Materials Science and Engineering C 45 (2014) 261–269

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Improvement of the activation of lipase from *Candida rugosa* following physical and chemical immobilization on modified mesoporous silica

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ARTICLE INFO

Article history:
Received 28 December 2013
Received in revised form 3 September 2014
Accepted 14 September 2014
Available online 16 September 2014

Keywords:
Co-template
Post synthesis-grafting
Co-condensation
2-Octanol
Enzymatic resolution

ABSTRACT

Lipase from *Candida rugosa* (CRL) was chemically and physically immobilized onto four types of rod-shaped mesoporous silica (RSMS). RSMS prepared using surfactant P123 and poly(ethylene glycol) as co-templates was functionalized with (3-aminopropyl)triethoxysilane (APTES) to obtain P-RSMS by post-synthesis grafting. Tetraethoxysilane was hydrothermally co-condensed with APTES to obtain C-RSMS. A two-step process using APTES and glutaraldehyde was also performed to obtain G-RSMS. The effects of modification methods (including post-synthesis grafting and co-condensation) and glutaraldehyde on the mesoscopic order, interplanar spacing d_{100}, cell parameter a_0, mesoporous structure, and wall thickness of RSMS were studied in detail. Results showed that all samples were mesoporous materials with 2D mesostructures ($p6mm$). Pore size and d_{100} decreased, whereas the wall thickness increased after different modifications. CRL was used as a model enzyme to determine the effect of physical and chemical adsorption on loading amount and enzymatic activity. The possible mechanism of CRL immobilization on G-RSMS by chemical adsorption was systematically investigated. The chemical immobilization of CRL on G-RSMS increased the loading amount, hydrolytic activity, thermal stability, and reusability. Moreover, immobilized CRL was employed to catalyze the resolution of 2-octanol by esterification with caprylic acid. The enantiomeric excess of 2-octanol was 45.8% when the reaction was catalyzed by G-RSMS-CRL and decreased to about 38%–39% using the physically immobilized CRL, after 48 h of reaction in hexane.

1. Introduction

Lipase from *Candida rugosa* (CRL) is an enzyme with considerable physiological and industrial significance because of its high reactivity. However, the activity of lipase gradually decreases in hydrothermal solutions because of its instability. Thus, lipase immobilization on solid support structures is a popular but challenging approach in designing alternative biocatalysts with potentially unlimited applications [1]. Three traditional methods can be used to immobilize lipase: support binding, entrapment (encapsulation), and cross-linking [2]. Support binding can be physical (e.g., hydrophobic and van der Waals interactions), ionic, or covalent in nature. In entrapment, lipase is enclosed in membrane structures (e.g., hollow fibers or microcapsules) or polymer networks (e.g., organic polymer or silica sol–gel). In cross-linking, lipase aggregates or crystals are chemically cross-linked using a bifunctional reagent to prepare carrier-free macroparticles.

Silica-based mesoporous materials have recently been regarded as suitable hosts for large molecules, such as lipases, because of their high surface area, tunable pore diameter, and abundant silanol (Si–OH) groups [3–8]. Zhou et al. [5] immobilized CRL using mesoporous, vesicle-like silica and observed successfully increased CRL activity and applicability. However, the interaction between the enzyme and hydrophilic lipid substrate interface is insufficient. Numerous hydrophobic functional reagents that provide a large number of amines, chlorides, and phenyls for covalent cross-linking with immobilized lipase are used for functionalization reactions to improve the stability of the immobilized enzyme [9]. A previous research has shown that mesoporous silica can be readily functionalized because of the abundant Si–OH groups on its surface. Polyethylene glycol (PEG) can easily be cross-linked with hydrogels and its molecular weight can be controlled. Polymer–surfactant complexes have been used as templates to synthesize mesoporous materials because their synergic structures are distinct from those of pure polymers or surfactant systems [6]. Surfactants and PEG can be used as stabilizers for crystals to prevent direct precipitation and aggregation. These substances can also maintain the structural stability of the template and exhibit an important function in the uniformity and polydispersity of materials. Post-synthesis grafting and co-condensation are commonly used for specific functionalization. Amino-functionalized mesoporous silicas fabricated by post-synthesis grafting have been traditionally prepared by coupling reactions between surface Si–OH groups and aminopropyltrialkoxysilane. However, grafted amine species in multiple states have been found; most of these species are hydrogen-bonded to one another or to surface Si–OH

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http://dx.doi.org/10.1016/j.msec.2014.09.026
0928-4931/

groups, which are not effective active sites [10–14]. Moreover, physical bonding (physical adsorption) is generally extremely weak to maintain a fixed lipase on the support. This characteristic leads to low stability caused by the leaching of adsorbed lipases. Meanwhile, co-condensation enables better control of pore size [15,16]. The stability of co-condensation-synthesized mesoporous materials is also higher than that of materials synthesized by post-synthesis grafting. However, modified materials prepared by co-condensation have poorly ordered mesostructures.

Poor structural ordering and adsorbed enzyme leaching can be solved by lipase immobilization through chemical adsorption. In this process, enzyme molecules are covalently bound onto ordered silica surfaces after surface functionalization with (3-aminopropyl) triethoxysilane and glutaraldehyde. Organo-functionalized mesoporous silicas obtained from the cross-linking method have their functional groups spatially dispersed on the surface [17–19]. The glutaraldehyde technique is highly versatile in immobilizing and stabilizing lipases [20–25]. In particular, glutaraldehyde treatment of lipases that were previously immobilized onto carriers bearing primary aminopropyl groups produces highly stable materials. The high stability is due to the cross-linking between glutaraldehyde molecules bound to the lipase and glutaraldehyde molecules bound to the support; such interaction demonstrates that the lipase surface can be chemically modified [26]. However, the strong interactions between lipase molecules and the support surface can affect lipase activity [12,19]. Therefore, the effect of support surface functionalization on the activity of immobilized lipase should be evaluated on a case-to-case basis. Immobilization by chemical adsorption using glutaraldehyde was also performed based on previously described immobilization protocols. The loading amounts and activity of CRL were compared with those of free and physically adsorbed CRL. Unlike in our previous study [6], the RSMS was prepared using P123 and PEG as co-templates and was functionalized with APTES and glutaraldehyde to obtain P-RSMS, C-RSMS, and G-RSMS by post-synthesis grafting, co-condensation, and a two-step process using APTES and glutaraldehyde, respectively. The effects of the modification methods (including post-synthesis grafting and co-condensation) and glutaraldehyde on the mesoscopic order, interplanar spacing d_{100}, cell parameter a_0, mesoporous structure, and wall thickness of the RSMS were studied in detail. The grafting ratios of P-RSMS, C-RSMS, and G-RSMS were calculated by thermogravimetric analysis (TGA). In addition, the enzymatic activity and possible mechanism of CRL immobilization on G-RSMS by chemical adsorption were systematically investigated. Immobilized CRL was also employed to catalyze the resolution of 2-octanol by esterification with caprylic acid.

2. Materials and methods

2.1. Materials

Triblock copolymer Pluronic P123 poly(ethylene glycol)-*block*-poly (propylene glycol)-*block*-poly(ethylene glycol), Lipase from *Candida rugosa* (CRL), (3-aminopropyl)triethoxysilane, and triacetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Poly(ethylene glycol) and glutaraldehyde solution (25%) were purchased from Sinopharm Chemical Reagent Company (Shanghai, China). 2-Octanol was purchased from Aladdin Chemical Co. (Shanghai, China). Tetraethoxysilane (TEOS), caprylic acid, and other chemicals were obtained from Tianjin Kermel Chemical Company (Tianjin, China). All chemicals were employed without further purification.

2.2. Methods

2.2.1. Preparation of RSMS and P-RSMS

RSMS and P-RSMS were fabricated according to the methods published by our group [6]. The molar composition of the mixture was 1 TEOS: 0.017 P123:5.71 HCl:192 H_2O:0.0096 PEG. P123 and PEG were dissolved in a mixture of water and 2 M HCl aqueous solution. The resulting solution was stirred at 313 K until a clear solution was obtained. TEOS was added dropwise into this solution with vigorous stirring. After stirring at 313 K for 24 h, the reaction solution was transferred into a Teflon-lined autoclave and stored at 383 K for 24 h under static conditions. The white solid products were collected by filtration, washed with water, air-dried at room temperature, and then calcined at 873 K in flowing air for 5 h in a tube furnace to remove the organic template. The sample was designated as RSMS.

Post-synthesis grafting was performed as follows [27]. RSMS was suspended in anhydrous toluene and then added with APTES. The reaction mixture was refluxed for 2 h, and the resulting amino-functionalized material was collected by filtration and washed properly with toluene to remove any unreacted reagent. The white solid was dried under vacuum and designated as P-RSMS.

2.2.2. Co-condensation synthesis of C-RSMS

The co-condensation synthesis of C-RSMS was conducted as follows. P123 and PEG were dissolved in a mixture of water and HCl solution. TEOS was added dropwise into the solution with vigorous stirring. The resultant solution was pre-hydrolyzed for 2 h and then slowly added with APTES. The molar composition of the mixture was 0.85 TEOS:015 APTES:5.71 HCl:0.017 P123:192 H_2O:0.0096 PEG. The resulting mixture was stirred at 313 K for another 18 h, transferred into a Teflon-lined autoclave, and then stored at 383 K for 24 h under static conditions. The solid products were collected by filtration, washed with water, and then dried at room temperature. The surfactant in the products was removed through Soxhlet extraction. The as-synthesized sample was added with ethanol and then refluxed for 24 h. The final white solid material was denoted as C-RSMS.

2.2.3. Glutaraldehyde synthesis of G-RSMS

G-RSMS was synthesized using a two-step process. In the first step, P-RSMS was prepared. In the second step, G-RSMS was prepared by soaking P-RSMS (2.5 g) in a mixture of 25% aqueous glutaraldehyde (2 mL) and 0.1 M phosphate buffer solution (18 mL, pH 7.5) for 1 h at room temperature. The product was washed exhaustively with distilled water and finally with the same buffer on a filter. The product was dried at room temperature and designated as G-RSMS [17].

2.3. Preparation of CRL immobilized in four supports

CRL powder was dissolved in 0.02 mol L^{-1} of phosphatic buffer (pH 6.0) to obtain a 2 mg mL^{-1} concentration. Lipase solution was mixed with RSMS [m (CRL): m (carrier) = 1:1] and then stirred magnetically at 298 K for 6 h. Afterward, the supernatant was separated from the solid by centrifugation at 6000 rpm for 10 min at 277 K. The CRL concentration of the supernatant was measured using a UV spectrophotometer at 258 nm, and the CRL content of the supernatant was recalculated. A mass balance for lipase solution before and after immobilization was applied to calculate the adsorbed lipase amount using the Bradford method according to Eq. (1) [28]:

$$P = \frac{C_i - C_f}{W} V \tag{1}$$

where P is the amount of bound enzyme onto the supports (mg g^{-1}, mg CRL per gram RSMS), C_i and C_f are the initial and final lipase concentrations in the reaction medium (mg mL^{-1}), V is the volume of the reaction medium (mL), and W is the weight of the support (g).

P-RSMS, C-RSMS, and G-RSMS were used similarly as described above for CRL immobilization. The CRL samples immobilized onto RSMS, P-RSMS, C-RSMS, and G-RSMS were designated as RSMS-CRL, P-RSMS-CRL, C-RSMS-CRL, and G-RSMS-CRL, respectively.