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Immobilization of laccase on carbon nanomaterials

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Abstract−Laccase from *Trametes versicolor* was readily immobilized on carbon nanomaterials including multiwalled carbon nanotubes (MWNTs), carboxylated multiwalled carbon nanotubes (MWNT-COOHs), and graphene oxides (GOs), by physical adsorption without using coupling agents. The immobilized amount of laccase strongly depends on the pH of the aqueous buffers of the immobilization mixture. As the pH of the aqueous buffer for immobilization increases, the immobilized amount of laccase decreases. The activity of the immobilized laccase on the three carbon nanomaterials exhibits a bell-shaped dependence on the pH of the immobilization solution with maximum activity at pH 6 or 7. When the immobilization solution becomes acidic or basic, the activity of the immobilized laccase declines significantly. The amount and the activity of immobilized laccase were maximum for graphene oxides as substrate material for immobilization.

Key words: Carbon Nanotubes, Graphene Oxides, Immobilization, Laccase, Oxidized Carbon Nanotubes

INTRODUCTION

Enzymes are known to catalyze various kinds of chemical conversions under mild reaction conditions of room temperature and atmospheric pressures near 1 atm. Therefore, as compared to the processes using chemical or metallic catalysts, enzymatic processes are environmentally benign, efficient in energy consumption, and selective toward the specific target-compounds. Due to such advantages, the utilization of enzymes as catalysts for electro-chemical processes has been of great interest. Representative examples of enzymatic processes are the development of enzymatic transformations [1,2], biosensors to detect environmentally harmful compounds or bioactive molecules of low concentrations [3-5], and enzymatic biofuel cells [6]. In these enzymatic processes, efficient immobilizations of enzymes are essential for their successful performance.

Recently, carbon nanomaterials including carbon nanotubes, graphene, and their oxidized derivatives have been acknowledged to possess great potential to develop materials for a variety of electrochemical applications such as Li-ion batteries, capacitors, fuel cells, polymer composites, due to their large surface areas and excellent mechanical and electronic properties [7]. Carbon nanotubes are divided into multiwalled carbon nanotubes (MWNTs) of diameters from 2 to 25 nm and single walled carbon nanotubes (SWNTs) of diameters of 1-2 nm depending on the number of concentric layers [8]. Graphene, especially, has the thickness of a single atomic layer and thus possesses the largest specific surface areas among the carbon nanomaterials. Multiwalled carbon nanotubes and graphene are also oxidized to produce carboxylated multiwalled carbon nanotubes (MWNT-COOHs) and graphene oxides (GOs), respectively. These oxidized carbon nanomaterials have abundant oxygen containing surface functional groups surface such as hydroxyl, carboxylic, and epoxy groups, thereby, enabling the facile binding of various molecules including proteins, enzymes, DNA, surfactants, and metals, etc., with or without coupling agents [9]. These carbon nanomaterials also have been utilized as the solid substrate materials to immobilize various enzymes including horseradish peroxidases and glucose oxidases [9-11].

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are multi-copper ion-containing oxidases that are widely distributed among plants, insects, and fungi. Laccases catalyze the one-electron oxidation of a variety of organic and inorganic substrates including phenols, aromatic amines, and ascorbate with the simultaneous reduction of oxygen to water by four electrons. Especially, laccases are used to synthesize conducting polymers including polypyrolles, polythiophenes, and polyanilines with the addition of redox mediators [12]. Other applications of laccases include delignification of lignocelluloses, bioremediation, detoxification, and biosensors [13,14].

In this report, we present the optimal conditions for the immobilization of laccase from *Trametes versicolor* on three different carbon nanomaterials, MWNTs, MWNT-COOHs, and GOs, without using any cross-linking reagents. Another carbon nanomaterial, reduced graphene, was excluded from this study due to its strong tendency to form aggregates in the aqueous solutions. The optimally immobilized laccase has potential applications as electrode material for the development of biosensors, enzyme fuel cells, and more recently supercapacitors.

MATERIALS AND METHODS

1. Materials

MWNTs (CM-95) with 95% purity prepared by the chemical vapor deposition method were purchased from Hanwha Nanotech (Korea). Expandable graphite (Grade 1721) was supplied by Asbury Carbon. Laccase from *Trametes versicolor* and other chemicals were purchased from Sigma and used as received.

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2. Carboxylation of MWNTs

MWNT-COOHs were prepared according to the previous method as follows [11]. MWNTs (200 mg) were refluxed with 3 M HNO_3 (200 mL) at 120 °C for 12 h. The MWNT-COOHs formed were filtered on a filter paper and washed with copious amount of double distilled deionized water until the pH of the final filtrate became nearly neutral. Filtered and washed MWNT-COOHs were then dried overnight at 80 °C.

3. Preparation of Graphene Oxides

Graphene Oxides (GOs) were synthesized from expandable graphite by a modified Hummers method [15]. Briefly, a sample of graphite was expanded about 150 times of its initial volume by heating for 10 s in a microwave oven. Five grams of expanded graphite was slowly added into 500 mL of concentrated H_2SO_4 with stirring to make a suspension, then chilled in an ice bath. Into the graphite suspension, 30 g of $KMnO_4$ was slowly added, maintaining the temperature below 20 °C. The temperature of the mixture was increased to 35 °C and then stirred for 2 h. After that, the mixture was chilled again in the ice bath and 6 L of deionized water was slowly added with stirring for 1 h. The temperature of the mixture was maintained below 70 °C. Fifty milliliters of H_2O_2 (30 wt%) was slowly added to the mixture until the color of the suspension changed from dark brownish to yellow. Finally, the suspension was filtered and washed with 5 L of 10% HCl solution four times followed by washing with copious amount of deionized water until the pH of the GOs dispersion became 6. The synthesized GOs paste was dried at 50 °C under vacuum for 24 h before being used for the immobilization of the enzyme.

4. Immobilization of Laccase on Nanocarbons

All the immobilization procedures of laccase were performed at 4 °C. Typically, 5 mg of carbon nanomaterials except GOs was pretreated by sonication for 30 min in 1 mL DMF, then washed with 1 mL aqueous buffer five times. For GOs, 5 mg of GOs particles were sonicated for 30 min in 1 mL aqueous buffer, then washed with 1 mL of the same aqueous buffer five times. Samples of 5 mg of the pretreated carbon nanomaterials were mixed with 5 mg of laccase in a 1.5 mL of aqueous buffer. These mixtures were incubated for 4 h at 50 rpm then centrifuged to remove supernatant. Loosely bound laccase was removed from carbon nanomaterials by repeatedly washing the sample with buffer and centrifuging until negligible activity of laccase was detected in the supernatant. Typically, six to seven repeated washings were required to completely remove the loosely bound enzyme. All washing solutions were gathered to measure the amount of dissolved unbound laccase.

5. Measurement of the Amount of Immobilized Laccase

The bicinchoninic acid (BCA) method was used to measure the amount of laccase contained in the washing solutions. The amount of immobilized laccase was calculated by subtracting the amount of laccase dissolved in the total washing solution from the initially added amount of laccase for the immobilization.

6. Measurement of the Activity of Free Laccase

The activity of free laccase was measured spectrophotometrically with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as the substrate at 25 °C. The assay mixture contained 50 μM ABTS and 30 μg enzyme in 1 mL aqueous buffers. The initial rate of the enzyme reaction was determined from the linear increase of the absorbance at 415 nm within 60 s after the initiation of the reaction (ε=36,000 M^{-1} cm^{-1}) [16]. One unit of the activity of laccase was defined as the amount of laccase required to oxidize 1 μmole ABTS per 1 min.

7. Measurement of the Activity of Immobilized Laccase on Nanocarbons

The activity of the immobilized laccases was also measured spectrophotometrically at 25 °C in 50 mL sodium phosphate buffer (0.1 M, pH 6) containing 50 μM ABTS and approximately 1 mg of each immobilized enzyme. Reaction samples containing immobilized enzyme were sonicated briefly to improve the dispersion of the carbon nanomaterials. Reactions were initiated by adding ABTS and samples were periodically withdrawn then filtered. The absorbance of the filtered samples was measured at 415 nm to determine the initial rate of the reaction by the immobilized enzymes.

RESULTS AND DISCUSSION

1. Effect of pH on the Activity of Free Laccase

The activity of free laccase from *Trametes versicolor* was measured over the pH range from 4 to 7. Fig. 1 shows that the activity of free laccase is strongly influenced by the pH of the aqueous buffers exhibiting a bell-shaped pattern with the maximum activity at pH 6 and decreased activity at pHs, which are either higher or lower than the optimum pH of 6. Fungal laccases are known to be more active with ABTS as substrate in acidic solutions than in neutral or basic solutions [17]. Hydroxyl ions (OH^-) are known to irreversibly inactivate laccase by inhibiting the binding of the enzyme's substrate [18]. Therefore, laccase from *Trametes versicolor* is presumed to be very vulnerable to the inhibition by hydroxyl ions of even very low concentrations.

One of the major beneficial effects of immobilizing enzymes on solid substrates is to stabilize the enzymes against environmental agents which are detrimental to the enzymes' activity and stability. Subsequent immobilization experiments for the laccase on three

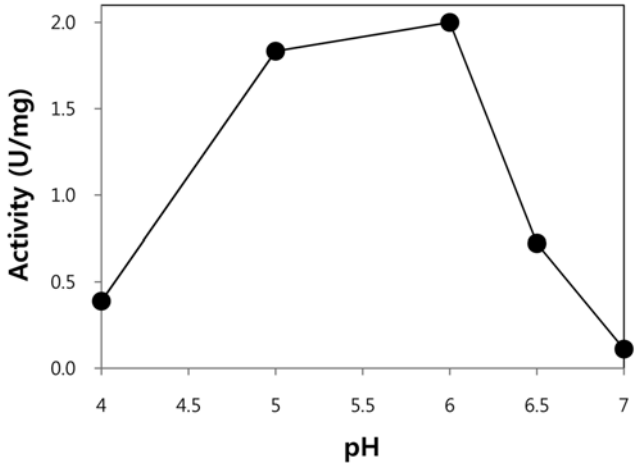

Fig. 1. Dependence of the activity of free laccase from *Trametes versicolor* on pH of the aqueous buffers at room temperature. Reactions were performed with 50 μM ABTS and 30 μg of laccase in 1 mL aqueous buffers. Data are the averages of duplicated measurements with errors smaller than 5%. One unit of laccase was defined to oxidize 1 μmole ABTS per 1 min.

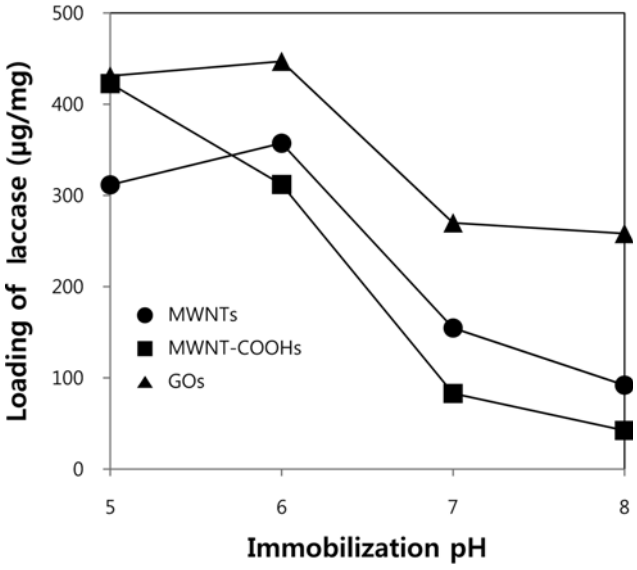

Fig. 2. Dependence of the loading of laccase on carbon nanomaterials on pH of the immobilization solution (For immobilization, 5 mg of laccase was mixed with 5 mg of each carbon nanomaterial. Data are the averages of duplicated measurements with errors smaller than 10%).

different carbon nanomaterials including MWNTs, MWNT-COOHs, and GOs were performed to investigate pH effects on the immobilization efficiency in terms of the loaded amount of the enzyme immobilized on each carbon nanomaterial and the activity of the final immobilized enzymes.

2. Immobilization of Laccase on Carbon Nanomaterials

Laccase was immobilized on carbon nanomaterials in the aqueous solutions of different pHs ranging from 5 to 8. The immobilization of laccase was performed by incubating each carbon nanomaterial (5 mg) with the enzyme (5 mg) in 1.5 mL phosphate buffer solution (10 mM) at 4 °C. The laccase was spontaneously immobilized on the carbon nanomaterials in the absence of any coupling reagents required for the covalent bonding of the enzyme onto the carbon nanomaterials. Fig. 2 shows that the loading of laccase on all three carbon nanomaterials decreases with increasing pH over 6. At pH 5 and 6, similar amount of laccase was immobilized on the carbon nanomaterials. Overall, GOs were most efficient to immobilize laccase, presumably due to the highest surface area and abundant surface functional groups such as epoxy, hydroxyl, and carboxylic groups. Zhang et al. [9] reported that GOs are assumed to have a net negative charge in the aqueous buffers with pH higher than 4. Therefore, in the pH range used for the immobilization of laccase in this study, GOs always have a net negative charge. The loading of laccase will be enhanced when the enzyme has a net positive charge at pHs below its pI and experiences consequent electrostatic attractions with GOs. Usually, laccases from *Trametes* species were found to have acidic pIs below 6 [19,20]. Similar rationale can be applied to the decreased loading of laccase on MWNT-COOHs with increasing pH of the aqueous solution. Dependence on the solution pH of the laccase loading on MWNTs is less severe than on the oxidized carbon nanomaterials (MWNT-COOHs and GOs), presumably due to the lack or little presence of the oxidized surface functional groups on MWNTs. The amount of loaded laccase on MWNTs also decreased with increasing pH of the immobilization solution. This may be due to the weakening attractive interactions between the negatively charged enzyme molecules and neutral MWNTs at higher pH. In general, less amount of laccase is immobilized on carbon nanomaterials at higher pH values of the immobilization solution.

3. Activity of Immobilized Laccase on Carbon Nanomaterials

The activity of laccase immobilized on each carbon nanomaterial in the aqueous buffers of pHs ranging from 5 to 8 was measured. The pH of the aqueous solution for the activity measurement of the immobilized enzymes was 6, which is the optimum pH for the activity of free laccase. The initial oxidation rates of ABTS catalyzed by the immobilized laccases were determined from the linear increases of the absorbance at 415 nm over time during the initial periods of reaction. The activities of the immobilized laccases were represented as the units per 1 mg enzyme immobilized on carbon nanomaterials, where one unit of the activity was defined to oxidize 1 μmole ABTS per 1 min. Fig. 3 shows that the laccase retained the maximum activity when it was immobilized at pH 6 and 7, and almost negligible activity when immobilized at acidic pH of 5 or basic pH of 8, respectively. These results indicate that the laccase was inactivated while being immobilized at pHs which were different from its optimal pH for the activity of free laccase. As shown in Fig. 3, among the tested carbon nanomaterials, GOs are most effective in retaining high activity of laccase. Among the three carbon nanomaterials used in this study, GOs have the largest specific surface area per unit mass due to their thin platelet structures [21]. Therefore, immobilized laccases on GOs are assumed to be most exposed to solution and hence available to ABTS oxidation. Fig. 4 shows the examples of the absorption spectra of the reaction mixtures 24 h after the initiation of reactions where ABTS (50 μM) was oxidized by laccase immobilized on MWNTs in solutions of different pHs ranging from 5 to 8. When the immobilization pH was 5 or 8, significant amount of unreacted ABTS was detected at 340 nm, indicating that the immobilized enzymes lost most of their activity dur-

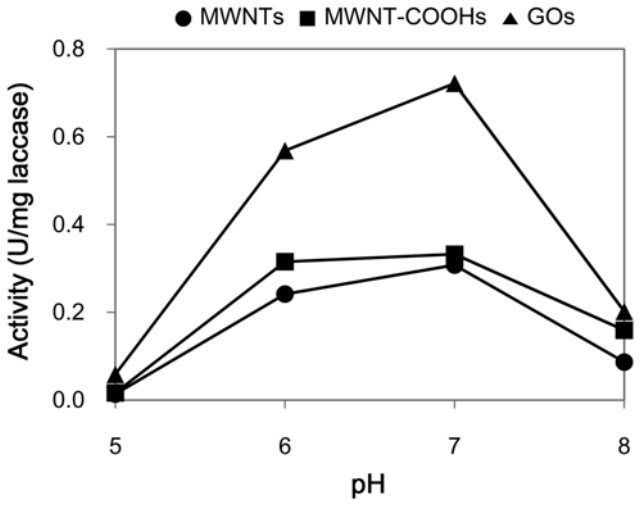

Fig. 3. Effects of the immobilization pH on the activity of the immobilized laccases on carbon nanomaterials. The activity of the immobilized laccase was measured at pH 6 and represented by the units per 1 mg of immobilized laccase.

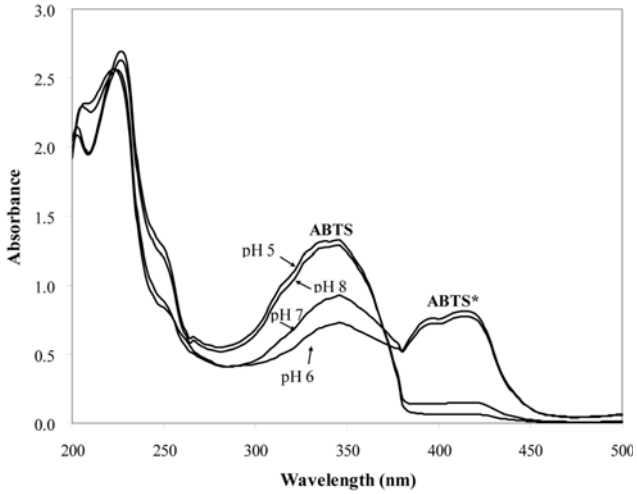

Fig. 4. Absorption spectra of reaction mixtures for the oxidation of ABTS (50 μM) 24 h after the initiation of the reaction. Laccase was immobilized on MWNTs in the solutions of different pHs (5 to 8). ABTS and its oxidation product, ABTS*, have absorption peaks at 340 nm and 415 nm, respectively.

ing the immobilization processes. However, the reaction mixtures with the laccases immobilized in pH 6 and 7 had less amount of remaining unreacted ABTS and higher amount of the oxidation products, ABTS*, near 415 nm after 24 h of reaction period. Similar results to Fig. 4 were also found for laccase immobilized on MWNT-COOHs and GOs.

CONCLUSION

Laccase was immobilized on the three different carbon nanomaterials including MWNTs, MWNT-COOHs and GOs. GOs were most efficient to immobilize the maximum amount of laccase and retain high activity of the immobilized enzyme. The pH of the immobilization solution was found to be the predominant factor to determine the loaded amount and activity of the immobilized laccase. As the pH of the immobilization solution increased, less amount of laccase was loaded on carbon nanomaterials. The activity of the immobilized enzyme was maximum when the enzyme was immobilized at pH 6 or 7. When the pH of the immobilization solution was either acidic or basic, the activity of the immobilized laccase dropped significantly.

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