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Can Laccase-Assisted Processing Conditions Influence the Structure of the Reaction Products?

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Laccase is a promiscuous enzyme that catalyzes the polymerization of a wide range of phenolic substrates. Diverse poly(catechol) products can be obtained depending on the reactor employed and modifications of the laccase enzyme. The generation of these different reaction products may be attributed to changes in the geometry of the enzyme active site induced by different environmental conditions.

Catalytic Properties of Laccase Enzyme

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are biological catalysts belonging to the polyphenol oxidase family that contain copper atoms in the catalytic center, and are often termed multicopper oxidases [1–3]. These enzymes have wide application in industrial biochemistry, and for example have created a major breakthrough in reducing pollution through environmentally neutral production of materials. Laccases are widely distributed in higher plants, fungi, insects, and bacteria [4] where they function in metal oxidation, morphogenesis, stress defense, and lignin degradation [5,6], as well as in the detoxification of antimicrobial agents [7]. The enzyme couples four-electron reduction of oxygen with the oxidation of a broad range of organic substrates, including phenolic and non-phenolic compounds such as catechin, catechol, gallic acid, ferulic acid, syringaldehyde, vanillin, acetovanillone, coniferyl alcohol, rutin, and others [1,8], and even oxidizes some inorganic compounds via a one-electron transfer mechanism [9,10]. Laccase uses dissolved oxygen to oxidize its substrates. In nature, laccase participates in lignin biodegradation, breaking down lignin in woody substrates to generate phenolic compounds that can be further oxidized into polyphenolics with antimicrobial properties. These polyphenolic surfaces are normally found at open wood cuts in nature, where they play a biological role in preventing microbial degradation.

Small oligomers are widely generated by laccase-assisted reactions because enzymatic polymerization via oxidative coupling is constrained by mass transfer and saturation limitations [6–8]. Because high molecular weight polymers are less well generated by native laccase-mediated catalysis, the structural features of the polyphenolics obtained and the necessary processing conditions are poorly described in the literature [6–8].

Different Approaches to Laccase-Assisted Polymerization of Catechol: Protein Modifications and Altered Processing Conditions

Enhanced polymerization, using laccases as catalysts, has been studied using compounds such as polyethylene glycol (PEG). Both free PEG and PEGylation of laccase enzyme accelerate the rate of conversion, leading to an increased yield of poly(catechol) product with a range of different structures (Table 1). Immobilization of laccase in epoxy resins, with or without PEG as a spacer between the resin and the catalyst, leads to the generation of additional structures [11,12]. Processing conditions have also been investigated in the laccase-assisted

Table 1. Characterization of Polymers Obtained Using Different Enzyme Forms and Reactors[a]

Reactor[b]	Enzyme	Repeating unit[c]	Free OH group content[d]	Possible structure of the repeating unit[e]	^{1}H NMR spectra of polymers obtained using different enzyme forms	Color of the mixture
WB	Native laccase	92/108	0.51–0.55			
	PEGylated laccase	106/107	0.29–0.33			
	Epoxy-native laccase	198/199	0.19–0.27			
	Epoxy-PEGylated laccase	161/162	0.21–0.25			
	Epoxy-PEG-laccase	116/117	0.07–0.16		Unpublished	
US	Native laccase	154	0.24–0.31		Unpublished	
HPH	Native laccase	233/234	0.32–0.39		Unpublished	

[a]Data reproduced, with permission, from [12,13].
[b]Abbreviations: HPH, high-pressure homogenizer; US, ultrasonic bath; WB, water bath.
[c]Calculated by MALDI-TOF spectra analysis.
[d]Values normalized to the total content of free OH of catechol (=1.00; 100%).
[e]Analysis by ^{1}H NMR of the powder fraction obtained after washing with water and methanol (in DMSO-d_6).

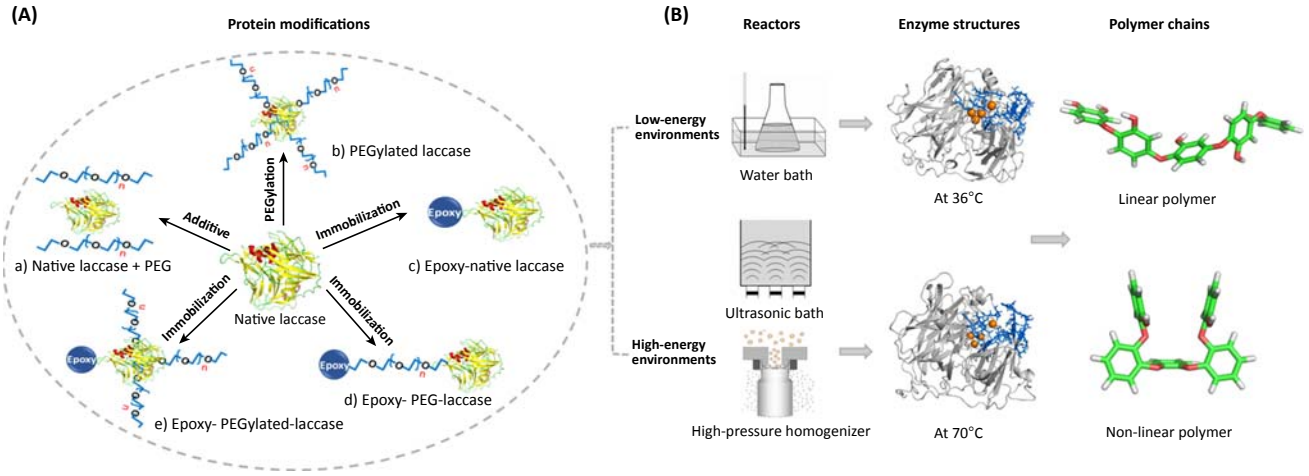

Figure 1. Different Laccase Modification and Processing Conditions Using High-Energy and Low-Energy Reactors for Catechol Polymerization. (A) Different laccase modifications: (a) native laccase + polyethylene glycol (PEG), (b) PEGylated laccase, (c) epoxy-native laccase, (d) epoxy-PEG-laccase, and (e) epoxy-PEGylated laccase, using different reactors (water bath, ultrasonic bath, and high-pressure homogenizer) for the polymerization reaction. (B) Different processing conditions: the high-energy and low-energy reactors used for the laccase-mediated polymerization of catechol are shown in the left-hand panel. The central panel shows the structures of laccase at 36 °C and 70 °C in cartoon representations; active site and cavities for catechol access to the T1 copper site are highlighted (amino acid side chains in blue; laccase is represented in grey, and copper atoms in orange). The right-hand panel shows the proposed structures of poly(catechol) after polymerization, as obtained by quantum calculation at the B3LYP 6-311 + +G(d,p) level (above, proposed linear polymer; below, proposed non-linear polymer) (image adapted, with permission, from [12,13]).

polymerization of catechol. Three types of reactor – water bath, ultrasonic bath, and high-pressure homogenizer – each with specific agitation, pressure, and cavitation characteristics, have been employed for polymerization (Figure 1A) [13]. These different processing conditions, focusing on low- and high-energy environments, yield several different poly(catechol) structural units that have been identified and characterized [13].

Table 1 summarizes the characterization of polymers produced using modified laccase forms and different reactors. The color variations of the reaction mixtures are displayed macroscopically, and these correspond to different UV/visible spectra that differ depending on the form of the enzyme and the type of reactor used. Quantifying the total content of free OH groups of poly(catechol) is crucial in evaluating the type of polymer that is generated. Normalizing the level of free OH groups in catechol to 1.0, the total content of free OH groups in new poly(catechol) polymer is generally much lower (0.5 or less), demonstrating that the enzymatic reaction is likely to take place via an oxidation cascade that either forms ether bonds in the polymeric structure or that ablates OH groups from the phenolic structures. Therefore, determining the total content of free OH in the polymer mixtures facilitates the identification of the different polymeric structures generated by different forms of laccase. In a water bath reactor [12], ultrasonic bath, or high-pressure homogenizer [13], the use of a modified enzyme led to a decrease in the total content of free OH. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) analysis of the reaction products revealed that different forms of laccase in the same reactor can generate distinct repeating units, indicating that PEGylation and immobilization greatly influence the catalytic behavior of the laccase enzyme [12,14]. In addition, using the same enzyme in the context of different reactors can generate different repeating units because acoustic and hydrodynamic cavitation produced by ultrasound and high-pressure homogenization alters the behavior of the active-site cavity of the enzyme, and thereby influences polymer production.

Catechol units in the polymer are most commonly connected by ether linkages [1], a reaction that takes place through O–C bonding to the *para* position of another monomeric unit. The formation of both quinoid derivatives and homomolecular dimers has also been reported after an extended reaction time, and these units are linked by C–C or C–O bonds in the oligomers or polymers [15].

The ^{1}H nuclear magnetic resonance (NMR) spectra (Table 1) of the polymers produced by the different enzyme forms revealed that the products are a mixture of oligomeric and polymeric species, making it difficult to accurately predict and identify their structures. Different possible structures have been proposed based on MALDI-TOF analysis, and these differ according to the form of the enzyme and the reactor used (Table 1).

Polymer Structure Prediction: Different Structures or Different Stages of Oxidation?

Predicting the structure of a polymer generally requires accurate ^{1}H NMR and ^{13}C NMR spectra that are consistent with each other. Previous studies on the enzymatic polymerization of catechol did not provide NMR spectra of the polymers generated or information about how the final polymers were fractionated. Furthermore, even after removing enzyme and unreacted substrate, the structures obtained do not always match the structures proposed in the related literature. Therefore, it remains extremely difficult to confirm the precise structure of a given polymer.

However, information obtained from MALDI-TOF analysis and from molecular dynamic simulations have provided a new perspective on the reported data [12,13]. The MALDI-TOF spectra of the final polymers indicate that modified laccases and high-energy environments favor the polymerization reaction, enhancing the yield of product with a high degree of polymerization (DP), but where the precise structure of the repeating unit depends on the enzyme and reactor used. These enhanced laccase reactions generally lead to a lower level of free OH, which suggests that the different polymeric structures might reflect the same stage of oxidation. Molecular dynamic simulation studies using the simulated annealing method to predict the behavior of the enzyme at different temperatures revealed that the enzyme adopts a more open and stable structure at higher temperatures [13] that are associated with high-energy environment reactors. In addition, increased substrate accessibility to the active site in high-energy reactors may generate a broader mixture of linear and branched polymers of higher molecular weight than can be generated using low-energy reactors (Figure 1B). Furthermore, complex polymers of different lengths and structures can be produced when the reaction is catalyzed by modified laccases in high-energy reactors. However, the identification of these oligomers/polymers is still a major difficulty that remains to be overcome. Further in-depth studies will be necessary to explain whether the different structures correspond to the final reaction product or if they represent intermediate stages of oxidation.

Concluding Remarks

Laccase enzyme is of exceptional interest because of its broad substrate specificity, and because laccase-mediated catalysis can generate diverse complex polymeric structures. Recent studies on poly(catechol) oxidation have

demonstrated that both enzyme modifications and different reactors can lead to the formation of different polymers. Although a large number of publications have addressed the production of different poly(catechol) structures, it is possible that some of these structures may be reaction intermediates at different stages of oxidation.

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