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Screening of New Mediators for Lignin Degradation Based on Their Electrochemical Properties and Interactions with Fungal Laccase

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ABSTRACT

This study was performed to evaluate extensive electrochemical characteristics of 23 commercially available mediators for laccase. Electrochemical properties, interactions with laccases, and ability to degrade lignin were compared for selected mediators. Among them, NNDS has very similar electrochemical properties in terms of reversibility and redox potential (about 470 mV vs. Ag/AgCl at pH=7) compared to ABTS which is a well-known mediator.

Specific activity of purified laccase from Cerrena unicolor was determined by both 2,2'-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) and 1-nitroso-2-naphthol -3,6-disulfonic acid (NNDS). The specific activity of the laccase was 23.2 units/mg with ABTS and 21.2 units/mg with NNDS. The electron exchange rate for NNDS with laccase was very similar to that for ABTS, which meant that NNDS had similar mediating capability to ABTS. Determining methanol concentration after reacting with laccase compared to lignin degradation capabilities of both ABTS and NNDS. ABTS or NNDS alone cannot degrade lignin, but in the presence of laccase enhanced the rate of lignin degradation. ABTS showed better activity in the beginning, and the reaction rate of NNDS with lignin was about a half of that of ABTS at 10 minute, but the final concentration of methanol produced in 1 hour was very similar each other. The reason for similar methanol concentration for both ABTS and NNDS can be interpreted as the initial activity of ABTS was better than that of NNDS, but ABTS would be inhibited laccase activity more during the incubation.

Keywords : electrochemical studies, mediators, laccase, Cerrena unicolor, NNDS, ABTS, redox potential, lignin, methanol

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1. Introduction

Laccases (p-diphenol:dioxygen oxidoreductase, EC 1.10.3.2) are in multi-copper oxidase family with ascorbate oxidase, ceruloplasmin, etc. and can be grouped into two categories, plant and fungal laccases. Multi-copper oxidases catalyze the oxidation of wide range of phenols and other substrates with concomitant reduction of dioxygen to water (Fig. 1) (1). Most plant laccases are capable of oxidative coupling monolignols to dimers and trimers, while peroxidase has a much greater activity toward higher oligomers (2), leading to a proposal that laccase catalyzes the initial polymerization of monolignols into oligolignols (3-7), while peroxidases then synthesize the extended polymeric lignin from oligolignols. The possible physiological substrates that are precursors to lignin formation are coniferyl alcohol, urishiol, etc. Lignin is present in wood tissue (30~45 wt%) as a natural macromolecule. Fungal laccase has been detected and purified from many species, some of which produce multiple isozymes (8-10). Most of fungal laccases are monomers or homodimers and are glycosylated, but generally to a lesser extent (10-25%) than the plant enzymes. Laccase is thought to be nearly ubiquitous among fungi. Most, but not all, are extracellular, and a given species may produce isozymes of both extra- and intra-cellular types (11).

So far, lignin degradation, detoxification and pigment formation are main functions of fungal laccases probed. Among them lignin degradation is the most important and interesting role of laccase. Peroxidase also plays an important role, and some fungi apparently produce no laccase, and yet are able to degrade lignin. Furthermore, although laccase can catalyze degradation of high-molecular weight lignin models *in vitro*, lower molecular weight compounds are repolymerized (12). Dimer models can be cleaved or oligomerized, depending upon the compound. This problem can be overcome by pairing laccase with a mediator (13-18) or another ligninolytic enzyme (19-21). On this basis, it has been suggested that, *in vivo*, several different enzymes, including laccase, operate synergistically to degrade lignin.

For applying laccase to pulp and paper industry, development of efficient and cheap mediator is essential (18). Since the enzyme is too big to penetrate into wood cell walls to react with lignin, a low molecular mediator to relay electrons from lignin to the enzyme is essential (13, 22-32). The mediator should have reasonably high redox potential and produce stable cation radical to transfer electrons from lignin to the enzyme, and then to oxygen which is a final electron acceptor in lignin degradation (Fig. 2).

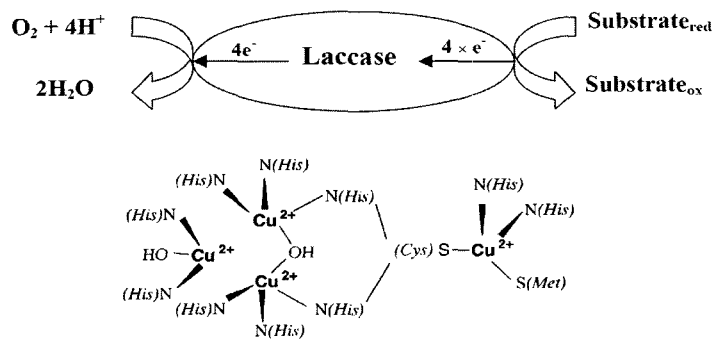

Fig. 1. Schematic diagram of catalytic reaction of laccase and proposed active site structure.

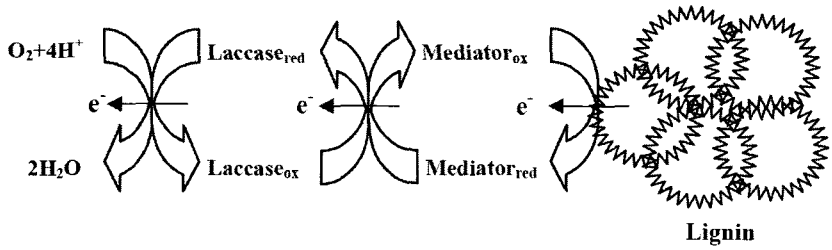

Fig. 2. Schematic diagram of lignin degradation by laccase-mediator-system (LMS).

The mediator can be produced internally by initial degradation of the lignin and other wood components. Veratryl alcohol, oxalate, 3-hydoxyanthranilic acids are possible candidate of low molecular weight components. Mediators can be added externally for the efficient lignin degradation. One of the most well-known and well-studied mediator is 2,2'-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) (33,34). ABTS has a good redox potential to interact with lignin and laccase, and produces stable radical which has a green color with a strong absorption (ε_{405} = 35 $mM^{-1}\cdot cm^{-1}$) to be followed effectively by absorption spectrum for the reactivity (as shown in Fig. 4). Although ABTS has a quite good ability as a mediator, the price is very expensive and its commercial use is impossible.

We extensively studied electrochemical properties of commercially available mediators and found that NNDS has quite similar electrochemical properties with ABTS. We compared interactions of laccases with ABTS and 1-nitroso-2-naphthol-3,6-disulfonic acid (NNDS). The lignin degradation capability for both mediators were determined by measuring the amount of methanol produced after incubating laccase, mediator, and lignin together.

2. Materials and Methods

Commercially available mediators were purchased from Aldrich and Sigma, except 2-Nitroso-1-naphthol-4-sulfonic acid (HNNS) and NNDS which are from Tokyo Chemical Industry (TCI). Other chemicals are all reagent grade and used without further purification. Acetate lignin was employed in this experiment and purchased from Aldrich.

The laccase from *Cerrena unicolor* was purified by following method (7-10). First, crude laccase was diluted 5 times by pure water and centrifuged to remove insoluble material (Vision Scientific Co. Ltd. high speed refrigerated centrifuge VS-21 SMTN, 7,000 rpm, 10 min). The supernatant was dialyzed against buffer (stirred

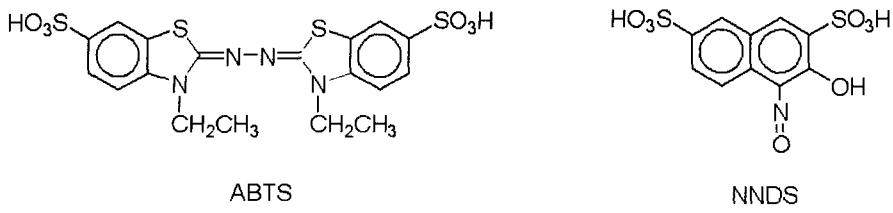

Fig. 3. Molecular structures of ABTS and NNDS.

ultrafiltration cell, Amicon. Inc. 8400, YM 30 membrane). The filtrate were applied to DEAE Sephacel column and eluted by a linear gradient 0.05 - 0.5 M $NaClO_4$. The fractions having laccase activity were applied to CM Sepharose column. The purified enzyme was dialyzed against buffer and kept frozen at -70℃. Laccase activity was determined by oxidation of ABTS. The assay mixture contained 1 mM ABTS, 8 mM MES buffer (pH 5.3), and a suitable amount of enzyme. Oxidation of ABTS was monitored by determining the increase in A_{405} (ε_{405} = 35 $mM^{-1} \cdot cm^{-1}$).

Electrochemical experiments were performed by CV-27 Potentiostat with PC interfaced by PCL-711S ADC board. The electrochemical cell is one-compartment with glassy carbon (0.070 cm^2) working, platinum counter, and Ag/AgCl reference electrodes. All potentials are reported vs. Ag/AgCl, which is +200 mV vs. NHE. Argon gas was bubbled into the buffer solution to remove oxygen before each mediator was dissolved to make 1.0 mM solution. Argon gas was being passed over the solution while taking electrochemical measurements. UV/Vis absorption measurements were taken with HP 8452 diode array spectrometer.

Hydrolytic lignin purchased from Aldrich was used for lignin degradation experiment. Five ml of 20 mg/ml of the lignin solution was prepared in 0.1 M acetate buffer (pH 6.0) in 10 ml round bottom flask and stirred under 1 atm oxygen. The reaction was initiated by adding 5 U of laccase with mediators, 1 mM ABTS and 1 mM NNDS, at room temperature. The methanol concentration was determined by Gas Chromatography(HP 5890 Series II plus) with chromosorp® 102(Johns-Manville Corp., 80/100) column. The oven temperature was increased from 75 to 95℃ by 5℃/min, and from 95 to 200℃ by 20℃/min, and kept constant at 200℃ for 5 minutes. The injector and flame ionization detector temperature were maintained 180℃ and 150℃, respectively. The flow rate of carrier gas was 60 mL/min. The methanol concentration was measured every 10 minutes.

3. Results and Discussions

Laccase is evaluated one of the best enzyme in terms of environmentally benign processes, since the enzyme uses oxygen as an oxidant to degrade lignin and produces no hamful products. We purified the laccase homogeneously from *Cerrena unicolor* in a very active state. It showed characteristic absorption feature with blue band at λ_{max} = 604 nm. Molecular weight of the enzyme was 57,608 which could be accurately determined by MALDI/TOF MS. The enzyme had 2.8 copper ions per enzyme implying apoenzymes might exist together. The enzyme was very active in lignin degradation and the activity increased 4 times in the presence of ABTS as a mediator (7-10).

We obtained cyclic voltammograms for 23 different mediators at 3 different pHs (pH=4, 7, 10) and redox potential values were shown in Table 1, which are organized, by their reversibility and redox potential values. Among them, NNDS had a good reversibility and similar redox potential (about 470 mV vs. Ag/AgCl at pH=7) to ABTS, which is very well-known mediator (Fig. 4).

The thermodynamic criterion for mediator is that the redox potential should exist between that of oxygen reduction(+620 mV vs. Ag/AgCl, pH=7.0) and that of substrate oxidation(more negative than around +200 mV depending on the kinds of substrate). The redox potential of laccase also should be in this range, and it is known that higher redox potential laccase has better catalytic activity (17, 18, 23, 25). If this is true, the redox

Table 1. Redox potential values of mediators (mV *vs.* Ag/AgCl)

Mediator	Epa (pH4)	Epc (pH4)	E0 (pH4)	Epa (pH7)	Epc (pH7)	E0 (pH7)	Epa (pH10)	Epc (pH10)	E0 (pH10)
ABTS (2,2'-azino-bis(3-ethyl benzthiazoline-6 -sulfonic acid))	527	432	475	543	423	477	549	430	490
HNNS (2-Nitroso-1-naphthol-4-sulfonic acid)	763	641	702	630	515	573	641	524	583
RBBR (Remazol Brilliant Blue R)	680	643	662	586			549		
CPZ (Chlorpromazine)	685	591	638	663			604		
NNDS (1-Nitroso-2-naphthol-3,6-disulfonic acid)	675	595	635	508	438	473	519	420	470
PZ (Promazine)	600	530	565	608			555		
SGZ (Syringaldazine)	413	406	410	230	181	206	168	87	123
L-DOPA (3-(3,4-Dimethylphenyl)-L-alanine)	417	296	357	205	156	180	14		
p-Phenylene diamine	382	310	346	178	133	156	100		
2,7-Diaminofluorene	336	277	307	243	179	211	217		
4-Methylcatechol	343	257	300	212	47	130	6	-55	-25
BT (Benzotriazole)	1554			1473			1288		
HBT (1-Hydroxybenzotriazole)	893			888			860		
Acetovanillone	753			569			550		
Vanillic acid	749			641			397		
DMAB (3-Dimethyl aminobenzoic acid)	700			617			605		
2-Naphthol	681			492			377		
Feruic acid	586			376			279		
Guaiacol	577			470			240		
Coniferyl alcohol	506			335			212		
3-HAA (3-Hydroxyanthranilate)	429			261			90		
Gallic acid	359			209			108		
MBT (3-Methyl-2-benzothiazoline)	351			210			20		

potential of mediator should be near to that of laccase to minimize thermodynamic loss during electron transfer. ABTS and NNDS are very high potential mediator can be fitted to the above criteria (Fig. 5).

The well-known mediator, ABTS, can be oxidized easily by laccase to produce dark green cation radical (ε_{405} = 35 $mM^{-1}\cdot cm^{-1}$), but no absorption characteristics for NNDS can be found from literature. We tried to find what kind of absorption behavior could be followed for NNDS. Fresh 0.20 mM NNDS solution (in 0.1 M KPi, pH=7.0) was prepared and small amount of laccase from *Cerrena unicolor* was added, and UV/VIS absorption spectra were recorded as time goes on (Fig. 6). A band around 350 nm was developed and a band around 430 nm diminished, which meant that the oxdized NNDS absorbed around 350 nm and NNDS absorbed around 430 nm. The band around 430 nm was better to be followed, and molar extinction coefficient was calculated from this

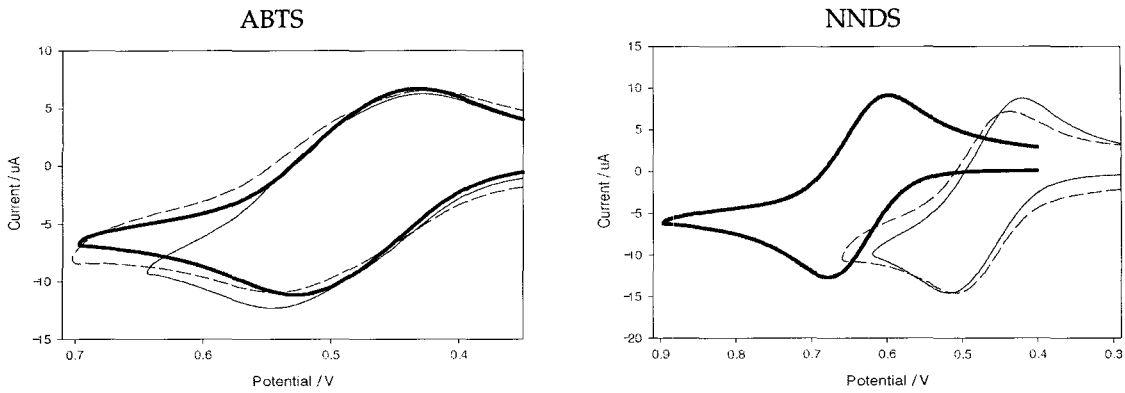

Fig. 4. Cyclic voltammograms of ABTS and NNDS. pH = 4.0 (bold line), at pH = 7.0 (dotted line) and at pH = 10.0 (solid line)

band to be ε_{428} =3.83 $mM^{-1} \cdot cm^{-1}$.

Fig. 6. Changes in absorption spectra of NNDS upon adding laccase,measured after 0, 2, 4, 8, 14 and 100 min.

Specific activity of purified laccase (8-10) from *Cerrena unicolor* was determined by both ABTS and NNDS using extinction coefficients of both compounds. The specific activities of the laccase were 23.2 units/mg with ABTS and 21.2 units/mg with NNDS. Therefore, the electron exchange rate for NNDS with laccase is very similar to that for ABTS, which means that NNDS has similar mediating capability to ABTS and very cheap mediator compared to ABTS.

Lignin degradation capabilities of both ABTS and NNDS were compared by determining methanol concentration after reacting with laccase. ABTS or NNDS alone could not degrade lignin, but laccase alone degraded lignin as shown in Fig. 7. ABTS and NNDS enhanced the rate of lignin degradation, and ABTS showed better activity especially in the beginning. The reaction rate of NNDS with lignin was about a half of that of ABTS at 10 minute, but the final concentration of methanol produced in 1 hour was very similar each other. It would be noticed that the laccase activity was inhibited upon incubating with mediators (33,34). Our data

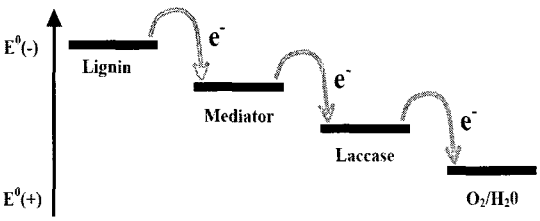

Fig. 5. Thermodynamic criteria for lignin degradation by laccase-mediator-system (LMS).

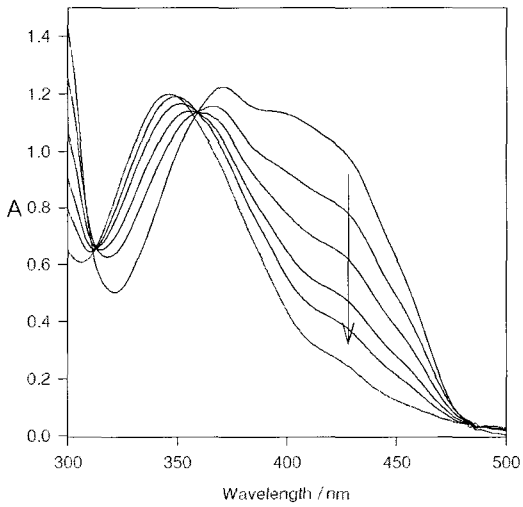

Fig. 6. Changes in absorption spectra of NNDS upon adding laccase, measured after 0, 2, 4, 8, 14 and 100 min.

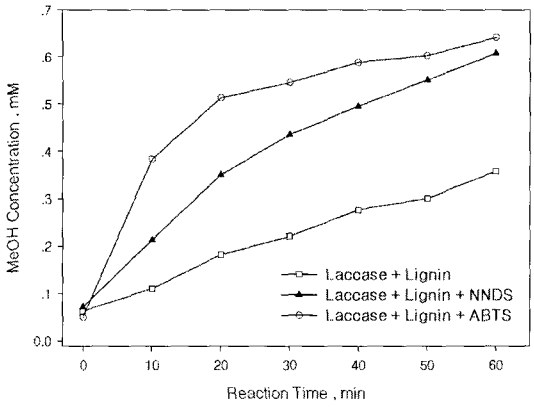

Fig. 7. Effect of mediators on lignin degradation measured by produced methanol amount.

showed that ABTS inhibited laccase more than NNDS. Therefore, the reason for similar methanol concentration for both ABTS and NNDS could be interpreted, as the initial activity of ABTS was better than that of NNDS, but ABTS inhibited laccase activity more during the incubation.

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