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Influence of the porous texture of silica gels on the enzymatic activity of lipases in esterification reactions

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Abstract

Pseudomonas Cepacia lipases were encapsulated in hybrid silica-polyvinyl alcohol gels, which were dried either supercritically in order to form aerogels or by evaporation so as to obtain xerogels. In each case, the catalytic activity of the encapsulated enzymes was studied and compared to free enzyme biocatalysis. This study demonstrates that the activity of the enzyme is increased when the procedure used allows it to resist capillary stresses occurring during the drying of the gel. That is, esterification rates are higher when the gels are synthesized with a base catalyst, such as NaF, in the presence of polyvinyl alcohol and then dried supercritically.

Keywords: *Pseudomonas Cepacia*; Silica gels; Esterification reactions

1. Introduction

Recent developments in biocatalysis have increased the activity of enzymes by encapsulating them inside sol–gel materials. Reetz et al. [1] have, for instance, increased the activity of certain lipases by immobilizing them inside hybrid silica-organic gels. The increase was even more important when a hydrophobic group, such as methyl or propyl, is used in the gel precursor, and when an organic component, such as polyvinyl alcohol, is added to the structure.

In this work, we analyze the effect that the pore texture of the gel has, on the activity of a specific lipase; *Pseudomonas Cepacia*, by modifying the technique used to dry the gels synthesized by various hydrolysis and condensation procedures. Two techniques were studied: drying by evaporation which leads to the formation of xerogels, and supercritical drying in CO_2 which produces aerogels.

2. Experimental procedure

The reactants used in this study are polyvinyl alcohol (PVA) with an average molar mass of $M =$ 15,000 (termed PVA in this paper), methanol (R.P. Normapur-Prolabo), ammonia (minimum 28% Prolabo), hydrochloric acid (minimum 36% Prolabo), sodium fluoride technical grade (Prolabo), methyltrimethoxysilane (MTMS, 98% — Aldrich) and tetramethoxysilane (TMOS, 98% — Aldrich). The enzyme studied is *P. Cepacia* (P.C.) and comes from Fluka.

This is a carboxyesterase which belongs to the class of hydrolases and the subclass of lipases; it contains divalent metal cations (Mg^{2+} or Ca^{2+}) in its active center. A 0.01 M phosphate buffer solution

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of pH 6.18 used to suspend the enzyme was also made by mixing aqueous solutions of sodium dihydrogen phosphate dihydrate and disodium hydrogen phosphate dodecahydrate. Both sodium phosphates used were of microselect grade and came from Fluka.

Fresh enzyme suspensions were prepared by dispersing 3 mg/ml of P.C. either in the buffer solution discussed above or in deionized water. Suspensions were used instead of solutions because solubilization experiments in excess water followed by the elimination of the unsolubilized part by centrifugation showed that only a small fraction of enzyme ($\approx 10\%$ by mass) can be solubilized. The remaining $\approx 90\%$ could not be solubilized even by further dilution. Use of suspensions with higher contents of enzyme could therefore be better compared to free enzyme catalysis. It also permitted to see by ^{29}Si NMR possible interactions between the silica gel network and the enzyme. It is important to note that for a given fresh lot of enzyme used in a relatively short span of time (a few days), the catalysis data are quite reproducible. The samples in which the enzymes were found to have the highest catalytic activity were reproduced using solutions instead of suspensions. In these cases, the concentrations of the enzymes were determined by measuring the protein concentration using the commercial test "BCA-200 Protein Assay Kit®" from Pierce. Solutions of about ≈ 0.9 mg/cm^3 were made by centrifuging a given suspension.

In the first set of samples, hydrophobic silica aerogels were prepared according to Schwertfeger et al. [2], in a procedure that consisted in dissolving MTMS and TMOS in methanol, in quantity such that the molar ratio of Si provided by MTMS, to the total Si provided by MTMS + TMOS, was $r_M \approx 0.2$. The molar ratio of methanol to total Si was $r_{meth} \approx 2$. Samples AMV and XMV, both containing PVA and P.C., were obtained by hydrolysis of the previous silica solution with a 4% (by mass) PVA aqueous solution and with a buffered enzyme suspension. The enzymatic solution provided $\approx 56\%$ by mass of the total water used in hydrolysis. Samples containing enzymes but not PVA, termed AM and XM, were also made by replacing the PVA solution used in hydrolysis by an equivalent volume of buffer solution without enzyme. In all cases, the total molar hydrolysis ratio of water to Si was $r_w \approx 6.8$. The enzyme suspension was always added to the silica solution just prior to gelation. Pure silica samples similar to the ones mentioned above but without enzymes and respectively termed as AsM, XsMV, AsM and XsM were also prepared. In these samples, the enzyme suspension was replaced by an equivalent volume of buffer.

The aim of this study is to assess the effect that PVA and the enzymes have on the porous structure and texture of the gels, and also, to primarily study the effect that different drying procedures have on the catalytic activity of P.C. The xerogels termed

Table 1
Specific surface area, A_{sp}, and specific pore volume, V_{sp}, of samples as derived from their adsorption isotherms

Samples	A_{sp} (m^2/g)	% contribution of mesopores to A_{sp}	V_{sp} (cm^3/g)	% contribution of mesopores to V_{sp}
with enzymes				
AMV	712	69	1.5	90
XMV	543	3.7	0.32	9.5
AM	788	69	1.6	87
XM	491	2.9	0.28	9.5
ARV[a]	108	0	0.06	0
XRV[a]	1	0	0	0
without enzymes				
AsMV	877	62	1.7	86
XsMV	495	0.6	0.28	8.8
AsM	932	73	1.34	85
XsM	488	1.2	0.26	3.6

[a] Data given for indication.

XMV and XM when they contain enzymes, or XsMV and XsM when they do not, were dried by evaporation at room temperature. The aerogels AMV and AM with enzyme, and AsMV and AsM without, were dried supercritically in CO_2 in the following manner. First, water is replaced by acetone through dialysis, then, the acetone is exchanged for liquid CO_2 according to a diffusion process taking place in an autoclave.

A sample similar to the aerogel AMV, which, as explained further, gives one of the highest reaction rate, was made using an enzyme solution instead of the suspension. This sample is called ZAMV. The equipment used for this supercritical drying technique was a "Supercritical Point Drier" of Polaron®, an autoclave able to stand pressures and temperatures above the critical point CO_2.

In order to compare the results of this study with other published ones, another set of gels was prepared from only MTMS by a technique described by Reetz et al. [1]. This set comprised aerogels ARV and AsRV, respectively, with and without P.C. and the corresponding xerogels XRV and XsRV. In samples ARV and XRV, the total molar ratio of hydrolysis water to Si was $r_w \approx 7.4$. 12.5% by mass of this water came from a 1 M NaF solution, 25% from a 4% by mass PVA solution, and 50% from the enzyme suspension. The remaining was deionized water. In the corresponding samples AsRV and XsRV, which do not contain any enzyme, the enzyme suspension was replaced by deionized water. The sodium fluoride used was of technical grade from Prolabo. To avoid any spontaneous heating, which could be harmful to the enzymes, these samples were kept in

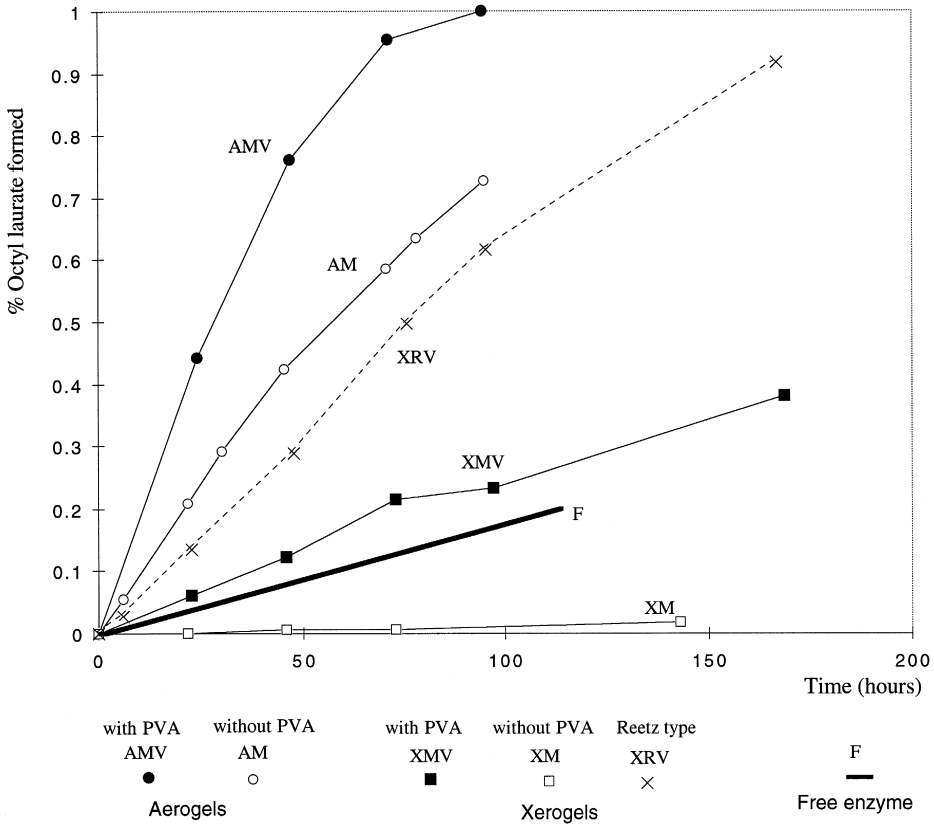


Fig. 1. Esterification kinetics for the transformation of 1-octanol to octyl laurate of the AMV and AM aerogels and XMV and XM xerogels prepared in this study. The kinetics of a xerogel sample prepared according to Reetz (XRV) as well as the initial rate for 0.7-mg free enzyme catalysis (F) are also indicated. The AM, AMV and XMV samples contained ≈ 0.6 mg, while the XM and XRV samples contained ≈ 0.7 mg.

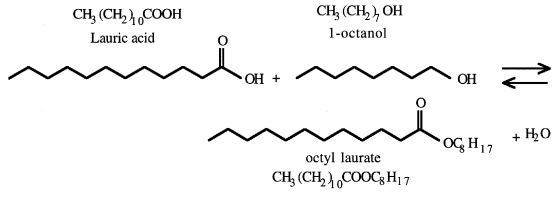
Table 2
Reaction rates of encapsulated enzyme and reaction rates relative to free enzyme catalysis

Material	Esterification rate ν (μmol h^{-1} mg^{-1})	Relative esterification rate ν/ν (free enzymes)	
	Total enzymes	Total enzymes	Enzymes' soluble part
Free enzyme	1.3	1	
Aerogels			
Enzyme suspension			
AMV	15.2	11.7	117[a]
AM	8	6.2	62[a]
ARV	16.1	12.4	124[a]
Enzyme solution			
ZAMV	350	269	269
ZARV	240	185	185
Xerogels			
XMV	2.4	1.9	19[a]
XM	0.09	0.07	0.7[a]
XRV	4.7	3.6	36[a]

[a] Estimated if 10% of the enzymes of the suspension are actually solubilized.

an ice bath during gelation. Here again, a sample similar to aerogel ARV, which as shown later, has one of the highest catalytic activity, was made using an enzyme solution instead of the suspension.

The pore texture of each sample was characterized by the Brunauer Emmett and Teller (BET) method after desorption at 150°C. The network structures were analyzed by ^{29}Si solid-state NMR spectroscopy on a Bruker DSX-400 spectrometer at 400 MHz. The samples were spun in the magic angle at ca. 10 kHz using a pulse technique with a pulse interval time of 5 s. A free induction decay of 4096 was taken for ^{29}Si. The catalytic activity of the P.C. enzyme was tested on the esterification of 1-octanol with Lauric acid which produces octyl laurate according to the following reaction also used by Reetz et al. [1]:



(1)

For this purpose, a gel sample of about 300 mg was grounded to powder and added to 9.84 ml of a water saturated isooctane in a 25-ml flask containing 0.5 mmol of lauric acid and 1.0 mmol of 1-octanol. In all samples, grinding produced powder with the same average size, that is to say ≈ 15 μm. The enzyme content of the gel sample was estimated from the enzyme suspension volume proportion used for the hydrolysis of silica. The catalytic reaction was carried out at 30°C with magnetic stirring at 180 rpm. At determined times, 0.15-ml aliquots were taken from the reaction mixture and analyzed by gas chromatography. The sample that produced the highest catalytic activities were remade to test the synthesis reproducibility; the effect of their repeated used was also studied.

3. Results

During gelation, the XM xerogels remained transparent while the XR xerogels and AR aerogels became white. The AM aerogels dried supercritically with CO_2 became partly transparent. These visual characteristics were the first indication that these samples had very different porous textures. Another one was the shrinkage occurring during drying. XM

xerogels shrunk a lot during drying while AM aerogels almost did not change in volume. There was also practically no shrinkage in the white XR xerogels and AR aerogels made according to Reetz.

More detailed data on the texture of the samples after catalysis, derived from nitrogen adsorption isotherms, are gathered in Table 1. The specific surface area A_{sp} and specific volume V_{sp} were higher in the aerogels than in the xerogels synthesized by the same procedure (e.g. AM aerogel vs. XM xerogel). There is no consistent effect of PVA nor of the enzymes on V_{sp}, that it be of xerogels or of aerogels. However, a systematic decrease of the order of 100 m^2/g in A_{sp}, due to the presence of enzymes, occurs in AM and AMV aerogels when compared respectively to AsM and AsMV samples.

Regarding the pore size distribution, AMV aerogels had a type IV nitrogen adsorption isotherm in the BDDT classification, which corresponds to a mesoporous structure with a mean radius of 8 nm. This pore size distribution is not significantly affected by PVA nor by the enzyme. Moreover, it practically does not change even after subsequent catalysis experiments and washing and drying the aerogels by evaporation. The nitrogen adsorption isotherms of XMV xerogels were of type I in the BDDT classification, which is due to a major contribution of micropores [3]. The pore size was therefore very different in XMV xerogels and AMV aerogels. The ARV aerogels had a specific surface area determined by the Horvath and Kawazoe method [4], $A_{sp} \approx 100$ m^2/g due to pores that are very difficult to access such as hollow spheres with a narrow opening. The XR xerogels were virtually unporous.

The kinetics of the esterification reaction (1) are reported in Fig. 1 for M-type gels made with compa-

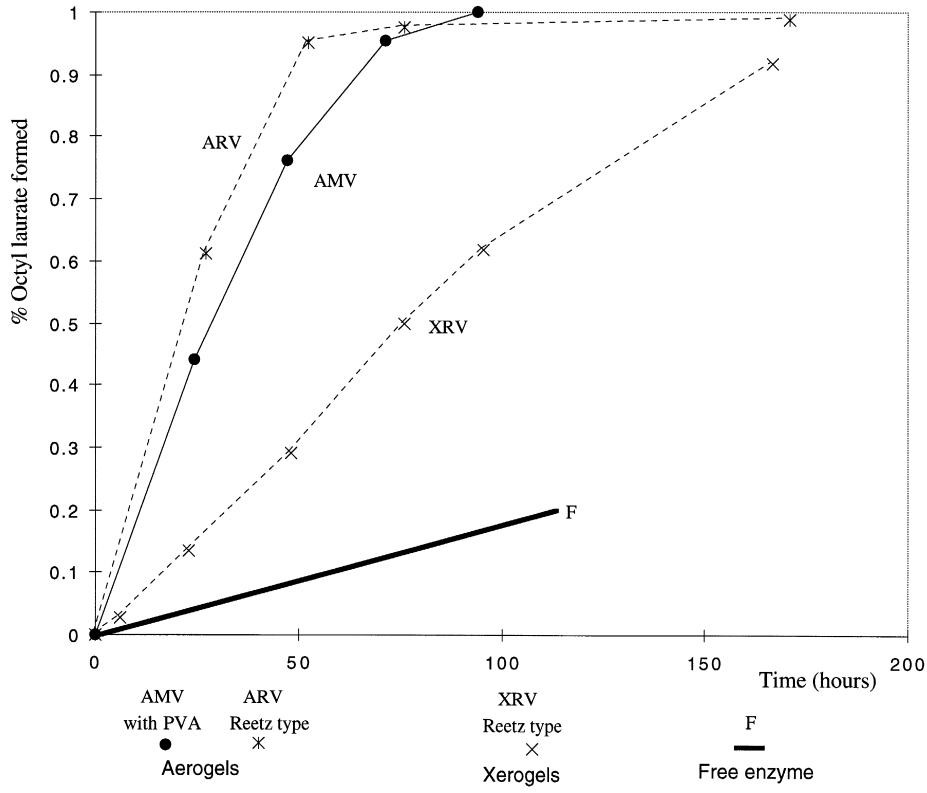
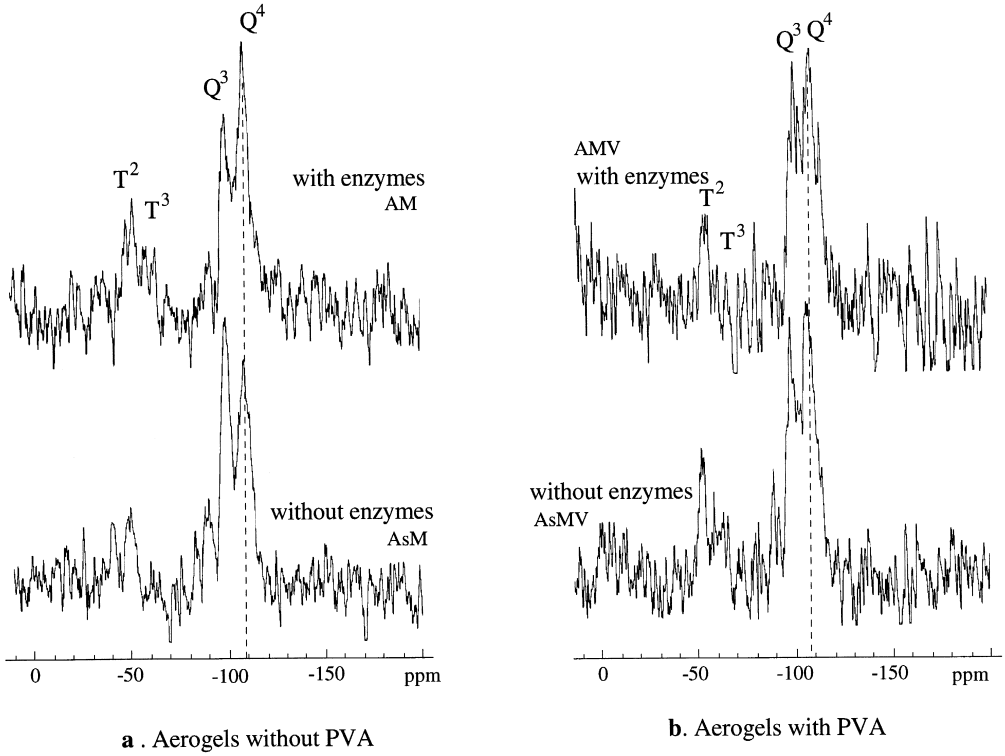


Fig. 2. Esterification kinetics for the transformation of 1-octanol to octyl laurate of samples made according to Reetz and dried to a xerogel (XRV) or to an aerogel (ARV). Both samples contained ≈ 0.7 mg of enzymes. The average initial rate for a 0.7 mg free enzyme catalysis (F), as well as the kinetics for an AMV aerogel containing 0.6 mg of enzymes, are also indicated.

rable enzyme contents of ≈ 0.6–0.7 mg. The absolute activities were determined from the initial slopes and the relative activities were derived by dividing the absolute activities by the absolute activity of free enzymes at the same concentration. The corresponding results are reported in Table 2. These data were found to be reproducible in the case of encapsulated enzyme, while they were much more scattered for free enzyme catalysis. The data obtained shows that, for the same wet chemical procedure and enzyme content, if evaporating the gel to a xerogel has a negligible effect on the enzyme activity, drying it supercritically to form an aerogel can multiply the reaction rate by a factor of up to 12. This same trend is observed for Reetz-type xerogels XRV and aerogels ARV as it is illustrated in Table 2 and Fig. 2. It is always the aerogel that gives better kinetics. The positive effect of PVA first reported by Reetz et al. [1] is also confirmed in this study. This positive effect is, however, similar in xerogels and aerogels. The effects of supercritical drying and PVA on the catalytic activity of the enzyme are apparently additive.

In the comparison of the R-type and M-type gels, the Reetz-type XRV xerogels with PVA performed similarly to the AM aerogels without PVA, while the XMV xerogels with PVA behaved similarly to free enzymes. The XM xerogels (that is, without PVA) were the only samples which performed much less efficiently than free enzymes.

In Table 2, the esterification rates of aerogels made with enzyme suspensions are also compared to those made with solutions. Reaction rates per milligram of enzymes show that samples obtained from the enzyme solutions were more active than when they were made from enzyme suspensions. But if one considers that in the suspension, there are about 10% of the enzymes that are solubilized and that the reactivity of non-solubilized enzymes are negligible in front of solubilized ones, the data obtained are



a . Aerogels without PVA

b. Aerogels with PVA

Fig. 3. ^{29}Si NMR spectra of M-type aerogels with and without enzymes: (a) without PVA; (b) with PVA.

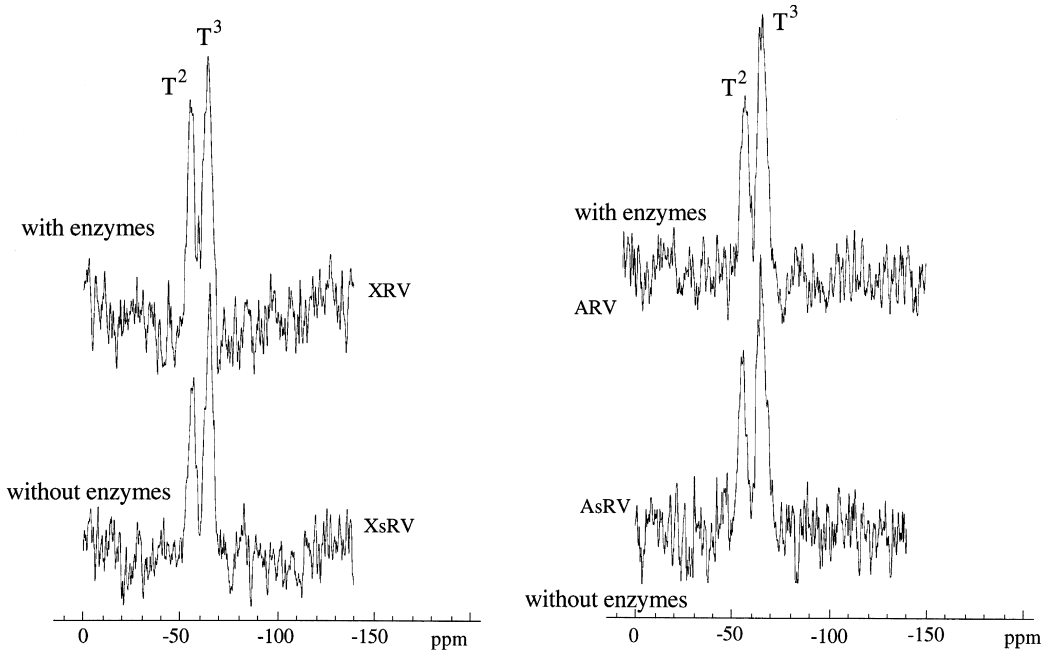


Fig. 4. ^{29}Si NMR spectra of R-type gels with and without enzymes: (a) xerogels; (b) aerogels.

consistent. The catalytic activity of AMV aerogels decreased by a factor of two when it was used for the second time. This seems to indicate that part of the most dispersed enzymes (the enzymes that were not well-encapsulated) was washed away when the sample was washed. This loss of activity was not so important when the sample was washed and used for a third time. Still, this must certainly be improved for industrial applications.

The ^{29}Si NMR spectra of AM aerogels and R-type gels are respectively reported in Figs. 3 and 4. The XM xerogels spectra are similar to those of AM aerogels showed in Fig. 3a and they show a systematic increase of the Q^4 Si sites, as compared to the Q^3 ones, when the enzymes are present in the gels. This finding is true whether PVA was used or not, and it therefore suggests that the lipase and silica gel network interacts with each other. However, the NMR spectra of AMV aerogels were not modified by the presence of lipase. As for the R-type samples (Fig. 4), the NMR spectra did not show Q^4 and Q^3 sites, but only T^3 and T^2 ones. Here again, some interaction between the enzyme and the silica network was observed, as the presence of enzymes somewhat decreased the relative magnitude of the T^3 sites by comparison with the T^2 ones.

4. Discussion

This study first confirmed important results already described by Reetz et al. [1]. The first one is that the presence of hydrophobic groups in a gel is important to enhance the catalytic activity of lipase in esterification reactions. However, this does not require special hydrophobic interactions between the enzyme and the gel network, as the simple presence of hydrophobic sites on the gel is sufficient to expel water from its network. The esterification equilibrium (1) is therefore displaced towards an accelerated formation of ester. This study demonstrated that using 20% of MTMS as silica precursor is sufficient to provide a good encapsulating silica gel. This is

consistent with the data from Schwertfeger et al. [2], which showed that such a proportion of MTMS makes the silica gels fully hydrophobic.

Another result that was confirmed is that an organic additive, such as PVA, enhances the activity of enzymes encapsulated in silica gels. The last one is that there is no direct relationship between the enzyme activity and the gel's pore texture, as characterized by either its specific surface area A_{sp} or its specific pore volume V_{sp}. The XRV xerogels, for instance, were not porous but very active, while the XMV xerogels, were very porous and inactive.

The most important result demonstrated by this study is that the procedure used to dry the gel has an effect as important as using PVA on the catalytic activity of P.C. The effects of these two factors (use of PVA and supercritical drying) were actually cumulative. They can possibly be interpreted by the intricate structure and microporous texture of the enzymes. An enzyme can have a much higher catalytic activity when it has a specific conformation. It can therefore be interpreted that supercritical drying systematically enhances the catalytic activity, because such a drying submits both the gel and the enzyme to much lower drying stresses than does drying by evaporation. This result is true for both the AMV and ARV aerogels, respectively, compared to the XMV and XRV xerogels. Lower drying stresses also considerably attenuates the shrinkage of the sample during drying. Hence, the lower stresses of the supercritical drying allows, for a given chemical procedure, the pore's volume and enzymes conformation to be modified in the same manner.

This same interpretation explains the difference between gels made by different protocols but dried by the same procedure, such as the XMV and XRV xerogels. While the XMV xerogels considerably shrunk during evaporation, the white XRV ones almost did not shrink. This difference is explained by the use of NaF as a Lewis basic catalyst for silica precursor condensation in the XRV samples. Such a gelation catalyst is known to produce a silica material with a coarse colloidal texture [5], which is well illustrated by the white color of these samples; in comparison, the XMV xerogels were fully transparent. The coarser XRV gels had stronger solid branches, which were able to withstand without shrinking the capillary drying stresses. These capillary stresses were themselves smaller than those in the XMV gels because the residual pores were coarser. The enzymes could therefore place themselves in a configuration where they could resist these capillary stresses, thereby preventing any deterioration of their conformation.

This study has also demonstrated through ^{29}Si NMR and specific surface area data the presence of interactions between the enzymes and the silica network. It appears difficult to understand the mechanism of this interaction and how it affects the enzymes' activity. But these data are consistent with each other, as a more condensed silica network with a higher Q^4 peak intensity is expected to result in a lower specific surface area. A weak nucleophilic–electrophilic linkage between the enzyme proteins and some sites of the silica network is often considered to explain these interactions. It could also be these interactions which help the enzymes resist capillary drying stresses in a different manner depending on the gel type.

5. Conclusions

P. Cepacia lipases encapsulated in hybrid silica-PVA aerogels show a higher catalytic activity than when they are encapsulated in xerogels. This increase is apparently not due to a particular pore texture as obtained from a given procedure of gel synthesis, but results from a reduction in the capillary stresses during drying. Furthermore, according to ^{29}Si NMR and specific surface area data, the enzymes not only interact with the silica network, but this interaction depends on the procedure used to synthesize the gels.

Acknowledgements

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References

[1] M.T. Reetz, A. Zonta, J. Simpelkamp, Biotechnol. Bioeng. 49 (1996) 527.
[2] F. Schwertfeger, N. Hüsing, U. Schubert, J. Sol–Gel Sci. Technol. 2 (1994) 103.
[3] S.J. Gregg, K.S.W. Sing, Adsorption, Surface Area and Porosity, 2nd edn., Academic Press, London, 1982.
[4] G. Horvath, K. Kawazoe, J. Chem. Eng. Jpn. 16 (6) (1983) 470.
[5] A. Pierre, Introduction to Sol–Gel Processing, Kluwer Academic Publishing, Boston, 1998.