Preliminary

Peroxidizable Compounds Produced by Selective White-rot Fungus, *Ceriporiopsis subvermispora*

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Ceriporiopsis subvermispora, the best biopulping fungus so far examined, is known to decompose lignin located in wood cell walls and middle lamellae without erosion of the wood cell walls even after fiber separation[1,2]. Since enzymes are too large to penetrate inside of wood cell walls without erosion, degradation of lignin by *C. subvermispora* must be catalyzed by low molecular mass agents[2]. In the wood decay by *C. subvermispora* extensive damage of middle lamellae has been observed even in an early stage of wood decay[1], suggesting that the lignin-degradation of this fungus is catalyzed by low molecular mass oxidants generated near the substrate. This is because active oxidants formed in cell lumina should react with the substrate first encountered and long distance between cell lumina and middle lamellae limits free radicals available in the lignolysis due to their short life time. Based on this discussion, we have analyzed peroxidizable compounds produced by this fungus.

Materials and Methods

Milli-Q^{TM} water and peroxide-free organic solvents were used throughout this study. Lipoxygenase from soybean (100,000 U/mg) was obtained from Nacalai Tesque (Japan). NMR spectra were recorded with a JEOL λ-400 NMR spectrometer (^{1}H : 400 MHz) in $CDCl_3$ at 22°C using TMS as an internal standard. GC-MS was obtained with Shimadzu QP-1000 on Silicone OV-101 (50 m×0.25 mm, GL Science, Japan). GC was recorded with Shimadzu GC-14A on OV-101 and CP-Sil-8 (50 m×0.25 mm, Chrompack, Netherlands). Manganese peroxidase (MnP) and laccase activities were measured with 2, 6-dimethoxy phenol (2, 6-DMP) at 470 nm. One unit (U) of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of 2, 6-DMP in one minute.

C. subvermispora FP90031 was cultivated from blended mycelial inocula at 28°C for two weeks in 300 ml Erlenmeyer flasks containing 15 ml of chelator-free (CF) growth medium which was modified from BIII medium[3]. $(NH_4)_2SO_4$ was used as a nitrogen source instead of ammonium tartrate. Nitrilotriacetic acid (NTA) was omitted from the medium. Glucose (10 g/l) was used as a carbon source. The preculture was suspended in 250 ml of the CF medium and blended for 10 sec 3 times. Five milliliters of the suspension was inoculated onto 5 g of extractive-free beech wood meal in 300 ml Erlenmeyer flasks and incubated at 28°C. After incubation, the cultures were extracted with 60 ml of Milli-Q^{TM} water, washed five times with the water and then extracted with 60 ml of a peroxide-free $CHCl_3$/MeOH (2 : 1) solution per flask. The aqueous extract was separated into higher (HMW) and lower molecular weight (LMW) fractions by ultrafiltration (Cut off 10,000).

The organic extracts obtained were methylated with diazomethane and analyzed by NMR and GC-MS. ESR spectra were recorded on a JEOL FR-30 X-band ESR spectrometer after reaction of the organic extract with soybean lipoxygenase (2,000 U) in 20 mM Tris-HCl buffer (pH 9.0) containing 100 mM 4-POBN.

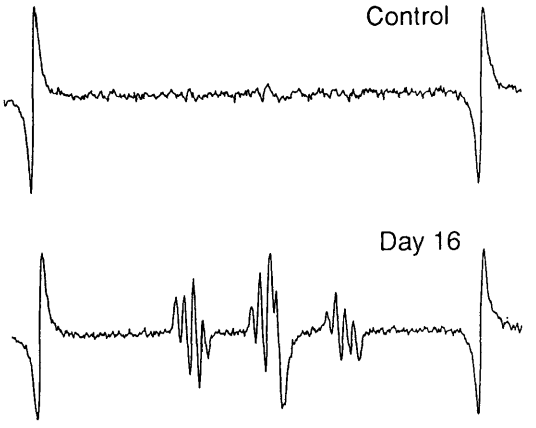

Fig. 1. ESR spectra of spin adducts formed by the reaction of lipoxygenase and organic extracts from 16 day-cultures of *C. subvermispora*. Control: Reaction of lipoxygenase with organic extracts from wood meal medium without inoculation.

Results and Discussion

In wood decay by *C. subvermispora*, lignin degradation is observed without cell wall erosion even after extensive fiber separation. This indicates that the low molecular mass diffusible compounds play a major role in the lignin biodegradation[2]. In this study, we focused on a free radical generation system from peroxidizable compounds

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produced by *C. subvermispora*. When wood meal cultures of *C. subvermispora* were extracted with $CHCl_3$/MeOH after removal of water-soluble materials, free unsaturated fatty acid (USFA), 9, 12-octadecadienoic acid was detected, together with saturated fatty acid (SFA), hexadecanoic acid. The production of USFA was also confirmed by ESR after peroxidation with soybean lipoxygenase which specifically oxidizes USFAs containing a *cis*, *cis*-1, 4-pentadiene group. GC and ESR spectra demonstrated that no distinct correlation exists between incubation time and the amount of intact peroxidizable USFAs. The free USFA and SFA were produced predominatly in the initial stage and then decreased with concomitant production of lipid hydroperoxide.

Lipid peroxidation is a process initiated by hydrogen transfer from methylene group of 1, 4-pentadiene moiety in USFA to yield lipid hydroperoxide. Lipid hydroperoxide is stable enough for diffusing into middle lamellae and it generates peroxyl, alkoxyl and carbon-centered radicals by the reaction with transition metal complexes or active oxygen species. Recently we reported that non-phenolic lignin was intensively decomposed by lipid hydroperoxide model and copper in the presence of fungal metabolite pyridine at room temperature in aqueous solutions[4]. This suggests that free radicals from lipid hydroperoxide is capable of degrading lignin located far from enzymes. It has been reported that MnP-dependent lipid peroxidation decomposed recalcitrant aromatic compounds *in vitro*[5–7]. In the wood cultures, however, activity of MnP reached to the maximum at day 4 and then decreased. This suggests some other initiation system is involved in the lipid peroxidation. Reaction of 9, 12-octadecadienoic acid with the aqueous LMW fraction in the presence of H_2O_2 indicated that simple Fenton system is not involved in the initiation system. Further research is in progress to elucidate the unique extracellular peroxidation processes of this fungus.

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