Process Biochemistry 47 (2012) 953–959

Contents lists available at SciVerse ScienceDirect

Process Biochemistry

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

Active biocatalysts based on *Candida rugosa* lipase immobilized in vesicular silica

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ARTICLE INFO

Article history:
Received 18 January 2012
Accepted 13 March 2012
Available online 20 March 2012

Keywords:
Vesicular silica
Candida rugosa lipase
Immobilization
Activity
Thermal stability

ABSTRACT

Vesicular silica (VS) with hierarchical structure was prepared by utilizing cationic surfactant cetyltrimethylammonium bromide (CTAB) and anionic surfactant sodium dodecyl sulfate (SDS) as the structure directing agents, and 1,3,5-triisopropylbenzene (TIPB) as the micelle expander. The resulting unilamellar and multilamellar VS with interlamellar mean mesopore size of 15–20 nm and shell thickness of 5–15 nm were used as supports for immobilization of *Candida rugosa* lipase (CRL) through physical adsorption. Possible mechanisms for the formation of VS and the immobilization of CRL on VS are proposed. N_2 adsorption-desorption experiments and Fourier transform infrared spectroscopy (FT-IR) measurements demonstrated that CRL was adsorbed into the curved channels of the VS. The catalytic activity, thermal stability, and reusability of VS immobilized CRL were assayed in phosphate buffer medium by hydrolysis of triacetin. The effects of pH and temperature on enzyme activity were also investigated. We report that VS immobilized CRL exhibited outstanding adaptability at higher pH and temperature, and excellent thermal stability and reusability compared with free CRL.

1. Introduction

The past decade has witnessed a rapid evolution in the synthesis of silica mesoporous materials with high specific surface areas, large pore volumes and diverse morphologies such as rod-like [1], fibers [2], cage-like [3], hollow spheres [4], films [5], and vesicle-like [6–9] by choosing various templates, swelling agents and using different synthesis approaches. Jensen et al. [10] first reported the biomimetic synthesis of mesoporous amorphous silica in aqueous solution at room temperature. These materials have been extensively applied in diverse areas such as immobilization techniques [11,12], catalysis [13], drug delivery [14], sensors [15] and separation process [16].

In 1996, Pinnavaia et al. [6] first demonstrated the feasibility of vesicular silica with a high degree of framework cross-linking, and high specific surface area and pore volume by employing bolaamphiphiles $H_2N(CH_2)_nNH_2$ (n = 12–22) as the structure directors through a biomimetic templating approach. Subsequently, silica materials with unique vesicle-like morphology and lamellar framework have been prepared by using various surfactants as templates [7–9]. Zhou et al. [11] used 1,3,5-triisopropylbenzene (TIPB) as a hydrophobic agent for adjusting the packing parameter of the surfactant to tune the size and morphology of the mesopores which resulted in a multilamellar vesicular silica with large uniform intershell spaces. They also immobilized enzyme in mesoporous vesicle-like and rod-like silica by physical adsorption. The properties of the lipase immobilized into the vesicle-like mesoporous silica are superior to that into the rod-like mesoporous silica. Besides the advantages of tunable pore size, large surface area and pore volume, vesicular silica possesses curved interlamellar mesochannel and multilamellar structure as well as the added inter-shell flexibility arising from the special vesicular structure. The features increase the affinity interaction between the silanol groups of vesicular silica and the lipase, resulting in a slower lipase leakage during the recycling process and are helpful to applications in proteins adsorption and enzyme catalysis. The higher stability and activity of the lipase immobilized in vesicular silica make it an excellent candidate for a new bioimmobilization host. However, they have not been widely studied in applied technological areas because of small pore size (interlamellar space) non-uniform morphologies.

Candida rugosa lipase (CRL) is a stable mesophilic lipase that has high activity and broad specificity in reaction medium. However, free lipase is often unstable and possesses low activity in organic solvents or in a harsh environment such as high temperature or extreme pH. Immobilization of CRL on various supports promotes the separation of products and lipases and improves catalytic activity, stability, and recyclability of immobilized lipase in continuous operations. A series of recent studies have reported investigating immobilization of CRL on different carriers [17–22]. Dyal et al. [17] reported immobilization of CRL on γ-Fe_2O_3 magnetic nanoparticles and studied the stability and enzymatic activity of the immobilized lipase. In 2010, effects of pore diameter and cross-linking method on the immobilization efficiency of CRL in SBA-15

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doi:10.1016/j.procbio.2012.03.004

with different pore sizes were investigated by Gao et al. [20]. Ye et al. [22] demonstrated the feasibility of covalently immobilizing CRL onto the nanofibrous poly-(acrylonitrile-co-maleic acid) (PANCMA) membrane formed by the electrospinning procedure. Moreover, they explored the effects of fiber diameter on kinetic parameters, enzyme loading, and activity of the immobilized lipase.

In this paper, we report the synthesis of vesicular silica (VS-n (n = 0, 1), where n is the molar ratio of 1,3,5-triisopropylbenzene (TIPB)/sodium dodecyl sulfate (SDS)) in aqueous solution by utilizing cetyltrimethylammonium bromide (CTAB) and SDS as co-surfactants, and a small amount of the hydrophobic additive TIPB. VS-1 was prepared in presence of TIPB and VS-0 was synthesized in absence of TIPB. These materials were used as carriers for the immobilization of CRL by physical adsorption. The optimum catalytic conditions for free and immobilized CRL to function as biocatalysts in the hydrolysis of triacetin were determined. Simultaneously, the thermal stability and reusability of different immobilized CRL were also compared. The results indicate that the properties of lipase adsorbed on VS-1 surpass the properties of those absorbed on VS-0. The differences in the amounts of CRL adsorbed and the activities of CRL between VS-0 and VS-1 may be attributed to their pore sizes. The pore diameter of VS-1 by adding swelling agent TIPB is increased, which may improve the transport of the CRL molecule, making it favorable for the enzymatic reaction. Vesicular silica with large pores prepared in this manner has potential applications in separation, catalysis and drug delivery. However, both two catalysts had low stability and the further cross-linking among the adsorbed CRL in supports would be attempted to strengthen the stability of immobilized CRL.

2. Materials and methods

2.1. Chemicals

C. rugosa lipase (CRL), 1,3,5-triisopropylbenzene (TIPB), sodium dodecyl sulfate (SDS), and triacetin (99%) were purchased from Sigma–Aldrich. Cetyltrimethylammonium bromide (CTAB) was obtained from Shanghai Chemical Reagent Inc. of Chinese Medicine Group. Tetraethoxysilane (TEOS), and other chemicals were of analytical grade and were used as received without any further purification.

2.2. Synthesis of VS

In a typical synthesis, 0.28 g of SDS was dissolved in 26.65 g of deionized water with continuous stirring at 35 °C to form a clear aqueous solution, and 0.61 g of CTAB was dispersed into the solution. After stirring for 2–3 h, specific amounts of TIPB (molar ratios of TIPB/SDS being 0 and 1) were added drop-wise into to the mixture for the preparation of two samples. The aqueous solution was stirred for another 18 h. Then, 4.2 g TEOS was added under vigorous stirring. The final molar ratio is 1 SDS/1.72 CTAB/x (x = 0, 1) TIPB/20.76 TEOS/1524.77 H_2O. This reaction mixture was constantly stirred at 35 °C for 24 h, then sealed within a Teflon autoclave and heated at 100 °C for 24 h. The solid products were collected by filtration, washed with water, and dried at room temperature. Finally, the resultant powder was calcined at 550 °C for 6 h in air to remove the surfactant template. The surfactant-free samples were denoted as VS-n (n = 0, 1), where n is the molar ratio of TIPB/SDS.

2.3. Characterization

Field emission scanning electron microscopy (FESEM) was performed with a JEOL JEM-6700F microscope operating at an accelerating voltage of 5 kV and electric current of 10 μA. Samples were mounted on the surface of a silicon wafer and sputtered with a thin film of gold to avoid charging under the electron beam prior to examination. Transmission electron microscopy (TEM) was carried out on a JEOL JEM-1400 microscope (accelerating voltage of 120 kV). Samples for TEM measurements were suspended in ethanol by ultrasound for 15 min and subsequently deposited on a carbon-coated copper grid. N_2 adsorption-desorption isotherms were measured at 77 K using a Gemini V 2380 system. Before measurement, samples were outgassed at 120 °C for at least 10 h. The specific surface areas, S_{BET}, were calculated using the Brunauer–Emmett–Teller (BET) method based on the adsorption data, while the pore diameter distribution was derived from adsorption branch using the Barrett–Joyner–Halenda (BJH) model. Fourier transformed infrared (FT-IR) spectra were collected on samples in KBr tablets using a Bruker Tensor 27 Fourier transform infrared spectrophotometer.

2.4. Preparation of VS-n immobilized CRL

The immobilization of CRL was performed as following: first, 40 mg of VS-n was suspended in 20 ml of CRL phosphate buffer (pH 6.0) solution with a concentration of 2 mg/ml in a 50 mL capped vial. The mixture was stirred by a magnetic stirrer at 25 °C for 6 h and was then centrifuged at 6000 rpm and 4 °C for 10 min for obtaining CRL immobilized VS. All the protein concentrations were determined by the Bradford method [23], using bovine serum albumin (BSA) as standard. The immobilization yield (IY) and the amount (P) of CRL loaded in support were calculated as follows:

$$P = \frac{(C_i - C_f) \times V_i - C_w \times V_w}{W} \quad (1)$$

$$\mathrm{IY}(\%) = \frac{(C_i - C_f) \times V_i - C_w \times V_w}{C_i \times V_i} \quad (2)$$

where IY is the immobilization yield (%); P is the amount of CRL loaded in support ($mg_{protein}/g_{support}$); C_i, C_f, and C_w are the protein concentrations of initial solution, final solution, and wash solution of CRL, respectively (mg/ml); V_i and V_w are the volume of initial solution and wash solution of CRL (ml); W is the weight of support (g).

After immobilization of lipase, samples were designated as VS-0-CRL or VS-1-CRL depending on the molar ratio of TIPB and SDS used in the preparation.

2.5. Activity assays

A triacetin emulsion was prepared by vigorously stirring a mixture of triacetin (1.0 g), deionized water (25 ml), and phosphate buffer (pH 7.0, 12 ml) at 35 °C for 10 min. Then free or immobilized CRL was added to the emulsion once pH was stabilized. The mixture was continuously titrated with 0.02 M NaOH solution for 10 min to maintain a constant pH. The volume of NaOH consumed in 10 min was recorded to calculate the specific activity of free or immobilized CRL. CRL activity unit was denoted as U/g, with one lipase unit (1 U) defined as the amount of enzyme required to hydrolyze 1 μmol triacetin per min at 35 °C.

Simultaneously, blank experiments were performed through the same procedure using the same amount (40 mg) of VS-n or without adding any VS. The volumes of NaOH solution consumed by blank experiments were eliminated. Our results showed that neither support nor substrate could consume significant amounts of NaOH solution. All of the experiments were repeated in triplicate. The absolute activity of enzyme was calculated using the equation:

$$E_a = \frac{(V_i - V_0) \times C}{t \times m} \quad (3)$$

where E_a is the specific catalytic activity of enzyme ($U/g_{protein}$); V_i and V_0 are the volumes of NaOH solution consumed in the lipase solution and blank experiments (ml), respectively; C is the concentration of NaOH solution (mol/l); t is the reaction time (min), i.e. 10 min and m is the weight of protein (g).

Relative activity (R) of immobilized CRL was calculated according to the equation:

$$R(\%) = \frac{E_a}{E_m} \times 100\% \quad (4)$$

where R is the relative activity of immobilized CRL (%); E_a is the specific activity of immobilized CRL ($U/g_{protein}$); E_m is the maximal specific activity of immobilized CRL ($U/g_{protein}$).

2.6. Effects of pH and temperature on CRL activity

The activity of free or immobilized CRL at different pH and temperature was measured as described previously. Optimum pH were compared in different buffers within the pH range of 5.0–9.0 (0.02 M K_2HPO_4–$C_6H_8O_7$ buffer solution for pH 5.0, 0.02 M phosphate buffer solution for pH 6.0–8.0, and 0.02 M Na_2CO_3–$NaHCO_3$ buffer solution for pH 9.0) at 35 °C. Thermal inactivation experiments were performed in phosphate buffer (pH 7.0) at temperatures ranging from 30 °C to 70 °C.

2.7. Reusability and deactivation stability of free and immobilized CRL

Phosphate buffer of free or immobilized CRL was heated in capped cuvettes in a water bath at 60 °C for desired time (15, 30, 60, 90, 120, and 150 min). Free or immobilized CRL was collected by centrifugation, and their relative activities were calculated as described above. The reusability of immobilized CRL was studied using the same substrate concentration, temperature, and reaction time as mentioned above. The initial activity of immobilized CRL was compared with the activity of the used CRL obtained after repeated use for 6 cycles.