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Influence of gelation time on the morphological and physico-chemical properties of the sol–gel entrapped lipase

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Abstract

Different gelation times (4, 18, 24 and 48 h) were used for the preparation of silica sol–gel supports and encapsulated *Candida rugosa* lipase using tetraethoxysilane (TEOS) as precursor. The hydrophobic matrices and immobilized lipases produced were characterized with regard to pore volume and size by nitrogen adsorption (BJH method), weight loss upon heating (TGA), differential scanning calorimetry (DSC), scanning electron microscopy (SEM), chemical composition (FTIR) and percentage of hydrolysis (POH%) of olive oil. These structural parameters were found to change with the gelation time, but no direct relation was found between the percentage of oil hydrolysis (POH%) and the gelation time. The best combination of high thermal stability and high POH% (99.5%) occurred for encapsulated lipase produced with 24 h gelation time.

Keywords: Gelation time; Sol–gel; *Candida rugosa*; Entrapped lipase; Hydrolysis

1. Introduction

The sol–gel process, in which inorganic oxides can be prepared from liquid precursors at low temperatures, allows biological molecules to be immobilized in amorphous inorganic matrices. The successful encapsulation of biomolecules such as enzymes and other proteins within the pores of sol–gel derived glasses has been well documented [1–3]. The sol–gel immobilized enzymes retain biological activity, and in some cases, proteins experience an increased stability upon encapsulation. Moreover, the encapsulation approach produces a more robust biologically active material. However, the nature of the solid network generated by aggregation of colloids or polymerization of alkoxides depends on the diffusion of the elemental species and the growing clusters. It also depends on the probability of aggregation during the contact between species in the procedure [4–6].

In a previous study [7], biocatalysts with encapsulated lipase in silica gels produced by acid- or base-catalyzed hydrolysis of silane compounds such as tetraethoxysilane (TEOS) or methyltrimethoxysilane (MTMS), in the presence of the additive polyethylene glycol (PEG) were developed. These gels were characterized with regard to mean pore diameter, specific surface area, pore size distribution (B.E.T. method), weight loss upon heating (TGA) and chemical composition (FTIR). The behavior of the sol–gel encapsulated lipase systems depends on the physical and structural properties of the support, and the biochemical properties of the lipase. The conformation of the encapsulated lipase in a gel can largely be improved by grafting appropriate functionality, such as alkyl-substituted silanes and additives, on the gel network, which surrounds the enzyme. The encapsulation of *Candida rugosa* lipase (CRL) in sol–gel prepared by the hydrolysis of alkyl-substituted silanes like TEOS, in the presence of PEG showed considerable hydrolytic and esterification activity, depending on the water content of the immobilized enzyme [7,8]. On the other hand, the encapsulation of CRL in sol–gel prepared by the hydrolysis of MTMS, in the presence of PEG only showed high activity for esterification [8]. This was related to the interactions caused by the resulting hydrophobic–hydrophilic nature of the support. The most efficient systems appeared to be worthy of further study, owing to their potential application as biocatalysts. In addition, further characterization of the gel structural properties was warranted, given the difficulty in correlating these properties with the enzymatic activity. The esterification or hydrolytic activity

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and the functional stability of *C. rugosa* lipase immobilized by the sol–gel process, using precursor MTMS or TEOS, in the presence of PEG, were analyzed in the light of the gel texture and structure which was characterized by ^{29}Si and ^{13}C NMR [9].

After these studies, it was required to determine the ideal gelation time to enable an adequate aggregation of the solid network generated during the contact between species of the procedure. The stability of the sol–gel structure due to the gelation time and the characteristics of the hydrophobic matrices and immobilized preparations were characterized with regard to the mean pore volume, specific surface area, pore size distribution (B.E.T. method), weight loss upon heating (TGA), chemical composition (FTIR). The catalytic activities of the immobilized preparations were assayed by the hydrolysis of olive oil.

2. Experimental procedures

2.1. Enzyme and chemicals

Commercial *C. rugosa* lipase (CRL, Type VII, product No L1754) was purchased from Sigma Chemical Co. (St Louis, MO, United States). This lipase is substantially free of α-amylase and protease, and contains lactose as an extender. Nominal specific lipase activity was 104.94 U mg^{-1} protein. Polyethylene glycol (PEG, MW 1450, Merck) was used as stabilizing agent. The silane precursor tetraethoxysilane (TEOS) was supplied by Across Organic (New Jersey, United States) and used without further purification. Ethanol (minimum 99%), ammonia (minimum 28%), hydrochloric acid (minimum 36%) and, gum Arabic were from Synth (São Paulo, Brazil). Olive oil was purchased at a local market. Water was purified by reverse osmosis and deionized through a Milli-Q four-cartridge organic-free water purification system. Other chemicals were of analytical grade and used as received.

2.2. Encapsulation of lipase from C. rugosa (CRL) in sol–gel matrices and gelation time

The methodology previously established by the Patent PI0306829-3 [10] was used and is briefly described as follows: 30 mL of TEOS were dissolved in 36 mL of absolute ethanol under nitrogen inert atmosphere. To this, 0.22 mL of hydrochloric acid dissolved in 5 mL of ultra-pure water was slowly added and the mixture was agitated (200 rpm) for 90 min at 35 °C. Then, 10 mL of lipase solution (18.29 mg mL^{-1}), PEG solution (5 mg mL^{-1}, 8 mL added) and 1 mL of ammonium hydroxide dissolved in 6 mL of ethanol were added (hydrolysis solution) and the mixture was kept under static conditions for gelation times of 4, 18, 24 and 48 h, to proceed with the chemical condensation. The bulk gel was washed with heptane and acetone and dried under vacuum at room temperature for 24 h.

2.3. Determination of the percentage of hydrolysis

The methodology previously described by Pinheiro et al. [11] was used. The substrate was prepared by mixing 50 mL of olive oil with 50 mL of gum Arabic solution (7%, w/v). The reaction mixture containing 50 mL of the oil emulsion, 10 mL of 100 mM sodium phosphate buffer (pH 7.0) and either free (1 mL, 5 mg mL^{-1}) or immobilized (≈250 mg) lipase was incubated in a thermostated batch reactor for 60 min at 37 °C. After this period, the percentage of hydrolysis was determined by titration of the released fatty acids and defined as the percentage weight of free fatty acids in the sample, in comparison with the maximum theoretical amount of free fatty acids that could be produced if all the oil in the sample was hydrolyzed:

$$\mathrm{POH}\,(\%) = \frac{\mathrm{Na} \times 0.02 \times 10^{-3} \times \mathrm{MM}}{W_\mathrm{t} \times f_0 \times f_1} \times 100 \quad (1)$$

where f_0 is the fraction of oil in the sample at the start of reaction ($g_{\mathrm{lipids}}/g_{\mathrm{sample}}$), f_1 the ratio of the mass of fatty acids to the mass of oil, after total oil hydrolysis ($g_{\mathrm{fatty\ acids}}/g_{\mathrm{lipids}}$), MM the average molecular weight of fatty acids in the olive oil (825.30 g mol^{-1}), N_a the volume of sodium hydroxide solution required during fatty acids titration (mL), and W_t is the weight of the sample (g).

2.4. Sample characterization

The characterization of the porosity of hydrophobic matrices and immobilized biocatalysts is a complex issue when the total porosity, the pore size and the pore size distribution should be further analyzed. Methods based on gas adsorption are the most convenient for the study of microporous and mesoporous materials, using volumetric measurements of the adsorbed gas quantities. Pure silica gel or encapsulated lipase samples were characterized based on the BJH calculations [12] which were evaluated by the B.E.T. (Brunauer–Emmett–Teller) apparatus software (NOVA 1200-Quantachrome).

The samples weight loss upon heating was determined in a thermo gravimetric analysis-TGA apparatus (TGA-50 Shimadzu-Thermogravimetric Analyzer) over the range 25–1000 °C, with a heating rate of 20 °C min^{-1}, using air as the purge gas. Differential scanning calorimetry data were taken in a PerkinElmer DSC-50 differential scanning calorimeter. Free lipase, pure silica or immobilized lipase samples weighting 6 mg were put in a sealable aluminum pan, which was heated from 25 to 500 °C at a rate of 10 °C/min. Free lipase, pure silica and immobilized lipase samples were also submitted to the Fourier transform infrared—FTIR analysis (Spectrophotometer FTIR BOMEM MB-100). The spectra were obtained in the wavelength range from 400 to 4000 cm^{-1} for evaluation of the immobilization procedures, in accordance with Soares et al. [7].

Scanning electron microscopy (SEM) was used to characterize the surface of pure silica matrices and immobilized lipase samples.

3. Results and discussion

3.1. Characterization of the porosity of hydrophobic matrices and immobilized biocatalyst

Data for pore volume distributions obtained for the different gelation times and the percentage of micropores and mesopores