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Enhancing oxidation activity and stability of iso-1-cytochrome c and chloroperoxidase by immobilization in nanostructured supports

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ABSTRACT

The immobilization of enzymes in inorganic materials has been widely used because it can produce an enhancement of the catalytic stability and enzymatic activity. In this article, the effect of the immobilization of iso-1-cytochrome c (CYC-Sc) from *Saccharomyces cerevisiae* and chloroperoxidase (CPO) from *Caldariomyces fumago* on the enzyme stability and catalytic oxidation of styrene was studied. The immobilization was carried out in three silica nanostructured supports with different pore size MCM-41 (3.3 nm), SBA-15 (6.4 nm) and MCF (12.1 nm). The adsorption parameters and leaching degree of immobilized enzymes were determined. Catalytic parameters of immobilized and free enzymes were determined at different temperatures (20–60 °C) and in different acetonitrile/water mixtures (15–85% of acetonitrile). The results show that there is low leaching of the enzymes in the three supports assayed and the adsorption capacity (q_{max}) was higher as the pore size of the support increased. The pore size also produces the enhancement of peroxidase activities on the styrene oxidation. Thus, CPO adsorption into SBA-15 and MCF showed remarkable thermal and solvent stabilities at 40 °C showing a total turnover numbers of 48,000 and 54,000 times higher than free CPO, respectively. The enhancement of activity and stability doubtless is interesting for the potential industrial use of peroxidases.

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1. Introduction

During last twenty years the use of enzymes as catalyst in industrial processes has experienced a significant growth. The high-level of quimio-, regio- and stereo-specificities of active sites of enzymes offers a new alternative to classic chemical modifications in different areas such as pharmacology [1], agriculture, food industry, fuel refinement [2] and fine chemical production [3,4], as well in bioremediation [5,6]. Furthermore, these enzymatic processes tools represent an attractive alternative to improve environment, being a fundamental part of the green chemistry [7].

Peroxidases are oxidoreductases that act with hydrogen peroxide or alkyl peroxides as electron acceptors. These enzymes have been used in the bioremediation and show potential applications for biotechnological processes in the petroleum industry, such as desulfurization, aromatic oxidation, asphaltene transformation, and others [8,9]. Beside activation with hydrogen peroxide, the trend of the reaction of these proteins depends on the active-site environment, metal coordination and axial ligands nature, redox potential, etc.

Yeast iso-1-cytochrome c (CYC-Sc) is an intermembrane mitochondrial heme protein. *In vivo*, electrons are transported from cytochrome c reductase to cytochrome c oxidase, followed by reduction of oxygen molecule to water, which provides the driving force for ATP synthesis [10]. It is well known that CYC-Sc is able to catalyze peroxidase like-reactions on several types of organic compounds [11]. Despite the diminished rates of the transformation, the main advantage of this peroxidase model is the higher stability of heme group which it is covalent-linked to the protein main chain. Chloroperoxidase from *Caldariomyces fumago* (CPO) is other peroxidase, which has been widely used in the biotransformation of different organic substrates. CPO is a versatile heme enzyme because of its catalytic diversity. CPO is a peroxide-dependent chlorinating enzyme and it also catalyzes peroxidase-, catalase- and cytochrome P450-type reactions of dehydrogenation, H_2O_2 decomposition and oxygen insertion, respectively [12–15].

CYC-Sc and CPO has been reported to catalyze the oxidation of a wide variety of organic substrates, including aromatic hydrocarbons [16–22]. In general, the aromatic hydrocarbons are insoluble in water and their low diffusion rate can produce problems during the biocatalysis in aqueous solution. In addition, one central

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problem arises from the intrinsic instability of peroxidases and hemeproteins. Peroxidases and heme-proteins are swiftly inactivated in the presence of catalytic amounts of hydrogen peroxide and this low stability restricts their applications on a large scale. Although the inactivation mechanism is not completely explained, several events such as heme destruction, intermolecular crosslinking and oxidation of low redox potential amino acid residues are known to lead to activity loss [23]. The inactivation is considered a suicide, as the main inactivating species are the enzymatic intermediates involved in the catalytic cycle. Several approaches have been developed in order to overcome these problems such as: the utilization of surfactants, the use of water-miscible organic solvents, the raising of reaction temperature, the chemical modification of enzyme surfaces and site-directed mutagenesis [21,24]. Several reports have showed that immobilization of cytochrome c into mesoporous silicates improve their stability and catalytic activity, still in extreme conditions [25]. Recently, Lee et al. [26], have shown that the immobilization of cytochrome c into nanostructured aluminosilicates increases the activity in the oxidation of pyrene and anthracene compared with the free enzyme.

The enzyme immobilization allows the incorporation of proteins between or within a larger structure, through simple adsorption or covalently bonded encapsulation [27,28]. This process may increase the enzyme stability due to the interaction between the support and protein surfaces, reducing protein denaturation [28]. In addition, immobilization can increase resistance to denaturation originated by the presence of either organic solvents and of water–organic solvent mixtures.

Silicates with mesoporous structure are a kind of materials that can provide a good support for the immobilization of enzymes. These supports have pore diameters ranging between 2 and 50 nm, known as mesoporous materials, which have a high surface area, high pore volume and an appropriate pore structural order [29,30]. Mesoporous materials can be synthesized through the polymerization of silica around a regular aligned mycelial template and subsequently removed by extraction or calcinations.

Enzyme immobilization in MCM-41 was first reported by Diaz and Balkus in 1996 [31]. They found that the immobilization is dependent on the molecular size of the enzyme. The immobilization of large enzymes such as horseradish peroxidase (spherical molecule of 4.6 nm diameter) was insignificant into MCM-41, whose average diameter is 4 nm. Thus, MCM type materials will be most successful for the immobilization of enzymes which possess a size smaller than the pore diameter of the support.

The development of supports like SBA-15 (pore size: 5–13 nm) and mesocellular foam (MCF, pore size: 15–40 nm) have solved the critical problem of pore size that have the MCM-type materials [32,33], becoming an alternative for the immobilization of large enzymes. Along MCM, MCF and SBA other mesoporous materials have been recently synthesized by Park et al. These authors have reported the synthesis of hydrophobic periodic mesoporous organosilicas, which were successfully employed for the adsorption of proteins.

Many studies of enzymes adsorbed discuss the intrinsic activity of the enzyme from the relationship between pore size and the enzyme size. Recent publications have suggested that the improvement of the intrinsic activity is due to the agglomeration of a high amount of enzyme molecules in a single pore, and that this confinement prevents unfolding or denaturing of the enzyme [34–36]. This feature is true whenever the pore size of the mesoporous material is greater than the dimensions of the immobilized enzyme [27,33,34]. Another important factor that determines the enzyme stability in the mesoporous materials are the electrostatic interactions [37–40]. If the net charge of the enzyme is opposite to the charge of pore wall of mesoporous materials, not only an easy adsorption occurs, but also more stable enzyme–mesoporous system will be, due to attractive interactions. To keep the charge differences between the pore wall of mesoporous materials and enzyme the pH of solutions must be adjusted [39,40] or the mesoporous materials can be functionalized with amino or carboxylic groups to achieve an appropriate charge difference with the immobilized enzyme [38]. In the case of CPO has been determined that the pH optimum for the enzyme immobilization into nanostructured mesoporous silicates approximately is 3.4, a pH slightly lower than the isoelectric point of the enzyme [37]. On the other hand, the immobilization of cytochrome c into mesoporous materials is pH dependent, obtaining a greater loading in a range between 6 and 10 pH units [40].

Mesoporous materials, MCM-41, SBA-15 and MCF appeared as good candidates to immobilize CYC-Sc from *Saccharomyces cerevisiae* and CPO from *C. fumago*, because the dimensions of these proteins are 2.6 nm × 3.2 nm × 3.3 nm [41] and 3.1 nm × 5.3 nm × 5.5 nm [42], respectively. These proteins do not exceed the average size of the pores of such materials (4 nm MCM-41, 6 nm SBA-15 and 12 nm MCF), with the exception of chloroperoxidase in MCM-41. We postulated that the immobilization of CPO and CYC-Sc onto these silica nanostructured supports can increase the activity and stability for the catalytic oxidation of aromatic molecules. Thus, in this work we have studied the effect of the immobilization of CYC-Sc and CPO onto nanostructured supports MCM-41, SBA-15 and MCF on their catalytic activity and stability. The oxidation of styrene was used as reaction test because the importance of aromatic oxidation reactions in areas as fuel refinement and bioremediation. The oxidation reaction has been carried out to different temperatures (20–60 °C) and in different mixtures of acetonitrile–water.

2. Materials and methods

2.1. Chemicals

Styrene was purchased from Sigma–Aldrich (St. Louis, MO). Cytochrome c from *S. cerevisiae* was obtained from Sigma–Aldrich (St. Louis, MO) and chloroperoxidase from *C. fumago* was obtained and purified as previously reported [43].

2.2. Preparation of mesoporous silica supports

SBA-15 mesoporous silica was synthesized according to the procedure reported by Zhao et al. [44] using the gel composition of 6.96×10^{-4} P123:4.08×10^{-2} TEOS:0.24 HCl:1.67 H_2O. This synthesis solution was prepared by mixing appropriate amounts of amphiphilic triblock copolymer, Pluronic P123 ($EO_{20}PO_{70}EO_{20}$, Mw = 5800, BASF); 2 M HCl solution, tetraethyl orthosilicate (TEOS 98%, Aldrich), and distilled water. The resulting solution was submitted to an aging period at 40 °C for 24 h with stirring. After that, the solution was added to Teflon-lined stainless steel autoclaves and hydrothermally treated at 100 °C for 48 h under static conditions. In order to prepare the large-pore size mesocellular foam silica (MCF) [33], 1,3,5-trimethylbenzene (TMB) was used as pore expanding agent. TMB was added on the predissolved P123 surfactant by using a TMB/P123 molar ratio of 1.0. After hydrothermal period the solid products were filtered, washed with distilled water, and dried overnight at 100 °C. The materials were calcined [45] in air at 550 °C for 8 h, at a heating rate of 1 °C/min to decompose the organic templates and obtain white powders (SBA-15, MCF). The synthesis of MCM-41 was carried out in a similar form, according to procedures reported in the literature [29,46].

All the mesoporous materials were characterized by N_2 adsorption, transmission electronic microscopy and XRD. The adsorption–desorption isotherms were obtained at 77 K with an

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