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Catalytic activity of mesoporous silicate-immobilized chloroperoxidase

Yong-Jin Han, Jordan T. Watson, Galen D. Stucky, Alison Butler*

Department of Chemistry and Biochemistry, Materials Research Laboratory, University of California at Santa Barbara, Santa Barbara, CA 93106, USA

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Abstract

A versatile enzyme, FeHeme chloroperoxidase (CPO) from *Caldariomyces fumago*, is immobilized in the mesoporous silicate material, mesocellular foam (MCF). MCF is a promising material for immobilizing enzymes, due to its large pore structure and high loading capacity compared to other mesoporous materials, such as MCM-48, SBA-16 and SBA-15. The immobilized CPO in MCF retains its activity. The optimal pH at which the maximum amount of enzyme is immobilized was determined to be pH 3.4, slightly below the isoelectric point of the enzyme. A weak ionic interaction between the enzyme and the surface of the inorganic substrate is thought to be critical in maintaining the activity of the immobilized enzyme. The loading capacity of MCF is 122 mg protein per 1 g of MCF. We demonstrate the advantage of MCF as an inorganic substrate for immobilization of enzymes. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Immobilization; Chloroperoxidase; Mesoporous silicate; MCF; SBA-15

1. Introduction

Immobilization and encapsulation of enzymes on solid inorganic materials have been the focus of intense studies due to potential applications in biocatalysis [1] and biosensors [2,3]. Inorganic supports with favorable surfaces for the immobilization of enzymes, which result in high enzyme activity have been highly sought [4]. Sol–gels have been on top of the short list since the surprising findings of Ottolenghi and co-workers [5] (and references therein) that encapsulated enzymes inside sol–gel matrices maintained their activities. In some cases, sol–gel encapsulation was even found to increase the stability of the enzymes and proteins [6]. As Ottolenghi and co-workers have found, substrates diffuse into the network of pores and channels of the sol–gel matrix to reach the active sites of the encapsulated enzymes to produce the product. However, the disordered network of pores and channels in the sol–gel matrix limits the reactions allowed. In a typical synthesis of sol–gels, tetramethylorthosilicate (TMOS) or tetraethylorthosilicate (TEOS) is hydrolyzed and then condensed to make SiO_2-based materials. In this synthesis, however, formation of the channels and the pores is not controlled, and various sizes of pores and channels are formed, ranging from 0.1 to 500 nm in size. Often interconnected micropores and channels are formed, allowing only the smallest of the substrates to penetrate, while the bigger substrates clog the channels, slowing the reactions. Favorable interaction between enzymes and the SiO_2 surface in the sol–gel encapsulation method

* Corresponding author. Tel.: +1-805-893-8178; fax: +1-805-893-4120.
E-mail address: butler@chem.ucsb.edu (A. Butler).

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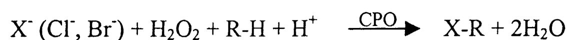

Scheme 1.

have been observed [7–11]. Controlling the pores and channels of defined size to guide the substrates to the active sites of the encapsulated/immobilized enzymes, while maintaining the support materials of the sol–gel encapsulation method would be a great improvement to the proven system.

Previously, Balkus and co-workers [12,13], and others [14–16] have immobilized enzymes onto MCM-type mesoporous silicate materials. While they have been successful in immobilizing small enzymes, MCM-type materials are still restrictive due to the limit of their pore diameter (ca. ∼80 Å). The recent discoveries of various mesoporous silicate materials, such as SBA-15 [17] (pore size ca. 50–130 Å) and mesocellular foam [18,19] (MCF, pore size ca. 150–400 Å), provide new avenues for encapsulation/immobilization processes and solve the problems mentioned above. We have shown previously [20] that these mesoporous silicate materials, with variable pore sizes and susceptible surface areas for functionalization, can be utilized as good separation devices for proteins, where the proteins are sequestered and released depending on their size and charge, within the channels. We have expanded the encapsulation technique further by utilizing the advantages of the MCF mesoporous silicates to immobilize various biomolecules within the galleries. In particular, FeHeme chloroperoxidase (CPO) (*Caldariomyces fumago*) [21–26,35] is being studied for its catalytic activity and stability upon immobilization. CPO catalyzes peroxidative halogenation reactions (Scheme 1), as well as epoxidation of alkenes in the absence of halide ion. Further versatility can be achieved by immobilizing CPO, leading to new industrial and biocatalytic applications. While few examples of CPO immobilization are known [27–30], we report here the first immobilization of CPO into mesoporous silicate materials.

2. Method and materials

All of the surfactants in the experiments were used as-received. P123 and F108 were obtained from BASF, and B50-6600 was obtained from Dow. CPO and monochlorodimedone (MCD) were obtained from Sigma and cetyltrimethylammonium bromide (CTAB), trimethylbenzyene (TMB), TEOS and fumed silica were used as obtained from Aldrich.

2.1. Instrumentation

Small-angle X-ray analysis was performed on a Scintag PADX diffractometer using Cu Kα radiation detected by a Si (Li) solid-state detector cooled by a Peltier cell. Pore sizes of the materials were determined by BdB-FHH pore size analysis [31] from the data obtained by nitrogen sorption, carried out on a Micromeritics ASAP 2000 system at 77 K with samples outgassed at 180–200 °C under high vacuum for at least 10 h, which also gave BET surface areas and pore volumes. Enzyme assay and other UV–VIS experiments were performed with Varian Cary 300 UV–VIS spectrophotometer.

2.2. Synthesis of mesoporous materials

MCM-48 materials were prepared according to the methods reported by Sayari [32]. CTAB of 5.89 g was dissolved in H_2O (37.1 ml) containing trimethylammonium hydroxide (TMAOH, 25 wt.%, 3.85 g). This mixture was stirred for 15 min, after which 2.0 g of fumed silica was added. The entire mixture was stirred for additional 45 min and then the mixture was transferred to an autoclave and thermally treated at 130 °C for 96 h. The final product is filter dried and calcined at 550 °C for 12 h. SBA-16 type cubic materials [33] were synthesized using non-ionic surfactant mixtures of Pluronic F108 (poly (ethylene oxide)-*block*-poly(propylene oxide)-*block*-poly(ethylene oxide), EO_{132}-PO_{50}-EO_{132}) and B50-6600 (poly(ethylene oxide)-*block*-poly(butylene oxide)-*block*-poly(ethylene oxide), EO_{39}-BO_{47}-EO_{39}).[1] A typical synthesis consisted of 1.2 g of F108 and 0.6 g of B50-6600 dissolved in HCl (65 g of 1.85 M) at RT, after which 4.25 g of TEOS was added. This mixture was then thermally treated at 100 °C for 48 h, and calcined at 550 °C for 6 h. The detailed synthesis method of SBA-15 [17] and MCF

[1] Modified method from [33] to obtain high ordered SBA-16 at room temperature using mixed surfactants.

[18,19] type materials has been reported elsewhere. In a typical preparation, triblock copolymer Pluronic P123 (poly(ethylene oxide)-*block*-poly(propylene oxide)-*block*-poly(ethylene oxide), EO_{20}-PO_{70}-EO_{20}, $M_{AV} = 5800$, BASF/Aldrich, 2.0 g, 0.4 mmol) was dissolved in HCl (1.6 M, 75 ml, 120 mmol) at 37–40 °C. The pore size can be increased by adding an appropriate amount of 1,3,5-trimethylbenzene (TMB, 0–5 g) to the polymer solution, after the polymer has completely dissolved in the aqueous solution. After 1 h, tetraethyl orthosilicate (TEOS, 4.25 g, 21 mmol) is added. After stirring for 24 h at 37–40 °C and aging at 100 °C for 24 h, the solids are collected by filtration and dried under vacuum in air. The isolated white powders are calcined at 500 °C for 8 h.

2.3. Immobilization of CPO to mesoporous materials

A concentrated CPO solution (1250 U/ml) was diluted with 5 mM citrate buffer, pH 3.4, to make a stock solution (30 μl enzyme per buffer of 30 ml).[2] This stock solution (5 ml) of the diluted enzyme was added to 10 mg of mesoporous material. The mixture was stirred for 1 h for the immobilization to take place at room temperature. The solids with the immobilized enzyme were recovered through centrifugation. The supernatant of the enzyme solution was saved and also assayed for CPO activity. The recovered solid was washed once with the same buffer solution (5 mM citrate buffer) and then resuspended with 5 ml of the citrate buffer (pH 3.4) and kept at 4 °C until use. Immobilization of CPO at various pH values were performed with citrate buffer (pH 2.75, 3.0, 3.4, 4.3, 5.0, 5.6, 6.45), phosphate buffer (pH 7.0) and tris buffer (pH 8.0).

2.4. Enzyme activity assay

The MCD assay [34] was performed to determine the enzyme activity of immobilized CPO. Typically, 0.970 ml of MCD and KCl solution (0.1 mM of MCD and 20 mM KCl in 5 mM citrate buffer at pH 2.75), along with 10 μl of the immobilized enzyme solution were added to a quartz cuvette (path length = 1 cm). The reaction was initiated with the addition of 20 μl of 0.09M H_2O_2.

[2] One unit of CPO will catalyze the conversion of 1.0 μmol of monochlorodimedon to dichlorodimedon per min at pH 2.75 at 25 °C in the presence of potassium chloride and H_2O_2 (Sigma Catalog).

3. Results and discussion

3.1. Effect of pH on immobilization

The optimum pH at which CPO binds to MCF while maintaining maximum activity was determined by performing the immobilization process at various pH values. As seen from Fig. 1, the maximum activity of the immobilized enzyme is observed at pH 3.4 (5 mM citrate buffer). The isoelectric point (pI) of CPO is ∼4.0, thus at pH 3.4, the overall net charge of the protein is slightly positive. At this pH, the surface of the SiO_2 framework of MCF has an overall negative charge since the isolectric point of SiO_2 is pH ∼2. Therefore, an electrostatic interaction between the two is expected. When the immobilization is carried out at pH values less than 3.4 (i.e. pH 2.75 and 3.0), the same amount of enzyme is immobilized, however, the activity of this immobilized enzyme is lower than when the immobilization process is carried out at pH 3.4.[3] It should be noted that although the immobilization process took place at different pH values, the enzyme assay for each sample was performed under the standard CPO assay conditions at pH 2.75. The decrease activity is possibly due to strong interactions of the charged CPO with the framework; CPO would have a greater positive charge at pH 2.75 versus pH 3.0 or 3.4.[4]

When the pH of the immobilization is increased above pH 3.4 (i.e. pH 4.3) less CPO is immobilized. The decrease in immobilization is apparent from the increased activity remaining in the supernatant (Fig. 1) compare to the process carried out at pH 3.4. At pH 5.5, i.e. above the isoelectric point of CPO, CPO is not

[3] When immobilization is carried out at pH 2.75, 3.0 and 3.4, all of the CPO is sequestered by MCF, as shown by the lack of CPO activity in the supernatant.

[4] This result is also supported by the fact that when CPO is immobilized at higher pH (7) with primary amine-functionalized MCF, the interaction between negatively charged CPO and positively charged MCF is very strong, and also lowers the activity of the immobilized enzyme dramatically.

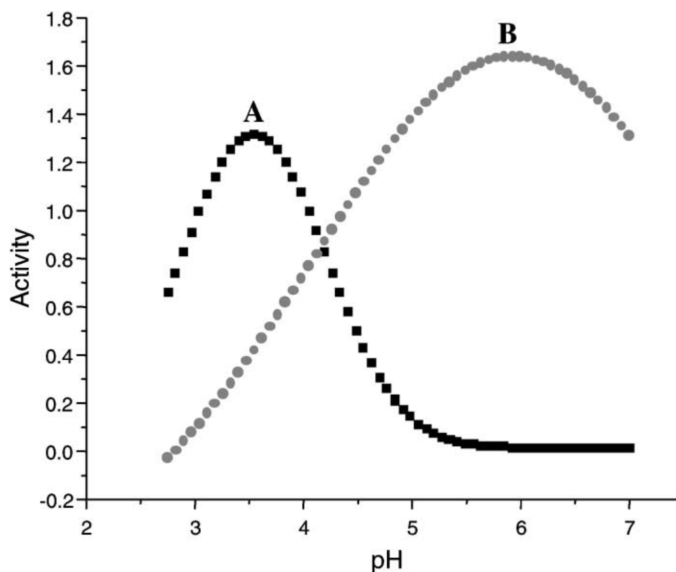

Fig. 1. Effect of pH on immobilization of CPO in MCF. Activity of (A) MCF-CPO; (B) supernatant. 0.2 μM of CPO in various buffer solutions (Section 2) incubated with 10 mg of MCF for each buffer solution for 1 h. The solids and the supernatant were separated by centrifugation and MCD assays were performed at pH 2.75.

immobilized by MCF, which is likely a result of the net negative charge of both the silicate framework and CPO. The immobilization of CPO was also attempted in pure water without success; all CPO activity remained in the supernatant. Thus from these results, we observed that the isoelectric point of SiO_2 and the overall framework charge play important roles in immobilizing enzymes. The strength of the electrostatic interaction between the enzyme and the silicate surface is very important in maintaining the overall activity of the enzyme. This method can be easily adopted for immobilization of various types of enzymes in mesoporous silicates; however, the condition at which the maximum amount of enzyme is immobilized with highest activity will vary depending on the pI and the surface characteristics of the enzymes, assuming the sizes of the enzymes are appropriate.

Leaching of the enzyme from the MCF–CPO material was tested with water by repeatedly washing the immobilized enzymes and measuring the activity of the washed solution. The activity obtained from the washed solutions was minimal.

3.2. *Effect of various mesoporous silicates*

Immobilization of CPO was attempted in various mesoporous silicate materials, including MCM-48, SBA-15, SBA-16, and MCF. As seen from Table 1,

Table 1
Physical properties of the mesoporous materials[a]

Mesoporous materials	Pore size (Å) (window size)	BET surface area (m^2/g)	Pore volume (cm^3/g)
SBA-15–42	42	550	0.4467
SBA-15–70	70	523	0.7619
MCF-150	150 (115)	517	1.553
MCM-48–32	32	1172	1.1023
SBA-16–82	82 (47)	786	0.6543

[a] See Section 2 for details of the synthesis and characterization of these silicate materials.

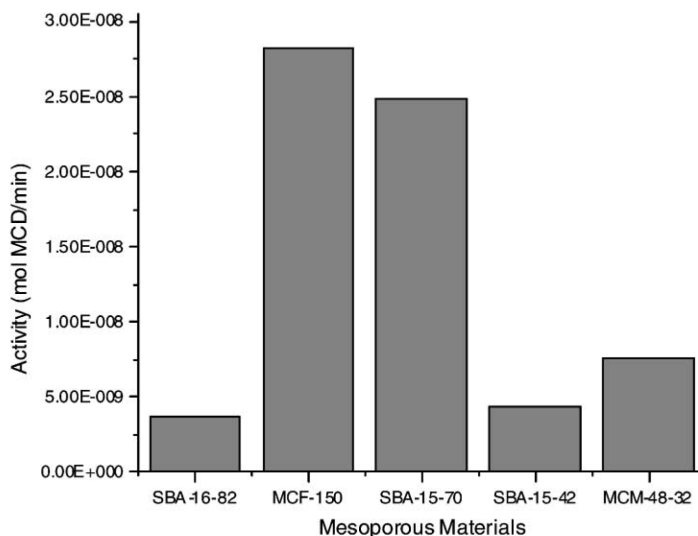

Fig. 2. Comparisons of mesoporous materials for immobilization. The same amount (10 mg) of different mesoporous materials was incubated with CPO (0.2 μM) for 1 h in 5 mM citrate buffer pH 3.4. The solids were separated and their activities were measured by MCD assay at pH 2.75.

the pore sizes of these materials varied from 32 to 150 Å. After the immobilization process, the solids and the supernatants were tested for CPO activity. Fig. 2 shows the activity of the immobilized enzymes with different mesoporous materials. It is clear from this figure that the pore sizes of the materials are crucial in allowing the enzyme to be incorporated within the channels of the mesoporous materials. As demonstrated by the activity of the immobilized enzymes on MCM-48–32 Å, and SBA-15–42 Å, small pore size limits the immobilization process and most of the enzyme is left in the supernatant. However, for SBA-15–70 Å and MCF-150 Å, the activities of the solids are much improved. We can attribute this result to the sufficiently large pores of SBA-15–70 Å and MCF-150 Å, which allow the enzyme to diffuse freely maximizing the number of enzymes immobilized. Large pores also allow substrates easy access to the immobilized enzymes during the assay, thus increasing the activity. Although SBA-16–82 Å has a large enough pore size to incorporate the enzyme, the pore structure of SBA-16 has proven to be a bottle-neck system [35], where a cell and a window type pore exists. Further studies of the pore sizes revealed that the window size of SBA-16 used was approximately 47 Å, which is too small for the enzyme to be incorporated deeper into the matrix, therefore, showing low enzyme activity. The same can be said about the pore structure of MCF materials, but MCF-150 has a window size (115 Å) much larger than the enzyme, which made it possible for the enzyme to diffuse through them.

3.3. Loading capacity of MCF

The loading capacity of mesoporous silicate MCF-150 was determined for protein immobilization using conalbumin. [5] Conalbumin is a good replacement for CPO since it has a similar pI to CPO, as well as similar size and MW [36]. The immobilization was performed under the same conditions with citrate buffer solution at pH 3.4. The loading of the conalbumin was monitored with UV–VIS at $\lambda_{max} = 280$ nm, until no apparent decrease in absorbance was detected in the supernatant (Fig. 3). The loading capacity of MCF-150 was calculated to be 122 mg of protein per gram of MCF-150, which is similar to the numbers obtained by Inagaki and co-workers [16] with the

[5] The amount of CPO needed to test the loading capacity of MCF would not have been cost efficient, hence a cheap and readily available conalbumin was chosen.

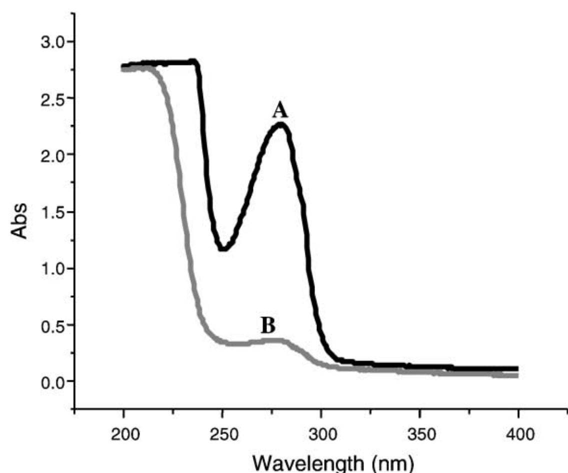

Fig. 3. Loading capacity of MCF with conalbumin. Absorbance of (A) protein solution and (B) protein solution after the addition of MCF. 25 μM of conalbumin in 5 mM citrate buffer solution (pH 3.4) was incubated with 50 mg of MCF for 1 h. The absorbance of the protein solution was measured by UV–VIS at $\lambda_{max} = 280$ nm intermittently after centrifugation until no change in absorbance occurred.

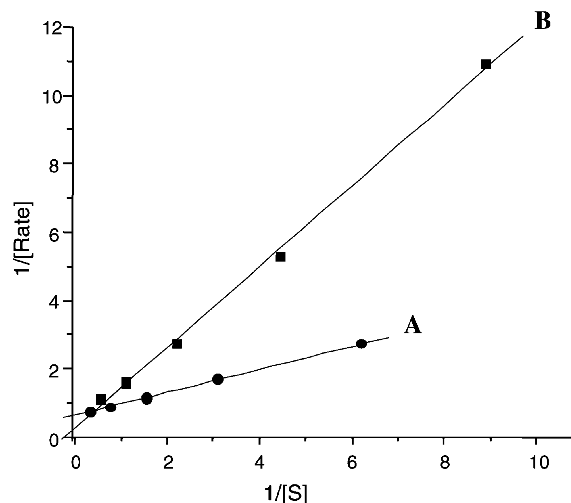

Fig. 4. Lineweaver–Burk plot of MCF-CPO. (A) native-CPO and (B) MCF–CPO. CPO immobilized on MCF-150 at pH 3.4 were assayed with various concentrations of H_2O_2. The inverse of the corresponding activities (1/rate) and H_2O_2 concentrations (1/[S]) are plotted.

MCM materials.[6] However, where as Inagaki and co-workers observed very low loading capacity for SBA-15 type materials (10–24 mg/g of SBA-15), we observed a much greater loading capacity for MCF and SBA-15 materials.

3.4. H_2O_2 dependency of the immobilized enzyme on MCF

The effect of H_2O_2 concentration on the rate of the CPO-immobilized-MCF material was investigated. The rate of the immobilized CPO showed Michaelis–Menton saturation kinetics, $K_m^{H_2O_2}$, demonstrating that the immobilized enzyme still reacts similarly to CPO in solution. However, further analysis of the kinetic data shows that the reaction rate of the immobilized enzymes is reduced compared to the CPO in solution, which is a commonly observed phenomenon with immobilized enzymes. As seen from the kinetic constants obtained from the Lineweaver–Burk plot (Fig. 4), the overall reactivity of the immobilized enzyme (Fig. 4B) is about half of that of CPO in solution (Fig. 4A).[7] The V_{max} of immobilized CPO (1.91 μM/s) and CPO in solution (2.28 μM/s) are comparable, however the $K_m^{H_2O_2}$ value of immobilized CPO (1.08 mM) is higher than the CPO in solution (0.148 mM). The specific activity of the enzymes is also reduced (i.e. immobilized CPO, 361.85 μmol min^{-1} mg^{-1} versus solution CPO, 693.8 μmol min^{-1} mg^{-1}).

Several reasons can account for the decrease in activity and $K_m^{H_2O_2}$ upon immobilization. Firstly, some of the active sites of the immobilized enzymes are probably not available or oriented improperly for them to part take in the reactions. Secondly, the electrostatic interaction of the enzyme with the SiO_2 surface might have changed the conformation of some of the enzymes to inactive forms. The immobilization process does not control the orientation of the enzyme and how it is attached to the surface; therefore, perhaps only half of the immobilized enzymes are oriented properly with their active sites exposed for

[6] The particle sizes of the materials (i.e. SBA-15, MCF and MCM) used typically range 20–80 μm, and are comparable. However, the particle size of the powders does not affect the loading capacity greatly, since the majority of the large surface area of the mesoporous materials arises from the internal surface.

[7] Constants were calculated with an assumption that all of the immobilized enzymes were active.

the reactions to occur, which is the a likely scenario contributing to the lower reactivity of the immobilized enzymes.

3.5. Aging of immobilized enzyme

Stability of MCF-immobilized-CPO, which was stored at 4 °C in citrate buffer solution (pH 3.4), was investigated over several weeks. Over that time period, a small but constant drop in activity occurred. However, the majority of the activity of the enzyme was retained for the duration of the experiments. We investigated whether the loss of the enzyme activity is cause by leaching of the enzyme. Therefore, CPO activity of the supernatant containing the solids was tested. After centrifugation, the supernatant with enzyme immobilized in MCF showed some activity after 12 days of aging time, due to the leaching of the enzyme. However, the supernatant was inactive for SBA-15–70 Å. The large pores of MCF provide easy access for the transport of the enzyme, but the smaller pore sized SBA-15 was more restricting for the immobilized enzyme. Therefore, we conclude that the majority of the activity loss for the immobilized enzyme was due to something other than the leaching effect. This loss of activity is currently being investigated.

3.6. Stability of the immobilized enzymes

The stability of the immobilized enzymes against the denaturants, urea and guanidine salt was also investigated. The immobilized CPO was incubated in solution containing varied concentrations of the denaturants, 2 h in guanidine salt solutions, and 24 h in the urea solutions. As seen from Figs. 5 and 6, the immobilized enzymes behaved in the same manner as CPO in solution enzymes. Immobilization of CPO did not improve the stability of the enzyme against these particular denaturants. Thermo-stability was also tested on the immobilized enzyme. However, after incubation at 70 °C for 1 h, the immobilized CPO lost all of its activity as did native-CPO in solution. According to Inagaki and co-workers [16], thermo-stability of horseradish peroxidase (HRP) was improved upon immobilization by restricting the conformational change of the enzyme in the confined space of the channels of the mesoporous materials. HRP is more inherently thermally stable than CPO in solution, thus, less susceptible to loss in activity upon heating. CPO is more sensitive to heat, and easily loses its activity, therefore, confining the enzyme inside a constricting environment did not improve its stability. Thus, the

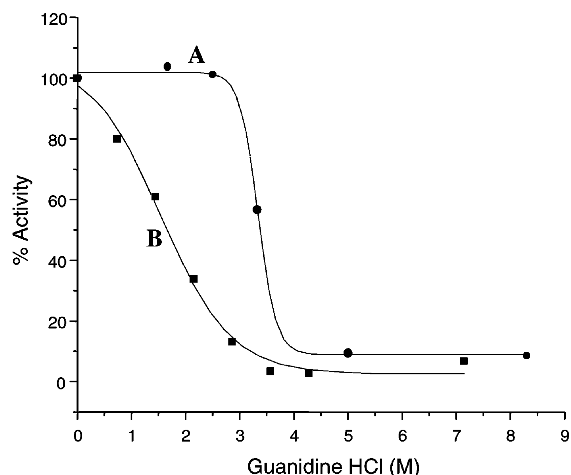

Fig. 5. Comparison of the effect of Guanidine–HCl on (A) native-CPO and (B) MCF-immobilized-CPO. For (A), 1 μM of native-CPO was incubated in various concentrations of guanidine–HCl for 2 h. The activities of the enzymes were monitored with MCD assay at pH 2.75. The same experiments were performed for MCF-immobilized-CPO (see Section 2 for immobilization process).

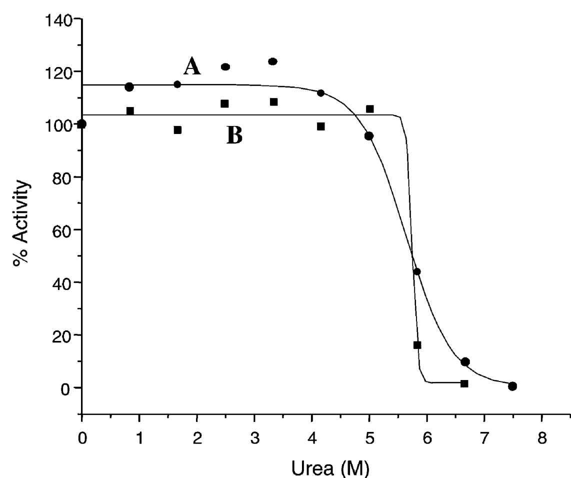

Fig. 6. Comparison of the effect of urea on (A) native-CPO and (B) MCF-immobilized-CPO. An amount of 1 μM of native-CPO was incubated in various concentrations of urea for 20 h. The activities of the enzymes were monitored with MCD assay at pH 2.75.

stability of immobilized enzymes varies depending on the type of enzyme, and thus cannot be generalized.

4. Conclusion

A versatile enzyme, CPO, was immobilized in various mesoporous silicate materials. Mesoporous silicates with large-pore-size structures, SBA-15–70 Å and MCF-150 Å, are best suited for this purpose, since more enzymes can be immobilized and the large porosity of the materials provide better access for the substrates to the immobilized enzyme. The immobilized CPO retains its function and behaves similar to CPO in solution. The pH at which the immobilization takes place is very important in maintaining the enzyme function as well as maximizing the amount of enzyme immobilized. The conditions for immobilization vary depending on the pI, the surface characteristics, and the size of the enzyme, among other factors. Further experiments to control the orientation of the enzymes during the immobilization process are currently in progress. The use of MCF will expand the range of not only biomolecules, but also other materials for immobilization.

Acknowledgements

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