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Immobilization of lipase on surface modified magnetic nanoparticles using alkyl benzenesulfonate

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Abstract−The surface of nano-sized magnetite (NSM) particles synthesized by coprecipitation method was modified by alkyl benzenesulfonate (ABS) as a coating material. ABS on the NSM was expected to form a spacer between the surface of the NSM particles and the enzyme adsorbed and to play a role of strong enzyme adsorption onto a hydrophobic surface. Transmission electron microscopy showed that the NSM particles had an average size of 10 nm. Magnetic measurement revealed that the nanoparticles were superparamagnetic and the saturation magnetization was about 68 emu/g. Porcine pancreas lipase (PPL) was immobilized onto the ABS-NSM, which was to catalyze hydrolysis of olive oil and showed enhanced durability in the reuse after being recovered by magnetic separations.

Key words: Alkyl Benzenesulfonate, Lipase, Nano-sized Magnetite, Enzyme Immobilization

INTRODUCTION

Magnetic nanoparticle of iron oxide is one of the renowned materials in the field of biotechnology and medicine [1]. Bioactive substances such as enzymes, proteins [2-11], have been bound to it. Iron oxide nanoparticles have been widely used as magnetic resonance (MR) contrast agents, and important studies such as cancer, gene expression, angiogenesis imaging, and cellular trafficking have been well performed [12-16]. Using magnetic nanoparticles as a support for the enzyme immobilization is the important focus in enzyme-catalyzed reactions.

Lipases are ubiquitous enzymes with various biological activities, including triacylglycerols hydrolysis, esterification between fatty acid and alcohol, and other enzymatic reactions [17-19]. In practical applications, the activity recovery and repeated use of lipase are very important in the economic point of view. Hence, the immobilization technique has been attractive for enzyme engineering. In order to improve the stability, separation, and enzyme reusability, the surface of magnetic nanoparticles is modified by hydrophobic polymers or sol-gel techniques [2,5,6,23-29]. These applications offer the magnetic feature of the solid-phase that enables us to achieve a rapid separation in a magnetic field. Therefore, our intention was to synthesize hydrophobic magnetite nanoparticles for enzyme immobilization. In this point of view, in our previous work, porcine pancreas lipase (PPL) was immobilized on sodium dodecyl sulfate-coated NSM particles (SDS-NSM) by physisorption method [30].

In this study, alkyl benzenesulfonate modified NSM (ABS-NSM) particles were synthesized to improve the immobilization of PPL using hydrophobic surface. Generally, immobilization methods decrease the enzyme activity. However, it has been reported that in case of lipase the immobilization by adsorption increases the activity [20-22], and we could confirm the above results in this study. In addition, the catalytic ability and reuse of the immobilized lipase for the hydrolysis of olive oil were evaluated.

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EXPERIMENTAL

1. Materials and Methods

Crude porcine pancreas lipase (E.C.3.1.1.3), alkyl benzenesulfonate with dodecyl carbon chain and aqueous ammonia (28% (w/w)) were purchased from Sigma-Aldrich Co. (USA). All other materials were of analytical grade and used without any further purification, including ferric sulfate ($Fe_2(SO_4)_3 \cdot nH_2O$), ferrous sulfate ($FeSO_4 \cdot 7H_2O$), ammonium oxalate ($(NH_4)_2C_2O_4$), olive oil, gum arabic, and ethanol. The size and morphology of magnetic nanoparticles were assessed by HR-TEM (High Resolution TEM) using a JEOL model JEM-3010 (JPN) at 300 kV. The sample for HR-TEM analysis was obtained by placing a drop of the magnetic nanoparticle solution onto a copper grid and evaporating it at room temperature. FT-IR analysis was performed on a Varian Excalibur Series (Varian, Inc. USA). The magnetic property was evaluated by using a vibration sample magnetometer (VSM; Lake Shore Model 7300; USA).

2. Synthesis of Alkyl Benzenesulfonate Coated Nano-sized Magnetic Particles

ABS-coated NSM particles were synthesized by using the following method, which is similar to that used in our previous studies [30-33]. Briefly, oxalate-coated NSM particles were prepared by adding NH_4OH to a solution containing ferric sulfate, ferrous sulfate, and ammonium oxalate in 100 ml of ultra pure water at the molar ratio of 2 : 1 : 0.1 (pH 9) at 75 °C for 1 hr. Ligand exchange of oxalate-NSM was carried out with 0.1 M ABS at the molar ratio of 10 : 1 in an acidic condition (pH 5).

3. Lipase Immobilization

200 mg of ABS-NSM particles were added to 50 ml of 0.85% (w/v) sodium chloride solution containing 100 mg of lipase. The solution was mixed for 4 h with an over-head stirrer. After washing twice with 0.85% (w/v) sodium chloride solution, the immobilized lipase was separated by magnetic decantation of the supernatants; concentration of ABS-NSM particles was adjusted to 4 mg/ml and stored at 4 °C prior to being used. The amount of proteins adsorbed on the supports was determined by measuring the protein concentration of the lipase solution and the supernatant by Lowry method

[34].

4. Assay of Enzyme Activity

The activity of lipase was determined by measuring the degree of oil hydrolysis. 1% (w/v) olive oil (Sigma Chemical Co.) was mixed with an aqueous solution of NaCl (20 mM), $CaCl_2$ (1 mM), and gum arabic (1% (w/v)) as an emulsifier. Hydrolytic reaction was initiated by adding 2 mg of the lipase-immobilized ABS-NSM particles to the oil emulsion at pH 7.7 and 37 °C. Degrees of oil hydrolysis were determined by measuring fatty acids released from the oil hydrolysis by titrating with 10 mM NaOH using a pH-stat titrator (718 Stat Titorino, Metrohm, Switzerland).

5. Recycling Process

Lipase-immobilized ABS-NSM particles could be easily recovered from the reaction mixture by magnetic decantation. The recovered particles were washed several times with NaCl solution. Then, a fresh oil emulsion was added to the solution with the recovered particles and hydrolysis was carried out again. The recycling process was repeated for five times. In this study, the independent experiments were performed repeatedly until the three duplicated results were obtained.

RESULTS AND DISCUSSION

1. Characterization of Immobilized Enzyme Catalyst

The schematic diagram to prepare the surface-modified magnetite nanoparticles is shown in Fig. 1. The surfactant was attached to the magnetite nanoparticles via a chelation of sulfonate group of ABS. To accomplish this, we followed a multistep procedure consisting of (1) preparation of magnetite nanoparticles covered by oxalate, and finally (2) the exchange of oxalate with surfactant on the surface of the nanoparticles. The morphology of the sample was characterized by high resolution transmission electron microscope and shown in Fig. 2. It shows that the ABS-NSM particles have a diameter size of 10±3 nm. This suggests that the magnetic particles prepared in this work are superparamagnetic because their sizes are all less than 20 nm. To further demonstrate the superparamagnetic

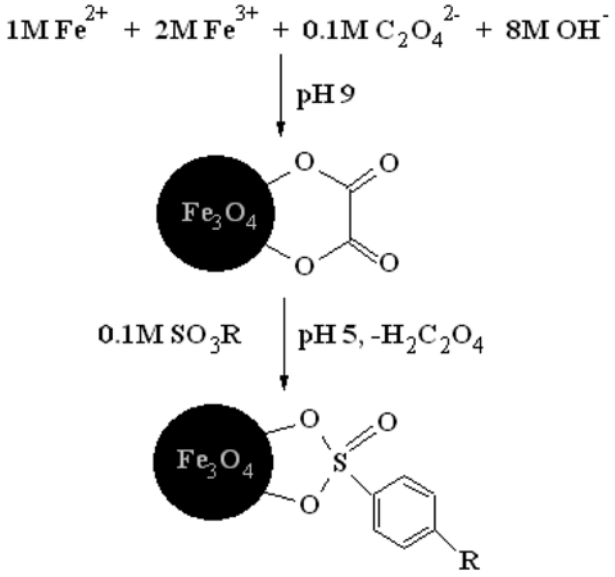

Fig. 1. Synthesis of ABS-NSM particles: R=dodecyl carbon chain.

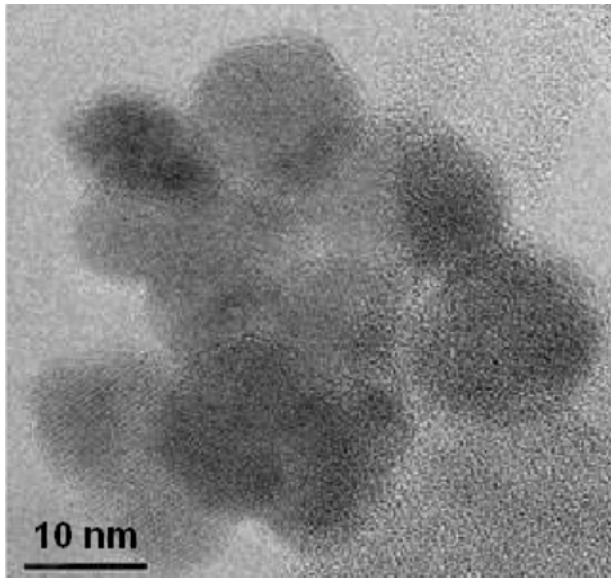

Fig. 2. HR-TEM image of ABS-NSM particles.

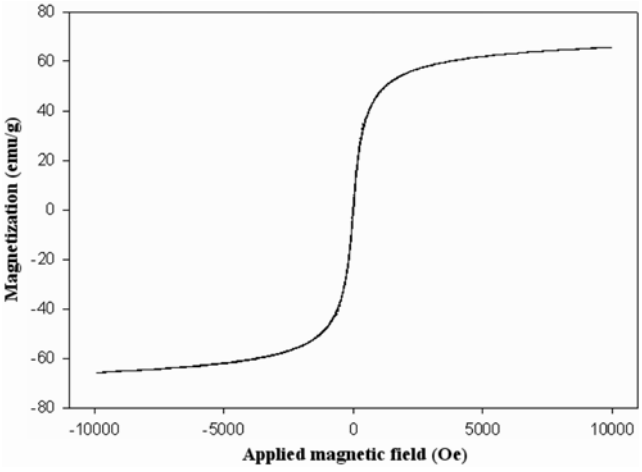

Fig. 3. Magnetization curve of ABS-NSM particles.

property of the magnetic particles, the magnetization of ABS-NSM particles was measured and it showed 68 emu/g as shown in Fig. 3. The magnetization curve exhibits zero remanence and coercivity, which shows that these nanoparticles have superparamagnetic properties. The superparamagnetism enables the nanoparticles to respond to an applied magnetic field without any permanent magnetization and re-disperse rapidly when the magnetic field is removed. This large saturation magnetization of magnetic nanoparticles makes them very susceptible to magnetic fields, and therefore makes the solid and liquid phases separate easily.

The binding of ABS to the surface of the nanoparticles was confirmed by infrared spectroscopic analysis (Fig. 4a). The spectrum obtained for the sample ABS-NSM shows a band characteristic for -CH vibration of ABS at 2,900 cm^{-1}. This indicates that ABS was bound on the surface of magnetic nanoparticles. Fig. 4b represents the IR spectrum of immobilized lipase on the NSM particles. The characteristic bands for protein appear in the region of 1,650 and 1,535 cm^{-1} [27], which shows that lipase is immobilized on the ABS-

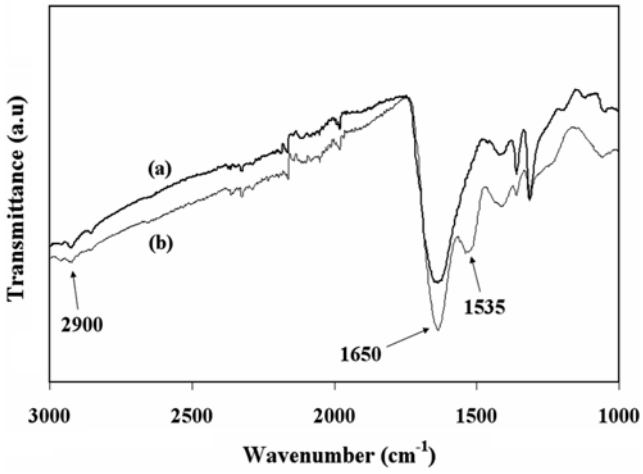

Fig. 4. FT-IR spectra of ABS-NSM without (a) and with (b) immobilized enzyme.

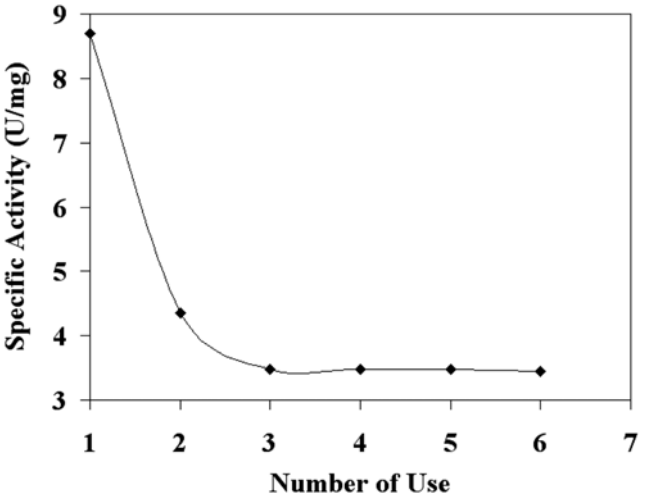

Fig. 5. Changes in the specific activity of immobilized ABS-NSM along the reuse.

NSM particles.

2. Activity of Immobilized Enzyme on ABS-NSM

PPL was immobilized on the surface of the ABS-NSM particles by physisorption techniques. Hydrophobic chain of ABS is expected to form a spacer arm and to improve physisorption affinity between lipase and NSM. The enzymatic activity of the immobilized enzyme was determined by following the hydrolysis of olive oil via pH-stat titrator. The activity attained a maximum value at an initial lipase concentration of 2 mg/ml and the protein amount was found as 37.6 μg/mg. Increasing the initial lipase concentration above 2 mg/ml decreased the activity of immobilized enzyme. The excessive enzyme loading is known to hinder the substrate conversion due to the increased protein-protein interaction [4,35]. The enzymatic activity of immobilized enzyme was greater than that for the free enzyme. In physisorption technique, the enzyme is just adsorbed on the hydrophobic surface without chemical activation. Hence, the activity was higher than that of free enzyme. The specific activity of immobilized enzyme was 8.7 U/mg, whereas that of the free enzyme was 6.37 U/mg. In the previous report [30], the specific activity of immobilized SDS-NSM was also higher than that of the free enzyme. From the results, ABS-NSM particles could also be used for the enzyme immobilization and for oil hydrolysis as like as SDS-NSM.

By magnetic separation, the used immobilized enzyme was recovered and washed several times with 0.85% sodium chloride solution, and supplied again to the fresh reaction solution to find out the enzymatic activity. It was found that the specific activity of the immobilized ABS-NSM decreased significantly up to 50% from first use (Fig. 5). Then, after the specific activity in the third reuse decreased slightly from that in the second reuse, it showed constant activity with a continuous reuse. Diminishing activity of immobilized lipase may be due to desorption of protein from support's surface during the reaction time. Enzymes immobilized onto hydrophobic support materials through physical adsorption are known to be subject to desorption and exhibit a continuous decrease in activity [36]. However, after certain number of reuses, the remaining proteins on the ABS-NSM seem to be immobilized strongly on the surface.

Such phenomenon was also observed in the result of SDS-NSM [30]. Compared with the specific activity between two kinds of immobilized enzymes, the immobilized SDS-NSM showed slightly higher specific activity (the lipase concentration for SDS-NSM was 0.8 mg/g and specific activity was 9.87 U/mg) than the immobilized ABS-NSM. However the activity of ABS-NSM (8.7 U/mg) was almost higher than that of SDS-NSM (8.54 U/mg) at 2 mg/g of lipase concentration. Moreover, in the result of reuse, the decrease of relative specific activity of the immobilized ABS-NSM was higher than that of the immobilized SDS-NSM. From the viewpoint of chemical structure in the surface modifiers, the only difference between ABS and SDS is a benzene ring. Therefore, it is presumed that the aromatic ring of the hydrophobic modifier is slightly less favorable to immobilize PPL on the NSM while the initial concentration of lipase is below 2 mg/g.

CONCLUSION

A simple protocol for enzyme immobilization has been developed by physisorption method and has the following benefits: (1) ABS-NSM particles with a diameter size of about 10±3 nm, together with a hydrophobic surfactant, allows protein loading, (2) convenient recovery using magnetic separation, (3) their reusability is promising for potential application in industry, and (4) in addition, the use of surfactant, which is a cheap and abundant chemical, as a support for immobilization would result in overall cost reduction. However, since the immobilized amount and affinity of enzyme on the hydrophobic surface shows the dependence of applied surfactant, the selection of surfactant acts as one of key factors in the reusable enzyme catalyst.

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