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The Catalytic Activity of Laccase Immobilized in Sol-Gel Silica

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Abstract: The removal potentially harmful chemicals such as substituted phenols and aromatic amines by laccase oxidative biocatalyst has been attracting interest, particularly in view of certain unsolved problems of oxidation reactions in conventional chemical process. These environmental pollutants mostly do not dissolve in an aqueous solution due to their high hydrophobicity, hence organic solvents are required to dissolve and concentrate them. The biodegradation of these pollutants using enzymatic process has been hindered by instability of enzyme in organic solvents. In order to increase enzyme stability, several methods of stabilization through immobilization and encapsulation have been reported successfully for various enzymes. Enzyme immobilization by physical entrapment has a wide benefit and may provide relatively small perturbation of the enzyme native structure and function. Therefore, in afford to enhance the laccase activity and stability; encapsulation of laccase by sol-gel silica method was studied. The catalytic activity of laccase immobilized in sol-gel silica was demonstrated by an enzymatic assay using 2, 6-dimethoxyphenol as a substrate. After immobilization, the laccase was proven to be active in a wider pH range and the laccase loading in sol-gel silica has a significant effect on laccase activity.

Key words: Pollutants, laccase, immobilization, sol-gel silica, biodegradation

INTRODUCTION

The environmental is continuously polluted by a large array of hazardous chemicals with different structures and different toxicity levels that are released from several anthropogenic sources. Phenol and several of its derivatives such as dioxins, Polycyclic Aromatic Hydrocarbon (PAHs), amines and chlorophenols have a large group of organic pollutants, which are widely distributed throughout the environment. They are used in a variety of industrial processes, for instance, in the manufacture plastics, papers, dyes, drugs, pesticides and antioxidants.

An enzymatic treatment is currently considered as an alternative method for the removal of toxic xenobiotics from the environment (Dick and Tatabai, 1993; Gianfreda and Bollag, 1996; Karam and Nicell, 1999). Laboratory experiments have demonstrated that phenol and its derivates may be removed by the application of various oxidoreductases such as laccase (Dec and Bollag, 1992). The removal of organic pollutants involves enzymatic oxidation to free radicals or quinones that subsequently undergo polymerization and precipitation (Dec and Bollag, 1992; Huttermann *et al*., 1989).

Oxidoreductase enzyme such as laccase was observed as a great variability for the induction mechanism, degree of polymorphism, physicochemical and enzyme activity (Rodakiewicz-Nowak *et al*., 2000; Dec and Bollag, 1992). Over the last decades, the use of laccase has been explored for wide applications. Such applications include the detoxification of industrial effluents, mostly from the paper and pulp, textile and petrochemical industries, use as a tool for medical diagnostics and as a bioremediation agent to clean up herbicides, pesticides and certain explosives in soil. Laccase are also used as cleaning agents for certain water purification systems, as catalysts for the manufacture of anti-cancer drugs and even as ingredients in cosmetics. In addition, their capacity to remove xenobiotic substances and produce polymeric products makes them a useful tool for bioremediation purposes (He *et al*., 2006).

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However, enzymes have been modified during biological evolution in order to optimize their behavior in the framework of complex catalytic chains, inside living things under stress and needing regulation. Obviously, enzymes have not been optimized to work inside industrial reactors. In this way, enzymes, in addition to their excellent catalytic properties, also have some characteristics that are not very suitable for industrial applications. Enzyme may be unstable, difficult to handle under non-conventional conditions, may be inhibited by substrates and products and enzyme only work well on natural substrates and under physiological conditions. From this point of view, the transformation from the laboratory to industry of chemical processes catalyzed by enzymes and cells may be one of the most complex and exciting goals (Compas and Marty, 2006).

Therefore, in order to increase the potential use of enzymes in industrial process the immobilization is absolutely necessary for biochemical stability and reuse. By using these immobilized forms, adequate characteristics including high resistance to thermal denaturation, significant improvement of the enzymatic activity and its preservation for long periods have been frequently reported (Peralta-Zamora *et al*., 2003; Baker *et al*., 1996).

The encapsulation is well suited for the entrapment of sensitive molecules such as enzymes in porous matrix. Sol-gel technology posses interdisciplinary research domain involving application of the principle of chemistry, physics, engineering and material science (Baker *et al*., 1996). It is carried out under aqueous conditions and at ambient temperature and based on the formation of silica matrices of metal or semi-metal oxide through the aqueous processing of hydrolytically labile precursors (Compas and Marty, 2006). Indeed, many different biological which are sensitive molecule, including DNA, RNA, enzymes, antibodies, living cells, etc., have been encapsulated in glasses of silica or other metallic oxides, organosiloxanes and many other sol-gel hybrids or composite polymers (Avnir *et al*., 1994; Avnir, 1995; Gill, 2001). Nevertheless, the experimental conditions of the sol-gel technique do still require some optimization to better preserve the conformation of the most delicate biomolecules during encapsulation and to recover a higher fraction of their natural activity. Besides, shrinking and pore collapse and the mechanical stability of the matrix have to be improved to guarantee a fast response, long term stability and reusability of biomaterials.

The purpose of this study was to improve the enzyme catalytic activity of laccase by encapsulating into sol-gel silica. Herein, we report on the effect of encapsulation, pH and loading on the catalytic activity laccase.

MATERIALS AND METHODS

Materials: Type 1 reagent water was obtained using Nanopure deonizer, purchased from Purite Ltd. (England). All gels were prepared using tetraethyl orthosilicate (TEOS) (99% purity) purchased from Fluka (Switzerland). 2,6-dimethoxylphenol, Potassium dihydrogen phosphate (KH_2PO_4) and di-Potassum hydrogen phosphate (K_2HPO_4) were purchased from Sigma (St. Louis, MO, USA). Laccase enzyme from Tremetes sp. was purchased from Daiwa Kasei Co. Ltd. (Japan). Other chemicals were analytical grade reagents from various suppliers and use without further purification.

Methods

Sol-Gel Encapsulation: The immobilization of laccase was carried out according to Vera-Avila *et al*. (2004) for encapsulation of an antibody with very slight modifications. Briefly, 2.5 mL of TEOS, 0.4 mL of deionized water and 0.1 mL of 0.04 M HCl (corresponding to 10, 28 and 0.004 mmol, respectively) were mixed with stirring; the mixture was sonnicated under ice cooling for 30 min and left at ambient temperature until it was acquired an elastic consistency. Sonnification of the sol during the initial hydrolysis and condensation reactions eliminates the need for often used co-solvents such as alcohols, which can promote protein denaturation. Two milliliter aliquot of phosphate buffer (0.4 M, pH 7) was added to the sol to facilitate the gelation process followed by addition of the laccase solution (1 mL of lacccase solution, equivalent to 50 mg of laccase); both additions under vigorous stirring. As soon as gelation began (1-4 min), stirring was stopped to avoid the formation of bubbles in the gel. The gels were then lyophilized at -42°C for 24 h. The lyophilized gels were then crushed with mortar to get homogenous samples and stored in refrigeration to maintain enzymatic activity. Other reports have demonstrated that sol-gel encapsulated microorganisms cells can be wet stored for several months without significant loss of activity. However, dry storage complies better with distributed sensing systems, shipment requirements, field analysis and handling by non-experienced personnel (Vera-Avila *et al*., 2004).

Enzyme catalytic activity assays: Sol-gel and free laccase activity was assayed spectrophotometrically using 2, 6-dimethoxyphenol (2, 6-DMP) as a substrate (Leoniwicz *et al*., 1988). The change in absorbancy at 469 nm was recorded for 5 min and the catalytic activity was determined by measuring the initial linear portion of the kinetic curve. The reaction mixture contained 1 mM DMP, 10 mM phosphate buffer (pH 5). One unit of each

enzyme activity was defined as the amount of the enzyme producing 1 μmol of the quinone dimer per min from 2, 6-DMP.

RESULTS AND DISCUSSION

Effect of immobilization: The encapsulation of enzymes in sol-gel silica works well for a number of enzymes (Avnir *et al*., 1994; Avnir, 1995; Gill, 2001). Upon encapsulation, the enzymes and proteins will retain their native function and spectroscopic properties (Leoniwicz *et al*., 1988). Figure 1 shows that the encapsulation of laccase in sol-gel silica greatly enhanced and retained its enzyme catalytic activity. Through encapsulation, enzymes will find more stable environment in a silica host. The polymeric framework which grows around the enzyme will create a cage and thus protecting the enzyme from aggregation and unfolding (Vera-Avila *et al*., 2004).

The encapsulation is based on the entrapment of the biomolecules in a polymer matrix and there is no covalent association between the network and the biomolecules. The hydrophilic characteristics of silica will also help to minimize the partitioning of substrates or products between the gel surface and the bulk aqueous solution, which adversely affect the global reaction (Vera-Avila *et al*., 2004).

Other advantages of encapsulation are the permeability of the matrices will allow the transport of low-molecular-weight (MW) compounds without leaking of the entrapped biomolecules; the material porosity will be tuned to allow the accommodation of biomolecules of different size, the possibility of chemically modifying the matrix and the resistance to thermal and biological degradation (Compas and Marty, 2006).

The hydrophilic character of silica as support will minimize the partitioning of substrates or products between the gel surface and the bulk aqueous solution (Vera-Avila *et al*., 2004). A hydrophilic support was preferred since enzymes are spontaneously soluble in aqueous solutions. Such a support not only maximizes available area of enzyme attachment and improves the efficient immobilization of enzyme, but also keeps the essential water layer that surrounds the biocatalyst and prevents the impairment of catalytic activity (Karube *et al*., 1977; Lobarzewski, 1981).

Effect of pH: The effect of substrates' pH on catalytic activity of the free and sol-gel laccase was investigated using 2, 6-dimethoxyphenol as a substrate. The reactions were conducted using phosphate buffers at various pH values (pH 3-8) to determine the optimum pH for free and

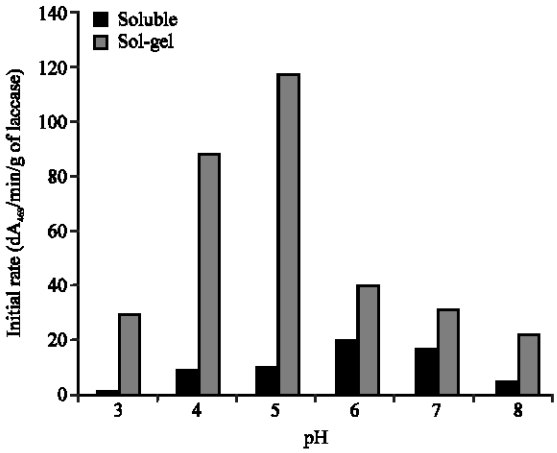

Fig. 1: Effect of encapsulation and pH on the activity of laccase

sol-gel laccase. Figure 1 shows that the optimal pH of sol-gel and free laccase is at pH 5 and 6, respectively. The results indicated that the optimal pH for sol-gel laccase shifts slightly toward a more acidic region compared to the free laccase.

Such shifts have previously been detected for various immobilized enzymes (Karube *et al*., 1977; Lobarzewski, 1981; Rogalski *et al*., 1985). This phenomenon can be explained by the partitioning of protons at the active site of an enzyme when affected by ionized active groups of the support (Trevan, 1980). When enzymes are immobilized in a charged matrix as a result of a change in the microenvironment of the enzyme, the apparent bulk pH optimum of the immobilized enzyme will shift from that of free enzyme. The charged matrix will repel or attract substrate, product, cofactor and H^+ depending on type and quantity of surface charge (Shuler and Kargi, 2005; Chin *et al*., 1994).

The low activity was explained by a change in protein behavior. Variations of the pH of the medium result in changes in the ionic form of the active site and changes in the activity of the enzyme and hence change the reaction rate. Changing the pH may alter the three-dimensional shape of the enzyme. For these reasons, enzymes are only active over a certain pH range. The pH of the medium may affect the maximum reaction rate and the stability of enzyme. In some cases, the substrate may contain ionic groups and the pH of the medium affects the affinity of the substrate to enzyme (Shuler and Kargi, 2005).

Effect of enzyme loadings: The effects of enzyme loading in sol-gel are shown in Fig. 2. The enzyme loading is defined as a laccase/TEOS ratio (μg mg^{-1}). The optimum loading was found to be at 2 μg mg^{-1}. The

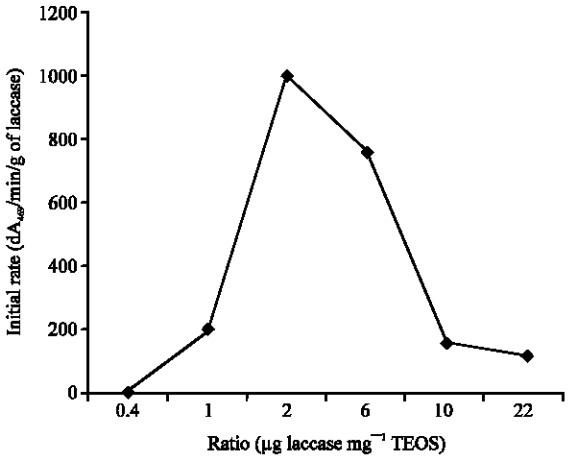

Fig. 2: The effect of loading on the activity of sol-gel laccase

catalytic activity of laccase (pH 5) at loading ratio of 2×10^{-3} mg mg^{-1} was found to be 1000 (dA_{469}/min/g of laccase), hundred time higher than the free laccase. So far, the causes of this enhancement of enzyme catalytic activity have not been determined.

CONCLUSION

The results reported so far were shown that the immobilized laccase in sol-gel silica is active in a wider pH range and the laccase loadings in sol-gel silica has a greatly influence the laccase activity. Thus, this study showed that the significant improvement on laccase activity could be achieved by choosing an optimized pH of substrate and laccase loadings.

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