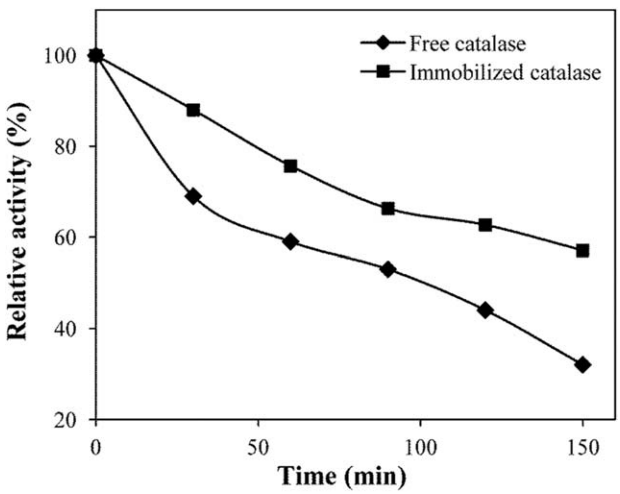

Figure 5. Thermal stability of free (◆) and immobilized (■) catalase determined by the decomposition of hydrogen peroxide at 50°C.

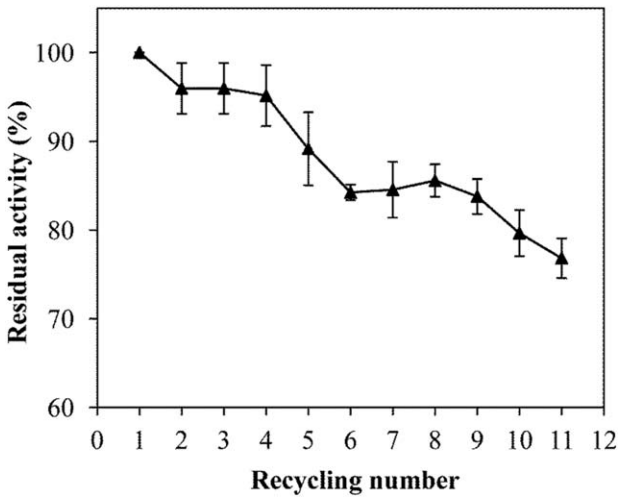

Figure 6. Reusability of immobilized catalase determined by the decomposition of hydrogen peroxide.

free catalase and immobilized catalase were found as 32% and 57% of their original activity, respectively (Figure 5). These results indicated that the thermal stability of immobilized catalase was higher than that of free catalase. It is probably because that EVOH monolith as a matrix preserved tertiary structure of the catalase and this protected the catalase from conformational changes caused from microenvironment changes.

Reusability of Immobilized Catalase

One of the important advantages of immobilized enzyme is that it could be reused for many times. The reusability of immobilized catalase is showed in Figure 6. The immobilized catalase retained 75% of its initial activity after being used for eleven times. The stable covalent bonds between catalase and EVOH monolith, was probably the reason for this high recycling stability. The high reusability of immobilized catalase is vital in consideration of their potential industrial applications.

CONCLUSIONS

In conclusion, hydrophilic EVOH monoliths have been prepared by a TIPS method. The monoliths were activated by CDI for catalase immobilization. Optimum pH value was found as 6.8 and optimum temperature value was determined as 30°C for both free catalase and immobilized catalase. The immobilized catalase retained 75% of its initial activity after eleven successive cycles of decomposition of hydrogen peroxide. The hydrophilic catalase-EVOH monolith with a three-dimensional continuous porous structure will find valuable applications in biotechnology.

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