Appl Biochem Biotechnol (2013) 171:1673–1680
DOI 10.1007/s12010-013-0463-0

Laccase-Catalyzed Oxidative Polymerization of Phenolic Compounds

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Received: 27 May 2013 / Accepted: 22 August 2013 /
Published online: 31 August 2013

Abstract Enzymatic polymerization of phenolic compounds (catechol, resorcinol, and hydroquinone) was carried out using laccase. The mechanism of polymerization and the structures of the polymers were evaluated in terms of UV–Vis and Fourier transform infrared spectroscopy. The molecular weights of the produced polyphenols were determined with GPC. The results showed that the phenolic monomers firstly turned into quinone intermediates by laccase catalysis. Through further oxidation, the intermediates formed covalent bonds. Finally, catechol units were linked together with ether bonds, and both resorcinol and hydroquinone units were linked together with C-C bonds. The number-average molecular weights of the polyphenols ranged from 1,000 to 1,400 Da (corresponding to the degree of polymerization that varied from 10 to 12) with a lower polydispersity value of about 1.10, showing selective polymerization of phenolic compounds catalyzed by laccase.

Keywords Catechol · Resorcinol · Hydroquinone · Laccase · Oxidation · Polymerization

Introduction

Enzymes have been widely used for the production of goods to meet various human needs. These biological catalysts can accelerate the chemical reactions by decreasing the activation energy used for triggering the reaction. With the rapid development of biotechnology, polymer synthesis via enzyme-catalyzed reaction has received much attention in the recent years. Compared with polymer synthesis which makes use of chemical catalysts, enzymatic polymerization takes place with higher efficiency and better selectivity under milder conditions [1–3].

Laccases (EC 1.10.3.2) are multicopper oxidoreductases which are capable of reducing oxygen to water and eliminating one electron from phenolic compounds to form phenoxy radicals in the meantime. Laccases exist in many plants, fungi, and microorganisms. As biological catalysts, laccases are useful for replacing expensive and harmful chemical catalysts with saving energy, and have been widely used in "green chemistry" and "cleaner

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production" [4–6]. In textile processing, laccases have been used for improving the fabric whiteness in bleaching process [7], decolorization of colored effluents [8], scouring of fibers, wool dyeing and wool anti-felting [9], and bio-washing finishing of denim fabrics to achieve the special worn look [10].

In fact, the research on laccase has been further extended in recent years. In a series of previous reports, the potential of laccase for polymerization, cross-linking, and functionalization of various phenolic compounds and lignin-derived aromatic compounds has been widely studied. For instance, Cavaco-Paulo et al. indicated that lignosulfonates, flavonoids, and phenolic compounds such as catechols could be polymerized via laccase-catalyzed oxidation, and the products could be applied for fiber dyeing [11–14]. Aktas et al. evaluated the biokinetic parameters and reaction conditions of oxidative polymerization of catechol catalyzed by laccase [15, 16] and characterized the structure of polycatechol [17]. Božič et al. revealed that the laccase-initiated reaction between phenolic acids and chitosan greatly improved 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical cation scavenging capacity of the phenolic acid-functionalized chitosans [18]. Synthesis of polymers by laccase has attracted much attention nowadays. Some related studies showed that this synthesis is a two-step process, i.e., enzymatic and nonenzymatic reactions [14, 19]. A possible disadvantage of laccase is that this enzyme creates reactive intermediates, the phenoxy radicals, which is not further controlled by the enzyme during the subsequent reaction steps and can further react spontaneously, leading to possible undesired side reactions [14, 20–23].

Catechol, resorcinol, and hydroquinone are polyphenol compounds which have the simplest structure of polyphenols. With the catalytic oxidation by laccase, they show different colors. In this study, catechol, resorcinol, and hydroquinone were used as the substrates to produce colored polymers via laccase-catalyzed oxidation. UV–Vis and Fourier transformed infrared spectroscopy (FT-IR) were employed to investigate the polymerization mechanism of phenolic compounds in laccase system and the structures of the products. The molecular weights of the produced polymers were determined with gel permeation chromatography (GPC)

Materials and Methods

Materials

Laccase Denilite II from *Aspergillus* (EC1.10.3.2) was kindly supplied by Novozymes (Shanghai, China). Tetrahydrofuran was HPLC grade from Merck (Darmstadt, Germany). Catechol, resorcinol, hydroquinone, and all other chemicals used were analytical grade from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China), except extra explanation.

Determination of Laccase Activity

The activity of laccase was measured spectrophotometrically by monitoring the oxidation of ABTS (ε_{420nm}=36,000 M^{-1} cm^{-1}) as substrate at 420 nm in 50 mM acetate buffer at pH 5.0 as described by Niku-Paavola et al. [24] with some slight modifications. Briefly, the reaction mixture contained 0.1 mL laccase of 5 g/L, 2.9 ml ABTS (0.5 mM), at pH 5, with 50 mM acetate buffer as solvent to make a final volume of 3 mL. The spectrometric measurements were carried out with a stopwatch by recording the time per 0.05 absorbance increment. The enzyme activity was expressed in units per milliliter. One unit of laccase was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute at room temperature.

Enzymatic Oxidative Polymerization of Phenols Using Laccase

For polymerization, 2.5 mmol of phenolic compounds (catechol/resorcinol/hydroquinone) was completely dissolved in 50 mL of 0.1 M sodium acetate buffer at pH 5.0 by vigorous stirring. The polymerization reaction was initiated upon the addition of enzyme (laccase) solution (approximately 50 mL with a total activity of 10 U). The reactor was immersed in a constant temperature water bath at 50 °C for 5 h and then boiled for enzyme inactivation.

The color of the medium would slowly turn into dark during the enzymatic incubation, which can be used as an indication of the progress of polymerization reaction. After the polymerization, the reactor was kept in a fridge at 4 °C for 24 h to allow the polymer to precipitate. Then, the residue was centrifuged and the polymer was removed and washed with 1:3 (v/v) acetone/deionized water solution several times to remove the unreacted phenolic compounds and laccases. The produced polymer was left in a lyophilizer through removing the solvent and then was kept in a desiccator for use [16].

UV–Vis Spectra Analysis

To monitor the reorganization of phenolic compounds in different periods of incubation, the UV–Vis spectra of prepared solutions were measured using a UV-2808S UV–Vis spectrophotometer (Unicosh, China) every few minutes during enzymatic treatment.

Fourier Transformed Infrared Spectroscopy

FT-IR spectra of the produced polymers were obtained with a Nicolet iS10 infrared spectrophotometer (Thermo Nicolet, USA), and the phenolic compounds without polymerization were also monitored as reference samples. Before collection, background scanning was performed using the KBr powder. The polymerized phenolic compounds, previously lyophilized, were mixed with a small amount of KBr that was used as matrix. At least 32 scans were performed to achieve an adequate signal-to-noise ratio. The spectra were obtained in the region of 450–4,000 cm^{-1} with a resolution of 8 cm^{-1} at room temperature.

Gel Permeation Chromatography

GPC chromatograms were obtained by using a Waters 1515 Isocratic HPLC pump (Waters Corporation, Milford, USA), with a Waters 2414 refractive index detector (Waters Corporation, Milford, USA), and a GPC KD-802 packed column (Shodex, Japan).

All samples were dissolved in tetrahydrofuran (HPLC grade), and the injection amount of the sample was 30 μg. The flow rate was maintained at 1.50 mL/min with a column temperature of 35 °C. A calibration curve of polyethylene glycols with molecular weight ranged from 400 to 612,000 Da was used to calculate the average molecular weight and polydispersity.

Results and Discussion

UV–Vis spectra of the Phenol Oxidase

The molecular reorganization of the phenolic compounds catalyzed by laccase after different periods of incubation was studied. The spectrum of each solution was monitored and interpreted by UV–Vis spectrophotometry (Fig. 1). The phenolic compound solutions

without laccase were also monitored as the reference samples. The results showed that laccase was highly active for the oxidative polymerization of the phenolic compounds.

For catechol (Fig. 1a), a new peak appeared at 408 nm at the beginning stage of laccase action. As the reaction continued, this peak disappeared. Meanwhile, the color of catechol solution changed from colorless to dark brown during enzymatic oxidation. It can be explained that in the first stage of oxidation, catechol formed phenoxy radical that turned into o-benzoquinone intermediates, which corresponded to the new peak [13]. These quinine intermediates have quite high activity. They could further react spontaneously, forming dimers, oligomers, and polymers [22, 23]. The peak representing the intermediates died away after a period of time due to the consumption of these "active" intermediates in further reaction. Moreover, the oxidative polymerization led to the generation of conjugate structure, making the color of solution become dark [17].

For resorcinol (Fig. 1b), the color of solution changed from colorless to dark orange during enzymatic oxidation, which corresponded to the increase of absorbance value. However, no remarkable absorption bands in the visible region were detected. It can be ascribed to the fact that two phenolic hydroxyl groups of resorcinol could not be oxidized into quinones at the same time, and there was no corresponding oxidative product of quinone for resorcinol. It seems that resorcinol presented slightly different polymerization mechanisms when compared with catechol.

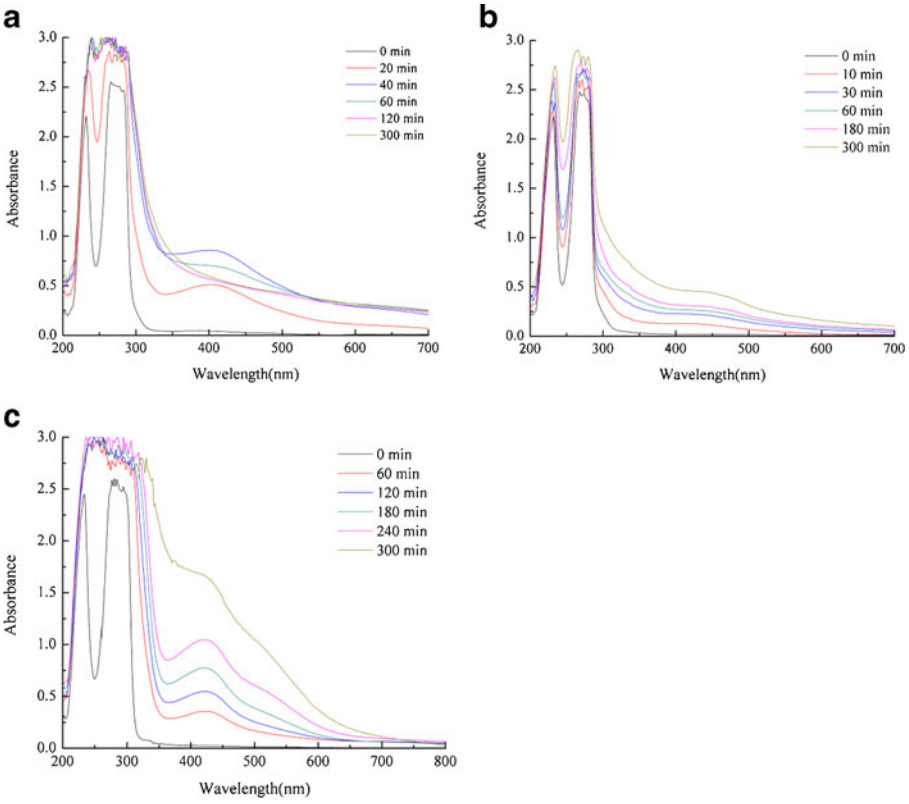

Fig. 1 UV–Vis spectra of **a** catechol, **b** resorcinol, and **c** hydroquinone oxidation in the presence of laccase monitored in different periods; solutions of monomer without laccase addition were used as controls

For hydroquinone (Fig. 1c), the reaction rate was relatively slow, causing no obvious change during the first hour. After that, a new peak appeared at 415 nm, which corresponded to the formation of p-benzoquinone, and the peak died away owing to further reaction of p-benzoquinone [14]. During enzymatic oxidation, the color of hydroquinone solution changed from colorless to dark wine red.

FT-IR Spectra of the Polyphenols

To evaluate the structural properties of the phenolic compounds and their polymers, corresponding FT-IR spectra were tested and presented in Fig. 2a–c, respectively. It can be observed that the structural changes from the monomers to the polymers (polyphenols) followed the same rule. The bands between 3,500 and 3,300 cm^{-1} which existed in all samples' FT-IR spectra and belonged to phenolic O-H vibration bands became broader after incubation with laccase. It might be caused by the laccase-catalyzed oxidative reaction. The bands at 1,600–1,450 cm^{-1} represented the aromatic skeletal vibrations. The bands between 1,300 and 1,000 cm^{-1} were formed due to the various vibration modes such as C-H, C-O, and C=O. All the samples presented these peaks. As the polymers have more rigid groups compared to the monomers, the intensities of the absorption bands between 1,600 and 1,000 cm^{-1} of the polymers tended to weaken. Meanwhile, the presence of bigger aggregates

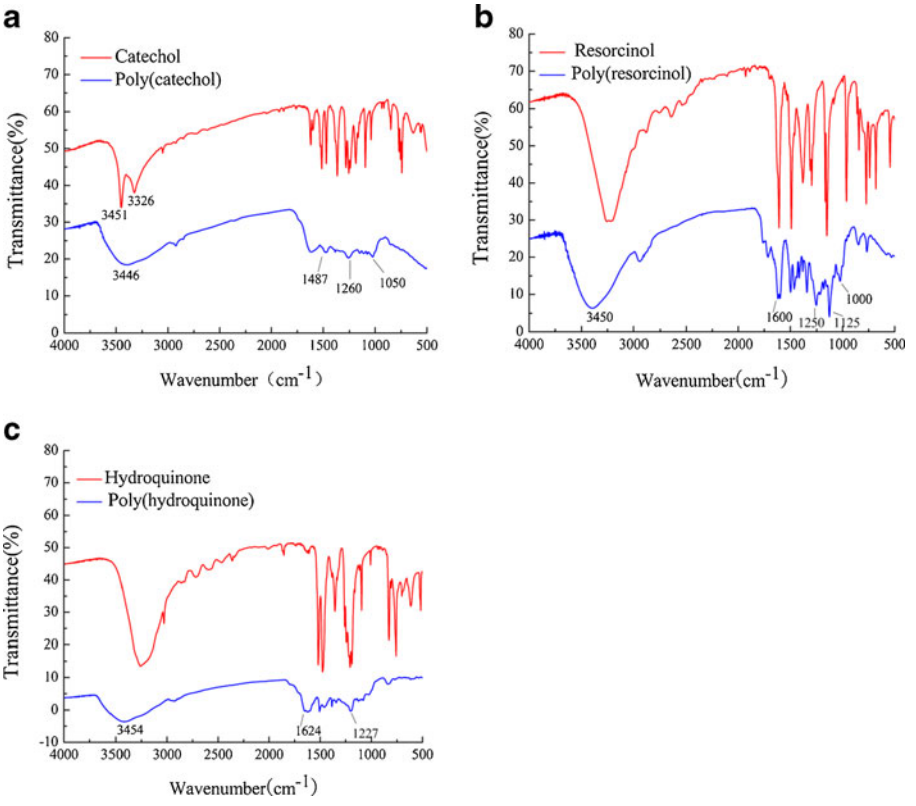

Fig. 2 FT-IR spectra of **a** catechol and polycatechol, **b** resorcinol and polyresorcinol, and **c** hydroquinone and polyhydroquinone

Table 1 Molecular weights of products obtained by laccases-catalyzed polymerization of phenolic compounds

Sample	$\overline{Mn}$ (Daltons)	$\overline{Mw}$ (Daltons)	Polydispersity
Polycatechol	1,172	1,268	1.082
Polyresorcinol	1,341	1,489	1.110
Polyhydroquinone	1,082	1,157	1.070

by polymerization might increase the steric hindrance and hamper the detection of the small groups [11, 17].

For polycatechol, the double peaks at 3,451 and 3,326 cm^{-1}, which attributed to the hydroxyl groups, transformed to one broad band at 3,446 cm^{-1} after enzymatic reaction. The most likely explanation was that the hydroxyl groups involved oxidation polymerization of catechol, which caused the change of the hydroxyl vibration bands. The peak at 1,487 cm^{-1} belonged to the ortho-substitute benzene ring. The peaks appeared at 1,260 and 1,050 cm^{-1} which corresponded to the C-O-C stretches. These strong absorption bands showed that catechol units in the structure of produced polymer connected to each other by ether linkages [17].

For polyresorcinol, the peak at 3,450 cm^{-1} represented the hydroxyl group, the peak at 1,600 cm^{-1} belonged to C=C aromatic skeletal vibration, and the peak at 1,125 cm^{-1} was attributed to phenolic C-O vibration band. The fourth C in resorcinol structure has high reactivity, and chemical reactions always initiate from this site. As suggested above, we

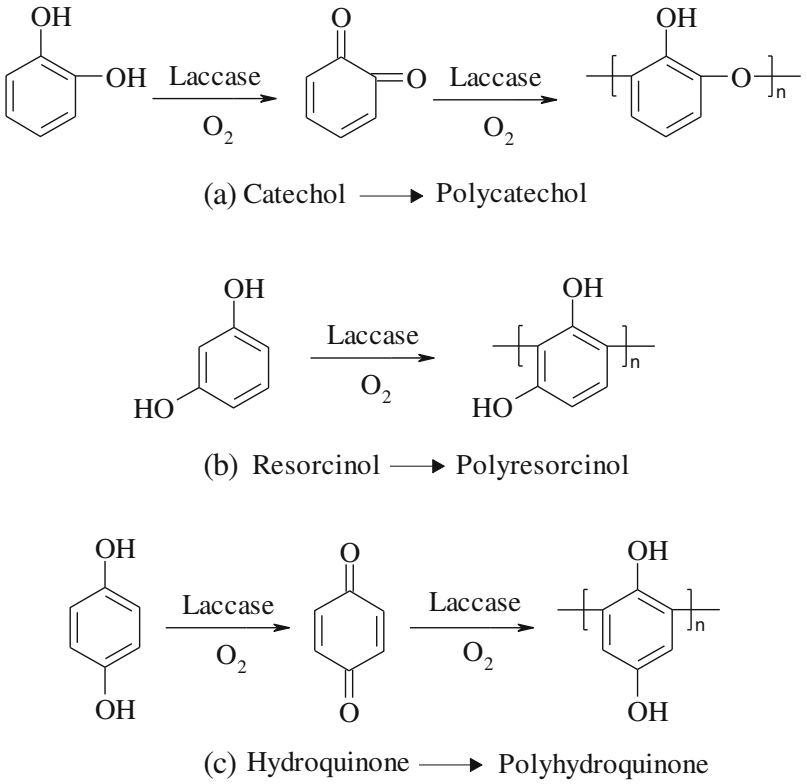

Fig. 3 Proposed schematic and chemical structure of laccase-catalyzed polymers

hypothesized that resorcinol units connected to each other by C-C linkages after laccase-catalyzed oxidative polymerization.

For polyhydroquinone, the peak at 3,454 cm^{-1} belonged to phenolic O-H vibration band, the peak at 1,624 cm^{-1} was attributed to quinones C=O vibration band, and the peak at 1,227 cm^{-1} corresponded to phenolic C-O vibration band [20]. There is no remarkable absorption band to demonstrate the existence of ether bond. Given all that, it can be inferred that hydroquinone units in the structure of produced polyhydroquinone connected to each other by C-C linkages.

Molecular Weights and Their Distributions Measured with GPC

The polyphenols produced in laccase system were not perfectly soluble in aqueous solutions. They were merely dissolved at pH 10–11. The weight-average molecular weights ($\overline{Mw}$) varied from 1,200 to 1,500 Da, and the number-average molecular weights ($\overline{Mn}$) ranged from 1,000 to 1,400 Da, which were assessed by GPC. The data on molecular weights are shown in Table 1. As the molecular weights of the monomers (catechol, resorcinol, and hydroquinone) are all 110 Da, it was easy to figure out that the degree of polymerization varied from 10 to 12. The polydispersity values of these polyphenols are close to 1.10, showing that their molecular weight distributions are narrow.

The proposed schematic of phenolic compounds polymerization in laccase system and the chemical structures of produced polyphenols are presented in Fig. 3 based on above data analysis [14, 17, 25, 26].

Conclusion

In this study, three phenolic compounds (catechol, resorcinol, and hydroquinone) were catalyzed by laccases. As an oxidoreductase, laccases initiated the oxidation and polymerization of these phenolic compounds to form colored polymers. The results presented in this work showed that phenolic compounds formed intermediates (quinones) in the first stage of oxidation and underwent further reaction spontaneously to link together with covalent bonds. The colors of polyphenols were dark brown, dark orange, and dark wine red that corresponded to polycatechol, polyresorcinol, and polyhydroquinone, respectively. Although the number-average molecular weights of the enzymatically synthesized polymers were low, their molecular weight distributions are narrow, showing selective polymerization of phenolic compounds initiated by laccase.

As polymeric dyestuffs, these new polyphenol compounds may be very useful for the dyeing of the fabrics made of natural or synthetic fibers. For its high selectivity, high efficiency, and mild conditions, this enzymatic polymerization method has incomparable advantages than conventional methods. In addition, the substrates for laccase-catalyzed polymeric dyestuffs could theoretically extend to other phenolic compounds. Laccase provides a new route for green synthesis of polymeric dyestuffs.

Acknowledgments Authors gratefully acknowledge the financial support of the National Natural Science Foundation of China (51173071,21274055), Program for New Century Excellent Talents in University (NCET-12-0883), The Natural Science Foundation of Jiangsu Province (BK2011157), Ph.D. Programs Foundation of Ministry of Education of China (20110093110003), the Fundamental Research Funds for the Central Universities (JUSRP51312B), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), and the Program for Changjiang Scholars and Innovative Research Team in University (IRT1135).

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