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Stabilization of *Candida rugosa* lipase on nanosized zirconia-based materials

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ABSTRACT

We synthesized and characterized three novel materials on the basis of zirconia. Despite their three-to six fold higher specific surface area, nanoZrO_2-CeO_2 (150 m^2/g) and nanoZrO_2-B (296 m^2/g) they proved to be less effective supports for a lipase from *Candida rugosa* than nanoZrO_2-A. For the last, we achieved protein loading of 23 mg/g, distributed in a monolayer, which means that 61% of the carrier surface was occupied by the protein. The immobilized on nanoZrO_2-A lipase (nanoZrO_2-A-CRL) exhibited enhanced thermal and solvent stability in comparison to the native enzyme. NanoZrO_2-A-CRL has a half-life at 55 °C of 73 min, while for the native enzyme it was only 5.3 min. The immobilized enzyme preserved 20% of its activity in six consecutive cycles of the reaction hydrolysis of tributyrin. The immobilization influenced the enantioselectivity of CRL. Using nanoZrO_2-A-CRL under optimal reaction condition in the reaction of esterification of lauric acid with (±)-menthol, we achieved menthol conversion of 43% (*i.e.* 82% of (−) methyl laurate), enantiomeric excess of 97% and enantiomeric ratio of 144. The conformational analysis proved that upon immobilization no serious change in the secondary structure of the lipase from *Candida rugosa* had occurred.

1. Introduction

Lipases (EC 3.1.1.3) are large group of enzymes that in aqueous media catalyze the hydrolysis of ester-bonds in triglyceride substrates to glycerol and free fatty acids. In non-aqueous media they can catalyze the reverse reaction, *i.e.* the reaction of esterification [1]. All lipases belong to "α/δ-hydrolase fold" family and most of them have a highly conservative pentapeptide sequence (Gly-X-Ser-X-Gly) that includes the active nucleophile serine residue [2]. The active site of lipases consists of three amino acid residues (serine, histidine, aspartate or glutamate) known as "catalytic triad". These enzymes share a common mechanism which includes steps of acylation and deacylation. During each step acyl-enzyme and tetrahedral intermediate are formed, the latter is stabilized by the amino acid residues assembling the "oxyanion hole" [3]. Another structural and mechanistic feature typical for most of the lipases is the presence of α-helical fragment ("lid") which covers the active site in absence of substrate and protect the enzyme. Upon contact with the hydrophobic substrates or organic solvents the "lid" undergoes a conformational change, moves away and the active site of the enzyme becomes accessible to the reagents. This phenomenon is known as "interfacial activation" [4]. Similar activation upon binding to hydrophobic surfaces has been observed for many lipases. For example, Palomo et al. reported enhanced thermal and solvent stability as well as hyperactivation of the immobilized on octadecyl-sepabeads lipase from Bacillus thermocatenulatus with respect to the soluble enzyme [5]. The same interfacial hyperactivation by physical adsorption on hydrophobisized vinyl-based amphiphilic polymers was reported for the lipase from *Candida rugosa* [6] and for many other lipases from various species (*Candida antarctica*, *Humicola lanuginosa*, *Mucor javanicus*, *Rhizomucor miehei*, *Pseudomonas fluorescens*, *Rhizopus niveus*) when immobilized on octyl-agarosa [7].

Lipases are enzymes of considerable industrial significance. They are widely applied in food, detergent, pharmaceutical and cosmetic industries as well as in paper, textile and leather processing [8]. Yet, their price is high in comparison with chemical catalysts and their utilization in several consecutive cycles is envisaged. Use of immobilized enzymes has many advantages over the soluble enzymes. They can be easily separated from the reaction mixture *i.e.* the reaction can be easily terminated, immobilized enzymes usually are more stable at higher temperatures and organic solvents, as well as they can be used several operation cycles [9].

Nanobiotechnology is a new fast developing trend of biotechnology. It has shown great potential in applications such as: biosensing,

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drug delivery, protein immobilization, and many others. Excellent enzyme stabilization upon immobilization on various nanomaterials (nanoparticles, nonofibres, nanopubes, nanopores, nanosheets and nanocoposites) has been reported and is the basis for the development of a new sub-field in biocatalysis, *i.e.* nanobiocatalysis [10,11]. In addition, systems with trapped in nanospaces several enzymes are good model for studying the protein-protein interactions inside the cells which still are not completely elucidated. For example, *in vivo* many enzymes are located in the organelles and they are catalytically active under crowded environment, however, *in vitro* most of enzymes are prone to easy aggregation and unfolding even at concentrations of 1 mg/mL. Within the aggregates, the enzyme active centre remains inaccessible to the substrate molecules and usually low activity is detected under such conditions. In some cases, the addition of small quantities of non-ionic surfactants to the enzyme solutions can be beneficial and prevent aggregation. Another successful strategy for protein stabilization is immobilization, especially on nanosized particles [12].

Inorganic nanostructures are characterized with large surface area, narrow particle size distribution, mechanical strength and resistance to microbial attack and organic solvents. In addition, they can be well-dispersed in liquid reaction media and thus the problem with low protein solubility in organic medium is overcome as well as good homogenization of the reaction mixture can be achieved [13]. Biocompatible particles on the basis of silica, iron oxides, carbon (grapheme, diamond), gold, *etc.* have been obtained in nanosize dimensions. Their surface and pore size can be easily modified by functionalization or/and coating with organic compounds which makes them applicable for immobilization and stabilization of various proteins and enzymes.

Zirconia is a polymorphic bioinert material that possesses the above discussed favourable characteristics of the inorganic materials such as: high thermal, pH- and solvent stability, *etc.* It occurs in three temperature-depending forms: monoclinic, tetragonal and cubic. There are several papers on application of zirconium dioxide in chromatography, preparation of enzyme-based reactors and biosensors. There are also reports on the utilization of zirconia nanoparticles as enzyme carriers. For example, the α-amylase form *Bacillus subtilis* immobilized on zirconia exhibited good stability and activity in the reaction of hydrolysis of starch [14]. Bellezza et al. reported on excellent catalytic efficiency of the myoglobin immobilized on phosphoric acid (or benzenephosphonic acid) grafted zirconia [15]. There is also a report on the selective adsorption of different isoforms of *C. rugosa* lipase on α-zirconium phosphate and phosphonates and an enhanced enantioselectivity of the immobilized lipase preparations in the reaction of hydrolysis of (±)-methyl-2-(4-chlorophenoxy) propionate [16]. We think that the results on zirconia-based biocatalysts found in the literature are very attractive but that the potential of zirconia as nanocarrier is not fully revealed and worth further investigations.

We synthesized and characterized three materials based on zirconia. They were tested as suitable supports for a lipase from *C. rugosa*. The lipase from the yeast *C. rugosa* is a glycoprotein that presents in different isoforms and isoenzymes which differ in their stability and specificity [17]. It is widely used for industrial production of highly pure unsaturated fatty acids, synthesis or kinetic resolution of racemic mixture of some therapeutics, synthesis of flavour esters for food and perfume industries, in detergent formulations, *etc.* [18].

We assessed the effect of surface morphology, particle and pore sizes and surface area of the carriers on their lipase loading capacity. The catalytic efficiency of the two promising immobilized enzymes was estimated in a hydrolytic reaction. Then, their thermo-, solvent, and operational stability was estimated. The novel most stable and active catalyst was tested in a reaction of enantioselective acylation of (±)-menthol.

2. Materials and methods

2.1. Materials

Lipase from *C. rugosa* (CRL) (MW 64 kDa, 30 U/mg (olive oil as substrate), 20% (w/w) protein content) was provided by Amano Pharmaceutical Co., Japan. Glyceryl tributyrate (99% purity), Folin & Ciocalteu's phenol reagent (2 N, suitable for determination of total protein by Lowry's method) were purchased from Sigma, Germany. Lauric acid (>98%), (±)-menthol (98%), (1R, 2S, 5R) (−)-menthol (99%), (1S, 2R, 5S)(+)-menthol (99%), zirconium (IV) chloride (>99.9% purity), cerium(III) chloride heptahydrate (99.9%) and hexadecyl-N,N,N-trimethylammoniumbromide (CTAB) were obtained from Sigma–Aldrich, Germany.

2.2. Synthesis of the nanosized ZrO_2-A, ZrO2-B and ZrO_2-CeO_2

For the synthesis of the nanostructured zirconia samples a template-assisted approach was used following a procedure reported by Tsoncheva et al. [19]. In principle, 12.0 g N-hexadecyl-N,N,N-trimethylammoniumbromide (CTAB) were dissolved in 100 mL distilled water. To this solution was added slowly and under vigorous stirring a second solution of $ZrCl_4$ (6.80 g) in 50 mL distilled water. In the case of the mixed oxide sample the second solution contained a mixture of $ZrCl_4$ (3.40 g) and $CeCl_3 7H_2O$ (5.40 g) in 50 mL distilled water. Then, the temperature was raised to 50 °C and the reaction mixture was stirred for 30 min before adding dropwise 40 mL NH_4OH (12.5%). The resulting mixture was stirred overnight at 50 °C. Then it was transferred into a polypropylene container and treated at 100 °C for 24 h. The so-prepared particles were then filtrated, washed with distilled water, then dried at room temperature and calcinated up to 300 °C (nanoZrO_2-B and nanoZrO_2-CeO_2) or 500 °C (nanoZrO_2-A) with a ramp of 1 °C/min and dwelling time of 15 h at the final temperature. For, nanoZrO_2-A the hydrothermal treatment step was skipped and an extraction of the organic template in absolute ethanol (100 mL per 1 g carrier) was conducted at 70 °C for 24 h. Then, the sample was filtrated and dried at room temperature before calcination.

2.3. Characterization of the particles

Powder X-ray diffraction patterns were collected within the range of 10–80° 2θ with a constant step of 0.02° 2θ and counting time of 1 s/step on Bruker D8 Advance diffractometer equipped with Cu Kα radiation and LynxEye detector. The size of the crystalline domains in the samples was determined using Topas 4.2 software with Rietveld quantification refinement for nanoZrO_2-A, and the Scherrer equation [20] in case of the other samples. Nitrogen sorption measurements were recorded on a Quantachrome NOVA 1200e instrument at 77 K. Before the physisorption measurements the samples were outgassed at 423 K overnight under vacuum.

2.4. Immobilization of lipase from Candida rugosa

In a typical preparation, 20 mg of carrier (nanoZrO_2-A, nanoZrO_2-B, nanoZrO_2-CeO_2) was mixed and was gently stirred with 5.0 mL solution of *C. rugosa* lipase (CRL) for 12 h at room temperature and then incubated overnight at 4 °C. The protein concentration in the loading solutions (50 mM sodium phosphate buffer, pH 7.0) was in the range 0.05–1 mg/mL. After the incubation, the immobilized biocatalysts were filtered and freeze-dried.