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Performance of Chloroperoxidase Stabilization in Mesoporous Sol-Gel Glass Using *In Situ* Glucose Oxidase Peroxide Generation

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Abstract

A unique mesoporous sol-gel glass possessing a highly ordered porous structure (with three pore sizes of about 50, 150, and 200 Å diameter) was used as a support material for immobilization of the enzyme chloroperoxidase (CPO). CPO was bound onto the glass via a bifunctional ligand, trimethoxysilylpropanal. *In situ* production of the cosubstrate, H_2O_2, was achieved using glucose oxidase. Solvent stability in acetonitrile mixtures was enhanced when a pore size larger than the size of CPO was used (i.e., 200 Å). From these results, it appears that the glass-enzyme complex developed through the present work can be used as high-performance biocatalysts for various chemical-processing applications, particularly in harsh conditions.

Index Entries: Sol-gel glass; chloroperoxidase; glucose oxidase; acetonitrile; horseradish peroxidase; thermostability.

Introduction

Chloroperoxidase (CPO) is a very versatile enzyme capable of carrying out a number of reactions including epoxidation *(1,2)*, sulfoxidation, alcohol oxidation, N-dealkylation, *(3)* and hydroxylation in the presence of a suitable reductant *(4–7)*. Most of these hydrophobic molecules require the use of an organic solvent in the reaction medium to enhance solubility. However, the enzyme has very low activity in organic solvents *(8)*, reducing its potential for industrial application.

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Factors that affect the activity of enzymes, in general, in an organic medium are structural denaturation, substrate desolvation, degree of hydration, and diffusional limitations (owing to the insolubility of native enzymes) *(9)*. For redox enzymes, another complication is the requirement that cosubstrates be provided (e.g., H_2O_2 or other cofactors). Various activation and stabilization methodologies for enzymes have been reported. These include covalently modifying enzymes with chemical groups *(10–13)*, complexation with surfactants *(14,15)* or polymers *(16)*, freeze-drying with inorganic salts *(17)*, and incorporation of enzymes into polymeric supports *(18–20)*. In particular, the covalent binding of enzymes to solid supports can effectively extend the lifetime of biocatalysts by protecting the native three-dimensional structure of enzyme molecules. Traditional enzyme immobilization technologies have been developed mostly based on consideration of the reuse of biocatalysts *(21,22)*, but the detrimental mass transfer effect usually leads to very low apparent enzyme activity and thus considerably limits the effectiveness of the biocatalysts. By contrast, attachment of enzyme to solid supports may result in enhanced enzyme activity in organic solvents as compared with that of the native enzyme in the same reaction medium. It has been demonstrated that the incorporation of enzymes into synthetic polymers, especially those via multiple covalent bonds, can significantly improve their activities in a nonaqueous environment *(18–20,23,24)*. Particularly, the plastic enzymes showed activities that were comparable or even higher than those of enzymes solubilized via ion pairing with surfactant in organic solvents *(18)*.

The *in situ* incorporation of enzymes into inorganic materials, as compared with organic materials, is rather difficult. Nevertheless, sol-gel silica materials have been investigated by different groups as an alternative to organic matrices *(25,26)*. The requirement for cosolvent to overcome the low aqueous solubility of alkyl silicate precursors and the formation of alcohols during the gelation reaction process, however, can adversely affect enzyme activity. Even though different precursors (such as poly-[glyceryl silicate]) have been examined in an effort to generate milder sol-gel conditions *(27)*, *in situ* sol-gel incorporation has been mostly applied in the development of enzyme-coated electrodes for analytical applications, in which enzyme is entrapped in a sol-gel thin film attached to electrodes *(26,28,29)*. In most of these applications, the effective enzyme loading is not as critical as in other bioprocessing applications.

Immobilization onto inorganic surfaces generally severely limits the possible enzyme loading. Recent breakthroughs, *(30–33)* in catalyst synthesis have resulted in a novel methodology for preparing mesoporous inorganic materials with extremely high surface areas and ordered mesostructure. Mesoporous silicon, aluminum, and transition metal oxides have been prepared. The essence of this new methodology is the use of molecular assemblies of surfactants or related substances as structure templates during the formation of oxides. The surfactants used in synthesis can be cationic, anionic, or neutral, depending on the charge of

the inorganic precursors. Unlike microporous materials, which can only interact with enzyme molecules on outer surfaces, mesoporous materials have larger pore openings that allow access to the inner pore surfaces. In a recently published article, mesoporous silica materials (pore diameter ranged from 27 to 92 Å) were used to physically absorb horseradish peroxidase (HRP), and improved enzyme activity in organic solvents and enhanced thermostability in aqueous solutions were demonstrated *(34)*. The use of highly porous glass may allow the enzyme to be hosted inside the microchannel via multiple point attachment, which is expected to improve enzyme structural stability *(35)*. In addition, the mass transfer of organic chemicals in these materials is far more efficient than in conventional microporous catalysts, such as zeolites, because of their unique mesoporous pore diameters (20–250 Å). Immobilization of other peroxidases into sol-gel material has been shown to improve their operational stability *(33,36,37)*. An HRP–sol-gel catalyst was shown to be recyclable for multiple reactions without loss of activity. These considerations prompted us to investigate immobilization of industrially useful enzymes in ordered mesoporous hosts. We recently reported immobilization of α-chymotrypsin into mesoporous silica *(38)*. Here, we extend that work to immobilize CPO into sol-gel and evaluate its thermostability and solvent stability.

A second hurdle in industrial applications of CPO is its sensitivity to H_2O_2, a required cosubstrate. The enzyme undergoes a mechanism-based inactivation in the presence of primary olefins and peroxide *(39)*. Small amounts of allylbenzene can result in formation of *N*-alkylporphyrin resulting in deactivation. This problem can be solved in two ways: first, by controlled addition or *in situ* generation of H_2O_2 and second, by modifying the enzyme (genetically) to prevent inactivation. Use of *glucose oxidase* (Go_x) allows *in situ* generation of H_2O_2. This is a convenient although more expensive, way of providing H_2O_2 compared to a pump-based continuous addition. A mutant enzyme that does not denature in the presence of excess peroxide has also been produced *(40)*. Another way to protect the enzymes from oxidation by peroxide is sol-gel immobilization *(41)*. The activity was reported to be unchanged apparently owing to preservation of the protein structure in the sol-gel matrix. We therefore were interested in evaluating the effect of sol-gel immobilization on CPO and report the results here.

Materials and Methods

CPO Assay

The standard chlorination assay for measuring CPO activity is as follows: 100 m*M* potassium phosphate buffer, pH 2.75; 0.16 m*M* monochlorodimedone (MCD); 0.3 mg/L of CPO, 10 m*M* KCl. The typical H_2O_2 concentration was 2 m*M*. The peroxide stability was studied by varying the concentration of H_2O_2 and by using *in situ* produced peroxide.

The assay procedure was modified to study the effect of solvent concentration and use of CPO with *in situ* H_2O_2. To produce H_2O_2 *in situ*, 24 µmol of glucose and 20 µg GOx were added in a 3 mL reaction mixture. The disappearance of MCD was monitored spectrophotometrically at 278 nm with an extinction coefficient of 12,200 M^{-1} cm^{-1}. The various enzymatic reactions are as follows:

CPO Standard Assay

$$MCD + Cl^- + H_2O_2 \rightarrow CPO \rightarrow Dichlorodimedone + H_2O + OH^-$$

GOx Standard Assay

$$\beta\text{-D-Glucose} + O_2 + H_2O \rightarrow GOx \rightarrow D\text{-Glucose-1,5-lactone} + H_2O_2$$

$$H_2O_2 + o\text{-Dianisidine (reduced)} \rightarrow HRP \rightarrow o\text{-Dianisidine (oxidized)}$$

Combined *in situ* H_2O_2 CPO-GOx Reaction

$$\beta\text{-D-Glucose} + O_2 + H_2O \rightarrow GOx \rightarrow D\text{-Glucose-1,5-lactone} + H_2O_2$$

$$MCD + Cl^- + H_2O_2 \rightarrow CPO \rightarrow Dichlorodimedone + H_2O + OH^-$$

The peroxidase activity of CPO was measured using other assays that utilize other substrates such as 2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and *o*-dianisidine. A typical assay consisted of 1 m*M* substrate in 100 m*M* potassium phosphate, pH 5.0, and 2 m*M* H_2O_2. The absorbance for ABTS was measured at 414 nm using an extinction coefficient of 3.6×10^4 M^{-1} cm^{-1}, and that for *o*-dianisidine was measured at nm using 11,600 M^{-1} cm^{-1}. Preliminary experiments with solvents indicated loss of activity at higher solvent concentrations and were therefore repeated using alternate substrates, in order to confirm that the loss of activity was not owing to substrate desolvation.

Solvent Stability

Acetonitrile (MeCN) was used as a model solvent to measure the stability of CPO in a solvent environment. Enzyme assays were performed as just described with the addition of MeCN. The experimental problem of salt precipitation was minimized by using citric acid (7.5 *M*) for pH adjustment and reducing the phosphate buffer concentration to 10m*M*. The concentration of the remaining reagents in the CPO assay remained the same.

Thermostability

The enzyme was incubated for a given amount of time at different temperatures, after which the activity was measured by the standard assay at 30, 40, and 50°C using MCD as the substrate. GOx was used for *in situ* production of H_2O_2.

CPO Immobilization in Sol-Gel

Preparation of Ordered Mesoporous Silica Support

The typical procedure *(42)* involved mixing 4 g of nonionic triblock copolymer L123 (BASF), 20 g of water, and 80 g of 2 *M* HCl. To this solution, 8 g of tetraethylorthosilicate was added at room temperature. The mixture was then stirred at room temperature for 24 h. The solid product was recovered by filtration. The polymer template was removed by calcination at 500°C for 5 h.

Ligand Synthesis

Trimethoxysilylpropanal was synthesized via a method similar to the one reported by Takeuchi and Soto (43). A solution of 20.0 g (135 mmol) of vinyltrimethoxysilane, 92 mg (0.01 mmol) of $RhH(CO)(PPh_3)$, and 0.21 g (0.8 mmol) of PPh_3 in 100 mL of toluene was placed in a 300-mL autoclave. The vessel was loaded with 600 psi of CO and stirred for 10 min followed by the addition of H_2 to 1200 psi total pressure ($CO/H_2 = 1/1$). It was then heated at 80°C for 4 h under stirring (70 rpm). The products (90%) were isolated by distillation under vacuum (0.5 torr, 45–50°C) and consisted of the mixture of two isomers (normal and iso) at a ratio of normal:iso of 95:5 (38).

CPO Immobilization

In a typical immobilization procedure, 0.5 g of porous silica glass was added to 5 mL of ethanol at 40°C. The mixture was stirred for 1 h to wet the glass thoroughly, and 0.2 g of the trimethoxysilylpropanal ligand and 0.5 mL of water were added to the mixture. One hour later, the mixture was centrifuged and the supernatant solution was then removed. The reagent-coated gel was then washed extensively with ethanol, then water, to remove residual reagent. The washed sol-gel was mixed with CPO (2.0 mL of 7.6 mg/mL solution in water) and stirred at 4°C for 48 h. The mixture was centrifuged, and the protein loading was determined by a difference between the enzyme activity of the supernatant solution and that of the initial enzyme solution. The enzyme-bound glass was washed five times with 25 mL of deionized water until no absorbance at 280 nm was observed in the washing solution and then dried by purging with N_2 gas. The enzyme activity of all washes was measured. The CPO was immobilized on sol-gels with three different pore sizes: 50, 150, and 200Å.

CPO Sol-Gel Activity Measurements

Sacrificial sampling was used to assess the activity of sol-gel–immobilized CPO over a 3-h period. H_2O_2 was provided via the *in situ* reaction with GOx. Experiments were conducted in by preparing solutions of sol-gel enzyme preparations in assay buffer in a centrifugal filter unit. About 10 mg of sol-gel enzyme was used for each assay. The purpose of the filter was to allow easy separation of the solid-phase enzyme for measurement

of changes in substrate concentration by spectrometry at the time of assay (5-min period). These mixtures were incubated for 30, 60, 100, and 180 min at 40 and 50°C, followed by the standard activity assay. Sample was centrifuged to separate the sol-gel from the supernatant, and the concentration of the substrate in the supernatant was measured every minute for 5 min. After absorbance measurement, the solution was returned to the centrifuge tube containing the sol-gel for another minute of incubation. The stability of the sol-gel enzyme in solvents was measured in a similar manner.

Results and Discussion

Effect of Peroxide

Experiments were conducted to evaluate two modes of supplying peroxide for the CPO reaction. In one reaction, it was supplied as an H_2O_2 solution, and in the second it was produced *in situ*. Figure 1 shows the progress of the reactions in the two modes. In the presence of 2 m*M* H_2O_2 (added externally), the enzyme lost than 50% of its activity in (Fig. 1A). This drop in activity was determined by taking the first derivative of the polynomial equation and then calculating the rate at time (x) = 1 and 60. The substrate:peroxide ratio under these conditions is 1:25. The use of 20 m*M* peroxide resulted in similar substrate disappearance curves (data not shown). Lloyd and Eyring *(41)*, who studied the stability of HRP, reported that a ratio of peroxide to substrate of 50 resulted in 90% loss of enzyme activity, whereas a ratio of 5 resulted in no loss of activity. To prevent enzyme inactivation, the alternate method of *in situ* peroxide production was tested. This would allow better control of both the peroxide substrate ratio and the excess peroxide concentration. The results (Fig. 1B) show that *in situ* production of the peroxide at a controlled rate did not result in enzyme deactivation. These results demonstrate the utility of the GOx method for supplying peroxide. We also studied the peroxide stability of HRP and found the results to be very similar (data not shown). The rate of H_2O_2 production was determined separately using an assay coupled with HRP. The rate under the conditions studied (0.013 mg/mL of GOx, 0.067 m*M* glucose, 3-mL reaction volume) was found to be 0.16 µmole/min. Under similar conditions, the substrate:peroxide ratio would not reach 1:25 for at least 30 min.

Effect of Solvent Concentration on Free CPO Reaction

The solvent stability of CPO was measured by varying the MeCN concentration. The enzyme ceased to be active above 40% MeCN (Fig. 2). The stability of GOx was also under question in the solutions with 40% MeCN or higher. This was verified by using HRP in place of CPO (Fig. 3B) along with GOx. The results indicate that GOx was active up to 70% MeCN. This proves that the drop in the rates at 40% and higher MeCN concentrations is owing to inactivation of CPO. Assays measuring peroxidase activity using the alternate substrates *o*-dianisidine and ABTS (Figs. 3A, and 4)

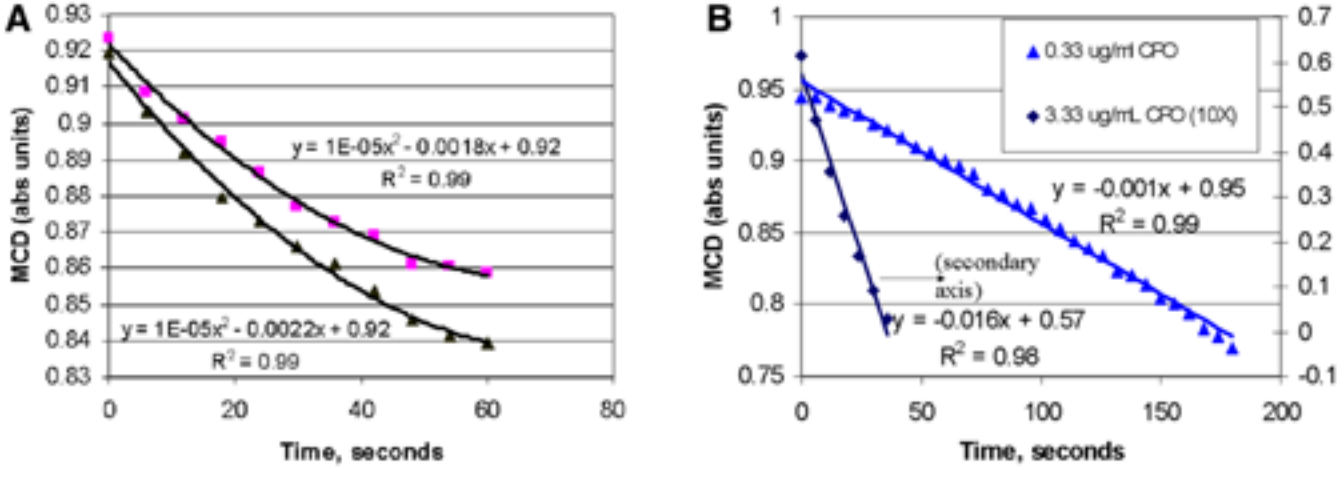

Fig. 1. Conversion of MCD in two different H_2O_2 supply modes: **(A)** direct addition of H_2O_2 to reaction solution in two replicate experiments (CPO concentration = 0.33 µg/mL); **(B)** *in situ* H_2O_2 production using GOx.

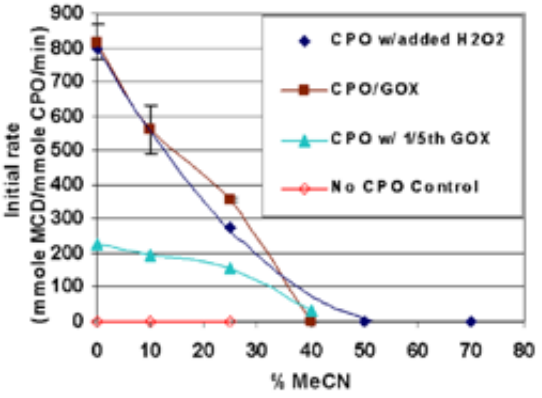

Fig. 2. Conversion of MCD by CPO in MeCN.

in MeCN also demonstrated that CPO was the enzyme that was inactivated at and above 40% MeCN.

CPO assays with added H_2O_2 and combined CPO/GOx assays in 100% aqueous buffer showed a rate of conversion similar to that of MCD. Note that the rates reported in Fig. 2 are initial rates. Similar initial rates for *in situ* and added H_2O_2 suggest that the amount of peroxide generated by GOx was at least equal to the stoichiometric amounts required for reaction with CPO. To verify this, an experiment was conducted with a lower amount of GOx. Reducing the amount of GOx to one-fifth reduced the rates of the CPO reaction proportionately, indicating that the rate was controlled by the rate of peroxide production. This also implies that the peroxide produced would be immediately consumed, and no significant accumulation would occur.

Sol-Gel CPO Immobilization

The extent of CPO immobilized on the sol-gel was determined by the difference between the activity of the initial enzyme solution and that measured in cumulative washes. Based on the cumulative activity lost in six washes, a second preparation of the CPO-bound sol-gel contained 10, 24, and 55 mg of CPO/g of sol-gel for the 50-, 150-, and 200-Å CPO sol-gels, respectively. In prior experiments, the total activity was measured and an estimated 80% of the bound CPO was active. The sol-gel immobilization is expected to limit the unfolding of the protein bound inside pores of the sol-gel. Thus, immobilization is expected to affect solvent stability and thermostability. Immobilization would probably not impact peroxide stability, since the mechanism of peroxide inactivation is associated with changes in the redox properties and oxidation state of the heme iron and the active center, which cannot be protected by immobilization. Experimental studies of immobilized CPO were therefore limited to temperature and solvent stability.

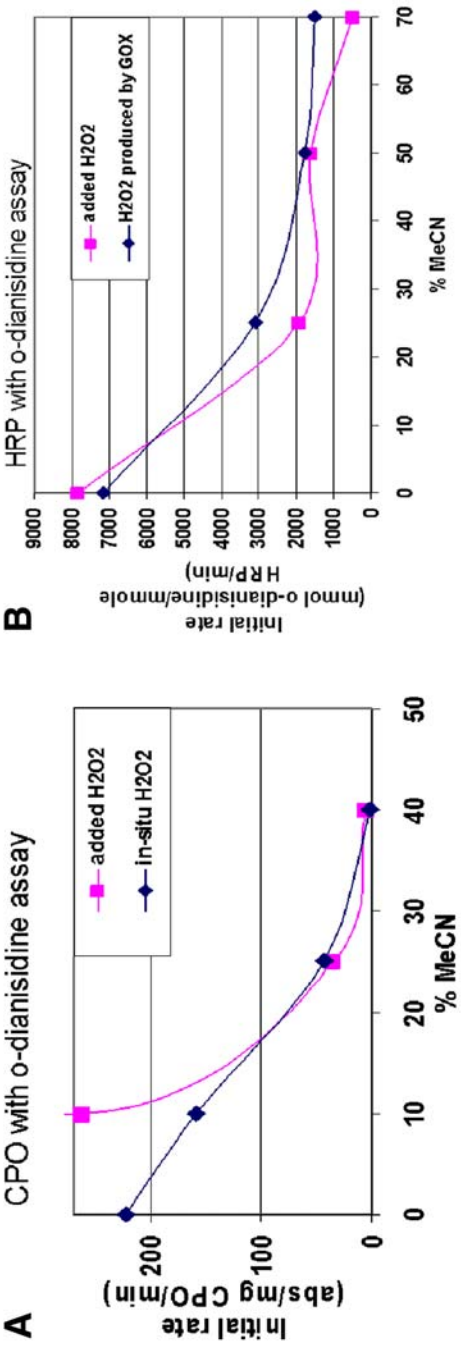

Fig. 3. Conversion of *o*-dianisidine by **(A)** CPO and **(B)** HRP in % MeCN.

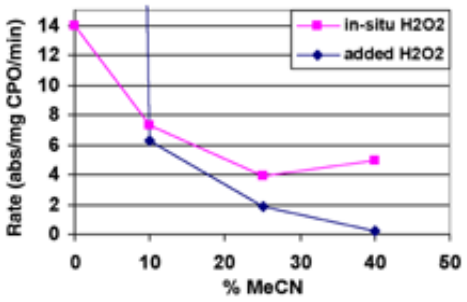

Fig. 4. Conversion of ABTS by CPO at various MeCN concentrations. The maximum activity of CPO was 260 abs units/(mg of CPO·min) and was observed in the absence of MeCN but in the presence of added peroxide.

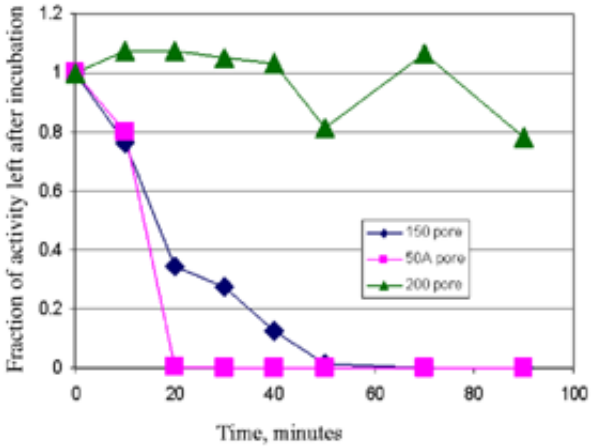

Fig. 5. Effect of solvent on stability of sol-gel–immobilized CPO. The highest stability displayed in 200-Å pore sol-gel may be owing to immobilization of the enzyme within the pores of the sol-gel, whereas it may have been immobilized effectively only on the surface in the case of the other pore sizes.

Effect of Pore Size on Solvent Stability

The 50-Å sol-gel CPO showed no protective effect from immobilization to MeCN. Both free and sol-gel CPO had no activity above 40% MeCN. This is not surprising given that the size of CPO (59×71×92 Å) is larger than the pore size of this sol-gel and the CPO must be bound to the outer gel surface. The largest stabilization was observed with 200-Å sol-gel (Fig. 5). This must be owing to immobilization of the enzyme within the pores of the sol-gel as compared to just surface immobilization, as must be the case with the 50-Å sol-gel. Partial stabilization was observed with the 150-Å sol-gel enzyme. The stabilization of the enzyme within the larger pores is probably owing to multipoint attachment of the enzyme to the glass surface. A convex surface such as in a pore provides several points of attachment as compared to a flat glass surface, where typically a single bond is realized *(38)*.

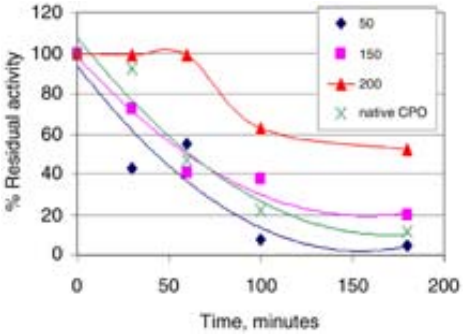

Fig. 6. Thermostability of CPO immobilized on sol-gel material of different pore sizes.

Thermostability

Free CPO lost 95% activity within 3 h when incubated at 40ϒC, whereas sol-gel CPO lost only 50% activity under this condition (Fig. 6). At an incubation temperature of 50ϒC, no protection was observed by sol-gel immobilization for CPO. Only the CPO immobilized in 200-Å sol-gel retained activity over 3 h at 40ÀöC.

Conclusions

Nonaqueous enzymatic redox reactions have been limited by stability owing to solvents and highly reactive substrates (H_2O_2). Here we have shown evidence of methods to alleviate these concerns for reactions with CPO. In experimental systems, the *in situ* production of H_2O_2 by GOx was shown to function equally well and more reproducibly than added H_2O_2. *In situ* production is experimentally easier and prevents enzyme deactivation owing to high peroxide levels. GOx was more solvent stable than CPO; therefore, the GOx system may be useful for this and other redox systems.

Immobilization has also been shown to stabilize against solvent denaturation of enzymes. However, here we presented suggestive data on the mechanisms of this stabilization. Only the CPO immobilized in 200-Å sol-gel showed any solvent or temperature stabilization. CPO bound to matrices with pores smaller than the protein showed little or no stabilization effect owing to surface immobilization alone. This supports the concept that steric hindrance to protein unfolding within a pore is part of the stabilization mechanism. An unresolved question for the future applications of this research is to increase the overall enzyme activity or loading.

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