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Covalent immobilization of chloroperoxidase on silica gel and properties of the immobilized biocatalyst

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Abstract

Immobilization of chloroperoxidase (CPO) isolated from the mold *Caldariomyces fumago* on silica gel was performed. The support was derivatized with 3-glycidoxypropyltrimethoxysilane by using a reported method and the enzyme was covalently bound via reaction with epoxide groups under mild conditions. The enzyme was still active after the immobilization process. No leaching of the enzyme from the support was detectable after repeated washings. The halogenation reaction catalysed by the immobilized enzyme in buffer was compared to that catalysed by the free enzyme. The immobilization of CPO enhanced the stability of the enzyme with respect to the effect of pH and oxidizing agent concentration.

Keywords: Chloroperoxidase; Immobilization; Silica gel; Enzymatic activity; Enzyme stability

1. Introduction

In the last decade, heme peroxidases and notably chloroperoxidase (CPO, E.C. 1.11.1.10) from *Caldariomyces fumago* were extensively used [1,2]. CPO is a heme-containing enzyme that exhibits catalase, peroxidase and cytochrome P450 activities besides the halogenation reaction [3]. Among the wide variety of CPO catalysed reactions, very important biotransformations are the stereoselective epoxidations, hydroxylations and sulfoxidations which allow to obtain very useful synthons for the synthesis of important chiral substances [4,5]. Even if the use of CPO as catalyst looks very promising, till now it has not been used on a large scale in part because its high cost, a highly acidic pH optimum (pH 3), instability at high temperatures and deactivation at high concentrations of oxidizing agents. To overcome these drawbacks and enhance the operational lifetime, CPO has been immobilized on different supports [6–10]. Indeed the immobilization on an insoluble support converts an enzyme to a recoverable solid catalyst which offer several advantages such as enhanced stability, easier product recovery and purification, the possibility of continuous processes and repetitive enzyme use. So far, three approaches have been used for the immobilization of CPO, i.e. (i) covalent binding to porous glass beads [6] and polyurethane foams [7], (ii) noncovalent adsorption onto talc [8] and celite [9] and (iii) encapsulation in mesoporous silicate material [10]. Covalent binding of an enzyme to a macroscopic carrier is probably the most interesting method of immobilization from an industrial point of view. Following this approach, the activated groups of the support react with some external functional groups of the enzyme with the formation of stable linkages thus inhibiting leakage almost completely. A disadvantage of this method is that rather harsh conditions are often employed since the biocatalyst undergoes chemical reaction. Consequently, some loss of activity of the enzyme is often observed.

We have shown previously [11] that activated silica gel can serve as a useful enzyme support in an enantioselective synthesis application. The enzyme Lipase from *Pseudomonas cepacia* was covalently bound on an epoxide derivatized silica gel and successfully used in the preparation of both the enantiomers of 2-subsituted 1,3-propanediol monoacetates. The immobilized enzyme is still active after months from the first use either in aqueous or in organic media.

These results suggested that utilization of CPO bound to silica in stereoselective reactions and in particular in

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enantioselective epoxidation could enlarge the industrial applications of this enzyme. We report herein the first covalent immobilization of CPO onto silica gel and our investigations on its activity and stability.

2. Materials and methods

2.1. Materials

Chloroperoxidase from *C. fumago* was obtained as a suspension from Fluka. One unit of chloroperoxidase catalysed the conversion of 1.0 μmol of monochlorodimedon (MCD) to dichlorodimedon (DCD) per min at pH 2.75 and 25 °C in the presence of KCl and H_2O_2 [12]. *tert*-Butyl hydroperoxide (*t*-BuOOH, aqueous solution) and 3-glycidoxypropyltrimethoxysilane were obtained from Aldrich. Monochlorodimedon (1,1-dimethyl-4-chloro-3,5-cyclohexanedione) was obtained from Sigma. Dichlorodimedon (4,4-dichloro-1,1-dimethyl-3,5-cyclohexanedione) was prepared according to a reported procedure [12]. Solvents were of analytical grade or superior. Silica gel (5 μm, specific surface of 340 m^2/g) was from Alltech.

2.2. Instrumentation

Enzyme assay and other UV experiments were performed on a Varian CARY 4E spectrophotometer at 25 °C using quartz cuvettes. A spectral bandwidth of 1.0 nm was used.

2.3. Methods

2.3.1. Immobilization of CPO onto silica gel

Epoxide derivatized silica gel was prepared according to the methods reported [13]. Four milliliters of 0.05 M phosphate buffer solution, pH 6, containing ammonium sulphate 2 M and 250 μl of CPO suspension (12.6 mg/ml) were added to 0.5 g of functionalised silica gel. The mixture was stirred at room temperature for 2 h. The solid with the immobilized enzyme was then recovered through centrifugation and the UV spectrum of the supernatant was recorded at 400 nm. The recovered solid was washed with the same buffer (0.05 M, pH 6) until the disappearing of the maximum at 400 nm in the UV spectrum (ε_{400} = 91 mM/cm) [14]. The amount of enzyme bound to the support (2.71 mg CPO/g of silica gel) was calculated from the UV absorbance by subtracting the absorbance of the supernatant plus washings from the initial absorbance of the enzyme solution. The experiment was carried out in duplicate. The SiO_2-CPO preparations were stored at −20 °C until use.

2.3.2. Enzyme activity assay

The MCD assay was performed to determine the enzyme activity of immobilized CPO. Typically, 5 ml of 0.1 M phosphate buffer, pH 2.75, containing MCD 0.05 mM and KCl 20 mM, along with 20 mg of the immobilized enzyme suspension were added to a quartz cuvette (path length = 20 mm). The reaction was initiated with the addition of 200 μl of 1 M *t*-BuOOH. The enzyme activity was calculated from the rate of disappearance of MCD at 25 °C determined by measuring the absorbance at 278 nm [14].

2.3.2.1. Stability of SiO_2-CPO: influence of pH. Five milliliters of 0.1 M phosphate buffer containing 20 mg of SiO_2-CPO or 50 μg of free CPO at selected pH (2.75, 4.0, 5.0, 6.0, 7.0) in 10.0 ml vials were incubated at 25 °C on a rotational shaker (350 rpm). After different times of incubation (0–15 days), samples were withdrawn and MCD assay was performed at pH 2.75. Each experiment was carried out in triplicate.

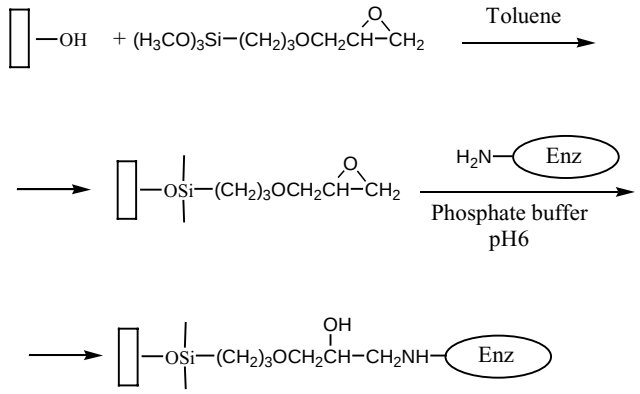
2.3.2.2. Stability of SiO_2-CPO: influence of t-BuOOH. Five milliliters of 0.1 M phosphate buffer containing 20 mg of SiO_2-CPO or 50 μg of free CPO and 50 μl of *t*-BuOOH solution at selected concentrations (0.1, 0.5, 1, 5, 10 mM in the reaction medium) in 10.0 ml vials were incubated at 25 °C on a rotational shaker (350 rpm). After different times of incubation samples were withdrawn and MCD assay was performed at pH 2.75. Each experiment was carried out in triplicate.

3. Results and discussion

3.1. Immobilization of CPO onto epoxide activated silica gel

Activated silica gel has been successfully used in the covalent immobilization of proteins and enzymes and the resulting preparations have been widely utilized in the development of chiral separation systems in liquid chromatography [15]. In general, this approach involves two steps, i.e. (i) activation of the carrier with a reactive ‘spacer’ group such as epoxy, diol, aldehyde, tresyl groups and (ii) enzyme or protein attachment via functional groups belonging to aminoacid residues. Among the several examples reported, it has been shown that the use of an epoxide activated silica gel for enzyme-based chiral stationary phases (CSP) give a good immobilization ratio and ability of the enzyme for structural recognition [16,17]. As already mentioned [9], a Lipase-based stationary phase was prepared by coating the enzyme on epoxide silica and successfully used as a bioreactor. We used the method developed by Felix [13] for preparing epoxide activated silica gel and the reaction scheme followed for the immobilization of CPO to epoxy-silica is reported in Scheme 1.

The immobilization yield was 43% and the amount of enzyme bound to the carrier was 2.7 mg CPO/g silica gel. The immobilization efficiency, which represents the residual activity of the bound enzyme, was 19.5%: indeed, the specific activity of the SiO_2-CPO preparation was reduced (i.e. immobilized CPO, 18.1 U/mg versus solution CPO, 93.7 U/mg:



Scheme 1. Reaction scheme for covalent immobilization of CPO on silica.

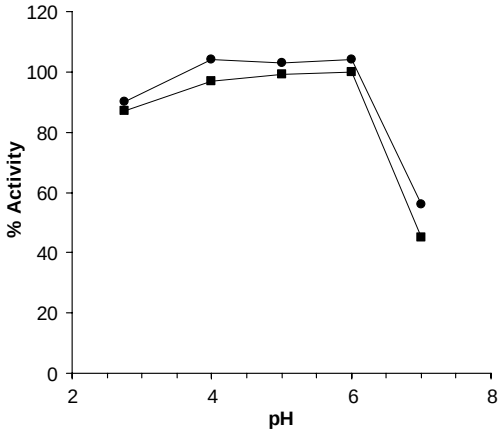


Fig. 1. Comparison of the effect of pH on the activity of soluble and immobilized CPO. Soluble (■) and immobilized (●) CPO were incubated at 25 °C in 100 mM phosphate buffer at different pH values: 2.75, 4, 5, 6, 7 for 24 h. The activities of the enzymes were monitored with MCD assay at pH 2.75 and were set to 100% at $t = 0$.

these specific activities were determined with the MCD assay described in Section 2). In general, several reasons can account for the decrease in activity after immobilization [18]. Perhaps only part of the immobilized enzymes is oriented properly with their active sites exposed for the reaction to occur. In addition the immobilization process might have changed the conformation of some of the enzymes to inactive forms. Leaching of the enzyme from the SiO_2-CPO material was tested by repeatedly washing of the immobilized enzyme and measuring the activity of the washed solution. No residual activity was observed in the supernatant solutions.

3.2. Properties of immobilized CPO

The influence of pH and *t*-BuOOH concentration were mainly investigated. Since the conditions for maximum catalytic rates are not necessarily optimal for enzyme stability, the effects of reaction conditions (pH, oxidizing agent) on the stability of immobilized CPO were investigated. The activity of free and immobilized CPO was compared by performing the MCD halogenation assay [14]. While many CPO-mediated reactions involve H_2O_2 as the oxidant, this study utilizes *t*-BuOOH instead. The first reason is the well known tendency of the enzyme to generate O_2 consuming H_2O_2 in a catalase-type reaction [19], which is slower with alkyl peroxides [20,21]. Furthermore, CPO is quite sensitive to H_2O_2 [22], loosing activity rapidly in the presence of excess reagent. So, especially for larger scale reactions and in view of recycling the enzyme, the use of *t*-BuOOH appears more suitable.

The specific activities of soluble and immobilized CPO were determined at selected pH values ranging from 2.75 to 7.0. The pH-activity profiles (Fig. 1) obtained after 24 h of incubation in the absence of the substrate are almost superimposable with a slight increase in the activity of the immobilized CPO. As it was expected from the reported stability properties of native CPO [14], both systems were stable at 25 °C over a pH range from 2.75 to 6 while at pH 7 about 50% of the initial activity was lost.

The stability of CPO and SiO_2-CPO against pH was investigated over several weeks. In Fig. 2A and B, the effect of 1 week storage is reported.

It has to be noted that over a pH range from 4 to 6 the initial activity of the immobilized preparation was unaffected, whereas the native species retained less than 90% of its activity after the same period. At acidic pH (2.75), the activities of SiO_2-CPO and native CPO were respectively 71 and 47%. At pH 7, the activity of both systems was dramatically affected, but there appeared to be an enhancement in the stability of the immobilized preparation. After the initial loss, about 40% activity was retained in the SiO_2-CPO, while the activity of soluble CPO dropped to less than 10%. These results seemed to indicate that the rate of alkaline denaturation was reduced by the immobilization of the enzyme. The effect of pH on stability was studied also for long-term storage: after 6 months at −20 °C and pH 6 more than 80% activity was recovered for the immobilized enzyme.

The effect of *t*-BuOOH concentration on the stability of CPO was investigated by incubating native and immobilized enzyme in buffer 100 mM at pH 6 containing different amounts of the oxidant. At selected time of incubation the reaction was started by addition of MCD. In Fig. 3 the comparison of the activities of free and immobilized CPO after 1 h of incubation is reported.

At concentrations of oxidant below 1 mM, immobilized CPO retained almost completely its initial activity. At higher concentrations (5 and 10 mM) respectively 70 and 40% activity was retained by the SiO_2-CPO preparation. It was also found that at 10 mM the immobilized CPO was still active (>20%) after 4 h of incubation, while the soluble enzyme was almost completely inactive after 2 h. The stability of SiO_2-CPO in *t*-BuOOH 0.1 mM was also investigated over a longer period of time. After 24 h the activity of the immobilized enzyme was unaffected, while soluble CPO lost more than 50% of its original activity.

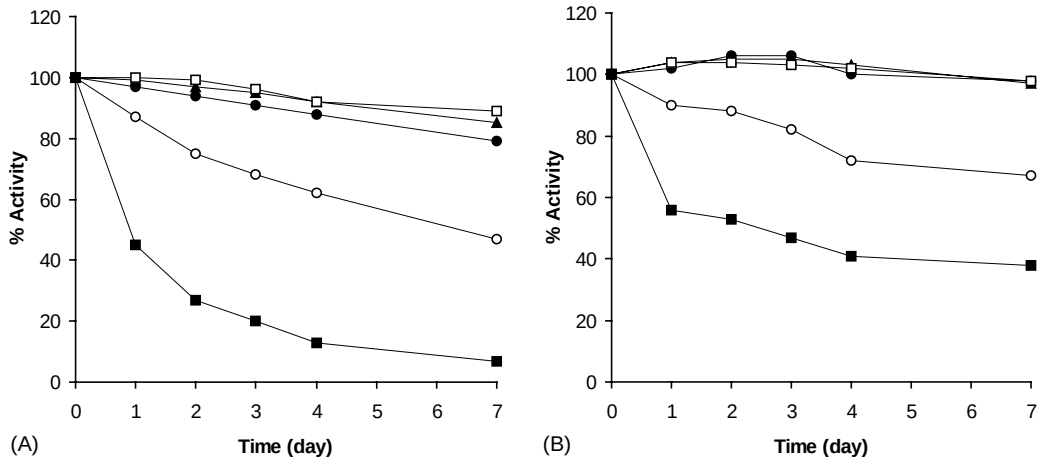


Fig. 2. Effect of storage on the pH-dependent stability of CPO at 25 °C. Soluble (A) and immobilized (B) CPO were stored at 25 °C in 100 mM phosphate buffer at different pH values: 2.75 (○), 4 (●), 5 (▲), 6 (□), 7(■). The activities of the enzymes were monitored periodically with MCD assay at pH 2.75.

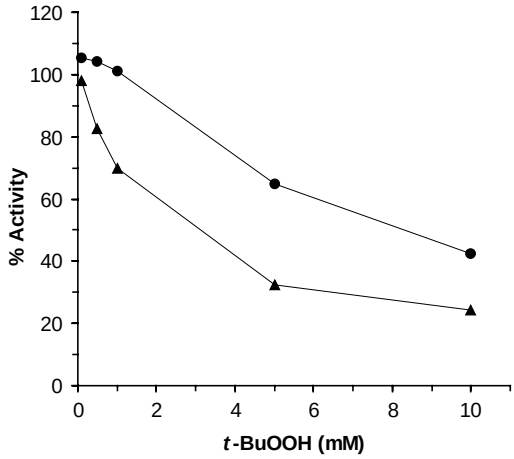


Fig. 3. Comparison of the effect of *t*-BuOOH on soluble and immobilized CPO. Soluble (▲) and immobilized (●) CPO were incubated in various concentrations of *t*-BuOOH at 25 °C in 100 mM phosphate buffer for 1 h. The activities of the enzymes were monitored with MCD assay at pH 2.75.

4. Conclusion

A convenient and efficient heterogeneous biocatalyst was prepared by immobilization of the enzyme CPO onto epoxide derivatized silica gel. The immobilized enzyme was easily obtained by a reported procedure and no leaching of CPO from the support was detected. The loss of activity upon immobilization was compensated by a better enzyme stability as functions of pH and oxidant concentration. Therefore, the SiO_2-CPO promises a wide variety of applications and merit further investigation for large-scale reactions and long-term use.

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