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Lipase entrapment in a zirconia matrix: Sol–gel synthesis and catalytic properties

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ABSTRACT

Zirconia-based sol–gels have been used for lipase entrapment, to avoid the biocatalyst inactivation associated to the use of basic or acid catalysts. The method developed allows a single-step entrapment of the enzyme in a zirconia sol–gel with mesopores of diameter in the range 2–4 nm, and a surface area of 219 $m^2\ g^{-1}$. The zirconia matrix is substantially unaffected by the presence of the lipase. The size of the pores, though smaller than that of the enzyme, seems large enough to minimise the effect of the internal diffusional limitations on the overall reaction kinetics, as demonstrated by the substantial equivalence between the activation energies measured with the free and with the immobilised lipase. The thermal inactivation of the zirconia-entrapped lipase, characterised by a mathematical model, is significantly reduced as compared to the inactivation of the free enzyme.

1. Introduction

Sol–gel nanomaterials are gaining a growing importance as solid supports for the immobilisation of biomolecules to be used for biocatalysis, biosensors, and biomedical applications, offering a useful alternative to the traditional polymer technology [1–5]. As a matter of fact, thanks to their porous structure in nanometer dimensions, sol–gel materials offer unique intrinsic properties, such as high surface to volume ratio, large surface area and porosity. In addition, the sol–gel process offers higher flexibility as regards the surface, composition and properties. Furthermore, nanoporous materials, especially inorganic nanoporous materials, which are made mostly of metal oxides, are usually nontoxic, inert, chemically and thermally stable, so they have wide applications where biocompatibility and thermal stability requirements are essential.

Due to the low temperature processing, the sol–gel technology offers useful methods to immobilise heat-sensitive biomolecules. Nevertheless, the structure/activity of encapsulated biomolecules could be affected by the presence of enzyme-denaturing and cytotoxic alcohol in the reaction media, both as cosolvents or as products of the hydrolysis/condensation reactions. In addition, when carrying out the synthesis of silica sol–gel materials, such reactions are usually too slow and need basic or acid catalysis, causing extreme pH values, giving rise to biocatalysts inactivation.

In this study, the entrapment of lipases in zirconia-based sol–gel material has been undertaken to improve the enzyme stability. The sol–gel method allowed to make the support material and to entrap the enzyme in a single step, starting from a solution containing both the matrix precursors and the enzyme, so that when the gelation occurs the enzyme remains entrapped and uniformly dispersed in the gel. Moreover, due to high hydrolysis and polycondensation rates offered by zirconia precursors, a neutral pH could be adopted, avoiding any possible configurational modification of the enzyme induced by acid or basic environments.

So far, zirconia has been used as a support for enzyme immobilisation only in the form of a zirconia/nafion composite [6,7], obtained by casting on an electrode a solution containing zirconia, nafion and an enzyme.

Lipases, chosen as model enzyme, are used in a growing number of industrial applications, both in aqueous and in nonaqueous media. In particular, immobilised lipases have been successfully used in organic synthesis [8–10]. Also, lipases have been recently used in biosensors for the determination of lipids for clinical purpose [11,12], and in biosensing [13,14]. So far, immobilisation of lipases by the sol–gel technique has been carried out only using silica-based materials [2,15–18].

2. Experimental

2.1. Enzyme encapsulation in sol–gel material

In a typical test, 45.0 mg of lipase from Rhizopous oryzae and 15 mg of PEG (MW 1450) were dissolved in 3.0 mL of distilled water and the mixture was stirred by hand up to obtaining a clear

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Nomenclature

a	specific activity of the total enzyme
a_N	specific activity of the native enzyme, N
a_X	specific activity of the partially inactivated form of the enzyme, X
A, B	mathematical constants
D	completely inactivated form of the enzyme
k, k_1, k_2	first order inactivation constants
N	native form of the enzyme
[N]	concentration of the native form of the enzyme, N
t	time
X	partially inactivated form of the enzyme
[X]	concentration of the partially inactivated form of the enzyme

solution. Subsequently, 2.0 mL of supernatant were drawn and added to 8.5 mL of 1-propanol (99.80+%). The resulting solution was added to a solution formed by 10 mL of Zirconium(IV) propoxide (70 wt.% in 1-propanol) and 1.5 mL of acetylacetone (99+%). The final solution was vigorously stirred at room temperature and the gelation occurred in about 20 min. A transparent and homogeneous slightly yellow coloured gel was obtained. The gel was left at room temperature for 3 h and then lyophilized, in vacuum at −50 °C for 20 h. The lyophilized material was washed three times by distilled water to remove the lipase not entrapped. The enzyme concentration in the washing liquid, estimated by the Lowry method [19], was very low, corresponding to a 99% entrapment yield. The measured lipase loading in the biocatalyst was 8.8 mg lipase/g zirconia, whereas the PEG loading was 2.9 mg PEG/g zirconia.

In the same conditions was prepared a zirconia matrix, as reference material both for the activity tests and for surface area measurements. The chemicals were provided by Aldrich, the lipase by Fluka.

2.2. Structure and thermal behaviour

The nature and temperatures of the various reactions occurring during the heating of the biocatalyst and the corresponding weight losses were evaluated by a Netzsch simultaneous thermoanalyser STA 409 PC with Al_2O_3 as reference material. The TG/DTA curves, recorded in N_2 from room temperature up to 900 °C at a heating rate of 10 °C min^{-1}, were carried out on 50 mg of the bulk sample.

The structure of the biocatalyst as well as that of the zirconia matrix were studied by FTIR spectroscopy. FTIR absorption spectra were recorded, in the 4000–400 cm^{-1} range, using a spectrometer equipped with a DTGS KBr (deuterated triglycine sulphate with potassium bromide windows) detector. A spectral resolution of 2 cm^{-1} was chosen. 4.0 mg of each test sample were mixed with 200 mg of KBr in an agate mortar and then pressed into pellets of 13 mm diameter. The spectrum for each sample represents an average of 64 scans, which were corrected for the spectrum of the blank KBr pellet.

2.3. Textural properties

N_2 adsorption–desorption isotherms at −196 °C were obtained by a Micromeritics Gemini II apparatus model 2370. The samples were previously treated at 50 °C for 2 h under N_2 flow. The N_2 adsorption–desorption isotherms were elaborated by the Brunauer–Emmett–Teller (BET) [20] method for the calculation of the surface areas. Pore volumes were determined from the amounts of adsorbed N_2 at $P/P^0 = 0.98$ (desorption curve), assuming the presence of liquid N_2 (density = 0.807 g cm^{-3}) in the pores under these conditions. The pore distribution was evaluated by the Barrett–Joiner–Halenda (BJH) method [20].

2.4. Activity assay

The activity of the immobilised enzyme as well as that of the free enzyme was assayed by monitoring the amount of *p*-nitrophenol released upon hydrolysis of a 0.5 mM solution of *p*-nitrophenyl butyrate (pNPB) in 50 mM of phosphate buffer, usually at pH 7.5. In a typical test, 10 mg of immobilized biocatalyst, 10 mg of ZrO_2 matrix or 0.1 mg of free lipase were added to 2 mL of the reaction mixture. The mixture was incubated in 5 mL test-tubes, using a thermostatic shaker. After the fixed reaction time, the increase in absorbance at 410 nm was measured for 1 min using an apparent extinction coefficient of 1.33×10^4 M^{-1} cm^{-1}. One unit of hydrolase activity was defined as the amount of enzyme or biocatalyst required to transform 1 μmol of *p*-nitrophenyl butyrate to *p*-nitrophenol per min at room temperature.

The catalytic activity of the ZrO_2 matrix was tested to consider the possible contribution to the reaction rate. It appeared to be so low to be neglected in comparison with that of as the biocatalyst and the free enzyme.

2.5. Thermal stability and reusability tests

Operational stability tests on lipases were carried out keeping entrapped or free enzyme samples immersed in 50 mM of phosphate buffer, pH 7.5, at constant temperature. At given deactivation times, enzyme samples were withdrawn and assayed for activity at 37 °C.

Reusability tests on the entrapped lipase were carried out keeping the enzyme samples immersed in 50 mM of phosphate buffer, pH 7.5, at constant temperature for a fixed period (4 days), then removing the lipase from the solution, removing external water with filter paper and keeping it 3 days over silica gel.

3. Results and discussion

3.1. Structural and textural characterisation

TG/DTA curves of biocatalyst bulk sample are displayed in Fig. 1. The overall weight loss given by the TG curve was 44.4 wt.%. The majority of the weight loss takes place from room temperature to about 400 °C. In this range, for a typical gel-derived sample, the evaporation of the solvents and the subsequent pyrolysis and/or

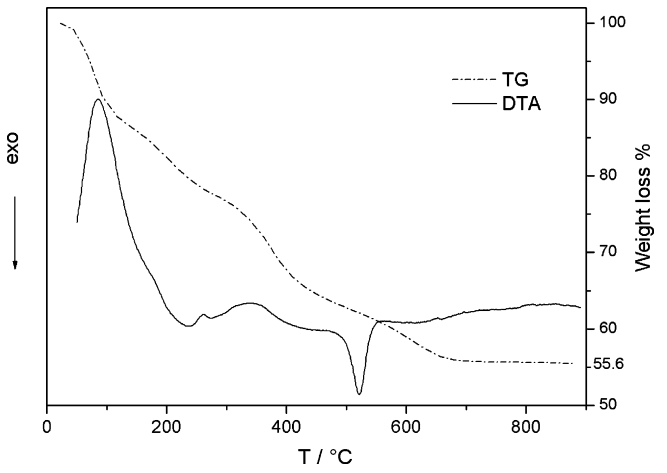

Fig. 1. TG/DTA curves of the zirconia-immobilized lipase, recorded in N_2 at 10 °C min^{-1}.