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Encapsulation of chloroperoxidase in novel hybrid polysaccharide-silica biocomposites: Catalytic efficiency, re-use and thermal stability

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ABSTRACT

Chloroperoxidase was encapsulated into hybrid polysaccharide-silica matrices by means of sol-gel processing. Chitosan was found to be the most effective additive to improve enzyme catalytic performances. At the standard initial experimental conditions, enzyme was able to perform five complete reaction cycles and a substrate conversion of 60% at the sixth cycle. The optimization of the procedure, which consisted in the reduction of reaction time, the elimination of washes between cycles and the increase of the chitosan concentration in matrices, allowed a considerably higher reusability of the biocatalyst, up to 18 consecutive reaction cycles.

Moreover, immobilized enzyme displayed an excellent thermal stability and even at 70 °C its activity remained higher than 95% also after 2 h of incubation.

These results paved the way to the application of chloroperoxidase for academic synthesis and industrial production of chemicals.

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1. Introduction

Enzyme catalysis in organic synthesis is widely recognized as potentially the first choice in the preparation of a wide range of chemical compounds, not only for academic synthesis, but also for industrial-scale applications. Nevertheless, the employment of enzymes in their native form for industrial applications is often hindered by high costs, low operational stability and difficulties in recovery and re-use of the biocatalyst. Enzyme immobilization onto solid supports represents one of the most attractive methods to overcome these drawbacks.

To date, an ever increasing number of enzymes, applications, supports and methods of immobilization are reported in literature (see for example [1–4]). A variety of synthetic inorganic or organic polymers and biopolymers can be used as supports, and enzymes can be immobilized by physical adsorption, covalent binding on carrier surface, cross-linking or by encapsulation. The support and the method of immobilization are of fundamental importance for a successful large-scale application of biocatalysts, since the actual process used for each enzyme must be carefully chosen and depends on the selected enzyme.

Chloroperoxidase (CPO) from *Caldariomyces fumago*, a heavily glycosylated heme enzyme, is a highly versatile enzyme that catalyses different reaction types, many of which are of industrial interest, i.e. sulfoxidation, hydroxylation and epoxidation [5]. Although these advantages, enzyme deactivation at high temperatures and in the presence of concentrated oxidizing agents limits the CPO synthetic applications. Different efforts have been directed to improve CPO catalytic properties, the simplest among them being the use of additives in water solution. The presence of PEG 200 [6], for example, allowed a better substrate solubilization in the reaction mixture and the enzyme to retain more of its initial activity, with respect to pure buffer. An increase in CPO productivity was also observed due to an enzyme greater stability. Moreover, CPO chlorination and oxidation activity was enhanced 32 and 20%

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by chitosan, and oxidation activity 24% by thioglycolic acid, respectively, compared to that in pure buffer [7].

CPO performances can be greatly enhanced by immobilization and several methods and supports are reported in literature. CPO has been immobilized by physical adsorption on talc [8], on mesoporous materials [9–14] and on amino-agarose gel [15]. The main disadvantage of these methods is the leaching of the enzyme from the support due to desorption.

Covalent attachment on mesoporous silica [11,13,16,17], on methacrylate [18] and chitosan membranes [19], on aminopropyl-glass [20], on magnetic beads [21] and on amino-agarose gel [15] typically involved the formation of covalent bonds between the amino or carboxylic groups on the enzyme surface and active functionalities present on the solid support. In this case, leakage of enzyme is often minimized but, as a consequence of the binding reactions, a loss of enzymatic activity can be observed due to linking conditions (pH, temperature, additives, medium), to an increase in enzyme rigidity or in steric hindrance of the active site.

Other methods commonly used to increase the stability of CPO involve the formation of cross-linked aggregates [22], encapsulation on polymersome nanoreactors [23] or entrapment in silica materials [24,25]. The entrapment of CPO in microporous silica cage allowed the enzyme to retain a sufficient stability in the presence of hydrogen peroxide and the oxidation of some organic sulfides was performed with high activity [24].

In our previous paper PEG-modified silica matrices were found to be excellent supports for CPO immobilization by entrapment [25]. An optimization of the sol-gel process was performed, several parameters were modified and the reusability of entrapped enzyme was maximized by controlling the oxidant/substrate molar ratio at each cycle. In fact, in the first cycle, a greater amount of oxidant was required (oxidant/substrate molar ratio equal to 2.6), compared to the next cycles, probably because of oxidation of some hydroxyl groups of the additives and/or silanols on the silica surface could occur and, at lower oxidant/substrate molar ratio, i.e. 1.6, conversion was incomplete. By adjusting these parameters, encapsulated CPO was reused for four consecutive reaction cycles with high yields, while inorganic silica matrix-based biocatalyst was able to convert only a 20% of substrate at the first cycle. Moreover, enzyme stability towards both oxidant agents and high temperatures was greatly increased as compared to the free form and a stable and easily recyclable biocatalyst was obtained, probably thanks to more suitable physico-chemical properties of the support: in fact the structure of the network strongly depends on the interactions between the additive and the silica precursor.

Composite organic-inorganic hybrid supports are considered an optimal starting point for the development of advanced functional materials, since they present new chemico-physical properties with respect to pure inorganic materials [26]. In the case of silica supports, their major drawback is represented by their lack of biocompatibility that in some cases does not allow a fully maintenance of biological activity of the immobilized biomacromolecules [27]. The addition of organic additives can significantly increase protein stability [25,28,29]. Among them, polysaccharides have been reported to radically change the structure of the biosilicate owing to the formation of hydrogen bonds between hydroxyl groups in macromolecules and the silanol groups produced by the hydrolysis of precursor [30,31]. Hybrid-polysaccharide silicates have been successfully used to immobilize α-galactosidase [32] and 1,3-β-D-glucanases [33]; both enzymes retained their catalytic activity and became more thermally stable.

Here, we report results obtained after CPO encapsulation into hybrid polysaccharide-silica matrices and the biocatalyst efficiency in a chlorination reaction has been investigated. CPO molecules were encapsulated into a matrix formed in situ by polycondensation reactions. The properties of the material depended on the type of polysaccharide used, on its concentration and charge. Two linear polysaccharides, sodium alginate and chitosan, which differ in their functional groups, i.e. carboxyl and amino respectively, were selected. Structural characterization of hybrid silicates was accomplished by SEM and the effect of the polysaccharide-silica supports on CPO efficiency, re-use and thermal stability were evaluated.

2. Experimental

2.1. Materials and Instruments

Chloroperoxidase (CPO, EC 1.11.1.10) from *Caldariomyces fumago*, as a crude suspension (26,776 U/mL), the silica precursor, tetramethylorthosilicate (TMOS, 99%), chitosan (low-viscous), xanthan and dextran were purchased from Fluka. Monochlorodimedon (MCD, 2-chloro-5,5-dimethyl-1,3-cyclohexanedione), hydrogen peroxide (30%) and alginic acid sodium salt (from brown algae) were purchased from Sigma–Aldrich, while potassium chloride and phosphoric acid from Carlo Erba. All the chemicals were of analytical grade. MilliQ water was used in all experiments.

Quantitative determinations of chloroperoxidase activity have been carried out with a Shimadzu UV-160 A UV–Vis spectrophotometer. Images of the sol–gel materials were recorded by a Philips XL30-CP Scanning Electron Microscope (SEM). The samples were coated with a thin layer of gold in a customized coating machine before the imaging process.

2.2. Encapsulation of CPO in doped sol-gel matrices

Silica sol was prepared by following a published procedure [25]; it will be referred to "standard protocol" in the following part of the paper. TMOS was mixed with a phosphate buffered solution (0.01 M, pH 2.75) in a 1:2 stoichiometric molar ratio. The two-phase mixture was vigorously stirred for a few minutes, until a homogeneous and transparent phase was obtained. For each matrix, 2.5 mL of the just formed sol were put in a glass vial and the suitable amount of polysaccharide aqueous solution and CPO suspension (1 μL) was sequentially added. The vials were sealed, shaken and kept in a water bath at 25.0 °C until gelation occurred. Then, the wet-gels were left aging for 24 h, crushed in a mortar and the resulting powders were allowed to air-dry for 2 days. Gels and powders were always maintained at 25.0 °C. The final biocatalytic materials weighed 1–1.4 g, depending on the type of additive and on its concentration.

2.3. Entrapped CPO catalytic efficiency assay

In the standard protocol, before each reaction cycle, matrix was transferred in a 25 mL round-bottomed flask, suspended in 10 mL 0.01 M phosphate buffer solution at pH 2.75, left in magnetic stirring for 30 min and then centrifuged for 30 min at 3000 rpm. The recovered matrix was mixed with the buffered reaction mixture containing 1.15×10^{-4} M MCD, 1.15×10^{-2} M KCl and 2.99×10^{-4} M H_2O_2, for the first cycle and 1.84×10^{-4} M H_2O_2 for the following cycles, kept in magnetic stirring for 30 min and then centrifuged for 15 min at 3000 rpm to separate the immobilized biocatalyst from the reaction mixture. The efficiency of entrapped CPO was determined as ratio percentages between the reaction mixture absorbance at 278 nm ($\varepsilon = 12{,}000\ M^{-1}\ cm^{-1}$) before and after any reaction cycle. If substrate conversion was complete, the matrix was washed with buffered solution, as already described, centrifuged and mixed with a freshly prepared reaction mixture to carry out a further reaction cycle. On the other hand, if conversion was incomplete, the reaction mixture and the matrix were left to react for a longer time (up to 4 h) and then assayed again. All sets of

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