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Immobilization of lipase onto micron-size magnetic beads

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Abstract

A novel and economical magnetic poly(methacrylate-divinylbenzene) microsphere (less than 8 μm in diameter) was synthesized by the modified suspension polymerization of methacrylate and cross-linker divinylbenzene in the presence of magnetic fluid. Then, surface aminolysis was employed to obtain a high content of surface amino groups (0.40–0.55 mmol g^{-1} supports). The morphology and properties of these magnetic supports were characterized with scanning electron microscopy, transmission electron microscopy, Fourier transform infrared spectroscopy and a vibrating sample magnetometer. These magnetic supports exhibited superparamagnetism with a high specific saturation magnetization (σ_s) of 14.6 emu g^{-1}. *Candida cylindracea* lipase was covalently immobilized on the amino-functionalized magnetic supports with the activity recovery up to 72.4% and enzyme loading of 34.0 mg g^{-1} support, remarkably higher than the previous studies. The factors involved in the activity recovery and enzymatic properties of the immobilized lipase prepared were studied in comparison with free lipase, for which olive oil was chosen as the substrate. The results show that the immobilized lipase has good stability and reusability after recovery by magnetic separation within 20 s.
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Keywords: Magnetic microsphere; Synthesis; Suspension polymerization; *Candida cylindracea* lipase; Immobilization

1. Introduction

Lipases (E.C. 3.1.1.3) are ubiquitous enzymes with various biological activities, including triacylglycerols hydrolysis, esterification between fatty acid and alcohol, and other enzymatic reactions [1–3]. In practical applications the activity recovery and repeated use of lipases are very important for the process economy. Many immobilization techniques of enzymes have been employed and reviewed recently [4]. There are many factors affecting the activity recovery and reusability of enzymes in immobilization process. Some of the most important factors are the choice of a support and the selection of an immobilization strategy. Thus, exploiting good supports and immobilization strategy has been an attractive work for enzyme engineering. Magnetic supports have been used in enzyme immobilization [5–7] and cell separation [8], which were first applied to immobilize enzymes in 1973 [9]. Besides the merits of other solid supports, lipases immobilized by magnetic supports can be more easily recovered from a reaction system, and stabilized in a fluidized-bed reactor by applying an external magnetic field. The use of magnetic supports can also reduce the capital and operation costs. However, for these presently available magnetic supports, complex preparation process, insufficient enzyme loading capacity and high cost restrict their wider applicability in enzymatic engineering [10,11].

Magnetic polymeric supports are often prepared by the co-polymerization of monomers (one of them is the functional monomer) including suspension polymerization, emulsion polymerization, dispersion polymerization and two-step swelling [12]. Among them suspension polymerization is simple and easy to scale up, hence, more suitable for mass

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production. However, the magnetic polymeric supports made by conventional suspension polymerization were mostly in the size of several hundred micrometers with a very broad size distribution [13,14]. During polymerization process, moreover, a large amount of functional groups were buried in the polymer with only a small part localized on the surface [10]. The main disadvantages of those magnetic supports are their large size, low density of surface functional groups and weak magnetism. It is hardly reported in literature that micron-size (several microns) magnetic polymeric supports with high density of surface functional groups could be prepared by suspension polymerization.

Choosing a suitable method of lipase immobilization also enables an increase in the stability without negative influence on their catalytic activity. Covalent immobilization of enzymes onto spacer-arm attached magnetic supports can lead to a high activity yield and stability [5]. Since the interactions between lipases and magnetic supports are not sufficiently clear, the study of immobilization process is important for optimizing conditions for preparation and application of the immobilized lipases.

In this study, we develop an economical, high loading capacity magnetic support, which could be chemically derivatized for covalent immobilization of lipase. First, micron-size magnetic polymeric spheres were synthesized by the modified suspension polymerization of methacrylate (MA) and cross-linker divinylbenzene (DVB) in the presence of oleic acid-coated magnetite nanoparticles. Then, surface aminolysis was employed to introduce functional groups (–NH_2). *Candida cylindracea* lipase (CCL) was covalently immobilized onto the amino-functionalized magnetic supports by the glutaraldehyde method. The magnetic supports were characterized with scanning electron microscopy (SEM), transmission electron microscopy (TEM), diffusive reflectance infrared spectroscopy (DR-IR) and a vibrating sample magnetometer (VSM). The factors affecting the activity recovery and properties of the immobilized lipase were investigated.

2. Experimental

2.1. Materials

Commercial lipase (E.C. 3.1.1.3) from *C. cylindracea* and bovine serum albumin (BSA) were obtained from Sigma. Other chemicals were generally of reagent grade and purchased from Beijing Chemical Reagent Company. Methacrylate and DVB were distilled under a reduced pressure to remove the inhibitor prior to use. All other materials were of analytical grade and used without any further purification, including ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), ferrous chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$), poly(vinyl alcohol) (PVA-1788), aqueous ammonia (25% (w/w)), oleic acid, benzoyl peroxide (BPO), ethylenediamine, dimethylformamide (DMF), glutaraldehyde, and ethanol.

2.2. Synthesis of magnetic PMA-DVB microspheres

The oleic acid-coated Fe_3O_4 was obtained by the method described previously [15]. Magnetic PMA-DVB microsphere was prepared by modified suspension polymerization. Methacrylate (95 ml), DVB (5 ml), magnetic fluid (30 g) and BPO (4.0 g) were mixed to form the organic phase. PVA-1788 (25 g) was dissolved in 1000 ml deionized water to form the aqueous phase. They were mixed together and transferred to a 2-l beaker equipped with four vertical stainless steel baffle-plates, a condenser, a nitrogen inlet, and a four-paddle stirrer. The mixture temperature was maintained at 45 °C for 45 min and then increased to 60 °C within 10 min. Finally, the temperature was increased to 70 °C and the reaction was carried out for two more hours with the stirring speed of 1000 rpm. The resulting magnetic PMA-DVB microspheres were isolated by magnetic decantation and washed with deionized water and ethanol several times.

2.3. Surface functionalization and activation

Magnetic PMA-DVB (10.0 g) was washed with DMF twice and then mixed with DMF (100 ml) and ethylenediamine (100 ml). The mixture was shaking gently at 110 °C for 12 h. After washing with deionized water and ethanol two times, the amino-functionalized magnetic microspheres were obtained. To facilitate the covalent attachment of enzyme, the amino groups on the surface were transferred to aldehyde groups by the glutaraldehyde method [16]. After agitating at 30 °C overnight, the glutaraldehyde-activated magnetic supports were washed with deionized water three times and stored for future use.

2.4. Characterization of magnetic microspheres

The morphology and structure of the magnetic microspheres were observed by scanning electron microscopy (SEM; JSM-6700F, JEOL, Japan) and transmission electron microscopy (TEM, H-8100, Hitachi, Japan). The DR-IR spectra were recorded in KBr on a Fourier transform infrared spectrophotometer (FT-IR; Vecter 22, Bruker, Germany). The sample was placed in the sample port of an integrating sphere (diameter 110 mm), and the diffuse reflectance was measured at 610 nm with a Varian cary 5 spectrophotometer. The magnetization curves of samples were measured with a vibrating sample magnetometer (VSM; Model-155, Digital Measurement System, USA). The amount of amino group of on the surface of magnetic microspheres was determined from elemental analysis device (CHNS-932, Leco, USA).

2.5. Lipase immobilization

All lipase immobilization experiments were carried out batchwise in 5 ml of 0.1 M phosphate buffer pH 7.0 at continuous shaking of 150 rpm at room temperature. In a typical experiment, 50 mg magnetic support was dispersed in 5 ml of 0.1 M buffer and a predetermined amount of lipase powder

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