Immobilization of lipase on silicas. Relevance of textural and interfacial properties on activity and selectivity

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Two lipases from *Mucor miehei* have been immobilized by adsorption in MCM-41 materials featuring different hydrophilic/hydrophobic surfaces and by encapsulation either in hydrophobic silica sol-gel or in Sponge Mesoporous Silicas (SMS), a new procedure based on the addition of a mixture of lecithin and amines to a sol-gel synthesis to provide pore-size control. The resulting biocatalysts have been evaluated for various ester hydrolysis reactions and compared with commercially available immobilized lipases in silica sol-gel (Sol-gel AK-Fluka) and in ion-exchange resin (Lipozyme-Fluka). Too hydrophilic (pure silica) or too hydrophobic (butyl-grafted silica) supports are not appropriate to develop high activity for lipases. An adequate hydrophobic/hydrophilic balance of the support, such as supported-micelle, provides the best route to enhance lipase activity. The SMS encapsulation procedure enables the highest activity for the lipases. The lecithin/amines mixture structuring the pore network leads to a suitable phospholipids bilayer-like environment, which avoids the necessity to create an interface by substrates assembly. The specificity of the lipase towards middle ester chain length is lost for immobilized lipases, but the activity of lipase towards short ester chain length is considerably increased. This typoselectivity change is more likely related to a strained configuration of the immobilized enzyme.

Introduction

In the last years, many research efforts have been devoted to the development of carrier-bound immobilized enzymes with the aim of facilitating their use in continuous processes. Such systems were expected to reduce costs by enabling more efficient separation, recycling and reuse of costly enzymes, and it was also anticipated that they could provide better process control.[1,2] Moreover, improved enzyme performance, such as activity, stability and selectivity can often be achieved by enzyme immobilization.[3–5] The most general methods to immobilize enzymes are adsorption on a carrier material, inclusion or encapsulation in a solid or a polymer and covalent attachment to an activated matrix. The physical adsorption of an enzyme on a carrier material is one of the most widely used methods due to its simplicity. The size of the protein to be adsorbed as well as the specific surface area of the carrier material and the nature of its surface (type of porosity, pore size, hydrophobic/hydrophilic nature of surface) are crucial parameters for successful immobilization. Micelle-Templated Silicas (MTS), such as MCM-41[6] and related materials, with their well-defined pore size and high surface areas have been used, since 1996 as carrier materials for protein immobilization.[7–17] Small globular enzymes, cytochrome *c*, papain and trypsin were immobilized by physical adsorption in MCM-41.[13] Cytochrome *c* was also immobilized into MCM-48 and SBA-15 by adsorption followed by silylation of the pore openings[14] to block the pore aperture and into SBA-15 with functionalized surfaces which present higher retention of the enzymes than purely siliceous SBA-15.[15] The sequestration and release of conalbumin, ovalbumin, trypsin inhibitor protein or lysozyme proteins into SBA-15 and MCF with varying pore sizes and derivatized surface silanol groups was also studied.[16] 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.[17] The resulting immobilized enzymes 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.[18]

A variety of techniques has been applied for the immobilization of 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 large number of publications dealing with all these techniques. Most of them were excellently reviewed by Villeneuve *et al.*[19] The sol-gel process developed by Reetz *et al.*[20] in 1995 is the most famous technique of lipase encapsulation and showed that the introduction of hydrophobic functionalities on the inorganic network of silica magnified the esterification activity of the enzyme relative to the

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traditional use of lipase powders in organic solvents. The enzymes are hydrophilic so they show very low activity in organic media. Their immobilization in a controlled hydrophilic/hydrophobic environment inside a solid allows greatly enhanced activity. This makes the entrapment of biocatalysts very useful in organic chemistry,[21] and is at the origin of the commercially available immobilized lipases. Nevertheless the sol-gel method does not allow control of the pore size of the resulting solid which should play an important role in enzyme accessibility, in substrates and in products diffusivity and, accordingly, in its activity.

This study is aimed at investigating the relevance of textural and interfacial properties on the activity and selectivity of encapsulated enzymes. For this purpose lipases have been immobilized by adsorption on different types of MCM-41 materials featuring various hydrophilic/hydrophobic surfaces and by encapsulation in our recently reported Sponge Mesoporous Silica (SMS) prepared using a mixture of lecithin and dodecylamine as templating agents.[22] MCM-41 supports have been chosen for enzyme adsorption rather than other ordered mesoporous silica (SBA-15, MCM-48, MCF), because they are the only one readily available to date to offer a wide range of pore size from 2 to 15 nm for a similar surface area of 1000 m^2 g^{-1}. The catalytic activity of ester hydrolysis of these immobilized enzymes was compared with that of immobilized lipases obtained by the well known method of entrapment in hydrophobic silica sol-gel introduced by Reetz[20] and to that of commercially available immobilized lipases: Sol-Gel AK and Lipozyme from Fluka. Lipases are relatively robust enzymes and for this reason we have chosen them as a models for enzymes encapsulation. The catalytic activity of lipases for ester hydrolysis has been studied instead of the ester synthesis, firstly, because the hydrolysis reaction is most demanding in terms of stability of the encapsulated enzyme and secondly, because it is difficult to compare activity of a free enzyme to that of the immobilized one for the ester synthesis reaction, as the free enzyme has an activity close to zero in organic media. The hydrolytic activity of the lipases was evaluated for esters with different chain lengths to compare the specificities of immobilized enzymes to those of the free ones.

Experimental

Materials

Lipases. The enzymes used were two lipases from *Mucor miehei* (also called *Rhizomucor meihei*) obtained from two different suppliers: (1) a first one from Gist-Brocades named Esterase 30 000 referred hereafter as GB, and (2) the second from Fluka, referred hereafter as MM. Both lipases come from the same strain but were conditioned differently by the suppliers. The conditioning effect remains a subject of debate.

GB was purified before immobilization: 500 mg of crude enzyme was dissolved in 8 mL of Tris buffer at pH 8.08 (hydroxymethyl aminomethane, HCl 0.1 M), shaken for 15 min and centrifuged to remove insoluble components. The supernatant was then purified by G25 Sephadex exclusion chromatography. The lipase content in the resulting solution was determined using the Bradford method.[23] Five enzymatic GB solutions were prepared: GB1, GB2, GB3 and GB4 containing 0.74, 7.16, 3.50 and 5 mg enzyme mL^{-1}, respectively. A solution of lipase MM at 4 mg protein mL^{-1} was prepared in a Tris buffer at pH 8.08 without further purification.

The commercial immobilized enzymes used for comparison were the lipase from *Mucor miehei* (Fluka) immobilized in sol-gel-AK (Fluka) and in an ion-exchange resin called Lipozyme (Fluka).

MCM-41 supports. Different MCM-41 materials were used as supports for enzyme immobilization by adsorption. They were prepared with various pore sizes and various hydrophobic/hydrophilic balance. MCM-41 with 37, 62 and 100 Å pore sizes were prepared accordingly to refs. 24 and 25, using Aerosil 220 V (Degussa) as source of silica, cetyltrimethylammonium bromide (CTAB) (Aldrich), 1,3,5-trimethylbenzene (TMB) (Aldrich), NaOH (SDS) and deionized H_2O, in the molar ratio 1 SiO_2/0.1 CTAB/0, 0.27 and 1.3 TMB/0.25 NaOH/20 H_2O, respectively. The syntheses were performed at 115 °C for 24 h in an autoclave. The resulting products were filtered, washed with water, dried at 115 °C overnight and calcined at 550 °C for 8 h. Both as-synthesized and calcined materials were used as supports for the enzyme adsorption.

MCM-41 with 44 Å pore size was synthesized with the same procedure as above, but with octadecyltrimethyl ammonium bromide instead of CTAB. The resulting calcined MCM-41 was further grafted with butyl chains according to the procedure described in ref. 26 by using chlorodimethylbutyl silane and pyridine in toluene. This grafted MCM-41 was used as a carrier for enzyme adsorption. In summary, four materials with varying pore sizes (37, 44, 62, 100 Å) were available as well as three states of surface: hydrophilic (calcined MCM-41), hydrophilic/hydrophobic (as-synthesized MCM-41) and hydrophobic (grafted MCM-41).

Immobilization of lipase by adsorption in MCM-41s

The immobilization of the lipase by adsorption into MCM-41 materials was performed using the GB1 solution (0.74 mg enzyme mL^{-1}) for the MCM-41 materials with pore sizes of 37, 44 and 62 Å and using the GB4 solution (5 mg protein mL^{-1}) for MCM-41 with 100 Å pore diameter. 5 mL of the lipase solution GB1 were added to different amounts of MCM-41 (0.50 g of as-synthesized MCM-41, 0.25 g of calcined MCM-41 and 0.30 g of grafted MCM-41) in order to reach equivalent amounts of 14.9 mg of enzymes per g of pure SiO_2 depending on the organic content of each support (as-synthesized and grafted MCM-41 contain 50 and 20 wt% of organics, respectively). 2 mL of the lipase solution GB4 were added to 1 g of calcined MCM-41(100 Å) corresponding to 10 mg of proteins per g of pure silica. The suspensions were mixed for 2 h at room temperature. The resulting products were then filtered and washed 5 times with distilled water until no leaching of enzyme was observed, dried for 24 h at 50 °C.

The amount of immobilized enzyme was determined by dosing the quantity of enzyme contained either in the supernatant or in the solid after its dissolution in alkaline solution at pH 9. Before performing the Bradford test, the two resulting

solutions were separated from other organic molecules by elution through G25 Sephalex column.

Immobilization of lipase by sol-gel silica method

The immobilization of the lipase by encapsulation *via* a classical sol-gel silica was performed using the enzymatic solution GB2 and following the procedure described in literature by Reetz *et al.*[27] 564 μL of the lipase solution were added to a mixture of 100 μL aqueous sodium fluoride (1 M) (Aldrich) and 200 μL polyvinyl alcohol (PVA) (MW 15 000) (Merck) (4% w/w in water). The solution was shaken and 875 μL (5 μmol) propyltrimethoxysilane (PTMS) (Fluka) was added followed by 148 μL (5 μmol) tetramethylorthosilicate (TMOS) (Aldrich). The final ratio R = total water/silane was equal to 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 up, it was placed in an ice bath (for about 10 min) until gelation occurred and then aged at room temperature for 24 h. The resulting material was then dried at 37 °C for 3 days, and then ground in a mortar. The solid was then suspended in 4 mL of water and shaken for 2 h (350 rpm). The product was then collected on a glass frit and washed with 20 mL of water, 20 mL acetone and 20 mL of pentane. The resulting product was then dried at 37 °C for 24 h and ground in a mortar. The enzyme content was determined in the washing water using the Bradford method.

Immobilization of lipase by direct synthesis of Sponge Mesoporous Silica (SMS)

The biocatalysts named SMS-GB3, SMS-GB4 and SMS-MM were synthesized by using the enzymatic solutions GB3, GB4 and MM, respectively. The strategy which led us to adopt the SMS procedure is described in detail in ref. 22.

The synthesis of SMS was performed by using the following reactants: TEOS (Aldrich), dodecylamine (Aldrich), 3-sn-phosphatidylcholine from egg yolk (L-α-lecithin) (Fluka), β-D-lactose (Aldrich) in the molar ratio: 1 TEOS/0.04 dodecylamine/0.05 lecithin/0.03 lactose/28 H_2O/8.5 ethanol. A first solution of 0.15 g lactose and 7 mL of aqueous enzymatic solution (in Tris buffer) was prepared and added under stirring to a second solution containing 0.5 g lecithin and 0.1 g dodecylamine in 5.2 g ethanol. 2.7 g TEOS were added under stirring for 15 min, and, the mixture was left for 24 h in static conditions at room temperature. The resulting powder was then washed 5 times with 10 mL of ethanol/water mixture (1/1, v/v) at room temperature, followed by centrifugation, until no leaching of enzyme is observed. The sample was then dried for 24 h at 50 °C.

The amount of immobilized enzyme was determined by titrating both the supernatant solution and the solid as described above.

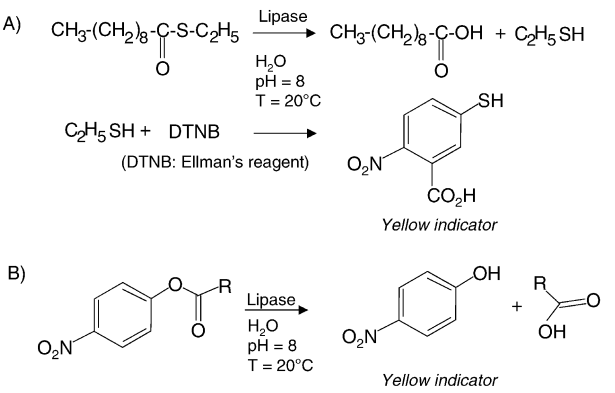
Measurements

Nitrogen adsorption–desorption isotherms at 77 K were recorded using a Micrometrics ASAP 2010 apparatus. Samples were outgassed either at 250 °C (for calcined samples) or at 180 °C (for as-synthesized and grafted samples) until a stable static vacuum of 3×10^{-3} Torr was reached. Specific surface areas were calculated by the BET method using the isotherm adsorption data in the range from $p/p_0 = 0.12$ to 0.25 just below capillary condensation. Pore diameters were evaluated from the isotherm desorption branch by the Broekhoff and de Boer method[28] which has been demonstrated as one of the most reliable for MCM-41 materials.[29] The pore volume was taken at the top of the step corresponding to the pore filling. Measurements of pH were performed using a G 810 pH-meter (Schott). UV-Vis spectroscopy measurements were recorded with a PU 8625 UV/VIS Spectrophotometer (Philips) coupled to a BD 40 recorder (Kipp & Zonen). Thermogravimetric analyses were performed under an oxygen flux using a TG 209C analyser (Netzsch Proteus).

Lipase catalytic tests

Hydrolysis of ethylthiodecanoate. A first catalytic test chosen for the evaluation of the activity of the immobilized lipases was the hydrolysis of ethylthiodecanoate (Scheme 1A). The thioethanol formed during the reaction can be easily detected by UV/VIS spectroscopy from the yellow colour developed in the presence of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), (Lancaster synthesis) according to ref. 30. The enzymatic catalytic tests were performed as follows:

- For the enzymatic solution: 100 μL of the aqueous enzymatic solution were added to a measuring cuvette containing 2.7 mL Tris buffer 0.05 M (pH 8), 100 μL of DTNB solution (20 mg mL^{-1} in DMSO) and 100 μL of ethylthiodecanoate solution (0.2 mg mL^{-1} in DMSO). The absorbance corresponding to the yellow product at 412 nm (ε = 14 800 L mol^{-1} cm^{-1}) was recorded immediately. The specific activity of the lipase is given in International Unit (I.U.) per mg of enzyme (I.U.: μmol of substrate transformed per min).
- For the immobilized lipase: 0.05 g of the solid was added to a mixture containing 28.7 mL Tris buffer 0.05 M (pH 8), 0.7 mL DTNB solution (20 mg mL^{-1} in DMSO), 1.5 mL ethylthiodecanoate (0.2 mg mL^{-1} 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 and 2.7 mL



R = CH_3 (C2), CH_2CH_3 (C3), $(CH_2)_6CH_3$ (C8), $(CH_2)_{10}CH_3$ (C12), $(CH_2)_{14}CH_3$ (C16)

Scheme 1

Tris buffer were added. The absorbance was measured at 412 nm to evaluate the activity of the biocatalysts (I.U. per g solid). Then, the specific activity (I.U. per mg enzyme) was determined to calculate the relative specific activity, which is the percentage of specific activity of immobilized lipase *versus* the specific activity of the lipase in solution.

Hydrolysis of esters of *p*-nitrophenol. To compare more directly our results with literature data, we have used a second catalytic test: the hydrolysis of the esters of *p*-nitrophenol (Scheme 1B). This reaction, more precisely the hydrolysis of the propionate of *p*-nitrophenol, was used as a test reaction by Reetz for characterizing the activity of the immobilized lipase prepared by silica sol-gel encapsulation.[31]

In addition, in order to test the specificity of the lipases towards the chain length of esters, various substrates with five alkyl chain lengths were used: *p*-nitrophenylacetate (named C2) (Fluka), *p*-nitrophenylpropionate (named C3) (Biochemika), *p*-nitrophenylcaprylate (named C8) (Fluka), *p*-nitrophenyllaurate (named C12) (Fluka), *p*-nitrophenylpalmitate (named C16) (Biochemika).

Each substrate was dissolved in *N*,*N*-dimethylethylurea (DiMEU) (Biochemika) to obtain 40 mM solutions of substrate.

First, the activities of the free enzymes for each substrate were measured before evaluation of the solid biocatalysts. In a 3 mL measuring cuvette, 2.93 mL of Tris buffer (pH = 8.08) were added to 50 μL of GB4 or MM solutions and 20 μL of 40 mM substrate solution. The apparition rate of *p*-nitrophenol was determined by UV-Vis by measuring the optical density variation at 495 nm.

The activity of the solid biocatalysts was measured as follows: 300 mg of biocatalysts were added to 28 mL of Tris buffer and 1.6 mL of the 40 mM substrate solution. Every minute, aliquots of 0.3 mL of supernatant were taken and put in a measuring cuvette filled with 2.7 mL of Tris buffer. The optical density was determined at 495 nm to follow the formation of *p*-nitrophenol.

Results and discussion

Immobilization of lipase by adsorption into MCM-41s

Mesoporous silicate materials formed by self-assembly of surfactants and silicas[6] exhibit a highly ordered pore structure, very narrow pore size distributions and large surface areas. Owing to their silicate inorganic framework, they are mechanically stable and are resistant to microbial attack. In addition, it is possible to chemically modify their surface with various functional groups, enabling different interactions with the enzyme of interest. In all the reported methods for enzyme immobilisation in mesoporous silicate materials,[7–17] enzyme immobilisation occurs after the synthesis of the support by adsorption, to avoid its denaturation due to the use of harsh conditions or reagents that are detrimental to the enzyme. Adsorption is one of the simplest methods of physical immobilisation of enzyme but leaching of adsorbed enzyme into solution is a common disadvantage of this technique, particularly when the enzymatic catalysis is performed in aqueous media.

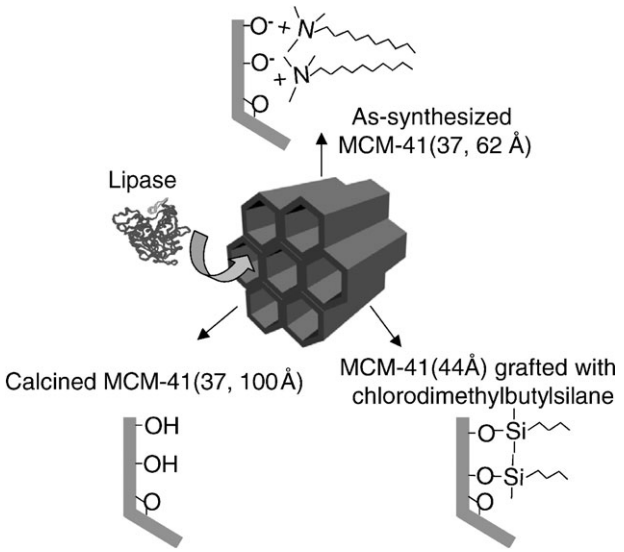


Fig. 1 Schematic representation of the various MCM-41s (as-synthesized, calcined, grafted) with different pore sizes and surface properties used for the immobilization of lipase by adsorption.

MCM-41 characterizations. In order to immobilize lipases by adsorption, a series of parent and modified MCM-41 materials have been synthesized with different pore sizes (37, 44, 62, 100 Å) and various surface states: hydrophilic (calcined MCM-41), hydrophilic/hydrophobic or micelle-like (as-synthesized MCM-41) and hydrophobic (dimethylbutyl-grafted MCM-41) (Fig. 1). The pore size, the pore volume, the surface area and the relative polarity[26] of the surface monitored by means of C_{BET} parameter of the different MCM-41 supports have been determined by nitrogen sorption isotherms and are presented in Table 1. All calcined MCM-41 supports exhibit the expected well-defined pore structure with narrow pore size distributions, high pore volume and high surface area. The pore diameters of as-synthesized and grafted MCM-41s are smaller than the corresponding calcined forms because the organics present in the solid are lying on their surface. For as-synthesized MCM-41(37 Å), synthesized without swelling agent, the pore size cannot be evaluated by nitrogen adsorption, because nitrogen can not penetrate inside the organic chains of surfactant at the temperature of measurement (77 K). Therefore as-synthesized MCM-41(37 Å) appears as a non porous material from the point of view of nitrogen adsorption, but pores are accessible at room temperature to organic molecules and behave like supported-

Table 1 Results of nitrogen sorption at 77 K of MCM-41 supports used for lipase adsorption: pore volume (V), BET surface area (S), pore size (D) and C_{BET} parameter

Materials	V/mL g^{-1})	S/m^2 g^{-1}	D/Å	C_{BET}
MCM-41(37 Å)-calcined	0.74	964	37	100
MCM-41(37 Å)-as-synthesized	0	144	np	31
MCM-41(44 Å)-grafted	0.19	288	34	20
MCM-41(62 Å)-as-synthesized	0.43	368	49	27
MCM-41(100 Å)-calcined	2.00	985	100	85

[a] np: non porous to nitrogen.

Table 2 Lipase loading and enzymatic activity for the hydrolysis of ethylthiodecanoate for lipase immobilized by adsorption in MCM-41 supports, by encapsulation by sol-gel and by SMS direct synthesis and comparison with commercially immobilized lipase in silica sol-gel (sol-gel-AK). The enzymatic solutions used for the different immobilizations are specified

Biocatalysts	Enzyme loading (mg enzyme per g solid)	Specific activity (I.U.per g solid)	Relative specific activity (%)
MCM-41(37 Å)-calcined-GB1	4.4	4.1	2.6
MCM-41(37 Å)-as-synthesized-GB1	5.0	9.3	5.2
MCM-41(62 Å)-as-synthesized-GB1	7.2	3.0	1.1
MCM-41(44 Å)-grafted-GB1	3.2	1.3	1.1
sol-gel-GB2	4.8	13.9	29
sol-gel-AK (Fluka)	4.2	1.0	—
SMS-GB3	1.3	20.7	46

Specific activity of lipase solutions: GB1 (0.74 I.U. per mg enzyme), GB2 (7.16 I.U. per mg enzyme), GB3 (3.50 I.U. per mg enzyme).

micelle materials with the same solubility properties as those of the micelles themselves.[32] The C_{BET} parameters of the MCM-41 supports vary from 100 for hydrophilic silica (calcined MCM-41) to 20 for hydrophobic silica (grafted MCM-41). Calcined MCM-41(100 Å) exhibits a lower C_{BET} (C_{BET} = 85) as do the usual calcined MCM-41s due to the presence of some hydrophobic patches on its hydrophilic surface, induced by the use of TMB in its synthesis.[33,34] As-synthesized MCM-41s reveal a dual hydrophilic/hydrophobic character of the surface characterized by a C_{BET} value around 30.

Lipases loading in MCM-41 supports. The enzymes are adsorbed efficiently into all the different MCM-41s supports with loadings, after the 5 washing steps, comprised between 3 to 10 mg of enzyme per gram of solid (Tables 2 and 3). The lipase size is *ca.* 30–40 Å, which is quite compatible with the pore sizes of the MCM-41 supports. Both determinations of enzyme loadings, by difference from the enzyme remaining in the supernatant solution or by dosing the amount of enzyme adsorbed in the solid after its dissolution in alkaline solution, led to self-consistent results. In the MCM-41s materials with pore sizes around 40 Å *ca.* 4 mg of enzyme per g of solid have been adsorbed, whereas higher values, 7.2 and 9.9 mg proteins per g solid, have been adsorbed for MCM-41s with larger pores, as-synthesized MCM-41(62 Å) and calcined MCM-41(100 Å), respectively. The values obtained for the larger pore materials correspond to a total adsorption of the enzyme present in the solution. If the enzyme amount is expressed on a per g basis of silica alone, it is apparent that either hydrophilic (calcined MCM-41(37 Å)) or hydrophobic (grafted MCM-41(44 Å)) materials adsorbed much less enzyme (4 mg g^{-1} silica) than a support of similar pore size with a dual hydrophobic/hydrophilic character (as-synthesized MCM-41(37 Å). The latter leads to a loading of 10 mg of enzyme per gram of silica. There is therefore a greater affinity of the lipase for the support with a hydrophilic/hydrophobic surface. The lipases adsorbed inside the supported-micelle were not leached by the successive washings.

A hydrophilic/hydrophobic support is in adequation with the dual nature of the lipase itself, which is mostly hydrophilic with some hydrophobic domains surrounding its active site. Thus, lipase recognizes the hydrophobic patches of the support similarly to those of their natural substrates.[35] However, a strongly hydrophobic support (grafted-MCM-41) does not allow enzyme to migrate significantly inside the pores. Hydrophobic interactions alone are not prone to promote enzyme adsorption. Similarly, lipases adsorbed on a pure hydrophilic support (calcined MCM-41(37 Å)) are leached from the support by lack of hydrophobic interactions. The additional hydrophobic patches on the surface of calcined MCM-41(100 Å) are, on the other hand, effective enough to stabilize lipase in the support. The adsorption of enzymes limited at the sole pore openings of MCM-41 materials is an hypothesis which can not be definitively proved nor disproved. However two arguments are in favor of adsorption in pores, even in the presence of encapsulated micelles: (i) the contribution of external surface (pore openings and silica walls) to the total surface is negligible in comparison to the surface developed by the pores, (ii) the fact that for a given pore diameter the amount of enzyme adsorbed is a function of the polarity of the surface and for a given surface polarity the amount adsorbed increases with pore size is a strong argument in favor of adsorption inside the pores.

Table 3 Lipase loading and enzymatic activity for the hydrolysis of the propionate of *p*-nitrophenol for lipase immobilized by adsorption in MCM-41, by encapsulation by SMS direct synthesis and comparison with commercially immobilized lipases in silica sol-gel (sol-gel-AK) and in cationic resin (Lipozyme). The enzymatic solutions used for the different immobilizations are specified

Biocatalysts	Enzyme loading (mg enzyme per g solid)	Specific activity (I.U. per g solid)	Relative specific activity (%)
MCM-41(100 Å)-calcined-GB4	9.9	0.71	163
SMS-GB4	4.9	1.95	911
SMS-MM	3.2	0.55	154
Lipozyme (Fluka)		0.25	
Sol-gel-AK (Fluka)	4.2	0.021	4.4

[a] Specific activity of lipase solutions: MM (0.11 I.U. per mg enzyme), GB4 (0.044 I.U. per mg enzyme).

Catalytic activity of MCM-41-based biocatalysts

Hydrolysis of ethylthiodecanoate. The catalytic activity of lipase GB adsorbed in as-synthesised, calcined and grafted MCM-41 supports have been measured first for the ethylthiodecanoate hydrolysis (Table 2, Figs. 2 and 3) to explore the possibility of a correlation between the catalytic activity and the hydrophobic/hydrophilic nature of the support, and

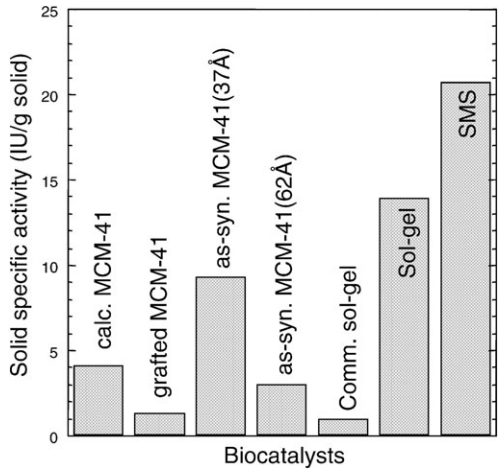


Fig. 2 Specific activities of lipase GB per g of biocatalyst towards ethylthiodecanoate hydrolysis as a function of the different procedure of lipase immobilization: by adsorption in MCM-41 supports (as-synthesized, calcined, grafted), by sol-gel encapsulation (commercial sol-gel and as-made), by entrapment in sponge mesoporous silica (SMS) by direct synthesis.

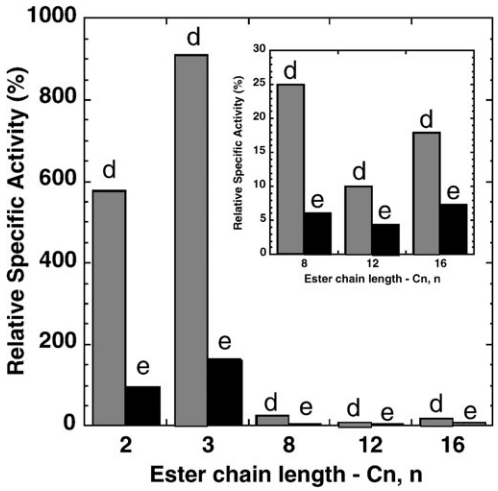


Fig. 4 Relative specific activity of lipase GB encapsulated in (d) SMS and (e) calcined MCM-41(100 Å) for the hydrolysis of the esters of *p*-nitrophenol as a function of the ester chain length Cn. Insert $n = 8$ to 16.

eventually verify the statement that hydrophobic materials lead to the best supports for enzyme immobilization.[36]

The catalytic activity of the immobilized enzymes for the hydrolysis of ethylthiodecanoate (Table 2) was expressed in terms of the overall activity of the solid biocatalyst (I.U. per g solid) (Fig. 2) and in terms of relative specific activity, which is the percentage of the specific activity of the solid biocatalysts (I.U. per mg protein) $_{\text{materials}}$ compared to the specific activity of the enzyme in the solution (I.U. per mg protein) $_{\text{solution}}$ (Fig. 3).

In our case, the most hydrophobic support (grafted-MCM-41) gives the lowest activity compared to the other supports (Table 2). For an equivalent enzyme loading, the lipase immobilized inside hydrophilic support (calcined MCM-41(37 Å)) shows a relative specific activity nearly 3 times higher than that immobilized in the hydrophobic support. This is most likely due to the hydrophobic barrier for water on the latter. The relative specific activity (3%) of lipase immobilized in the hydrophilic support is nevertheless quite low, probably on account of an inadequate conformation of the enzyme resulting from interactions with silanol groups. The relative specific activity (5%) of lipase immobilized in the hydrophilic/hydrophobic support (as-synthesized MCM-41(37 Å)) is the highest, though moderate, which confirms that the lipase environment generated by a hydrophobic/hydrophilic surface, such as a micelle-like environment, is more favourable for the lipase activity. Indeed, lipases are known to be more active at the hydrophobic/hydrophilic interface, especially when the concentration of their substrates reaches their critical micellar concentration. However, the relative specific activity of the lipase adsorbed in as-synthesized MCM-41(62 Å), shows a drastic decrease. This suggests an inhibition of the enzyme activity in this support arising from the interaction of lipase with ammonium surfactant head groups, which are inhibitors of lipase.[22] Indeed, in MCM-41 synthesized with micelles swelled with TMB, different conformations of surfactant chains and head groups have been found.[33,34] Some surfactants interact with the surface by hydrophobic interaction and therefore their head groups are pointing inside the cavity and can interact with lipase and inhibit their activity.

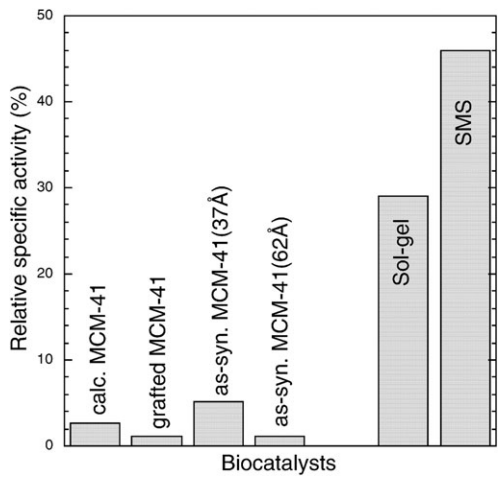


Fig. 3 Percentage of relative specific activities of lipase GB towards ethylthiodecanoate hydrolysis $((\text{I.U. per mg lipase})_{\text{solid}}/(\text{I.U. per mg lipase})_{\text{liquid}}) \times 100$ as a function of the different modes of lipase immobilization: by adsorption in MCM-41 supports (as-synthesized, calcined, grafted), by sol-gel encapsulation (as-made), by entrapment in sponge mesoporous silica (SMS) by direct synthesis.

Hydrolysis of the esters of p-nitrophenol. In order to check whether the low relative specific activities (1 to 5%) observed for all biocatalysts were arising from the chain length of the substrate (C10), another catalytic reaction was used, namely the hydrolysis of esters of *p*-nitrophenol with different chain lengths from C2 to C16 (Figs. 4–7). As mentioned under the experimental section, the hydrolysis of the propionate of

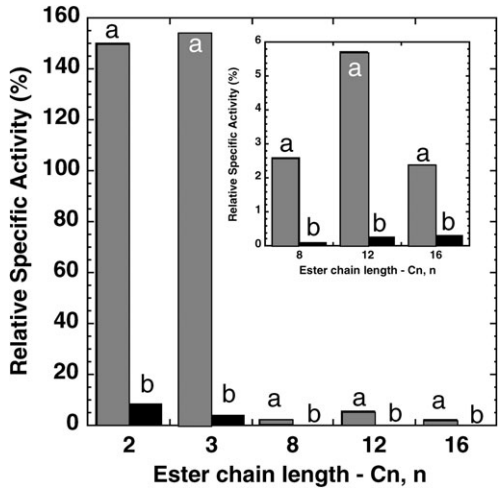


Fig. 5 Relative specific activity (%) of the lipase MM encapsulated in (a) SMS and (b) commercial sol-gel biocatalyst (Fluka) for the hydrolysis of the ester of *p*-nitrophenol as a function of the ester chain length Cn. Insert $n = 8$ to 16.

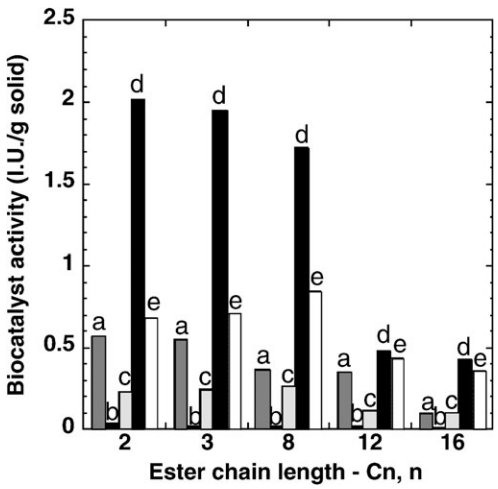


Fig. 7 Biocatalyst activity (per g of solid) of the lipase MM encapsulated in (a) SMS, (b) commercial sol-gel biocatalyst (Fluka) and (c) commercial Lipozyme (Fluka) and of the lipase GB encapsulated in (d) SMS and (e) calcined MCM-41(100 Å) for the hydrolysis of the ester of *p*-nitrotoluene as a function of the ester chain.

p-nitrophenyl (C3) was used by Reetz *et al.* for evaluating biocatalysts prepared by sol-gel encapsulation, thus allowing results to be further compared.[31] In order to avoid eventual diffusion limitations, experiments have been performed using the support with the largest pores, MCM-41(100 Å) (Table 3, Figs. 4 and 7).

Before studying the solid biocatalysts, the activity of the native lipase MM from *Rhizomucor miehei*, from Fluka, has been compared to that of the native Esterase 30 000 from Gist-Brocades in solution in the hydrolysis of same esters of *p*-nitrophenol (Fig. 8). Both lipases show the same profile of selectivity as a function of ester chain length with a strong specificity for the ester chains with 8 carbon atoms and a very low activity for the hydrolysis of short chain esters. This similarity confirms that both enzymes have a close relationship and come from the same strain. However, the specific activities of both enzymes are very different, the specific activity of the lipase commercialized by Fluka is about twice that of Esterase 30 000 for each chain length.

The catalytic activity of lipase GB adsorbed in MCM-41 (100 Å) is reported in Fig. 4. The data reveal a dramatic change of the typoselectivity of the enzyme, resulting most likely from a modification of its conformation upon adsorption. The solid biocatalyst is much less effective than the native enzyme for converting the long alkyl-chain esters but shows a remarkable activity for the short chain analogues, equal to 96% of relative specific activity for the acetate of *p*-nitrophenol (C2) and 163% for the propionate (C3).

Immobilization of lipase by sol-gel

Hydrolysis of ethylthiodecanoate. Lipases can be entrapped in so called hydrophobic sol-gel materials,[27] in which the

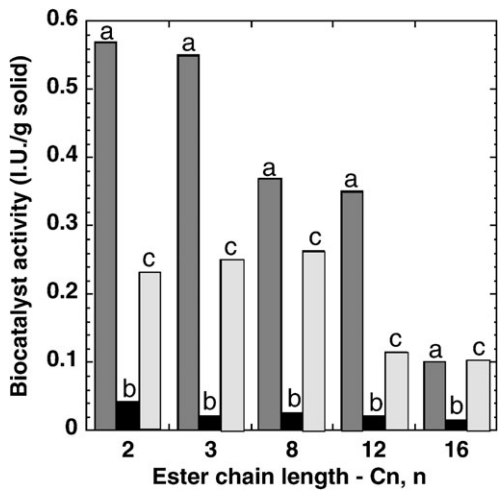


Fig. 6 Biocatalyst activity (per g of solid) of the lipase MM encapsulated in (a) SMS, (b) commercial sol-gel biocatalyst (Fluka) and (c) commercial resin Lipozyme (Fluka) for the hydrolysis of the ester of *p*-nitrotoluene in function of the ester chain length Cn.

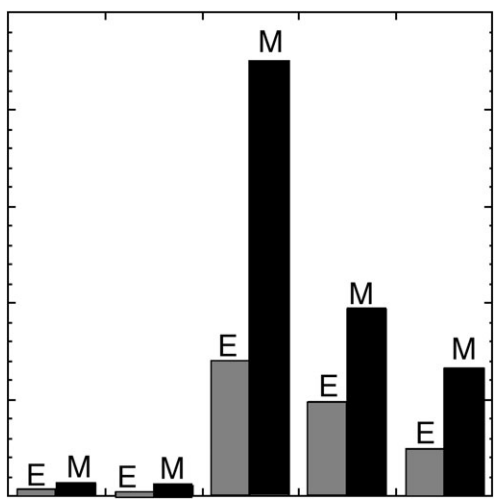


Fig. 8 Specific activity of (E) lipase GB Esterase 30 000 and (M) lipase MM for the hydrolysis of the esters of *p*-nitrophenol as a function of the ester chain length Cn.

hydrophobic/hydrophilic balance is controlled by the nature and ratio of alkoxides. The highest activity of lipases materials produced by sol-gel encapsulation were obtained using a mixture of propyltrimethoxysilane and tetramethoxysilane in a ratio PTMS/TMOS = 1/3. The resulting heterogeneous biocatalyst displays long-term stability as well as enhanced activity in esterification and transesterification reactions in organic solvents.[27] It was further demonstrated that some lipases entrapped in this type of hydrophobic sol-gel material are active heterogeneous catalysts for ester hydrolysis reactions in aqueous medium for the hydrolysis of propionate of *p*-nitrophenol.[31] The lipase from GB2 solution was entrapped by this reported sol-gel procedure (sol-gel-GB2) and tested in the hydrolysis of ethylthiodecanoate (Table 2, Figs. 2 and 3). The amount of immobilized enzyme corresponds to 4.8 mg protein per g of support (5.7 mg per g of silica), which is close to the amount of enzyme immobilized by adsorption in MCM-41(37 Å) (Table 2). However, the relative specific activity of sol-gel-GB2 is much higher (29%) than those of lipase adsorbed in MCM-41 supports, although all lipase encapsulated in the sol-gel material are not accessible due to the non-control of porosity. This means that the cavity built by sol-gel encapsulation provides a better structural and chemical environment for the enzyme—for preserving its conformation, and therefore its activity—than a pre-formed pore.

As a reference, a commercial sol-gel-AK from Fluka synthesized in accordance with the Reetz method with lipase from *Rhizomucor miehei*, as described above, was also evaluated in the ethylthiodecanoate hydrolysis. The amount of lipase (4.2 mg enzyme per g solid) was determined by the Bradford method after dissolution of the silica matrix in basic medium and separation on G25 column and was found similar to that of our silica sol-gel preparation. However, the activity of this commercial biocatalyst is much lower than that of our sol-gel preparation: 1 I.U. per g of solid compared to 13.9 I.U. per g of solid, respectively (Table 2, Fig. 2). The explanation for this discrepancy probably lays in the fact that the two lipases, coming from the same strain, have been prepared, extracted and purified differently by Gist-Brocades and Fluka.

Hydrolysis of the esters of p-nitrophenol. The commercial biocatalyst sol-gel AK (Fluka), synthesized using the lipase MM from Fluka, has been evaluated in the hydrolysis of the esters of *p*-nitrophenol with different chain lengths (Figs. 5–7). The results lead to a relative specific activity sequence of: 8.3%, 4.4%, 0.1%, 0.3% and 0.3% for the esters: C2, C3, C8, C12, and C16, respectively. The catalytic activity is maximum for short chain esters (C2 and C3), but, as noticed previously for the materials prepared by adsorption in MCM-41(100 Å), drastically decreases for long chain esters. Lipase immobilized either by sol-gel or by adsorption feature therefore different typoselectivities than their native counterparts. The result found in the hydrolysis of the propionate of *p*-nitrophenyl (C3), 4.4% of relative activity, is of the same order of magnitude as the result reported by Reetz in the literature.[31] Among the different lipases tested by Reetz, the best relative specific activity measured was 107% for the lipase of *Humicola lanuginosa*.[31] In our case however, the lipase encapsulated by sol-gel shows a lower activity (4%) than the lipase immobilized by adsorption in MCM-41(100 Å) (163% activity for C3) (Table 3). This difference could come from the difference of lipase conditioning, although in solution lipase MM is two times more active than lipase GB. The two lipases MM and GB have been further immobilized by our new SMS encapsulation route and compared in the different ester hydrolysis reactions.

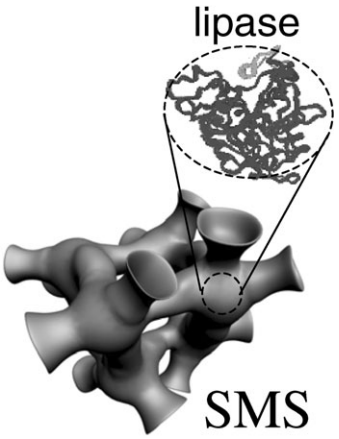


Fig. 9 Schematic representation of the Sponge Mesoporous Silica (SMS) pore structure, showing a cavity of 40–50 Å of diameter, used for lipase encapsulation by direct synthesis.

Immobilization of lipase by direct synthesis of Sponge Mesoporous Silica (SMS)

The lipases MM and GB were encapsulated following a new one-step procedure, *viz*. Sponge Mesoporous Silica (SMS) direct synthesis, recently introduced and described in detail in reference 22 (Fig. 9). This preparation method adds to the sol-gel procedure the possibility to control the pore size, thanks to the use of gentle surfactants (lecithins (phospholipids) and dodecylamine) as templating agents. The objective is to protect the enzyme by a silica matrix and, at the same time, to allow a good accessibility to substrates for catalysis by creating a very open porous structure. The SMS direct synthesis method is based on mild conditions of preparation that do not affect the structure and the activity of the enzyme. The SMS direct synthesis was achieved using tetraethoxysilane as silica precursor and: (i) a natural surfactant, egg lecithin, to avoid direct interaction between silanol groups and the enzyme which may denatures its activity by changing its conformation, (ii) amines to create a porous structure thanks to their curvature effect on the phospholipid bilayer, which should enhance the diffusivity of substrate compared to the conventional sol-gel technique, and (iii) lactose to preserve the enzyme activity by replacing the conformational water of the protein. The backbone of SMS features a 3-dimensional sponge-like isotropic structure with interconnected spherical pores of *ca.* 40–50 Å (schematically represented in Fig. 9), such as a disordered SBA-16[37] mesostructure, with a typical surface area of 600 $m^2\ g^{-1}$ and a pore volume of 0.8 $mL\ g^{-1}$.[22,38]

The lipase from the GB3 solution was encapsulated by this new SMS route, corresponding to 1.3 mg of enzyme per g of

solid. It revealed a very high relative specific activity towards the hydrolysis of ethylthiodecanoate corresponding to 46% of relative specific activity (Table 2, Fig. 3) compared to 29% for the biocatalyst obtained by the sol-gel technique and 5% for that prepared by adsorption in as-synthesized MCM-41. These figures reveal the benefit of adding pore size control into sol-gel chemistry. The lipase from solution GB4 was encapsulated by the SMS route, corresponding to 4 mg of proteins per g of solid, and the resulting biocatalyst has been tested in the hydrolysis of the esters of *p*-nitrophenol with different chain lengths (Table 3, Figs. 4–7). As already observed for the materials prepared by adsorption in MCM-41 or *via* the sol-gel encapsulation, the lipase encapsulated by SMS route shows low activity for the esters with alkyl chain with 8 or more carbon atoms but a remarkable activity for the short alkyl-chain esters, corresponding to six fold and nine fold the activity of the free lipase in solution for the C2 and the C3 esters, respectively.

In order to compare with commercial biocatalysts, lipase MM from Fluka has been also encapsulated by the SMS route, leading to a material with 3.2 mg of protein per gram of solid. The resulting biocatalyst demonstrated also an enhancement of the hydrolysis activity of the esters with short alkyl chains equal to 150% of relative activity in the case of the C3 ester. The same lipase immobilized by the sol-gel procedure led to a maximum of 9% relative specific activity.

The activity of the biocatalyst obtained by the SMS route has been compared to another commercially available immobilized lipase, called Lipozyme, where the lipase has been immobilized on an anionic resin. The activity results are presented on the basis of the activity per gram of biocatalyst (Figs. 6 and 7) because the amount of lipase present in the Lipozyme is unknown and difficult to evaluate by the usual procedure. The Lipozyme shows a higher activity than biocatalyst prepared by the sol-gel encapsulation method, but almost half the activity of the lipase encapsulated by the new SMS route.

If all biocatalysts are compared in terms of activity per weight of biocatalyst (Fig. 7), we can see that the lipase GB (Esterase 30 000) encapsulated by the SMS route leads to the most active biocatalyst, while in solution the lipase of MM from Fluka had an activity twice that of lipase GB. Encapsulation by the SMS route clearly resulted in an enhancement of the activity of the lipase for the hydrolysis of esters with short alkyl chains.

Conclusion

Different techniques for immobilization of lipases from *Mucor meihei* such as adsorption in mesoporous silica supports, in polymers, by silica sol-gel encapsulation, or by entrapment in sponge mesoporous silica (SMS), have all produced active biocatalysts for ester hydrolysis. The two latter procedures lead to the more efficient systems due to a high substrate accessibility and an adequate balance of the hydrophilic/hydrophobic character of the surface. Several recent papers[39–42] discussed the improvement of enzyme stability and intrinsic activity upon confining them in mesoporous materials. This confinement can restrain enzyme unfolding or denaturation when it is located in a pore of similar dimension.

The sol-gel method is known to result in a fairly stable form of the immobilized enzyme because of a close fit between the enzyme molecule and the sol-gel pore which is likely to prevent unfolding and denaturation of the encapsulated enzyme.[43] Gil *et al.*[44] observed moreover an increase of the specific activity of *Candida rugosa* lipase by immobilization in poly(hydroxymethyl siloxane) and a change in the enantioselectivity.

In our case it can be proposed that the typoselectivity for long chain fatty acid esters is inverted because of the unfolding of the entrapped enzyme. It has been established that better recognition of long chain fatty acid by lipase is due to the presence of a lid that undergoes a conformational rearrangement in the presence of this type of substrate, making the active site accessible.[45] As this rearrangement is partly blocked, it is conceivable that the hydrolysis activity of long chain fatty esters is decreased, and concurrently the one for short chain fatty acids is increased.

Acknowledgements

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