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New Potential Biocatalysts by Laccase Immobilization in PVA Cryogel Type Carrier

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Abstract Laccases are enzymes belonging to the *Oxidoreductases* class. These enzymes may be good biocatalysts for different processes, at laboratory and industrial levels. A successful use at industrial scale demands a higher stability of the enzyme. As an easy way to obtain longer life biocatalysts, the immobilization process is recommended. Thus, the paper presents different ways of obtaining new biocatalysts by a laccase covalent immobilization on a macroporous carrier based on poly(vinyl alcohol) cryogel. Different procedures of covalent immobilization are described, the newly obtained biocatalysts being characterized. According to the experimental data, the stability of the immobilized enzyme increased and the pH profile changed, compared with those of the free enzyme.

Keywords Laccase covalent immobilization · Poly(vinyl alcohol) cryogel

Introduction

A sustainable development demands new green technologies. Biotechnology seems an appropriate solution for such purpose. The use of enzymes supposes low consumptions of raw materials and energy besides diminishing the pollution [1].

Laccases are multicopper oxidases (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) oxidizing different substrates with aromatic moieties, like lignin, dyes, polyaromatic hydrocarbons, etc. These enzymes have found lately various applications, in food, pulp and paper, and textile industries as well as in solving environmental problems by pollutant

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biodegradation [2–6]. New applications, such as biofuel cells [7] or biotransistors [8], based on laccases have been also developed.

This enzyme family has the advantage to be widely spread in nature (bacteria, plants, and insects) being mainly obtained from *basidiomyceteous* and *ascomyceteous* fungi [9]. Studies of laccase structure have revealed a monomer glycoprotein chain with three spectroscopically distinct copper atoms in the catalytic site: one type 1 (T1) blue copper, one type 2 (T2) normal copper, and two type 3 (T3) antiferromagnetically coupled copper atoms [10]. The T1 site oxidizes the substrates by taking out, one by one, four electrons and four protons from the substrate molecules, directed then by the internal cluster T2/T3, to an oxygen molecule which is turned into water [11,12]. The substrate loses first a single electron giving a free radical which is stabilized by different kind of reactions, like hydration, elimination, polymerization, etc. [13]. Laccases catalyze the oxidation of substrates simultaneously with the reduction of molecular oxygen to water [11].

$$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$$

For large-scale applications, some properties of free laccases are not desirable, sensitivity to denatured processes and the non-reusability being the major impediments. These constraints could be eliminated by the immobilization process. The process enlarged the area of application, immobilized enzymes being used as: heterogeneous biocatalysts, analytical devices, controlled released drugs, etc. [14]. The new biocatalysts may be evaluated according their catalytic (activity, selectivity, stability) and non-catalytic (geometry, mechanical, and chemical stability) properties, general criteria for evaluation of both functions being established [15].

Literature presents a number of applications of laccases immobilized on different supports [16–18]. According the described examples, immobilization of laccases on different solid carriers improved their stability and made possible the reuse of the biocatalyst, leading to more economic processes.

The non-catalytic function is mainly determined by the carrier, its structure, and stability being important. The choice of the matrix was based on previous results. Insoluble matrix, based on poly(vinyl alcohol) (PVA) as support for laccase immobilization, was used successfully by D. Yinhui and coworkers [19]. The advantages of this type of immobilization, making the enzyme stable and reusable, were underlined by the authors. Thus, the preparation of new biocatalysts by laccase immobilization on PVA carriers seems of interest.

The present study describes the immobilization of a commercial laccase *Roglyr Lite 1540* provided by Hungarian Industry-KFT on PVA *cryogel* type support used with success as carrier for other enzymes or cells immobilization [20–25]

Materials and Methods

Chemicals

N-(3-Dimethylaminopropyl)-*N*′-ethyl-carbodiimide hydrochloride (DCD), β-alanine, and 2,2′-azinobis-(3-etylbenzothiazoline-6-sulfonate) (ABTS) were provided by Sigma. Glutaraldehyde (50% aqueous solution) was purchased from Aldrich. Poly(vinyl alcohol) (PVA) with molecular weight of 86 kDa and deacetylation degree of 99–100% was supplied by Acros Organics, Belgium. All the chemicals used were of analytical grade.

Polymer Carrier

PVA cryogel particles (diameter 0.8–1.6 mm) were prepared using cryogranulating setup "CryoMat" (BioChimMac Co. Moscow, Russia) based on the original procedure described before [26–28].

Enzyme

Laccase Roglyr Lite 1540 was a commercial product supplied by Hungarian Industry Products KFT and produced by Rotta Manheim.

Enzyme Protein Content

The protein content of the commercial laccase was determined by the Bradford method, measuring the complex of Coomassie Brilliant Blue G250 with the protein at 595 nm. The standard protein solution was bovine serum albumin [29].

Enzyme Activity Assay

The activity of the enzyme free or immobilized was assayed with a solution of 60 mM ABTS as substrate, at 37 °C, by monitoring the absorbance at 420 nm ($\varepsilon_{420}=3.6\cdot10^4 M^{-1} cm^{-1}$) which is characteristic for the ABTS radical-cation oxidation product [7,30]. The measurements have been done at several pH values in: 100 mM citrate buffer for pH 3.8, 100 mM acetate buffer for pH 4.7, and 100 mM phosphate buffer for pH 5.8. For the free commercial laccase, the solution of 15 mg mL^{-1} concentration has been used while for the immobilized enzyme, the equivalent quantities of beads, precisely weighed, were soaked in the ABTS solutions. Enzyme-specific activity was expressed as units per protein content (U g^{-1}). One unit of laccase activity (U) is defined as the amount of enzyme required for oxidizing 1 μmol of ABTS per minute. Each determination was done in triplicates.

PVA-Cryogel Carrier Pretreatment

The necessary amount of PVA cryogel beads (1 g) was suspended in a solution of 100 mM hydrochloric acid and shaken for 15 min. The PVA carrier was then functionalized by treatment with 5 mL of 7% glutaraldehyde solution in 100 mM hydrochloric acid (pH ~ 1), with gentle stirring, for 1 h at 20 °C. The activated CHO-containing matrix was rinsed with water to remove the remaining unreacted aldehyde.

Enzyme Immobilization

The enzyme immobilization was performed as follows:

Procedure (a) The activated PVA cryogel (1 g of wet weight) was treated with 1.5 mL of commercial laccase solution (15 mg mL^{-1}) in 100 mM phosphate buffer, pH 6.0. The suspension was mixed by shaking at room temperature (20 °C) for 6 h. After 24 h of staying in a refrigerator (4 °C), the shaking process was resumed for 2 h at room temperature. The mixture was filtered and the beads washed with buffer solution for eliminating the trace of free enzyme. Finally, the biocatalyst was stored in 100 mM phosphate buffer pH 6.0 solution, at a

temperature of 4 °C. The amount of immobilized laccase was measured by making the difference between the protein contents of the initial commercial enzyme solution and that of the reunited washing solutions.

Procedure (b) In this procedure, the same experimental conditions have been preserved, the difference consisting in a concomitant treatment of the carrier (1 g of wet beads) with the same amount of enzyme [1.5 mL of commercial laccase solution (15 mg mL^{-1}) in 100 mM phosphate buffer, pH 6.0] and DCD (0.1 μmol DCD per 1 g activated wet carrier). The same workup procedure, previously described for procedure (a), has been applied.

Procedure (c) In the third procedure of immobilization, the activated carrier (4.03 g wet beads) was first treated with β-alanine (50 mL distilled water solution of 100 mM β-alanine) for 2 h at room temperature with shaking. The suspension was kept overnight at the refrigerator and then filtered. After washing with water of the filtered carrier for elimination of the unreacted β-alanine, the enzyme immobilization was performed by a stepwise reaction. The carrier with β-alanine spacer was suspended in 100 mM phosphate buffer of pH 6.0, and 0.1 μmol DCD per 1 g wet beads was added. The suspension was shaken for 20 min at 20 °C. The solution was removed through filtering at vacuum. The obtained carrier was used for the immobilization process without any intermediate washing step. A 1.5-mL enzyme solution (15 mg mL^{-1}) in 100 mM phosphate buffer, pH 6.0 per 1 g wet carrier was added, and the reaction mixture was shaken for 6 h at 20 °C. Then, it was kept for 24 h at 4 °C, filtered, washed with buffer (measuring the unreacted enzyme by its protein content), and stored (4 °C).

Stability of the Free and Immobilized Enzyme

Solutions of free and immobilized enzyme in acetate buffer of pH 4.7 were prepared and stored at 25 °C. By measuring the enzyme-specific activity at different times, the stability of the free and immobilized enzyme was evidenced (Fig. 1).

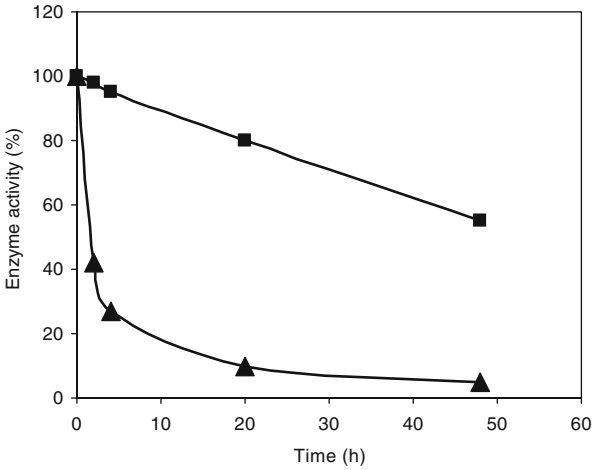

Fig. 1 Stability of the free enzyme (*filled triangles*) and immobilized enzyme (*filled squares*) (procedure [a])

Results and Discussion

The macroporous polymer carrier was prepared originating from the 100 g/L aqueous PVA solution by a freeze-thawing process [28]. The carrier, thus, formed was of a spherical shape having diameters of 0.8–1.6 mm and, more important, presenting a macroporous structure. The characteristic feature of the gel matrix in PVA cryogels is the supramolecular network, possessing viscoelastic properties that provide both excellent rigidity (elastic module up to 10–20 kPa and higher) and high resistance to abrasive erosion even under intense stirring. These properties, in combination with the macroporosity of the polymer [31–33], facilitate the non-hindered diffusion of solutes within the carrier's bulk [34]. The hydrophilic properties of the PVA carrier change the microenvironment of the enzyme improving the catalytic function [14].

A commercial laccase *Roglyr Lite 1540* was immobilized using three different procedures in the PVA cryogel type matrix, activated by treatment with glutaraldehyde (see experimental data). In the first procedure (a), the activated matrix was treated directly with a solution of enzyme:

$$\text{Polymer} - \text{CHO} + \text{H}_2\text{N} - \text{Enz} \rightarrow \text{Polymer} - \text{CH} = \text{N} - \text{Enz}$$

DCD was used in a second procedure (b) for obtaining better yield, by elimination of the water resulted in the condensation reaction of amino groups of the enzyme with the free carbonyl groups from the glutaraldehyde moiety of the polymer.

In a third procedure (c), by a multistep synthesis, β-alanine was introduced as a spacer:

$$\begin{aligned}&\text{Polymer} - \text{CHO} + \text{H}_2\text{N} - \text{CH}_2\text{CH}_2 - \text{COOH} \rightarrow\\ &\text{Polymer} - \text{CH} = \text{N} - \text{CH}_2\text{CH}_2 - \text{COOH} + \text{H}_2\text{N} - \text{Enz} \rightarrow\\ &\text{Polymer} - \text{CH} = \text{N} - \text{CH}_2\text{CH}_2 - \text{CONH} - \text{Enz}\end{aligned}$$

The protein content of the free enzyme, established based on the Bradford method, is of 25.7±1.2 mg protein/g commercial product (average value of triplicate experimental determinations) [29]. The commercial product contains a large quantity of other components than the enzyme.

The amount of enzyme fixed on the polymeric support was measured related to the protein content of the samples of enzyme before and after immobilization [29].

The quantities of enzyme (by the protein content) immobilized in each of the three procedures are presented in Table 1.

According the experimental data, the amount of enzyme in the biocatalysts varies from 0.23 to 0.57 mg/g carrier. The small quantity of immobilized enzyme in all the experiments is most probably due to the reduced enzyme content into the commercial product (25.7± 1.2 mg protein/g commercial product). The inclusion of the β-alanine spacer leads to the reduction of enzyme content most probably due to the multistep synthesis.

Table 1 Protein content in the biocatalysts.

Immobilization procedure	Protein/carrier (mg g^{-1})
Procedure (a)	0.57
Procedure (b)	0.41
Procedure (c)	0.23

The specific activity of the free enzyme and the immobilized samples has been determined by a well-known procedure [30] using ABTS as substrate (see experimental data). The measurement of the absorbance values at 420 nm, specific to ABTS radical-cation, evidenced the enzyme activity. The obtained values (U g^{-1}) for the free and immobilized enzymes, at different pH, are included in Table 2.

The specific activity of the immobilized enzyme is lower compared with the free enzyme. One advantage concerning the catalytic function is the change of pH domain which has been displaced toward less acidic values. The immobilized enzyme has an optimum pH value of 4.7 while the free enzyme is more reactive at a pH of 3.8. According to the literature, by immobilization, the optimum pH may be changed by as much as 2 pH units. Such a change is explained by the influence of the carrier microenvironment on the reaction conditions [35,36]. Similar behavior has been also observed in the case of an immobilized protease [37] as well as an immobilized laccase [38].

The β-alanine spacer does not give changes in enzyme activity reported to protein content (U g^{-1}). Usually, spacer changes the microenvironment, orientation, and conformational flexibility of the enzyme, but for having visible effects, a longer spacer seems to be needed. In the presented experiments, the biocatalyst with spacer has similar behavior with the biocatalyst without spacer. These results confirm previous data presented in literature [14,39].

Another advantage of immobilization is the higher stability of the immobilized enzyme confirmed by the values of the half-lives for a sample of covalent immobilized enzyme synthesized by the procedure (a), compared with the free commercial enzyme incubated in the same conditions (pH 4.7, 25 °C).

The obtained experimental values of the half-lives, for the free and immobilized enzyme, are 1.5 h and more than 50 h, respectively.

The improved stability and pH range modification give the opportunity for new applications using laccase as catalysts. For better results, enzyme from a different source, with higher protein content, will be studied.

Table 2 Specific activity of free and immobilized enzyme.

Biocatalyst	pH	Specific activity (μmol $min^{-1}g^{-1}$)
Free enzyme	3.8	1,301.2
	4.7	1,017.1
	5.8	965.0
Immobilized enzyme [procedure (a)]	3.8	196.5
	4.7	268.4
	5.8	76.7
Immobilized enzyme [procedure (b)]	3.8	207.7
	4.7	285.0
	5.8	106.3
Immobilized enzyme [procedure (c)]	3.8	158.0
	4.7	263.3
	5.8	143.6

Conclusions

New biocatalysts have been prepared by covalently immobilizing laccase in PVA cryogel type carrier. The immobilized enzymes have a lower activity compared with the free enzyme but are more stable and can be stored longer without losing their activity. The optimum pH value is higher for the immobilized enzyme compared with the free enzyme, facilitating reactions in milder conditions. The introduction of a short chain spacer does not change the catalyst specific activity. A commercial enzyme, having a small content in the active compound, is not the best solution to be used as raw material for the immobilization process despite the advantage of easy availability. Taking into account these preliminary results, further experiments with enzymes from other sources will be performed.

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