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Enantioselective hydrolysis of rasemic naproxen methyl ester with sol–gel encapsulated lipase in the presence of sporopollenin

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ABSTRACT

Sporopollenin is a natural polymer obtained from *Lycopodium clavatum*, which is highly stable with constant chemical structure and has high resistant capacity to chemical attack. In this study, the *Candida rugosa* lipase (CRL) was encapsulated within a chemically inert sol–gel support prepared by polycondensation with tetraethoxysilane (TEOS) and octyltriethoxysilane (OTES) in the presence and absence of sporopollenin and activated sporopollenin as additive. The catalytic properties of the immobilized lipases were evaluated into model reactions, i.e. the hydrolysis of *p*-nitrophenylpalmitate (*p*-NPP), and the enantioselective hydrolysis of rasemic Naproxen methyl ester that was studied in aqueous buffer solution/isooctane reaction system. The results indicated that the sporopollenin based encapsulated lipase particularly had higher conversion and enantioselectivity compared to the sol–gel free lipase. In this study, excellent enantioselectivity ($E > 400$) has been noticed for most lipase preparations ($E = 166$ for the free enzyme) with an ee value ~98% for *S*-Naproxen. Moreover, (*S*)-Naproxen was recovered from the reaction mixture with 98% optical purity.

1. Introduction

Sporopollenin is a natural biopolymer which occurs in the outer membranes of moss and fern spores and most pollen grains. It has been shown that spore and pollen membranes have two layers, an inner one known as intine, and an outer one the exine containing a material to which the name sporopollenin was given. Sporopollenin is stable and highly resistant to chemicals. It has a constant chemical structure and exhibits very good stability even after a prolong exposure to mineral acids and alkalies [1]. The mechanism of its synthesis and its consolidation are not yet understood [2]. In the past two decades modified forms of sporopollenin have been utilized as anion, ligand [3], and as cation exchangers for removal of heavy metal ions from aqueous solutions [4].

Sporopollenin offers several advantages over man made and other naturally occurring materials (e.g. acrylic resins, chitosan). It is easily extracted from its natural source, spores or pollen, using cheap non-toxic reagents which are used in the food industry. It forms microcapsules which have a large internal cavity available for encapsulation with very high loadings. The particles are monodispersed and, from one species, uniform in size, morphology and chemical composition, i.e. sporopollenin contains only carbon, hydrogen and oxygen. It is thus free from any allergens. Such uniformity is difficult and expensive to achieve in man made products, especially possessing a large cavity capable of being filled with a wide range of polar and non-polar materials. Particular encapsulants previously used for macromolecules are relatively expensive with lower loadings than sporopollenin [5].

Sol–gel encapsulation has proven to be a particularly easy and effective way to immobilize enzymes [6]. Following isolated reports describe the specific examples; it was the seminal work of Avnir and co-workers that led to the generalization of this technique [6–8].

A well-established sol–gel processing technique consists in hydrolyzing adequate precursors in aqueous solutions to produce soluble hydroxylated monomers, followed by polymerization and phase separation to produce a hydrated metal or semi-metal oxide hydrogel [9]. Removal of water from the wet gel, which is usually accompanied by changes in the structure of the pores and of the gel's network, results in a porous xerogel. The most widely used precursors are alkyl-alkoxysilanes. These precursors were used already in the mid-1980s these precursors were used to prepare organically modified silicates for the successful encapsulation of antibodies and enzymes [10,11]. Although the final structure of the material is basically determined by the differences in chain length, functionality and hydrophobic character of the precursors, it can be tailored *via* the addition of a wide range of molecules. Examples include surfactants [12–14], room-temperature ionic liquids [15], crown ethers, β-cyclodextrins or porous solid supports like Celite [16]. The commonly used catalysts are weak acids or bases [9,16]. Recent research describes the use of other species that include

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peptides like silaffins [17], polyamines [18] or enzymes such as hydrolases [19] and silicateins [20].

Candida rugosa is an important industrial lipase due to its wide application in oil hydrolysis, transesterification, esterification and enantioselective biotransformation [21,22]. Thus, lipase is becoming one of the most industrially used enzymes due to its high stereoselectivity, regioselectivity, and low price [23]. (*S*)-(+)-2-(6-Methoxy-2-naphthyl)propionic acid (Naproxen) is a non-steroidal anti-inflammatory drug that belongs to the family of 2-aryl propionic acid derivatives, it is widely used as a drug for human connective tissue diseases. The physiological activity of the *S* form of Naproxen is 28-fold that of the *R* form [24]. Hence, only the *S* form is used as a drug for humans [25–27]. Of the profen drugs, an important family of non-steroidal anti-inflammatory drugs, Naproxen is now the only member to be sold as a single enantiomer.

In the case of Naproxen, lipase has been used to prepare optically pure Naproxen by enantioselective hydrolysis of its racemic esters [28–30]. In addition, the preparation of Naproxen ester prodrugs has been accomplished by resolution of racemic Naproxen mixtures [31–33]. More recently, alcohol and buffer or acetone treatment method has been developed to improve the enantioselectivity of CRL towards the hydrolysis of racemic Naproxen methyl ester [34,35]. The changes in the enantioselectivity of CRL for *S*-Naproxen with the alterations in its microenvironment by changing the medium properties [36], by immobilization [37] and by introducing additives into the reaction medium [38] have also been reported.

To the best of our knowledge, there exists no report on the use of sporopollenin from *Lycopodium clavatum* as support for immobilization of lipase. It has been thought that the sol–gel procedure could be interesting for lipase immobilization. Therefore, we now report the use of sporopollenin (Spo) and activated sporopollenin (Spo_{act}) as additives on lipase immobilization made by sol–gel process and explore the effect of these materials in the enantioselective hydrolysis of (*RS*)-Naproxen methyl ester. The effect of temperature, pH and thermal/storage stability was also investigated.

2. Materials and methods

2.1. Materials

C. rugosa lipase (*CRL*) was a commercial enzyme obtained from Sigma-chemical Co. (St. Louis, MO) used in the immobilization. *Lycopodium clavatum* with a particle size of 25 μm was purchased from Fluka Chemicals. Bradford reagent, Bovine Serum Albumin 99% (BSA), *p*-nitrophenylpalmitate (*p*-NPP), TEOS (tetraethoxysilane) and OTES (octyltriethoxysilane) were purchased from Sigma-chemical Co. (St. Louis, MO). Pure *S*-Naproxen, was purchased from Sigma (USA). The solvents used in HPLC analyses were HPLC grade (Merck, Germany). All aqueous solutions were prepared with deionized water that had been passed through a Millipore Milli-Q Plus water purification system. All other chemicals (Merck, Darmstadt, Germany) were of analytical grade and used without further purification.

2.2. Instrumentation

FT-IR spectra were recorded on a PerkinElmer 1605 FT-IR spectrometer as KBr pellets. UV–vis spectra were obtained on a Shimadzu 160A UV-vis recording spectrophotometer. High-performance liquid chromatography (HPLC) Agilent 1200 Series was carried out using a 1200 model quaternary pump, a G1315B model Diode Array and Multiple Wavelength UV–vis detector, a 1200 model standard and preparative auto sampler, a G1316A model thermostated column compartment, a 1200 model vacuum degasser, and an Agilent Chemstation B.02.01-SR2 Tatch data processor.

The enantiomeric excess determination was performed with HPLC (Agilent 1200 Series) by using a Chiralcel OD-H column at the temperature of 25 °C with n-hexane/2-propanol/trifluoroacetic acid (100/1/0.1, v/v/v). The flow rate of 1 mL/min; the UV detector was fixed at 254 nm.

The surface morphology of samples was examined by scanning electron microscope (SEM, Jeol, JSM 5310, Japan).

2.3. Preparation of activated sporopollenin

The sporopollenin exines (25 μm) were extracted from *L. clavatum* as follows: raw *L. clavatum* spores (powdered form, 50 g) were suspended in acetone (150 mL) and stirred under reflux for 6 h. The defatted spores were filtered and then treated with 8% (w/v) potassium hydroxide (150 mL) solution. After that the mixture was refluxed for 15 h. This base-hydrolyzed sporopollenin was filtered and washed with hot water and hot ethanol. It was finally suspended in 85% (w/v) *ortho*-phosphoric acid (150 mL) and stirred under reflux for 7 days. This acid-hydrolyzed sporopollenin was filtered, washed with water, acetone, 2 M hydrochloric acid solution, 2 M sodium hydroxide solution, water, acetone and ethanol and dried at 60 °C until constant weight [39].

2.4. General procedure for sol–gel encapsulation of lipases

Sol–gel encapsulated lipases were prepared according to a modified method of Reetz et al. [14]. A commercial lipase powder (lyophilizate) such as CRL type VII (60 mg) was placed in a 50-mL Falcon tube (Corning) together with phosphate buffer (390 μL; 50 mM; pH 7.0) and the mixture was vigorously shaken with a Vortex-Mixer. The Spo or Spo_{act} (0.05 g) was included. Then 100 μL of aqueous polyvinyl alcohol (PVA) (4% W/V), aqueous sodium fluoride (50 μL of 1 M solution) and isopropyl alcohol (100 μL) were added, and the mixture homogenized using a Vortex-Mixer. Then the alkylsilane (2.5 mmol) and TMOS (0.5 mmol; 74 μL; 76 mg) were added and the mixture agitated once more for 10–15 s. Gelation was usually observed within seconds or minutes while gently shaking the reaction vessel. Following drying overnight in the opened Falcon tube, isopropyl alcohol (10 ± 15 mL) was added in order to facilitate removal of the white solid material (filtration). The gel was successively washed with distilled water (10 mL), isopropyl alcohol (10 mL). The resulting encapsulated lipases were lyophilized and stored at 4 °C prior to use.

2.5. Determination of enzyme activity

Activity of the free and encapsulated lipases were assayed using 14.4 mM *p*-nitrophenyl palmitate in 2-propanol as substrate. The reaction mixture consisting of 1 mL of 50 mM phosphate buffer (pH 7.0 for immobilized lipase) containing 25 mg of immobilized lipase (or 0.1 mL free lipase) was initiated by adding 1 mL of substrate and mixed for 5 min at 30 °C. The reaction was terminated by adding 2 mL of 0.5 N Na_2CO_3 followed by centrifuging at 4000 rpm for 10 min. The increase in the absorbance at 410 nm produced by the release of *p*-nitrophenol in the enzymatic hydrolysis of *p*-NPP was measured in a Shimadzu UV-160A (Japan) spectrophotometer. A molar extinction coefficient (ε410) of 15,000 M^{-1} cm^{-1} for *p*-nitrophenol was used [40]. One unit (U) of lipase activity was defined as the amount of enzyme necessary to hydrolyze 1 μmol/min of *p*-NPP under the conditions of assay. All measurements were performed in triplicate and an average was taken as final result.