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Methods and Supports for Immobilization and Stabilization of Cyclomaltodextrin Glucanotransferase from *Thermoanaerobacter*

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Abstract *Thermoanaerobacter* cyclomaltodextrin glucanotransferase (CGTase) was immobilized using different supports and immobilization methods to study the effect on activity recovery. The enzyme covalently attached into glyoxyl-silica showed low activity recovery of 1.5%. The hydrophobic adsorption of the enzyme on Octadecyl-Sepabeads yielded also low activity recovery, 3.83%, and the enzyme could easily leak from the support at low ionic strength, although the immobilization yield was satisfactory, approximately 76%. The CGTase encapsulated in a sol–gel matrix gave an activity recovery of 6.94% and maximum cyclization activity at 60 °C, at pH 6.0. The half-time life at 60 °C, pH 6.0, in the presence of substrate was 100 min, which was lower than that of the free enzyme. The best activity recovery in this work (6.94%) is approximately five times smaller than that obtained previously using glyoxyl-agarose as support and covalent immobilization. Thus, the best support and method we tested so far for immobilization of CGTase is covalent attachment on glyoxyl-agarose.

Keywords CGTase · Toruzyme® · Glyoxyl-silica · Octadecyl-sepabeads · Sol–gel encapsulation · Multipoint attachment · Hydrophobic adsorption

Introduction

Cyclomaltodextrins (CDs) are cyclic oligosaccharides that are produced by the action of the CGTase enzyme (CD glucanotransferase, EC 2.4.1.19) on liquefied starch [1–3]. They have

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countless applications in various industries [4–8] because they have a nonpolar cavity, which favors the encapsulation of a great variety of organic molecules [3, 6] conferring improved physicochemical properties, such as greater chemical resistance to environmental factors, higher solubility, and reduced volatility.

Immobilization of the CGTase enzyme has been pursued as a means of insolubilizing and stabilizing the enzyme molecule to allow using its catalytic activity continuously and repeatedly. However, the activity recovery of the immobilized CGTase is usually very low [9]. Therefore, we have set a research line with the main focus of immobilizing the *Thermoanaerobacter* extremely heat-stable CGTase [10], by different methods and supports, seeking high activity recovery. In this article, we report the immobilization of CGTase by three different methods: covalent multipoint attachment on glyoxyl-silica, physical adsorption on Octadecyl-Sepabeads, and sol–gel encapsulation.

Enzyme immobilization by multipoint attachment into macroporous solid supports (for example, silica) activated with glyoxyl groups (monolayer of aliphatic aldehydes moderately away from the support surface) is a technique that has been extensively used since 1987 for the immobilization of different enzymes. For example, Pereira et al. [11] immobilized Penicillin G acylase on glyoxyl-silica (140 μmol of aldehyde groups per gram of silica) and obtained 100% activity recovery. Silica is an adequate support because it is inexpensive and easily regenerated. The formation of glyoxyl (aldehyde) groups on the silica is achieved by silanization with 3-glyxidoxypropyl-trimetoxysilane (GPTMS), followed by acidic hydrolysis to open the oxirane ring and to obtain vicinal hydroxy groups. Finally, the aldehyde groups are formed by oxidation with sodium periodate. The degree of activation of the support (i.e., the number of aldehyde groups on the silica surface) is related to the intensity of the multi-interaction between enzyme and support [11].

Physical adsorption of enzymes into solid supports is an immobilization technique commonly used, mainly when the enzyme has a low cost or is thermostable. The advantages of this technique are that reaction conditions are gentle and, when inactivated, the enzyme can easily be eluted from the support, which can be regenerated by a new charge of active enzyme. Its chief disadvantage is the fact that under the reaction conditions, the enzyme may be eluted from the support, and this is highly undesirable.

Octadecyl-Sepabeads is an epoxy-acrylic resin, which has octadecyl hydrophobic groups covering its surface. In comparison with conventional hydrophobic supports, it has the advantage of having pores with large surfaces that facilitates intense interaction with proteins. In addition, it becomes a very rigid support after extensive intercrosslinking with porousgenic agents, hence appropriate for application in packed-bed or stirred-tank reactors.

Octadecyl-Sepabeads particles do not swell when transferred from a buffered aqueous solution to an anhydrous medium, and they can be used in all reaction media [12]. The adsorption of enzymes in Octadecyl-Sepabeads was first proposed by Palomo et al. [12] for the immobilization of lipases. The idea behind this method was to take advantage of the enzyme affinity for hydrophobic interfaces as a strategy of immobilization.

In our work, CGTase immobilization into Octadecyl-Sepabeads by adsorption was tried hoping to obtain a biocatalyst with higher activity recovery, given the gentle conditions for obtaining this derivative (low ionic strength, pH 7.0, and room temperature).

The encapsulation of enzymes in a sol–gel matrix is an immobilization method in which the enzymes are incorporated into hybrid organic–inorganic hydrophobic materials (alkylsilanes) [13]. The sol–gel process can easily be recognized because it is the synthesis route in which at a certain time, there is the transition from the sol to the gel system. In contrast to the conventional methods, which require high temperatures for the fusion of vitreous silica, the sol–gel technique uses low temperatures for the hydrolysis and

condensation reactions, which are ideal for a great variety of organic molecules sensible to high temperatures, as the class of proteins that can denature and loose their biological activity [14]. Enzyme immobilization by the sol–gel process has been extensively used because the physicochemical characteristics of the biocatalysts obtained by this method qualify the sol–gel matrices as ideal supports for the encapsulation of biological materials and enzymes [14, 15], giving very satisfactory results.

Materials and Methods

Materials

Toruzyme® 3.0 L (a liquid preparation of CGTase from *Thermoanaerobacter* sp.) was a kind gift from Novozymes A/S (Bagsvaerd, Denmark). It contained 5.0 mg of protein/mL and had a specific activity of 32.26 U/mg of protein, previously determined by Tardioli et al. [9]. One unit was defined as the amount of enzyme that produces 1 μmol of β-CD per min at 60 °C, using 0.5% w/v dextrin solution prepared in 10 mM sodium citrate buffer, pH 6.0. Dextrin 10 from cornstarch was supplied by Fluka Chemie AG (Buchs, Switzerland), and soluble starch was supplied by Acros Organics (New Jersey, USA). Octadecyl-Sepabeads (epoxy-acrylic resin covered with octadecyl groups) was a gift from CSIC-ICP (Madrid, Spain). Controlled pore silica (CPS) was a gift from Sucrerie Vanciennes (France). The CPS particles had an average diameter of 0.42 mm, with a particle-size distribution range of 0.351–0.589 mm, previously determined by Tardioli et al. [16]. GPTMS was purchased from Aldrich. Sodium borohydride was supplied by Sigma Chemical (St. Louis, MO), and sodium periodate was purchased from Nuclear (Diadema, Brazil). Tetraethylortosilicate (TEOS), the encapsulation reagent, was purchased from Across Organic (New Jersey, USA). β-CD and γ-CD were acquired from Sigma Chemical and Wacker Consortium (Munich, Germany), respectively. All other reagents were of analytical grade.

β-CD Colorimetric Assay

The concentration of β-CD was measured by the dye-extinction colorimetric method using phenolphthalein, as described by Tardioli et al. [9].

Enzymatic Activity Assays

Enzymatic activity of soluble and immobilized CGTase was measured at 60 °C and pH 6.0 by assaying the initial reaction rate of β-CD production using 0.5% (w/v) maltodextrin solution as substrate. The full protocol can be accessed from Tardioli et al. [9].

Preparation of CGTase Glyoxyl-Silica Derivative

Support Activation The glyoxyl-silica support with high concentration of aldehyde groups was prepared as described by Pereira et al. [11] with slightly modifications.

The silica was washed with nitric acid solution (10%, v/v) for 30 min at room temperature. Then, the silica was washed with the same acidic solution and with acetone/water solutions at increasing concentrations (25, 50, 75, and 100% v/v of acetone). The treated silica was

dried in an oven for 1 h at 40 °C. The silanization reaction with GPTMS was accomplished at 25 °C, pH 8.5, for 1 h under gentle agitation, using 30 mL of 5% (w/v) GPTMS solution per gram of dried silica. The solution pH was constantly adjusted with diluted KOH solution. Then, the silanized support was washed with distilled water and acetone/water solutions at increasing concentrations (25, 50, 75, and 100% v/v of acetone) and dried at 40 °C for 1 h.

The epoxy groups formed during the silanization step were hydrolyzed by sulfuric acid (0.1 M) for 2 h at 85 °C, using 30 mL of acid solution per gram of dried silica. The glyceryl-silica support was washed with distilled water and acetone/water solutions, as described above, over a sintered glass filter under vacuum. After the last washing, the support was dried at 40 °C for 1 h. The glyoxyl-silica support was finally obtained by oxidation of the glyceryl groups to glyoxyl groups, using sodium periodate (1 h of reaction at room temperature, 200 μmol of periodate per gram of glyceryl-silica support). Under these conditions, the support had 113.4 μmol of aldehyde groups per gram of silica.

CGTase Immobilization Five grams of glyoxyl-silica support were added to 49 mL of enzyme solution in 0.1 M sodium bicarbonate, pH 10.05, at 25 °C and kept under gentle stirring. Enzymatic activities from the initial enzyme solution and from the final supernatant, after 5 h of reaction, were assayed. The initial enzyme solution had an activity of approximately 3.93 U/mL. The immobilized enzyme derivative was reduced with sodium borohydride (1 mg/ml, 25 °C, 30 min), as described by Blanco and Guisán [17]. After 30 min of reduction, the CGTase-glyoxyl derivative was washed with an excess of distilled water.

A blank, under the same immobilization conditions, using untreated silica instead of activated silica, was used to verify if CGTase was adsorbed on silica. It was observed that the nonactivated silica-CGTase suspension, after 5 h at 25 °C, pH 10.05, still showed 96% of the initial activity present in the suspension. After reduction with sodium borohydride, washing, and suck drying under vacuum, the resulting solid had no enzymatic activity.

Preparation of CGTase Octadecyl-Sepabeads Derivative

Hydration of the Support Eight grams (dried mass) of octadecyl-Sepabeads support were suspended in 100 mL of acetone/water solution (80% v/v). The air was removed from the suspension using ultrasound. Then, the support was washed with 100 mL of acetone/water solution (50% v/v), acetone/water solution (20% v/v), distilled water in excess, and phosphate buffer (10 mM, pH 7.0). The hydrated support was filtered under vacuum and suck dried. The mass of the hydrated support was 18.104 g.

CGTase Adsorption Hydrated support (6.79 g) was added in 73 mL of an enzymatic solution (7.88 U/mL of solution) prepared in phosphate buffer (10 mM, pH 7.0). The suspension was gently agitated for 2 h at room temperature. Then, the immobilized enzyme was washed with distilled water, filtered under vacuum and suck dried.

Enzymatic activities from the initial enzyme solution and from the supernatant after 30, 60, and 120 min of reaction were assayed.

Desorption Assay One gram of CGTase–Sepabeads (CGTase immobilized on octadecyl-Sepabeads) was suspended in 20 mL of citrate buffer (10 mM, pH 6.0) and kept under

agitation for 5 h. The supernatant activity was assessed in a span of 1 h to verify the amount of enzyme that leaked from the support.

Preparation of CGTase Encapsulated into a Sol–Gel Matrix

The protocol used was described by Soares et al. [13]. Sixty-two milliliters of TEOS (manipulated under argon atmosphere because it is highly hydroscopic) were diluted in 72 mL of absolute ethanol (99%) inside a 250-mL three-neck round-bottom flask that was then connected to a fractional distillation apparatus. The flask was half-immersed in glycerin at 45 °C, and the ethanolic solution was stirred for 5 min. Then, 0.22 mL of hydrochloric acid (37%) diluted in 10 mL of ultrapure water (prehydrolyzing solution) was added slowly through a funnel, drop by drop, for about 1 min. The quantities of TEOS and hydrochloric acid were taken as to give the molar ratio of 2.1:0.01. After completing the addition of the prehydrolyzing solution, the mixture was kept under agitation for 90 min at 45 °C. Then, 6 mL of the CGTase stock solution diluted in 14 mL of ultrapure water (30 mg of protein, 960 U) were added. Finally, 2 mL of ammonium hydroxide diluted in 12 mL of absolute ethanol (hydrolyzing solution, 1:6) were added slowly through a funnel, drop by drop, for approximately 1 min, and a transparent homogeneous solution was obtained. After this latter addition, the solution was left at rest (without agitation), at 45 °C for 60 min under an inert atmosphere. After the resting period, the material was sealed off inside the flask and kept for 18 h (aging time), at 4 °C. The biocatalyst was then transferred to a Buchner funnel and washed with 3 vol of 60 mL heptane. The material was sucked dry for 10 min for removing residual water, then washed with 90 mL acetone, and after being further sucked dry for 10 min, it was left in a desiccator for 24 h.

The biocatalyst mass obtained was 90.01 g, with 23% humidity, determined by the Karl Fischer automated titration model D18, Mettler.

Morphologic Characterization of Sol–Gel-encapsulated CGTase

The biocatalyst morphology was characterized in relation to the superficial area, pore diameter, and volume of pores.

Physical Characterization with the BET Equipment

Samples of pure silica matrix and the sol–gel-encapsulated CGTase were analyzed with the NOVA 1200 Quantachrome equipment to obtain the particles' superficial area, pore mean diameter, and volume.

Thermoporometry

The mean pore diameter of the pure silica matrix and the sol–gel-encapsulated CGTase was determined by thermoporometry, according to the method described by Iza et al. [18]. The water freezing and boiling temperatures were analyzed with a Shimadzu calorimeter, model DSC-50, equipped with an accessory section containing liquid nitrogen used for the cooling of water located inside the pores of the sample material. The samples with 10 to 20 mg were conditioned in aluminum capsules and an excess of solvent (water) was maintained. The material was cooled up to the freezing point of nitrogen (−30 °C) and latter kept at −20 °C for 10 min. The temperature was then reduced to −30 °C and subsequently raised by

1°C/min, up to the temperature of the thermodynamic equilibrium for the solidification of water inside the pores (from 0 to −5 °C). From the thermogram obtained, the sample mean pore diameter was determined according to Eq. 1:

$$\mathrm{Dp}\ (\text{Å}) = 0.02 \times \left[\left(\frac{64.67}{\Delta T}\right) + 0.57\right] \quad (1)$$

where Dp is the mean pore diameter and $\Delta T = T - T_o$ is the porous material temperature triple point of saturation with water.

Physicochemical Characterization of the Sol–Gel-encapsulated CGTase

The hydrophobic matrix (pure silica) and the sol–gel-encapsulated CGTase, produced from the precursor TEOS were characterized by thermogravimetric analysis (TGA) and Fourier transform infrared spectroscopy (FTIR).

Thermogravimetric Analysis

Samples of the pure silica sol–gel matrix and encapsulated CGTase were taken for TGA with a TGA-50 Shimadzu Thermogravimetric Analyzer, and the mass loss as a function of temperature was registered at a heating rate of 20 °C/min from room temperature to 1,000 °C. Initial mass samples varied from 2 to 6 mg.

Fourier Transform Infrared Spectroscopy

FTIR was used for characterization of the pure silica sol–gel matrix and encapsulated CGTase with a FTIR BOMEM MB-100 spectrophotometer in the wavelength range 400–4,000 cm^{-1}.

Catalytic Properties of the Soluble and Encapsulated CGTase

Soluble and sol–gel-encapsulated CGTase were characterized with respect to thermal stability and enzymatic activity as a function of temperature and reaction pH.

Activity as a Function of Temperature and Reaction pH

CGTase activity was measured at the following temperatures (40, 50, 70, 80, and 90 °C) and pH 6.0 (pH of maximum catalytic activity), keeping fixed the biocatalyst mass (307.5 mg). Samples of the reaction medium were taken in duplicates at the times of 0 and 25 min and added to a test tube containing 20 μL of 5 M HCl. These tubes were immersed in boiling water for 5 min to inactivate the enzyme. After cooling, a sample was taken to determine β-CD concentration using the phenolphthalein colorimetric method. The same assay was conducted from pH 4.0 to 10.0 and 60 °C, the temperature for maximum enzyme activity.

Biocatalyst Thermal Stability

Suspensions of the immobilized CGTase were prepared in sodium citrate buffer 10 mM, pH 6.0, containing maltodextrin 0.5% (w/v) and incubated at 60 °C for 3 h. At regular time intervals, the residual activity of the immobilized enzyme was measured.

Results and Discussion

Immobilization of CGTase on Different Supports and Methods

The results for the immobilization of CGTase from *Thermoanaerobacter* sp. in glyoxyl-silica, hydrophobic adsorption in Octadecyl-Sepabeads, and encapsulation into the sol–gel matrix are shown at Table 1.

Usually, activity loss occurs as a consequence of enzyme immobilization because of many causes, for example, steric hindrances (the immobilized enzyme molecule may be inappropriately oriented in relation to the porous surface, and the active site may not be accessible to the substrate), intraparticle diffusional resistance (an effect more severe in the case of sol-gel encapsulation), or tridimensional conformal changes in the enzyme molecule (induced by enzyme-support multipoint covalent link attachments).

In a previous work, Tardioli et al. [9] have immobilized CGTase from *Thermoanaerobacter* sp. by covalent attachment into glyoxyl-agarose particles and obtained an activity recovery of about 32%, that is, five times the highest values obtained in this work (6.94% with sol–gel encapsulation). It is thought that with the latter method, in addition to the causes listed above for immobilized enzyme activity loss, the immobilization conditions and reagents used by the sol–gel method contribute to enzyme deactivation.

Although the activity recovery of the immobilized CGTase in glyoxyl-silica was low, a result of the same magnitude, 2.5%, was obtained by Tardioli et al. [16] using CGTase from *Bacillus* sp. and silica silanized with γ-amino-propyl-trietoxi-silane (γ-APTS) and activated by glutaraldehyde.

The activity recovery of the CGTase adsorbed in Octadecyl-Sepabeads was higher than that obtained with covalent attachment in glyoxyl-silica, but the latter support can be considered more appropriate for immobilization of this enzyme because it was weakly adsorbed into the Octadecyl-Sepabeads, being easily desorbed even at low ionic strength (25% desorbed with at 10 mM buffer).

In general, results of the same magnitude as in this work were obtained for the activity recovery of immobilized CGTase by many different authors [9]. There are three exceptional cases reported of activity recovery equal to or higher than 74%: two of them use adsorption

Table 1 Activity results for the immobilization of CGTase from *Thermoanaerobacter sp.* using different supports and methods.

Method (Support)	Conditions	U_I (U/g)	U_{IT} (U/g)	RI (%)	U_{EI} (U/g)	RA (%)
Covalent attachment (silica-glyoxyl)[a]	25 °C, pH 10.05, 5 h	38.4	38.4	100	0.59	1.54
Adsorption (Octadecyl-Sepabeads)	25 °C, pH 7.0, 2 h	84.7	64.3	75.9	2.46	3.83
Encapsulation (sol–gel)	Note 1	10.7[b]	10.7	100	0.74	6.94

U_I The enzymatic charge offered for immobilization in U/g of support, U_{IT} the enzymatic charge theoretically immobilized in U/g of support, *RI* the immobilization yield, defined as $(U_{IT}/U_I)\times 100$, U_{EI} the measured immobilized enzyme activity, in U/g of biocatalyst, *RA* the activity recovered in the immobilized enzyme, defined as $(U_{EI}/U_{IT})\times 100$, *Note 1* gelation/encapsulation=45 °C, ethanolic/acid medium, 155 min, aging=18 h at 4 °C, drying=suck dried by vacuum, followed by a 24-h resting period in a desiccator

[a] All the results of this immobilization are the averages of duplicates.

[b] The enzymatic charge per gram of support, U_I, was calculated from the biocatalyst mass obtained after the process of gelation/encapsulation (90.01 g) and the offered enzymatic charge (960 U).

in resins as the immobilization method, and a third uses a high-silica fabric treated with γ-APTS and glutaraldehyde. The highest activity recovery reported is 95%, and in this case, CGTase was fused with ten lysine residues and electrostactically immobilized in a cation exchanger resin [19]. For the case of CGTase from *Thermoanaerobacter* sp., the highest activity recovery reported is 10.2%. The hydrophobic support Eupergit C was used, and the enzyme was immobilized by covalent attachment [20]. From these observations and the results of our work, it may be concluded that CGTase is very sensitive to its microenvironment of immobilization, and it seems worthy protecting the enzyme, such as in the case that attached ten lysine residues to the enzyme.

Temperature Dependence of the CGTase Activity

Figure 1 shows the relative activity of the soluble and sol–gel-encapsulated CGTase, measured at pH 6.0 and temperatures from 40 to 90 °C. The soluble enzyme showed a maximum activity in the temperature range of 80–90 °C, which is in accord with Norman and Jorgensen [10] and Tardioli et al. [9]. However, the sol–gel-encapsulated CGTase has shown a shift in the maximum temperature range to around 60 °C, which demonstrates that the conformal changes that occurred at the tridimensional enzyme shape made it more susceptible to thermal inactivation.

The thermal stability of an immobilized enzyme is another important factor for the selection of an immobilization method. A method that shifts the maximum catalytic activity for a region of higher temperatures is undoubtedly preferred. Tardioli et al. [9] obtained greater thermal stability for a *Thermoanaerobacter* sp. CGTase covalently bound to glyoxyl-agarose; the temperature range shift for the maximum activity was from 80 to 85 (for the free enzyme) to just above 90 °C (for the immobilized enzyme).

pH Dependence of the CGTase Activity

Figure 2 shows that the CGTase from the *Thermoanaerobacter* sp. in the soluble form has the maximum activity in the pH range from 5.0 to 6.0 at 60 °C, corroborating data from Norman and Jorgensen [10], but the sol–gel-encapsulated enzyme showed maximum

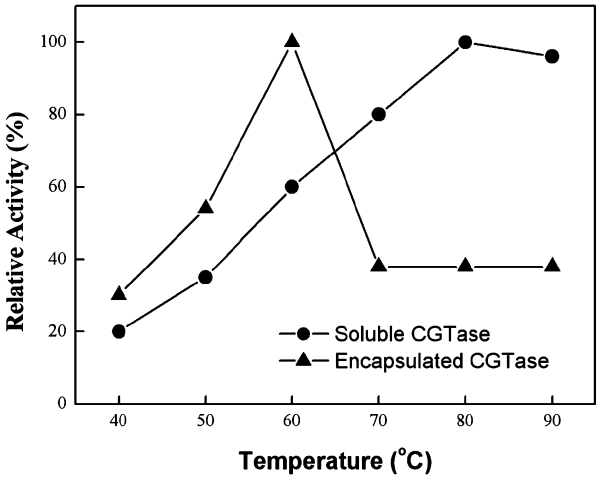

Fig. 1 Effect of temperature on the cyclization activity (Effect of temperature on the cyclization activity (β-CD formation rate) of the free and sol–gel encapsulated CGTase at pH 6.0. The maximum catalytic activity in each case was normalized to 100%

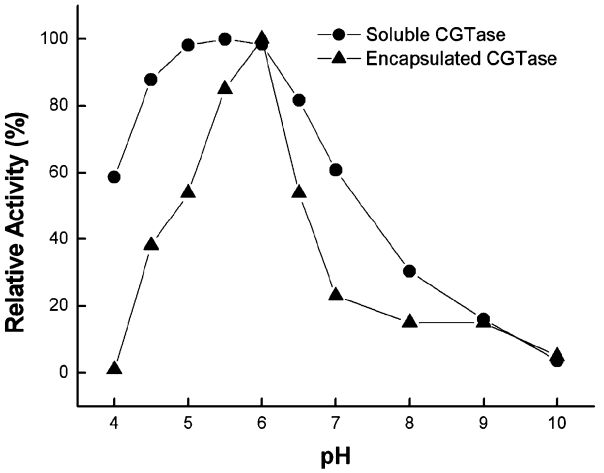

Fig. 2 Relative cyclization activity (β-CD formation rate) for free and sol–gel-encapsulated CGTase at 60 °C and pH 4.0 to 10.0 with 10 mM buffers: sodium citrate (pH 4.0–6.5), Tris–HCl (pH 7.0–9.0), and bicarbonate (pH 10.0). The substrate was maltodextrin 5 g L^{-1}, and the maximum catalytic activity in each case was normalized to 100%

activity at pH 6.0, and below or above this pH value, the immobilized enzyme activity has an abrupt drop that is not observed around the maximum region of the free enzyme. This sharp drop in activity adds strength to the conclusion that CGTase encapsulation by the sol–gel technique has caused detrimental conformal changes to the enzyme.

Thermal Stability of the Sol–Gel-encapsulated CGTase

Figure 3 presents the thermal deactivation as a function of time for the sol–gel-encapsulated CGTase incubated at 60 °C and pH 6.0 (citrate buffer). These results demonstrate a low thermal stability of the encapsulated enzyme that at 60 °C has a half-life of approximately 100 min and is practically inactivated after 3 h. In addition, they are consistent with the shift of the maximum catalytic activity to a lower temperature range, as the enzyme was encapsulated, and reinforce the conclusion that deleterious conformal changes occurred

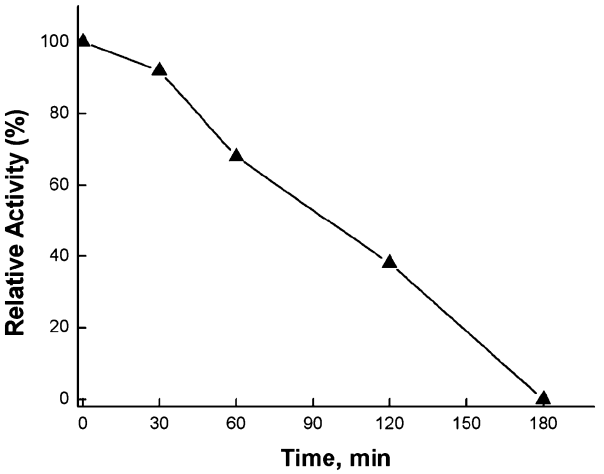

Fig. 3 Thermal deactivation as a function of time for the sol–gel-encapsulated CGTase incubated at 60 °C and pH 6.0 (citrate buffer 10 mM and maltodextrin 5 g L^{-1})

when the enzyme was encapsulated. Norman and Jorgensen [10] informed that the CGTase from *Thermoanaerobacter* sp. in soluble form retains more than 95% of its activity when incubated at 75 °C in acetate buffer, pH 5.5, for 60 min in the absence of substrate.

Morphologic Characterization of Sol–Gel-encapsulated CGTase

The morphologic characterization of the immobilized enzyme is important to correlate the biocatalyst performance with porous structure parameters. BET analysis, which is usually based on N_2 isothermal adsorption at 77 K, allows determining the solid-specific surface area, total pore volume, pore size distribution, and mean pore diameter. It is not recommended for solids with a low specific surface area (<5 m^2 g^{-1}). Table 2 shows the specific surface area, mean pore diameter, and total pore volume determined by BET for the pure sol–gel silica matrix having TEOS as the precursor and the same matrix with the encapsulated CGTase.

For the encapsulated CGTase, a significant reduction in surface area was observed, while pore diameter and volume remained practically constant.

With the objective of studying the biocatalyst textural properties, N_2 adsorption isotherms were obtained (Fig. 4), and according to the International Union Pure and Applied Chemistry nomenclature, they are of type IV, which indicates that the solid contains a large proportion of mesopores (1.8–6 nm), and this facilitates the substrate access to the catalytic site of the immobilized enzyme.

TGA and FTIR Analyses of the Sol–Gel-encapsulated CGTase

The encapsulated CGTase mass loss as a function of temperature, calculated by TGA [21], is shown in Fig. 5. The temperature range used was divided in three regions for interpretation: Region I (0 to 130 °C) shows a relatively large mass loss, probably caused by loss of water and organic residues, region II (130 to 620 °C) has also a large mass loss, probably caused by decomposition of organic compounds including the enzyme, and region III (620 to 1,000 °C) shows sample mass stabilization corresponding to the final carbonization of the residual organic mater.

FTIR analysis was also used to study the efficiency of the sol–gel enzyme encapsulation method that uses TEOS as precursor. The silica matrix gelation procedure produces a tridimensional reticulate formed by interacting polymeric inorganic chains that form a holding net around the enzyme, and FTIR can be used to follow this encapsulating process. The FTIR spectrograms obtained for the pure silica matrix and the encapsulated CGTase are shown at Fig. 6.

The CGTase characteristic peaks are the bands at 1,650 and 1,600 cm^{-1} related to primary and secondary amino groups. They can be seen at the biocatalyst spectrogram (Fig. 6) and are in accord with the bands described in the literature [22].

Table 2 Morphologic characteristics of the support prepared with TEOS as precursor (pure silica matrix) and the biocatalyst (sol–gel-encapsulated CGTase).

Material	Superficial area (m^2 g^{-1})	Mean pore diameter (nm)	Total pore volume (cm^3 g^{-1})
Pure silica	607	1.80	0.37
Sol–gel-encapsulated CGTase	484	1.79	0.36

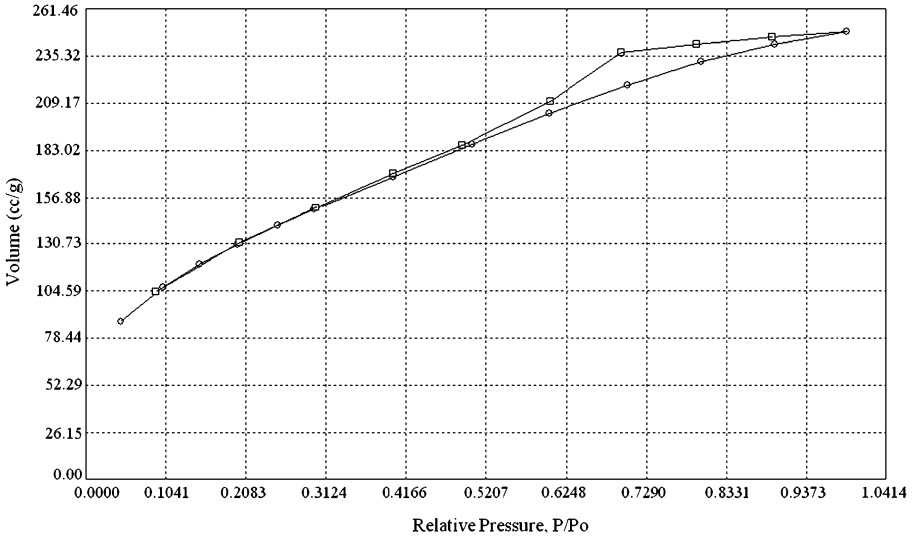

Fig. 4 Nitrogen adsorption (*squares*) and desorption (*circles*) isotherms measured at 77 K for the sol–gel-encapsulated CGTase

Figure 6 shows in addition the characteristic bands of pure silica, that is, 950 (Si-O-Si, axial deformation), 810 (Si-O-Si, axial deformation), and 600 cm^{-1} (Si-O-Si, angular deformation) [23, 24]. The characteristic peaks for the hydroxyl group bonds can also be observed in the range of 3,400 cm^{-1} [23].

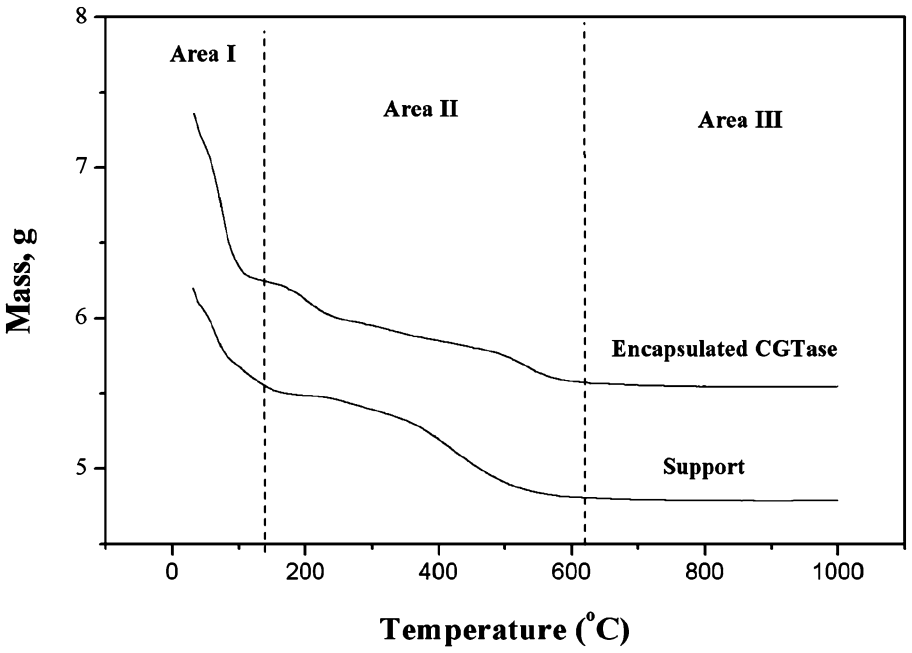

Fig. 5 TGA of the pure silica matrix (*support*) and the sol–gel encapsulated CGTase, prepared using TEOS as precursor

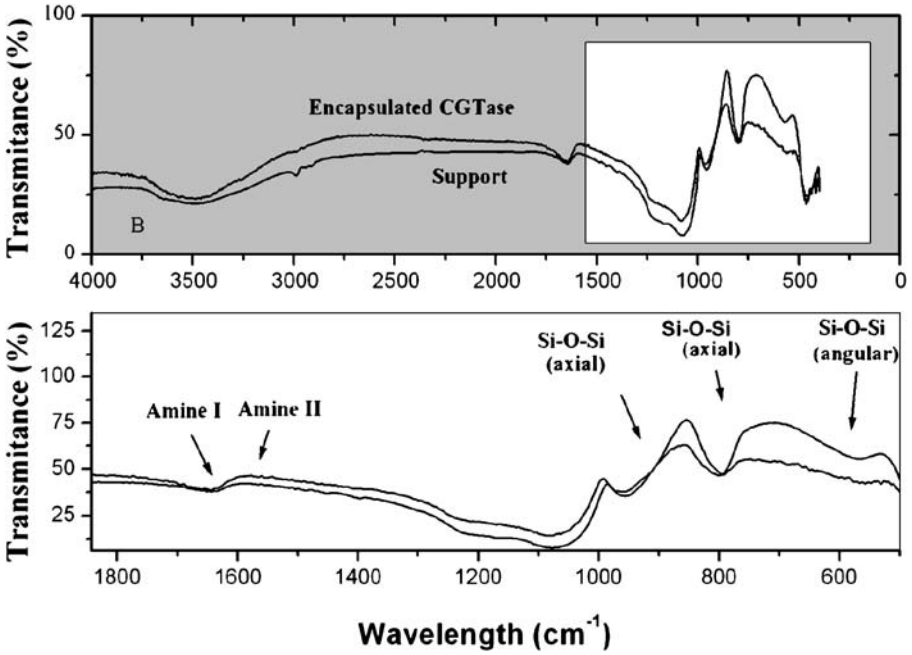

Fig. 6 FTIR spectrograms for the pure silica matrix and the sol-gel encapsulated CGTase, prepared with TEOS as precursor

Conclusions

The covalent immobilization of CGTase into silica particles activated with linear aldehyde groups (glyoxyl-silica) at 25 °C, pH 10.05, by 5 h resulted in a yield of immobilization of approximately 100% and activity recovery of 1.54%. Hydrophobic adsorption on Octadecyl-Sepabeads at 25 °C, pH 7.0, by 2 h gave approximately 76% of immobilization yield and 3.83% of activity recovery. In addition, the enzyme-support binding was shown to be very weak, in this case, losing approximately 25% of the enzyme from the support under low ionic force. Encapsulation of CGTase using the sol–gel technique produced the best results: 100% yield of immobilization and 6.94% of activity recovery.

The temperature of maximum cyclization activity downshifted from 80–90 (for the soluble enzyme) to 60 °C (for the sol–gel-encapsulated CGTase) at pH 6.0. However, both, soluble and encapsulated CGTases exhibited almost the same pH for maximum cyclization activity.

The thermal stability of the sol–gel-encapsulated CGTase was not satisfactory. The approximate half-life time was only 100 min at 60 °C, pH 6.0, in the presence of substrate (maltodextrin 0.5%, w/v). After 3 h of incubation, the enzyme was nearly completely inactivated.

Encapsulation of CGTase by the sol–gel technique strongly influenced the superficial area of the formed gel, but the influence on pore average diameter and total pore volume was negligible.

Among all the methods and supports already tested by our group for the immobilization of CGTase, the covalent link of the enzyme to agarose activated with glyoxyl groups (glyoxyl-agarose) was the most efficient, giving an immobilization yield of practically

100% and an activity recovery of 32% [9]. Consequently, this method warrants further studies that will be carried out by adding enzyme active site protectors, such as polyethylene glycol, β-CD, or acarbose, which could lead to higher activity recoveries that we seek.

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