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Formation of enzyme polymer engineered structure for laccase and cross-linked laccase aggregates stabilization

Thanina Hassani [a], Sidy Ba [b], Hubert Cabana [a,c,*]

[a] Environmental Engineering Laboratory, Department of Civil Engineering, University of Sherbrooke, 2500 Boulevard de l'Université, Sherbrooke, Quebec, Canada J1K 2R1
[b] Department of Chemical Engineering, University of Sherbrooke, 2500 Boulevard de l'Université, Sherbrooke, Quebec, Canada J1K 2R1
[c] Étienne-Le Bel Clinical Research Centre, Centre Hospitalier Universitaire de Sherbrooke, 3001, 12e Avenue Nord, Sherbrooke, Quebec, Canada J1H 5N4

HIGHLIGHTS

- The organic/inorganic network stabilized the enzyme versus harsh environments.
- These structures did not limit the mass transfer of the substrate.
- Applications of these biocatalysts are promising for bioprocesses.

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ABSTRACT

Laccase and laccase-based cross-linked enzyme aggregates (CLEAs) were stabilized through the formation of a surrounding polymeric network made of chitosan and 3-aminopropyltriethoxysilane. The thermoresistance of the resulting enzyme polymer engineered structures of laccase (EPES-lac) and CLEAs (EPES-CLEA) were more than 30 times higher than that of free laccase and CLEAs at pH 3 and 40 °C. The EPES showed higher residual activity than the unmodified biocatalysts against chaotropic salts (up to 10 times), EDTA (up to 5 times), methanol (up to 15 times) and acetone (up to 20 times). The Michaelis–Menten kinetic parameters revealed that the affinity for 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) has doubled for the EPES-lac and EPES CLEA compared to their unmodified forms. The EPES-lac structures acted optimally at pH 4 and their activity was nearly temperature-independent, while the laccase activity of EPES-CLEA was optimal at pH 4 and 60 °C. Globally, the EPES have shown significantly improved properties which make them attractive candidate for the development of laccase-based applications.

1. Introduction

Laccases (polyphenoloxidase, EC 1.10.3.2) mainly produced by plants, fungi, bacteria and insects, are able to catalyze the oxidation of many organic compounds such as methoxyphenols, phenols, o- and p-diphenols, aminophenols, polyphenols, polyamines, molecules from lignin and some inorganic ions (Rodríguez Couto and Toca Herrera, 2006; Burton, 2003; Call and Mucke, 1997). These multicopper oxidases offer various applications including, cosmetics, food, textile and paper industry, wastewater treatment, and soil bioremediation (Call and Mucke, 1997; Selinheimo et al., 2006; Hou et al., 2004; Pointing, 2001).

The use of free laccases in such applications is hampered by their relatively short lifetime and their instability under harsh environment (e.g., temperature, organic solvents, and salts) (Brady and Jordaan, 2009). In addition, the difficulty of retaining the free enzymes in a continuous flow bioreactor makes the use of laccases a costly alternative to conventional industrial processes (Osma et al., 2011).

These drawbacks have impelled the search for strategies to enhance the stability of the enzyme such as enzyme immobilization (with or without support), enzyme modification, genetic modification and medium engineering (Kim et al., 2006; Burton et al., 2002; Livage et al., 2001; Durán and Esposito, 2000; Koeller and Wong, 2001; Schmid et al., 2001).

Abbreviations: EPES, enzyme polymer engineered structure; SENs, single enzyme nanoparticles; CA, carbonic anhydrase; APTES, 3-aminopropyltriethoxysilane; CLEAs, cross-linked enzyme aggregates; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; ABTS, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid); EPES-lac, enzyme-polymer engineered structure of laccase; EPES-CLEA, enzyme-polymer engineered structure of CLEAs.

* Corresponding author at: Department of Civil Engineering, Environmental Engineering Laboratory, University of Sherbrooke, boulevard de l'Université, 2500 Sherbrooke (Qc), Canada J1K 2R1. Tel.: +1 819 821 8000x65457; fax: +1 819 821 7974.
E-mail address: Hubert.Cabana@USherbrooke.ca (H. Cabana).

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These stabilization methods enable to improve enzyme stability, produce reusable biocatalysts, and maintain good catalytic efficiency (Majeau et al., 2010) but complications such as lower specific activity, enzyme activity decrease, and mass transfer limitation still hamper the development of enzyme based bioprocesses (Majeau et al., 2010).

One way to overcome the low specific activity of the biocatalysts is the formation of cross-linked enzyme aggregates (CLEAs). Since this technique does not require a solid support, it produces biocatalysts with high enzyme activity per unit volume (Sheldon, 2007).

On the other hand, the enzyme polymer engineered structure (EPES) approach, based on the grafting of a polymer at the surface of enzymes through the formation of covalent bounds between non-essential amino acids residues and a selected polymer also increases the stability of the resulting biocatalysts. This improvement has been obtained, in some cases, without steric hindrance which could reduce the mass-transfer of the substrate into the enzymes active site.

Kim and Grate (2003) and Hegedus and Nagy (2009) prepared single enzyme nanoparticles (SENs) with proteolytic enzymes using a three-step procedure involving the anchorage of a vinyl group at the surface of the enzyme, polymerization of free vinyl groups and trimethoxysilane under UV radiations, and an orthogonal polymerization between the polymer chains. The resulting SENs showed a half-life of more than 140 days comparatively to 12 h for their free counterparts (Kim and Grate, 2003). Yadav et al. (2011a, 2011b) stabilized carbonic anhydrase (CA) through the formation of a hybrid polymer of chitosan and 3-aminopropyltriethoxysilane (APTES) and showed that their modified CA remained stable for more than 100 days at either −20, 4 or 20 °C.

Thus, the formation of a new generation of biocatalysts based on a combination of CLEAs and EPES techniques is a way to enhance enzyme stability. The first objective of the present work was to synthetize EPES of laccase (EPES-lac) and EPES of laccase-based CLEAs (EPES-CLEA). As the synthesis of CLEAs and EPES requires the use of a cross-linking agent and a polymer, the use of the renewable biopolymer chitosan was due to its protein affinity, good mechanical strength and its resistance to chemical degradation (Arsenault et al., 2011). The second objective was to characterize the formed biocatalysts including, thermal storage, chemical stability, Michaealis-Menten kinetics, and optimal pH and temperature.

2. Methods

2.1. Chemicals

Laccase from *Trametes versicolor* and all other chemicals were from Sigma–Aldrich (Saint-Louis, MO, USA). All solvents were of HPLC grade. Chitosan (mean molecular weight of 750 kDa and 64% deacetylated) was solubilized in 0.1 M HCl.

2.2. Preparation of the biocatalysts

The preparation of EPES-lac and EPES-CLEA was performed in two stages. The first one consists of the formation of CLEAs and laccase–chitosan bioconjugate followed by the synthesis of EPES-lac and EPES-CLEA.

2.2.1. Chitosan grafting

2.2.1.1. Laccase–chitosan bioconjugate formation. For the conjugation reaction a 10-mL solution containing chitosan (1.5 g/l), laccase (1 U/mL), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (100 mmol/l) and sodium phosphate buffer pH 5 (0.1 M) was stirred at 150 rpm with a magnetic stirrer at room temperature (20 °C) for 10 min. After incubation at 4 °C for 24 h, the laccase conjugates were precipitated by adding 100 mM Tris buffer (pH 7.5), centrifuged at 7000×*g* and 4 °C, and washed 3 times with 2 mL of Tris buffer to remove unreacted chemicals. The precipitate was dissolved in 10 mL of 100 mM sodium acetate buffer (pH 4) and stored at 4 °C.

2.2.1.2. Formation of CLEAs. Ten milliliters of a solution containing 550 g/l of ammonium sulfate and 1000 U/l of laccase was left to precipitate for 1 h at 20 °C. Subsequently, chitosan (1.5 g/l), EDC (100 mM), and sodium phosphate buffer (500 mM) at pH 5 were added and the mixture was incubated at 4 °C for 48 h. CLEAs were recovered by centrifugation at 9000×*g* for 10 min, repeatedly washed with 3 mL of deionized water and centrifuged at 9000×*g* for 10 min until no laccase activity was detected in the supernatant. Deionized water was added to the precipitate to a final volume of 10 mL.

2.2.2. Synthesis of EPES-lac and EPES-CLEA

Ten milliliters of a solution containing 3-aminopropyltriethoxysilane (APTES) and dichloromethane (1:10 v:v) was added to 10 mL of laccase–chitosan bioconjugate or CLEAs solution, vortexed at 20 °C (500 rpm) for 5 min and centrifuged at 9000×*g* for 10 min. The aqueous phase was recovered and 2 mL of deionized water was added to the lower dichloromethane fraction. This solution was again vortexed and centrifuged for the recuperation of the EPES. This process was repeated until no laccase activity was detected in the deionized water used to extract biocatalysts from the dichloromethane fraction. The final volume of the aqueous fractions was adjusted to 16 mL with deionized water. The solutions were then stored 3 days at 4 °C for hydrolysis and crosslinking.

2.3. Enzyme activity assay

Laccase activity was measured by monitoring the oxidation of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) to its cation radical ($ABTS^{\cdot+}$) at 420 nm ($\varepsilon_{max} = 3.6 \times 10^4$ M^{-1} cm^{-1}) (Johannes and Majcherczyk, 2000; Bourbonnais and Paice, 1990). The assay mixture contained 0.5 mM ABTS and a pH 3 citric acid (50 mM)/disodium hydrogen phosphate (50 mM) buffer. The temperature was set at 20 °C. One unit (U) of activity was defined as the amount of enzyme forming 1 μmol of $ABTS^{\cdot+}$ per min.

2.4. Stability of the biocatalysts

The stability of the biocatalysts was determined by incubating 750 μl of free laccase, CLEAs, EPES-lac and EPES-CLEA solutions (initial laccase activity of 0.5 U/mL) at 40 °C in 250 μl of pH 3 citric acid (50 mM)/sodium phosphate (50 mM) buffer for 2 h to 22 days. The samples were assayed as described in section 2.3.

Stability against different chemical denaturants was also tested. The denaturing solutions consisted of 10 μM $CaCl_2$, 10 μM $ZnCl_2$, 10 μM EDTA, 25% (v/v) methanol or 25% (v/v) acetone, prepared in citric acid (50 mM)/disodium hydrogen phosphate (50 mM) buffer at pH 3. Two hundred fifty microliters of each biocatalyst incubated for 4 h in 750 μl of each of these denaturing solutions.

2.5. Biocatalysts kinetics

Kinetic parameters of the biocatalysts were determined by using ABTS concentrations in the range of 0.05–2 mM in 50 mM pH 3 citric acid/disodium hydrogen phosphate buffer at 20 °C. Michaelis–Menten constants were obtained by curve fitting the