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A novel approach for bioconjugation of *Rhizomucor miehei* lipase (RML) onto amine-functionalized supports; Application for enantioselective resolution of *rac*-ibuprofen

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ABSTRACT

Since the amine groups are highly reactive toward various functional moieties, the formation of covalent bonds between a biomolecule and an insoluble amine-functionalized support is the most frequently used technique in immobilization protocols. A new approach based on the Ugi four-component reaction was used for immobilization of *Rhizomucor miehei* lipase (RML) as a model enzyme on amine-functionalized silica and silica nanoparticles (SBA-15). For this, the amine-modified supports were prepared and the structural properties of the functionalized supports, prior to and after functionalization were characterized by using IR, SEM, TGA, DTA, TEM. Immobilization of RML on the aminated carriers was performed under extremely mild conditions (25 °C, pH 7).
The results revealed very rapid immobilization of 150 and 200 mg of RML on 1 g of silica-NH_2 and SBA-NH_2, respectively, producing 95–100% of immobilization yield. The specific activity and optimum pH of the immobilized preparations and the effect of temperature and co-solvents on their stabilities as well as the reusability of the derivatives were tested. The immobilized preparations were also used as enantioselective catalyst in kinetic resolution of racemic ibuprofen. Among them, Silica-RML showed the best selectivity with 92.2% enantiomeric excess (*ee*) and E-value of 33.9.

1. Introduction

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are the most important group of enzymes which are useful for the synthesis of many pharmaceutical intermediates [1]. They have the natural catalytic function of hydrolyzing fatty acid ester bonds in water and are the most commonly used enzymes in organic synthesis because of their capacity of catalyzing different reactions such as esterification, transesterification and hydrolysis [2,3]. Lipases are available from several (micro) organisms and normally accept a wide range of non-natural compounds as substrate without requiring cofactors. However, industrial applications of the free form of lipases remain controversial because of their poor stability and prohibitive cost [4]. Therefore, there is a great interest in methods trying to develop useful biocatalysts for industrial applications by improvement of their catalytic properties such as activity, stability or recycling capacity [5]. Numerous attempts have been carried out on the preparation of immobilized lipases, which involves a variety of immobilization techniques and new support materials [6–10]. Among them, irreversible covalent attachment of enzymes on a solid support is an efficient and popular method and is usually preferred when leaching of enzyme from the support is a main concern [11]. Moreover, if the immobilization protocol is properly designed to give an intense covalent attachment, the immobilization will be a powerful way to improve the enzyme rigidity, which increases enzyme stability against any distorting agents. However covalent attachment is often time consuming, needs harsh condition (for example high pH) and generally results in reduced activity of the immobilized enzyme [12]. Therefore, it is still a big demand for discovery of new simple strategies for the covalent binding to improve enzyme catalytic functions. Recently we have reported the interesting results of using the multi-component reaction for enzyme immobilization on aldehyde, epoxy and acid functionalized supports [8,9,13]. High capacity of loading, simple and rapid immobilization and significant improvement in activity of the immobilized enzyme are the main advantages of this novel procedure. In this procedure the support supplies the aldehyde, epoxy or acid groups, the enzyme molecules supply the carboxylic or amine groups and the other missing component is added to the reaction medium. However this protocol offers great flexibility to use different functional groups

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of many supports. In fact by utilizing this method a large number of supports and functional groups can be used for immobilization of enzymes. One of these supports is amine-functionalized supports which are widely used for immobilization of biomolecules [5,14]. Covalent bonding of proteins on these supports is usually performed via diimide-activated amidation of protein-bound carboxylic acids. However, this process requires more time/chemical reagents and also causes to undesirable side reactions of intermolecular conjugation of proteins [15]. Herein, we describe a simple, efficient and general method for immobilization of proteins on amine-functionalized supports without introducing time-intense process or harsh chemical/physical condition. For this, silica nanomesopurus materials (SBA-15) were synthesized and fully characterized by different methods (BET, TEM, XRD, TGA, SEM, IR) and then used together with silica particles as carrier for immobilization of RML as a model enzyme. The obtained biocatalysts were further studied in terms of activity and stability. The selectivity of the immobilized enzymes was also studied in kinetic resolution of (*R,S*)-ibuprofen as a model reaction. Ibuprofen belongs to non-steroidal anti-inflammatory drugs (NSAIDs) which are known as one of the most commercially successful and important classes of analgesic anti-inflammatory drugs used in the treatment of headache, rheumatoid arthritis, cephalgia and muscular strain [16]. Ibuprofen has a stereogenic center which is the carbon bearing carboxyl group. The (*S*)-enantiomer of ibuprofen has the desired therapeutic effect (160 times more active than it's (*R*)-enantiomer) in the in vitro inhibition of prostaglandin synthesis, while the (*R*)-ibuprofen is inactive and can cause side effects affecting to the gastrointestinal tract, normal lipids metabolism and membrane function [17].

2. Experimental

2.1. Materials

Ibuprofen was extracted from the readily marketed tablets. The lipase from *Rhizomucor miehei* (RML), *p*-nitrophenyl butyrate (*p*-NPB), cyclohexyl isocyanide, acetaldehyde, tetraethyl orthosilicate (TEOS), polyuronic acid (P123) and N-[3-(Trimethoxysilyl) propyl] ethylenediamine (APTMS) were from Sigma (Steinhiem, Germany). Silica gel (70–230 mesh), dioxane, 1-propanol and 2-propanol were purchased from Merck. Other used reagents and solvents were of analytical grade. Fourier transform infrared spectra (FT-IR) were recorded on a Bomen FT-IR-MB-series instrument with a KBr pellet technique. Thermogravimetry (TGA) and differential thermal analysis (DTA) were carried out from 10 °C to 800 °C at a heating rate of 20 °C/min in air atmosphere using a STA 503 M system from Bähr GmbH, Germany.

2.2. Preparation of the silica nanoparticles

Based on the reported procedure in literature [12] for the preparation of pure siliceous SBA-15, 4 g of pluronic P123 triblock copolymer (EO20–PO70–EO20, BASF) was added to 144 mL of an aqueous solution of HCl (2 M) at 40 °C. Successively, TEOS was added dropwise (mass ratio TEOS/P123 = 2:1) and stirring was continued for 2 h. Afterwards the mixture was transferred to Teflon-lined sealed container and kept at 100 °C. After 48 h, the white solid was filtered and washed with distilled water. At the end, in order to remove the template, the white solid was calcined at 550 °C.

2.3. Functionalization of the supports

The dry silica gel particles (1 g) or SBA-15 (0.5 g) was dispersed in 50 mL of dry toluene, and then 1 mL of APTMS was added. The resulting mixture was refluxed under nitrogen atmosphere and vigorously stirring for 4 h. The modified support was collected by filtration and washed thoroughly with ethanol. Finally the modified particles were dried at 120 °C for 8 h. IR spectroscopy and TGA-DTA analysis confirmed the successful functionalization of the supports.

2.4. Enzyme immobilization on the amine-functionalized supports

For the immobilization of RML on silica-NH_2 and SBA-NH_2, a solution containing a certain amount of RML in distilled water was prepared; afterwards 100 mg of the support and 6 μL of acetaldehyde were added to the mixture. In order to start the immobilization reaction, 12 μL of cyclohexyl isocyanide was added to the protein solution under gently stirring at 25 °C. For monitoring the reaction, samples from the supernatant were withdrawn periodically, and then the protein concentration was determined by the Bradford's method [18]. Finally the immobilized RML derivatives were filtered and washed by distilled water and stored at 4 °C.

2.5. Enzymatic activity assay

The activity assay of RML and its immobilized preparations was performed spectrophotometrically based on the increment in absorbance at 348 nm ($\epsilon = 5150\ M^{-1}\ cm^{-1}$) since hydrolysis of *p*-NPB in buffer (25 mM sodium phosphate buffer, pH 7, 25 °C) released *p*-nitrophenol and it made an increment in the absorbance.

Briefly, 0.05–0.2 mL of the lipase suspension or solution (blank or supernatant without further dilution) was added to 1.25 mL of substrate solution (0.8 mM) under magnetic stirring [13]. Enzymatic activity is given as 1 μmol of *p*-nitrophenol released per minute per mg of the enzyme (IU) under the conditions described above.

2.6. Determination of the amount of protein bound to the carriers

The amount of protein in supernatant and blank was determined based on the Bradford's method. The immobilization yields were calculated and reported as the ratio of the amount of protein attached to the support to the initial amount of protein.

2.7. Thermal inactivation of RML immobilized preparations

Thermal stability of the immobilized enzyme was investigated by the incubation of free RML and its immobilized preparations in 25 mM sodium phosphate (pH 7) at different temperatures. The suspension of each sample was withdrawn periodically and their activities were measured by using the *p*-NPB assay.

2.8. Co-solvent stability of the free and immobilized preparations of RML

In order to investigate the stability of the immobilized enzyme in the presence of various organic solvents, different solutions of sodium phosphate buffer (25 mM, pH 7, total volume of 1 mL) containing 20% (v/v) of dioxane, 1-propanol and 2-propanol were prepared. Afterwards, free RML and its immobilized preparations were added to these solutions and incubated at 25 °C for 24 h. The activity of each sample was measured by using the *p*-NPB assay.

2.9. Determination of the optimum pH activity

For determining the optimum pH activity, first sodium phosphate buffers (25 mM, total volume of 1 mL) with different pH ranging from 5 to 9 were prepared. Then, RML and the immobilized preparations were added to these buffers and incubated at 25 °C for 2 h. At the end of incubation, the activity of each sample was measured by using the *p*-NPB assay.