Journal of Molecular Catalysis B: Enzymatic 95 (2013) 9–15

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

The study on effective immobilization of lipase on functionalized bentonites and their properties

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ARTICLE INFO

Article history:
Received 8 January 2013
Received in revised form 27 March 2013
Accepted 20 May 2013
Available online 31 May 2013

Keywords:
Functionalized bentonite
Lipase
Immobilization
Interfacial activation
Kinetic parameters

ABSTRACT

Three different functionalized bentonites including acid activated bentonite (B_a), organically modified bentonite with cetyltrimethyl ammonium bromide (B_{CTMAB}) and the composite by acid activation and organo-modification ($B_{a\text{-}CTMAB}$) were prepared, and used for immobilization of lipase from bovine pancreatic lipase by adsorption. The amount of lipase adsorbed on the functionalized bentonites was in the following sequence: $B_a > B_{CTMAB} > B_{a\text{-}CTMAB}$, showing the strongest affinity of B_a for lipase among the three supports. However, the immobilized lipase on $B_{a\text{-}CTMAB}$ showed the highest activity in the hydrolysis of olive oil by 1.67 times of activity of free lipase due to the hydrophobically interfacial activation and enlarged catalytic interface. While, the activity of immobilized lipase on B_a was lower than 20% of free lipase's activity due to the absence of hydrophobic activation and negative impact of excessive hydrogen ions on the surface. The K_m values for the immobilized lipase on $B_{a\text{-}CTMAB}$ (0.054 g/mL) and B_{CTMAB} (0.074 g/mL) were both lower than that of free lipase (0.115 g/mL), and the V_{max} values were higher for the immobilized lipases, exhibiting a higher affinity of the immobilized lipase toward olive oil than free lipase. In comparison to free lipase, the better resistance to heating inactivation, storage stability and reusability of the immobilized lipases on $B_{a\text{-}CTMAB}$ and B_{CTMAB} were also obtained. The results show that the efficient and stable biocatalysts for industrial application can be prepared by using the low-cost bentonite mineral as the supports.

1. Introduction

Enzyme-mediated reactions always have been gained an enormous attention depending on their higher catalytic efficiency and specificity under milder conditions in comparison with chemical catalysts [1]. Lipase is one class of the important enzymes with practical significance, and widely applied in a variety of industries, such as food, fine chemistry and pharmaceutical industries [2,3]. However, their widespread application is often hampered by low catalytic and operational stabilities. Among the methods for stabilization of enzymatic activity [4–7], the immobilization of lipase has been extensively studied because it also can provide the following crucial advantages for the continuous fabrication on a large-scale: reutilization of lipase, facilitating the separation and preparation of aim products, enhancing the lipase's stability under changeable and extreme conditions [7–9]. Of the main immobilization methods including adsorption, entrapment, covalent bonding and cross-linking [10–12], physical adsorption is universally acknowledged as a simpler one without introducing redundant and toxic reagents [13,14]. More importantly, physical adsorption shows less negative affect on the conformation of lipase, and a high catalytic activity is always obtained [13,15,16]. Numerous supports for the immobilization of lipases from different sources by adsorption have been investigated [15–17]. Comparative studies have indicated that the marked differences in the activities of immobilized lipases on different supports are observed [15–18]. The catalytic performance of immobilized enzyme can be affected by the support through the following ways: diffusion of substrate and product, the conformation of enzyme and the interaction between the support and the reaction medium [19,20].

Nowadays, relying on the advantages of mechanical stability, high adsorption and availability of reactive functional groups, various inorganic support materials are more and more popular in the application of enzyme immobilization [3,16,18–21]. In the past decade, functionalizations of inorganic materials for lipase immobilization have been frequently performed by different reactive compounds with unique properties [13,15,16,20]. Some studies have showed that preparation of composite supports with hydrophilicity–hydrophobicity balanced surfaces could adjust the structures of supports to be appropriate for certain

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http://dx.doi.org/10.1016/j.molcatb.2013.05.018

biocatalytic applications [14,18,22], and promote the "interfacial activation" by shifting the lipase's active center from closed form (inactive form) toward open form (active form) [15,16,23]. Furthermore, a hydrophilicity/hydrophobicity balanced support with high specific surface can enlarge the effectively catalytic area of immobilized lipase, improve the affinity between hydrophobic substrate and lipase's active center, and decrease the diffusion resistance of substrate [16]. Therefore, preparation of suitable supports for enzyme immobilization has become an important subject.

Bentonite, a layered material constituting primarily of montmorillonite, has the potentially interesting properties including high specific surface, thermal stability, natural abundance, chemical and bacterial resistance, and electrostatic interactions [24,25]. The original bentonites are easily obtainable and cheaper than other inorganic materials such as carbon nanotubes and nano silica. Moreover, the presence of silanol groups provides the active sites for structural and functional adjustment to tailor-make suitable supports for enzyme immobilization [13,14,26]. The original bentonites have been applied to support the enzymes to improve the reusability and thermal stability of lipase and α-amylase [24,27]. However, their activities were observed to decrease markedly mainly resulting from the absence of hydrophobic interfacial activation for lipase, the block of the active sites, and low affinity to hydrophobic substrates [16,28]. Ghiaci et al. prepared the organo-bentonites for enzyme immobilization, showing better biocompatibility with enzymes than original bentonites [29]. However, the catalytic activities of immobilized lipases were still low, possibly due to the absence of effectively adjusting the balance of hydrophilicity/hydrophobicity, surface property and structure of bentonite for the catalytic performance of guest lipase. To the best of our knowledge, there has been no report concerning the application of functionalized bentonites by acid activation, organo-modification and combination of both methods in the immobilization of lipase by adsorption, for the purpose of its catalytic performance enhancement.

In this study, the different functionalized bentonites were prepared including organo-bentonite, acid activated bentonite and the composite bentonite by acid activation and organo-modification, and used for immobilization of lipase from bovine pancreatic lipase by adsorption. Olive oil was used as a model substrate to investigate the catalytic activities of the free and immobilized lipases. The adsorptions of lipases on these prepared supports were investigated to evaluate and compare the affinity between lipase and the selected supports. The catalytic efficiencies of the immobilized lipases were compared to get the truth how the organobentonite and composite affected the activity of lipase. The catalytic kinetics of the free and immobilized lipases were investigated, and their reusability and storage stability were also studied.

2. Materials and methods

2.1. Materials

The bovine pancreatic lipase (15–35 U mg^{-1}), olive oil and polyvinyl alcohol were purchased from Aladdin Chemicals, Shanghai, China. Bovine serum albumin and cetyltrimethyl ammonium bromide (CTMAB) were obtained from Aldrich. All other chemicals were of analytical grade. The original bentonite (denoted Na-bentonite) consisting primarily of montmorillonite was purchased from Inner Mongolia, China. The cationic exchange capacity (CEC) of dried Na-bentonite (NB) was determined to be 1.15 mmol g^{-1} by ammonium acetate method [30].

2.2. Functionalization of bentonites

The acid treated bentonite (B_a) was prepared as follows: 10 g Na-bentonite was dispersed in 200 mL sulfuric acid (15%, v/v) in 250 mL round bottom flask, and the mixture solution was refluxed at 80 °C for 2 h. Afterwards, the supernatant was removed by vacuum filter, and the resulting residue was washed repeatedly by deionized water to remove the excessive acid and determined to be neutral. The obtained acid treated bentonite was dried under 110 °C and ground to obtain the powder support. This support was denoted as B_a.

B_a was further modified with CTMAB to prepare $B_{a\text{-CTMAB}}$ as following steps: 5 g B_a was dispersed in 100 mL deionized water, and 2.0 g CTMAB was introduced into the solution, this mixture was continuously stirred at 60 °C for 15 h. The solid support was obtained by centrifugation, washed with deionized water, and dried at 60 °C. The resulting solid was ground and denoted as $B_{a\text{-CTMAB}}$.

The CTMAB modified bentonite (B_{CTMAB}) was prepared according to the following steps: 4.2 g CTMAB was added into 200 mL aqueous suspension of Na-bentonite (5%, w/v). The mixture was continuously stirred at 60 °C for 15 h. Then the solid was obtained by centrifugation, and washed with deionized water three times to remove the surfactant loosely attached to the bentonite. The moist solid was dried at 60 °C and ground to obtain the support of B_{CTMAB}.

2.3. Characterization of functionalized bentonites

The X-ray patterns of the supports were performed on a Panalytical diffractometer with CuKα radiation (40 kV, 40 mA) over a 2θ range from 2.5° to 40°. Fourier transform infrared spectroscopy (FTIR) was recorded on Thermo Nicolet Nexus between 4000 and 400 cm^{-1}.

2.4. Methods for immobilization of lipase

The immobilization of lipase onto B_a, B_{CTMAB} and $B_{a\text{-CTMAB}}$ was carried out as follows: 0.05 g lipase powder and 0.5 g each support were orderly dispersed in 5 mL corresponding buffer solution at the optimum pH, and each mixture solution was stirred at 200 rpm for every optimum time at room temperature [16]. After that, the supernatant solution was collected by centrifugation at 15,000 rpm for 15 min, the resulting immobilized lipase was washed three times by deionized water to remove impurities and dried under vacuum at room temperature.

The amount of protein before and after immobilization was measured by Bradford method using bovine serum albumin as the standard [31]. The amount of protein loaded on the support was determined by comparing the difference between the total amount of protein and the amount of protein in the washing solutions as the following the formula.

$$\text{Protein loading (mg g}^{-1}) = \frac{(C_0 - C_e)V}{W} \quad (1)$$

where C_0 and C_e are the concentration of lipase in the initial solution before immobilization and the concentration of lipase in the supernatant solution after immobilization (mg/mL), respectively, V is the volume of immobilization medium (mL), W is the dry weight of the support (g).

The effect of pH of immobilization medium on loading amount of lipase onto Na-bentonite and functionalized bentonites was investigated at room temperature, under the variety of pH 4.0–9.0.