Available online at www.sciencedirect.com
ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 44 (2007) 117–127
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Immobilization of a lipoxygenase in silica gels for application in aqueous media

Ali Karout [a], Claude Chopard [b], Alain C. Pierre [a,*]

[a] *Institut de Recherches sur la Catalyse, CNRS-UPR 5401, Université Claude Bernard-Lyon I, 2 Avenue Albert Einstein, 69622 Villeurbanne Cedex, France*
[b] *Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, UMR 8601, Université Paris V-René Descartes, 45 rue des Saints Pères, 75270 Paris Cedex 06, France*

Received 29 June 2006; received in revised form 31 August 2006; accepted 25 September 2006
Available online 27 October 2006

Abstract

The encapsulation of soybean lipoxygenase-1 (LOX-1) in silica gels and its application in an aqueous medium, were studied. The main silica precursor was tetramethoxysilane (TMOS) but the introduction of hydrophobic $SiCH_3$ groups brought with methyltrimethoxysilane (MTMS) was evaluated. Other sol–gel synthesis parameters investigated comprised partial or complete drying by evaporation and CO_2 supercritical drying. The influence on LOX-1 activity of the various chemicals with which the enzyme was in contact during encapsulation (acetone, methanol, polyvinyl alcohol), as well as the temperature and pH, were examined. The activity of free and encapsulated LOX-1 was assayed on the oxygenation reaction of linoleic acid by dioxygen from air dissolved in aqueous medium, in a UV–vis spectrophotometer. With free LOX-1, the reaction advancement could be followed in continuous in the spectrophotometer. With the gels, in a first approach, the conversion was simply determined after 15 min reaction after filtration of the liquid, to discriminate between active and inactive gels. For the most interesting gels, the kinetics were then assessed by continuous recording in the UV spectrophotometer, after placing a small piece of gel (≈15 mg) directly in the cell. The best gels had an activity ≈30% of free LOX. The present studies, supplemented by characterization of the gels texture and structure, respectively by nitrogen adsorption and ^{29}Si MAS NMR, showed that drying a gel before use in aqueous media was detrimental to the activity. This effect is due to a contraction of the gel network which occurs when a dry aerogel sample is dipped in water after drying. Hence gels containing LOX-1 enzyme must not be dried but kept in water impregnated state, for optimum use.

Keywords: Lipoxygenase; Silica; Gel; Immobilization

1. Introduction

Lipoxygenases (LOX) (E.C.1.13.11.12) are dioxygenases which belong to the enzyme class of oxydoreductases [1]. They are able to catalyze the stereospecific incorporation of molecular oxygen in unsaturated fatty acids which comprise a *cis*,*cis*-1,4-pentadiene unit, to give hydroperoxides with a conjugated *cis*,*trans* diene [2,3]. Multiple isoforms of LOX-1 have been detected in a wide range of plants fungi and animals.

Originally, mammalian cells were thought to contain three types of lipoxygenases (5-, 12- and 15-lipoxygenase) corresponding to their predominant regiospecificities toward arachidonic acid. More recent investigations have lead to discriminate, independent of the variation of species, at least six types of mammalian lipoxygenases: the 5-lipoxygenases, the platelet-type 12-lipoxygenases, the leukocyte-type 12-lipoxygenases, the epidermal-type 12-lipoxygenases in mice, the reticulocyte-type 15-lipoxygenases and the human 15-lipoxygenase of type2 [4]. The hydroperoxides these enzymes produce from arachidonic acid or others polyunsaturated fatty acids or derivatives, operate as regulatory mediators and/or precursors of other bioactive compounds such as leukotrienes, lipoxines and hepoxilins. In humans, they have an important role in physiological reactions, such as the immunologic response and the inflammatory process, in cancer and atherogenesis [5].

In higher plants, LOX enzymes have been known for over 70 years [6,7]. Their predominant substrates are α-linolenic and linoleic acids[1] and they are usually classified according to the

* Corresponding author.
E-mail address: apierre@catalyse.cnrs.fr (A.C. Pierre).

[1] Arachidonic acid is rarely found in higher plants.

1381-1177/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2006.09.008

specific carbon atom of linoleic acid which they predominantly oxidize: the 9- and 13-LOX enzymes mainly oxidize the carbon atoms #9 and 13, respectively. The success of research on mammalian lipoxygenases has also stimulated research on the biological functions of lipoxygenases in higher plants. At that time the involvement of lipoxygenases in the biosynthesis of jasmonic acid and traumatin considered as vegetal hormones and in the biosynthesis of volatile compounds such as hexanal, hexenal and hexenol was established [8]. Many available data suggest that plant oxylipins[2] are potent bioregulators playing important role in signalling cascades, plant growth and development, senescence, organogenesis, maintenance of homeostasis [9]. However, their physiological importance is far from being completely understood at the present time. The functions of volatile organic compounds (VOCs) in plant–insect interactions [10,11] are now investigated with molecular perspectives.

Some of these oxylipins present an industrial interest: they are used in agriculture as fungicides [12–15]. The reduced hydroperoxides can replace the ricinoleic acid in industrial applications as lubricants, greases, thickening agents and dry oils [16]. Some hydroperoxides are also interesting as precursors of flavour components such as ketones, aldehydes or alcohols. Two of the latter compounds are of more particular interest: the *cis*-3-hexenol and the *trans*-2-hexenal, respectively, known as the leaves alcohol and aldehyde [17]. Hence the interest in the regio- and enantioselective production of fatty acid hydroperoxides and their derivatives is expanding.

Soybean seed is the best known natural source of lipoxygenases and, even if six isoenzymes are mentioned in the literature, termed LOX-1 to LOX-6 [17], the most widely studied is LOX-1. Data for the other isoenzymes are less abundant, perhaps because LOX-2 and -3 are more difficult to purify and LOX-4, -5 and -6 appear in the seed only during germination [18]. The pH of maximum activity of LOX-1 is in the range 9–9.5, by comparison with 6–6.5 for LOX-2 and -3; this is of some help to discriminate their activities. Moreover, LOX-1 predominantly produces 13-hydroperoxide, although the ratio of 9-hydroperoxide increases as the pH decreases, by comparison with the lower specificity of LOX-2 and -3. LOX-1 is generally considered as a good structural model for the lipoxygenase family.

LOX-1 consists of a single chain of 839 amino acids giving a mass of 94,262 g mol^{-1} according to Boyington [19]. When crystallized, it forms a prolate ellipsoid of dimension 9 nm × 6.5 nm × 6 nm comprised of two distinct domains. The smaller N-termination one is a polypeptidic chain of 146 aminoacids, arranged in a largely hydrophobic tertiary structure with a drum shape. The larger C-termination domain is a chain of 639 aminoacids which contains the enzyme active site [19]. This active site includes a non-hemic Fe atom in an octahedral coordination sphere composed by the side chains of three histidines (His^{499}, His^{504} and His^{690}), Asn^{694}, the terminal carboxylate residue of Ile^{839} which binds as monodentate ligand and one water molecule [20]. Iron is essential for the enzymatic activity, and shuttles between Fe^{2+} and Fe^{3+} oxidation states in the catalytic cycle [21].

A good immobilisation may possibly combine the achievement of a high substrate conversion, an easy product separation, and a moderate loss of enzyme and enzyme activity. Nevertheless, an efficient LOX-1 immobilization technique must take into account the amphiphilic nature of this enzyme. Efficient supports must have an appropriate hydrophobic/hydrophilic balance [22]. Given the importance of LOX-1 applications, a number of immobilisation methods and supports have been studied, in particular by adsorption on glass wool [23], porous glass beads [24], glutenin [25], covalent grafting on agarose [26,27], several types of commercial organic polymers [1,15,28,29] and magnesium silicate (talc) [30]. In the latter case, immobilisation was controlled by ionic interactions between the negatively charge support silanols and the positively charged proteins. The gel types studied as immobilisation media comprise polyacrylamide gels [3], hybrid silica–alginate gels [31] and silica gels loaded with montmorillonite [16,32] to which it is possible to add gel derived glass beads with a controlled pore size [24].

As a general fact, enzyme desorption easily occurs in polar solvents such as water when this enzyme is adsorbed on a support [12]. Covalent grafting of the enzyme on a support makes it possible to improve the enzyme stability during storage, at the cost of reducing its activity [27]. Besides, covalent grafting usually requires a relatively complex and costly multi-step process. An improvement in the immobilised activity has been achieved by ionic immobilization of the LOX-1 from other sources than soybean [30], but not with the soybean LOX-1 [33]. At last, encapsulation, or entrapment, in the network of a gel is an interesting technique with relatively big molecules such as enzymes, which does not require bonding between the enzyme and the encapsulation medium. Nevertheless, the diffusion of products and/or substrates inside the gel may slow down the kinetics of a reaction, when the heterogeneous catalysis is further used in water. With LOX-1, encapsulation in silica–alginate beads resulted in a lower activity, although the storage stability at room temperature was improved [32]. The addition of montmorillonite in silica gel made it possible to both reinforce a gel and introduce a macroporosity which facilitated the diffusion of products [22]. This also resulted in an improved storage stability and reusability according to the authors. Similarly, the use of porous glass beads resulted in an improved mechanical stability [24].

The objective of the present work was to study the immobilization of soybean LOX-1 in silica gels, for use in aqueous media. Silica gels, including xerogels dried by evaporation and aerogels dried by the CO_2 supercritical method, have made it possible to significantly increase the activity of lipases in organic solvents, by comparison with free lipases [34–36]. This activity improvement was mainly due to the fact that the enzyme was maintained in a dispersed state by the gel network, when applied in an organic solvent, while it agglomerated when used without any immobilization. On the other hand, for lipases used in aqueous media, sol–gel immobilization usually resulted in an activity at best similar to that of the free enzyme and only with a few

[2] The term "oxylipins" designates all the products of fatty acids oxidation irrespective of their chain length and other structural peculiarities: hydroperoxy and hydroxy derivatives, aldehydes, diols, divinyl ethers, ketols, double dioxygenation products, etc.; they comprise jasmonoids and some are also volatile organic compounds (VOCs).

lipases [37]. Immobilization of enzymes other than lipases, for use in an aqueous medium, therefore appears very challenging.

2. Experimental

2.1. Enzymes and chemicals

The reactants used in this study were methyltrimethoxysilane (MTMS, 98%, Aldrich) and tetramethoxysilane (TMOS, 98%, Aldrich), methanol (R.P. Normapur-Prolabo), polyvinyl alcohol (PVA) with an average molar mass of $M = 15{,}000$ (Fluka), deionized water, diethyl ether 99.8% (Carlo Erba), hexane 99% (SDS), NH_4OH 28% (Prolabo), linoleic acid 99% (Acros), hydrochloric acid 37% (Prolabo). Tris–HCl buffer (pH 9; pH 8) were prepared by mixing HCl (Prolabo) with tris (hydroxymethyl)aminomethane (Fluka). Phosphate buffer (pH 6; pH 7) was prepared by mixing sodium dihydrogen phosphate dehydrate (Fluka) with di-sodium hydrogen phosphate dodecahydrate (Fluka). A phosphate buffer (pH 5) was prepared by mixing potassium dihydrogen phosphate (Fluka) with disodium hydrogen phosphate (Fluka). A carbonate buffer (pH 10.6) was prepared by mixing anhydrous sodium carbonate (Fluka) with sodium hydrogen carbonate (Riedel-de-Haën).

The purification protocol of the enzyme from soybean seeds was mainly based on the procedure of Slappendel [38]. It involved fractionation by ammonium sulfate precipitation, followed by chromatography with CM C50 Trisacryl (IBF) then with DEAE Trisacryl (IBF). All steps were made at 4 °C and diethylenetriaminepentaacetic acid, a heavy metal chelator (1 mM), was added in all buffers. The active fractions were pooled, then lyophilized and the enzyme was stored at −20 °C under argon. This purification procedure gave ≈0.8 mg of LOX-1 for 1 g of soybean seeds. The enzyme purity was checked by SDS-PAGE. RP-HPLC analysis on an AX 300 column (Brown Lee) showed that this enzyme was free from other isoenzymes contrary to commercial LOX. The purity of this enzyme preparation, by comparison with commercial LOX, must be outlined. The present aqueous LOX solutions had a specific activity of ≈200 U/mg (1 U = 1 μmol 13-HPOD formed by minute) just after preparation, of the order of 30–40 U/mg in the stored state where they were used. Typically, tests on commercial LOX showed a specific activity of the order of a few U/mg, with the same definition of 1 U.

For catalytic tests, the enzyme concentration in an aqueous solution was determined by measuring the optical density (OD) in a UV–vis spectrophotometer Lambda 35 of Perkin-Elmer, at the wavelength of 280 nm. A solution of LOX-1 (1 mg ml^{-1}) in a quartz cell with an optical pathlength $l = 1$ cm, was known to have an OD of 1.6 ± 5% [39] at 280 nm. Using this data, the LOX-1 concentration C of any enzyme solution could be derived by measuring its OD. Considering that the molar mass M_r of LOX-1 is 96,500, which corresponds to a molar absorbtivity $\varepsilon_{280} = 154{,}400\ M^{-1}\ cm^{-1}$, the mole number of lipoxygenase could also be derived from this OD by applying the Beer–Lambert's law.

The above UV–vis spectrophotometer was also used to follow the biocatalytic reaction described further on, in situ. For this purpose, the sample and a reference were placed in 3.5 ml quartz cells from Hellma, equipped with a micro-magnetic agitation system. The spectrophotometer was also equipped with an optical fibre (external fibre diameter 6.5 mm, length 15 cm), but it could only be used in liquid without any abrasive component, hence not in the presence of silica gel.

Linoleic acid was purified by filtration under argon on a Sep-Pak solid extraction silica column (Waters) according to Mulliez [40]. An aqueous linoleic acid solution (as ammonium linoleate) was finally obtained. This solution was stored in 1–2 ml microtubes at −20 °C. Its concentration (≈10 mM) was determined from the concentration of HPOD obtained after its transformation in the presence of LOX-1.

2.2. Gel encapsulation technique

In a preliminary investigation, the technique used in previous studies to encapsulate a lipase from *Burkholderia cepacia* [35] was directly applied. According to this protocol, the two silica precursors MTMS and TMOS were dissolved in 5 mmol methanol in a first vial. Several proportions of MTMS were used, namely 0%, 20% and 40%, for a total Si precursor content of 2.5 mmol. In a second vial, 10 mmol of water containing 4% (by mass) polyvinyl alcohol (PVA) was mixed with 15 mmol of water to which 10 μl of an aqueous solution containing ≈0.8 mg LOX-1 per millilitre was added. The content of the two vials were then mixed under magnetic agitation until gelation occurred, generally after ≈2 h. However, this protocol did not produce any active gel.

In a successful adaptation, which defines our so-called "reference" protocol further on, methanol was no longer used as a solvent. Rather, an acidic pre-hydrolysis was applied to a mixture of TMOS and MTMS in molar proportions 80% and 20% respectively. In details, a series of gels were for instance prepared by mixing 11.82 mmol, TMOS, 3.02 mmol, MTMS, 144.4 mmol distilled water and 19.16 mmol. H_2O acidified at pH 2.8 with HCl, in the flask of a rotating evaporator. After 15 min magnetic agitation, the methanol produced by hydrolysis of TMOS and MTMS was evaporated at 30 °C during ≈4 min, before mixing with the enzymatic solution. The mass loss (≈1.7 mg), essentially methanol, was compensated by addition of ≈60 μl distilled water, first to recover more fluidity in the silica sol before casting for gelation, secondly to maintain a sensibly constant number of Si atoms per volume of silica sol ($n_{Si} \approx 3$ mmol ml^{-1} in final sol sample just before gelation).

In practice, a total volume of ≈2.5 ml aqueous silica sol without methanol was obtained. Five hundred microlitres aliquots of this sol were then distributed in a series of vials. A 500 μl of a pH 9 Tris–HCl buffer, plus 10 μl of the LOX-1 solution were added each vial. Agitation during 20 s on a vortex mixer induced gelation in 30–40 s. The gels obtained were moreover aged for another 15 h in a pH 9 Tris–HCl buffer and finally stored at +4 °C. The wet gels obtained are termed "reference gels" further on. Overall, a typical reference gel sample contained ≈8 μg LOX-1 enzyme according to the OD, in a silica gel made from 14.84 mmol Si atoms equivalent to ≈100 mg dry SiO_2. This was consistent with the typical mass of such a wet gel (≈1 g), con-

sidering the water volume contained in its pore was ≈90% of the sample volume.

In the present study, other gels were also made by modifying one parameter at a time, such as for instance applying a dialysis step in acetone, a drying stage by evaporation, CO_2 supercritical drying, adding some polyvinyl alcohol (PVA) or modifying the molar proportion of MTMS and TMOS. The parameters modified are described altogether with the results in the following sections.

2.3. Enzyme activity assay

The catalytic activity of free or immobilized LOX-1 preparations was evaluated from the formation of the product hydroxoperoxyoctadienoate (HPOD) as a result of the oxygenation of linoleic acid by the O_2 from air dissolved in aqueous medium. Two positional isomers of HPOD exist. However, both have the same effect on the OD, as the absorption is due to absorbance by the conjugated double bond at a wavelength of 234 nm [7].

This reaction was run in Tris–HCl buffer solution (50 mM pH 9), at room temperature. For this purpose, 3 ml of the buffer was placed in the cell of the UV–vis spectrophotometer, to which 25 μl of the substrate linoleic acid was added. The reaction was always tested by addition of either 10 μl of a LOX-1 solution, or a small piece of gel in which the immobilized LOX was provided by addition of 10 μl LOX-1 solution before gelation.

Formation of the product HPOD was characterized by the appearance of an absorption band centred at 234 nm. In each case, the measured optical density (OD) was corrected by subtracting the OD of the equivalent liquid without the substrate. The mole number of product formed could be derived from the OD at 234 nm by applying the Beer–Lambert's law, in which $\varepsilon = 25{,}000\ M^{-1}\ cm^{-1}$ is the product molar absorbtivity at 234 nm [7].

For free LOX-1, reliable data could always be obtained by in situ continuous recording of the OD as a function of time. In this case the activity v_0 was determined as the initial formation rate of the product v_0, derived from the initial slope d(OD)/dt by applying Beer's law. It was expressed in units (U), with $1\ U = 1\ \mu mol\ min^{-1}$ of HPOD product formed. The specific activity $v_{0,sp}$ in $U\ mg^{-1}$ could then be calculated by dividing v_0 by the mass of LOX-1 added in the UV cell.

In an aqueous homogeneous medium, without any gel, it appeared that the LOX-1 catalyzed reaction was complete in a time <5 min. A continuous recording could also be achieved with a gel. The most practical technique in this case proved to consist in adding a very small piece of wet gel (≈15 mg) taken from the same wet gel (typical 100 mg sample containing 8 μg LOX), directly in the UV cell, as done for instance by Chikere et al. [1]. Nevertheless, it appeared that the reaction was at best much slower. As an example, the variations of the OD as a function of time with 2 μl free LOX and ≈15 mg of a reference wet gel, are shown in Fig. 1.

With gel encapsulated LOX, an adaptation of the UV technique could be considered. For instance, the activity of larger monoliths could be measured in an external reactor, with the help of an optical fibre. Nevertheless, preliminary experiments showed that the gel needed to be separated in a basket inside the reactor, to not deteriorate the fibre tip by abrasion. The substrate concentration itself needed to be adapted, because the optical fibre was found to considerably attenuate the signal magnitude. Oxygen consumption could also be measured with a Clarkson type oxygen electrode. However, again, a large cell with the gel monolith separated from the electrode membrane would need to be designed. Otherwise, preliminary experiments showed that the gel tended to stick to the electrode membrane and attenuate the oxygen consumption signal. In any case, the present aim was to determine the main characteristics of active gels with encapsulated LOX and the technique presently applied showed to be quite sufficient for this purpose.

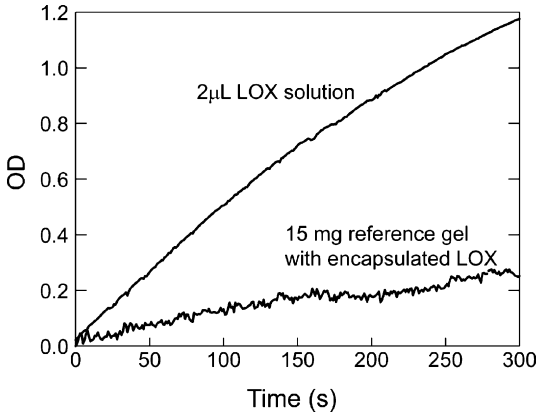

Fig. 1. Examples of the variation of the OD as a function of time, with 2 μl free LOX and ≈15 mg of a reference wet gel, by continuous recording in the UV–vis cell.

Indeed, in a first step, it appeared more interesting to determine a preliminary "figure of merit" of each type of gel. For this purpose, with each type of gel, a reaction was performed in a separate flask and maintained under magnetic agitation. The liquid medium was separated by filtration from the gel after 15 min reaction, that is to say a time much longer than that necessary to attain a constant final conversion with the free Lox, before analysis of its absorption in the UV cell. This did not make possible to derive a true catalytic transformation rate, but at least to discriminate between the samples which showed a significant activity, and those which did not do so. The catalytic activity of only the best gels was then analyzed by continuous UV recording.

2.4. Gels characterization

The structure of un-dried gels was characterized by direct MAS ^{29}Si NMR (no proton coupling). The spectra were recorded on a 400 MHz BRUKER DSX-400 spectrometer equipped with a 4 mm cell. The spinning frequency was 8 kHz, the pulse time: 7 μs, the repeat time: 40 s, and the number of acquisition scans: 1024. All chemical shifts were referenced to TMS ($Si(CH_3)_4$). The spectra were fitted to Lorentz/Gauss profiles using WINFIT.

Pore texture analysis required first to dry some samples, in particular by the CO_2 supercritical method, so as to examine this pore texture in a state as close as possible to that prevailing in the wet gels used in catalysis. Each nitrogen adsorption isotherm

was recorded after desorbing a dry sample under vacuum during 6 h at 200 °C, on a custom made equipment. The pore size distribution was determined from the desorption branch of the isotherms down to the Kelvin limit, using the Roberts' method [41].

3. Results and discussion

3.1. Free enzyme kinetics parameters

The initial product (HPOD) formation rate was found to be a linear function of the mass of enzyme in the range of 0–10 μg (Fig. 2). The latter enzyme mass was calculated from the volume of LOX-1 solution added in the reaction UV cell, and the OD of this solution previously determined as explained before. In this linear range, the specific activity of a newly prepared LOX-1 solution was found to be $v_{0,sp} = 37 \pm 8\ \mathrm{U\ mg_{(enzyme)}^{-1}}$. This corresponds to a turnover number (TON), in terms of number of HPOD molecules formed per unit active centre, of ≈60 $\mathrm{s^{-1}}$. The Michaelis parameters known as V_{max} and K_m were determined with 8 μg LOX-1 solutions, from experimental data points providing the initial product formation rate v_0, as a function of linoleic acid concentration. These data points are reported in Fig. 3. A statistical best fit of these experimental points with a simple Henri–Michaelis–Menten law provided specific constant

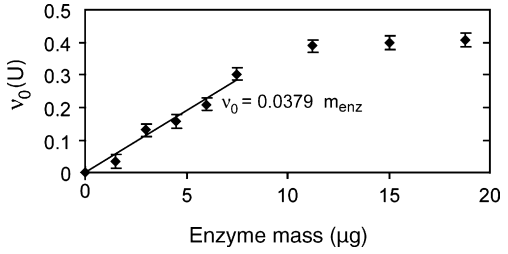

Fig. 2. Initial product formation rate, in units (1 U = 1 μmol $\mathrm{min^{-1}}$ HPOD produced), as a function of the mass of free LOX-1 in the solution.

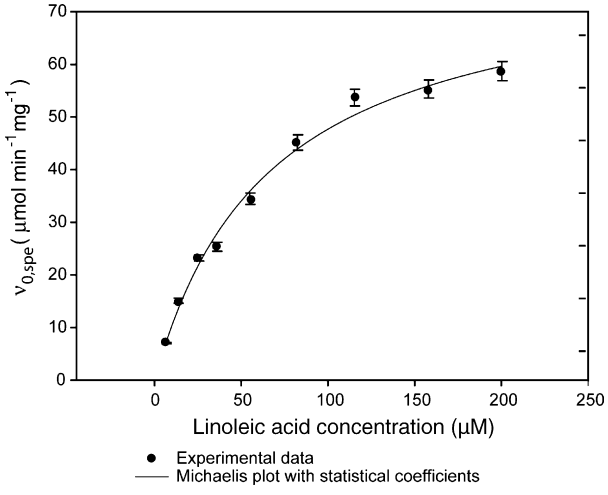

Fig. 3. Experimental activity data points, and curve obtained by plotting a theoretical Michaelis function with the statistical V_{max} and K_m values determined from the experimental points.

per mg of LOX-1: $V_{max} = 80 \pm 4\ \mathrm{U\ mg^{-1}}$ and $K_m = 67 \pm 8$ μM ($R^2 = 0.990$). The theoretical Michaelis plot derived when using these statistical data is reported on the same graph.

3.2. UV–vis spectra of solutions and gels. Effect of methanol, ethanol and acetone additives

Analysis of the product formed with gel encapsulated LOX was performed at the end of each reaction, in practice after 15 min reaction, when the OD reached a constant and similar value in each experiment. In this way, no reactant addition was needed to stop the reaction. When the filtrate was taken with a syringe without any filtration, in order to perform it rapidly, the presence of gel, either with encapsulated LOX-1 or without it, modified the filtrate UV spectrum. A general shift of the OD baseline by ≈0.5 could be observed, independent of the presence or absence of free enzyme in the solution. Nevertheless, the OD maximum at 234 nm had the same value after correction for this baseline shift for: (1) free LOX-1 without any gel in solution and (2) free LOX-1 in solution to which gel without LOX-1 was added. The enzyme concentration itself in the reaction medium was impossible to determine by UV absorption, because the LOX-1 absorption band at 280 nm was very weak by comparison to the product.

The above OD baseline shift was found to be due to the presence of a residual quantity of gel in the liquid analyzed. It was no longer observed when the gel liquid was first filtered on a filter paper from MACHEREY-NAGEL at the end of the reaction, an operation which took a few seconds more.

Besides, it appeared that other factors than the gel could significantly modify the UV–vis spectra: in particular acetone. For instance, spectra were recorded with the filtrates obtained after 15 min in a reaction medium, with: (1) a reference gel; (2) a reference gel dialyzed for 5 h in acetone before being again equilibrated at pH 9 before the catalytic test; (3) a reference gel dried by the CO_2 supercritical method. The latter drying technique required dialysis in acetone, as an intermediate exchange medium between water and liquid CO_2. The supercritically dried reference aerogel (3) showed no observable band at 234 nm for the product HPOD. This suggested that no substrate transformation had occurred. On the other hand a new significant absorption band centred at ≈264 nm could be observed. This band was also present in the reference gel (2) dialyzed in acetone (but not supercritically dried) before use. Nevertheless it was absent with the reference gel, which had not been in contact acetone. This band at 264 nm was therefore due to the acetone adsorbed in the supercritically dried aerogel as well as in the acetone dialyzed wet gel. Indeed, it was checked that adding a small quantity of acetone in the range from 0 to 100 μl, to 3 ml of free LOX-1 solution in a pH 9 buffer, did not attenuate the enzyme activity. Simply, the OD at 234 nm increased by a magnitude which was proportional to the volume of added acetone (Fig. 4). This increase in OD appeared to be independent of the catalytic reaction advancement. A linear regression provided the estimate:

$$\Delta(\mathrm{OD}) = 0.0165 v_{\mathrm{acetone}}\ (\mu\mathrm{L}) \quad (1)$$

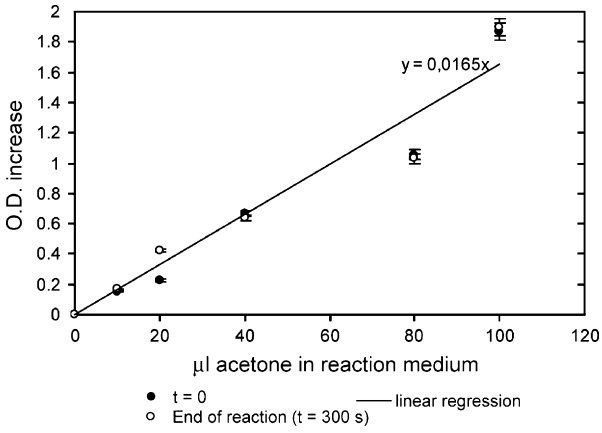

Fig. 4. Increase of the OD at 234 nm at the beginning and the end ($t = 300$ s) of the reaction, with free LOX-1 (homogenous conditions) as a function of the volume of added acetone.

In spite of the above shift, the initial slope d(OD)/dt was not modified for addition of a small volume of acetone, which led to measure the free LOX-1 activity by the initial d(OD)/dt slope. The latter one was determined by linear regression from the experimental OD data between 0 and 20 s. Fig. 5 shows that a small volume of ethanol or methanol, as well as acetone, did not alter the free LOX-1 activity. On the other hand, a higher concentration of these liquids progressively annihilated this activity. Fig. 5 also shows that addition of an increasing volume of 4% polyvinyl alcohol (PVA) aqueous solution did not modify the free LOX-1 activity.

3.3. Influence of the gel processing on the encapsulated LOX-1 activity

As mentioned in the experimental section, the preliminary encapsulation method in methanol as a solvent did not produce any active gel. On the contrary, the reference gel obtained by the reference protocol (methanol pre-evaporated before adding the LOX-1 solution) and directly used in catalysis without any drying stage was active. An average final conversion of $\approx 86.3 \pm 4\%$ was achieved after 15 min. The initial specific activity determined with a very small mass of gel ($m_{\text{wet gel}} \approx 15$ mg) placed in the UV cell, by continuously recording the OD as a function of time, provided a relative immobilized specific activity

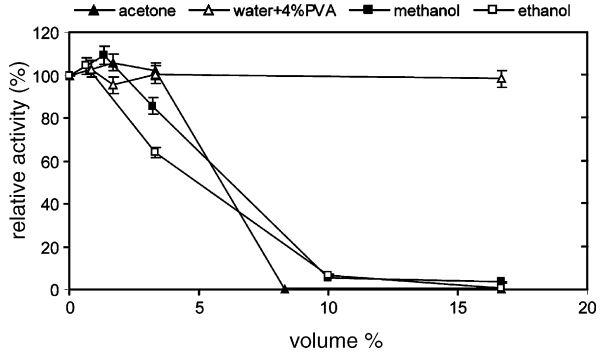

Fig. 5. Effect of acetone, methanol, ethanol and a 4% aqueous PVA solution, on the initial activity of free LOX-1.

Table 1
Conversion after 15 min reaction with a reference gel submitted to a progressive drying by evaporation

Evaporation time (day)	Conversion after 15 min (%)
0	83.6 ± 4
1	78 ± 3.8
2	24 ± 1.5
3	0

$v_{0,\text{sp}}$ (gel)/$v_{0,\text{sp}}$ (free LOX-1) $\approx 22\%$. On the other hand, a similar reference gel dialyzed for 5 h in acetone without any further drying before use in catalysis, did not show any conversion at all. Similarly, a reference aerogel (i.e., reference gel dried by the CO_2 supercritical method) as well as a reference xerogel (i.e., a reference gel dried by evaporation) did not show any activity at all.

3.4. Influence of partial drying a reference gel by evaporation

As the previous results showed that a reference gel was active, while the reference xerogel was inactive, partial drying by evaporation was attempted. The conversions measured after 15 min reaction in a filtrate taken from the reaction medium, are reported in Table 1. This table clearly shows that the gel activity decreased as the evaporation time increased, as a consequence of an increasing gel shrinkage. After 3 days evaporation, a inactive xerogel was obtained. This suggests that the lack of activity of a xerogel encapsulated LOX-1 could be due to a lack of substrate or product diffusion inside the gel network, or to a progressive enzyme inactivation due to compression by the gel network. As a consequence, all other results presented in the next sections address the use of gels derived from the reference gel by modification of one parameter, but always used without any prior drying.

3.5. Effect of modifying the MTMS proportion in a gel

Results on the conversion achieved after 15 min reaction with wet gels, modified from the reference gel by adding an increasing proportion of MTMS, are reported in Table 2. The results are expressed as % of the conversion achieved with free LOX-1. In all cases, this conversion was slightly lower than with free LOX-1, possibly because of product adsorption in the silica gel. Moreover, a very small quantity of substrate was used, which may have induced relatively large measurements dispersions.

Table 2
Conversion after 15 min reaction with powders of wet gel (gel not dried before use) synthesized with an increasing proportion of MTMS

% MTMS	Conversion after 15 min (%)
0	92.5 ± 4
20 (reference gel)	86.3 ± 4
40	50 ± 2.4
60	8.7 ± 0.5
80	6 ± 0.3

Nevertheless, it appeared that the gel activity decreased as the proportion of MTMS increased. The latter result could be explained by a decreasing gel porosity, which made diffusion of the substrate and product inside the gel more difficult. Inactivation of LOX-1 may also occur during the sol–gel process.

When some TMOS is replaced by MTMS, it was known that TMOS hydrolyzes and condenses first to silica. MTMS condenses more slowly and finally coats the silica network derived from TMOS [42,43]. The latter phenomenon was well described in a detailed texture study of similar gels (solvent molar ratios $H_2O/Si > 10$ and methanol/Si < 1), dried by the CO_2 supercritical drying technique. This study reported that the specific surface area remained relatively unaffected up to 40% MTMS, while it rapidly decreased for a higher MTMS proportion. Actually, it was impossible to analyze the texture by nitrogen adsorption beyond 60% MTMS because there was virtually no nitrogen adsorption. The specific pore volume also decreased rapidly as the MTMS content increased, while the pore size shifted from the mesoporous to the microporous range [43]. These data were moreover consistent with other data previously reported by Reetz et al. on similar xerogels dried by evaporation [44]. Actually, ^{29}Si NMR data showed that an increasing proportion of MTMS was poorly hydrolyzed and condensed. Especially beyond 60% MTMS, the corresponding "dried" gel samples kept a viscous touch: they did not really dry. This gel did not shrink when trying to evaporate the solvent. They were full of poorly condensed MTMS.

The situation could be very different with other types of silica aerogels, for instance synthesized with high methanol/Si and low H_2O/Si molar ratios and dried by a high temperature (>260 °C) supercritical method in methanol [42]. Nevertheless, the latter sol–gel technique does not appear interesting to apply to enzyme encapsulation. First because a low H_2O/Si molar ratio would imply a low enzyme loading, since the enzyme is in solution in water. Moreover the enzymes would unlikely withstand such a high temperature treatment, especially in methanol.

An increased hydrophobicity due to MTMS may also decrease the diffusion coefficient of hydrophilic substrates and products inside the gel. Indeed, Table 2 shows that the gel initially chosen as a reference (made from 20% MTMS) achieved a lower HPOD conversion after 15 min reaction, than the more hydrophilic gel made only from TMOS. For these two types of gel, the activity was also determined by continuously recording the OD in the UV cell, after adding ≈15 mg gel. Table 3 shows that the gel made from only TMOS was ≈50% more active than the reference gel. This table also shows that pre-evaporation of methanol before adding the LOX-1 significantly improved the activity in both type of gels. The best initial specific activity of encapsulated LOX-1 was ≈30% of the free LOX-1 activity. Such a relative activity may seem low, but it is comparable or superior the best ones previously reported, in particular 29% by adsorption on Eupergit C250L/EDA [45], 30% on silica–alginate [31], 1.8% on Dowex50WX4-200 applied in ternary micellar systems [28]. In other cases where LOX was immobilized by covalent grafting, the relative activity was not reported because of uncertainty in the data [12,15,46].

Table 3
Relative initial activity of gel encapsulated LOX-1, compared to free LOX-1, by direct continuous measurement in the UV spectrophotometer (enzyme load in the gel: 8 μg LOX-1 for 100 mg SiO_2)

Gel type	Relative initial specific activity $v_{0,sp}$ (gel)/$v_{0,sp}$ (free) (%)
Free enzyme ($v_{0,sp}$ (free) = 36.6 U mg^{-1})	100 ± 3
No methanol evaporation	
Reference gel (80% TMOS; 20% MTMS)	12 ± 0.5
Gel from 100% TMOS	25 ± 1
Methanol evaporation	
Reference gel (80% TMOS; 20% MTMS)	22 ± 1
Gel from 100% TMOS	30 ± 1.5

The LOX-1 powder loading in the gels was ≈10 μg for ≈100 mg SiO_2. Nevertheless, despite this low enzyme loading as explained in introduction, a total activity comparable or even better than other previously mentioned results, corresponding to a much higher LOX loading, were obtained. The present results are due to the high purity of the LOX preparation used, as detailed in the section on chemicals and enzymes.

Such performance is not yet sufficient to apply the present gel immobilized LOX industrially. An economical use could be considered if extensive repeated use of this catalyst with limited loss of activity could be demonstrated. Moreover, the relative activity by comparison with free LOX should be increased to 60% at least. Considering that a large range of silica gels with different porous textures is feasible, the pore size distribution could possibly be improved. For this purpose, it should combine large pore channels in order to facilitate the diffusion of substrates, linking smaller mesoporous domains where the enzyme would be entrapped. Hence, efforts in this direction must be maintained, but it requires more involved sol–gel work.

3.6. Effect of gel particle size

A wet reference gel can be used as a powder or as small monoliths. The diffusion of the substrate and product may also be expected to take a longer time when the monolith size increases. For reference gels, Table 4 provides a comparison of the HPOD conversions which were achieved with a cylindrical monolith (diameter ≈12 mm, height ≈6 mm), small cylinders (diameter ≈4 mm, height ≈5–6 mm) and a similar wet gel reduced to a powder, for a same total gel mass. Obviously, the

Table 4
Activity of LOX-1 encapsulated in reference gels of different particles size, for a same total mass of gel

Reference gel shape	Conversion after 15 min (%)
Powder	83.6 ± 4
Small monoliths	38 ± 1.9
Single monolith	33 ± 1.6
Small monoliths without LOX-1 + free LOX-1	100 ± 4

conversion after 15 min reaction decreased as the size of the gel particles increased from powder to small cylinders, then to a single larger monolith. These "figures of merit", seem to indicate that the reaction may be rate limited by diffusion of the substrate (a C_{18} compound) inside the monolith, so that it is not complete with monoliths after 15 min. Secondly, some LOX-1 may be leached out from the wet gel for the sample which was crushed to a powder. In this case, the high conversion measured with the powder could be due to free LOX-1 in solution.

3.7. Loss of enzyme

In order to determine whether some enzyme was leached out of a reference gel reduced to powder during a test, a series of wet gels containing an increasing mass of immobilized LOX-1, from 10 to 2500 μg, were prepared. Each sample was reduced to powder. Then, it was maintained under magnetic agitation during 15 min in a buffer solution without any substrate. Hence no reaction could occur, although enzyme leaching could proceed. After 15 min, each filtrate was tested in continuous analysis directly in the UV cell, by adding 25 μl substrate to the cell solution. In the full range of enzyme loading, the filtrate activity measured corresponded to ≈1.1 ± 0.3 % of the encapsulated LOX-1. Hence, these experiments prove that only ≈1.1% of the encapsulated LOX was leached out after 15 min, for the full range of enzyme loading tested.

3.8. Aging studies

The relative specific LOX-1 activity $v_{0,sp}/v_{0,sp(unaged)}$, calculated from the initial OD curves, after aging at 4 °C or at room temperature and pH 9, is shown in Fig. 6. It appeared that the free LOX-1 activity continuously decreased with time. For LOX-1 encapsulated in reference gels, Fig. 6 illustrates that the conversion achieved after 15 min reaction decreased only moderately with an increasing aging time at 4 °C. Yet, it is not possible to conclude that gel encapsulation improved the storage protection at 4 °C, because of the different nature of the two types of results. On the other hand at room temperature, the decrease in activity was much more important for the free LOX-1 than for the gel encapsulated one. Hence, some improvement in the protection at room temperature was achieved by gel encapsulation.

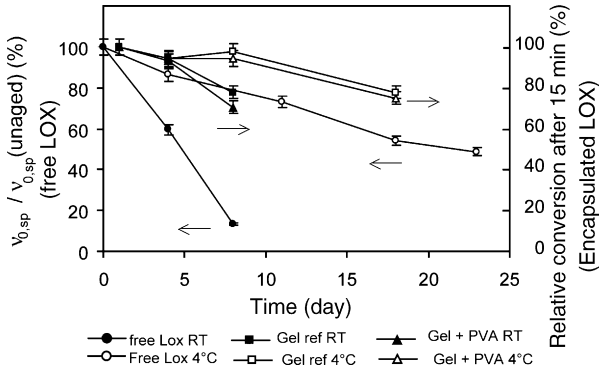

Fig. 6. Evolution as a function of aging time at 4 °C and room temperature of free LOX-1 activity and the conversion to HPOD after 15 min reaction with a reference gel. LOX-1 was immobilized in a gel made with 80 μl of a 4% PVA solution, or without any PVA (reference gel). Aging pH was ≈9.

3.9. Effect of the temperature on the activity

The activity of free LOX-1 was measured by maintaining the reaction medium in a thermal bath at controlled temperature. In this case, the OD was measured with an optical fibre connected to the spectrophotometer. This activity went through a maximum at a temperature $T_{max} \approx 40$ °C. Below this temperature, an Arrhenius plot (Fig. 7a) showed that the enzyme activity increased with temperature with an activation energy $\Delta G_a \approx 37$ kJ mol^{-1}. Above this temperature, the enzyme deactivated with a deactivation free energy $\Delta G_d \approx 25$ kJ mol^{-1}.

Reference gels were also applied in reaction media maintained at a constant temperature in a range from 5 to 60 °C. Fig. 7b shows that the conversions achieved after 15 min reaction also passed through a slight maximum near 40 °C. Of course, the latter conversions only represent "figure of merit" of the gels, not the true initial reaction rate. Hence they could not be used to determine true activation and deactivation free energies. But the decrease in conversion was very moderate from 40 to 60 °C. Hence, it seems that the gel somewhat helped to stabilize the LOX encapsulated activity.

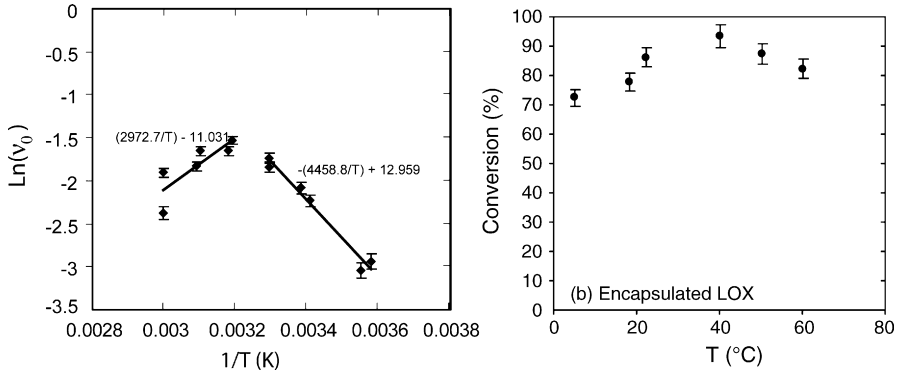

Fig. 7. (a) Arrhenius plot of the variation of the free LOX-1 initial activity v_0, as a function of the temperature; (b) conversion achieved after 15 min reaction with a reference gel, as a function of the temperature.

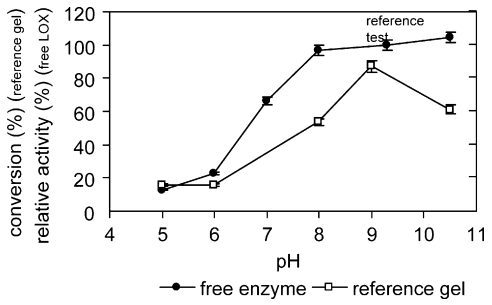

Fig. 8. Conversion after 15 min reaction achieved with reference gels, and relative activity of free LOX-1 (reference pH 9.3) as a function of the reaction medium pH.

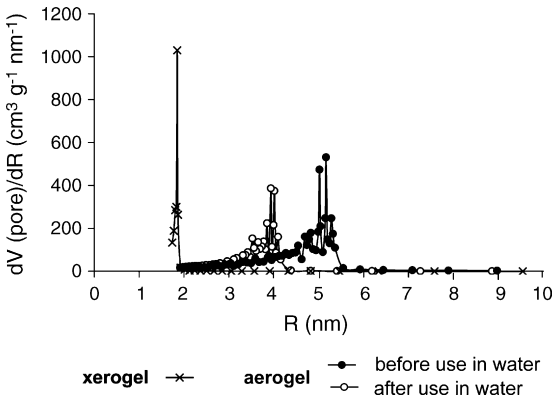

Fig. 9. Pore size distribution of a reference aerogel before and after use in aqueous medium at pH 9 and of a reference xerogel.

3.10. Effect of the pH on the activity

The effect of pH on a reference gel activity was determined by adjusting the reaction medium at a fixed pH value in a range from 5 to 10.5, with appropriate buffers. The conversions achieved after 15 min reaction are reported in Fig. 8, altogether with free LOX-1 relative initial activity, as a function of pH. The optimum activity was achieved at ≈pH 9 in both cases.

3.11. Recycling

A reference gel powder was submitted to seven successive catalytic tests. After each test, the wet gel powder was washed with a pH 9 buffer solution. The conversion achieved after 15 min was found to regularly decrease during these repeated tests (Table 5). By measuring the mass of a wet gel before and after a test, an average mass loss of gel of ≈0.1 g was also observed after each test, corresponding to ≈10% of a reference gel mass. Hence, the latter mass loss can be considered as a major contributor to the loss of activity of encapsulated LOX, although some inactivation may eventually occur. Nevertheless, some potential to improve the material loss upon recycling, exists. A gel can be reinforced with ceramic fibres for instance, as this was done with success for a lipase [47].

3.12. Textural characteristics

Analysis of nitrogen adsorption isotherms showed that a reference aerogel, obtained by drying a reference gel in supercritical CO_2, was characterized by a pore size distribution narrowly centred about an average radius ≈5 nm just after drying.

Table 5
Conversion achieved after 15 min reaction, in repeated catalytic tests, with a reference gel

Test number	Conversion/coversion (test 1) (%)
1	100 ± 4
2	97.8 ± 4
3	93.2 ± 4
4	82.2 ± 4
5	57.4 ± 2.8
6	55.6 ± 2.7
7	39.2 ± 1.9

Nevertheless, after dipping in the aqueous reaction medium, the average pore radius decreased to ≈4 nm (Fig. 9). Simultaneously, its specific pore volume, decreased from 2.65 to 1.72 $cm^3\ g^{-1}$. These data indicate that a significant gel contraction occurred when dipping a dry aerogel in the aqueous medium. Considering that a LOX-1 molecule has approximate dimensions of ≈9 nm × 6.5 nm × 6 nm, this contraction was in itself sufficient to explain the inactivity of dry aerogels. In turn, this result was consistent with the present strategy of using a wet gel without any drying, in order to keep an acceptable activity.

Similarly, a reference xerogel dried from a reference gel by evaporation was completely microporous, which was also sufficient to explain its lack of activity. A reference gel (made with 20% MTMS) had a slightly smaller average pore radius (≈5 nm) than the gel made from only TMOS (average radius ≈6 nm), for a similar total pore volume (≈2.65 $cm^3\ g^{-1}$). This again correlated well with a somewhat higher activity of the gel made from only TMOS. Even evaporation of the methanol produced during the pre-hydrolysis step resulted in a significant elimination of the smaller pores <5 nm, which correlated with a higher activity. At last, the addition of PVA was not found to induce a significant modification in the pore texture of a reference gel, which correlated with a constant activity.

3.13. Structural characteristics

Direct MAS ^{29}Si NMR spectroscopy (no proton coupling) was done on a series of gel samples comprising a reference gel, a similar gel with PVA added, a similar gel without pre-evaporation of methanol and a similar gel made only from TMOS. The spectra showed some differences in the connectedness of the silica network. The gels modified by pre-evaporation of methanol, resulted in relatively less intense Q^4 signals, by comparison with a reference gel. Similarly, the gel made from only TMOS (no MTMS) had a higher Q^4 proportion than the reference gel. Such Q^4 signals come from the fully hydrolyzed and condensed products of TMOS (≡Si(–OSi≡)$_4$ units). Also, the total area of $T^3 + T^2$ signals coming from MTMS was slightly higher in the reference gels made with MTMS, than in the other gels. Such results are consistent with a disappearance of the

smaller pores in favour of larger mesopores, as the MTMS content decreased, as observed in the texture analysis by nitrogen adsorption.

3.14. Overall analysis of LOX sol–gel encapsulation

The main question concerns the design of gels to encapsulate enzymes for use in aqueous medium. Such gels, and hence the enzyme encapsulated inside, are submitted to very different mechanical stresses when they are immersed in water after drying, instead of being immersed in an organic solvent with low gel–solvent interfacial tension. This explains that the technique of storing the gel in a wet state, instead of drying it, was an appropriate one.

Silica gels made from only TMOS are hydrophilic. When such gels were dried by the CO_2 supercritical method and carefully placed on the top surface of water contained in a dish, they sunk to the bottom of the dish. They also broke to very small pieces under the capillary contraction forces induced by the water penetrating the pores. MTMS introduced hydrophobic functions on the gel pore surface. Nevertheless, in a reference gel made from 20% MTMS dried by the CO_2 supercritical method, the density of such hydrophobic groups was too low to prevent sinking and collapsing when placed on top of water. This observation, which added up to the inactivation effect of the acetone used for CO_2 supercritical drying, was in itself sufficient to explain that a reference aerogel was inactive, while the reference (un-dried) gel was active. With 40% MTMS, the aerogel sunk in water but the collapse was partial. With 60% MTMS, part of the aerogel sunk and part remained at the water surface because the condensation of MTMS was incomplete. In the latter gel, uncondensed MTMS may have been partially washed out in some parts, which may explain a mixed behaviour. Only with 80% MTMS, the aerogel kept floating and did not break or shrink in water. Unfortunately, it was also inactive due to a lack of porosity accessible to polar liquids such as water [43], hence a severe limitation of the reaction by diffusion.

Besides, for an enzyme which is soluble in water and to be used in water, encapsulation in a gel does not bring any advantage in terms of dispersion of the enzyme. The situation is indeed very different from that of lipases to be used in organic solvents where they would aggregate if they were free. For such lipases, encapsulation in a gel makes it possible to maintain good enzyme dispersion, even for use in an organic solvent. Eventually, using the presently gel encapsulated LOX-1 in such an organic solvent could also be worth to examine. Besides, a gel with pore size as large as possible is necessary to minimize the limitation by diffusion. Nevertheless in simple gels, there is a trend, as the pores must be small enough to restrain the enzyme from leaching out. Besides, larger pores make a gel weak, unless it is reinforced by fibres or particles. But more involved architectures can be designed. For the present LOX-1 to be used in water, as a another potential advantage concerns shaping. Honeycomb structures for instance could be coated with a thin gel layer. Hence they would offer a combination of large pores for substrate transport (the honeycomb) with a small diffusion distance to the enzyme, in a thin micrometer gel layer. Such a structure would also be easy to recycle, while the gel thin layer would enhance enzyme stability, at room temperature.

4. Conclusions

In summary, lipoxygenase (LOX-1) from soybean was successfully encapsulated in a silica gel, for use in an oxidation reaction in aqueous medium. Contrary to previous very successful aerogel encapsulation studies of lipases used in organic solvents, the application of supercritical drying to silica gel did not appear to be efficient. The reason is that the dry aerogel porous texture was shown to collapse under capillary contraction forces, when it was dipped in water after drying. On the other hand, the wet (un-dried) gel was quite active, in spite of a lower relative activity (≈30%) than that of free LOX. This lower activity, also contrary to what was observed with lipases used in organic solvent, can be explained by an enzyme dispersion which is not better in a gel than in water. Overall, the optimum type of encapsulation gels to use in aqueous media appeared to be quite different from that applicable in organic solvents. Further studies are necessary to improve the results already achieved in the present study, in particular to permit a repeated use with limited enzyme inactivation, as well more complex material architectures.

References

[1] A.C. Chikere, B. Galunsky, V. Schuhemmann, V. Kasche, Enzyme Microbial. Technol. 28 (2001) 168.
[2] A.S. Bommarius, B.R. Riebel, Biocatalysis: Fundamentals and Applications, Wiley-VCH, 2004, p. 611.
[3] M.C. Pinto, P. Macias, Appl. Biochem. Biotechnol. 59 (1996) 309.
[4] T. Schewe, C.R. Pace-Asciak, S. Nigam, in: S. Nigam, C.R. Pace-Asciak (Eds.), Lipoxygenases and Their Metabolites: Biological Functions (Proceedings of the First Conference on lipoxygenases, Held in Malta, 17–21 May, 1997). Advances in Experimental Medicine and Biology, vol. 447, Kluwer, New York, NY, 1999, pp. 1–4.
[5] S. Nigam, C.R. Pace-Asciak (eds.), Lipoxygenases and Their Metabolites: Biological Functions (Proceedings of the First Conference on Lipoxygenases, Held in Malta, 17–21 May, 1997). Advances in Experimental Medicine and Biology, vol. 447, Kluwer, New York, NY, 1999, 230 p.
[6] E. Andre, K. Hou, CR Acad. Sci. (Paris) 194 (1932) 645.
[7] J.N. Siedow, Ann. Rev. Physiol. Plant. Mol. Biol. 42 (1991) 145.
[8] D. Shibata, B. Axelrod, J. Lipid-Mediat. Cell Signal 12 (1995) 213.
[9] A. Grechkin, Progr. Lipid Res. 37 (1998) 317.
[10] I.T. Baldwin, R. Halitschke, A. Kessler, U. Schittko, Curr. Opin. Plant Biol. 4 (2001) 351.
[11] E. Pichersky, J. Gershenzon, Curr. Opin. Plant Biol. 5 (2002) 237.
[12] F.P. Cuperus, G.F.H. Kramer, J.T.P. Derksen, S.T. Bouwer, Catal. Today 25 (1995) 441.
[13] E.A. Emken, J. Am. Oil. Chem. Soc. 55 (1978) 416.
[14] T. Kato, H. Ohta, K. Tanaka, D. Shibata, Plant Physiol. 98 (1992) 324.
[15] D. Parra-diaz, D.P. Brower, M.B. Medina, G.J. Piazza, Biotechnol. Appl. Biochem. 18 (1993) 359.
[16] S.Y. Shen, A.F. Hsu, T.A. Foglia, S.I. Tu, Appl. Biochem. Biotechnol. 69 (1998) 79.
[17] M. Fauconnier, M. Marlier, Biotechnol. Agron. Soc. Environ. 1 (1997) 125.
[18] C.X. Wang, K.P.C. Croft, U. Jarlfors, D.F. Hildebrand, Plant Physiol. 120 (1999) 227.
[19] J.C. Boyington, B.J. Gaffney, L.M. Amzel, Science 260 (1993) 1482.

[20] W. Minor, J. Steczko, B. Stec, Z. Otwinowski, J.T. Bolin, R. Walter, B. Axelrod, Biochemistry 35 (1996) 10687.
[21] J.F.G. Vliegenthardt, G.A. Veldink, in: W.A. Pryor (Ed.), Lipoxygenases, Academic Press Inc., 1982, p. 29.
[22] S. Shen, A.F. Hsu, T.A. Foglia, S.I. Tu, US Patent # 6,180,378 (2001).
[23] J.C. Allen, Eur. J. Biochem. 4 (1968) 201.
[24] E. Santano, M.D. Pinto, P. Macias, Enzyme Microbial. Technol. 30 (2002) 639.
[25] A. Graveland, Biochem. Biophys. Res. Commun. 41 (1970) 427.
[26] S. Grossman, M. Trop, M. Perl, A. Pinsky, P. Budowski, Biochem. J. 127 (1972) 909.
[27] S. Laakso, Lipids 17 (1982) 667.
[28] S. Kermasha, N. Dioum, B. Bisakowski, M. Vega, J. Mol. Catal. B: Enzyme 19 (2002) 305.
[29] M.D. Pinto, J.L. Gata, P. Macias, Biotechnol. Prog. 13 (1997) 394.
[30] S. Battu, H. Rabinovitchchable, J.L. Beneytout, J. Agric. Food Chem. 42 (1994) 2115.
[31] A.F. Hsu, T.A. Foglia, G.J. Piazza, Biotechnol. Lett. 19 (1997) 71.
[32] A.F. Hsu, S.Y. Shen, E. Wu, T.A. Foglia, Biotechnol. Appl. Biochem. 28 (1998) 55.
[33] C. Chebli, Interactions talc/enzymes—Réactivité de la lipoxygénase et de la chloroperoxydase de caldariomyces fumago absorbées en milieux aqueux et organique. Doctorate Thesis, Universite Paul Sabatier, Toulouse, France, 1993.
[34] A.C. Pierre, Biocatal. Biotrans. 22 (2004) 145.
[35] M. Pierre, P. Buisson, F. Fache, A. Pierre, Biocatal. Biotrans. 18 (2000) 237.
[36] M.T. Reetz, P. Tielmann, W. Wiesenhofer, W. Konen, A. Zonta, Adv. Synth. Catal. 345 (2003) 717.
[37] M.T. Reetz, R. Wenkel, D. Avnir, Synthesis (Stuttgart) 6 (2000) 781.
[38] S. Slappendel, Magnetic and Spectroscopic Studies on Soybean Lipoxygenase-1, Rijks-Universiteit te Utrecht, Utrecht, 1982.
[39] M.C. Feiters, G.A. Veldink, J.F.G. Vliegenthart, Biochim. Biophys. Acta 870 (1986) 367.
[40] E. Mulliez, J.P. Leblanc, J.J. Girerd, M. Rigaud, J.C. Chottard, Biochim. Biophys. Acta 916 (1987) 13.
[41] B.F. Roberts, J. Colloid Interf. Sci. 23 (1967) 266.
[42] R.A. Venkateswara, D. Haranath, Micropor. Mesopor. Mater. 30 (1999) 267.
[43] H. El Rassy, P. Buisson, B. Bouali, A. Perrard, A.C. Pierre, Langmuir 19 (2003) 358.
[44] M.T. Reetz, A. Zonta, J. Simpelkamp, A. Rufinska, B. Tesche, J. Sol–Gel Sci. Technol. 7 (1996) 35.
[45] M. Vega, S. Karboune, S. Kermasha, Appl. Biochem. Biotechnol. 127 (2005) 25.
[46] G.J. Piazza, D.P. Brower, D. Parra-Diaz, Biotechnol. Appl. Biochem. 19 (1994) 243.
[47] A. Karout, P. Buisson, A. Perrard, A.C. Pierre, J. Sol–Gel Sci. Technol. 36 (2005) 163.