Bioresource Technology 100 (2009) 505–508

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Short Communication

Determination of reactive oxygen species generated in laccase catalyzed oxidation of wood fibers from Chinese fir (*Cunninghamia lanceolata*) by electron spin resonance spectrometry

Guanwu Zhou [a,b,1], Jianing Li [a,b,c,1], Yongsheng Chen [a], Baolu Zhao [a], Yongjian Cao [b], Xinfang Duan [b,*], Yuanlin Cao [a,*]

[a] Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, PR China
[b] Research Institute of Wood Industry, Chinese Academy of Forestry, Beijing 100091, PR China
[c] College of Mechanical and Electronic Engineering, Northwest A&F University, 712100 Yangling, Shaanxi, PR China

ARTICLE INFO

Article history:
Received 4 December 2007
Received in revised form 10 June 2008
Accepted 10 June 2008
Available online 22 July 2008

Keywords:
Reactive oxygen species (ROS)
Electron spin resonance (ESR)
Wood fibers
Laccase
Lignin

ABSTRACT

The aim of the present study was to determine whether the radical reaction intermediates – reactive oxygen species (ROS) were formed during the laccase-catalyzed oxidation of wood fibers from Chinese fir (*Cunninghamia lanceolata*) and to quantify tentatively its production with electron spin resonance (ESR) spectrometry. To investigate the activation pathways triggered by laccase, ESR spin-trapping techniques using *N-tert*-butyl-α-phenylnitrone (PBN) as spin trap followed by ethyl acetate extraction were employed to identify and quantify the free radical intermediates. ROS such as the superoxide and hydroxyl radical was detected and quantified in the laccase catalyzed oxidation of wood fibers, suggesting that ROS is the main free radical intermediates for laccase reaction. Based on the findings of the presence of ROS and previous literature on the free radical reaction of laccase oxidation of wood fibers, a possible reaction mechanism involving ROS-mediated attack on the domains of lignin which is not directly accessible for the enzyme and solubilized low-molecular mass lignins which function as reactive compounds like adhesives and may cling back to the fiber surface, could accordingly describe laccase-catalyzed oxidation of Chinese fir wood fibers.

1. Introduction

Conventional approaches to the manufacture of fiberboards involve the application of thermosetting resins (e.g. urea–formaldehyde resin and phenol–formaldehyde resin) to wood fibers. The bonding is achieved not only by the addition of adhesive but also by the auto-adhesive properties of the wood components. Wood scientists have performed studies to determine if the auto-bonding of wood fibers through using oxidoreductases such as laccase without synthetic resins can be achieved (Felby et al., 1997b, 2002; Kharazipour et al., 1997). Oxidoreductases including laccase and peroxidase catalyze in vivo polymerization of lignin through free radical reactions, and thus it may be feasible to simulate the naturally growing process of wood and to bond the lignocellulosic materials together in vitro.

Commonly found in the white-rot fungi and plants, laccase (EC 1.10.3.2) plays an important role in the synthesis and degradation of lignin (Ander and Eriksson, 1976; Ishihara, 1980; Morohoshi and Haraguchi, 1987; Kawai et al., 1988). Laccase has been found causing polymerization of lignin compounds through free radical reactions. Previous investigations on free radical reaction intermediates in laccase catalyzed reaction have focused on isolated lignin polymers and model compounds. Laccase has been found to generate phenoxy radicals on solubilized milled wood lignin (Ferm et al., 1972). Felby et al. (1997b) reported that high concentrations of radicals were stabilized in the lignin polymer of beech wood fibers subjected to laccase-catalyzed oxidation.

Reactive oxygen species (ROS), including superoxide anions and hydroxyl radicals, produced by a variety of biochemical reactions, has been postulated to mediate varying metabolic processes related to proton leakage from electron transport chains in biological systems. ROS has been detected in fungal degradation of lignin (Amer and Drew, 1980; Bes et al., 1983; Faison and Kirk, 1983). Milstein et al. (1994) reported immobilized laccase acting on technical lignin to yield superoxide as a reaction intermediate, but the results were only gained from indirect methods based on

* Corresponding authors. Tel.: +86 10 64888577; fax: +86 10 64889872 (Y. Cao), tel.: +86 10 62888324; fax: +86 10 62881937 (X. Duan).
E-mail addresses: xfduan@caf.ac.cn (X. Duan), caoyl@moon.ibp.ac.cn (Y. Cao).
[1] The first and second authors contributed equally.

0960-8524/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2008.06.010

a cytochrome *c* assay. Felby et al. (1997a) compared the cytochrome *c* assay with the ROS-detecting method using spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) for the determination of ROS generation in the laccase and wood fiber system, and no superoxide or hydroxyl radicals was detected, suggesting that cytochrome *c* assay for detection of superoxide in systems involving lignin oxidized by oxidoreductases should be used with caution (Felby et al., 1997a).

There are few reports (Milstein et al., 1994; Felby et al., 1997a) on measuring ROS in laccase catalyzed oxidation of wood fibers by electron spin resonance (ESR) spectrometry at room temperature. Questions arise as to what free-radical reaction intermediates formed in the laccase-catalyzed oxidation. The study reported here has two objectives: (i) Determination and quantification of ROS in laccase catalyzed oxidation of wood fibers by ESR at room temperature; and (ii) suggesting possible hypothesis on the reaction mechanism for the laccase activation of wood fibers from Chinese fir.

2. Methods

2.1. Materials

Chinese fir (*Cunninghamia lanceolata*) wood fibers, produced by an Asplund process, were obtained from Jinggu forest products Co. Ltd. (Yunnan province, China). The fibers (MC, ca 10%) were ground into powder that passes through a 60-mesh screen but not a 80-mesh screen for the convenience of laccase treatment and ESR measurement. Fungal laccase (EC 1.10.3.2) from *Agaricus bisporus* was purchased from Sigma–Aldrich Inc. (Beijing, China). The enzyme is deep-brown powder with an enzyme activity of 4 units (U)/mg using catechol as substrate according to the supplier.

The spin trap *N*-benzylidene-*tert*-butylamine *N*-oxide (PBN) was purchased from Sigma–Aldrich Inc. (Beijing, China). Hydrogen peroxide solution (30 wt.% in H_2O, analytical grade) was from Beijing Chemical Technology Plant. All other reagents were purchased from China and were analytical grade.

2.2. ROS standard curve preparation

Hydrogen peroxide solution (30 wt.% in H_2O) was freshly diluted to a 50 mM with double distilled water. Ferrous sulphate was prepared as 0.2 M solution. The traditional Fenton system was used to generate hydroxyl radical. Varying concentrations (final concentrations 20–320 μM) of diluted hydrogen peroxide were added to the mixtures of ferrous sulphate (final concentrations 80–1280 μM) with the spin trap PBN (final concentration 4 mM). The total volume of each sample was 500 μl. After the termination of the reaction, 300 μl ethyl acetate was added to the mixture, shaken for 1 min, and centrifuged at 10,000*g* for 4 min. The organic solvent layer was withdrawn into a quartz tube with 2.5 mm diameter for determination of ROS on an ESR spectrometer.

2.3. Extraction power of various organic solvents concerning PBN–ROS spin adducts

Diluted hydrogen peroxide (final concentration 500 μM) was added to the mixtures of ferrous sulphate with the spin trap PBN (final concentration 4 mM). The total volume of each sample was 500 μl. After the termination of the reaction, 300 μl various organic solvents including ethyl acetate, butyl acetate, isoamyl acetate, glycerol triacetate, and *n*-butanol, were added to each sample respectively. The reaction system was shaken and centrifuged as above mentioned. Then the extractions were withdrawn and detected by ESR under same conditions.

2.4. The stability of spin trap complex in ethyl acetate

For the determination of the stability of spin trap complex in ethyl acetate, 1 mM PBN–OH complex from Fenton reaction was extracted into 300 μl ethyl acetate. The organic solution of PBN–OH adduct was then exposed to incandescent lamp light with a luminous intensity of 139 cd for different periods of time. Light intensity was measured using a light meter (model: Panlux, type Electronic 2, Gossen, Germany), which had a circular light sensor with a diameter of 20 mm. After the exposure, samples were taken and measured by ESR. For the stability of PBN–OH adduct dissolved in ethyl acetate, the extraction was kept in dark at 0–4 °C for different periods, then the extraction was detected by ESR.

2.5. Laccase treatment of fibers

0.045 g powder of wood fiber (or precisely fine wood fiber segment; approximately 10% moisture content) was suspended in 400 μl 0.2 M di-sodium hydrogen phosphate–citric acid buffer (pH 5). 40 mM PBN solution (final concentration 4 mM) and laccase were added at 10^{-3} U/g fiber dry substance. The suspension with a total volume of 500 μl was vortically mixed up and then incubated at 50 °C for 120 min. 300 μl ethyl acetate was added to the reaction mixture, shaken for 1 min, and centrifuged at 10,000*g* for 4 min. The organic solvent layer was withdrawn into a quartz tube with 2.5 mm diameter for determination of ROS on an ESR spectrometer.

2.6. Determination of ROS by ESR spectrometer

ESR measurements were performed on a spectrometer (200D-SRC; Brucker Instruments, Germany) at room temperature. The PBN–ROS complex in the organic solvent layer was measured in a 2.5 mm internal diameter quartz tube at 25 °C. During measurement the effective volume for sampling organic solvent was 60 μl. The conditions for ESR detection were as follows: X-band; 100 kHz modulation with 3.2 G amplitude; microwave power, 20 mW; central magnetic field 3,385 G, scan width 400 G, time constant 0.3 s, and scan time 4 min. Unless otherwise stated, all of the ESR measurements described in this paper were conducted under the above conditions. The whole height of triplet hyperfine structure, namely the three peaks in each ESR signal, was taken as the relative intensity of ROS signal. Every test has at least three replications.

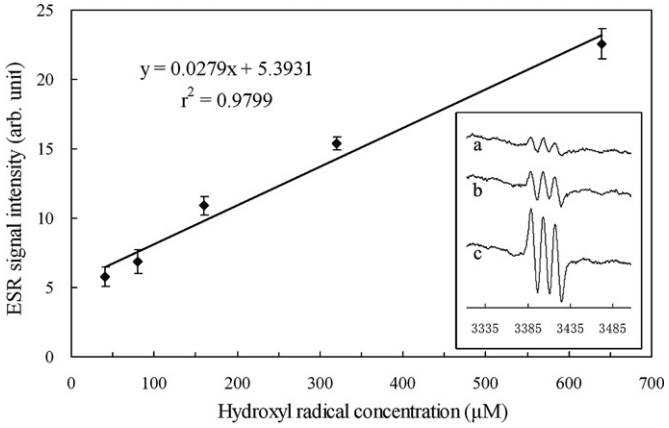

Fig. 1. Standard curve of PBN–OH adduct that was obtained by Fenton reaction using PBN as spin trap. Insert is the ESR spectra which represent ·OH concentrations at (a) 40 μM (b) 80 μM and (c) 640 μM.

3. Results and discussion

3.1. ROS standard curve

The height of the triplet hyperfine structure increased linearly with H_2O_2 concentration within the current experimental range. After linear regression analysis, the determination coefficient of the two variables with positive linear correlation was 0.9799 (Fig. 1). With the regression equation for the hydroxyl radical concentrations in the Fenton system and ESR signal intensities, the ESR signal strength quantitatively corresponds to the level of ·OH, namely the number of unpaired spins. The amount of PBN–ROS detected with our method may be quantified as the number of unpaired spins, for the ESR spectra of both PBN–OH and PBN–ROS adducts are of the identical hyperfine pattern and line width. The standard curve from Fenton reaction makes the quantification for ROS determination possible.

3.2. Extraction power of various organic solvents concerning PBN–ROS spin adducts

The optimum organic solvent may be of three properties: (i) a high partition coefficient to extract most of the PBN–ROS from water phase into organic phase; (ii) the ESR signal of the PBN–ROS complex need to be strong when the complex dissolved in the organic solvent and detected by ESR spectrometer; (iii) the organic solvent should be readily separated from water, and effectively separate PBN–ROS complex from the remaining substances in the reaction mixture. In order to find the optimum organic solvent, the ESR signal intensities for the same concentration of PBN–ROS complex in different organic solvents were determined. The results showed that different kinds of organic solvents gave various ESR signal strengths. Under the same experimental conditions, a maximum of ESR signal intensity was reached when ethyl acetate (density: 0.899 mg/ml) was used, followed by butyl acetate, isoamyl acetate, glycerol triacetate, and *n*-butanol. Ethyl acetate, therefore, was utilized to extract PBN–ROS complex from the reaction mixture of laccase and wood fiber.

3.3. The stability of spin trap complex in ethyl acetate

It was found that the ESR signal intensities for the ROS complex decreased significantly when exposed to incandescent lamp light with a luminous intensity of 139 cd. After 25 min of exposure, the radical intensity decreased about 50%. After 3 h of exposure, the radical intensity almost disappeared. These results indicated that PBN–ROS complex was sensitive to light; therefore, the whole process of application of the method to detecting ROS may be kept from light.

When the extraction of PBN–ROS complex were kept in dark at 0–4 °C for some time, a time curve of the radical intensity in ethyl acetate showed that the radicals had little change for up to 3 days, suggesting that PBN–ROS spin adduct was at a stable level under this condition. Therefore, the stability of PBN–ROS complex in ethyl acetate allows adequate time to measure radicals' intensities in the ESR spectrometer.

3.4. Radicals on solid fibers and detection of ROS in laccase catalyzed oxidation of wood fibers

ESR measurement on solid fibers gave a characteristic powder-type spectrum with no hyperfine structure. The ESR spectrum of free radicals generated during laccase catalyzed oxidation of wood fibers trapped by PBN had a triplet at $g = 2.005$ with $a_N = 15.0$ G, which was identical to PBN–ROS complex reported in biological systems (Cao et al., 2005). Superoxide anions, hydroxyl radicals, and other reactive oxygen free radicals can be trapped by PBN and the ESR signal of PBN–ROS complex is the same as the one found in our work (Capani et al., 2001). This indicated that the signal came from PBN–ROS spin adduct and ROS was generated when wood fibers were incubated with laccase. By comparing the signal from the supernatant of the enzyme-fibers solution to that from the Fenton reaction, the number of spins shown in Table 1 was obtained. With the standard curve from Fenton reaction, the amount of ROS reaction intermediates of laccase-catalyzed oxidation of wood fibers from Chinese fir was quantified.

Previously several methods (Widsten et al., 2002; Milstein et al., 1994) have been utilized to identify the free radical intermediates generated during the laccase activation of wood fibers and isolated lignin. Felby et al. (1997a), however, showed that the presence of superoxide detected by the cytochrome *c* assay could not be confirmed by the ESR spin-trapping technique using DMPO. In general, ESR is considered the most direct and efficient technique currently available for detection and measurement of free radicals.

In fungal transformation of lignins, a previous series of studies have suggested the mediation of short-lived radicals or activated low-molecular-mass intermediates (Hall, 1980). However, the role of separate enzymatic systems, especially laccase, in the generation of the superoxide radical was not proved yet.

We have gained evidence for the presence of ROS such as $O_2^{\cdot-}$ and ·OH, which was generated by laccase during lignin–laccase interaction. PBN is insensitive to light, heat, oxygen and steam, and can be dissolved in a wide variety of solvents. The incubation temperature is up to about 60 °C when wood fibers are treated with laccase. The water in samples, however, has a negative effect on ESR detection of free radicals. Therefore it lowers the sensitivity of ESR and restricts its application. PBN–ROS spin adduct is a hydrophobic complex that can be extracted by organic solvents. An approach using ethyl acetate as an extract solvent to concentrate PBN–ROS spin adduct from the aqueous supernatant and to eliminate the water's negative effect on ESR detection was developed, and ROS content in the laccase–wood fibers reaction mixture was detected successfully.

For ROS detection in the laccase treatment of wood fibers, the ESR spin trap PBN may outperform DMPO. DMPO is very sensitive to light and heat, which makes the ESR spin trap technique with DMPO have several limitations, among others, the susceptibility of DMPO-adducts to decomposition reactions and metal ion-catalyzed air oxidation (Finkelstein et al., 1982; Pou et al., 1989). Since an overwhelming majority of free radicals, including ROS, is very reactive, they do not normally occur in high concentrations in biochemical reactions. With the help of spin trap PBN, it became possible to detect the low concentrations of ROS intermediates in the laccase and wood fibers reaction system, for PBN stayed in the suspension throughout the incubation waiting for the ROS to come up and capturing them one by one. What spin trap technique with PBN caught was the total amount of free radicals generated during the laccase catalytic reaction; therefore the concentrations of PBN–ROS spin adducts reached the extent to which ESR spectrometer could give signals. The level of instantaneous ROS is much lower

Table 1
Characterization of radicals formed in the suspension liquid

ROS	g-Value	Spins/g dry substances
Suspension liquid, 0 U/g fiber	–	–
Suspension liquid, 10^{-3} U/g fiber, 60 min after addition of laccase	2.005	$3.74 \pm 0.05 \times 10^{18}$

The enzyme dosage in the suspension liquid was 10^{-3} laccase units (U)/g fiber[a].
[a] Data are given as mean values ± S.E.M.

than that of the overall amount of free radicals, which may accordingly explain the negative results of DMPO method.

The ROS detected via the PBN spin trap method has been accordingly quantified. Ferm et al. (1972) measured the amount of free radicals formed in milled wood lignin treated with laccase and peroxidase. The amount of phenoxy-type radicals generated in the suspension liquid and on the solid fiber subject to laccase treatment has been measured through comparing the signal to a weak pitch sample, which was supplied by Bruker and contained a known amount of unpaired spins (Felby et al., 1997a). The amount of radicals in green wood, chemical pulps and technical lignins has been reported previously (Rex, 1960; Steelink, 1966). A direct comparison of the level of radicals may not be possible, because the radicals detected – ROS in the present experiments and phenoxy-type radicals reported before – are of different type and reactivity. The problem on the quantification of the free radicals deserves more research work such as distinguishing various types of free radicals and quantifying each type generated.

4. Conclusions

The results of this ESR study of laccase catalyzed oxidation of wood fibers suggest that ROS such as $O_2^{\cdot-}$ and ·OH, is generated during lignin–laccase interaction. ROS is the main free radical intermediates for laccase activation of wood fibers. The ROS detected via the PBN spin trap method was quantified as $3.74 \pm 0.05 \times 10^{18}$ spins/g wood fiber dry substances, using ROS standard curve. On the basis of the direct ESR measurement of the ROS intermediates and former research findings (Kharazipour et al., 1997, 1998), a possible reaction mechanism involving ROS-mediated attack on the part of lignin indirectly accessible for the enzyme and solubilized low-molecular mass lignins which function as reactive compounds like adhesives, clinging back to the fiber surface, could accordingly describe laccase-catalyzed oxidation of Chinese fir wood fibers.

Acknowledgement

This study was supported by the Project 30471352 from National Natural Science Foundation of China.

References

Amer, G.I., Drew, S.W., 1980. The concentration of extracellular superoxide radical as a function of time during lignin degradation by the fungus *Coriolus versicolor*. Dev. Ind. Microbiol. 22 (3), 479–484.

Ander, P., Eriksson, K.E., 1976. The importance of phenol oxidase activity in lignin degradation by the white-rot fungus *Sporotrichum pulverulentum*. Arch. Microbiol. 109 (1–2), 1–8.

Bes, B., Ranjeva, R., Boudet, A.M., 1983. Evidence for involvement of activated oxygen in fungal degradation of lignocellulose. Biochimie 65 (4), 283–289.

Cao, Y.L., Guo, P., Xu, Y.C., Zhao, B.L., 2005. Simultaneous detection of NO and ROS by ESR in biological systems. In: Packer, L., Cadenas, E. (Eds.), Methods in Enzymology: Nitric Oxide, Part E, vol. 396. Academic Press, San Diego, pp. 77–83.

Capani, F., Loidl, C.F., Aguirre, F., Piehl, L., Facorro, G., Hager, A., De Paoli, T., Farach, H., Pecci-Saavedra, J., 2001. Changes in reactive oxygen species (ROS) production in rat brain during global perinatal asphyxia: an ESR study. Brain Res. 914 (1–2), 204–207.

Faison, B.D., Kirk, T.K., 1983. Relationship between lignin degradation and production of reduced oxygen species by *Panerochaete chrysosporium*. Appl. Environ. Microbiol. 46 (5), 1140–1145.

Felby, C., Hassingboe, J., Lund, M., 2002. Pilot-scale production of fiberboards made by laccase oxidized wood fibers: board properties and evidence for cross-linking of lignin. Enzyme Microb. Technol. 31 (6), 736–741.

Felby, C., Nielsen, B.R., Olesen, P.O., Skibsted, L.H., 1997a. Identification and quantification of radical reaction intermediates by electron spin resonance spectrometry of laccase–catalyzed oxidation of wood fibers from beech. Appl. Microbiol. Biotechnol. 48 (4), 459–464.

Felby, C., Pedersen, L.S., Nielsen, B.R., 1997b. Enhanced auto adhesion of wood fibers using phenol oxidases. Holzforschung 51 (3), 281–286.

Ferm, R., Kringstad, K.P., Cowling, E.B., 1972. Formation of free radicals in milled wood lignin and syringylaldehyde by phenol oxidizing enzymes. Sven. Papperstidn. 14 (12), 859–865.

Finkelstein, E., Rosen, G.M., Rauckman, E.J., 1982. Production of hydroxyl radical by decomposition of superoxide spin-trapped adducts. Mol. Pharmacol. 21 (2), 262–265.

Hall, P.L., 1980. Enzymatic transformations of lignin. Enzyme Microb. Technol. 2 (3), 170–176.

Ishihara, T., 1980. The role of laccase in lignin biodegradation. In: Kirk, T.K., Higuchi, T., Chang, H.M. (Eds.), Lignin Biodegradation: Microbiology, Chemistry and Potential Applications, vol. 2. CRC Press, Boca Raton, Fla, p. 17.

Kawai, S., Umezawa, T., Higuchi, T., 1988. Degradation mechanisms of phenolic β-1 lignin substructure model compounds by laccase of *Coriolus versicolor*. Arch. Biochem. Biophys. 262 (1), 99–110.

Kharazipour, A., Hüttermann, A., Luedemann, H.D., 1997. Enzymatic activation of wood fibers as a means for the production of wood composites. J. Adhes. Sci. Technol. 11 (3), 419–427.

Kharazipour, A., Bergmann, K., Nonninger, K., Hüttermann, A., 1998. Properties of fiber boards obtained by activation of the middle lamella lignin of wood fibers with peroxidase and H_2O_2 before conventional pressing. J. Adhes. Sci. Technol. 12 (10), 1045–1053.

Milstein, O., Hüttermann, A., Fründ, R., Ludemann, H., 1994. Enzymatic co-polymerization of lignin with low-molecular-mass compounds. Appl. Microbiol. Biotechnol. 40 (6), 760–767.

Morohoshi, N., Haraguchi, N., 1987. Degradation of lignin by the extracellular enzymes of *Coriolus versicolor* III. Mokuzai Gakkaishi 33 (2), 143–150.

Pou, S., Hassett, D.J., Britigan, B.E., Cohen, M.S., Rosen, G.M., 1989. Problems associated with spin trapping oxygen-centered free radicals in biological systems. Anal. Biochem. 177 (1), 1–6.

Rex, R.W., 1960. Electron paramagnetic resonance studies of stable free radicals in lignin and humic acids. Nature 188 (4757), 1185–1186.

Steelink, C., 1966. Stable free radicals in lignin and lignin oxidation products. Adv. Chem. Ser. 59 (1), 51–64.

Widsten, P., Laine, J.E., Tuominen, S., 2002. Radical formation on laccase treatment of wood defibrated at high temperatures part 1: studies with hardwood fibers. Nord. Pulp Pap. Res. J. 17 (2), 139–146.