INFLUENCE OF THE DRYING TECHNIQUE OF SILICA GELS ON THE ENZYMATIC ACTIVITY OF ENCAPSULATED LIPASE

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Recent studies by Reetz et al. (Reetz, M.T., Zonta, A. and Simpelkamp, J. (1996a) Biotechnology and Bioengineering, 49, 527-534) have shown that the catalytic activity of lipase encapsulated in sol–gel materials, in esterification reactions, depends on many parameters such as the presence of hydrophobic groups grafted on the gel network and of an organic component in the gel network. In the present study, we have examined the effect of the gel pore texture which can be modified by varying the drying technique, for a given silica precursor composition and hydrolysis–condensation procedure. For a given mixture of two silane precursors, propyltrimethoxysilane and tetramethoxysilane, we compared the effects of the presence or absence of an organic component such as polyvinyl alcohol, in combination with drying either by evaporation which leads to the formation of xerogels, or by supercritical drying in CO₂ which leads to the formation of aerogels. For this last technique, the exchange of liquid is also an important step and its effect on the enzyme activity has been examined. The gel pore texture was characterized by nitrogen absorption according to the Brunauer Emmett and Teller method. The catalytic activities of the materials were compared in the esterification of lauric acid by 1-octanol.

Keywords: Lipase; Pseudomonas cepacia; Esterification; Silica; Aerogel; Xerogel; Drying

INTRODUCTION

Lipases have proven to be versatile and efficient biocatalysts which can be used in a broad variety of esterification and ester hydrolysis reactions. The commercial application of lipases as biocatalysts for organic synthesis requires simple but efficient methods to immobilize the enzyme, yielding

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highly stable and active biocatalysts, because process economy demands among other things recyclable biocatalysts. Hence a broad variety of immobilization techniques have been studied or applied, including for example: physical adsorption onto solid supports; cross-linking to a suitable carrier matrix; entrapment and micro-encapsulation in polymeric matrices. A recent extensive review of this field was published by Malcata et al. (1990).

Recently, new developments in biocatalyst immobilization have appeared, in which the enzymes were encapsulated inside sol–gel materials (Avnir et al., 1994; Avnir, 1995; Dave et al., 1994; Gill and Ballesteros, 1998). In this way, biocatalysts can be used as if they were heterogeneous catalysts. That is to say, they can cleaned after service and reused for further catalysis reactions. One of the most interesting developments concern lipases, encapsulated in hybrid silica-organic gels (Reetz et al., 1996a,b,c). According to these authors, one likely reason for the unusually high relative enzyme activity is the high dispersion of the lipase in the sol–gel matrix. Moreover they have shown that successful sol–gel immobilization of lipases requires to use modified hydrophobic silica matrices prepared from alkyl-substituted silane precursors, such as with methyl or propyl groups, and that organic additives such as polyvinyl alcohol enhance the activity of lipases. Hence, an additional favorable factor may be a possible interaction between the lipophilic domains of the lipases and the hydrophobic regions of the organic/inorganic sol–gel matrix.

On the other hand, Schwertfeger et al. (1994) studied organically modified silica aerogels prepared by aqueous NH₃ catalyzed hydrolysis and condensation in methanol of Si precursors mixtures comprising in part RSi(OMe)₃ compounds, followed by supercritical drying. They showed that 20% of RSi(OMe)₃ as Si precursors was sufficient to provide the gels with a permanent hydrophobicity.

In the present study, we examined the effect of the gel pore texture which can be modified by varying the drying technique, for a gelation chemistry related to the synthesis procedure by Schwertfeger et al. The results obtained on materials dried by evaporation which leads to the formation of xerogels, are compared with those on materials dried by supercritical drying in CO₂ which leads to the formation of aerogels.

EXPERIMENTAL PROCEDURE

The main reactants used in this study were: the silane precursors, propyltrimethoxysilane (PTMS) and tetramethoxysilane (TMOS) both 98% from
Aldrich; the solvent, methanol R.P. Normapur from Prolabo or deionized water; the catalyst, aqueous NH$_3$ min 28% R.P. Normapur or NaF technical grade both from Prolabo; the lipase of *Pseudomonas cepacia* from Fluka; and polyvinyl alcohol with average molar mass 15,000 (termed PVA) from Prolabo.

For a first series of samples, silica gels were prepared according to a method by Schwertfeger *et al.* (1994), from solutions of PTMS and TMOS in methanol, according to the flow chart shown in Fig. 1. The silicon molar ratio coming from PTMS was $r_{SI, P} \approx 0.2$. Separately, about 2 mg of enzyme was dispersed in a pH 6.18 buffer solution containing a concentration of 0.01 M phosphate, to which a 4% (by mass) aqueous solution of PVA was added. The enzyme alone in suspension was agitated for one half hour before mixing in the silica sol just prior to gelation. The proportions of each

![Flow chart](image_url)

**FIGURE 1** Flow chart of the gel samples made in the present study.
TABLE I(a) Proportions of the chemical components used in the synthesis of samples in the first series for 1 mg of enzyme

<table>
<thead>
<tr>
<th>Samples</th>
<th>Drying procedure</th>
<th>Lipase (mg)</th>
<th>NH₄OH 0.01 M (µL)</th>
<th>4% PVA pH 6.18 (µL)</th>
<th>Deionized water (µL)</th>
<th>MeOH (µL)</th>
<th>PTMS (µL)</th>
<th>TMOS (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP</td>
<td>Aerogel</td>
<td>1</td>
<td>120</td>
<td>75</td>
<td>138</td>
<td>61</td>
<td>207</td>
<td></td>
</tr>
<tr>
<td>DP</td>
<td>Direct aerogel</td>
<td>1</td>
<td>120</td>
<td>75</td>
<td>138</td>
<td>61</td>
<td>207</td>
<td></td>
</tr>
<tr>
<td>XP</td>
<td>Xerogel</td>
<td>1</td>
<td>120</td>
<td>75</td>
<td>138</td>
<td>61</td>
<td>207</td>
<td></td>
</tr>
<tr>
<td>Samples without NH₄OH or PVA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP (−NH₄)</td>
<td>Aerogel</td>
<td>1</td>
<td>—</td>
<td>75</td>
<td>120</td>
<td>138</td>
<td>61</td>
<td>207</td>
</tr>
<tr>
<td>AP(−PVA)</td>
<td>Aerogel</td>
<td>1</td>
<td>120</td>
<td>—</td>
<td>75</td>
<td>138</td>
<td>61</td>
<td>207</td>
</tr>
</tbody>
</table>

Note: 1 mmol TMOS = 148 µL; 1 mmol PTMS = 176 µL; 1 mmol methanol = 40.5 µL.

TABLE I(b) Proportions of the chemical components used in the synthesis of samples in the second series for 1 mg of enzyme

<table>
<thead>
<tr>
<th>Samples</th>
<th>Drying procedure</th>
<th>Lipase (mg)</th>
<th>NaF 1 M (µL)</th>
<th>4% PVA (µL)</th>
<th>Deionized water (µL)</th>
<th>PTMS (µL)</th>
<th>TMOS (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR</td>
<td>Aerogel</td>
<td>1</td>
<td>50</td>
<td>100</td>
<td>282</td>
<td>428</td>
<td>82</td>
</tr>
<tr>
<td>XR</td>
<td>Xerogel</td>
<td>1</td>
<td>50</td>
<td>100</td>
<td>282</td>
<td>428</td>
<td>82</td>
</tr>
</tbody>
</table>

reactant used for all samples in this study are shown in Table I(a). The fraction of hydrolysis water provided by this buffer solution was ≈0.38, the remaining water to achieve silica gelation being provided by a 0.01 M NH₄OH solution. The total molar ratio of water to silicon was r_w ≈ 6.3. The samples differed in the solution in which the enzyme was dispersed before gelation. In some cases, no PVA or no aqueous NH₃ was added, but the total aqueous volume was maintained at the same value by adding deionized water. The aim was to compare the effect of PVA or aqueous NH₃ with the effect of the drying method.

A second series of samples containing dispersed enzyme was prepared by a technique detailed by Reetz et al. (1996a), with a silicon molar ratio r_sil,P ≈ 0.8 coming from PTMS, a total water to silicon molar ratio r_w ≈ 8, and with NaF as the gelation catalyst. The proportions of each reactant used for this second series are gathered in Table I(b).

As can be seen in Tables I(a) and (b), the main difference between these samples was the drying procedure. Xerogels XR and XP were dried by evaporation at room temperature. On the other hand, some aerogels were dried by supercritical drying in CO₂ without intermediate solvent exchange for acetone (DP samples), while other aerogels were previously dialyzed in acetone before drying by the supercritical method with CO₂ (AP and AR samples).
The equipment used for this supercritical drying technique was a “Supercritical Point Drier” of Polaron®, which is an autoclaveable to withstand a pressure and a temperature slightly above the critical point of liquid CO₂. After the autoclave was closed, liquid CO₂ was progressively introduced while air was slowly purged through an exhaust valve so as to facilitate filling the drier with liquid CO₂. Once a sample was completely covered with liquid CO₂, all valves were closed and the autoclave was left to stand for about 30 min so that liquid CO₂ could exchange for the gel liquid. Next, liquid CO₂ mixed with acetone, was evacuated through a drain valve while maintaining the liquid CO₂ level above the sample to avoid any capillary contraction. As the evacuated CO₂ sublimated, only the liquid acetone remained at the autoclave drain valve. When this liquid could be deemed to be entirely recovered, the autoclave was closed and heated up to a temperature \( T \approx 35^\circ\text{C} \) and pressure \( P \approx 8 \text{ MPa} \) in about 30 min, so as to reach conditions above the critical point of CO₂ with critical temperature \( T_c = 31.0^\circ\text{C} \) and critical pressure \( P_c = 7.37 \text{ MPa} \) (Matson and Smith, 1989), without cutting the liquid–gas coexistence line of CO₂. Once above this critical point, the pressure was very slowly released to evacuate supercritical CO₂ as a gas, while avoiding any condensation by adiabatic expansion. Finally, only a dry aerogel remained in the autoclave.

The porosity of the samples was characterized by the Brunauer, Emmett and Teller (BET) method (Gregg and Sing, 1982) after desorption at 200°C, on part of the samples used further on for catalysis. The catalytic activity was tested on the remaining part of the samples in the esterification of lauric acid by 1-octanol, which produces octyl laurate according to the following reaction used by Reetz et al. (1996a):

\[
\text{CH}_3\left(CH_2\right)_7\text{COOH} + \text{CH}_3\left(CH_2\right)_7\text{OH} \rightarrow \text{CH}_3\left(CH_2\right)_7\text{CH}_2\text{COOC}_\text{H}_{17} + \text{H}_2\text{O}
\]

For this purpose, a known mass fraction of a gel sample containing the corresponding mass fraction of the total enzyme was ground to a powder and added to 10 mL of a water saturated isooctane in a 25 mL flask, containing 100 mg lauric acid (0.5 mmol) and 158 \( \mu \text{L} \) 1-octanol (1 mmol). Isooctane was selected as the solvent to perform the esterification reaction in conditions similar to Reetz et al. (1996a). The saturation of this solvent with
water insured a constant water concentration so as not to artificially displace the chemical equilibrium reaction (1) and interfere in a complex manner with the catalytic activity of the encapsulated lipase. The catalytic reaction was carried out at 30°C with agitation on a magnetic stirrer at 180 rpm. At intervals, 50 µL aliquots were taken and analyzed by gas chromatography. The catalytic activity of each sample was compared with the homogeneous activity of 2 mg fresh enzymes, in a polypropylene reactor.

RESULTS

All dry gels of the second series (XR and AR gels) were white and did not shrink during drying. Xerogels were non-porous while aerogels had a small total pore volume due to micropores (Table II). On the other hand, for the first series, XP xerogels and DP aerogels remained transparent and shrank a lot during drying. As for AP aerogels submitted to acetone exchange before CO2 supercritical drying, they shrank only moderately and remained partly translucent in the dry state. They had a type IV isotherm in the BDDT (Brunauer, L. Deming, W. Deming and Teller) or IUPAC classification (Brunauer et al., 1940; Gregg and Sing, 1982) as shown in Fig. 2, with a mesopore average radius rather well centered in a range from 8 to 10 nm (Fig. 3). The previous figures also show that XP xerogels were microporous with type I isotherms and DP aerogels had an isotherm intermediate between types I and IV, comprising two hysteresis loops corresponding to two types of pores: mesopores contributing about 30% of the pore volume and micropores. The mesopore radius was rather narrowly centered around ≈ 3 nm. In AP aerogel samples, it also appeared that PVA and aqueous NH3 had a minor effect on the adsorption isotherms which remained of type IV

<table>
<thead>
<tr>
<th>Samples of the second series*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP (-NH4) Aerogel</td>
</tr>
<tr>
<td>≈ 0.5</td>
</tr>
<tr>
<td>≈ 0.0015</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Samples of the second series*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR Xerogel</td>
</tr>
<tr>
<td>≈ 1</td>
</tr>
<tr>
<td>≈ 0.0023</td>
</tr>
</tbody>
</table>
FIGURE 2 Nitrogen adsorption isotherms according to the BET method of gel samples made with the same wet chemistry and dried by different techniques: (AP) aerogel dried by the CO₂ supercritical method after liquid exchange for acetone; (DP) aerogel dried by the supercritical method without acetone exchange; (XP) xerogel dried by evaporation at room temperature.
Gels pore size distributions

FIGURE 3 Pore size distributions derived from the isotherms in Fig. 2.

(Fig. 4), although the pore radius decreased from the range 8–10 to 6–8 nm without PVA and was more scattered without aqueous NH₃ (Fig. 5). The effects of PVA and aqueous NH₃ were minor, on the specific surface area $A_{sp}$ and specific pore volume $V_{sp}$ gathered in Table II.
Aerogels BET adsorption isotherms

**FIGURE 4** Nitrogen adsorption isotherms according to the BET method of aerogel samples made with a different wet chemistry and dried after liquid exchange for acetone: (AP) aerogel made with aqueous NH$_3$ and PVA; (AP–PVA) aerogel made without PVA; (AP–NH$_4$) aerogel made without aqueous NH$_3$; (AR) Reetz type aerogel.
The esterification kinetics of gels from the first series made with aqueous NH$_3$ and PVA, depending on their drying procedure, are reported in Fig. 6, with the estimated mass of enzyme which was close to 1.5 mg. This figure shows that supercritical drying after intermediate liquid exchange for acetone...
INFLUENCE OF DRYING TECHNIQUE

Gel activity and drying procedure

FIGURE 6 Progress of the fraction of lauric acid transformed to octyl laurate as a function of time for gel samples made with aqueous NH₃ and PVA and dried by a different technique: (AP) aerogel submitted to liquid exchanged for acetone before supercritical drying; (DP) aerogel directly placed in the autoclave without liquid exchange before supercritical drying; (XP) xerogel. The mass of enzyme in the samples are indicated. The data for XR xerogel and the initial kinetic rate for a similar mass of free enzyme (F) are also reported.

dramatically improved the catalytic activity of the free enzymes and a similar improvement was found for gels of the second series. The esterification kinetics of all “A type” aerogels are reported in Fig. 7, which shows that all such aerogels behaved at first view similarly, except for those made without PVA. The absolute esterification rate reported in Table III appears to be low because they were calculated per mg of total enzyme content. However, lipase solubilization experiments, followed by elimination of the suspended part by centrifugation and measurement of the lipase concentration in solution with the commercial test “BCA-200 Protein Assay Kit®” (Pierce), showed that only about \( \approx 10\% \) of the total enzyme content was in solution. Hence, absolute reaction rates per mg of enzyme in solution would be \( \approx 10 \) times higher and of the same order of magnitude as those reported by Reetz et al. (1996a) for the best samples. Relative activity data in the esterification reaction, defined as the initial rate of esterification, divided by the initial
FIGURE 7 Progress of the ratio of lauric acid transformed to octyl laurate as a function of time, for different aerogel samples dried after liquid exchange for acetone: (AP) aerogel made with aqueous NH$_3$ and PVA; (AP-PVA) aerogel made without PVA; and (AP-NH$_3$) aerogel made without aqueous NH$_3$. The mass of enzyme in the samples are indicated. The initial kinetic rate for a similar mass of free enzyme (F) is also reported.

TABLE III Reaction rates of encapsulated enzymes and relative reaction rates by comparison with homogeneous catalysis

<table>
<thead>
<tr>
<th>Material</th>
<th>Mass of Lipase in esterification reaction (mg)</th>
<th>Esterification rate $\nu$ (µmol/h/mg)</th>
<th>Relative esterification rate $\nu/\nu$ (F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F (Free enzyme)</td>
<td>≈ 2</td>
<td>≈ 1.3*</td>
<td>1</td>
</tr>
<tr>
<td>AP</td>
<td>≈ 1.5</td>
<td>≈ 10.7</td>
<td>≈ 8.4</td>
</tr>
<tr>
<td>DP</td>
<td>≈ 1.2</td>
<td>≈ 2.8</td>
<td>≈ 2.2</td>
</tr>
<tr>
<td>XP</td>
<td>≈ 1.6</td>
<td>≈ 0.14</td>
<td>≈ 0.1</td>
</tr>
<tr>
<td>AP (−NH$_3$)</td>
<td>≈ 1.1</td>
<td>≈ 12.5</td>
<td>≈ 9.8</td>
</tr>
<tr>
<td>AP (−PVA)</td>
<td>≈ 1.5</td>
<td>≈ 3.5</td>
<td>≈ 2.8</td>
</tr>
<tr>
<td>AR</td>
<td>≈ 1.5</td>
<td>≈ 11.5</td>
<td>≈ 9</td>
</tr>
<tr>
<td>XR</td>
<td>≈ 1.5</td>
<td>≈ 5.4</td>
<td>≈ 4.2</td>
</tr>
</tbody>
</table>

*Free enzyme: average of several experiments.

The rate of esterification in free enzyme catalysis, are gathered in Table III. This table shows that all “A” type aerogels dried after acetone intermediate exchange, were more active than homogeneous catalysis, with the DP aerogel having an intermediate behavior between the AP aerogel and free
enzymes. On the other hand, within the xerogels, the poor performance of XP xerogel and the good performance of XR xerogel, by comparison with free enzyme catalysis, were confirmed. This traditional XR xerogel has a performance intermediate between the “A” type aerogels and the DP one.

DISCUSSION

As illustrated in Fig. 7, this study confirmed that lipases encapsulated in hydrophobic gels can be substantially more active than when free. This behavior makes sense, as a hydrophobic gel is expected to expel the water produced by esterification reaction (1), hence to displace the equilibrium in favor of ester production, inside the gel medium where the enzyme was localized. This study also confirmed the importance of PVA to enhance the activity of enzymes encapsulated in sol–gels, possibly by an equilibrium displacement effect similar to hydrophobicity. However, as a new result, it showed that the gel drying procedure had an effect of equivalent importance and cumulative to PVA.

The pore volume was much higher in the AP type aerogels than in the XP type xerogels. However, it was considerably lower in the AR type aerogels than in the AP ones, which behaved roughly similarly, so that the enzyme activity cannot be directly related to the pore volume in agreement with previous conclusions by Reetz et al. This is also consistent with the fact that AP aerogels without PVA had a similar pore volume but a significantly lower activity than equivalent AP aerogels with PVA. A similar comment can also be made regarding the importance of the specific surface area.

The usual interpretation is therefore to consider that a higher catalytic activity must be explained in terms of enzyme conformation, that is to say on intricate microporous texture and structure characteristics of the enzymes. It is possible that this conformation can be improved by appropriate hydrophobic groups grafted on the silica network and organic additives, but as mentioned before, such groups and additives can also simply displace the equilibrium reaction. On the other hand, this study showed that the activity can also be improved by using supercritical drying instead of drying by evaporation, for an identical gelation chemistry and hydrophobic groups, which can only be explained by changing the conformation of enzymes.

Obviously, in the P type samples as in the R ones, supercritical drying systematically enhanced the catalytic activity, reflecting the submission of the enzymes to much lower drying stresses than by evaporation. These lower
drying stresses resulted in a considerable attenuation in sample shrinkage during drying, hence a much higher pore volume in an aerogel than in a xerogel for a given chemistry. However, the pore volume and the enzyme conformation were not directly related to each other. Rather, they both appeared to be parallel consequences of the lower drying stresses during supercritical drying.

Indeed, this view can also be extended to a comparison between the XR and the XP xerogel, for instance. While the XP xerogel shrank considerably during evaporation, this was not the case for the white XR ones, which virtually did not shrink. This absence of shrinkage is the common point between all “A” type aerogels and the XR xerogel. In the latter samples, the absence of shrinkage was due to the base catalyst NaF used for silica precursor condensation, which is known to produce rather dense hydrated globular silica particles, in which the enzymes were possibly helped in resisting the capillary stresses by being somewhat adsorbed on the surface of the particles. The fact that when comparing AR aerogels with XR xerogels, supercritical drying drastically increased the enzyme activity while there was virtually no change in the pore texture of the material, appears to support this view.

This study has, therefore, shown that the activity of lipases encapsulated in sol–gels, for esterification reactions, can largely be improved by minimizing the capillary stresses to which they are submitted during drying. This can be achieved either by performing supercritical drying instead of drying by evaporation, or by gelation of the silica network with a base catalyst such as NaF which considerably reduces the porosity. It is proposed that decreasing the drying stress had two parallel effects of a similar nature. First, it increased the pore volume and specific surface area of the silica gel network for a given wet chemistry protocol. Secondly it also improved the enzyme conformation by limiting contraction.

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**References**


