Ormosil gels doped with engineered catechol 1,2 dioxygenases for chlorocatechols bioremediation

Abstract

Enzymes entrapped in wet, nanoporous silica gel have great potential as bioreactors for bioremediation because of their improved thermal, chemical, and mechanical stability with respect to enzymes in solution. The B isozyme of catechol 1,2 dioxygenase from *Acinetobacter radioresistens* and its mutants of Leu69 and Ala72, designed for an increased reactivity toward the environmental pollutant chlorocatechols, were encapsulated using alkoxysilanes and alkyl alkoxysilanes as precursors in varying proportions. Encapsulation of the mutants in a hydrophobic tetramethoxysilane/dimethoxydimethylsilane-based matrix yielded a remarkable 10- to 12-fold enhancement in reactivity toward chlorocatechols. These gels also showed a fivefold increase in relative reactivity toward chlorocatechols with respect to the natural substrate catechol, thus compensating for their relatively low activity for these substrates in solution. The encapsulated enzyme, unlike the enzyme in solution, proved resilient in assays carried out in urban wastewater and bacteria-contaminated solutions mimicking environmentally relevant conditions. Overall, the combination of a structure-based rational design of enzyme mutants, and the selection of a suitable

encapsulation material, proved to be a powerful approach for the production and optimization of a potential bioremediation device, with increased activity and resistance toward bacterial degradation.

Abbreviations
Iso B C1,2O

B isozyme of catechol 1,2 dioxygenase

ormosils

organically modified silanes

TMOS

tetramethoxysilane

DMDMS

dimethoxydimethylsilane

1. Introduction

Enzymes offer a huge spectrum of reactivity, which can be further enhanced by protein engineering. Their selectivity and flexibility led to several applications in organic chemistry, food chemistry, and analytical chemistry, either as purified preparations or as part of the whole protein pool of living microorganisms. In particular, enzymes involved in catabolic reactions have a huge potential in the remediation of soils, sediments, or wastewater from pollutants [1, 2]. The use of purified enzymes for bioremediation can be significantly enhanced if coupled with immobilization techniques [3-5]. Immobilization usually results in the possibility of reuse and produces an increase in protein stability and *in situ* persistence [6].

Within the enzyme immobilization matrices currently available, encapsulation in wet silica gel through the sol–gel method [7, 8] proved to be a powerful approach to develop bioreactors [9-11], biosensors, and biodevices [6, 12, 13]. The appealing features of encapsulation in silica gel for bioremediation are manifold. First of all, the aqueous microenvironment around individual encapsulated protein molecules allows for the full retention of their biological properties [6, 7], and the preparation-dependent nanoporosity of the gels makes the enzyme active sites freely accessible to small reagents that diffuse from the solvent phase. In addition to the physical confinement, which allows for enzyme recovery and reuse, encapsulation of proteins usually enhances their thermal, chemical, and mechanical stability with respect to solution, due to the sterical inhibition of the large conformational changes associated with protein denaturation [14, 15]. The optimization of storage conditions can further increase the durability of silica gel-based enzymes [16]. Recently introduced organically modified silanes (ormosils) allow for the preparation of a number of polymeric matrices endowed with different physicochemical properties, particularly in terms of matrix hydrophobicity [17].

The B isozyme of the catechol 1,2 dioxygenase (Iso B C1,2O) [EC 1.13.11.1] from *Acinetobacter radioresistens* S13 [18-22] is of great potential relevance in bioremediation, as it catalyzes an intradiol cleavage of the aromatic ring of catechol and, with a lower efficiency, of its chloro derivatives, yielding *cis–cis* muconic acid or its chloro-substituted derivatives, respectively. This enzyme exhibits relatively high stability and high catalytic rate constants, at least with respect to

other catechol dioxygenases. Moreover, unlike other oxidizing enzymes with potential applications as biocatalysts [23], Iso B C1,2O does not require reducing agents to restore its activity at each catalytic cycle [24] because the Fe(III) ion merely acts as a catalyst and is not an electron acceptor.

As with other phenols, catechol is regarded as a hazardous pollutant by both U.S. and European Union regulatory authorities [25-28]. The presence of catechol in the environment stems from its past use as a photographic developer, a developer of fur dyes, an intermediate for antioxidants in rubber and lubricating oils, and a polymerization inhibitor. Catechol is also a by-product of the biodegradation of other aromatic pollutants. Halogenated catechols are significantly more toxic than catechol [25, 29-34] and are equally present in the environment because of their wide range of industrial applications. Their higher persistence in the environment with respect to non-chloro-substituted catechols is due to a lower rate of microbial degradation [35]. However, some microorganisms have evolved specific pathways for chloroaromatic degradation and use these compounds as the sole carbon sources [36, 37]. The enzymes responsible for these reactions have been studied with particular interest [38-40].

To improve the reactivity of wild type (WT) *A. radioresistens* Iso B C1,2O toward chlorocatechols, mutants with a larger and more hydrophobic ligand binding pocket were generated [41]. Iso B C1,2O Leu 69A showed an inversion of specificity from catechol to 4-chlorocatechol with respect to the WT enzyme [41], due to fine reshaping of substrate recognition sites [41, 42] and to subtle changes in the metal coordination sphere—resulting in a higher propensity to generate O_2–Fe adducts [43]; in contrast, mutants of Ala72 did not show an inversion of substrate specificity but an increased k_{cat} for 4-chlorocatechol. Furthermore, A72S showed a 33% increase in activity for 4,5-dichlorocatechol with respect to the WT enzyme [41].

In this work, the WT enzyme and mutants selected for higher specificity toward chlorocatechols in solution were encapsulated in silica and ormosil gels and tested for durability, thermal stability, and reactivity toward catechol, 4-chlorocatechol, and 4,5-dichlorocatechol. The most promising combination of mutants and encapsulation matrix was tested in wastewater to assess the interference of the most common contaminants on the enzyme activity, considering that iron-containing proteins and catechol dioxygenase in particular might be inactivated by chelants. Finally, to test the protection offered by the gel matrix to the enzyme against microbial degradation, bacterial challenge experiments were performed.

The feasibility of the biotechnological approach proposed in this article, including enzyme entrapment to generate efficient and long-lasting catalysts is a hot topic for bioremediation of catechol/phenol polluted sites and water, as attested by very recent papers [44-46].

2. Materials and Methods

2.1. Reagents

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) ≥95% (Sigma–Aldrich, St. Louis, MO, USA), pyrocatechol ~99% (Sigma–Aldrich), 4-chlorocatechol 97% (Sigma–Aldrich), 4,5-dichlorocatechol 97% (Sigma–Aldrich), tetramethoxysilane (TMOS) ≥99% (Sigma–Aldrich), and dimethoxydimethylsilane (DMDMS) ≥99.5% (Sigma–Aldrich) are used in this study. All other chemicals were of the best commercially available quality.

2.2. Enzymes

His-tagged WT Iso B C1,2O from *A. radioresistens* S13 was expressed and purified as previously described [20, 21]. His-tagged mutants L69A, A72G and A72S were prepared, expressed, and purified as described [22, 41].

2.3. Protein encapsulation

The Iso B C1,2O and its mutants were encapsulated in TMOS-based and in TMOS/DMDMS-based silica gels. For TMOS-based gels, samples were prepared according to a modified version of the protocol first proposed by Ellerby et al. [7]. Briefly, 750 µL of TMOS were mixed with 169 µL of water and 11 µL of 40 mM HCl and sonicated for 20 Min to promote acidic hydrolysis of TMOS. The sol was then diluted with an equal volume of a 50 mM HEPES solution at pH 6.0, followed by nitrogen bubbling for 40 Min to remove the methanol produced during the hydrolysis step. An aliquot of the sol was then mixed with an equal volume of 2 mg/mL protein solution containing 50 mM HEPES at pH 7.2. Ten-liter aliquots of the mixture were poured in individual microtubes. Upon gelation, which occurred in 10–15 Min, a storage solution containing 100 mM HEPES at pH 7.0 was added to each tube. Gels were stored at 4 °C, unless otherwise stated. For TMOS/DMDMS-based gels, the protocol was modified by using a 4:1 mixture of TMOS and DMDMS. Gels were aged for at least 24 H before carrying out activity assays unless otherwise stated. Lower ratios of TMOS/DMDMS, leading to lower polarity, caused enzyme aggregation.

2.4. Activity assays

The enzyme activity of Iso B C1,2O and its mutants in solution and encapsulated in silica or Ormosil gels was assayed at saturating concentrations of either catechol, 4-chlorocatechol, or 4,5-dichlorocatechol in a solution containing 50 mM HEPES at pH 7.2. The assays were carried out at 30 °C, unless otherwise stated. The reaction rate was determined spectrophotometrically, using a Cary400 spectrophotometer (Varian Ltd., Palo Alto, CA, USA), monitoring the time-dependent formation of *cis, cis*-muconic acid, or its chloro derivatives at 260 nm, based on an extinction coefficient of 17,600 M^{-1} cm^{-1}. The initial concentration of the substrate and the enzyme in the assay mixture was 200 µM and 65 nM, respectively. For assays carried out using enzyme-doped gels, an equivalent amount of encapsulated protein was added to the assay mixture. To avoid diffusion-limited reaction rates, gels were micronized by sonication. The size of gel particles was evaluated with a microscopic analysis and found to be on the order of 2–5 µm. The size of the gel microfragments is such that the diffusion of substrates and products through the gel matrix does not rate limit the reaction catalyzed by the enzyme. The fragment critical thickness over which rates are diffusion controlled, the d_c, was determined for each catalyzed reaction according to Eq. (1) [12, 47]:

- $$d_c = \left[(K_m + [S_0])\, D'/k_{cat}[E] \right]^{1/2} \quad (1)$$

where k_{cat} and K_m are the catalytic parameters obtained in solution for the wild-type and mutated Iso B C1,2O, E is the enzyme concentration expressed in millimolarity, S_0 is the substrate concentration expressed in millimolarity, and D' is the diffusion coefficient of the substrate inside the gel, calculated from the equation

- $$D'/D = 1 - (a^2/r) \quad (2)$$

where a is the average molecular radius (4 Å for molecules with molecular weights in the range 200–350 Da), D (~6×10^{-6} cm^2 Sec^{-1}) is the diffusion constant in water for substances in this range of molecular weights, and r is the average pore radius of the gel, 40–50 Å. The calculated values of d_c for the different mutants are in the range 200–500 µm, two orders of magnitude higher than the

average gel particle size of 4 μm, indicating that diffusion of substrates within the gel microsuspensions cannot limit reaction rates. Magnetic stirring was maintained throughout the experiments. All measures were carried out at least in triplicate. For durability tests, aliquots of Iso B C1,2O either in solution or encapsulated in gel, stored under the same conditions, were periodically tested for enzymatic activity over a time range of weeks. For pH stability assays, gels were micronized and suspended in solutions buffered at different pH values for 24 H. The solution was then centrifuged and the pelleted gel removed. The catalytic activity of an aliquot of the supernatant and of the gel was then assayed. The determination of optimal activity temperatures was carried out by using the same procedure at controlled temperatures ranging from 10 to 60 °C.

2.5. Activity in wastewater

The enzyme activity of the most promising combination of mutation and encapsulation matrix (C1,2O A72S in a TMOS/DMDMS polymer) was tested in samples of urban wastewater collected in the city of Torino, Northern Italy, using 4,5-dichlorocatechol as substrate. As a reference, enzyme activities were tested in water of the same origin upon purification in a plant of Società Metropolitana Acque Torino (SMAT, Castiglione Torinese, Torino, Italy). Preliminary analysis of the urban wastewater revealed the presence of sediments, nitrites, nitrates, ammonium salts, sulfites, and a pH of 7.3. Activity assays were carried out as described using 4,5-dichlorocatechol as substrate.

2.6. Bacterial challenge

Bacterial challenge experiments were carried out by diluting an overnight culture of JM109 *Escherichia coli* to a final concentration of approximately 3×10^6 cells/mL in a 10 mM HEPES solution buffered at pH 7.2. Aliquots of the C1,2O A72S mutant free in solution and encapsulated in the TMOS/DMDMS matrix were exposed to the contaminated solution for up to 48 H under stirring. As a reference, both the free enzyme and the encapsulated enzyme were exposed to the same buffer without bacteria. At regular intervals, 4,5 dichlorocatechol was added to aliquots and the residual enzymatic activity was measured in the same conditions used for the previous enzymatic assays.

3. Results and Discussion

3.1. Substrate specificity in TMOS-based and TMOS/DMDMS-based gels

The results of the activity assays of Iso B C1,2O and its mutants L69A, A72G, and A72S toward catechol, 4-chlorocatechol, and 4,5-dichlorocatechol in solution, in TMOS gels, and in TMOS/DMDMS gels are reported in Fig. 1 and Table 1. Measurements of the encapsulated enzymes were carried out after 24 H from encapsulation, a time required for the stabilization of the gel matrix.

Table 1. Relative activity of WT Iso B C1,2O, Iso B C1,2O L69A mutant, Iso B C1,2O A72G mutant, and Iso B C1,2O A72S mutant in two different silica gel matrices

	Substrate	k_{cat} in solution (Sec^{-1})	TMOS gel (after 1 day)	TMOS/DMDMS gel (after 1 day)	TMOS gel (after 7 days)	TMOS/DMDMS gel (after 7 days)
Iso B C1,2O (WT)	Catechol	5.770 ± 0.058	105%	236%	61%	112%
	4-Chlorocatechol	0.440 ± 0.005	148%	342%	102%	187%
	4,5-Dichlorocatechol	0.150 ± 0.015	300%	731%	207%	425%
Iso B C1,2O L69A mutant	Catechol	3.090 ± 0.08	139%	219%	86%	105%
	4-Chlorocatechol	0.110 ± 0.005	295%	1230%	174%	945%
	4,5-Dichlorocatechol	0.190 ± 0.005	280%	973%	195%	563%
Iso B C1,2O A72G mutant	Catechol	0.900 ± 0.02	123%	211%	78%	117%
	4-Chlorocatechol	0.260 ± 0.005	185%	746%	107%	358%
	4,5-Dichlorocatechol	0.200 ± 0.005	156%	845%	110%	470%
Iso B C1,2O A72S mutant	Catechol	0.730 ± 0.007	132%	287%	103%	158%
	4-Chlorocatechol	0.16 ± 0.01	185%	800%	170%	456%
	4,5-Dichlorocatechol	0.210 ± 0.005	150%	710%	122%	290%

1. Activity measurements after 12 H and 7 days are reported. Percentages are relative to the activity measured in solution for the same enzyme immediately after thawing assayed with the same substrate.

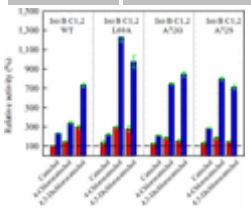

Figure 1. Relative activity of WT Iso B C1,2O, Iso B C1,2O L69A mutant, Iso B C1,2O A72G mutant, and Iso B C1,2O A72S mutant in TMOS-based gel (red bars) and TMOS/DMDMS-based gel (blue bars) tested for catechol, 4-chlorocatechol, and 4,5-dichlorocatechol. Percentages are

relative to the activity measured in solution for the same enzymes and substrates. Assays were carried out in 50 mM HEPES buffer, pH 7.2.

In solution, the WT enzyme shows a k_{cat} for catechol consistent with that reported in the literature, considering that the assays were carried out at pH 7.2 and not at the reported optimal pH of 8.0 [41]. The k_{cat} measured with 4-chlorocatechol as substrate is around 10% that of catechol, in line with measurements in solution and for C1,2O from *Pseudomonas putida* encapsulated in alginate gel [48]. The enzyme also shows appreciable activity toward 4,5-dichlorocatechol, a recalcitrant pollutant. The A72G and A72S mutants show a lower k_{cat} for catechol with respect to the WT enzyme, but an overall increase in relative activity for the mono and dichlorocatechol with respect to that measured for catechol (Fig. 1). It is known that several bacterial C1,2Os can also recognize 4-methyl catechol, although the bond cleavage is catalyzed at a much lower reaction rate [49, 50].

For A72G mutant encapsulated in the TMOS-based gels, the k_{cat} for all substrates are essentially retained with respect to solution with a slight increase in the relative reactivity toward chloro-substituted substrates (Fig. 1). The WT enzyme and the L69A mutant show a significant increase in k_{cat} for the chloro-substituted catechols when encapsulated in the TMOS-based gel. In particular, the L69A mutant exhibits a threefold increase in reactivity toward 4-chlorocatechol and 4,5-dichlorocatechol. The encapsulated WT enzyme shows a slight decrease in k_{cat} for catechol, but a threefold increase in k_{cat} when assayed with 4,5-dichlorocatechol. The reactivity of catechol dioxygenases toward a bis-substituted catechol is normally much lower, thus making the activity gain by encapsulation potentially interesting from a biotechnological point of view.

When encapsulated in the more hydrophobic TMOS/DMDMS-based gel, the reactivity systematically increases for all enzymes assayed with the substrates (Fig. 1). In measurements carried out after 24 H from encapsulation, the increase is twofold and threefold for catechol, and reaches 10-fold for chlorocatechols, particularly 4,5-diclorocatechol. These results suggest that all mutants encapsulated in TMOS/DMDMS-based gel become the elective enzymes for the reaction with 4-chlorocatechol and 4,5-dichlorocatechol, as the k_{cat} for chlorocatechols becomes very close to that measured for catechol itself. Specifically, the A72S and A72G mutant gels become nonselective for the three substrates, whereas the WT enzyme and the L69A mutant gels still retain a preference for catechol with respect to its chloro- derivatives.

The systematic increase in catalytic activity for WT Iso B C1,2O and its mutants in the hydrophobic gel matrix calls for speculations about the molecular basis of such an effect. The hydrophobic environment might stabilize a particularly active conformation showing an altered pattern of protein surface hydrophobicity, in agreement with experiments using hydrophobic fluorophores [19]. The stabilization, by interactions with the gel matrix, of more active (open) conformations might also explain the higher relative increase in activity toward the more bulky chloro-substituted substrates with respect to catechol. Alternatively, the hydrophobic matrix might interfere with the inhibitory effect because of a substituted glycerolphospholipid bound to *A. radioresistens* Iso B C1,2O [42] and also found in other catechol dioxygenases [51-53]. The lipophilic silica matrix might destabilize its interaction with the enzyme, thus reducing its inhibition. No hyperactivating effect such as the one here described (activity increase up to 13-fold) was observed on catechol-dioxygenase-based alginate gel-entrapment systems [48] and only a threefold enhancement of activity toward 4-chlorocatechol was reported on immobilized systems employing other matrices with tighter cross-linking, such as nanosponges [54]. Reactivity of catechol 1,2-dioxygenase from *Arthrobacter chlorophenolicus* was retained upon immobilization on fulvic acid-activated montmorillonite [55].

3.2. Optimal temperature for catalytic activity

The temperature optimum for WT Iso B C1,2O in solution is around 20 °C at pH 7.2, confirming previous determinations [21]. The enzyme encapsulated in both TMOS-based and TMOS/DMDMS-based gels shows an increased optimal temperature around 30–35 and 25 °C, respectively (data not shown). Similar shifts (10–20 °C) of the optimal temperature were observed for catechol dioxygenases encapsulated in other immobilization systems [48].

3.3. Chemical and pH stability of encapsulated C1,2Os

Silica gel is documented to undergo significant basic hydrolysis at a pH higher than 7.5. Leaking of the enzyme was determined to be less than 6% in 1 week at pH 6.0–7.5, whereas incubation of both TMOS and TMOS/DMDMS-based gels at pH 7.5 resulted in a loss of more than 12% of the encapsulated protein within 24 H. The pH optimum for the enzyme in solution is around 8.0, a value not compatible with the integrity of the gel matrix and, thus, the retention of the protein in gels. However, this behavior opens the possibility for a dual application of the enzyme encapsulation. The enzyme could be released in the environment as stable gel particles when the soil or wastewater exhibits acidic or neutral pH or as a delivery system for the free enzyme when the pH is basic.

3.4. Enzyme activity in solution and silica gels as a function of time

WT Iso B C1,2O catalytic activity, measured over time for the soluble form and the encapsulated one in either TMOS-derived silica gels or TMOS/DMDMS-derived gels (Fig. 2), exhibits the same time dependence. Similar time dependencies of enzyme activity were observed for the mutants L69A, A72G, and A72S (Table 1). The loss of the catalytic activity during time is probably associated with protein instability rather than iron loss [19]. Actually, incubation of aged gels (70% residual activity with respect to fresh gels) with either ferric or ferrous chloride failed to restore the full activity. Similarly, addition of 0.3 mM iron salts to the storage buffer did not prevent the loss of activity over time, with an activity decrease of around 20% in 24 H for gels stored both in the presence and absence of ion salts. Nevertheless, given the initial increased activity measured for the encapsulated enzyme, particularly for the mutants in the TMOS/DMDMS-derived gels, the residual activity after 1 week of storage is significantly higher than that of the enzyme in solution immediately upon thawing. For instance, the enzyme activity, measured for the L69A mutant using 4-chlorocatechol as substrate after 1 week, is still 10-fold higher than the reference activity measured in solution (Table 1).

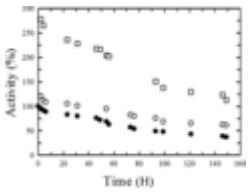

Figure 2. Time dependence of WT C1,2O activity toward catechol in solution (closed circles), encapsulated in a TMOS-based gel (open circles) and in the TMOS/DMDMS-based gel (open squares). All assays were carried out at pH 7.2. All values are normalized to the initial activity of the enzyme in solution.

3.5. Enzyme reuse

The enzymatic activity of WT C1,2O gels was measured in subsequent cycles of reuse. Each cycle consisted of an enzymatic assay followed for 10 Min, a centrifugation step, the separation from the reaction medium, and the resuspension in a fresh solution containing catechol as substrate. After 10

cycles of centrifugation and resuspension of both TMOS-based and TMOS/DMDMS-based WT C1,2O-doped gels, 90% of the activity was retained (data not shown). This shows high stability of the enzyme–ormosil matrix system, whereas the alginate gel-based system [48] shows a loss of activity (less than 80% retained activity) over just five reuse cycles.

3.6. Enzyme activity in contaminated urban wastewater and bacterial challenge

Assays in wastewater showed that neither the free soluble enzyme nor the enzyme encapsulated in the TMOS/DMDMS matrix undergo activity decrease in the wastewater sample as compared with a buffered solution and the purified wastewater (data not shown). Therefore, the contaminants present in the polluted sample do not interfere with the catalytic cycle of either WT catechol dioxygenase or its mutants.

Challenge tests showed that the free enzyme is markedly affected by the presence of relatively low amounts of viable bacteria (approximately 10^6 cfu/mL), with its activity disappearing in a matter of hours (Fig. 3). On the other hand, the enzyme encapsulated in the TMOS/DMDMS matrix is not affected by bacteria, and its activity only decreases within several days, with the same inactivation profile observed in reference conditions (Fig. 2). Therefore, encapsulation not only dramatically enhances the activity of the enzyme but also protects it from bacterial inactivation. Considering that bacteria are among the most represented items in waste-water, this constitutes a successful means to preserve enzyme activity.

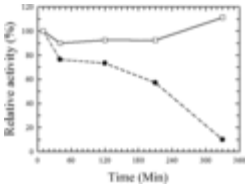

Figure 3. Inactivation of free (closed circles) and TMOS/DMDMS encapsulated C1,2O A72S (open squares) as a function of time when incubated in a solution containing 10 mM phosphate, pH 7.2, with the addition of aliquots from an overnight culture of E. coli (JM109 strain) to give a final concentration of approximately 3×10^6 cells/mL. Prior to enzymatic assays, samples were incubated at 20 °C under stirring. Values were normalized to the initial activity.

This result, together with the hyperactivating effect of the matrix on enzyme catalysis toward mono and bis-chlorinated catechols, stresses the relevance of the ormosil entrapping system for application in polluted wastewater treatment. Although ormosils have been proposed as matrices for pollutants and drug biosensing [56, 57], this entrapping system can also be very promising for the development of stabilized biocatalysts to be applied in bioremediation.

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