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Degradation of acenaphthylene and anthracene by chemically modified laccase from *Trametes versicolor*†

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We are studying the chemically modified laccase from *Trametes versicolor* for use in the *in vitro* oxidation of two polycyclic aromatic hydrocarbons (PAHs), acenaphthylene and anthracene, in combination with 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a redox mediator. The results indicate that the maleic anhydride modified laccase (MA-Lac) improved the stability of laccase to temperature, pH and storage time compared with the free enzyme. After incubation for 72 h, the MA-Lac–ABTS system oxidized acenaphthylene and anthracene to more than 70% from the reaction mixture.

Polycyclic aromatic hydrocarbons (PAHs) are highly toxic organic pollutants found at contaminated industrial sites around the world.[1,2] Due to their toxic effects, PAHs pose a serious health risk to microorganisms, plants and animals, including humans. Many compounds, such as acenaphthylene and anthracene, are admitted to be highly mutagenic and carcinogenic.

Many research efforts have been expended to find suitable methods for the remediation of soil and water environments contaminated with PAHs. In recent years, many studies on the biodegradation of PAHs became the focus of scientific research.[3,4] For example, the use of various white rot fungi for the biodegradation of PAHs has been extensively studied.[5,6] Unfortunately, these approaches could be very slow and may present some undesirable limitations and incomplete removal of pollutants. One of the strategies to overcome this limitation is through enzymatic treatment.[7]

Laccase (E.C. 1.10.3.2) is a copper-containing oxidoreductive enzyme that catalyzes one-electron oxidation of a broad range of polyphenols and aromatic substrates.[8] Using isolated laccase in its soluble form, the detoxifying effect of laccase in reaction with xenobiotics has been widely studied.[9–11] Radical mediator compounds acting as "electron shuttles" between the enzyme and the substrate extended the substrate spectrum of laccase; therefore, they could accelerate the laccase catalysis of a substrate.[12] The degradation of PAHs by a laccase mediator system was reported to be significantly effective in the presence of mediator compounds such as 1-hydroxybenzotriazole (HBT) and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).[13]

However, the practical application of laccase, mainly as an agent for environmental clean-up purposes, is still prevented by several limitations. In general, low stability and the potential for drastic reductions in enzymatic activity have always been considered as hindrances to the practical application of enzymatic systems.[14,15] Chemical modification is a rapid and inexpensive method for stabilizing the enzyme.[16] In this paper, chemical modification of laccase with maleic anhydride was performed to increase its stability. The oxidative potential of maleic anhydride-modified laccase mediator system for *in vitro* reaction with PAHs has not been studied. The objective of this study is to evaluate the potential of maleic anhydride modified laccase (MA-Lac) to oxidize acenaphthylene and anthracene in the presence of ABTS.

Ion exchange chromatography of free laccase and MA-Lac was performed on HiTrap DEAE FF, as described in the experimental section. The elution curves at 215 nm and enzyme activity are shown in Fig. 1(a) and (b), respectively. Three peaks appeared in the elution curve at 215 nm, as shown in Fig. 1(a) and only one peak of each enzyme activity is observed in

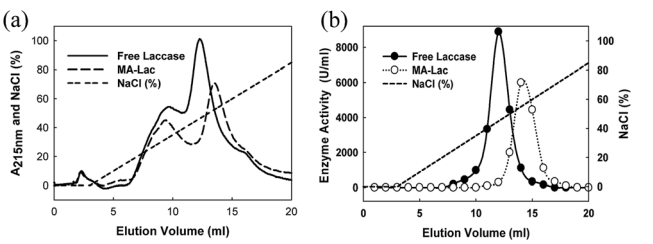

Fig. 1 Anion-exchange chromatography of free and modified laccase on HiTrap DEAE FF (1 ml columns); (a) protein content of the elution; (b) laccase activity of the elution.

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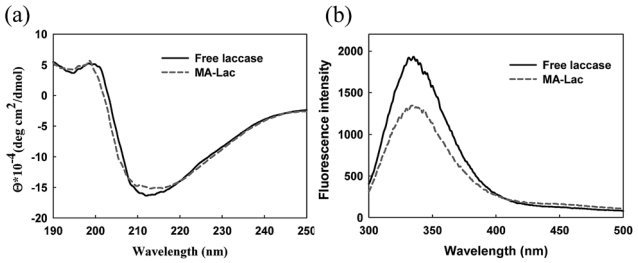

Fig. 2 (a) CD spectra; (b) fluorescence spectra.

Table 1 The enzymatic reaction parameters

Enzyme	K_m (mmol l^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mmol l^{-1})
Free laccase	0.68	9.04×10^6	13.29×10^6
MA-Lac	0.47	6.89×10^6	14.66×10^6

Fig. 1(b). The elution curve of MA-Lac in Fig. 1(a) showed that only the third peak shifted when the NaCl concentration increased from 0.0466 mol to 0.0524 mol, and this shift also reappeared in the enzyme activity of the elution (Fig. 1(b)). These results indicate that the structure of laccase had changed after the modification and confirm the success of the chemical modification with maleic anhydride.

The secondary and tertiary structures of the free and modified laccases were analyzed by circular dichroism (CD) and fluorescence emission spectroscopy to probe the structural differences caused by the chemical modification. The identical adsorption curves, as shown in Fig. 2, indicate that the maleic anhydride modified laccase retained its secondary and tertiary structure.

The determination of the reacted amino lysine groups was performed according to the method described in the experimental session. The modification ratio was determined to be 73.77% for maleic anhydride.

The biocatalytic activity of native and modified laccase was examined using ABTS as the substrate. Michaelis–Menten parameters, K_m and k_{cat}, interpreted from the Lineweaver–Burk plots are listed in Table 1. Here, the chemical modification leads to a reduction in K_m while an increase in k_{cat}/K_m, indicating that the modified laccase has an enhanced affinity and activity due to ABTS.

The thermal stability of the free laccase and MA-Lac was compared at 55 °C and at pH 7.0. During the experiment, the enzyme solution incubated without ABTS was sampled at given time intervals and subjected to activity assay using ABTS as the substrate. To magnify the difference in thermal stability, free laccase solution incubated at zero degree was used as a control. The residual activities are shown in Fig. 3, which indicate that MA-Lac has the highest stability. The half-lives of enzyme activity interpreted from the curves in Fig. 3(a) are 138.6 and 693 min for the free laccase and MA-Lac, respectively, *i.e.* a 5-fold increase in thermal stability is achieved by MA-Lac.

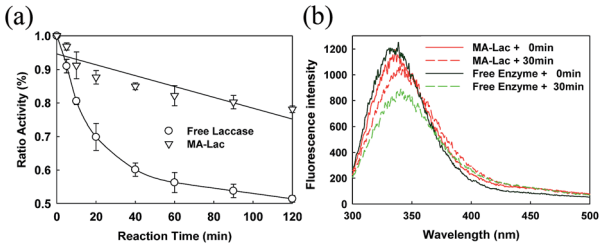

Fig. 3 Thermal deactivation of free and modified laccases at 55 °C; (a) deactivation plot; (b) fluorescence emission spectra.

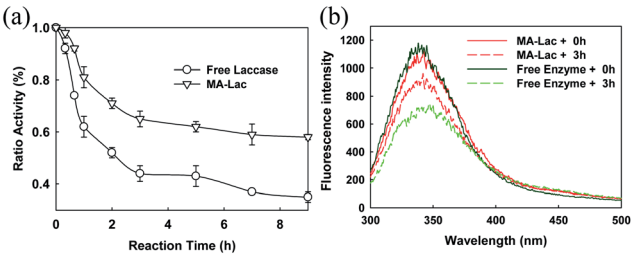

Fig. 4 Deactivation of free and modified laccases under acidic conditions (pH 3.5); (a) deactivation plot; (b) fluorescence emission spectra.

The stabilization of laccase under acidic conditions will extend its application spectrum and thus is constantly pursued. Here, the stability of free and chemically modified laccases was compared at pH 3.5 using 20 mM disodium hydrogen phosphate–citric acid as the buffer and during the experiment, the enzyme solutions were incubated at room temperature. The changes in the residual activity are shown in Fig. 4(a), in which MA-Lac exhibits improved stability with an increase in the half-life of enzyme activity from 6.45 h to 11.36 h.

The changes in the tertiary structure of the free laccase and MA-Lac at a high temperature of 55 °C or under acidic conditions (pH 3.5) were monitored by fluorescence intensity. As shown in Fig. 3(b) and 4(b), a more significant shift in the peak position of the fluorescence spectra was observed from free laccase, as compared to that of the MA-Lac, which indicates that the enzyme structure was more stable due to the chemical modification with maleic anhydride.

In the experiments, two compounds (acenaphthylene and anthracene) were chosen as representatives of PAHs. The degradation by free laccase and MA-Lac was conducted at pH 4.5, using 20 mM disodium hydrogen phosphate–citric acid buffer at a temperature of 30 °C. The metabolites were studied at different time points within the entire incubation time of 72 h. HPLC analysis indicated that the main products detected after the incubation of acenaphthylene were 1,8-naphthalic acid anhydride and 1,2-acenaphthenedione and that of anthracene was 9,10-anthraquinone.

The degradation ratios of acenaphthylene and anthracene are listed in Fig. 5. From the results, the presence of ABTS as an electron shuttle intensifies the oxidation of PAHs by both free and the chemically modified laccases *in vitro* within 72 h of incubation. The oxidation of acenaphthylene and anthracene

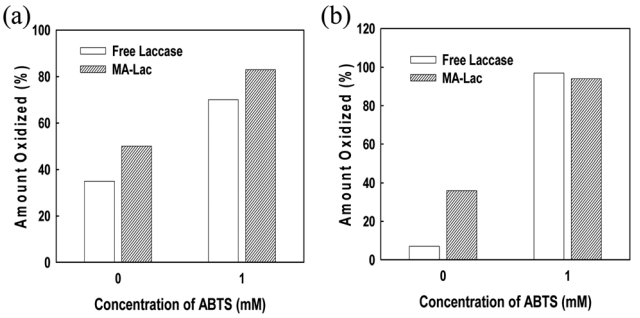

Fig. 5 Oxidation of PAHs; (a) acenaphthylene; (b) anthracene.

significantly increased when 1 mM of ABTS is added to the reaction mixture.

In the case of MA-Lac, the oxidation ratios of acenaphthylene and anthracene increased from 50% and 36% without ABTS to 83% and 94% in the presence of the mediator, respectively. The corresponding values for acenaphthylene and anthracene oxidation by free laccase increased from 35% and 7% without ABTS to 70% and 97% in its presence, respectively. After treatment for 72 h at 30 °C, the activities of the free and modified laccases were reduced by 82.8% and 43.3%, respectively.

The results presented indicate that the chemical modification of laccase with maleic anhydride increased the oxidization of acenaphthylene and anthracene. The modification of laccase could be responsible for the improved stability, induced by the introduction of new or additional electrostatic interactions. Lysine residues on the enzyme surface were chemically modified with maleic anhydride to create an Arg-like side chain. This may possibly be the reason for the improved stability of MA-Lac and the stimulation of the oxidization of PAHs.[17] The higher kinetic parameter k_{cat}/K_m values of the two PAHs after the modification also indicates higher substrate affinity (data presented in ESI†).

ABTS also played an important role in the oxidation of anthracene; however, it did not significantly influence the oxidation of acenaphthylene. Similar results were obtained in the case of oxidation of PAHs by free laccase, which significantly increased when mediator compounds were added to the reaction mixture, and the immobilized laccase-mediator system was as efficient as the free enzyme in oxidizing anthracene and benzo[*a*]pyrene.[18] After 24 h of incubation, the immobilized laccase-ABTS system oxidized more than 80% of the initial 70 μM of PAHs present. This result also agrees with Johannes *et al.* findings, which showed that after 72 h incubation, using ABTS (1 mM) as the mediator, the oxidization of anthracene by laccase increased from approximately 35% to 75%.[19]

To date, the mechanism of ABTS still remains unclear. Some evidence suggests that ABTS acts as a mediator that could increase the *in vitro* substrate-oxidizing capability of native or chemically modified laccase. Some evidence was provided to indicate that ABTS acts as an "electron shuttle" between the enzyme and the substrate, which transfers an electron to the enzyme, initiating the ability of the enzyme to accomplish electron transfer.[20]

Conclusions

Although the oxidation of PAHs by free laccase in the presence of an immobilized laccase-mediator system has been reported, this is the first report that showed how laccase modified with maleic anhydride oxidized acenaphthylene and anthracene in combination with ABTS. The results showed that the chemical modification of laccase with maleic anhydride could improve stability under acidic conditions and thermal stability compared with the free enzyme and also increase the oxidation of acenaphthylene and anthracene. In the presence of the ABTS mediator, both the free and chemically modified laccases increased the oxidation of anthracene, but had no significant influence on acenaphthylene. The results indicate a new opportunity in the practical application of laccase for bioremediation of environmental pollution from PAHs.

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