A New Mesoporous Micelle-Templated Silica Route for Enzyme Encapsulation

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A new mesoporous micelle-templated silica (MTS) route for enzyme encapsulation is presented. The pore structure is given by a new association of lecithin (double chain surfactant) and dodecylamine as cosurfactant. To enhance and to well protect the enzyme activity, lactose was loaded in the synthesis. The mixed-micelles give after the addition of tetraethyl orthosilicate a well-ordered mesoporous material with a spongelike rigid structure stable after calcination at 550 °C. The size of the pores lies between 30 and 40 Å, matching well with the size of the lipases. The activity of this heterogeneous catalyst was tested in the hydrolysis of the ethylthiodecanoate. These new biocatalysts were very active, more than hydrophobic sol–gel materials and commercially available sol–gel encapsulated lipase. This new MTS synthesis route allows one to encapsulate in one-step various enzymes, even those that are very fragile.

Introduction

Biocatalysts and biosensors are highly selective moieties, but by nature show limited stability and recyclability. For this purpose, many immobilization techniques have been investigated with different kinds of matrix to isolate the biomolecules. The main advantages expected from the immobilization are the separation from the final product, the reusability, and the use in unfriendly conditions. The fixation of biologically active molecules into inorganic materials combines the high selective activity of the active molecules such as enzymes or antibodies with the chemical and mechanical stability of the support. This combination is very useful for biocatalysis and chemical biosensing. Mesoporous silica materials with high surface areas and variable pore diameters are attractive candidates to host large molecules including proteins. The disclosure in 1992 by Mobil researchers of the mesostructured materials obtained by self-assembly of surfactants and silicates or aluminosilicates revealed new opportunities for immobilization. Mesoporous materials such as M41S and SBAs with high surface areas, high pore volumes, narrow pore size distribution, and tuneable pore size (between 20 and 150 Å) have aroused a great interest for the biological molecules immobilization. Small globular enzymes, Cytochrom C, Papain, and Trypsin, were immobilized by physical adsorption in MCM-41. Cytochrom C was also immobilized into MCM-48 and SBA-15 by adsorption followed by silylation of the pore openings to block the pore aperture and into SBA-15 with functionalized surfaces that present higher retention of the enzymes than purely siliceous SBA-15. The sequestration and release of Conalbumin, Ovalbumin, Tyspin inhibitor protein, or Lysozyme proteins into SBA-15 and MCF with varying pore sizes and derivatized surface silanol groups were also studied. Recently, horseradish peroxidase was adsorbed into hexagonal mesoporous silica materials (FSM-16, MCM-41, and SBA-15) with pore size controlled by the combined use of the surfactants having different alkyl chains length and swelling agents. The resulting immobilized enzyme catalysts were shown to be active and stable catalysts. Several lipolytic enzymes were immobilized in the pores of MCM-41 and Al-MCM-41 and used as catalysts in the gas-phase esterification of acetic acid with ethanol.

Lipases (triacylglycerol ester hydrolases, E.C.3.1.3) are enzymes that catalyze the chemo-, regio-, and stereo-selective hydrolysis of carboxylic acid esters in water or the reverse reaction involving esterification in organic solvent. It is known that lipases are serine-hydrolases, in which the catalytic active center is composed of an amino acid triad, generally asparagin, histidin, and serine. Lipases are interphase-active enzymes with hydrophobic domains; that is, they undergo not only ionic and polar interactions with other molecules, but also lipophilic interactions. The latter are believed to be responsible for

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the “activation” of the enzyme according to which hydrophobic domains of the substrate molecules interact with the lipase resulting in a conformational change with concomitant opening of the so-called “lid” and exposure of the active site. A variety of immobilization techniques have been applied to lipases, including adsorption on a carrier material, covalent attachment to a solid support, entrapment in an insoluble polymer, or microencapsulation in silica xerogels and aerogels. There is a great number of publications dealing with all of these techniques. Most of them were excellently reviewed by Villeneuve et al. The sol–gel process developed by Reetz et al. in 1995 is the most famous technique of lipase encapsulation and showed that in organic solvents, the introduction of hydrophobic functionalities on the inorganic network of silica magnified the esterification activity of the enzyme by 5 orders of magnitude relative to the traditional lipase powders. The enzymes are hydrophilic so they show very low activity in organic media; the immobilization in a controlled hydrophilic/hydrophobic environment inside a solid allows one to greatly enhance its activity. This makes the entrapment of biocatalysts very useful in organic chemistry and is at the origin of the commercially available immobilized lipases. Nevertheless, the sol–gel method does not allow one to control the pore size of the resulting solid, which should play an important role in enzyme accessibility and thus in its activity. Most of the immobilized lipase can only be used in nonaqueous system, so in ester synthesis, very few studies take into account the hydrolysis activity of the immobilized lipases. The high regioselective hydrolysis activity of lipases is also very important as, for instance, the selective synthesis of α-monoglycerides from fatty acids without forming β-monoglycerides or in the field of mutagenesis which applies the recognition of enantio- 

<table>
<thead>
<tr>
<th>enzyme</th>
<th>treatment</th>
<th>trade name</th>
<th>supplier</th>
<th>source</th>
<th>concentration</th>
<th>specific activity</th>
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<tbody>
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<td>Gist-Brocades</td>
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<td>Biochimika</td>
<td>Mucor miehei</td>
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</tr>
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<td>MY</td>
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<td>Lipase MY</td>
<td>Amano</td>
<td>Candida cylindracea</td>
<td>0.78</td>
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* Recombinant from Aspergillus oryzae.

Enzyme activity. This approach could overcome some of the disadvantages of entrapment techniques, such as the leaching of adsorbed molecules, the chemical degradation of the anchoring bond of covalently attached enzymes, and the barrier to diffusion of substrates and products which are encountered for large polymeric substrates in sol–gel preparations. Lipases are relatively robust enzymes, and for this reason we have chosen them as a model for enzymes encapsulation in a MTS-type mesoporous material using this new technique. The influence of different factors on the formation of the MTS structure and on the enzymatic activity has been determined. The measure of the catalytic activity of lipases for hydrolysis has been chosen rather than its activity for ester synthesis, because the hydrolysis reaction is the most demanding in terms of stability of the immobilized enzyme.

**Experimental Section**

**Measurements.** Powder X-ray diffraction patterns were collected on a CGR Thêta-60 diffractometer with Inel drive, using monochromated Cu Kα radiation and 0.025 mm slits. Nitrogen adsorption–desorption isotherms at 77 K were performed on calcined MTS corresponding to the inorganic backbone of the porous hosts (calcination was performed at 550 °C to remove all organics). The isotherms were recorded using a Micrometrics ASAP 2010 apparatus. Each calcined sample was outgassed at 250 °C until a stable static vacuum of 3 × 10⁻³ Torr was reached. Specific surface areas were calculated using the BET method using the isotherm adsorption data in the range p/p0 = 0.10–0.25 just below capillary condensation, and pore diameters were evaluated from the isotherm desorption branch by the Broekhoff and de Boer method, which has been demonstrated as one of the most meaningful for MCM-41 materials. The pore volume was taken at the top of the step corresponding to the pore filling. Measurements of pH data were collected on a G 810 pH-meter (Schott). UV–vis spectroscopy measurements were performed with a PU 8625 UV–vis spectrophotometer (Philips) coupled to a BD 40 recorder (Kipp & Zonen). Thermogravimetric analysis was performed under an oxygen flow on a TG 209C analyzer (Netzsch Proteus). Transmission electron microscopy (TEM) was done using a JEOL 1200 EX II instrument at an acceleration voltage of 120 kV. Scanning electron microscopy (SEM) was performed with a HITACHI instrument.

**Materials. Lipases.** Different lipases from different sources and delivered by different companies were used in this study (Table 1). The Esterase 30000 (from Mucor miehei) was a gift from Gist-Brocades, referred to later as GB. Esterase from Thermomyces lanuginosus recombinant from Aspergillus oryzae was a gift from Novo Nordisk, referred to later as N. Lipase MY (from Candida cylindracea) was a gift from Amano, referred to later as MY. The Lipase Mucor miehei recombinant from

Aspergillus oryzae was a gift from BioChemika, referred to later as MM. Except for the Mucor miehei lipase from BioChemika, which is pure and was used directly, the other enzymatic powders were purified by water washing before use: 500 mg of crude enzyme was dissolved in 8 mL of water, shaken for 15 min, and centrifuged to remove insoluble components. The Esterase 30000 from Gist-Brocades has been submitted to an additional treatment of purification where the supernatant was purified by G25 Sephadex exclusion chromatography; this lipase preparation will be referred to later as GB G25. The lipase content in each solution was determined through the resulting proteins concentration which is pure and was used directly, the other enzymatic powders added. The molar composition for the gel was: 1TEOS/0.05–0.2CTAB/0.05NaCl/143H2O. The mixture was then stirred for 18 h at 50°C. The white solid was filtered, washed with distilled water, and air-dried.

**Table 2. Protein Content and Enzymatic Activity toward the Hydrolysis of Ethylthiodecanoate of Lipase Immobilized by the New MTS Route and Characterization of Porosity of Resulting Calcedined Materials**: Pore Volume (V), Pore Size (D), BET Surface Area (S)\(^\text{a}\)

<table>
<thead>
<tr>
<th>samples</th>
<th>protein loading (mg protein/g)</th>
<th>specific activity (UI/g)</th>
<th>relative activity (%)</th>
<th>D (Å)</th>
<th>V (mL/g)</th>
<th>S (m²/g)</th>
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<tr>
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<td>18.5</td>
<td>np</td>
<td>np</td>
<td>57</td>
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</tbody>
</table>

\(^a\) 1, standard synthesis; 2, half water; 3, half TEOS; 4, glucose; 5, twice glucose; a, TEOS+lipase first; b, NaF (see text); np, nonporous after calcination.

The commercial immobilized lipase used for comparison was the lipase from Mucor miehei immobilized in sol–gel-AK (Fluka).

**Lipase Immobilized by the Sol–Gel Technique.** Esterase 30000 from Gist-Brocades, GB G25, purified by exclusion chromatography, was used for encapsulation by the sol–gel technique. The protein concentration of the enzymatic solution was 7.16 mg/mL. The encapsulation in the hydrophobic sol–gel technique. The protein concentration of the enzymatic solution was 7.16 mg/mL. The encapsulation in the hydrophobic sol–gel was performed as described in the literature by Reetz et al.\(^{29}\) 564 µL of the lipase solution (4.04 mg of proteins) was added to a mixture of 190 µL of aqueous sodium fluoride (1M) (Aldrich) and 1.0 mL of poly(vinyl alcohol) (PVA) (MW 15 000) (Merck) (4% w/w in water). The solution was shaken, and 875 g/L (5 µmol) of propyltrimethoxysilane (PTMS) (Fluka) was added followed by 145 µL (5 µmol) of tetramethyl orthosilicate (TMOS) (Aldrich). The final ratio R = total water/silane = 8. The reaction mixture was vigorously shaken for 5 s on a vortex mixer and then gently shaken by hand. After about 30 s, when the mixture formed a clear homogeneous solution and started to warm, it was placed in an ice bath until gelation occurred (about 10 min) and aged at room temperature for 24 h. The resulting material was dried at 37 °C for 3 days, and then ground in a mortar. The protein content was determined in the washing water using the Bradford method.

**Lipase Encapsulated by SBA-3 Direct Synthesis.** SBA-3 is a hexagonal MTS synthesized in acidic conditions\(^{30}\) and for which synthesis can be adapted to neutral conditions by replacing the acid by a salt like NaCl in the synthesis procedure.\(^{31}\) The Esterase 30000 (GB) was used for the immobilization by direct synthesis of SBA-3. The surfactants (CTAB) were dissolved in the mixture of the enzyme solution and NaCl. TEOS was then added. The molar composition for the gel was: 1TEOS/0.05–0.2CTAB/0.05NaCl/143H2O. The mixture was then stirred for 18 h at 50°C. The reaction mixture (samples referred to as MTS2 followed by the name of the enzyme used: GB G25, GB, N, MY, MM (Table 2). The amount of enzyme was quantified in the supernatant and in the solid. For the supernatant, the enzyme was separated from the other organic molecules via G25 exclusion chromatography. The enzyme in the solid was isolated by dissolution of the solid at pH 9 followed by elution through G25 column. The protein content of each phase was determined by Bradford method.

With these two determinations, the total amount of the initial enzyme was traced. Different modifications to the standard MTS synthesis (MTS1) procedure were performed either in composition of the reaction mixture (samples referred to as MTS2–MTSS hereafter) or in the order of addition of reactants (subscript a) or in the order of addition of NaF, a silica-condensation promoter (subscript b). Modifications of the MTS procedure reported in Table 2 are as follows:

- water amount, MTS2 uses half water in the synthesis (MTS2-GB);
- sugar amount and type, MTS4 and MTS5 use 0.2 mol of glucose (0.03 mol of glucose/TEOS) and 150 mg of glucose (0.06 mol of glucose /TEOS), respectively (MTS4-GB, MTS5-GB);


order of reactant addition, MTSa, TEOS was added in the first solution directly with the enzyme and the lactose (MTS1a-N); NaF addition, MTSb uses 200 µL of 0.1 M NaF in the synthesis (MTS1b-N and MTS3b-N); and washing step (not referred). In MTS1-GB G25 synthesis, one part of the sample was filtered and washed with 50 mL of ethanol, instead of a water/ethanol mixture and centrifugation.

**Lipase Catalytic Activity. Hydrolysis of the Ethylthio- decanoate.** The catalytic test chosen for these immobilized lipases was the hydrolysis of the ethylthio decanoate (Scheme 1). The ethylthio decanoate was synthesized according to ref 32. This reaction was selected because the resulting thioethanol formed during this reaction can be easily detected by UV−vis spectroscopy from the yellow color developed in the presence of 5,5'-dithiobis (2-nitrobenzoic acid), DTNB (Lancaster Synthesis).

The enzymatic catalysis tests were performed as follows:

For the enzymatic solution, 100 µL of the aqueous enzymatic solution was added to a measuring cuvette containing 2.7 mL of Tris buffer 0.05 M (pH 8), 100 µL of DTNB solution (20 mg/mL in DMSO), and 100 µL of ethylthio decanoate solution (0.2 mg/mL in DMSO). The absorbance corresponding to the yellow product at 412 nm (\(\lambda = 412 \text{ nm}\)) was recorded immediately and every 2−3 min intervals thereafter. The specific activity of the lipase is given in International Unit (I.U.) per mg (I.U.: \(\mu\text{mol of substrate transformed/min}\)).

For the immobilized lipase, 0.05 g of the solid was added to a mixture containing 28.7 mL of Tris buffer 0.05 M (pH 8), 0.7 mL of DTNB solution (20 mg/mL in DMSO), and 1.5 mL of ethylthio decanoate (0.2 mg/mL in DMSO). The mixture was vigorously shaken at room temperature at the same rate for all experiments. Every minute, aliquots of 0.3 mL were withdrawn, passed through a 0.2 µm filter to remove the solid material which interferes with the UV-analysis, 2.7 mL of Tris buffer was added, and the absorbance was measured at 412 nm. The specific activity (I.U./mg protein) was determined, as well as the relative specific activity, which is the percentage of specific activity of immobilized lipase toward the specific activity of lipase in solution.

**Results and Discussion**

**Entrapment of Lipase by MTS Direct Synthesis.** The lipases entrapment was attempted by using silica sources (alcoxides) similar to those used for the sol−gel procedure, but by adding the pore size control thanks to the use of surfactants as template agents. Entrapment of lipase in a MTS-type solid was performed by direct synthesis under mild conditions that do not affect the structure and the activity of the enzyme. First of all, the entrapment of Esterase 30000 (GB) was achieved by following a classical MTS synthesis procedure leading to SBA-3-type materials using CTAB surfactants in acidic and neutral conditions at room temperature. Well-ordered hexagonal mesoporous materials were obtained, but no enzymatic activity was revealed. It has been found that CTAB surfactants, even at low concentration and neutral pH, have an inhibitor effect on the enzymatic hydrolysis activity of lipase.

CTAB surfactants were then replaced in the synthesis medium by a natural surfactant containing a similar cationic headgroup: the lecithin from egg yolk, 3-sn-phosphatidylcholine (L-α lecithin). The composition of the synthesis mixture corresponded to the same molar ratio as for SBA-3 material. In this case, the irreversible inhibition of the lipase was avoided, probably because lecithin could be a lipase substrate and also because lecithin is a zwitterionic surfactant and not a purely cationic one as CTAB. Nevertheless, a very small amount of material was obtained, and its structure was revealed as a multilayer lamellar silica composed of a mixture of infinite plates and of vesicles. The lamellar structures collapsed after calcination at 550 °C leading to nonporous materials. The fact that lamellar structures are obtained when lecithin is used as surfactant is in accordance with its double-chained structure, giving a spontaneous curvature close to zero, which is favorable for lamellar phases formation.

A new method of MTS synthesis has been therefore developed to promote a porous structure instead of a lamellar one. To induce a curvature of the preferential lamellar structure of lecithin, cosurfactants were added. It is well known that neutral amines are good cosurfactants and furthermore they are very good nucleophilic catalysts for hydrolysis and condensation of silicon alcoxides. Different ratios of lecithin/decylamine have been tested. Pure decylamine as structure-directing agents has given a very well-structured mesoporous material with 25 Å pores diameter, like well-known mesostructured materials (HMS) in similar conditions, but a very small enzyme hydrolysis activity was obtained. The best results in terms of enzymatic activity and material structure were obtained for a lecithin/decylamine molar ratio of 1/0.8, corresponding to a synthesis mixture: 1TEOS/0.04decylamine/0.05lecithin/28H2O/8.49EtOH. A moderate activity was obtained. It is well known that the lack of stability of enzymes is one of the most difficult problems in protein chemistry. Various molecules like sorbitol, poly(ethylene glycol) (PEG), and different mono- or disaccharides are commonly used as protein stabilizers. So, to improve the enzymatic activity of the material obtained by the new MTS synthesis route, β-D lactose (one of the cheapest additives) was used as a protecting agent for the lipase, at a molar ratio of 0.03 as compared to Teos.

Hence, a very high enzymatic hydrolysis activity was obtained. The best results in terms of enzymatic activity and material structure were obtained for a lecithin/decylamine molar ratio of 1/0.8, corresponding to a synthesis mixture: 1TEOS/0.04decylamine/0.05lecithin/28H2O/8.49EtOH. A moderate activity was obtained. It is well known that the lack of stability of enzymes is one of the most difficult problems in protein chemistry. Various molecules like sorbitol, poly(ethylene glycol) (PEG), and different mono- or disaccharides are commonly used as protein stabilizers. So, to improve the enzymatic activity of the material obtained by the new MTS synthesis route, β-D lactose (one of the cheapest additives) was used as a protecting agent for the lipase, at a molar ratio of 0.03 as compared to Teos.

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(MTS1), were the following: 35 ± 7 Å pore diameter, 0.30 mL/g pore volume, and 300 m²/g surface area (Table 2). The pore size distribution of these MTS is larger, and the surface area and pore volume are lower than conventional MTS such as MCM-41 or MCM-48. In addition, they do not feature the long range ordering of the latter. TEM micrographs (Figure 3) reveal nevertheless a uniform porosity in the materials arranged in a spongelike structure, which can be seen as a disordered cubic structure with a pore size of 30 Å and wall thickness of 30 Å, in perfect agreement with XRD results (correlation distance of 60 Å) and nitrogen sorption measurements. The interconnected spongelike structure is the result of the mesophase transition from infinite plates (curvature nil or slightly positive (vesicles) obtained without amines as cosurfactants) to spongelike structure due to the slightly negative curvature effect induced by the addition of dodecylamine to the lecithin surfactant layer. SEM pictures of the MTS biomaterials (Figure 4) show that the samples are formed by an agglomeration of uniform microspheres of 2 μm. The presence of enzyme does not seem to play a very crucial role in the final structure of the material, but nevertheless some changes in surface area are observed depending on the type of enzyme: 350 m²/g are obtained for the blank MTS without enzyme, and the surface areas stand around 300 m²/g for Esterase GB, 400 m²/g for lipase N, 500 m²/g for lipase MM, while the structure formed with lipase MY collapsed during the calcination. This would mean that the final characteristic of the MTS (wall thickness and surface area of the material) is somehow influenced by the nature of the enzyme, but this point remains to be clarified. MTS with the highest pore volumes, the highest pore sizes, and the higher surface areas were obtained for the lipase N by using a different way of addition of TEOS (pore diameter of 65 Å) or by adding NaF in the synthesis, which is a good silica condensation catalyst (for MTS1a-N and MTS1b-N, respectively). A maximum of pore volume and surface area of 0.71 mL/g and 676 m²/g was obtained for the addition of NaF in the synthesis giving 44 Å pore size materials (MTS1b-N).

At this stage, the challenge with this new encapsulation procedure is to localize the enzyme inside the structure and to describe how the enzyme interferes with the material. The observed effects are consistent with the enzyme dispersed in the lecithin/dodecylamine bilayers of the same size, the spherical molecular diameter of the lipase being 30–40 Å, which corresponds to the pore size of the backbone of the mesoporous structure. Considering that an enzyme consists of a protein chain that is folded onto itself in a complex open architecture, it may act as a template for the silica network that builds a mesoporous structure. Around the enzyme, there is no porous micro-

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**Figure 1.** XRD pattern of as-synthesized MTS1-GB.

**Figure 2.** Nitrogen sorption isotherm at 77 K of MTS1-GB calcined at 550 °C for 8 h.

**Figure 3.** TEM micrograph of as-synthesized MTS1-GB.

**Figure 4.** SEM picture of as-synthesized MTS1-GB.
capsule as is the case for encapsulated lipase in aerogel, because the enzyme is in interaction with the lecithin used as surfactant and it is not entirely in direct interaction with the silica precursor. It is important that the interaction between the enzyme and the silica precursor or silica network not be strong. In these conditions, the enzymes remain free to adjust their conformation to perform a high catalytic activity.

Protein Loadings and Enzymatic Activity of the New MTS Biocatalysts. Generalities. The structure of the inorganic backbone of the MTS has been determined on materials after calcination, but the actual active biocatalysts are uncalcined materials that contain the surfactants. The as-synthesized MTS1s with encapsulated enzymes have the following composition (determined by chemical analysis): 1SiO2/0.04lecithin/0.04 amine/0.04lactose/0.30H2O, so there are 1 amine, 1 lactose, and 7 H2O per lecithin. All organics are necessary to generate an activity for the biocatalyst. Indeed, washing the biocatalyst with 50 mL of pure ethanol instead of 10 mL of ethanol/water removes more than half of the total organics initially present in the solid; all amines and lactoses are removed by this washing as well as a small portion of lecithin. Although the accessibility of the pores is increased by this supplementary organic removal, the relative enzymatic activity is much lower. Probably the loss of lactose is responsible of the decrease of the activity. The enzymes are really protected, thanks to the surrounding lecithin and lactose, from unfriendly environment. For instance, CTAB surfactants do not act anymore as enzymes inhibitors when enzymes are encapsulated by this new MTS route. The other advantage of this new method of encapsulation is that the biocatalyst can be recycled, and no decrease in activity has been observed after five reaction cycles using the same material, showing that the enzymes are really entrapped in the structure and not only adsorbed.

Activity of the Different Encapsulated Enzymes. Different enzymes listed in Table 1 were entrapped via this new MTS route, and they all show activity toward ester hydrolysis. Significant differences between relative enzymatic activities have been nevertheless found depending on the type of enzymes (Table 2): around 40% for esterase GB, around 20% for lipases N and MY, and around 4% for lipase MM. Also, different enzyme loadings have been obtained: around 0.5 mg protein/g solid for lipase N, 1 mg proteins/g solid for esterase GB and lipase MY, and 5 mg proteins/g solid for lipase MM. No clear relationship appears between enzyme loading and activity except for the lipase MM, for which low activity may be explained by a too large amount of enzyme entrapped in the solid that inhibits its activity. While the degree of immobilization is almost independent of the amount of protein used for entrapment, an increase in enzyme loading beyond a certain critical amount does not result in higher catalytic activity. The decrease in specific activity can be rationalized by diffusional limitations of the substrate into the porosity of the material, a lower degree of dispersion in the mesoporous matrix, or an insufficient lipase protection by sugar against the denaturing effects due to silanes used during the formation of the silica matrix. The difference in activity between the enzymes results essentially from their different size, from their intricate structure or microporous structure, or from their conformation, which can also change with the surrounding water available inside the material. The enzyme catalytic activity is essentially correlated with its conformation. To obtain a good activity, a lipase must be immobilized in such a way that it can easily change conformation. The different activities could also come from the presence of acids, products of the enzymatic reaction, in the very narrow environment of the enzyme. Indeed, even if the reaction occurs in a buffer media, the local pH around the enzyme could decrease depending on the relative diffusion rate of the acid product and of the buffer. As the pH of optimum activity for the lipase used in this study is at pH values above 8 and for the esterase at pH values around 6, consequently, esterase could be less affected by the pH decrease.

Optimization of the Enzymatic Activity. To optimize the activity of the biocatalysts, different variations in the synthesis have been performed. It is noteworthy that the changes in activities induced by different synthesis protocols can only be compared for the same enzyme. If different enzymatic solutions have been prepared as for esterase GB, it is preferable to compare the relative specific enzymatic activities than the specific activities (Table 2).

Decreasing the water amount by a factor of 2 (MTS2-GB) does not change the enzyme loading, but decreases slightly the enzymatic activity (MTS1a-N), although pore size and pore volume are a little bit larger by this method. Probably there is a denaturing effect of the silanes against the lipase that could not be avoided by the presence of sugar. The prior interaction of the enzyme/sugar solution with the lecithin is important to maintain a high catalytic activity. Decreasing the amount of TEOS in the MTS synthesis (MTS3-N and MTS3-MM) leads to very slight changes in pore structure but leads to an increase in enzyme loading and hence to a decrease in relative activity. Again, a too high enzyme loading in the biocatalyst inhibits its activity. The addition of NaF, which is a Lewis basic catalyst for silica precursor condensation, to the MTS synthesis mixture has been tested. In the case of MTS1b-N (Table 2), addition of NaF leads to a better structuration of the solid backbone: higher pore size, higher pore volume, and higher surface area, 44 Å, 0.71 mL/g, and 676 m2/g, respectively, but the enzyme loading has decreased and the relative specific activity remained the same. A better structuration does not enhance the enzyme activity. It seems that a pore size close to the size of the enzyme is more appropriate to obtain a good activity. In the case of a MTS synthesis using half TEOS in the synthesis mixture (MTS3b-N), the addition of NaF has not led to the expected better structuration of the solid, but the enzyme loading has decreased. A too fast structuration induced by the addition of NaF prevents the enzymes from being correctly incorporated in this solid.

(39) Fojan, P.; Jonson, Per H.; Petersen, M. T. N.; Petersen, S. B. Biochimie 2000, 82, 1033.

The type and the amount of sugar have been studied for this new encapsulation procedure. When esterase GB was entrapped in a MTS1 support without sugar, the enzymatic activity of the biocatalyst was lower as compared to the same esterase entrapped using lactose in the MTS synthesis mixture. Exchanging lactose by glucose (MTS4-GB) in MTS synthesis does not change the structure of MTS, but decreases the enzymatic activity and contributes to an increase in the enzyme loading. The type of sugar and/or the higher amount of enzyme loading could be responsible for the decrease in activity. Increasing the amount of glucose to have the same number of moles of sugar cycle (MTS5-GB) does not change the solid structure and enhances again the catalytic activity. The catalytic activity is nevertheless less than the biomaterial prepared using lactose, although it has a similar enzyme loading. The type and the amount of sugar are very crucial parameters to generate a high activity in MTS biocatalysts. Sugars are responsible for the maintenance of the three-dimensional structures of the enzymes, which are at the origin of their activity. Changes in their secondary and tertiary structure will decrease their activity. There should be an optimum in sugar amount to maintain a high catalytic activity of the enzymes. The optimization of this very important parameter for the enzymatic activity and the study of the effect of other polyhydroxy compounds on the activity of encapsulated lipase are in progress.

The synthesis pathway referred to as MTS1 constitutes the best compromise between well-ordered structure and high enzymatic activity. A higher degree of purification of the enzyme prior being entrapped can also enhance slightly the activity of the biomaterial. When the esterase GB, that shows the best hydrolysis activity, was purified by G25 gel-exclusion chromatography, both the protein loading and the relative activity increased (1.3 mg proteins/g solid and 46%, respectively). The purification enables to remove some small inauspicious impurities associated with the enzyme.

Comparison with the Entrapment of Lipase in Hydrophobic Sol–Gel Synthesis. Extensive research has been performed on the encapsulation of proteins by sol–gel chemistry for possible applications for biocatalysis and biosensors. However, a disadvantage in the use of sol–gels is their variability in pore size, which cannot be tailored to isolate specific proteins. Lipases can be entrapped in hydrophobic sol–gel materials resulting in heterogeneous biocatalysts, which display long-term stability as well as pronounced degrees of enhanced activity in esterification and transesterification reactions in organic solvents. Recently, it was proved that some lipases entrapped in hydrophobic sol–gel materials are also active heterogeneous catalysts for ester hydrolysis reactions in aqueous medium. So, it was important for the evaluation of our new biocatalysts to compare its activity with the well-established sol–gel procedure encapsulation. Using the reported procedure of sol–gel encapsulation, the esterase GB G25 was entrapped in a PDMS/TMOS (1/3) hydrophobic sol–gel material and its enzymatic activity toward the hydrolysis of ethylthiodecanoate was determined. No structural determination of the solid was possible because the material obtained by sol–gel synthesis collapsed after calcination. The enzymatic activity of the sol–gel material was compared to that of the biocatalyst obtained by the new MTS route and compared as well to the activity of a commercial immobilized lipase, which uses the same lipase encapsulated by a sol–gel route (sol–gel-AK) (Table 3).

<table>
<thead>
<tr>
<th>samples</th>
<th>protein loading (mg protein/g)</th>
<th>specific activity (UI/g)</th>
<th>relative activity (%)</th>
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</thead>
<tbody>
<tr>
<td>MTSI-GB G25</td>
<td>1.30</td>
<td>20.7</td>
<td>45.9</td>
</tr>
<tr>
<td>sol–gel-GB G25</td>
<td>4.75</td>
<td>13.9</td>
<td>29.0</td>
</tr>
<tr>
<td>sol–gel-AK commercial</td>
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</tr>
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</table>

Table 3. Protein Content and Enzymatic Activity toward the Hydrolysis of Ethylthiodecanoate of Lipase Mucor miehei Immobilized by the New MTS Route and in Hydrophobic Sol–Gels

To perform well, the lipases need the presence of an interface between a hydrophobic and a hydrophilic medium. For the entrapment of lipases in hydrophobic sol–gel materials, this interface can be tailored by introducing hydrophobic sites in an otherwise hydrophilic silica gel, or by adding a partially soluble organic material such as PVA. It appears, however, that these solutions are less efficient than the new MTS procedure presented here. For the new MTS route, the interface hydrophobic/hydrophilic medium is given by the zwitterionic lecithin, which at the same time provides a pore size control. The association of pore size control and enzyme environment control offers the best catalytic activity to the biocatalyst.

Conclusion

A new mesoporous micelle-templated silica route for enzyme encapsulation has been developed using: (i) natural surfactants such as lecithin to protect the enzyme and avoid the direct interaction between silica and enzyme, which usually denatures the enzyme activity, (ii) amines to create a curvature in the lecithin/amine system to generate pores and thus enhance the diffusivity of substrate as compared to the conventional sol–gel technique, and (iii) lactose to protect the enzyme activity. The lipases entrapped by this new MTS route are very active heterogeneous catalysts for ester hydrolysis. Their activity is higher than that of the lipases entrapped in hydrophobic sol–gel materials and that of commercially available encapsulated lipase. The various protections instituted in this new MTS synthesis allow the enzymes to be active even in an unfriendly environment such as in the presence of quaternary ammonium inhibitors. It is possible to recycle this heterogeneous catalyst without losing activity. This new MTS route leads to a well-structured mesoporous material with a spongelike structure, with controlled pore size and significant surface area. The synthesis can be easily adapted to other enzymes and proteins, even for very fragile enzymes. For instance, alcohol dehydrogenase (ADH) from baker’s yeast was successfully entrapped in MTS-type silica by this procedure.

References

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