Catalysis Today 243 (2015) 173–183

Contents lists available at ScienceDirect

Catalysis Today

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

Immobilization of lipase in cage-type mesoporous organosilicas via covalent bonding and crosslinking

Zhou Zhou, Frank Piepenbreier, V.R. Reddy Marthala, Karl Karbacher, Martin Hartmann*

Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen Catalysis Resource Center (ECRC), Egerlandstraße 3, 91058 Erlangen, Germany

ARTICLE INFO

Article history:
Received 9 June 2014
Received in revised form 21 July 2014
Accepted 22 July 2014
Available online 18 September 2014

Keywords:
Lipase
PMO
Enzyme immobilization
CLEA
MCF
Mesoporous

ABSTRACT

Lipase from *Thermomyces lanuginosus* (TLL) was immobilized in cage-type mesoporous materials via two different protocols, viz. covalent bonding and cross-linking. The comparison between the biocatalysts generated by the two methods on both pure silica mesocellular siliceous foam (MCF) and cage-type large pore mesoporous organosilicas (PMOs) disclosed that cross-linking results in the immobilization of a larger amount of TLL and reduced diffusion problems compared to covalent bonding. Benzene-bridged PMOs positively influence the activity of immobilized lipase due to the hydrophobic properties of the surface. Furthermore, a transesterification reaction in organic solvent was carried out to verify the biocatalytic performance of differently immobilized lipase in both batch and fixed-bed reactors. The results further confirm the superiority of the PMO support compared to MCF and also reveal that the different immobilization protocols strongly influence the activity, stability and specificity of immobilized TLL. Moreover, two commercial available immobilize formed TLL were also used in a fixed-bed reactor under the same condition for comparison.

1. Introduction

Enzymes are used industrially among others in the food, detergent, cosmetics textile, pulp and paper, animal feed, and pharmaceutical industry. Compared to chemical processes, enzyme-mediated reactions possess distinct advantages like mild reaction conditions, high specificity, and reduced side reactions, which attract growing interest both from academia and industry due to energetic and economic benefits. In order to increase the utilization of enzymes as industrial biocatalysts, it is crucial to obtain stable enzyme preparations with enhanced operational stability. Immobilization is one of the important pathways for stabilization of enzymes. It is generally found that a small energy changes of ca. −40 kJ/mol (Gibbs free energy between the native and the unfolded state of the protein) can overturn the stability as well as the activity of enzyme [1]. Thus forces, such as hydrophobic effects, van der Walls forces, electrostatic interactions, hydrogen bonds, and covalent crosslinking, can strongly participate in the stabilization of the enzyme and may have individual effects on the stability of a specific enzyme. Therefore, the empirical use of various immobilization techniques (physical adsorption, covalent bonding, cross-linking, etc.) and their influences on the activity, specificity and stability of enzyme molecules as well as the usability of biocatalysts for application-related reactions has been intensively studied in recent years [2].

Moreover, the properties of the chosen support also play a crucial role for the stabilization and the performance of the immobilized enzyme. Mesoporous materials with pore sizes between 2 and 20 nm have received considerable attention in applications involving large molecules. The first report on enzyme immobilization onto mesoporous MCM-41 appeared in 1996 [3]. Following the pioneering work by Balkus and co-workers, various enzymes have been immobilized in different mesoporous materials [4–6] and the generated bio-inorganic hybrid composites have been explored for many applications including bioanalysis [7], biocatalysis [8,9] and biofuel cells [10,11]. With respect to the support, many factors influence enzyme loading, activity and stability including pore size, pore volume, pore structure, the size and the morphology of particles and surface properties [4]. One of the important features of mesoporous materials is their variability in the above mentioned properties via simply controlling the synthesis conditions.

The chemical composition determines the surface property of the supports and therefore strongly influence enzyme immobilization [12–14]. The mesoporous materials can be varied in composition and surface properties via introducing inorganic or organic moieties [15,16]. Periodic mesoporous organosilicas (PMOs) as one of the important members of so called 'hybrid'

* Corresponding author. Tel.: +49 09131 85 28792; fax: +49 09131 85 67401.
E-mail address: martin.hartmann@ecrc.uni-erlangen.de (M. Hartmann).
URL: http://www.ecrc.uni-erlangen.de (M. Hartmann).

http://dx.doi.org/10.1016/j.cattod.2014.07.047
0920-5861/

organic/inorganic mesoporous materials have clearly shown their application potential in different high-end areas [17]. A particular feature of PMOs is the uniform distributions of organic groups inside the pore walls. Thus, the materials contain a large amount of organic functions which are not blocking the pores. Furthermore, the chemical and physical properties of PMOs such as reactivity, hydrophobicity and hydrophilicity, hydrothermal stability etc. are tuneable by changing the nature of the organic groups [18].

The use of PMOs for biomolecules immobilization has increased in recent years, which might be due to the recent developments of novel PMOs with large pore size, diverse structures and different morphologies [19]. Cytochrome *c*, xylanase and lysozyme were the first enzymes immobilized in hexagonally structured (ethane-bridged) PMOs via physical adsorption. However, this early study showed that these enzymes were preferentially adsorbed on the more hydrophilic surface of pure silica SBA-15 indicating the electrostatic interaction is the dominate force in these studies [20–22]. Later, Li et al. studied ethane-bridged PMO modified with 1,4-diethylenebenzene group in the framework and found a higher lysozyme capacity compared to the parent ethane-bridged PMO under the prevailing conditions [23]. The authors attributed their findings to stronger hydrophobic and hydrogen-bonding interaction between 1,4-diethylenebenzene and the enzyme compared to pure ethane bridges. Serra et al. studied the adsorption of lipase from *Candida Antarctica fraction B* on amorphous silica, ordered mesoporous SBA-15 and ethane-bridged PMO (hexagonal structure) [24]. The immobilization yields and rates are much higher on ethane-bridged PMO compared to SBA-15 and methyl-modified SBA-15. A similar observation has also been made in our previous study [25]. PMOs containing ethane, ethene and benzene-bridged organic groups were used for the adsorption of lipase from *Thermomyces lanuginosus* (TLL) and the most hydrophobic benzene-bridged PMO exhibited superior performance with enhanced adsorption, activity and stability of lipase. Hudson et al. introduced relative hydrophilic groups into the framework of PMO to improve the adsorption of the hydrophilic-dominated enzyme chloroperoxidase [26]. All above reports clearly confirm that the surface composition of the PMO strongly influences the physical adsorption of different enzymes on the support due to various interactions namely hydrophobic, electrostatic, hydrogen-bonding interactions.

Moreover, not only the nature of the framework but also the pore structure influences enzyme immobilization. Among the various structured mesoporous materials, cage-like large pore mesoporous silicas (viz. mesocellular siliceous foam (MCF) [27] and hierarchical mesocellular mesoporous silica (HMMS) [28]) with ultra-large mesocellular pores connected by meso-sized windows are superior for immobilization of different enzymes due to higher loading capacity [29], low mass transfer resistance [30] and reduced leaching [31]. These materials have received further interest as a proper support to form cross-linked aggregates (CLEAs) of enzymes in their cages [32–34], which show several advantages [35]: (1) no pre-precipitation of enzyme is necessary because the enzymes naturally aggregate in the cages of the solid through adsorption; (2) the growth of the aggregates is limited by the size of the cage; and (3) leaching can be suppressed due to size of the CLEAs, which exceeds the size of the entrances and (4) the diffusion of substrates is feasible through the interconnected cages.

Lipases belong to the enzyme family of hydrolases and catalyze the hydrolysis of triglycerides into diglycerides, monoglycerides, fatty acids and glycerol. In addition to their natural function in fat catabolism, they are gaining increasing attention for applications in food, detergent, cosmetics and pharmaceutical industries [36–39]. Lipase from *Thermomyces lanuginosus* (formerly *Humicola lanuginose*) containing a single chain protein is a 1,3-regiospecific enzyme responsible for the lipolytic activity of Lipolase, which was the first commercially available lipase used in detergents. The lipase has a molecular weight of 31.7 kDa consisting of 269 amino acids and is produced by Novozyme Corp. for several industrial applications [40]. TLL is a stable enzyme in aqueous solution and remains active over a large range of pH from 7 to 12 and maintains reasonable activity up to 60 °C [41]. X-ray crystallographic studies revealed that TLL is a typical lid-containing lipase. In its open form the lid is displaced and the active center is exposed to the medium, which often results in an increase in enzyme activity (interfacial activation) [42]. The active center of lipase is very sensitive to any changes in surrounding such as the experimental conditions, thus, the immobilization strategy employed etc. may greatly alter the performance of the enzyme.

In our previous study, we have described the synthesis of a series of PMOs with large cage-like pores [25] and the physical adsorption of TLL on these materials exhibiting improved capacity, activity and biocatalytic performance [43]. In the present work, two further immobilization techniques (covalent bonding and cross-linking) were continuously investigated for the immobilization of this lipase in cage-type pure silica MCF and benzene-bridged PMO, in order to systematically study and compare the features of immobilized TLL via different processes and on different supports as well as their performance in both batch and fixed-bed reactors.

2. Experimental

2.1. Materials and reagents

Glutardialdehyde (GA), aminopropyltriethoxysilane (APTS), tetraethyl orthosilicate (TEOS), Pluronic triblock copolymer (P123), 4-nitrophenyl palmitate, sodium chloride, *p*-nitrophenyl palmitate (pNPP) and 1,3,5-trimethylbenzene (TMB) and lipase immobilized on Immobead 150 were obtained from Sigma–Aldrich GmbH & Co. KG. Lipase from *Thermomyces Lanaginosus* employed in this work was produced by Novozyme Corp. and is also distributed by Sigma–Aldrich. Lipozyme TL IM (a silica granulated TLL) was kindly provided by Novozyme Corp. Hexane, butanol and vinylpropionate were obtained from VWR, Merck and Alfa Aesar, respectively. The precursor 1,4-bis(triethoxysilyl)benzene and the resulting benzene-bridged PMO used in this study were synthesized as described in our previous publication [25]. The MCF material employed was prepared as described by Stucky and coworkers [27]. For comparison, physical adsorption of TLL in PMO and MCF was performed as described in our previous publication [43] and the resulting catalysts were named MCF_Ad and PMO_Ad.

2.2. Physico-chemical characterization

The modified samples were characterized by FTIR spectroscopy (JASCO FT/IR-4100) equipped with an ATR-cell and a diffuse reflectance accessory with CaF_2 as background in the range from 4000 cm^{-1} to 500 cm^{-1} with a resolution of 4 cm^{-1}. The nitrogen sorption experiments were conducted in a Micromeritics ASAP 2010 analyzer at 77 K employing liquid nitrogen. Before the measurement all samples were degassed for 12 h at 110 °C under vacuum. Argon sorption experiments were performed on an Autosorb-1-MP by Quantachrome Instruments at 87 K using liquid argon. All samples were degassed for 12 h at 120 °C under vacuum prior to the adsorption of argon. The specific surface area was calculated by using the BET-formalism and the pore size distribution was determined by employing the NLDFT method. Elemental analysis was carried out by a Euro EA 3000 (Euro Vektor) instrument. Moreover, thermogravimetric analysis was performed in a TA Instruments SDT 2960 simultaneous TGA-DSC. The sample (10–20 mg) was heated from room temperature to 800 °C with a