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Immobilization of lipase from *Burkholderia cepacia* into calcium carbonate microcapsule and its use for enzymatic reactions in organic and aqueous media

Masahiro Fujiwara [a,*], Kumi Shiokawa [a], Koji Yotsuya [b], Kazutsugu Matsumoto [b,**]

[a] National Institute of Advanced Industrial Science and Technology (Kansai Center), 1-8-31 Midorigaoka, Ikeda, Osaka 563-8577, Japan
[b] Department of Chemistry and Life Science, Meisei University, 2-1-1 Hodokubo, Hino, Tokyo 191-8506, Japan

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ABSTRACT

We disclosed a useful procedure for the immobilization of a lipase from *Burkholderia cepacia* based on its encapsulation into vaterite calcium carbonate microcapsule. The immobilized enzyme had an adequate activity for the hydrolysis of 4-nitrophenyl acetate, which was preserved over 2 years in storage at 4 °C. The enantioselective esterification of (±)-1-phenylethanol with vinyl acetate using the immobilized lipase smoothly progressed to afford the corresponding optically active compounds, where the enzyme was easily recovered for reuse. Although the hydrolysis of (±)-2-acetoxyhexyl tosylate in aqueous solution was catalyzed by the enzyme, the phase transition of calcium carbonate from vaterite to poorly porous calcite during the reaction prevented the access of the reactant to the active site of the lipase, resulting in the significant decrease of the activity of the recovered enzyme. However, the treatment of the immobilized enzyme with zinc chloride solution inhibited the phase transition under the reaction conditions. The enzyme after the zinc treatment was recyclable for the hydrolysis of the substrate without significant decrease of the reactivity even after the uses in aqueous media.

1. Introduction

In our previous studies, we have reported on the enzymatic enantioselective hydrolysis of racemic 1,2-diol monotosylate derivatives with lipase PS (from *Burkholderia cepacia*, Amano Enzyme Inc.; Scheme 1) [1–4]. Since the molecular length of the R substituent group does not affect the enantioselelctivity and the gram-scale reactions smoothly proceeded, this method is applicable to the practical preparations of the various optically active 1,2-diol monotosylates. However, the enzymes used in the previous reactions were seriously wasted after the reactions, although the amount of the enzyme used (75 mg) was much lower than that of the substrate (ca. 300 mg, 1 mmol) [1–3]. Then, we are now focusing on the improvements that enable the recovery and recycling of the enzyme in order to establish the more effective and environmentally benign procedures.

Immobilization of enzymes is a typical and important technology for easy treatment, recovery and recycling of biocatalysts in practical reaction processes [5–7]. In particular, the immobilization of lipases, which are utilized not only to obtain optically active molecules in fine chemistry as mentioned above but also to produce various organic compounds such as agrochemical, pharmaceutical, food, biodiesel fuel and so on, in industrial chemistry, has significant advantages for achieving environmentally benign production and reducing their process costs [8–11]. Therefore, a number of studies for the immobilization of lipases have been developed. Among these procedures, the entrapment within a polymer lattice and the physical adsorption onto support materials are the representative techniques so far. Especially, in the latter case, some lipases immobilized onto diatomaceous earth and ceramics as the inorganic support matrices are easily obtained from commercial sources, and those immobilized lipases are useful for increasing the reactivity of transesterification in organic solvents and for easy recycling of the enzymes from organic solvents. However, the immobilized lipases by the physical adsorption are not suitable for the hydrolytic reactions in aqueous media, because the leaching of the lipases from the support matrices can easily occur under the reaction conditions to result in the decisive loss of the hydrolytic activity in the recovered materials.

* Corresponding author. Tel.: +81 72 751 9525; fax: +81 72 751 9628.
** Corresponding author. Tel.: +81 42 591 7360; fax: +81 72 751 7419.
E-mail addresses: m-fujiwara@aist.go.jp (M. Fujiwara), mkazu@chem.meisei-u.ac.jp (K. Matsumoto).

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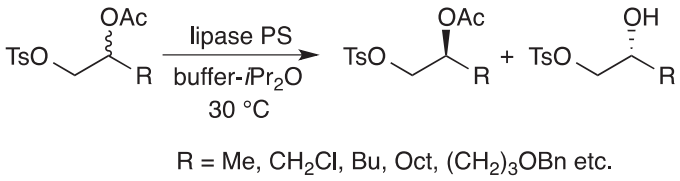

Scheme 1. Enantioselective hydrolysis of 1,2-diol monotosylate derivatives using lipase PS.

Calcium carbonate is a well-known biocompatible and environmentally-friendly material appropriate for an inorganic support matrix of enzyme immobilization, and some encapsulation techniques of proteins and enzymes have been studied [12–19]. Since calcium carbonate materials are generally non-porous to be unsuitable for the adsorption and inclusion of proteins, the additional components and processes such as carboxymethyl cellulose [15], silane coupling reagent [16], layer-by-layer techniques [17,18] and gold nanoparticles [19] are often required for immobilizing enzymes. Recently, porous calcium carbonate materials are prepared and utilized for the fixation of various molecules and proteins, where fine nanoparticles of calcium carbonate in amorphous [20] or calcite [21] are used. Lipases are also immobilized onto calcium carbonate related materials [22–25], and an immobilized lipase from *Rhizopus oryzae* onto calcium carbonate by adsorption was an active and stable catalyst, which could be used repeatedly in *n*-hexane solution [22]. However, a lipase-immobilized calcium carbonate material is reported to be difficult to recover in the case of olive oil hydrolysis in the presence of considerable amount of water [24], despite lipases are very useful for the reactions even in aqueous solutions. Thus, the further improvements of the lipase immobilization into calcium carbonate are desired especially for the utilization in aqueous solutions.

We have also studied the preparations and the applications of calcium carbonate materials prepared by using water/oil/water emulsion (interfacial reaction method) [26]. These calcium carbonate materials (microcapsules) are spherical and hollow, and its crystalline form is a metastable vaterite phase [27,28]. Recently, we found a simple and effective procedure of the protein encapsulation that the phase transformation of the vaterite calcium carbonate microcapsule to stable calcite phase in aqueous solutions with dissolved proteins achieved the inclusion of a wide variety of proteins into calcite calcium carbonate [29,30]. In this paper, we wish to report our successful approaches to immobilize lipase PS into vaterite calcium carbonate microcapsule using the phase transformation technique for the enzymatic enantioselective transesterification of racemic 1-phenylethanol (**1**) in organic media and the hydrolysis of racemic 2-acetoxyhexyl tosylate (**2**) in aqueous media. Furthermore, for improving the recyclability of the catalyst in the reaction of aqueous solutions, the treatment of calcium carbonate with zinc chloride to maintain the calcium carbonate in the vaterite crystalline phase was also described.

2. Experimental

2.1. Materials

The chemicals and proteins employed here are commercial available and listed as follows. $(NH_4)_2CO_3$, $CaCl_2{\cdot}2H_2O$, and 0.05 M Tris–HCl buffer (pH 7.6), 1 M Tris–HCl buffer (pH 7.0), (±)-1-phenylethanol (**1**) and 4-nitrophenyl acetate were obtained from Wako Pure Chemical Industries. Tween 85, vinyl acetate (monomer) and *n*-hexane were purchased from Kanto Chemical Co. Inc. Lipase PS from *B. cepacia* (534641) and lipase PS-IM (immobilized on diatomaceous earth, 709603) were obtained from Sigma–Aldrich Co. LLC. A substrate (±)-2-acetoxyhexyl tosylate (**2**) was synthesized by the procedure mentioned in our previous paper [3]. E. Merck Kieselgel 60 F_{254} Art.5715 was used for analytical TLC. Preparative TLC was performed on E. Merck Kieselgel 60 F_{254} Art.5744. Column chromatography was performed with Silica Gel 60N (63–210 μm, Kanto Chemical Co. Inc.). All other solvents were also obtained from commercially sources and were used without further purification.

2.2. Preparation of vaterite calcium carbonate microcapsule (μCap)

Vaterite calcium carbonate microcapsules (μCap) were prepared by a described method in our recent papers [28,29]. A typical procedure is as follows: an aqueous solution (32 mL) of 9.23 g (96 mmol) of $(NH_4)_2CO_3$ was mixed with a *n*-hexane solution (48 mL) of 1.0 g of Tween 85, and the resulting solution was emulsified with 8000 rpm for 1 min using a homogenizer (IKA-T25 digital ULTRA-TURRAX) with shaft generator S25N-25 F to form water/oil (W/O) emulsion. This W/O emulsion was poured into another aqueous solution (640 mL) of 28.2 g (192 mmol) of $CaCl_2{\cdot}H_2O$ in one portion at 30 °C. The final solution was stirred for 300 rpm at the same temperature for 5 min, and the white precipitate yielded was filtered, and washed with 1 L of deionized water twice and with 100 mL of methanol. Finally, the power samples were dried at 80 °C for 12 h.

2.3. Encapsulation of lipase PS into μCap

This process is generally analogous to reported one [29]. To a solution of lipase PS (1.0 g; 0.2 g per gram of μCap, 2630 units for the enzymatic activity) in Tris–HCl buffer (pH 7.0 or 7.6, 200 mL) was added 5 g of μCap. The resulting solution with μCap solid was stood at room temperature for 4 days. This solid was filtered, washed with enough amounts of fresh deionized water, and dried at room temperature for a few days (4.3 g; 731 units). The crystal phase and the morphology of these calcium carbonate materials (lipase PS μCap) were analyzed by XRD measurement and SEM observation, respectively. The presence of the enzyme in these calcium carbonate materials was ascertained by diffuse reflection UV spectroscopic analysis. For the enzymatic activity, see Section 2.6.

2.4. Zinc chloride treatment of μCap encapsulating lipase PS

To a solution of zinc chloride (0.46 g) in 1 M Tris–HCl buffer (pH 7.0, 96 mL) was added 2.4 g (408 units) of μCap encapsulating lipase PS (lipase PS μCap). After leaving the solution to stand for 1 day, the solid samples were filtered, washed with enough amounts of fresh deionized water, and dried at room temperature for a few days (1.7 g; 68 units).

2.5. Characterization of materials

X-ray diffraction patterns were recorded using Mac Science MXP3V diffraction meter with Ni filtered Cu Kα radiation (λ = 0.15406 nm). Scanning electron microscopy (SEM) images were measured using JEOL JSM-6390 microscope apparatus. For SEM observation, gold was deposited on the each sample using an ion sputtering device (JEOL JFC-1500). Powder UV spectrum measurement was carried out using JASCO V-550 spectrometer with an integrating sphere for diffuse reflectance UV spectroscopy. Kubelka–Munk functions were plotted against the wavelength.