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Resolution of (±)-menthol by immobilized *Candida rugosa* lipase on superparamagnetic nanoparticles

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Abstract

Lauric acid-stabilized magnetic particles were prepared by coprecipitation in the presence of lauric acid and used for the covalent immobilization of *Candida rugosa* lipase via carbodiimide activation. Size analysis by transmission electron microscopy (TEM) and measurement of magnetization curves revealed that the immobilized lipase was superparamagnetic. Resolution of (±)-menthol was performed by the immobilized lipase-catalyzed enantioselective esterification with propionic anhydride. Effects of various reaction parameters, such as enzyme load, solvents, water activity, substrate concentration, reaction time and temperature, on the conversion as well as enantioselectivity were investigated. As a result, (−)-menthyl propionate with a yield higher than 96% and over 88% enantiomeric excess of products was obtained. Better conversion and enantioselectivity could be expected for the immobilized lipase-catalyzed reaction performed at 30 °C for 2.5 h with 0.2 mol/l of (±)-menthol. Hexane was found to be the most suitable solvent, and the activity as well as enantioselectivity of the immobilized lipase decreased gradually with increasing water activity. Good durability of the immobilized lipase to catalyze the resolution of (±)-menthol was also observed.

Keywords: Immobilized lipase; Superparamagnetic nanoparticle; Resolution; Menthol; Enantioselectivity

1. Introduction

Biocatalytic revolution of racemic mixtures in organic media has become a potentially important method to acquire optically active enantiomers (Berglund, 2001; Persson, Costes, Wehtje, & Adlercreutz, 2002; Sakurai, Margolin, Russel, & Klibanov, 1988). Practical interests in this subject primarily arose from the fact that biocatalysis in organic media has many advantages such as higher solubility of hydrophobic compounds in the reaction system, good enantioselectivity and durability of biocatalyst, shifting many enzymatic reactions to the production of desired products and avoidance of bacterial contamination of bioreactors (Carrea, Ottolina, & Riva, 1995; Klibanov, 2001; Wescott, Noritomi, & Klibanov, 1996). Lipases (EC 3.1.1.3) are ubiquitous and highly stereoselective biocatalysts, which are of great value for the modern chemical and pharmaceutical industries, especially in enzymatic resolution of racemic mixtures of organic compounds (Ducret, Trani, & Lortie, 1998; Margolin, 1993; Wu, Xu, & Tsang, 2004). Therefore, there has been much work involving screening of lipases for racemate resolution, development of novel carriers for efficient application in enzymatic resolution, as well as optimization of reaction systems (Furukawa, Ono, Ijima, & Kawakami, 2001; Margolin, 1993; Wang, Nag, Lee, & Shaw, 2002).

Currently, the development of new immobilization methods and carriers remains one of the main subjects of research in enzyme engineering, because immobilization of lipase helps improve its stability, separation and reusability (Dyal et al., 2003; Guo, Bai, & Sun, 2003;

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Huang, Liao, & Chen, 2003). Of the various supports, magnetic nanoparticles have received considerable attention not only due to their higher specific surface area for the binding of larger amount of enzymes (Dyal et al., 2003), lower mass transfer resistance and less fouling (Curtis & Wilkinson, 2001; Huang et al., 2003), but also the ease in the separation of immobilized enzymes from a reaction mixture by the application of a magnetic field (Halling & Dunnill, 1980).

(*l*)-(−)-Menthol is widely used in industry (Gandhi, 1997) because of its refreshing flavor, whereas *d*-(+)-menthol has an undesirable taste. Previous work has demonstrated that *Candida rugosa* lipase can catalyze the enantioselective esterification of *l*-(−)-menthol from (*dl*)-menthol to obtain optically pure l-(−)-menthol (Wang et al., 2002; Wu, Akoh, & Phillips, 1996). However, the practical application of the methods depends on the yield and purity of product, reaction efficiency and enzyme stability. In this work, we developed lauric acid-stabilized Fe_3O_4 nanoparticles and coupled *C. rugosa* lipase for the enantioselective esterification of *l*-(−)-menthol. The effects of various reaction parameters on the enzymatic conversion and the enantioselectivity of the bioreaction as well as the stability of the immobilized lipase were investigated.

2. Materials and methods

2.1. Materials

(±)-Menthol, (−)-menthol, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), bovine serum albumin (BSA), coomassie brilliant blue G-250 and *C. rugosa* lipase (CRL, Type VII) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Propionic anhydride was obtained from Fluka (Buchs, Switzerland). $FeSO_4$, $FeCl_3$ and lauric acid were of analytic grade from local sources. All other reagents and solvents were obtained from local sources and purified before use. A permanent magnet (maximum field strength $3. \times 105$ A/m) provided by the Research Institute of Rare Earth Elements (Baotou, China) was used for magnetic separations.

2.2. Immobilization of lipase on magnetic nanoparticles and activity assay

$FeCl_3$ (100 ml of 0.2 mol/l), 100 ml of 0.32 mol/l $FeSO_4$ and 2.0 g lauric acid were mixed at 50 °C in a 1000 ml steeless reactor equipped with a jacketed heater by circulated water and mechanic agitator. Forty ml of 25% $NH_3 \cdot H_2O$ was slowly added to the reaction mixture under vigorous agitation and the reaction was allowed to proceed for 30 min. The magnetic particles were collected by magnetic separation and washed with 0.5% (v/v) ammonia. The resultant magnetic particles and 1.0 g lauric acid were dispersed in 100 ml distilled water at 80 °C and held at this temperature for another 30 min. The particles were finally harvested by magnetic separation and routinely washed to pH 5–6 with dilute HCl and distilled water. The magnetic particles were lyophilized and stored at room temperature for future use.

Enzyme coupling onto the magnetic nanoparticles was performed by the EDC activation method (Huang et al., 2003). The immobilized lipase collected in magnetic field was lyophilized and stored at −20 °C before use. Magnetic particles with or without lipase were subjected to magnetism characterization. The size and morphology of particles were observed by transmission electron microscopy (TEM) using a JEM-100CX II system (JEOL, Japan) as described previously (Guo et al., 2003). The magnetization curves of the particles were recorded with an LDJ 9600-1 vibrating sample magnetometer (LDJ Electronics, MI, USA). The magnetism of particles was measured with ST-I Tesla meter (Baotou Steel Plant, Inner Mongolia, China).

The amount of lipase protein in supernatant was determined by the Bradford method (Bradford, 1976) using BSA as a standard. The amount of protein bound onto the particles was calculated by mass balance. Activities of the native and the immobilized lipase were determined with the olive oil method (Cho & Rhee, 1993). One unit (U) of the activity was defined as the amount of lipase which liberates 1 μmol fatty acids per minute under the assay conditions.

2.3. Esterification of menthol and its assays

Esterification of (±)-menthol was performed in 10 ml screwed vials. In a typical experiment, 0.5 mmol menthol and 0.5 mmol propionic anhydride were dissolved in 3 ml solvent followed by the addition of 0.5 mmol $NaHCO_3$ and lipase. The reaction mixture was shaken at 200 rpm at a desired temperature (20–40 °C). The progress of the reaction was monitored by analyzing the aliquots of reaction mixture by gas chromatography on an Agilent 6590N system (Agilent Technologies, DE, USA) equipped with a splitless/split injector and a flame-ionization detector. A Cyclosil-B chiral column (30 m length, 0.5 mm I.D.) was used to analyze (±)-enantiomers of menthol and their respective esters. The injector was set at 200 °C and detector at 225 °C. The flow rate of the carrier gas N_2 was 2 ml/min. The initial column temperature of 110 °C was held for 12 min and then raised to 150 °C at a rate of 4 °C/min and finally held at 150 °C for 3 min.

Enantiomeric excess of menthyl propionate [*ee*(*P*)%] based on the GC analyses were calculated as described by Wang et al. (2002). Enantioselectivity (*E*) was then calculated by the following equation (Chen, Fujimoto, Girdaukas, & Sih, 1982):