Journal of Colloid and Interface Science 343 (2010) 359–365

Contents lists available at ScienceDirect

Journal of Colloid and Interface Science

www.elsevier.com/locate/jcis

Improved enzymatic activity of *Thermomyces lanuginosus* lipase immobilized in a hydrophobic particulate mesoporous carrier

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ARTICLE INFO

Article history:
Received 24 September 2009
Accepted 4 November 2009
Available online 10 November 2009

Keywords:
Lipase
Mesoporous
CLSM
Particulate
Carriers
Enzymatic activity
Supporting surfaces

ABSTRACT

Lipase from *Thermomyces lanuginosus* has been immobilized within particulate mesoporous silica carriers, with either hydrophilic or hydrophobic supporting surfaces, produced by the newly developed emulsion and solvent evaporation (ESE) method. The Michaelis–Menten model was used to calculate the parameters related to the enzymatic activity of lipase i.e. the turnover number, k_{cat}, and the specific activity. The specific activity was improved by immobilization of lipase onto the hydrophobic support, compared to lipase immobilized onto the hydrophilic support and lipase free in solution. The enhanced enzymatic activity of lipase onto a hydrophobic support was attributed to interfacial activation of the *Thermomyces lanuginosus* lipase when it is attached to a hydrophobic surface and a reduced denaturation. Confocal scanning laser microscopy (CLSM) studies, of fluorescently tagged lipase, showed that leakage of the lipase from the mesoporous particles was limited to an initial period of only a few hours. Both the rate and the amount of lipase leached were reduced when the lipase was immobilized onto the hydrophobic support.

1. Introduction

Lipases are used as versatile biocatalysts for ester synthesis, hydrolysis, alcoholysis, acidolysis, aminolysis and interesterfication; reactions of great importance in e.g. the food, detergent, leather, textile, cosmetic, paper and pharmaceutical industries [1]. The number of commercially available lipases has steadily increased since the first lipase developed for detergent industry, Lipolase®, was launched in 1988.

Heterogeneous catalysis, i.e. immobilization of the enzyme onto solid supports, e.g. different types of polymers, ceramics, and zeolites [2,3], have several advantages over homogeneous catalysis with respect to e.g. the ease of recovery and reusability [4–8]. The stability of biomolecules such as enzymes is also increased by the immobilization and it has been shown that the stability is improved by a close match of the pore size and the size of the enzyme [9–11]. While early work using sol–gel derived materials illustrated the importance of the pore size and the surface chemistry of the pore walls [12,13], it was not until the discovery of the surfactant-templated mesoporous materials [14], that a solid material with monodisperse and tuneable pore size became available. Immobilization of biomolecules into the mesoporous carrier may be carried out either by chemical binding or physical adsorption, e.g. through cross-linking of the enzyme onto the pore-walls or by encapsulation of the enzyme into the pore channels of the support [5,6,15] is also the simplest method and thus the most commercially viable alternative. The major drawback with physical adsorption is enzyme leakage due to weak interactions between the support and the enzyme. Attempts to reduce leakage by cross-linking of the enzyme inside the mesoporous carrier, or by silylation of the pore openings to reduce the size of entrance/exit of the pore channels, were only partially successful and frequently resulted in a reduction of the enzymatic activity [16–19]. On the other hand, hydrophobic treatments of the supports show a reduced leakage and studies also indicate an increase in the enzymatic activity [8,19–21].

In this work, lipase from the fungus *Thermomyces* (formerly *Humicola*) *lanuginosus* has been immobilized in mesoporous spherical particles produced by the newly developed emulsion and solvent evaporation (ESE) method [22]. The pore walls have been silylated and we have performed a systematic comparison of hydrophilic and hydrophobic mesoporous carriers. The specific enzymatic activity (EU/mass lipase) as well as the turnover number, k_{cat}, have been determined for both materials using a water soluble reactant, 4-nitrophenyl acetate. The spatial distribution of fluorescently tagged lipase within the mesoporous spheres and time dependent leaching of lipase have been quantified by confocal scanning laser microscopy (CLSM). The enzymatic activity of the immobilized lipases has been discussed and related to the effect

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doi:10.1016/j.jcis.2009.11.014

of the surface treatment of the pore walls on the possibility of interfacial activation of changes in the surface induced denaturation.

2. Experimental section

2.1. Materials

Tetraethoxysilane (TEOS (Purum >98%), $Si(OC_2H_5)_4$, Fluka), ethanol (99.7%), Pluronic block copolymer F127 ($[(EO)_{106}(PO)_{70}(EO)_{106}]$, BASF), poly(propylene glycol) (Mw 3000, Alfa Aesar), Arlacel P135 (Avecia), toluene (99.7%), trimethylchlorosilane (100%, Alfa Aesar), Alexa Fluor® 488 protein labelling kit from Molecular Probes Europe BV (Leiden, The Netherlands), Sephandex G-25M PD-10 column from Amersham Biosciences AB (Uppsala, Sweden), 4-nitrophenyl acetate (Sigma) and acetonitrile (100%, Alfa Aesar). Lipase from the fungus *Thermomyces* (formerly *Humicola*) *lanuginosus*, with the commercial name, Lipolase®, was kindly supplied by Novozymes A/S (Bagsvaerd, Denmark, $\varnothing$ = 46 Å, M_W = 31 kDa, c = 20 mg/ml). The lipase solution was used as received without further purification. Buffer solutions used were MOPS buffer pH 7.5 (0.2 M, MOPS + NaCl), sodium bicarbonate pH 7 (50 mM, $NaHCO_3$ + HCl) and pH 9 (50 mM, $NaHCO_3$ + Na_2CO_3). All water used for buffer solutions was of Milli-Q grade.

2.2. Synthesis of spherical mesostructured materials

Spherical mesostructured particles, templated by Pluronic F127 together with a swelling agent poly(propylene glycol), were produced by the emulsion and solvent evaporation (ESE) method [22]. This method consists of five distinct steps: (1) preparation of a precursor solution, (2) emulsification of the precursor solution, (3) evaporation of ethanol and water by applying vacuum, (4) separation of particles and (5) surfactant removal by calcination.

The precursor solution was prepared by first mixing 10.4 g of TEOS into a solution with 5.2 g of diluted hydrochloric acid (pH 2) and 12 g of ethanol. This solution was under vigorous stirring for 20 min to prehydrolyse the TEOS before it was mixed with a solution containing 3.2 g of the templating surfactant (F127), 0.3 g of the swelling agent (poly(propylene glycol)) and 8.0 g of ethanol.

The precursor solution was then emulsified in a continuous oil phase at a concentration of 44 wt.%. The emulsions were formed at room temperature by pouring the precursor solution into tetradecane, acting as the oil phase, under vigorous stirring for approximately 30 s. To stabilize the emulsions a polymeric dispersant, Arlacel P135, was dissolved in the oil phase at a concentration of 1 wt.% prior to emulsification.

The fresh emulsion was directly transferred to a 1000 ml round-bottom flask connected to a rotary evaporator with a vacuum pump and immediately submerged in a temperature-controlled water bath. The flask was rotated at a speed of approximately 150 rpm at 10 °C with vacuum applied for 120 min to remove ethanol and water from the hydrolysed precursor solution. Solid particles were formed and separated from the oil phase by centrifugation approximately 12 h after termination of the vacuum step. Finally, the particles were washed in ethanol and the template was removed by calcination at 550 °C for 4 h. The calcined mesoporous silica particles contain silanol groups and therefore have a hydrophilic pore surfaces.

2.3. Silylation of ESE-produced particles

Calcined mesoporous spheres (0.5 g) were dried at 600 °C for 1 h and thereafter cooled down to 300 °C before being removed from the furnace to further cool down in a desiccator. The dried particles were then soaked in a 50 ml solution of toluene with 5 wt.% of trimethylchlorosilane. The mixture was heated up to 70 °C under continuous stirring for 22 h. The hydrophobic mesoporous particles were separated from the solution through centrifugation and washed extensively in toluene and acetone.

2.4. Fluorophore tagging of lipase

The lipase solution (20 mg/ml) was diluted in sodium bicarbonate buffer pH 9 to a concentration of 2 mg/ml. A 0.5 ml of this lipase solution (2 mg/ml) was mixed with approximately 0.2 mg of Alexa Fluor 488. The solution was protected from light and stirred over night at 4 °C. The Alexa Fluor 488-labelled lipases were separated from the excess of the fluorescent probe through separation in a column. The lipase and fluorophore concentrations in the collected solution were determined from its absorbance at 280 nm and 494 nm according to following equations:

$$C_{\text{Lipase}} = \frac{A_{280} - A_{494} \cdot 0.11}{\in_{\text{Lipase,280 nm}}} \quad (1)$$

$$\text{Alexa488/Lipase} = \frac{A_{494}}{\in_{\text{Alexa488,494 nm}} \cdot C_{\text{Lipase}}} \quad (2)$$

where $\in_{\text{Lipase,280nm}} = 38440\ \text{cm}^{-1}\text{M}^{-1}$ and $\in_{\text{Alexa488,494nm}} = 71000\ \text{cm}^{-1}\text{M}^{-1}$. The concentration of lipase was measured to be 27.1 μM, and the fluorophore concentration was measured to be 0.27 Alexa Fluor 488 per enzyme.

2.5. Immobilization of lipase in hydrophilic mesoporous spheres

Two procedures were used for immobilization of lipase in calcined mesoporous spheres without any surface functionalisation:

2.5.1. Method (1a): immobilization of un-tagged lipase

The calcined mesoporous spheres (0.5 g) were dispersed in 12 ml of MOPS and sonicated for 2 min to prevent particle agglomeration. Thereafter 3 ml of lipase solution (20 mg/ml) was added to the dispersion and the mixture was stirred for 22 h at 4 °C. The particles were then separated through centrifugation and washed three times in 10 ml MOPS. Thereafter were the particles re-dispersed in MOPS and stirred for additionally 2 h at 4 °C to release all enzymes that were loosely attached to the particles. This was done to prevent release of lipase during the activity measurements. Finally, the spheres were dried in a vacuum chamber in 30 min.

2.5.2. Method (2a): immobilization of lipase tagged with Alexa Fluor 488

The calcined mesoporous spheres (25 mg) were dispersed in 0.41 ml of MOPS and sonicated for 2 min. Thereafter were 0.14 ml of lipase (20 mg/ml) and 0.2 ml of the tagged lipase (27.1 μM) added to the dispersion (same total concentration of lipase in the solution as in method (1a) and the mixture was stirred for 22 h at 4 °C. The particles were separated through centrifugation and washed six times in 5 ml of MOPS. Finally, the particles were dried for 30 min in a vacuum chamber. The final particles were protected from light.

2.6. Immobilization of lipase in hydrophobic mesoporous spheres

Improved lipase immobilization in hydrophobic particles has been shown through equilibration of the mesoporous particles in ethanol prior to addition of the lipase solution [23]. Two procedures were used for immobilization of lipase in the hydrophobic mesoporous sample: