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Journal of Molecular Catalysis B: Enzymatic 39 (2006) 69–76
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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Characterization of sol–gel encapsulated lipase using tetraethoxysilane as precursor

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Available online 3 March 2006

Abstract

Lipase from *Candida rugosa* was encapsulated within a chemically inert sol–gel support prepared by polycondensation of the precursor tetraethoxysilane (TEOS) in the presence of polyethylene glycol (PEG) as additive. The properties of silica and their derivatives with regard to mean pore diameter, specific surface area, mean pore size, weight loss upon heating (thermogravimetric analysis, TGA) and ^{29}Si and ^{13}C NMR are reported. The pH optimum shifted from 7.8 to 6.7 and optimum temperature jumped from 36 to 60 °C upon enzyme encapsulation. Encapsulated lipase in presence of PEG (EN-PEG) exhibited higher stability in the range of 37–45 °C, but from 50 to 65 °C the EN-PEG was inactivated after seven cycles. Hydrolytic activity during long-term storage at room temperature decreased to 50% after 94 days. High diffusional resistance was observed for large oil concentration reducing hydrolytic effectiveness by 60% in the case of the encapsulated lipase. NMR, pore size and specific surface area data suggested an active participation of the lipase enzyme during gelling of the silica matrix. This lead to reduction of available Si–OH groups, larger pores and smaller surface area. Larger pores increase substrate diffusion that correlates well with higher hydrolytic activity of the TEOS–PEG sol–gel matrix encapsulated enzyme in comparison with other sol–gel supports.

Keywords: Lipase; Encapsulated; Sol–gel; Tetraethoxysilane; Effectiveness

1. Introduction

Recently, an interesting approach to the entrapment of enzyme and others proteins concerns the use of inorganic matrices such as silica gel [1]. Accordingly, the so-called sol–gel process [2], initiated by the hydrolysis of $Si(OR)_4$, is performed in the presence of the enzyme and involves a low temperature of production. Hydrolysis and condensation of the Si-monomers in the presence of an acid or base catalyst trigger cross-linking reactions with formation of amorphous SiO_2, a porous inorganic matrix that grows around the enzyme in a three-dimensional manner.

$$n\,Si(OR)_4 + 2n\,H_2O \xrightarrow{\text{catalyst}} (SiO_2)_n + 4n\,ROH \quad (1)$$

Research has demonstrated that silicate glasses obtained by the sol–gel method can produce a supporting matrix, in which biomolecules can be encapsulated [1–9]. Various enzymes, antibodies and other proteins, DNA, RNA and antigens as well as more complex structures such as cell membranes and organelles, and even living microbial, plant and animal cells, have been entrapped in inorganic and inorganic–organic hybrid sol–gel polymers. These materials can be used as biocatalysts and highly specific sensors, for environmental, food and medical applications. Typical applications of sol–gel derived biomaterials include selective coatings for optical and electrochemical biosensors, stationary phases for affinity chromatography, immunoadsorbent and solid-phase extraction materials, controlled release agents, solid-phase biosynthesis and unique matrices for biophysical studies. Through careful selection of precursors and additives, these materials can be designed for specific applications, and can produce useful, robust devices. Although most applications are still at the developmental stage, these biocomposites promise to revolutionize the whole field of high-performance bioimmobilization [2,10,11,12,14]. The present article shows our efforts in this area and presents results concerning the entrapment of *Candida rugosa* lipase.

Natural fats and oils can be used directly in products, either individually or as mixtures. In many cases, however, it is

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doi:10.1016/j.molcatb.2006.01.005

necessary to modify their properties, particularly their melting characteristics, to make them suitable for special applications. Because of that, the oils and fats industry has developed several modifications applied to older processes, using new enzyme technology. In particular, lipases can be used for reactions such as esterification, interesterification and hydrolysis [13–16]. Although most lipases are more robust than many other enzymes, their industrial use as catalysts in synthetic organic chemistry has not reached a significant level [2,17]. This may be due to their limited long-term stability, difficulties in separating the products from the lipase and problems arising upon reusing the biocatalyst. In order to solve these drawbacks, a number of different strategies have been developed [3,5].

Many different methods have been described in this area [5,6] and some authors have suggested that hydrophobic materials are the most suitable supports for lipase immobilization [7,8], preferably by encapsulation within a polymer matrix, or silica glasses, obtained by sol–gel techniques [3,9,13]. This approach has several advantages because mechanical entrapment of enzymes using sol–gel materials allows stabilization of the protein tertiary structure caused by the tight gel network [3]. Moreover, good results were obtained in a number of studies in which enzyme entrapment concerns the use of inorganic matrices such as silica gel [3,14–17].

Interfacial enzymes act on insoluble substrates. Phospholipases and lipases are two important examples from this group of enzymes. Lipases, for example, hydrolyze the ester bond of triacyglycerols, which are insoluble in aqueous media [18]. During the digestion of lipids, triacyglycerols are emulsified by gum arabic solution, forming large emulsion droplets. For certain conditions, diffusional resistance arising from the transport of substrate from the bulk solution to the catalytic sites and from the diffusion of reaction products back to the bulk solution may reduce the rate of reaction. These diffusional resistances may be classified as [19]:

(i) Internal or intraparticle mass transfer effects, when the enzyme is located inside a porous medium.
(ii) External or interparticle mass transfer effects, which occur between the bulk solution and the outer surface of the enzyme-matrix particle.

The observed changes in enzymatic properties upon immobilization are the result of interactions of these factors, the exact contribution of each factor being difficult to determine, and frequently the conjoint effects of intraparticle and interparticle reaction rate diffusion limitations are measured together as we did in this work.

Table 1
Characterization of the pure silica gel matrix and encapsulated derivatives [9]

Precursor	Samples	Surface area[a] ($m^2\ g^{-1}$)	Mean pore diameter[a] (Å)	Pore volume[a] ($cm^3\ g^{-1}$)
TEOS	PS	607	18.23	0.37
	EN	382	49.99	0.48
	EN-PEG	348	56.59	0.43

[a] Measured by the BET technique.

1.1. Previous work of our research group

In our initial experiments, we have investigated the feasibility of encapsulating microbial lipases in hydrophobic matrices obtained by the sol–gel process. The enzyme used was the non-specific lipase from *C. rugosa* and silicate sol–gel particles were prepared by acid or base catalyzed hydrolysis of silane compounds as tetraethoxysilane (TEOS), methyltrimethoxysilane (MTMS), and poly(dimethylsiloxane) (PDMS) in the presence and absence of additives, such as polyethyleneglycol (PEG) or polyvinylalcohol (PVA) [7,9].

Systematic studies have been performed on the structure, morphology and pore size distribution of these hydrophobic sol–gel materials [7,9,20]. Soares et al. [9] characterized silica and their derivatives in regard to mean pore diameter, specific surface area, pore size distribution (Table 1), weight loss upon heating, analysis Fourier Transform Infrared (FTIR) and hydrolytic activity (Table 2).

The encapsulated lipase in presence of PEG (EN-PEG) showed considerable hydrolytic activity, which was explained by the increased mean pore size obtained with the use of an additive that inhibited gel contraction during support synthesis.

Comparative studies of free and encapsulated lipase in matrices obtained with MTMS as precursor were carried out as function of pH, temperature and thermal stability. Enzymatic hydrolysis with the immobilized enzyme in the framework of the Michaelis–Menten mechanism was also reported. The usefulness of thermoporometry in probing the structure of sol–gel matrices was demonstrated, in contrast to the classical techniques (like BET, which underestimates the pore size). Thermoporometry analysis allowed the determination of the porous dimensions in the presence of a solvent that swells the material. Further work is still needed to attain greater reliance on this kind of measurements [20].

Table 2
Hydrolytic activity of different gel types encapsulated *Candida rugosa* lipase [7]

Experiment	Hydrolytic activity ($\mu mol\ mg^{-1}\ min^{-1}$)	Sum of the relative percentages of the $Q^2 = [Si(OSi)_2(OH)_2]$ and $Q^3 = [Si(OSi)_3(OH)]$ sites (%)
Free lipase	152.77	–
Pure silica matrix	–	80
Encapsulated lipase (EN)	66.38	67
Encapsulated lipase in presence of PEG (EN-PEG)	128.92	70

The encapsulation of CRL in a sol–gel prepared by the hydrolysis of TEOS or MTMS [20], in the presence of PEG showed considerable hydrolytic and esterification activity that warranted the need for complementary studies about the influence of pH and temperature, storage, thermal and operational stability, mass transfer effects and sample characterization by NMR spectra of the encapsulated lipase obtained with TEOS. These studies are the objective of this paper.

2. Experimental procedures

2.1. Enzyme and chemicals

The same lipase as in previous biocatalytic esterification-hydrolysis studies [9,20] was selected: commercial *C. rugosa* lipase (Type VII – product No. L1754) was purchased from Sigma Chemical Co. (St Louis, MO, USA). 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 (MW 1450, Merck) was used as stabilizing agents. For sol–gel encapsulation, the silane precursor was tetraethoxysilane being supplied by Acros Organic (NJ, USA) 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 (low acidity) 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 (EN) in sol–gel matrices

C. rugosa lipase was encapsulated in a sol–gel silica matrix according to a modification of a published method [21]. Thirty milliliters of TEOS were dissolved in 36 mL of absolute ethanol under an inert nitrogen atmosphere. To this, 0.22 mL of hydrochloric acid dissolved in 5 mL of ultra-pure water was slowly added and the mixture 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 24 h to complete the chemical condensation [7,9]. The bulk gel was washed with heptane and acetone and dried under vacuum at room temperature for 24 h. Consequently, *C. rugosa* lipase was entrapped in the silicate sol–gel prepared by hydrolysis of TEOS in the presence of PEG, and the dried gels were crushed to a powder with particle size in the range of 180–250 μm, resulting in the encapsulated lipase in the presence of additive (EN-PEG) derivative.

2.3. Activity of lipase in the hydrolysis of emulsified olive oil

Hydrolytic activities of free and encapsulated lipase were assayed by the olive oil emulsion method according to the modification proposed by Soares et al. [7,22]. 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 5 mL of the emulsion, 4 mL of 100 mM sodium phosphate buffer (pH 7.0) and either free (1 mL, 5 mg mL^{-1}) or encapsulated (100–250 mg) lipase was incubated for 5 min at 37 °C. The reaction was stopped by the addition of 10 mL of acetone–ethanol solution (1:1). The liberated fatty acids were titrated with 25 mM potassium hydroxide solution in the presence of phenolphthalein as an indicator. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of free fatty acid per min (μmol min^{-1}) under the assay conditions (37 °C, pH 7.0, 150 rpm). Analyses of hydrolytic activities carried out on the lipase loading solution and bioencapsulates preparations were used to determine the coupling yield η (%) according to Eq. (2).

$$\eta\,(\%) = \frac{U_s}{U_o} \times 100 \tag{2}$$

in which U_s is the total enzyme activity recovered on the support and U_o is the enzyme units offered for immobilization.

2.4. Sample characterization

The structure of the gels samples was characterized by recording ^{29}Si and ^{13}C NMR spectra. The solid-state ^{29}Si CP-MAS NMR spectra were obtained on a RMN Inova 400 spectrometer at 300 MHz. The samples were spun in the magic angle at ca. 10 kHz. The pulse interval time was 10 s and the pulse duration 3 μs. For ^{13}C CP-MAS NMR, the equivalent numbers were 4.6 kHz, 2 s and 3.5 μs, respectively. For the two atom types, the chemical shifts were measured using $Si(CH_3)_4$ as a Ref. [23].

2.5. Effect of pH and temperature on the hydrolysis reaction rate

The influence of pH, and temperature on the rate of hydrolysis with the encapsulated (EN-PEG) derivative was measured with emulsified olive oil according to Section 2.3.

2.6. Thermal and operational stability

The thermal and operational stability (37–60°) of the encapsulated system (EN-PEG) was measured in a sequence of cycles with emulsified *p*-nitrophenyl palmitate (*p*-NPP) according to Kordel et al. [24]. One volume of a 16.5 mM solution of *p*-NPP in 2-propanol was mixed just before used with 9 volumes of 50 mM Tris–HCl buffer, pH according to the optimum pH of the biocatalyst and the solution also contained 0.4% (w/v) Triton X-100 and 0.1% (w/v) gum arabic. Then, for each cycle 1.35 mL of this mixture was pre-equilibrated at 37 °C in a 3 mL cuvette of a UV–vis spectrophotometer (Varian UV-Carry, Varian Corporation, USA). The reaction was started by addition of about 1 mg encapsulated lipase, the exact mass being determined by weighting the cuvette. The cuvette was shaken before returning it to the spectrophotometer and the absorbance at 410 nm, against a blank without enzyme, was monitored for 60 s. During reaction the encapsulated lipase remained at the bottom of the cuvette,

out of the optical path and the absorbance reading taken every 15 s, was due only to the absorbance of the reaction medium supernatant. The absorbance data was used to calculate the concentration of the reaction product, *p*-nitrophenol (*p*-NP), using a molar extinction coefficient of $1.32 \times 10^4\ \text{mol}^{-1}\,\text{L}\,\text{cm}^{-1}$. Then, a straight line was fit to this concentration data as a function of time. The slope of this straight line yields the enzyme activity that divided by the encapsulated lipase mass gives the enzymatic activity per mass of encapsulated lipase. One enzyme unit was the amount of protein liberating 1 μmol of *p*-nitrophenol per minute in the above conditions.

In each cycle, the encapsulated lipase remained incubated in the reaction medium for a period of 60 min and then, the supernatant was removed from the cuvette, and a fresh solution of the substrate was loaded and a new cycle for determination of the residual enzyme activity was started. The total period of this test was 420 min.

2.7. Mass transfer effects

The rate of olive oil hydrolysis was measured with free (FE) and encapsulated lipase (EN-PEG) at the same conditions as in the Section 2.3, for different oil concentrations (1, 2, 3, 4, 5, 10, 20, 30, 40, 50, 60 and 70%) and Michaelis–Menten kinetics was fitted to the rate data using the Enzyme Fitter® software.

To compare the relative efficiency of the free and encapsulated enzyme for hydrolysis, a relative effectiveness factor (η_{FE}^{EN}) was defined as:

$$\eta_{FE}^{EN} = \frac{-r_{EN}}{-r_{FE}} \tag{3}$$

where $-r_{EN}$ and $-r_{FE}$ are the rate of hydrolysis with the encapsulated and free enzymes, respectively. For obtaining the relative effectiveness factor, the reaction rate for the encapsulated enzyme was first multiplied by a factor so as to obtain the same initial rate as the free enzyme (FE) for low oil concentrations.

3. Results and discussion

The diffusional limitations for the hydrolysis of olive oil with the encapsulated lipase were characterized as described in Section 2.7. The results are given below.

3.1. Mass transfer limitations

Michaelis–Menten kinetics was fitted to the encapsulated lipase hydrolysis rate data (see Fig. 1) resulting the apparent kinetic parameters, $K_m = 0.08348\ \text{mol}\,\text{L}^{-1}$ and $V_{max} = 0.005029\ \text{mol}\,\text{L}^{-1}\,\text{min}^{-1}$, i.e.:

$$-r_{EN} = \frac{0.005029 C_{sb}}{0.08348 + C_{sb}} \tag{4}$$

where C_{sb} is the bulk olive oil concentration in $\text{mol}\,\text{L}^{-1}$. The above K_m and V_{max} values are apparent kinetic parameters because they have been measured in conditions in which they are affected by the conjoint effects of intraparticle and interparticle reaction rate diffusion limitations.

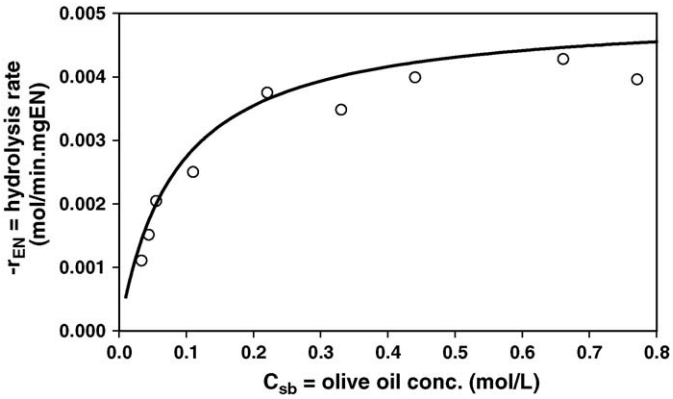

Fig. 1. Michaelis–Menten kinetics was fitted to the encapsulated lipase hydrolysis rate data (the substrate is emulsified olive oil at 37 °C, pH 7.0 and the enzyme is encapsulated CRL lipase in the TEOS–PEG matrix).

The corresponding results obtained with the free enzyme were (see Fig. 2), $K_m = 0.2474\ \text{mol}\,\text{L}^{-1}$ and $V_{max} = 1.290\ \text{mol}\,\text{L}^{-1}\,\text{min}^{-1}$, i.e.:

$$-r_{FE} = \frac{1.290 C_{sb}}{0.2474 + C_{sb}} \tag{5}$$

These reaction rates were obtained with 250 mg of encapsulated lipase and 5 mg of free lipase, respectively. To obtain the same initial reaction rate for low oil concentrations, the rate of hydrolysis with encapsulated lipase needs to be multiplied by 86.55, meaning that weight by weight it is necessary to use 86.55 times more encapsulated lipase to obtain the same initial rate for low oil concentrations. The relative effectiveness factor, η_{FE}^{EN}, in this normalized condition is then:

$$\eta_{FE}^{EN} = 0.3375 \frac{0.2474 + C_{sb}}{0.08348 + C_{sb}} \tag{6}$$

Fig. 3 is a plot of the relative effectiveness factor, η_{FE}^{EN}, for the hydrolysis of olive oil with the encapsulated (EN-PEG) and free enzyme (FE), as a function of the bulk substrate concentration C_{sb}. It can be observed that the relative efficiency of the encapsulated lipase decreases as the oil concentration increases and, in addition, it can noted that from an efficiency of nearly one at

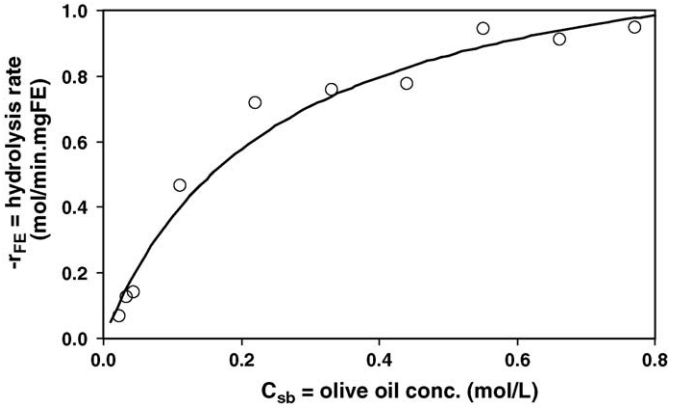

Fig. 2. Michaelis–Menten kinetics was fitted to the free lipase hydrolysis rate data (the substrate is emulsified olive oil at 37 °C, pH 7.0 and the enzyme is free CRL lipase).

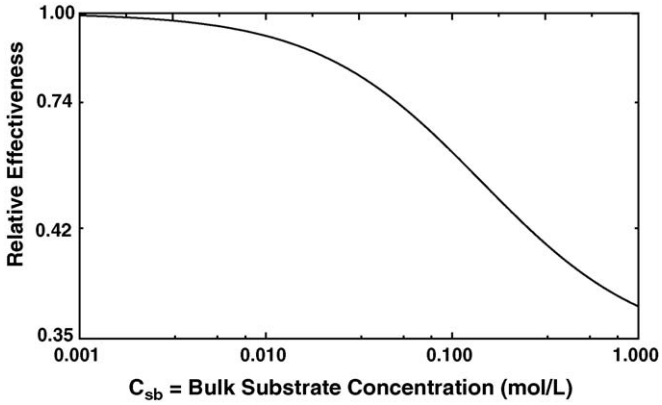

Fig. 3. Relative effectiveness factor η_{FE}^{EN} for the hydrolysis of olive oil at 37 °C, pH 7.0, with free and encapsulates lipase (EN-PEG), as a function of the bulk oil concentration (C_{sb}).

low oil concentration, it reduces to about 40% for the highest oil concentrations. As the particle size was kept constant for this study, the external and internal diffusion limitations were influenced only by changes in the properties of the reaction medium emulsified with different oil concentrations. Therefore, we may conclude that the overall diffusion limitations, including external and internal diffusion resistances, reduced the rate of hydrolysis for the encapsulated lipase, up to about 60%, as the oil in the reaction medium was increased up to 70%. Considering that 86.55 times more encapsulated enzyme was needed to give the same initial rate of hydrolysis at low oil concentration, Eq. (6) and Fig. 3 imply that for the highest oil concentration (70%) it is necessary to use 215 times more encapsulated lipase to reach the same initial rate of hydrolysis as with the free enzyme. This detrimental result is a consequence of the greater diffusion limitations for high oil concentrations.

3.2. pH and temperature dependence and thermal, operational and storage stability

The pH and temperature profiles of hydrolytic activities are shown in Figs. 4 and 5, respectively. Fig. 4 shows that the optimum pH for the EN-PEG was 6.7 while for the free enzyme it was 7.8. The optimum temperature for the encapsulated lipase (60–65 °C) was higher than for the soluble enzyme (36 °C), as shown in Fig. 5.

Our previous studies on the catalytic activity of the EN-PEG preparation for the hydrolysis of olive oil have shown that the hydrolysis reaction was diffusion-controlled for the gel prepared with MTMS as precursor owing to the small pore size of the samples and consequent substrate diffusion limitations. In addition, aggregation of the enzyme molecules might occur at very high enzyme concentrations, resulting in a lower degree of enzyme dispersion in the gel matrix. Therefore, for this work we decided to use a substrate with lower molecular weight such as *p*-nitrophenyl palmiltate to determine thermal stability and operational stability [20].

The EN-PEG was repeatedly used in batch hydrolysis of *p*-NPP at different temperatures (Fig. 6). The retention of the biocatalyst activity after repeated use was assessed in terms of *p*-

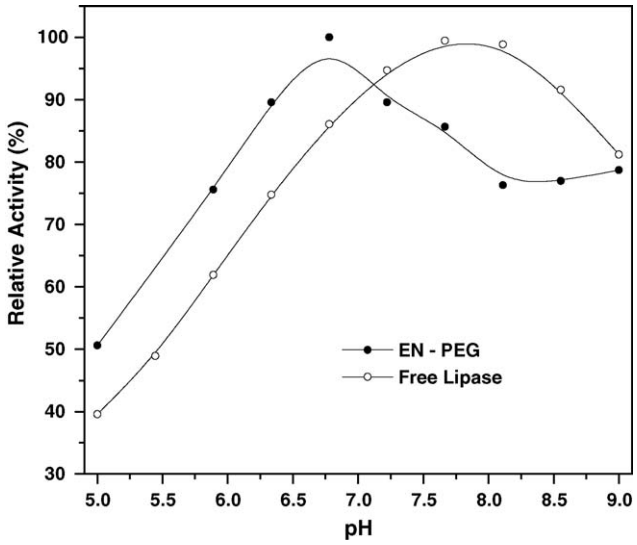

Fig. 4. Effect of reaction pH on hydrolytic activities of lipase preparations. Enzymes were assayed with olive oil as substrate at 37 °C, pH 7.0, (○) free lipase, (■) EN-PEG.

nitrophenol formation at the end of each cycle. Lipase entrapped in silicate sol–gel particles exhibited higher stability in the range of 37–45 °C, while at 50 and 65 °C the bioencapsulate was practically inactivated after seven cycles.

The results of the thermal stability tests have confirmed the usual trend of the enzyme immobilization process, that is, immobilization confers great stability to the immobilized enzyme. A higher thermal stability of the encapsulated derivative (EN-PEG) was also observed by thermogravimetric analysis (TGA) as described in our previous paper [9], in which the lower weight loss found for the encapsulated lipase was attributed to an increase in the thermal stability resulting from interactions between the silica matrix and organic components. To complete the characterization of the encapsulated lipase, the hydrolytic activity was measured as a function of long-term storage time at room temperature, and after 94 days the activity decreased by 50%. Results from literature, show that immobilized enzyme

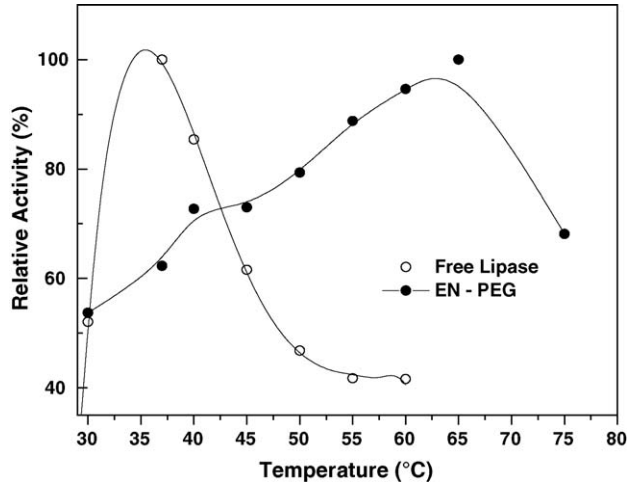

Fig. 5. Effect of reaction temperature on hydrolytic activities of lipase preparations. Enzymes were assayed with olive oil as substrate at pH 7.0 and 7.5, (○) free lipase, (■) EN-PEG.

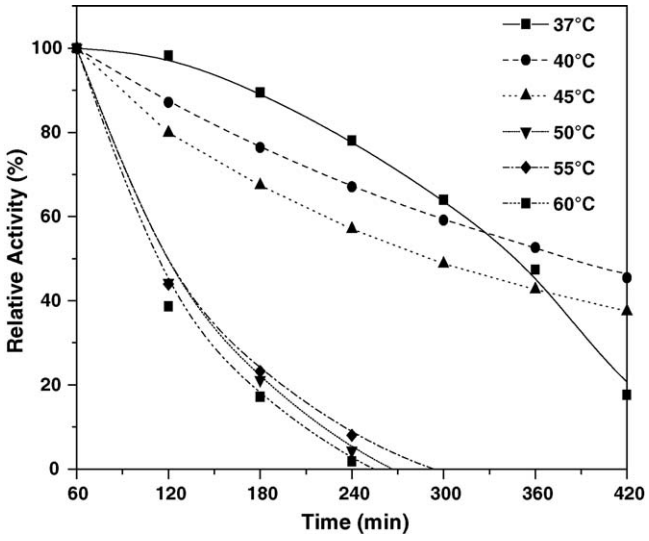

Fig. 6. Batch-operation stability tests at different temperatures, 37–60 °C, for the hydrolysis of *p*-nitrophenyl palmitate with the immobilized lipase (EN-PEG).

from *Rhizomucor miehei* in MTMS or MTMS/PDMS gel systems still retained up to 91% of their activity after storage in aqueous medium for 3 months [25]. In contrast, commercially available *R. miehei* lipase adsorbed onto an ion exchange resin (Lipozyme IM) retained only 6% of its initial activity under identical conditions [26].

3.3. ^{29}Si NMR and ^{13}C CP-MAS NMR spectra

The ^{29}Si NMR data (Fig. 7) can be analyzed according to the chemical shifts known for the Q^n and T^n Si sites [20], were Q refers to the presence of 4 oxygen first neighbors about a Si atom and T the presence of 3 oxygen first neighbors. The exponent *n* (from 0 to 4) represents the number of Si second neighbors. Fig. 7 shows the ^{29}Si CP-MAS NMR spectra exhibiting different chemical shift for the peaks between −80 and −120 ppm and clearly indicates that the presence of lipase increases the percentage of the Q^4 sites compared to the Q^2 and Q^3. The SiO_4 units originated from the hydrolysis of TEOS and in the case of pure silica matrix (Fig. 7A), the relative percentages of the Q^2, Q^3 and Q^4 sites were: 9.89, 69.29 and 26.82%, respectively. The sites Q^2 and Q^3 relative percentages for pure silica add up to approximately 80% (Fig. 7A) and because they contain Si–OH groups as $[Si(OSi)_2(OH)_2]$ and $[Si(OSi)_3(OH)]$, their sum correlates with the amount of the Si–OH groups.

For the encapsulated lipase derivatives, the amount of Si–OH groups obtained was smaller than for the pure silica matrix, that is smaller than 80%, as can be seen in Fig. 7B and C. The sum of the relative percentage of the Q^2 and Q^3 sites are: 67%, for EN, Fig. 7B and 70%, for EN-PEG, Fig. 7C. The decrease in the relative percentage sum of Q^2, and Q^3 sites, observed for the encapsulated enzyme derivatives are related to a reduction of Si–OH groups available in the matrices, which can be correlated with the hydrolytic activity (see Table 2). This study has demonstrated through ^{29}Si CP-MAS NMR and specific surface area data, the presence of interactions between the enzymes and

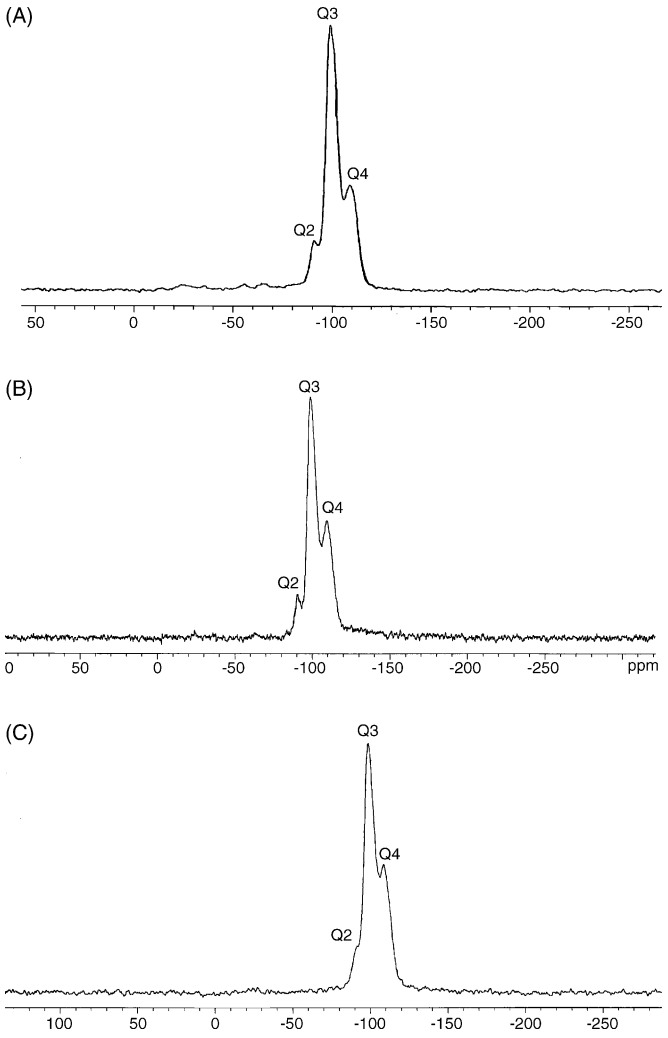

Fig. 7. ^{29}Si NMR spectra of the PS (pure silica, A), EN (encapsulated lipase, B) and EN-PEG (encapsulated lipase in presence of PEG, C).

the silica network. It is difficult to understand the mechanism of this interaction and how it affects the enzymes activity. But these data are consistent with each other, as a more condensed silica network with a higher Q^4 peak intensity is expected to give rise to a lower specific surface area for matrices obtained from TEOS. A weak nucleophilic–electrophilic linkage between the enzyme proteins and some sites of the silica network is often considered to explain these interactions. It could also be that these interactions help the enzyme molecules resist capillary drying stresses in a different manner depending on the gel type.

The nature of the gel solid backbone seems to explain the occurrence of such high specific surface areas, as illustrated in Soares et al. [20]. The scanning electron microscopy for the TEOS gel support (PS) and for lipase entrapped in the gel (EN and EN-PEG) showed that the surface of the PS (pure silica support) had extremely low porosity or practically did not have a porous structure, whereas the immobilized enzyme preparation using TEOS as precursor showed an irregular surface [7].

Finally, the solid-state ^{13}C CP-MAS NMR spectra for the TEOS gel, in spite of their general poor quality, clearly show a sharp peak near 0 ppm attributable to this group (Fig. 8A

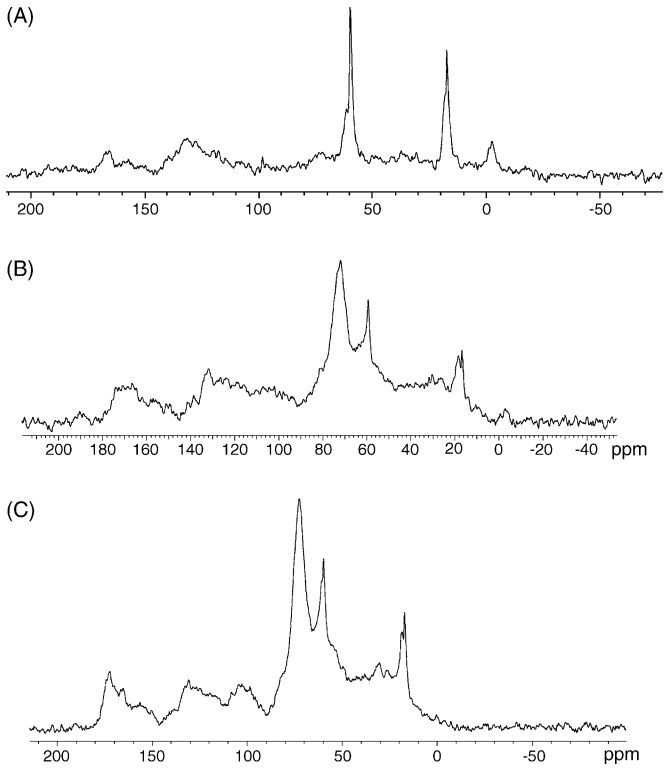

Fig. 8. ^{13}C NMR spectra of the PS (pure silica, A), EN (encapsulated lipase, B) and EN-PEG (encapsulated lipase in presence of PEG, C).

and B), which is normal because chemical shifts are also measured by comparison with $Si(CH_3)_4$ in ^{13}C NMR. As this is shown in Fig. 8A–C, in such gels made more recently with and without lipase from *C. rugosa*, addition of the enzyme in the same conditions (base catalysis) enhanced hydrolysis of the methoxy functionalities, showing efficient catalytic potential of the entrapped lipase in esterification and hydrolysis reactions, according to the water content of the biocatalyst. That is to say, a higher proportion of alkoxy groups were hydrolyzed to silanols, which could themselves participate in further siloxane condensation reactions, so as to build a larger pore silica structure [5]. Overall, the results indicate that the lipase could have a role in the hydrolysis–condensation reaction of silicon alkoxides. As lipase catalyzes hydrolysis as well as esterification reactions, it seems likely that the enzyme also catalyzed the alkoxide hydrolysis according to a reaction of types (7)–(9) to CRL in sol–gel prepared by the hydrolysis of TEOS [20]:

- Hydrolysis of TEOS:

$$Si(OEt)_4 + nH_2O \rightarrow Si(OH)_n\,(OEt)_{4-n} + n\mathrm{EtOH}(\mathrm{Et} = C_2H_5) \quad (7)$$

- Polycondensation:

$$(\text{–O–})_3\mathrm{Si–OH} + \mathrm{HO–(Si(Me)_2–O–)_x\,H\,(Me} = CH_3) \rightarrow (\text{–O–})_3\mathrm{Si–O–(Si(Me)_2\,–O–)_y\,–Si(–O–)_3} + [\mathrm{Si(Me)_2\,–O–}]_4\,(\text{cyclic}) + (\text{–O–})_3\,\mathrm{Si–O–(Si(Me)_2\,–O–)_z\,H} + H_2O\,(x > y,z) \quad (8)$$

- Self-condensation:

$$(\text{–O–})_3\,\mathrm{Si–OH} + \mathrm{HO–(Si–O–)_3} \rightarrow (\text{–O–})_3\mathrm{Si–O–Si(–O–)_3} + H_2O \quad (9)$$

4. Conclusions

The optimum pH shifted from 7.8 to 6.7 and the optimum temperature increased from 36 to 60 °C as lipase enzyme was encapsulated in a silica matrix produced with TEOS and PEG. This derivative exhibited higher stability in the temperature range of 37–45 °C, but from 50 to 65 °C the encapsulated lipase was inactivated after seven cycles. Hydrolytic activity during long-term storage at room temperature decreased to 50% after 94 days.

High overall diffusional resistance was observed for large oil concentration reducing hydrolytic effectiveness by 60% in the case of the encapsulated lipase (EN-PEG) and showing that if 70% oil reaction medium is used, about 215 times more encapsulated enzyme would be needed to obtain the same initial oil hydrolysis rate as the free enzyme.

The lipase enzyme seems to play an important role in the formation of the silica network, during gelling of the support matrix as implied by NMR, pore size and specific surface area. Lipase action seems to reduce Si–OH groups and produce larger pores and smaller surface area. Larger pores facilitate substrate diffusion and explain the higher hydrolytic activity of the TEOS–PEG sol–gel matrix encapsulated enzyme when compared to other sol–gel matrices. The more open porous structure allows higher substrate accessibility and correlates with higher hydrolytic activity observed for encapsulated lipase when TEOS is used as precursor and PEG as additive.

Acknowledgments

We acknowledge financial assistance from Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação Araucária.

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