Ultrasonics - Sonochemistry 48 (2018) 275–280

Contents lists available at ScienceDirect

Ultrasonics - Sonochemistry

journal homepage: www.elsevier.com/locate/ultson

The effect of high-energy environments on the structure of laccase-polymerized poly(catechol)

Jing Su[a,b], Tarsila G. Castro[b], Jennifer Noro[b], Jiajia Fu[a], Qiang Wang[a], Carla Silva[b], Artur Cavaco-Paulo[a,b,*]

[a] International Joint Research Laboratory for Textile and Fiber Bioprocesses, Jiangnan University, Wuxi 214122, China
[b] Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

ARTICLE INFO

Keywords:
Catechol
Polymerization
Laccase
Ultrasonic bath
High-pressure homogenizer

ABSTRACT

The laccase polymerization of catechol was performed using different reactors namely a water bath (WB), an ultrasonic bath (US) and a high-pressure homogenizer (HPH). The total content of free OH and the MALDI-TOF spectra of polymers obtained demonstrated that reactions are favored in the presence of high-energy environments. Higher conversion yields and polymerization degrees (DP) were obtained after polymerization using US or HPH. Molecular dynamic simulation studies supported these findings by revealing a more open enzyme active site upon environments with high molecular agitation. The higher mass transport generated by US and HPH is the main feature responsible for a higher substrate accessibility to the enzyme which contributed to produce longer polymers.

1. Introduction

Wastewater discharged from processing industries, like oil refineries and petrochemical, contain dissolved organic pollutants such as phenols and substituted phenolic compounds, which are toxic and hazardous to the environment, unless they are treated properly [1]. A number of technologies have been proposed for the removal of phenolic compounds, like catechol, from wastewater aqueous solutions. These include destructive processes such as chemical oxidation, coagulation, solvent extraction, liquid membrane permeation and adsorption, adsorptive micellar flocculation, ultrafiltration, and biological methods [2]. Although chemical methods are mostly applied, milder reaction conditions (like mild pH and low temperature) are often preferred. Due to their versatile biochemical properties, high protein stability, and breadth of substrate spectrum, laccases are the key promiscuous and environmentally friendly biocatalysts, able to catalyze various aromatic compounds [3]. Previous studies indicate that flavonoids, like catechol, represent important and versatile substrates which are polymerized via laccase-catalyzed oxidation, and the products of reaction are regarded as valuable redox polymers with excellent matrix functionalities applied in several fields [4,5]. In our previous studies we chemically modified laccase from *Myceliophthora Thermophila* by PEGylation with 20 kDa poly(ethylene glycol) methyl ether, and use it to polymerize catechol without solvents addition, using a water bath as reactor device under mild pH conditions (pH 5). We have found that it is possible to control the structure of the poly(catechol) produced by PEGylated laccase polymerization [6]. However, as other studies reported, low polymerization yields were obtained after laccase catalysis in the absence of external stimulus.

Ultrasound has been extensively used in enzyme-catalyzed biotransformations aiming to intensify the reaction processes and obtain higher yield of products in short periods of time [7–10]. Several works have been reporting laccase catalysis assisted by ultrasound however lacking information about the polymer structure obtained using differentiated devices [9,11,12].

High pressure homogenization as well as ultrasound are known to produce cavitation. Comparison studies have been performed between both methods which reveal that hydrodynamic cavitation offers a better control over operating parameters, being more energy-efficient and less sensitive to reactor geometry [13]. However, until now few reports have been presenting the use of hydrodynamic cavitation to assist polymerization being the acoustic cavitation the only tool used for this purpose.

Our aim in this study is to evaluate the role of different high-energy environments on the laccase-assisted polymerization of catechol. For this, three different reactors were used namely a water bath (WB), an ultrasonic bath (US) and a high-pressure homogenizer (HPH). The polymerization was followed during time by UV–Vis spectra analysis of the color change. The produced polymers were characterized by Matrix Assisted Laser Desorption/Ionization-Time of flight Mass spectrometry

* Corresponding author at: Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
E-mail address: artur@deb.uminho.pt (A. Cavaco-Paulo).

https://doi.org/10.1016/j.ultsonch.2018.05.033
Received 27 February 2018; Received in revised form 21 May 2018; Accepted 28 May 2018
Available online 29 May 2018
1350-4177/

(MALDI-TOF) and ^{1}H NMR. The activity and stability of laccase during processing were evaluated. Molecular dynamic simulations were also conducted to understand the molecular behavior of laccase under high-energy environments.

2. Experimental part

2.1. Materials

Laccase from *Myceliophthora Thermophila* was supplied by Novozymes, Denmark. Catechol, sodium carbonate, Folin-Ciocalteu reagent and MALDI-TOF matrices were purchased from Sigma Aldrich, Spain. Deuterated chloroform and dimethyl sulfoxide were obtained from Cortecnet, France.

2.2. Evaluation of enzyme stability and half-life time quantification

The effect of high-energy environments on the activity and stability of laccase was evaluated. For this, laccase was incubated under the same conditions used for catechol polymerization: the enzyme (100 U/ml) was incubated in acetate buffer (pH = 5) at 40 °C for 2 h using different reactors namely a water bath, an ultrasonic bath and a high-pressure homogenizer. Aliquots of enzyme solution were taken at different periods of incubation and the activity of laccase was measured against ABTS according to the methodology described by Childs and Bardsley [14]. The half-life time of native laccase was calculated according to the Eq. [15]:

$$K = (\ln U_0 - \ln U_t)/t$$

U_0: enzyme activity at time zero;

U_t: enzyme activity at time t;
t: time of incubation;
U: one U is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate (ABTS) per minute.

2.3. Laccase-assisted polymerization of catechol using different reactors

Catechol polymerization was processed by incubating 5 mg/mL of monomer with 100 U/mL laccase in acetate buffer (pH = 5). The reactions were performed in three different reactors namely a water bath (Grant, United Kingdom), an ultrasonic bath (USC600TH, VWR International Ltd., USA; frequency 45 kHz and power of 120 W) and a high-pressure homogenizer (Emulsifex-C3, Avestin, Canada; 500–2000 bar, 50 Hz), at 40 °C for 2 h. During reactions, the temperature of the homogenizer container was monitored using a thermometer. Afterwards the powder was washed with water by centrifugation to remove the maximum amount of protein and dried under vacuum for further ^{1}H NMR and MALDI-TOF characterization.

2.4. UV–Visible spectra analysis

The polymerization was followed by UV–Vis spectroscopy using a 96-quartz microplate reader (Biotek Synergy Mx, Shimadzu, Japan).

2.5. ^{1}H NMR spectra

The precipitates obtained after washing and centrifugation were dissolved in deuterated solvents, DMSO-d_6 and $CDCl_3$, for ^{1}H NMR evaluation. The spectra were acquired in a Bruker Avance III 400 (400 MHz) using the peak solvent as internal reference.

2.6. MASS SPECTRA analysis

The polymers were analyzed by Matrix-Assisted Laser Desorption/Ionization with time-of-flight (MALDI-TOF) using 2,5-dihydroxy benzoic acid (DHB) as the matrix (≥99.5 %). The mass spectra were acquired on an Ultra-flex MALDI-TOF mass spectrophotometer (Bruker Daltonics GmbH) equipped with a 337 nm nitrogen laser. For this, the samples were dissolved in a TA30 (30 % acetonitrile/70 % TFA) solution and mixed with a 20 mg/mL solution of DHB (1:1). Then a volume of 2 μL was placed in the ground steel plate (Bruker part n° 209519) until dry. The mass spectra were acquired in linear positive mode.

2.7. Determination of the total content of free OH

The total content of free OH groups of monomer and polymers was determined before and after polymerization using the Folin-Ciocalteu spectrophotometric method. The monomer and polymer solutions dissolved in DMSO (100 μL) were added to the mixture of Folin-Ciocalteu reagent (500 μL) and distilled water (6 mL), and the mixture was shaken for 1 min. Then Na_2CO_3 solution (15 wt %, 2 mL) was added to the mixture and shaken for 1 min. Later the solution was brought up to 10 mL by adding distilled water. After 2 h, the absorbance was measured at 750 nm (25 °C). The total content of free OH was assessed by plotting a gallic acid calibration curve (from 1 to 1500 μg/ml). The equation of the gallic acid calibration curve was $A = 0.2977c + 0.0368$, and the correlation coefficient was $r^2 = 0.999$.

2.8. Molecular modeling and molecular dynamic simulations

Molecular dynamic (MD) simulations were performed with the native laccase structure originated by homology modelling: the *Myceliophthora thermophila* amino acid sequence was obtained from the Gi number identifier (Gi 10058140) published in 2003 among several laccase structures [16] and accomplished by searching this number on UniProt server [17]. After that, we use the Swiss-model server [18,19] to determine the laccase 3D structure under study, building a model from the most similar laccase template, *Melanocarpus albomyces*, [20] with 75 % of similarity.

Laccase was modelled with the simple point charge (SPC) water model in an octahedral box with a hydration layer of at least 1.5 nm between the peptide and the walls. Na^+ ions were added to neutralize the simulation boxes. Three stages of energy minimization were performed using a maximum of 50,000 steps with steepest descent algorithm, due to the size of the system. Position restraints were applied in all heavy atom at the first step, followed by position restraints in the main chain atoms for the second step, and no restraints were used for the last step of energy minimization. The systems were initialized in a NVT ensemble, using Berendsen [21] algorithm, with the coupling constant $\tau_T = 0.10$ ps, to control temperature at 310 K (40 °C) and simulate the experimental conditions used. After that, a NPT initialization step was performed, with V-rescale [22] and Parrinello-Rahman barostat [23] algorithms to couple temperature and pressure at 313 K and 1 atm, respectively. The following coupling constants were considered: $\tau_T = 0.10$ ps and $\tau_P = 2.0$ ps. Position restraints (with force constant of 1000 kJ·mol^{-1}·nm^{-2}) were applied to all protein heavy atoms in NVT initialization, and in the main chain in NPT step. The positions of copper atoms in the active site were frozen to maintain the coordination with histidine. Simulated Annealing [24,25] method was performed during 10 ns to gently carry the system from 36 to 70 °C, simulating the experimental temperature conditions. After this procedure, one of the frames at 36 °C and other at 70 °C were submitted to classical molecular dynamics during 30 ns, without position restraints, and with the same NPT ensemble described above. All simulations were performed using the GROMACS 4.5.4 version [26–28], within the GROMOS 54a7 force field (FF) [29]. The Lennard-Jones interactions were truncated at 1.4 nm and using particle-mesh Ewald (PME) [30] method for electrostatic interactions, also with a cut-off of 1.4 nm. The algorithm LINCS [31,32] was used to constrain the chemical bonds of the enzyme and the algorithm SETTLE [33] in the case of water. From MD simulations, we computed the middle structure at 36 °C and 70 °C,