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

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We are studying the chemical modified laccase from the *Trametes versicolor* for use in the oxidation of the two polycyclic aromatic hydrocarbons (PAHs) acenaphthylene and anthracene in vitro in combination with 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as the redox mediator. Results indicated that the maleic anhydride modified laccase (MA-Lac) improved stability of laccase to temperature, pH and storage time compared with the free enzyme. After 72 h incubation, MA-Lac-ABTS system oxidized acenaphthylene and anthracene 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, and many, such as acenaphthylene and anthracene, are admitted to be highly mutagenic and carcinogenic.

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

Laccase (E.C. 1.10.3.2) are copper containing oxidoreductive enzymes that catalyze a one-electron oxidation of broad range of polyphenols and aromatic substrates [8]. Using isolated laccase in soluble form, the detoxifying effect of laccase in reaction with xenobiotics has been studied widely [9-11]. Radical mediator compounds, acting as "electron shuttle" between the enzyme and the substrate, extended the substrate spectrum of laccase; therefore, they could accelerate laccase catalysis with substrate [12]. The degradation of PAHs by laccase mediator system was reported to be significantly effectively 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 agents for environmental cleanup 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 to stabilize

enzyme [16]. In the paper, chemical modification of laccase to increase its stability by maleic anhydride was carried out.

The oxidative potential of maleic anhydride modified laccase mediator system for in vitro reaction with PAHs has not been studied. The objective of the present 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 session. The elution curves at 215nm and enzyme activity are shown in Fig. 1 (a) and Fig. 1 (b), respectively. Three pecks appeared in the elution curve at 215nm shown in Fig. 1(a) and only one peck of the each enzyme activity showed in Fig. 1(b). The elution curve of MA-Lac in Fig. 1(a) showed that only the third peck was shifted with the NaCl concentration from 0.0466mol to 0.0524mol and the accordingly shift also reappeared in the enzyme activity of the elution (Fig. 1(b)), which indicated the structure of laccase was changed after modification and the success of chemical modification with maleic anhydride.

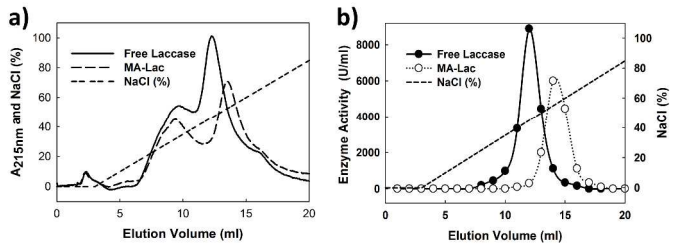

Figure 1. Anion-exchange chromatography of free and modified laccase on HiTrap DEAE FF (1ml columns); a) Protein content of the elution; b) Laccase activity of the elution.

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

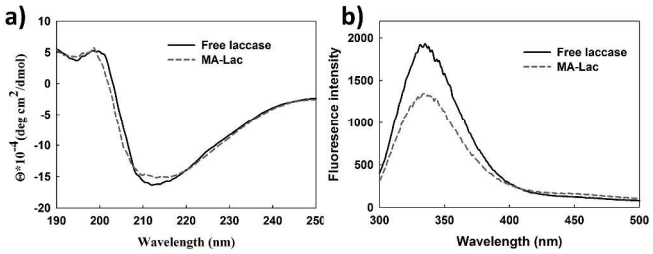

Figure 2. a) CD spectra; b) Fluorescence spectra.

Determination of the reacted amino lysine groups was performed according to the method described in the experimental session. The modification ratio was determined as 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 of K_m while an increase in k_{cat}/K_m, indicating that the modified laccase has an enhanced affinity and activity to ABTS.

Table 1 The enzymatic reaction parameters

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

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

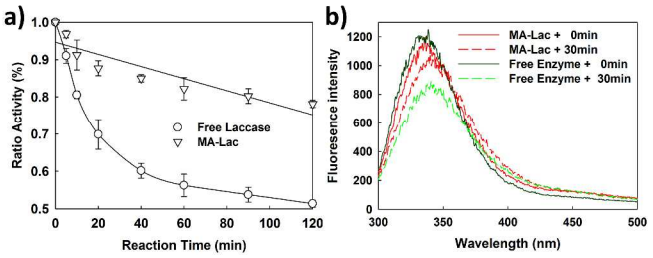

Figure 3. Thermal deactivation of free and modified laccases at 55°C; a) Deactivation plot; b) Fluorescence emission spectra.

Stabilization of laccase at an acidic condition will extend its application spectrum and thus be pursued constantly. Here the stability of free and chemical modified laccases was compared at pH 3.5 using 20mM di-sodium hydrogen phosphate-citric acid as buffer and during the experiment, the enzyme solutions were incubated at room temperature. The changes of the residual activity are shown in Figure 4(a), in which MA-Lac gives an improved stability with the half life of enzyme activity from 6.45h to 11.36h.

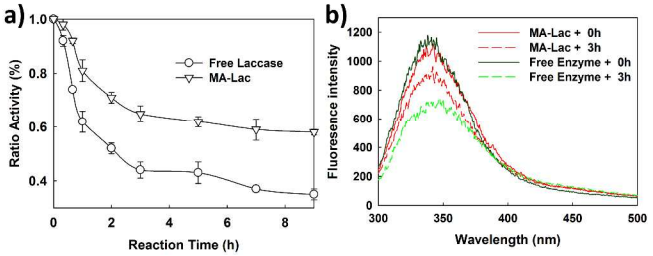

Figure 4. Deactivation of free and modified laccases in acidic condition (pH 3.5); a) Deactivation plot; b) Fluorescence emission spectra.

The changes in tertiary of the free laccase and MA-Lac at the high temperature of 55°C or in acidic condition (pH 3.5) were monitored by fluorescence intensity. As shown in Figs. 3(b) and 4(b), a more significant shift of the peak position of the fluorescence spectra was observed from free laccase, as compared to that of the MA-Lac, which indicated the enzyme structure was more stable through chemical modification by 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 pH4.5, 20mM di-sodium hydrogen phosphate-citric acid buffer at the temperature of 30°C. The metabolites were studied at different time points within the whole incubation time of 72 h. HPLC traces indicated that 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.

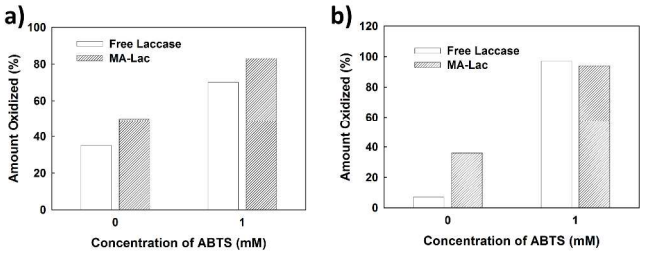

Figure 5. Oxidation of PAHs; a) acenaphthylene; b) anthracene.

The degradation ratio of acenaphthylene and anthracene are listed in Fig. 5. From the results, the presence of ABTS as electron shuttle intensifies the oxidation of PAHs by both free and the chemical modified laccases in vitro within 72 h of incubation. Oxidation of acenaphthylene and anthracene were significantly increased when 1mM of ABTS was added to the reaction mixture.

In the case of MA-Lac, the oxidiation ratios of acenaphthylene and anthracene increased from 50 and 36% without ABTS to 83 and 94%, respectively, in the presence of the mediator. Corresponding values for acenaphthylene and anthracene oxidation by free laccase were from 35 and 7% without ABTS to 70 and 97%, respectively, in its presence. After treatment for 72h 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 chemical modification of laccase with maleic anhydride increased the oxidization of acenaphthylene and anthracene. The modification of laccase could be responsible for the better stability, induced by the introduction of new or additional electrostatic interaction. Lysine residues of the enzyme surface were chemical modified with maleic anhydride to create an Arg-like side chain. This may possibly be the reason for the better stability of MA-Lac and the stimulation oxidization of PAHs. [17] The larger kinetic parameter Kcat/Km values toward the

two PAHs after modificatioin also indicated the higher substrate affinity (data presented in supplementary materials).

ABTS also played the important role in the oxidation toward anthracene, however, did not significantly influence toward acenaphthylene. Similar results were obtained in the case of oxidizing of PAHs by free laccase increased significantly 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 24h of incubation, 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 (1mM) as the mediator, the oxidization of anthracene by laccase was increased from about 35% to 75%. [19]

Up to now, however, the mechanism of ABTS is still not completely clear. Some evidences are suggested that ABTS, acted as mediator, could increase in vitro substrate-oxidizing capability of native or chemical modified laccase. Some evidences were provided to indicate that ABTS acts as "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 present of and immobilized laccase-mediator system have been reported, this is the first report that 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 at an acidic condition and thermal stability compared with free enzyme and also increased the oxidation of acenaphthylene and anthracene. In the presence of mediator, ABTS, both free and chemical modified laccases increased the oxidation of anthracene ability, and had no significantly influence toward acenaphthylene. The results indicated a new opportunity for practical application of laccase to bioremedy the pollution of environment by PAHs.

Notes and references

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Graphical Abstract