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Laccase-catalyzed polymerization of *m*-phenylenediamine in aqueous buffers

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Abstract–In the laccase-catalyzed polymerization of *m*-phenylendiamine in 100% aqueous buffers, the yield of the polymer was strongly influenced by various reaction conditions such as the solution pH and the concentrations of laccase and *m*-phenylendiamine. When the reaction was performed at pH 3, the 100% synthetic yield of the polymer was achieved. As pH increased, the yield of the polymer decreased significantly to only 4.4% at pH 9. Effects of solution pH on the morphology and the thermal stability of the polymer were investigated in detail. The polymer synthesized at pH 3 has the typical aggregated morphology of globular particles, but being synthesized at pH 7, it has non-aggregated morphology. The thermal stability of the polymer deteriorated as reaction pH increased.

Keywords: Laccase, Morphology, Polyphenylenediamine, Thermal Property

INTRODUCTION

Polymers of aromatic amines and diamines have attracted increasing interest because they have multiple functional amine groups and possess good thermal stability [1-4]. Among them, phenylenediamine polymers have been actively investigated for use as biochemical sensors and adsorbents for dyes or metal ions such as Cr(VI), Ag(I), Hg(II) or Pb(II) [5-8].

In general, most phenylenediamine polymers have been synthesized by chemical oxidation methods using ammonium persulfate as an oxidant. Contrary to the chemical methods using strong oxidants, enzymatic methods neither require toxic oxidants for polymerization nor result in environmentally harmful byproducts. Therefore, enzymatic methods have been attempted as alternatives to chemical synthetic methods to develop environmentally friendly processes. Until now, a variety of polymers including polyesters, polylactones, polyphenols, and conducting polymers have been synthesized by various enzymes including peroxidases, laccases, and lipases [9-12]. Horseradish peroxidase (HRP), in particular, has been frequently used as a catalyst to synthesize phenylenediamine polymers with H_2O_2 as an oxidant in aqueous buffer-dioxane mixtures [3,13-15] or in reversed micelles inside nonpolar organic solvents [16].

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are oxidoreductases containing multicopper ions at the active sites, which catalyze the oxidation of various organic and inorganic compounds with using molecular oxygen as an oxidant instead of H_2O_2 [17-20]. In the laccase catalysis, each oxygen molecule is reduced to water, while four monomeric substrates are oxidized to the corresponding cationic radicals. The enzymatically produced cationic radicals undergo subsequent radical reactions to form corresponding oligomers or polymers of the monomeric substrates.

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To date, however, the use of laccases to synthesize phenylenediamine polymers has not been reported. In this paper, we describe the synthesis of *m*-phenylenediamine polymer (poly(*m*-PDA)) using a laccase from *Trametes versicolor* as a catalyst in 100% aqueous buffer, therefore, without invoking secondary environmental issues as in the horseradish peroxidase-catalyzed synthetic methods where organic solvents are typically used as cosolvents.

EXPERIMENTAL

1. Materials

Laccase (Sigma 53739) from *Trametes versicolor*, *m*-phenylenediamine (*m*-PDA), and other chemicals were purchased from Sigma-Aldrich and used without further purification. Previously, native-PAGE [21] and sodium dodecyl sulfate (SDS)-PAGE [22] analyses have shown that this commercial laccase is pure having a molecular weight of about 66 kDa.

2. Enzymatic Synthesis of Polymers

The typical laccase-catalyzed synthesis of poly(*m*-PDA) in aqueous solutions was performed as follows. To a 10 mL buffer (10 mM citrate-phosphate buffer (CPB) for pHs from 3 to 7, 10 mM Trizma base for pH 9) which was bubbled with air beforehand, *m*-PDA was dissolved to a final concentration of 50 mM. Then, 10 mg of laccase was added to initiate the reaction. The reaction mixture slowly turned dark green. The reaction mixtures were shaken at 25 °C for 48 h. The final reaction mixture was filtered on a nylon membrane (0.2 μm, Whatman), and then washed repeatedly with distilled water and methanol to remove enzymes and remaining monomers [13,14]. The final black poly(*m*-PDA) precipitates were dried under vacuum. The synthetic yield of poly(*m*-PDA) was calculated as the percent of the initially added mass of *m*-PDA monomer.

3. Characterization of Poly(*m*-PDA)

Thermal degradation property of enzymatically synthesized poly (*m*-PDA) was examined by thermogravimetric analysis (TGA) on a TA instrument (TGA Q50). Dried poly(*m*-PDA) samples (~5 mg)

were heated from 25 °C to 800 °C under N_2 atmosphere at a rate of 10 °C/min. Structural nature of synthesized poly(*m*-PDA) was analyzed by Fourier transform infrared (FTIR) spectroscopy performed on a Nicolet 380 FTIR spectrometer (Thermo Electron Co.). The wave number range of FTIR was from 500 to 4,000 cm^{-1} at a 2 cm^{-1} resolution. Samples of poly(*m*-PDA) were mixed with KBr using a mortar and a pestle, then compressed into pellets for FTIR spectroscopy. The morphology of poly(*m*-PDA) was examined by scanning electron microscopy (SEM).

RESULTS AND DISCUSSION

1. Effect of Reaction Conditions on the Laccase-catalyzed Synthesis of Poly(*m*-PDA)

For most enzymatic polymerizations, either nonpolar organic solvents in the presence of small but enough amounts of water to retain enzyme's molecular flexibility and activity or mixtures consisting of water and polar organic solvents have been used as reaction solutions [23-25]. The major reason to employ organic solvents for enzymatic polymerization reactions is to increase the solubility of nonpolar substrates of enzymes for improved productivity of synthesized polymers. Unlike nonpolar monomeric substrates, *m*-PDA has a high solubility in water. In this study, hence, 100% aqueous buffers were used to polymerize *m*-PDA by laccase.

At first, various factors which could potentially affect the yield of poly(*m*-PDA) were investigated, including solution pH, the amount of laccase, and the initial concentration of *m*-PDA. Fig. 1 shows that the synthetic yield poly(*m*-PDA) decreased continually as the solution pH increased from almost 100% at pH 3.0 to only 4.4% at pH 9.0. This type of pH dependence is coincident with that of the laccase activity: The activity of laccase for the oxidation of ABTS (2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) was the maximum at pH 3.0, then declined sharply upon the increase of pH [22]. In a blank reaction at pH 3.0 where boiled laccase was used instead of the active laccase, poly(*m*-PDA) was not synthesized, indicating that the active laccase catalyzed the synthesis of the polymer. Unlike HRP catalysis, which showed the maximum yield of poly(*m*-PDA) near 80% in the presence of 20% 1,4-dioxane as a cosolvent and the lower yield of about 35% in 100% aqueous buffer [13], laccase-catalyzed poly(*m*-PDA) synthesis can attain 100% yield at the optimum pH without adding organic solvents as cosovents to the reaction media. Consequently, laccase-catalyzed synthesis of poly(*m*-PDA) can be performed efficiently in 100% aqueous buffer without using organic solvents.

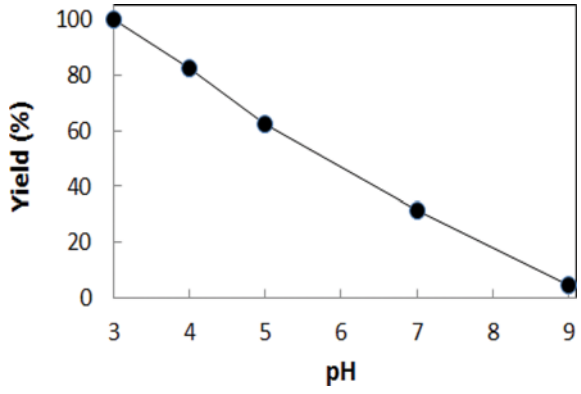

Fig. 1. Effect of initial pH on the yield of poly(*m*-PDA) catalyzed by laccase. Buffers are 10 mM CPB for pH 3-7 and 10 mM Trizma for pH 9. The initial concentration of *m*-PDA was 50 mM and 10 mg laccase was used in 10 mL aqueous buffers.

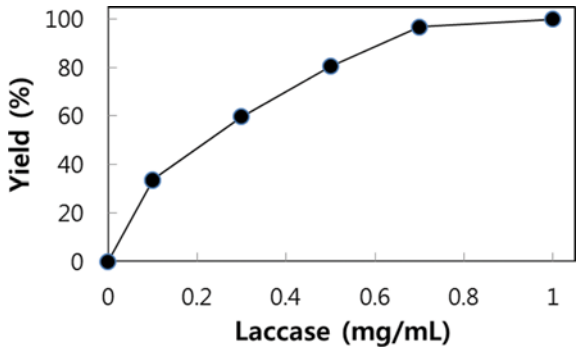

Fig. 2. Effect of laccase concentration on the yield of poly(*m* -PDA) catalyzed by laccase. Reactions were carried out in 10 mL buffer (10 mM CPB at pH 3) at 25 °C for 48 h. The initial concentration of *m*-PDA was 50 mM.

Fig. 2 shows the dependence of the synthetic yield of poly(*m*-PDA) on the amount of laccase at pH 3.0. The polymer yield increased steadily with the initial concentration of laccase up to 0.8 mg/mL, then leveled off for further increase of laccase concentration.

Fig. 3 shows the substrate inactivation of the laccase by *m*-PDA. When the initial concentration of *m*-PDA increased higher than 50 mM, the synthetic yield of poly(*m*-PDA) dropped markedly. These kinetic results suggest that various reaction conditions should be adjusted to achieve high synthetic yields of poly(*m*-PDA) catalyzed by laccase. Finally, Fig. 4 shows the change of poly(*m*-PDA) yield with reaction period. The polymer yield increased slowly until 24 h, then remained constant thereafter. As compared to the HRP catalysis for which the synthetic reaction was complete within 10 h [13], the laccase catalyzed synthesis of poly(*m*-PDA) proceeded slowly, indicating that the laccase activity for the polymerization of *m*-phenylendiamine is lower than that of HRP.

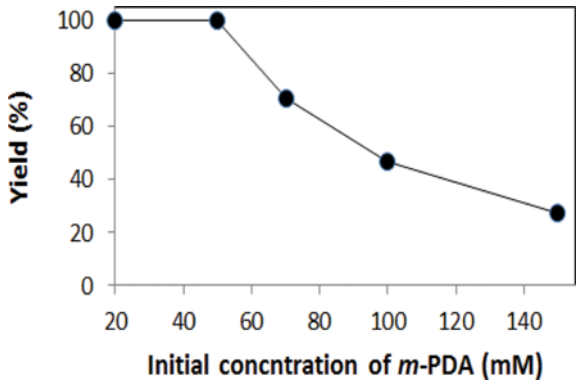

Fig. 3. Effect of initial concentration of *m*-PDA on the yield of poly (*m*-PDA) catalyzed by laccase. Reactions were carried out at 25 °C for 48 h with 10 mg laccase in 10 mL buffer (10 mM CPB at pH 3).

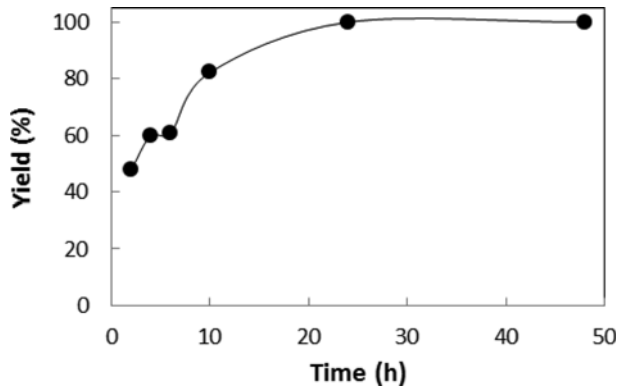

Fig. 4. Yield of poly(*m*-PDA) synthesized by laccase at pH 3 as a function of reaction time. The initial concentration of *m*-PDA was 50 mM and 10 mg laccase was used in 10 mL aqueous buffer (10 mM CPB at pH 3).

2. Characterization of Poly(*m*-PDA) Synthesized by Laccase-catalysis

Next, several physico-chemical properties of poly(*m*-PDA) synthesized by laccase were investigated. The solubility of poly(*m*-PDA) synthesized at pH 3 was measured in a variety of polar or nonpolar organic solvents and found to be negligible as also previously reported for the poly(*m*-PDA) synthesized by horseradish peroxidase [13,15]. Organic solvents tested in this study for the examination of the polymer's solubility are acetone, acetonitrile, ethanol, methanol, chloroform, N,N-dimethylformamide, 1,4-dioxane, dimethysolfoxide, tetrahydrofuran, and N-methyl-2-pyrrolidinone.

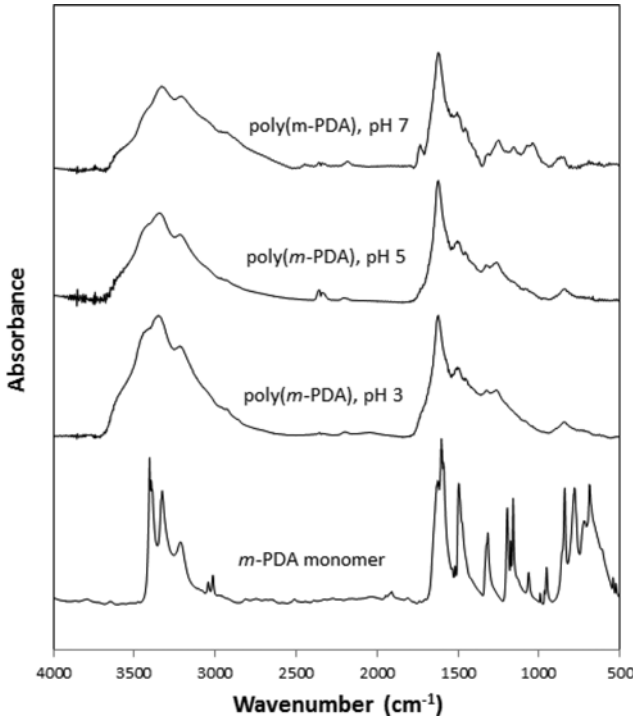

Fig. 5. FTIR Spectra of *m*-PDA monomer and poly(*m*-PDA) synthesized by laccase at different pHs.

Such low solubility of poly(*m*-PDA) in the various solvents made the determination of the molecular weight infeasible.

Fig. 5 shows FTIR spectra of *m*-PDA and poly(*m*-PDA) being synthesized at pH 3, 5, and 7, respectively. Two distinct differences between the spectrum of *m*-PDA and the spectra of poly(*m*-PDA) are observed. The characteristic absorption bands at 3,200-3,400 cm^{-1} for *m*-PDA due to N-H stretching of the primary amine (-NH_2) groups are broadened for poly(*m*-PDA) samples, indicating that the secondary amines (-NH-) are formed in poly(*m*-PDA) during the polymerization process through the formations of N-N or N-C bonds [14,16]. In addition, the bands at 600-900 cm^{-1}, which are attributed to C-H out-of-plane deformation of benzene rings of *m*-PDA, were diminished to a small broad band around 850 cm^{-1} for poly(*m*-PDA). This indicates that the substituents on the benzene rings of poly(*m*-PDA) are increased during polymerization by the formation of N-C or C-C bonds, resulting in the branched or cross linked structures [13-16].

These characteristics in the FTIR spectra of poly(*m*-PDA) synthesized by laccase suggest that the structural nature of the poly(*m*-PDA) is similar to that of poly(*m*-PDA) synthesized by horseradish peroxidase using H_2O_2 as the oxidant in that the polymer has complex structures through N-N, N-C, and C-C bonds [14,16]. Such a complicated irregular structure of the poly(*m*-PDA) may be responsible for the negligible solubility of the polymer in most organic solvents. The FTIR spectrum of poly(*m*-PDA) synthesized at pH 7 shows an additional C=O stretching peak at 1,750 cm^{-1} and C-O stretching peaks at 1,050-1,150 cm^{-1} which are absent in the FTIR spectra of poly(*m*-PDA) synthesized at pH 3 and 5. This indicates that one of the buffer components, citric acid, was bound to poly(*m*-PDA), presumably by the formation of amide bonds between C=O groups on citric acid and electron-donating -N- groups on *m*-PDA, when poly(*m*-PDA) was synthesized by laccase at pH 7. The absorption peak at 2,400 cm^{-1} is due to CO_2, which is contained in air and happened to be incorporated into the FTIR pellets [26].

The morphology of poly(*m*-PDA) was studied by SEM, and the micrographs of the polymers synthesized at different pHs are compared in Fig. 6. The micrograph of poly(*m*-PDA) synthesized at pH 3 clearly reveals that the polymer morphology is an aggregate of globular particles of the diameter of approximately ~1 μm. This morphology of poly(*m*-PDA) is similar to that of the chemically synthesized poly(*m*-PDA) [5,6]. In contrast, the micrograph of poly(*m*-PDA) synthesized at pH 5 shows that the polymer has less aggregated morphology of smaller granules. The aggregated globular particles become obscure for poly(*m*-PDA) synthesized at pH 7. It is known that the dimer formation reaction is the rate-limiting step for the solution polymerization reactions [27]. For this reason, polyanilines typically have the morphology of one-dimensional matrix of nanofibers without aggregated particles as the dimer formation rate is fast enough to enable homogeneous nucleation [28]. On the other hand, polypyrroles, for which dimer formation rate is slow, have the typical aggregated morphology of globules due to the heterogeneous nucleation of the dimers [29]. Tran et al. reported that the addition of small amounts of pyrrole dimers to the reaction mixtures changed the typical aggregated morphology of polypyrroles to one-dimensional morphology of nanofiber matrix, since

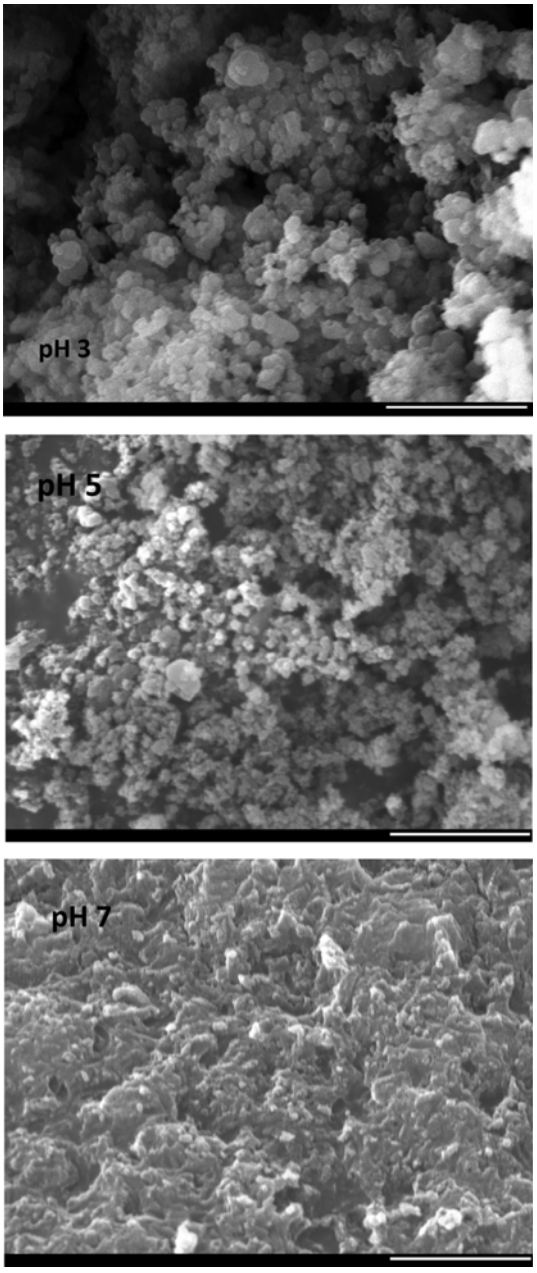

Fig. 6. SEM micrographs of poly(*m*-PDA) synthesized by laccase at different pHs. Magnification: 10 k. Scale bar: 5 μm.

the added extra dimers act as homogeneous nucleation sites for the polymer growth [27]. The intrinsic role of laccase is to produce *m*-PDA radicals. The actual synthesis of poly(*m*-PDA) is achieved by the subsequent non-enzymatic radical coupling processes among the *m*-PDA radicals. When laccase activity is high at pH 3, the formation of *m*-PDA radicals is assumed to be fast enough to make the dimer formation step as the rate-limiting step; thus, the aggregated morphology of poly(*m*-PDA) is obtained. But, as pH increases, the laccase activity decreases significantly, making the formation rate of *m*-PDA radicals much slower. Consequently, we presume that poly(*m*-PDA) synthesized by laccase at pH 7 has a non-aggregated morphology due to the homogeneous nucleation because of

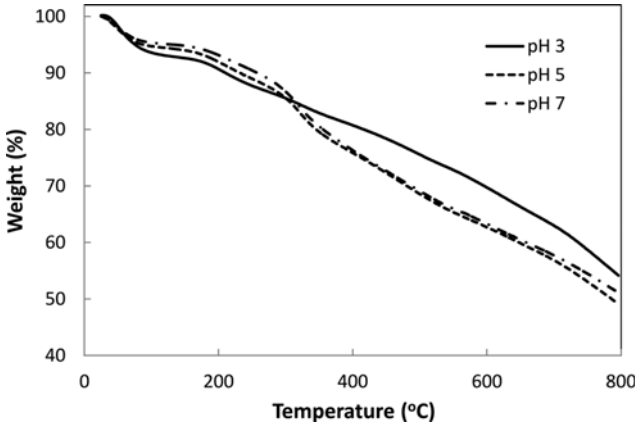

Fig. 7. Effect of pH on the TGA curves of poly(*m*-PDA) synthesized by laccase. TGA was performed under nitrogen atmosphere.

the diminished enzymatic production rate of *m*-PDA radicals. Since the morphological study of poly(*m*-PDA) synthesized by horseradish peroxidase-catalysis has not been reported yet, no direct comparison can be made between the morphology of the poly(*m*-PDA) synthesized by laccase to the same polymers synthesized by horseradish peroxidase.

As a final characterization of poly(*m*-PDA), the thermal stability of the polymers, which is a basic property of polymers to be considered for applications, was examined by TGA and the results are shown in Fig. 7. The poly(*m*-PDA) showed inferior thermostability when it was synthesized at pH 5 or pH 7 than when it was synthesized at pH 3. The char yields at 800 °C of the polymers synthesized at pH 3, 5, and 7 were 54.1%, 49.1%, and 51.1%, respectively.

CONCLUSIONS

Poly(*m*-PDA) was successfully synthesized by laccase in 100% water (10 mM CPB at pH 3) without using an organic solvent as a cosolvent. The synthetic yield of the polymer was found to be strongly affected by various factors, including initial pH, concentration of laccase, and initial concentration of *m*-PDA. The morphology of poly(*m*-PDA) was also affected by solution pH. As a consequence, careful selection of reaction conditions is required to optimize the laccase-catalyzed synthesis of the polymer to obtain high yield and targeted morphology.

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