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High operational stability in peroxidase-catalyzed non-aqueous sulfoxidations by encapsulation within sol–gel glasses

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Abstract

The peroxidase activity of immobilized horseradish peroxidase (HRP) has been employed for the asymmetric oxidation of thioanisole in acetonitrile with H_2O_2 (30%). Encapsulation of HRP by the sol–gel method considerably enhanced its operational stability by protecting the peroxidase activity under harsh conditions. The total rates of the encapsulated HRP increased up to six-fold (TTN $= 4.22 \times 10^3$) the rates observed with its homogeneous counterpart. The sulfoxide selectivity and the enantiomeric excess also increased greatly upon encapsulation. Coupling glucose oxidase reaction to the encapsulated peroxidase allowed high enantiomeric excesses (up to 56%) and sulfoxide as sole product by elimination of side non-enantioselective and overoxidation reactions. The heterogeneous catalyst can be recycled by simple filtration in successive runs.

Keywords: Horseradish peroxidase; Sol–gel; Bioencapsulation; Sulfoxidation; Glucose oxidase

1. Introduction

Peroxidases are potentially attractive biocatalysts for selective oxidations, and their applications have been extensively reviewed [1–3], being the asymmetric sulfoxidations of thioethers of particular synthetic interest since the resulting chiral sulfoxides are valuable pharmaceutical synthons [4]. However, the practical application of this enzymatically-assisted reaction is limited by inactivation of the heme enzymes by an excess of oxidant (i.e. H_2O_2) as well as by the low solubility of most reactants in aqueous solutions. While the in-situ generation of hydrogen peroxide using a bienzymatic system (Scheme 1) has been proposed to keep the concentration of the oxidant below toxicity levels [5–7], the solubility of the reactants has been recently improved by performing the oxidations in mixtures of aqueous buffers and water miscible organic solvents [1,5,8,9].

Enzyme immobilization techniques have also been assayed as an alternative to overcome these problems, i.e. serving as a protecting microenvironment and having beneficial influence on the partitioning of substrates and products [5,9–11]. Among other immobilization techniques, the sol–gel encapsulation of biological catalysts in silicate glasses has experienced a swift expansion due to the synthetic easy of enzyme entrapment procedures and to the significantly enhanced stability of the entrapped enzyme [12–17]. Horseradish peroxidase (HRP) has been widely used as a model for enzyme immobilization, and the catalytic activity of the HRP encapsulated in a sol–gel matrix has been demonstrated in aqueous solution or under mild conditions [18–22]. However, very little is known on the catalytic activity of sol–gel encapsulated HRP under harsh reaction conditions (e.g. presence of organic solvents and high oxidant and sulfide concentrations) where the free enzyme becomes readily inactive.

Here in, we report on the sol–gel encapsulation of horseradish peroxidase for the preparation of a heterogeneous biocatalyst. The model reaction to investigate the oxygen-transfer catalytic property of the encapsulated HRP under harsh conditions was the enantioselective sulfoxidation of thioanisole. Among other hydrophilic solvents, acetonitrile was selected since it causes rapid deterioration on

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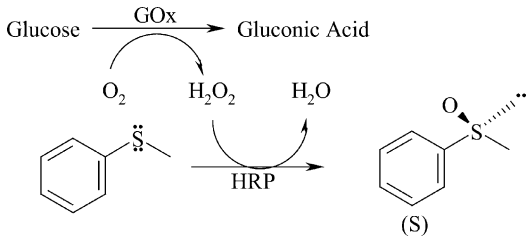

Scheme 1. Mode of action of coupled GOx-HRP.

HRP activity [11]. The effective protection of the enzyme activity upon encapsulation has been evaluated in terms of the total turnover number (TTN; mmol product/mmol catalyst), sulfoxide/sulfone selectivity and enantioselectivity. The glucose oxidase coupled enzymatic reaction has been used to improve enantiomeric excess values and to further evaluate the peroxidase activity protection. Reusability of the immobilized enzyme has also been demonstrated.

2. Experimental

2.1. Materials

Horseradish peroxidase (type II, E.C. 1.11.1.7; 1520 U/mg) and glucose oxidase (GOx, E.C. 1.1.3.4; 157.5 U/mg from *Aspergillus niger*) were used as received from Sigma, methyl phenyl sulfide (thioanisole), tetramethyl orthosilicate (TMOS), hydrogen peroxide (30% aqueous solution), acetonitrile (HPLC grade) and phosphate salts were used as received from Aldrich.

2.2. Enzyme immobilization

Silica sol stock solution was prepared by mixing TMOS (13.5 ml), double distilled water (2.94 ml) and HCl (0.18 ml of 0.04 M solution) at 4 °C. After 15 min of stirring, the clear sol was stored at −20 °C for 48 h. The HRP stock solution (8 mg/ml; 12.2 kU/ml) was prepared in sodium phosphate buffer (10 mM, pH 7.0) with deionized doubly distilled water. Peroxidase gels were obtained within 15 min by addition of 0.5 ml of peroxidase stock solution to 0.5 ml of the sol stock solution in a polystyrene container cooled in an iced bath. Gels were rinsed up to three times with 1 ml aliquots of the phosphate buffer, sealed with Parafilm and stored at 4 °C for at least 2 weeks for standard procedures and up to 6 months for stability experiments. The samples were further dried at room temperature for 24–48 h to ensure the xerogel formation. Blank gels for control experiments (undoped silica sol–gel glasses; 2.68 mmol Si) were prepared as above but using 0.5 ml of phosphate buffer solution instead the peroxidase stock solution. For absorption spectra, red transparent glass slides were prepared in polystyrene cuvettes of 1 cm path length containing a final volume of 0.3 ml (0.15 ml of each stock solution). Laying the cuvettes on their side prior to gelation resulted in the slide shape processed gels of 16 mm × 8 mm × 1 mm. Absorption spectra were carried out in a Varian Cary 50. Fluorescence spectra were achieved in diluted samples (2×10^{-6} M) of dimension 18 mm × 9 mm × 9 mm with a 48000 SLM Aminco spectrofluorometer as described elsewhere [23]. Xerogels were finely grinded (e.g. in the micron range) in a mortar for catalytic activity analysis.

2.3. Standard oxidation procedure

In a standard procedure, methyl phenyl sulfide (thioanisole; 0.8 mmol) and HRP (9.09×10^{-5} mmol) were added to acetonitrile (3 ml) in a flask equipped with a magnetic stirrer. The mixture was sonicated briefly and then warmed up to 40 °C. The oxidant H_2O_2 (1.6 mmol) was added at once and the reaction mixture stirred. After the selected reaction time, the heterogeneous biocatalyst was recovered by centrifugation and washed with acetonitrile. No attempts to recover the biocatalyst were performed on homogeneous reactions. Chemical yields and enantiomeric excess values of 2-methylphenylsulfoxide were measured by gas chromatography analysis (Hewlett-Packard 5890 II) with a chiral glass capillary column [24]. The absolute sulfoxide configuration was determined by ^{1}H NMR (Varian XR300 spectrometer) using (−)-MPPA ((*R*)-(−)-α-methoxyphenylacetic acid) as chiral agent [25]. The chemical shifts were referred to an internal standard (tetramethylsilane). A control experiment in absence of any catalyst (e.g. blank gels and HRP) showed no reaction. For every thioanisole oxidation condition, three runs were performed. The long-term stability of the prepared biocatalyst was examined by performing the oxidation reaction under the same conditions every 2 weeks.

The glucose oxidase coupled enzymatic reaction was carried out as follows. A solution of glucose (1.6 mmol) in 3 ml of 50% CH_3CN:phosphate buffer (10 mM, pH 7) was added to the encapsulated biocatalyst and the sulfide. In this case, the use of buffer was required to dissolve the glucose substrate. At this stage, addition of glucose oxidase (10 mg; 1.56 kU) made the reaction begin. The course of the reaction was followed as described above.

3. Results and discussion

3.1. Encapsulation of HRP

The heterogeneous biocatalyst subject of study in the current work was obtained through the encapsulation of HRP in silica sol–gel glasses. The absorption spectrum of the encapsulated enzyme was similar to that of the free enzyme in solution (Fig. 1), which indicates that the protein structural integrity is preserved upon the encapsulation process [19,23,26]. However, this yield of encapsulation does not provide an estimation of the amount of active protein, since the heme group remains trapped in the aged gels whether or

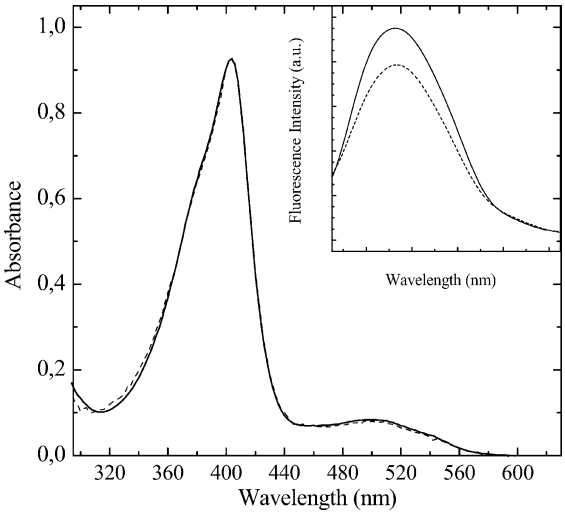

Fig. 1. Absorption spectra of HRP in solution (- - -) and in aged gel (——). Inset: Emission spectra of HRP in solution (- - -) and in aged gel (——).

not it is attached to the active site of the protein. Fluorescence spectroscopy presents a valid alternative to investigate this issue since tryptophan fluorescence is quenched in the native enzyme while is strongly fluorescent and red shifted in denatured samples [23]. Thus, while the shape and wavelengths of the fluorescence spectra of HRP in the aged gels are similar to those in solution, the slight increase of the fluorescence intensity of HRP in the aged gels is indicative of a partial relief of the heme group from the active site (∼15%, Fig. 1) [23].

3.2. Evaluation of the encapsulated HRP activity towards thioanisole oxidation

Data regarding the enantioselective sulfoxidation of thioanisole catalyzed by encapsulated HRP (e.g. conversion, selectivity and TTN) are shown in Table 1 (entry 3). It can be readily observed an overall improvement as compared to those obtained for free HRP under identical conditions (entry 1b). Such an improvement must be ascribed to a good individual dispersion of the immobilized peroxidase within the cages of the silica matrix, which prevents enzyme aggregation and stabilizes the enzyme conformation [10,11,13]. The preferred formation of (*S*) rather than (*R*)-sulfoxide with the encapsulated HRP confirms that the structure of the active site has not been changed upon encapsulation [9]. Moreover, the relatively high amount of (*S*)-sulfoxide recovered (31% ee) corroborates the protection of the peroxidase activity of encapsulated HRP by keeping the enzyme apart from the initial high H_2O_2 concentration. The high polarity of the matrix and the presence of an organic solvent also contributed to favor the catalytic performance of the encapsulated HRP through a favorable partitioning of the substrates (thioanisole, H_2O_2) and the product in the heterogeneous system [10]. The use of acetonitrile/buffer mixtures did not improve the operational stability under high oxidant concentration for free HRP as indicate the no-correlated increase of the TTN with the selectivity and enantioselectivity values (Table 1; entries 1b, 1c). This result is in agreement with data previously reported, where similar increase of the initial sulfoxidation rates in both enantiomers were obtained for free HRP using isopropyl alcohol as co-solvent [8]. As expected, application of milder conditions such as lowering the temperature to 20 °C did slightly improve the stability of the free HRP (i.e. the enantioselective enzymatic reaction versus the non-enantioselective oxidation). No significant reaction was observed for the encapsulated HRP at this temperature most likely due to diffusion limitations.

The catalytic behavior of an undoped silica sol–gel glass was also studied in order to evaluate whether or not background heterogeneous reactions took place in our system (Table 1; entry 2). It can be observed that the sulfoxide conversion in undoped silica sol–gel glass was just a 10% of the sulfoxide conversion obtained for encapsulated HRP (Table 1; entry 3). This value differs from that previously reported [20], as consequence of the different reaction conditions (e.g. buffer solution, low sulfide/catalyst ratio, and step-wise addition of diluted H_2O_2). Actually, much larger TTN has been found with our heterogeneous catalyst (4.2 ×

Table 1
Oxidation of thioanisole (0.27 M) catalyzed by sol–gel encapsulated HRP matrices at 40 °C during 17 h

Entry	Biocatalyst[a]	*T* (°C)	Solvent	Conversion[b] (%)	Selectivity[c] (%)	TTN	ee[d] (%)
1	Free HRP	20	CH_3CN	10	99	0.88×10^{3}[e]	15
1b	Free HRP	40	CH_3CN	8	64	0.70×10^{3}[e]	11
1c	Free HRP	40	CH_3CN:buffer[f]	32	65	2.82×10^{3}[e]	6
2	Silica (Sol–gel)	40	CH_3CN	5	95	1.50×10^{-2}[g]	0
3	Encapsulated HRP	40	CH_3CN	48	90	4.22×10^{3}[e]	31

Oxidant:sulfide molar ratio (2:1). The (*S*)-sulfoxide was predominantly formed in all cases.
[a] H_2O_2 (30 wt.%) was added at once.
[b] mol sulfide consumed/mol sulfide added.
[c] mol sulfoxide/mol sulfide consumed.
[d] ee determined by GC-MS and ^1H NMR.
[e] TTN (mol sulfide consumed/(mol HRP)).
[f] CH_3CN:phosphate buffer (10 mM, pH 7).
[g] TTN (mol sulfide consumed/mol Si).

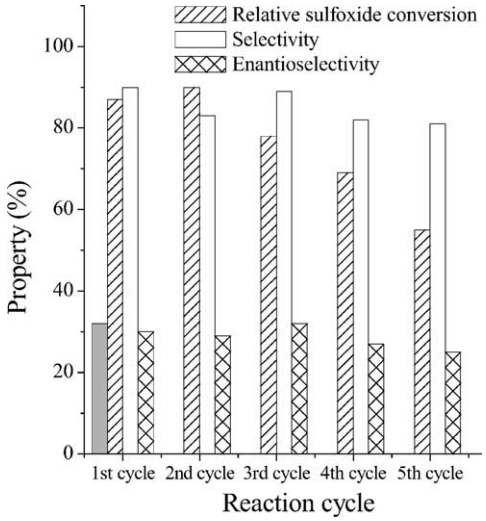

Fig. 2. Recyclability of the sol–gel encapsulated HRP. Relative sulfoxide selectivity (white bars), enantiomeric excess (squared bars) and conversions (lined bars) after 48 h of reaction at different reaction cycles. Relative sulfoxide conversion after 17 h (gray bar) is also showed for the first reaction cycle.

10^3) than for the aforementioned (~9). The threefold increase of the enantiomeric excess found for the encapsulated HRP as compared to the free HRP also confirms the predominant contribution of enzymatic catalysis, i.e. silica catalysis gives rise to non-enantioselective contribution.

3.3. Reusability of encapsulated HRP

An additional advantage of this heterogeneous catalyst is its capability to be successfully recovered by simple centrifugation once the reaction is concluded. No further conversion in the reaction system was observed after removal of the catalyst, which confirms the lack of active protein leaching to the reaction media. Otherwise, oxidation would continue after the removal of the heterogeneous catalyst. Fig. 2 shows the catalytic activity of the sol–gel encapsulated HRP towards the oxidation of thioanisole for up to five reaction cycles. The catalyst was recovered after every reaction cycle as described above. For every cycle, data regarding conversion, selectivity and enatioselectivity were obtained after 48 h of reaction. Similar recovered values of selectivity and enatioselectivity together with higher degrees of conversion than those obtained after 17 h (Table 1) confirm the robustness of the encapsulated biocatalyst. It is observed that both the selectivity and the enantioselectivity in regards to the sulfoxide oxidation are maintained all over the cycles, while the conversion begins to decrease at the fourth cycle. Since no significant changes on the enzymatic activity were found up to 6 months after preparation, the observed conversion decrease should be ascribed to partial heme leaching and/or heme degradation after successive reactions [18,23].

3.4. Study of the coupled GOx/HRP system

As mentioned above, the stereoselective biotransformations can be improved by the slow addition of the oxidant to the reaction bath. Coupled enzymatic systems have been successfully employed for the in-situ and slow generation of H_2O_2 in sulfoxidations carried out in buffer solutions [5–7]. Co-immobilization of glucose oxidase/soybean peroxidase in polyurethane foams has been shown to greatly induce not only the enantioselectivity but also the oxygen transfer activity with a significant increase of TTN as compared to its solution counterpart [5]. The simultaneous incorporation of HRP and GOx in a sol–gel matrix resulted in less than 5% of conversion (Table 2; entry 6), which is ascribed to diffusion limitations of glucose substrate in sol–gel matrices [27]. To avoid such undesired diffusion effects, we used the tandem GOx/HRP without co-immobilization of the glucose oxidase (Table 2; entry 5). In this case, a significant increase of both selectivity and enantiomeric excess were observed, while the conversion and TTN remained in range to those obtained for the encapsulated HRP (Table 1; entry 3). Note that the use of free GOx/HRP in acetonitrile/buffer solution with lack of pH-stat (Table 2; entry 4) results in a significant decrease of the conversion as compared to the encapsulated HRP + GOx (Table 2; entry 5). It is remarkable that the value of the obtained enantiomeric excess is comparable to that found for GOx/HRP in aqueous solution when pH-stat is used (64%) [7]. The overall behavior described above points out how effective is the HRP protection obtained upon encapsulation, i.e. the slow incorporation of the oxidant is unable to further protect HRP and it just results in the minimization of non-enantioselective side reactions.

Table 2
Oxidation of thioanisole (0.26 M) catalyzed by sol–gel encapsulated HRP at 40 °C in CH_3CN:phosphate buffer (10 mM, pH 7) during 17 h

Entry	Biocatalyst	Conversion[a] (%)	Selectivity[b] (%)	TTN[c]	ee[d] (%)
4	Free (GOx/HRP)	15	98	1.32×10^3	48
5	Encapsulated HRP + Gox	51	100	4.49×10^3	56
6	Encapsulated (GOx/HRP)	<5	–	–	–

Oxidant:sulfide molar ratio (2:1). The (*S*)-sulfoxide was predominantly formed in all cases.
[a] mol sulfide consumed/mol sulfide added.
[b] mol sulfoxide/mol sulfide consumed.
[c] TTN (mol sulfide consumed/mol HRP).
[d] ee determined by GC-MS and ^{1}H NMR.

4. Conclusions

We have reported on the encapsulation of HRP in a sol–gel matrix to obtain a heterogeneous catalyst, which has shown a high operational stability in the non-aqueous enzymatic asymmetric sulfoxidation of thioanisol under harsh experimental conditions. The reported increase in TTN, selectivity, and enantioselectivity has been correlated to the peroxidase activity protection exerted upon encapsulation. The effective protection of the encapsulated HRP allows the simultaneous addition of every reactive at the reaction bath even at synthetic scale conditions (i.e. high concentrations) with no degradation of the enzyme activity. The effective protection of the encapsulated HRP is corroborated by the lack of improvement of the operational stability when a coupled enzymatic system (e.g. glucose oxidase–peroxidase) is used. Furthermore, the enantiomeric excess value obtained in this case is comparable to those reported in solution under mild conditions. Reusing of the heterogeneous biocatalyst after reaction is demonstrated.

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