



FTIR, SEM and fractal dimension characterization of lipase B from *Candida antarctica* immobilized onto titania at selected conditions

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ABSTRACT

Lipase B from *Candida Antarctica* (also known as *Candida antarctica* lipase B or CALB) was immobilized onto titanium dioxide (TiO₂) in a buffer-free, bidistilled aqueous medium. The adsorption isotherm was determined by UV–vis analysis of supernatant solution at 280 nm, revealing that in 7 h 98% of the theoretical lipase monolayer on the TiO₂ (with 45.7 m²/g BET area) was achieved.

Samples withdrawn from the supernatant immobilization medium were analyzed by Fourier-transform infrared spectroscopy in the 1700–1600 cm^{−1} range (where the Amide I Proteins band appears) in order to obtain quantitative information on the evolution of the secondary-structure elements of the protein. The analysis performed revealed that lipase conformation suffers only minor changes during its adsorption onto TiO₂. However, water associated to the lipase may interact of several ways with the surface of the hydrated oxide.

Characterization of the immobilized biocatalyst (CALB/TiO₂) implied SEM, fractal dimension analysis and FTIR techniques. A proposal of lipase-hydrated oxide interaction is presented.

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1. Introduction

Lipases (EC 3.1.1.3) are a family of enzymes that in their natural environment catalyse the hydrolysis of fats, as well as synthetic reactions such as esterification of fatty acids, alcoholysis and transesterification. Lipase B from *Candida antarctica* (also known as *C. antarctica* lipase B or CALB) is one of the most widely used lipases with a broad range of applications [1–6]. CALB has been mostly used in an immobilized form, commercially available from Novo Nordisk (Novozym 435).

In this report immobilization of CALB on an alternative support such as fumed titanium dioxide (TiO₂) is assayed. Immobilization of another microbial lipase, from *Candida rugosa*, on other oxides like Al₂O₃ [7] and crystalline and amorphous hydrous niobium [8,9] has been reported. The benefits of lipase immobilization include the possibility of using the biocatalyst in continuous processes, easy separation from product and reuse, higher temperature stability and less sensibility to alkaline/acid pHs and to denaturation than their free counterparts.

There are several recent reports on the use of hydrophilic oxides as supports of lipases. Sedaghat et al. reported the immobilization of lipase from *C. rugosa* in bentonite (65% SiO₂, 13.6% Al₂O₃ and

21.4% other oxide compounds). By storing the immobilized enzyme at 30 °C, the hydrolytic activity (related to the hydrolysis of olive oil in hexane) of the enzyme adsorbed on bentonite was similar to its initial activity until 60 days [10]. Giordano et al. reported the encapsulation of lipase enzyme in highly ordered mesoporous matrix by a sol–gel method that involves the hydrolysis/polycondensation of a silica precursor at neutral pH and room temperature. The enzyme is encapsulated within the micellar phase of the surfactant that is self-assembled with silica. The encapsulated biocatalyst has been used for the transesterification reaction of triolein with methanol under solvent-free conditions. The highest fatty acid methyl esters yield (77%) was obtained after 96 h at 40 °C, with triolein:methanol molar ratio equal to 1:3. Total productivity of the immobilized enzyme is almost six times higher than the one obtained using free lipase [11]. The formation of a mesoporous bioreactor- or an inorganic–organic hybrid material-based on SBA-15 and porcine pancreatic lipase (PPL) has been reported by Guo et al. The assay of enzyme activity shows that the reduction in pore size by chemical modification prevents the PPL leaching as expected [12].

Batch hydrolysis of olive oil was performed by *C. rugosa* lipase immobilized on Amberlite IRC-50 and Al₂O₃. These two supports were selected out of 16 carriers: inorganic materials (sand, silica gel and Al₂O₃), inorganic salts (CaCO₃ and CaSO₄), ion-exchange resins (Amberlite IRC-50 and IR-4B, Dowex 2×8), a natural resin (colophony), a natural biopolymer (sodium alginate), synthetic

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polymers (polypropylene and polyethylene) and zeolites. Most of the immobilized lipase exhibited either a low enzyme activity or difficulties during the hydrolytic reaction. The immobilizate with Al_2O_3 was less stable and less active [13].

Kimoto et al. have published data on the selective hydrolysis of ketoprofen by *Mucor javanicus* lipase immobilized on Toyonite 200-M (9), Toyonite 200-A, or Hyflo Super-ccl (Wako, Osaka). Lipase M immobilized on Toyonite 200-A showed the best selectivity ($E = 55$) and reactivity. Moreover, the lipase could be recycled several times [14].

Toyonite 200-M-TN-M was prepared hydrothermally from the minerals of kaolinite. Compared with some other commercial solid supports, the TN-M one exhibited better stability and higher selectivity for lipase proteins, and lipase PS from *Pseudomonas cepacia* immobilized on the ceramics support showed higher reactivity for organic substrates than the free crude enzyme [15].

CALB/fumed silica preparations approached the catalytic activity of commercial Novozym 435 for a model esterification in hexane at 90 wt.% fumed silica (relative to the mass of the preparation) in the manuscript of Rezac et al. An intriguing observation was that the catalytic activity at first increases as more fumed silica was made available to the enzyme but then decreased precipitously when fumed silica exceeded 90 wt.% [16].

The search for alternative supports (different from commercial resins usually used for enzyme immobilization) motivated the assay of inorganic oxides as matrices for lipase immobilization. It has been reported that oxide supports in the presence of water generate a specific pH at the surface, which is measured as the surface pH at the point of zero charge (pzc) from electrophoretic measurements [17]. Since water is the immobilization medium used in this contribution, the inorganic oxides evaluated as potential matrices for lipases adsorption (MgO , $\alpha\text{-Al}_2\text{O}_3$, ZrO_2 , TiO_2 , Nb_2O_5 and SiO_2) were analyzed in terms of their surface pH [17]. Searching for a “neutral” surface that would not deactivate enzymes due to a high acidic or basic nature, TiO_2 , with a pH at pzc of 6.0–6.4, was the selected oxide. Although immobilization of other kinds of enzymes on TiO_2 and TiO_2 -coated surfaces has been reported (i.e. enzyme immobilized on polypropylene loaded titanium dioxide membrane [18], adsorption of horseradish peroxidase on TiO_2 nanotube arrays and on titania sol–gel thin films [19,20]). However, at the best of the authors’ knowledge, this is one of the few reports dealing with the immobilization of *C. antarctica* B lipase (CALB) onto titanium dioxide of the anatase type. The techniques and the approach reported have been established as common procedures in the field of the analysis and study of the lipase immobilization process [21].

Besides lipase immobilization, this contribution is devoted to the characterization of the biocatalyst synthesized (CALB/TiO_2) by means of FTIR, SEM and fractal dimension analysis. Immobilization of enzymes at solid surfaces can cause a change in the enzyme conformation [21]. Then, in the present work, the second derivative of the FTIR spectra and curve fitting procedures are used in order to obtain quantitative information on the relative contribution of the structural elements that constitute the secondary structure of lipase (α -helices, β -sheets, turns and unordered structures) during the immobilization course. This well-known procedure in the field has been applied to the different steps of the immobilization investigated in the present contribution.

2. Experimental

2.1. Materials

C. antarctica Lipase B (CALB) (35,500 g/mol) was purchased from Sigma Aldrich, Argentina (10.9 U/mg). High surface area and high purity titanium dioxide anatase, Degussa P-25 (TiO_2) was

kindly supplied by Lehigh University (USA). After calcination at 500 °C for 5 h, titanium dioxide with a Brunauer, Emmett and Teller (BET) area of 45.7 m^2/g was obtained. Only particles with a minimum diameter of 420 μm were contacted with the lipase solution. Bidistilled water (pH 5) used in the immobilization experiments was bought from Anedra. Special nylon filters for small particle’s powder (0.45 μm) were purchased from Osmonics.

2.2. Adsorption experiments

In order to prepare the lipase solution 20 mg of CALB were added to 25 ml of bidistilled water (pH 5) and subjected to magnetic stirring during 30 min in order to solubilise the lipase at room temperature. Bidistilled water instead of buffer was chosen as immobilization medium in order to avoid the adsorption of buffer’s ions onto the immobilization support as previously reported by some of us for buffered immobilization media [22]. Three millilitres of the lipase solution were withdrawn with a syringe and subsequently filtered for later UV–vis analysis. The impregnation process was performed by contacting 100 mg of TiO_2 with the lipase solution that is maintained at 25 °C and 180 rpm with a temperature controlled shaker-bath (Julabo SW22). Samples (typically, 3 ml) from the supernatant solution were withdrawn at 30 min, 1, 2, 4, 6 and 7 h during the immobilization. These samples were filtrated with nylon filters for small particle’s powder (0.45 μm). The solid retained at the filter is return to the TiO_2 -lipase system to minimize the variation of the amount of the oxide support.

At the end of the immobilization period (7 h), the catalyst (CALB/TiO_2) was washed once with bidistilled water and separated from the solution by centrifugation. Finally, it was dried to constant weight at 50 °C and kept refrigerated (5 °C) for further analysis. No evidence/report of conformational secondary changes by heating at 50 °C in air – no vacuum – is found for the free CALB, much less for the adsorbed, TiO_2 -immobilized CALB.

2.3. Immobilization course analysis

2.3.1. Quantitative analysis of immobilization through UV–vis spectroscopy

The absorbance of supernatant solution samples withdrawn during the immobilization was measured at 280 nm. Then, using a calibration curve, micromols of adsorbed lipase per nm^2 of TiO_2 were calculated as a function of time. The value obtained at the end of the immobilization period (7 h) was compared with a theoretical value of adsorbed lipase monolayer, calculated considering the specific BET area available of the used amount of TiO_2 and the average projected area of a molecule of lipase.

2.3.2. Secondary-structure analysis of supernatant solution through infrared spectroscopy

The evolution of the secondary structure of CALB during the adsorption process was followed through infrared analysis of samples withdrawn from the supernatant solution. A drop of sample of definite volume was placed between CaF_2 crystals and analyzed with a Bruker IFS 66 spectrometer. The infrared analysis was recorded with 100 scans in the absorption mode. The contribution of the infrared spectrum of H_2O was digitally subtracted from the spectra of the lipase, considering the spectrum of pure water. Similar maximum absorption intensity of all the spectra was verified, which indicates that a constant optical path length was maintained during the analysis of the liquid samples.

To estimate the secondary structure, peak fitting of the Amide I band ($1700\text{--}1600\text{ cm}^{-1}$) by Lorentzian-shaped components was performed on the non-deconvoluted spectra. The software used with this purpose was a special peak fitting module of Origin 5.0.

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