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Enzymatic Staining for Detection of Phenol-Oxidizing Isozymes Involved in Lignin-Degradation by *Lentinula edodes* on Native-PAGE

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1. Introduction

Lignocellulose is the most abundant organic compound in the terrestrial environment. Nonetheless, with the exception of basidiomycetous fungi, most organisms are either unable to degrade lignocellulose, or if they can, they do so with difficulty (Kirk & Fenn, 1982). Wood-decomposing basidiomycetes can be grouped into two categories: white-rot and brown-rot fungi. White-rot fungi have cellulases and lignin-degrading enzymes that decompose most cell wall components, whereas brown-rot fungi have enzymatic systems that selectively degrade cellulose and hemicelluloses, leaving brown shrunken lumps of tissue composed mainly of a loose lignin matrix (Enoki et al., 1988; Highley et al., 1985; Highley & Murmanis, 1987; Kirk & Highley, 1973). The name 'white-rot' is derived from the bleaching effect that this fungus has when degrading wood; the lignin-degrading enzymes that they secrete have the effect of promoting lignin loss and exposing the white cellulose fibrils. White-rot fungi are known to produce polyphenol oxidases (phenoloxidases), which, when the fungi are plated on agar media containing gallic or tannic acids, change the color of the agar to a dark reddish-brown in what is referred to as Bavendamm's polyphenol oxidase test or Bavendamm reaction (Bavendamm, 1928, as cited in Jørgensen & Vejlby, 1953). Based on this reaction, phenoloxidases are considered to be one of putative lignin-degrading enzymes (Higuchi 1990). Laccase (Lcc, EC 1.10.3.2), catechol oxidase (EC 1.10.3.1) and tyrosinase (monophenol monooxygenase, EC 1.14.18.1) are phenoloxidases with considerable overlap in their substrate affinities (Burke & Cairney, 2002). Lcc catalyze the reduction of O_2 to H_2O using a range of phenolics, aromatic amines, and other electron-rich substances as hydrogen donors (Thurston, 1994). Similar phenol-oxidizing activities are also observed in peroxidases (EC 1.11.1.x), which use H_2O_2 as an electron donor. Lignin peroxidase (ligninase, LiP, EC 1.11.1.14) was first discovered in *Phanerochaete chrysosporium* in which the H_2O_2-dependent C_α-C_β cleavage of non-phenolic lignin model compounds was first described (Tien & Kirk, 1983, 1984). Manganese peroxidase (MnP, EC 1.11.1.13) also strongly degrades lignin model compounds and the reaction is mediated by H_2O_2 and Mn^{2+} (Glenn et al., 1983; Glenn & Gold, 1985; Kuwahara et al., 1984). Whereas lignin can effectively be oxidized by LiP directly, as reviewed previously (Cullen & Kersten, 2004;

Gold & Alic, 1993), Mn^{2+} is considered to be an important physiological substrate for MnP. Further, while LiP expression has been observed in certain white-rot fungi (e.g. *Phanerochaete chrysosporium* and *Phlebia radiata*) under specific culture conditions (e.g., temperature, agitation, and nutritional constraints), MnP expression has been observed in a wide range of white-rot fungi (Gold & Alic, 1993), including cultivated edible fungi, such as *Agaricus bisporus* (Bonnen et al., 1994), *Ganoderma lucidum, Lentinula edodes*, and *Pleurotus* spp. (Orth et al., 1993).

The shiitake mushroom, *Lentinula edodes* (Berk.) Pegler, a white-rot basidiomycete, is one of the most valuable, cultured, edible mushrooms in the world (Chang & Miles 1989). Shiitake mushrooms were traditionally cultivated on Fagaceae logs, but they are now grown on sawdust-based media. The ability of white-rot basidiomycetes to degrade wood components, especially lignin, therefore affects both culture-time to harvesting and yields (Kinugawa & Tanesaka, 1990; Ohga & Kitamoto, 1997; Smith et al., 1988; Tanesaka et al., 1993). Although *L. edodes* secretes the lignin-degrading enzymes laccase (Lcc) and MnP when cultivated on sawdust-based media (Buswell et al., 1995; Leatham, 1985; Makker et al., 2001), it does not usually secrete these enzymes in liquid media. It was previously reported that the main isozyme produced by *L. edodes* cultured on sawdust was the manganese peroxidase, LeMnP2 (Sakamoto et al., 2009). In addition, we previously reported that a β-O-4 lignin model compound, 4-ethoxy-3-methoxyphenylglycerol-β-guaiacyl ether (Umezawa & Higuchi, 1985) was effectively degraded by *L. edodes* under MnP-induced conditions, but not under Lcc-induced conditions (Kochi et al., 2009). These observations supported the hypothesis that these enzymes, particularly MnP, play an important role in degrading sawdust during cultivation, and corroborating reports that the expression and properties of these enzymes is likely to influence mycelial growth and fruit body development (Smith et al., 1988; Wood et al., 1988). Several reports have been published on the purification and characterization of the lignin-degrading enzymes secreted by *L. edodes* using sophisticated biochemical procedures (Forrester et al., 1990; Nagai et al., 2002, 2003, 2007; Sakamoto et al., 2008, 2009). However, these methods are impracticable for routine isozyme analysis during breeding trials. Methods for isozyme detection by electrophoresis using enzyme catalytic properties - referred to as "protein activity staining" or "enzymatic staining" - are well established in histochemical studies and genetics (Pasteur et al., 1988). It was expected that Lcc, peroxidases (Per, EC 1.11.1.7), and MnP bands could be distinguished on the same gel by subtraction of newly appeared bands produced by sequential enzymatic staining. In practice, however, unexpected bands frequently appeared on gels exposed to conventional Lcc staining solutions. Indeed, in samples exhibiting strong MnP activity without Lcc activity, no additional bands appeared in subsequent staining procedures for either Per or MnP. We recently reported improved methods for enzymatic staining using native-PAGE to distinguish between Lcc and MnP isozymes induced in liquid cultures of *L. edodes* (Saeki et al., 2011).

In this chapter, we describe an assay system for the induction and identification of phenol-oxidizing enzymes produced by *L. edodes* grown under liquid culture conditions. In addition, the assay system was used to compare the glycosylation characteristics of these extra- and intracellular isozymes, as well as their modes of inheritance within monokaryotic progenies and β-O-4 lignin model compound degradation characteristics under Lcc- and MnP-induced conditions. Based these findings, the potential application of this assay system to elucidate the ligninolytic mechanisms employed by this fungus is also discussed.

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2. Experimental procedures

2.1 Terminology

We use the term "phenol-oxidizing enzymes" to describe all phenoloxidases and peroxidases. We do so because of the ability of these enzymes to utilize the same substrates and produce the same catalytic products as described in the Introduction.

2.2 Fungi and culture conditions

The Hokken 600 variety of *L. edodes* (Hokken Co., Ltd., Tochigi, Japan; hereafter referred to as H600) and monokaryotic progenies derived from basidiospores were used in this study. To induce the phenol-oxidizing enzymes, mycelia were cultured in MYPG liquid medium (2.5 g malt extract; 1.0 g yeast extract; 1.0 g peptone; 5.0 g glucose in 1,000 ml of distilled water) supplemented with sawdust extract (MYPG-S). This sawdust extract was produced by adding 1 g of *Castanopsis cuspidata* (Thunb. ex Murray) Schottky sawdust to 30 ml of distilled water and then autoclaving the mixture for 15 min before filtering through filter paper (No. 1, Advantec, Tokyo) and collecting the extract. MYPG-S liquid media samples were then prepared from MYPG liquid medium by adding half a volume of sawdust extract instead of distilled water, which gave an MYPG-S extract that contained 500 mg sawdust in 30 ml media. Mycelia were sub-cultured at 25°C on MYPG 1% agar plates. After 14 days, three mycelial disks measuring 3 mm in diameter were harvested from the plates and used to inoculate 30 ml MYPG-S liquid in a 100 ml flask which was then statically cultured at 25°C. MnP activity was induced during culture on MYPG-S. Lcc activity was induced by adding 2 mM $CuSO_4 \cdot 5H_2O$ to the same media seven days after initial inoculation.

2.3 Enzyme assay

A schematic representation of the strategies employed to distinguish between individual phenol-oxidizing enzymes by subtractive activity assays (Szklarz et al., 1989) and sequential enzymatic staining of gels using native-PAGE is shown in Fig. 1.
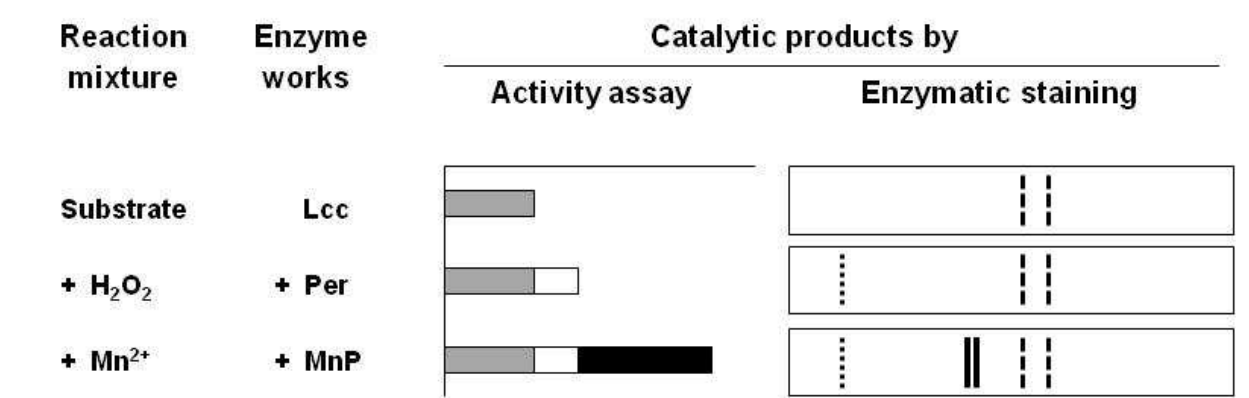

Fig. 1. Strategy underlying the subtractive activity assay and sequential enzymatic staining of a gel to distinguish between individual phenol-oxidizing enzymes. Grey, open, and solid squares represent the activities of Lcc, Per and MnP, respectively. Broken, dotted, and solid lines represent the sequential enzymatic staining of Lcc, Per and MnP in a gel, respectively.

To assay the activities of extracellular enzymes, 100 µl of culture liquid was sampled every two to three days during culture and centrifuged at 13,000 rpm for 10 min; this supernatant was used as a crude enzyme solution. The crude enzyme solution was assayed for Lcc, Per and MnP in identical 5 ml test tubes containing the following reaction mixtures: Lcc assay mixture consisted of 0.1 mM o-dianisidine in 0.1 M sodium tartrate buffer (pH 5.0), with additional H_2O_2 (final concentration 0.1 mM) added to the Lcc assay mixture to assess Per activity. Additional $MnSO_4 \cdot 5H_2O$ (final concentration 0.1 mM) was added to the Per assay mixture to assess MnP activity. Aliquots (20 µl) of crude enzyme solution were added to test tubes containing 980 µl of each reaction mixture, which were then incubated at 37°C for 10 min. The reactions were stopped by the addition of 50 µl of 40 mM NaN_3. To inactivate the enzymes in the control tubes, sodium azide was added to the control tubes containing the Lcc assay mixture before incubation. Catalytic products of the reaction were spectrophotometrically assayed using o-dianisidine as a substrate, and the activity of enzyme products was estimated by subtracting the respective absorbance values at 460 nm: i.e., Lcc activity = Lcc assay minus the control assay; Per activity = Per assay minus the Lcc assay; and MnP activity = MnP assay minus the Per assay, respectively. One unit (U) of enzyme activity was defined as the amount of enzyme required to catalyze 1 µmol of o-dianisidine in 1 min (ε_{460} = 29,400 $M^{-1}cm^{-1}$: Paszczynski et al., 1988).

2.4 Native PAGE and enzymatic staining

Each of the phenol-oxidizing isozymes was detected by native PAGE as described previously (Saeki et al., 2011). Briefly, whole cultures were filtered through a nylon stocking to separate the mycelia from the culture liquid. The collected mycelia were then ground with a ceramic mortar and pestle in two volumes (v/w) of crushing buffer (0.05 M Tris-HCl, pH 7.2, 0.1% β-mercaptoethanol) before being centrifuged at 12,000 rpm for 10 min. The resulting supernatant was considered to represent the intracellular enzyme sample. To prepare the extracellular enzyme sample, the culture liquid was centrifuged at 13,000 rpm for 10 min, and the supernatant was filtered (No.2 filter paper, Advantec) and then concentrated 15-fold by ultrafiltration using the centrifugal filter unit, Centriprep YM-10 (10-kDa cut-off membrane, Millipore, MA). Aliquots containing 15 µl enzyme sample, 1.5 µl glycerol and 1.5 mg bromophenol blue (BPB) as a dye marker were then loaded into the wells of 12.5% (for intracellular) or 17.5% (for extracellular) polyacrylamide gels. Native-PAGE gels were run at 15 mA for 15 min followed by 25 mA for 3−4 h. After electrophoresis, the gel was sequentially incubated at 37°C for 30 min in three different staining solutions. The first staining solution was an improved enzymatic staining solution containing additional ethylenediaminetetraacetic acid (EDTA) (for Lcc and Per) to remove the Mn^{2+} typically used in conventional staining solutions. Staining for enzymes was performed as follows. To the Lcc staining solution (LccS+EDTA), 1.8 mM o-dianisidine, 0.1 mM acetate buffer (pH 4.0) containing 130 mM EDTA (LccS+EDTA), an additional H_2O_2 (final concentration 1.0 mM) was added to produce the Per staining solution (PerS+EDTA). In the same way, additional $MnSO_4 \cdot 5H_2O$ (final concentration 0.1 mM) was added to the PerS without EDTA to produce the MnP staining solution (MnPS). The gels were rinsed with distilled water between each staining procedure to remove the previous staining solutions, particularly the EDTA from PerS+EDTA used for MnP staining. Isozyme nomenclature employed an (-e) or (-i) in Lcc-e, MnP-e or MnP-i to indicate whether the Lcc and MnP enzymes were extra- or intracellular. Numerals in parenthesis, e.g., MnP-e (52),

MnP-e (57) etc. indicated the relative mobility of each isozyme relative to the mobility of the bromophenol blue used as a dye.

2.5 Glycosidase treatment

To purify the enzymes in the crude enzyme solutions prior to electrophoresis, 1 ml acetone was added to a 100 µl aliquot of the enzyme solution and kept at −20°C for 3 h to precipitate the proteins. The proteins were then resuspended in 100 µl of 10 mM phosphate buffer (pH 6.0). To determine whether the enzymes were glycosylated, the protein suspension in phosphate buffer was incubated with glycosidase (Glycosidases 'Mixed', Seikagaku Biobusiness Corp., Japan) at final concentrations of 0.25−2.0% (w/v) with a protease inhibitor (Complete, Mini, EDTA-free; Roche Diagnostics, Germany) at 37°C overnight. Effects of the glycosidase treatment on activities of each of the isozymes were then examined by enzymatic staining after native-PAGE as described in section 2.4 above.

2.6 Identification of isozymes by mass spectrometry

Distinguishing between isozymes was performed as described previously (Saeki et al. 2011). Briefly, after native-PAGE had been conducted on the same sample solution in adjacent lanes, each gel was then subjected to enzymatic staining and Coomassie brilliant blue (CBB) staining. Bands of interest, such as those exhibiting the same mobility as bands in the enzymatic staining experiments, were then excised from the CBB-stained gel using a sterile surgical blade and placed in 1.5 ml microcentrifuge tubes. To remove the CBB dye, each polyacrylamide gel section was then repeatedly washed with 50, 30 and 50% v/v acetonitrile containing 25 mM NH_4HCO_3 under sonication for 20 min with a micromixer (Taitec, Tokyo), before finally being washed with 100% acetonitrile without NH_4HCO_3 for 5 min. The sections of polyacrylamide gel were then vacuum-dried for 5 min and recovered in 100 µl of 50 mM NH_4HCO_3 (pH 7.8) containing 10 ng/µl trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega, WI) on ice for 30 min. Any extra trypsin solution was then removed and the sections of gel were incubated at 37°C for 16 h. The tryptic fragments in a gel were then extracted by immersing the gel sections in 50 µl of extraction buffer consisting 50% acetonitrile and 5% trifluoroacetic acid under sonication (Ultrasonic cleaner, SU-3T, Shibata, Japan) for 20 min. The extraction buffer was placed into new tube and replaced with 25 µl of fresh extraction buffer. The extraction process was repeated a further three times and the collected buffer containing the tryptic fragments was finally concentrated to approximately 5 µl by drying under vacuum. Analysis of the tryptic peptides by tandem mass spectrometry was performed on a nanoelectrospray ionization quadrupole time-of-flight (Q-TOF) hybrid mass spectrometer (Q-TOF Premier, Waters Micromass, MA) coupled with a nano-HPLC (Cap-LC; Waters Micromass). The peptides were separated on a BEH 130-C18 column (1.7 µm, 100 µm × 100 mm, Nano Ease, Waters, MA) at flow rate of 0.2 µl/min according to the manufacturer's instructions. The peptide sequences thus obtained were then either matched automatically to proteins in a non-redundant database (National Center for Biotechnology Information, NCBI, www.ncbi.nlm.nih.gov) using the Mascot MS/MS ions search algorithm (Mascot Server version 2.2, Matrix Science), or BLAST searches were manually performed against the DNA Data Bank of Japan database (DDBJ, www.ddbj.nig.ac.jp). Mascot search was also performed to calculate the false discovery rate (FDR) on acquired MS/MS data against decoy database.

2.7 RNA isolation and Northern blot analysis

Total RNA was extracted from mycelia after varying incubation periods under MnP- and Lcc- induced conditions using TRIzol Reagent (Invitrogen, CA). cDNA was synthesized from total RNA using an RNA PCR Kit Ver.3.0 (Takara Bio, Japan), and amplified using the primer set LeMnP2En5f (5′-TCCGACAGTGTCAATGACCTCGCTC) and LeMnP2En13r (5′-GTCAGTGGTGAGATTTGGGAAGGGC), which were designed based on the highly conserved *lemnp2* region (DDBJ Acc. No. AB306944; Sakamoto et al., 2009). A fragment measuring approximately 700 bp was then extracted from the 1% agarose−formaldehyde gel, purified, and sequenced using an ABI Prism 3100-*Avant* Genetic Analyzer (Applied Biosystems, CA) according to the manufacturer's instructions. Fragments with sequences matching *lemnp2* were then labeled using a PCR-based digoxigenin (DIG)-dUTP labeling kit (PCR DIG Probe Synthesis Kit, Roche Diagnostics) according to the manufacturer's instructions. The resulting DIG-labeled probe, *lemnp2*N, was then used for Northern hybridization following blotting of 10 µg of total RNA onto a Hybond-N$^+$ membrane (GE Healthcare, Switzerland) using an established protocol (Sambrook et al., 1989).

2.8 DNA isolation and Southern blot analysis

Genomic DNA was extracted from mycelia using cetyltrimethylammonium bromide (CTAB) isolation buffer (J. J. Doyle & J. L. Doyle, 1987, as cited in Milligan, 1998). To prepare the DIG-labeled probe, genomic DNA was used as a template for PCR with the primer set, LeMnP2En5f2 (5′-TCAGGAAAATTCCCGACTAT) and LeMnP2En12r (5′-GAACCTCGATG CCATCAA); this primer set was designed to amplify the region from exon 5 to exon 12 of *lemnp2*, including introns (DDBJ Acc. No. AB306944; Sakamoto et al., 2009), and the resulting probe was named *lemnp2*S. In addition, to examine cross-hybridization between *lemnp2* and a relative of the manganese peroxidases LeMnP1 coded by *lemnp1*, a probe for *lemnp1* was also prepared as described above using the primer set LeMnP1f1 (5′-GATTCCTGAGCCTTTCG) and LeMnP1r (5′-TTCGGGACGGGAATAAC); this primer set was designed to amplify the regions from exon 7 to exon 15 of *lenmp1* including introns (DDBJ Acc. No. AB241061; Nagai et al., 2007) and the resulting probe was named *lemnp1*S. These two probes were then used for Southern blot analysis (Sambrook et al., 1989).

2.9 Degradation assay of β-*O*-4 lignin model compound

2.9.1 Culture experiment

To assay the abilities of MnP and Lcc to degrade the β-*O*-4 lignin model compound, 4-ethoxy-3-methoxyphenylglycerol- β-guaiacyl ether (Fig. 2; β-*O*-4 compound, hereafter) was synthesized according to the method previously described (Umezawa & Higuchi 1985).

To prepare media containing the β-*O*-4 compound to media, 300 µg of the β-*O*-4 compound was diluted in 50 µl acetone and added to 30 ml MYPG-S. Whole liquid culture media was collected 14, 21 and 42 days after inoculation and assayed for phenol-oxidizing enzyme activity. The β-*O*-4 compound was then recovered from the liquid culture media by the addition of two volumes of ethyl acetate to separate the aqueous phase, before evaporating the ethyl acetate off and then precipitating the compound. To improve subsequent chromatographic analysis, the recovered β-*O*-4 compound was silylated using N-*O*-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) to form a trimethylsilyl (TMS) derivative. This

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TMS derivative was then subjected to gas chromatography-mass spectrometry (GC-MS) analysis (6890N, Agilent Technologies, CA), which was fitted with an capillary column (HP-5 MS, 30 m × 0.25 mm i.d., 0.25-μm; J&W Scientific, CA) coupled to an MS (JMS-K9, JEOL, Japan) according to the manufacturer's instructions. Helium was used as the carrier gas at 1.5 ml/min. GC oven conditions consisted of 150°C for 1 min, initially ramped at 10°C/min to 200°C and then at 5°C/min to 250°C. The electron impact mass spectra were obtained at an acceleration energy of 70 eV. The degradation rate (%) of the β-*O*-4 compound was calculated using the rate of quantities of the TMS derivative before and after culture, compensating for the recovery of the β-*O*-4 compound with 4,4'-dimethoxybenzoin (anisoin), which was used as an internal standard.
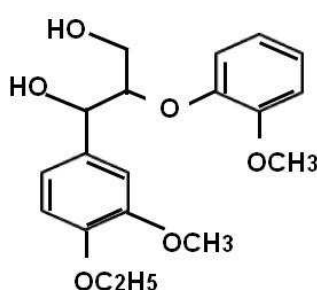
Fig. 2. β-*O*-4 lignin model compound used in this study; 4-ethoxy-3-methoxyphenylglycerol-β-guaiacyl ether (from Umezawa & Higuchi, 1985).

2.9.2 Incubation experiment

Degradation of the β-*O*-4 compound was also examined by incubation with the extracellular enzyme solution, which was prepared using the same procedures used for electrophoresis (section 2.4) with the following slight modifications. The extracellular enzyme solutions were diluted with 0.2 M sodium tartrate buffer (pH 5.0, final concentration of 0.1 M) and distilled water to bring the volume to 10 ml and keep the activity of MnP, Lcc and the mixed solution (MnP+Lcc) at 17 U/ml. For the MnP and MnP+Lcc reactions, additional H_2O_2 (final concentration 0.1 mM) and $MnSO_4 \cdot 5H_2O$ (final concentration 0.1 mM) were added to the reaction for Lcc. Then, 500 μg of the β-*O*-4 compound in 50 μl of acetone was added to the 10 ml enzyme solution and incubated at 37°C with agitation at 100 rpm for up to 10 days. The rate of degradation of the β-*O*-4 compound was evaluated using GC-MS as above.

3. Results and discussion

3.1 Selective induction of phenol-oxidizing enzyme

Phenol-oxidizing enzyme activities under different culture conditions are shown in Fig. 3. Neither MnP nor Lcc was induced when mycelia were cultured on MYPG liquid medium without sawdust extract (data not shown). Under MnP-induced conditions (i.e. when mycelia were cultured on MYPG+S), MnP activity increased suddenly on day 21, before reaching a maximum activity (95 U/ml) on day 35 and then decreasing thereafter (Fig. 3a).

We previously found that supplementing the MYPG liquid medium with wood chips or sawdust from members of the Fagacae, *C. cuspidata* or *Fagus crenata* Blume, induced MnP

activity (Yoshikawa et al., 2004). The results of the present study show that sawdust extracts produced by autoclaving sawdust in hot water (section 2.2) induce MnP activity (Fig. 3a). Compared with mycelial growth on the MYPG (without extract) medium, the sawdust extract also had a marked effect on the promotion of mycelial growth (MYPG-S). Although less marked than that observed on MYPG-S (100 mg/30 ml), extracts produced using 100 mg sawdust in 30 ml media also promoted mycelial growth; however, MnP activity was not induced in cultures grown in MYPG-S media with lower extract concentrations for up to 35 days (data not shown). These observations suggested that MnP was induced by specific functional compounds in the sawdust extract, and not only due to mycelial growth.
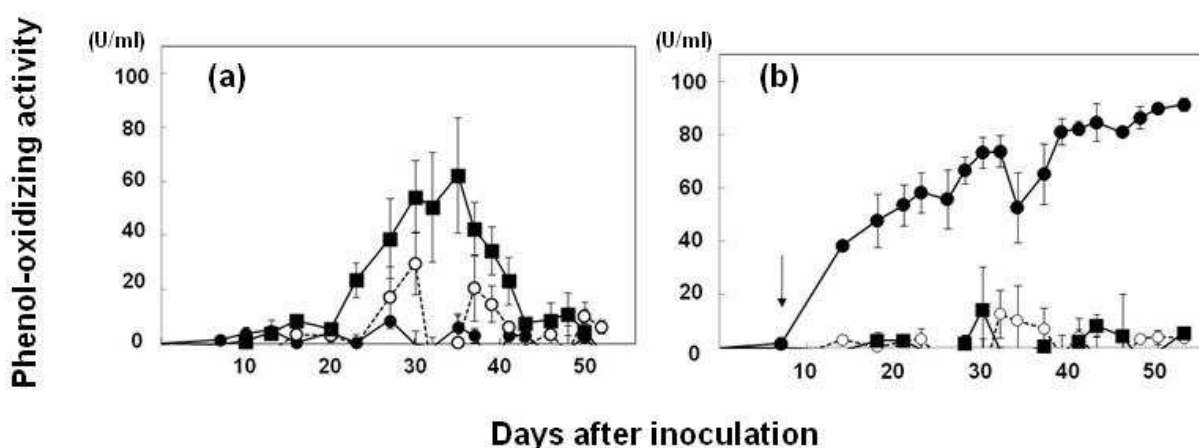
Fig. 3. Changes of phenol-oxidizing activities of (●) Lcc, (○) Per and (■) MnP in a liquid culture medium of *L. edodes* under (**a**) MnP-induced (MYPG-S) or (**b**) Lcc-induced conditions (MYPG-S with 2mM $CuSO_4 \cdot 5H_2O$) (reprinted from Saeki et al., 2011). The arrow in panel (**b**) indicates the day of 2 mM $CuSO_4 \cdot 5H_2O$ addition. Values are means with standard errors (vertical bars) for three replicate cultures.

Fourteen days after inoculation in MYPG-S containing Cu^{2+}, Lcc activity was detected (7 days after the addition of 2 mM $CuSO_4 \cdot 5H_2O$) (Fig. 3b). This Lcc activity increased gradually after day 52 while MnP activity was completely suppressed. Lcc has been shown to be induced by aromatic compounds and metallic ions such as copper (Collins & Dobson, 1997; Saparrat et al., 2002; Scheel et al., 2000; Shutova et al., 2008; Soden & Dobson, 2001). Indeed, copper has been reported to be a strong laccase inducer in the white-rot fungi *Pleurotus ostreatus* (Palmier et al., 2000), *Trametes pubescens* (Galhaup & Haltrich, 2001; Galhaup et al., 2002), and *T. versicolor* (Collins & Dobson, 1997). In *Trametes pubescens*, the transcription of the laccase gene is induced within 10 h after the addition of 2 mM $CuSO_4$ (Galhaup et al., 2002). Under the two culture conditions employed in this study, either Lcc or MnP activity were detected, but not both (Fig. 3). This finding suggests that the induction of MnP and Lcc are controlled by a negative feedback system, i.e., Lcc-induction suppresses MnP production, or more specifically, the addition of $CuSO_4 \cdot 5H_2O$ suppresses MnP production. Although the addition of several of the aromatic compounds that were tested did not induce Lcc - 2-methoxyphenol (guaiacol), 2,6-dimethoxyphenol (DMP), 4-anisidine, hydroquinone, or 1,2-benzenediol (catechol) - these substances except for DMP were observed to suppress MnP activity (data not shown).

3.2 Isozyme detection and identification

Of CBB staining bands subjected to protein identification, proteins with FDR (q≤0.05) were described below. Number of entry (MS/MS data) was ranged from 68 to 89. In the extracellular enzyme sample (culture liquid) prepared under MnP-induced conditions, two MnP-e isozyme bands, MnP-e (52) and MnP-e (57) in Fig. 4a, were detected. These two MnP isozymes were identified as the manganese peroxidase, LeMnP2, a major MnP isozyme that is secreted into sawdust medium by *L. edodes* (Sakamoto et al., 2009). Other enzyme, exo-β-1,3-glucanase, was also detected under MnP-induced conditions (Fig. 4b).   In the extracellular enzyme samples prepared under Lcc-induced conditions, two major Lcc isozyme bands, Lcc-e (61) and Lcc-e (67), were detected together with broad tailing smears (Fig. 4c). These two isozymes were identified as being laccases (Lcc1; Fig. 4d), and are known to be an extracellular laccase produced by *L. edodes* (Nagai et al., 2002; Sakamoto et al., 2008). These results, combined with enzyme assay data and results of isozyme detection using PAGE, indicate that MnP and Lcc isozyme detection using the improved LccS+EDTA, PerS+EDTA and MnPS enzymatic staining methods can be used to successfully distinguished between each of the phenol-oxidizing enzymes (Saeki et al., 2011).
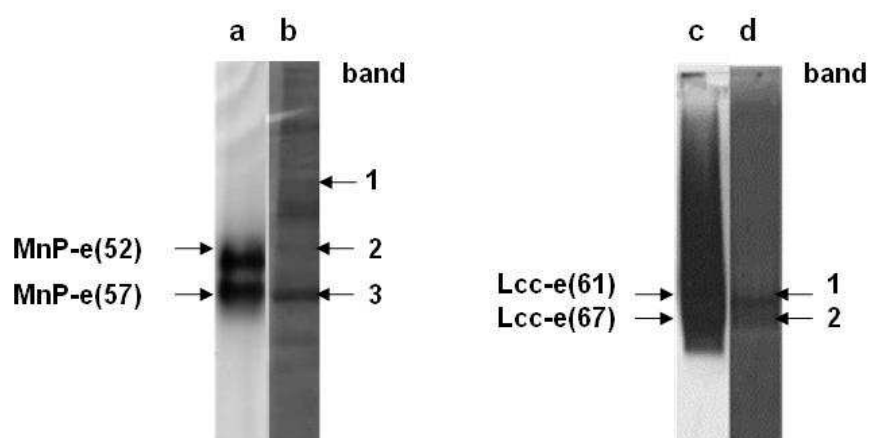

Fig. 4. Protein bands detected by (**a, c**) enzymatic staining and corresponding (**b, d**) CBB staining on native-PAGE (reprinted from Saeki et al., 2011). Lanes (**a**) and (**b**) show bands detected under MnP-induced conditions at 22 days; lanes (**c**) and (**d**) show bands detected under Lcc-induced conditions at 30 days. Protein bands identified by Q-TOF mass spectrometry in (**b**): band 1, exo-β-1,3-glucanase; band 2, manganese peroxidase (LeMnP2); and band 3, manganese peroxidase (LeMnP2) and in (**d**): band 1, laccase (Lcc1); band 2, laccase (Lcc1).

3.3 Comparisons of intracellular and extracellular Lcc isozymes

Expression patterns of extra- and intracellular Lcc isozymes during culture under Lcc-induced conditions are shown in Fig. 5.

Three major extracellular Lcc isozymes were detected: Lcc-e (61), which was expressed from day 12 (5 days after the addition of $CuSO_4 \cdot 5H_2O$), had a constant intensity with a broad smear tails, an Lcc-e (67) from day 17, and another Lcc-e (74) from day 22. All of these enzymes were expressed until the end of culture on day 47. The observed changes in the total band intensity of the three Lcc extracellular isozymes was generally associated with

changes in Lcc activity in the culture liquid (refer Fig. 3). Three major intracellular Lcc isozymes, Lcc-i (61), Lcc-i (67) and Lcc-i (74), were also detected, and all exhibited the same mobilities as their respective extracellular Lcc isozyme counterparts. The intracellular Lcc were coincidentally expressed with the extracellular Lcc isozymes.
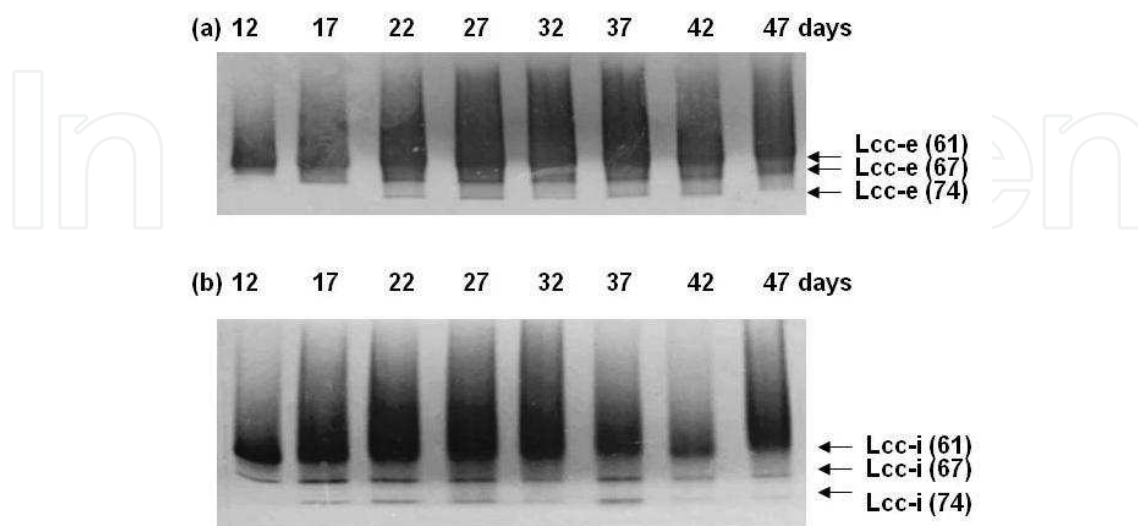

Fig. 5. Laccase isozyme banding patterns detected as (**a**) extracellular and (**b**) intracellular isozymes during culture under Lcc-induced conditions. Days after inoculation are shown above each lane.

Although we successfully extracted total RNA from mycelia under Lcc-induced conditions to examine the transcription of Lcc1, extraction of native (undigested) total RNA was unsuccessful. Native total RNA, which was prepared from mycelia under MnP-induced conditions (described in section 3.4 below), was degraded considerably quicker and to a greater extent after the addition of small amounts of cell lysate obtained under Lcc-induced conditions compared to when cell lysate obtained under MnP-induced conditions was added (data not shown). This relatively quicker degradation of native total RNA suggests a relatively high internal RNase activity in the cell lysate of the Lcc-induced condition, which may be attributed to the decrease observed in mycelial growth after the addition of $CuSO_4 \cdot 5H_2O$ and subsequent induction of Lcc, as well as the antagonistic expression of Lcc in the mycelial contact zone of any adjacent and competing basidiomycetes or other fungi (Iakovlev & Stenlid, 2000; Mercer, 1982; White & Boddy, 1992).

3.4 Manganese peroxidase gene transcription

The sequence of the fragment amplified from cDNA, which was prepared from a mixed pool of total RNAs obtained from the mycelia of 10-, 15- and 18-day-old cultures under MnP-induced conditions, was identical to that of *lemnp2a* but slightly different from *lemnp2b* (data not shown), corroborating the results obtained from the protein identification deduced by Q-TOF mass spectrometry (section 3.2). The finding that these sequences were similar also indicated that hot-water sawdust extracts induced the secretion of the same isozyme, LeMnP2, which is a major MnP isozyme that is secreted into sawdust media (Sakamoto et al. 2009). The results of the Northern blotting experiments with *lemnp2* are shown in Fig. 6. Under MnP-induced conditions, a detectable amount of *lemnp2* mRNA was present at

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10−19-days during the initial stage of culture, with transcription increasing from day 22 to day 25 and then decreasing at day 28. These changes in *lemnp2* transcription occurred several days prior to the changes observed in MnP activities in the liquid culture medium.
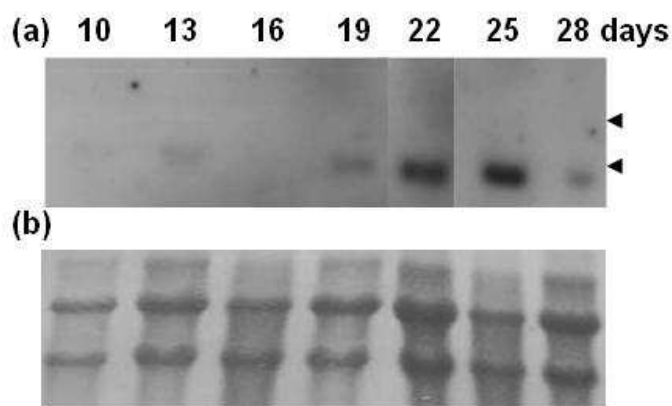

Fig. 6. Northern blot analysis of *lemnp2* gene transcript under (**a**) MnP-induced conditions and (**b**) ribosomal RNA used as a loading control. Days after inoculation are shown above each lane. Arrowheads indicate position of 26S and 18S rRNA.

3.5 Comparisons between intracellular and extracellular MnP isozymes

Expression patterns of extra- and intracellular MnP isozymes during culture under MnP-induced conditions are shown in Fig. 7. The extracellular MnP isozymes, MnP-e (52) and MnP-e (57), were strongly expressed during the initial stage of culture on days 11 to 19, before gradually decreasing until day 43. Four major bands were considered to be intracellular MnP isozymes, and of these, two bands, MnP-i (52) and MnP-i (57), exhibited the same mobility as extracellular MnP isozymes, while the other two bands, MnP-i (63) and MnP-i (66), were strictly intracellular. The intracellular MnP isozymes were expressed during the initial stage of culture, either several days before, or coincident with, the expression of the extracellular MnP isozymes. Compared to the intracellular MnP isozymes, the extracellular MnP isozymes maintained relatively high activities for up to 43 days of culture. However, changes in the intensities of bands that were neither extracellular nor intracellular MnP isozymes coincided with changes in MnP activity in the liquid culture medium during culture. Although we have no experimental data to explain why this may have occurred, it is worth noting that the intracellular enzyme solution (cell lysate) did not exhibit any phenol-oxidizing activities when assayed spectrophotometrically. In addition, the addition of intracellular enzyme solution to extracellular enzyme solutions caused marked inactivation of the latter (data not shown). Taken together, these observations either imply that the cell lysate contained a specific inhibitor of phenol-oxidizing enzymes when these enzymes and the inhibitor in cell lysate were not separated on a gel, or that there was an error in the manner in which the different experimental culture lots were processed, including the replicate flasks.

While treatment with glycosidase completely inactivated the two strictly intracellular MnP isozymes, glycosidase treatment had no effect on the activities of the extracellular MnP isozymes (Fig. 8). This finding indicates that the intracellular isozymes were active as

glycosylated proteins, and implies that a relationship exists between the secretion of MnP and the simultaneous expression of β-glucanase detected by Q-TOF mass spectrometry.
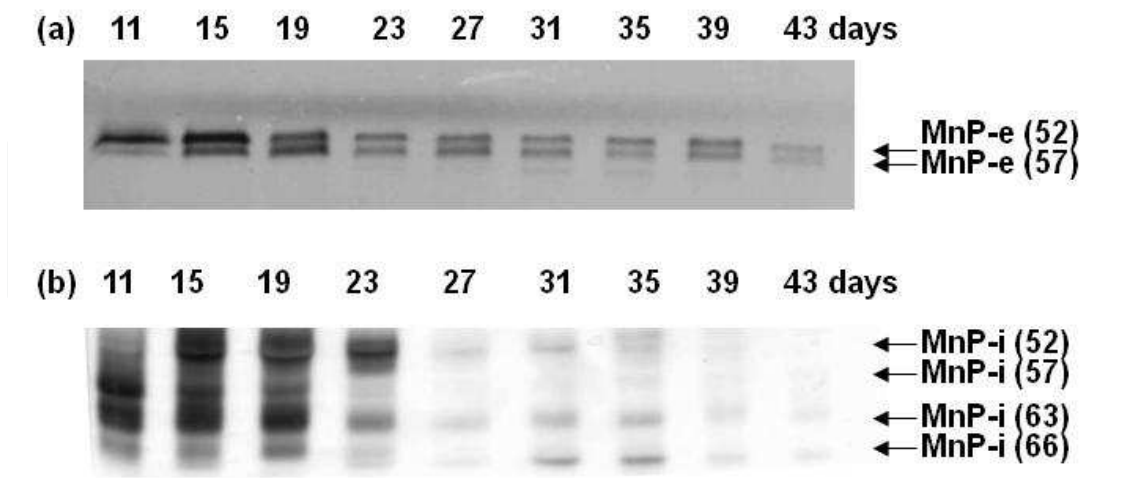

Fig. 7. Manganese peroxidase isozyme banding patterns detected as (**a**) extracellular and (**b**) intracellular isozymes during culture under MnP-induced conditions. Days after inoculation are shown above each lane.
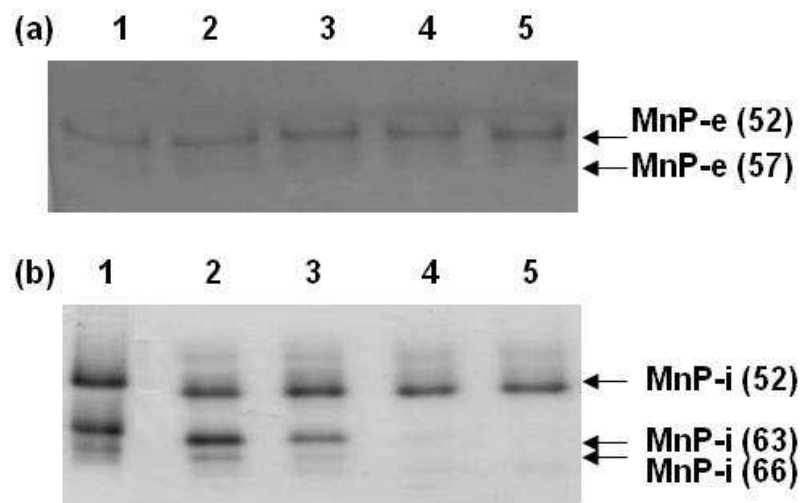

Fig. 8. Effects of glycosidase treatment on enzymatic staining of (**a**) extracellular and (**b**) intracellular MnP isozymes expressed under MnP-induced conditions (at 20 days). Lanes differ according to concentration of glycosidase: lane 1, control (0%); lane 2, 0.25%; lane 3, 0.5%; lane 4, 1.0%; and lane 5, 2.0%, respectively. MnP-i (57) was not detected.

3.6 MnP isozymes in monokaryons

Four monokaryotic strains (#317, #208, #305 and #105), each carrying the mating type factor A_1B_1, A_1B_2, A_2B_1 and A_2B_2, respectively, were derived from basidiospores from dikaryon H600. Although MnP activities of the monokaryons were very weak compared to the MnP activities of H600 (refer Fig. 7), both of the extracellular MnP isozymes (MnP-e (52) and MnP-e (57)) that were detected in the parent dikaryon were also detected in monokaryons, irrespective of their mating-type factors (Fig. 9). This finding suggests that, although these

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isozymes may be encoded by different loci, the isozymes are not under allelic control (i.e. they are not allozymes).
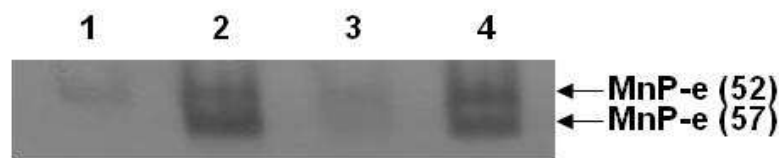

Fig. 9. Extracellular manganese peroxidase isozymes expressed by monokaryotic progenies of H600. Lanes represent strains with mating type factor in parentheses: lane 1, #317 (A_1B_1); lane 2, #208 (A_1B_2); lane 3, #305 (A_2B_1) and lane 4, #105 (A_2B_2).

The results of the Southern blotting experiment of *lemnp2* on *Hin*d III-digested monokaryon genomes are shown in Fig. 10. There was no *Hin*d III restriction site in the amplified region (*lemnp2*S) of the H600 genomic DNA, as expected from the database analysis of different *L. edodes* stock SR-1 (DDBJ Acc. No. AB306944, Sakamoto et al. 2009). Two *lemnp2* hybridization signals appeared at positions between 564-2322 bp in all four of the monokaryotic strains (lanes 1-4 in Fig. 10), and all of the strains exhibited the same two hybridization signals observed in the H600 parent dikaryon (lane 5 in Fig. 10). However, single intense and weak hybridization signals of *lemnp1* were observed using another probe, *lemnp1*S, at different positions between 2322-6557 bp in H600 (lane 6 in Fig. 10), indicating that the two probes did not cross-hybridize with each other. Conversely, it is likely that the weak hybridization signals that appeared between 2322-4631 bp (lane 2 in Fig. 10) were cross-hybridization products between the two probes (see lane 6 in Fig. 10). These observations, combined with the observation of two isozymes being expressed by all of the monokaryons assayed in this study, suggest that there are two copies of *lemnp2* in the haploid genome of *L. edodes*. Nevertheless, to confirm whether the lemnp2 gene is indeed duplicated as proposed here, further analysis will need to be undertaken to assign lemnp2 to a genetic linkage map or on chromosomal DNA which separated by contour-clamped homogeneous electric fields (CHEF) gel electrophoresis. Indeed, such attempts at combining assignments of quantitative trait loci (QTL) related to wood and lignin degradation in fungi would facilitate the identification of new genes involved in another ligninolytic system.
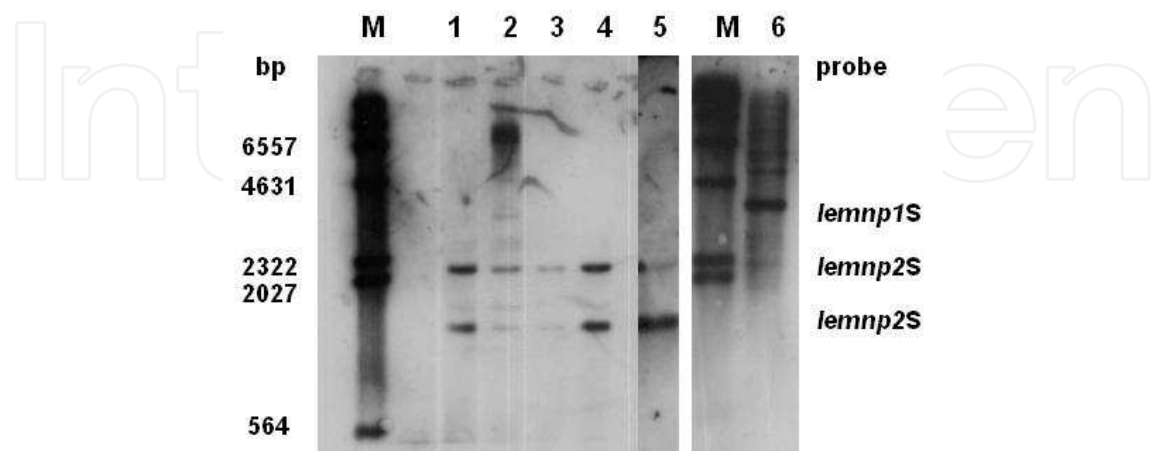

Fig. 10. Southern blot analysis with probe *lemnp2*S on genomic DNAs (digested with *Hin*d III). Lanes represent strains: lane M, size marker (λ/*Hin*d III); lane 1, #317; lane 2, #208; lane 3, #305; lane 4, #105, lane 5, H600; and lane 6, H600 (probed with *lemnp1*S).

3.7 Degradation of β-*O*-4 lignin model compound

We performed preliminarily examinations of the degradation of a β-*O*-4 lignin model compound under MnP- and Lcc-induced conditions (culture experiment) and the degradation of the model compound by incubation with enzyme solutions (incubation experiment) (Table 1).

Culture conditions	Days after inoculation in the culture experiment			Days of incubation in the incubation experiment[1]	
	14	21	42	4	10
MnP-induced	1.4 (nd)[3]	13.4 (32.2)[3]	20.0 (46.8)	16.8 (9.4)	23.8 (6.3)
Lcc-induced	1.1 (nd)	1.8 (47.0)	4.2 (58.5)	3.5 (8.0)	6.9 (3.1)
Enzyme mix[2]				16.0 (12.0 for MnP) (5.0 for Lcc)	22.9 (7.0 for MnP) (3.3 for Lcc)

[1]: Extracellular enzyme solution at an initial activity adjusted to 17 U/ml
[2]: Mixture of the extracellular enzyme solutions (MnP+Lcc), each at an initial activity of 17 U/ml
[3]: Numerals in parentheses represent enzyme activities (U/ml) , nd = not detected

Table 1. Degradation rate (%) of β-*O*-4 lignin model compound under MnP- or Lcc-induced conditions (culture experiment) and after incubation with enzyme solutions prepared from given culture conditions (incubation experiment) (Data from Kochi et al., 2009)

Under MnP-induced conditions, the β-*O*-4 compound was not degraded at all during the initial stages of the culture experiment. Indeed, effective degradation only occurred after day 21 when MnP activities suddenly increased; by day 42, 20.0% of the β-*O*-4 compound had been degraded. Conversely, no degradation of the β-*O*-4 compound was observed under Lcc-induced conditions until day 42 (4.2%). In the incubation experiment with MnP solution, the β-*O*-4 compound was effectively degraded in the initial 4 days of incubation (16.8%), with degradation increasing very gradually thereafter and then decreasing markedly near the end of the experiment; i.e., 23.8% at day 10 and only 7% of the compound was degraded in the latter 6 days. Conversely, degradation of the β-*O*-4 compound incubated with Lcc solution was detectable, but weak, until 10 days after inoculation (6.9%). The change in the degradation rates of the β-*O*-4 compound incubated with a mixture of the MnP and Lcc enzyme solutions (each at an initial activity of 17 U/ml) were similar to the degradation patterns of the MnP solution alone. This similarity indicated that no additive or multiplier effects could be attributed to the interaction of the two enzymes on the degradation of the β-*O*-4 compound. Compared to the initial period of the incubation experiment, the shallow slope of degradation rate in the latter period of the incubation was partly attributable to decreased enzyme activities over the course of the experiment (Table 1). Unfortunately, because we conducted this experiment without a protease-inhibitor, the decrease in enzyme activities was observed in enzyme solutions containing both MnP and Lcc, as well as the mixed MnP+Lcc solutions. In addition, laccase is also capable of degrading non-phenolic lignin model compounds in systems incorporating naturally

occurring or synthetic redox mediators (Johannes & Majcherczyk, 2000; Srebotnik & Hamel 2000; Tanaka et al., 2009). Nevertheless, based on the above results, manganese peroxidase (LeMnP2) appears to be more important than laccase (Lcc1) in lignin degradation by *L. edodes*. Prior to the discovery of Lip and MnP, one of the major catabolites formed by the degradation of the β-*O*-4 dimer by *P. chrysosporium*, 2-guaiacoxyethanol (II), was identified (Enoki et al., 1980). The results described above suggest that the assay system developed in this study is well suited for identifying phenol-oxidizing isozymes involved in the degradation of lignin model compounds. Further, these phenol-oxidizing isozymes have been effective for elucidating the mechanisms involved lignin degradation, and this role is likely to extend into the future (Cullen & Kersten, 2004).

Although we attempted to identify other MnP isozymes using another commercial Japanese Shiitake variety, "Bridge 32" (The General Environmental Technos Co. Ltd., Osaka, Japan), which is also used in sawdust cultivation, the variety exhibited the same extracellular MnP isozyme patterns as H600 (data not shown). The estimated heritability (h^2), which is the ratio of the additive genetic components of variance to the phenotypic components of variance, of the variety's wood-degrading ability was relatively low (32.2%) compared to the heritabilities estimated for other traits in crosses of H600 and Bridge 32 (Tanesaka et al., 2007). This low heritability may be attributable to the low allelic variation that exists between the MnP isozymes of the two varieties. The MnP that is produced by *L. edodes* when it is cultured on sawdust media (Buswell et al., 1995; Leatham 1985; Makker et al., 2001; Sakamoto et al., 2009), and which degrades the β-*O*-4 lignin model compound (Kochi et al., 2009), is likely to be critical for mycelial growth and fruit-body development during sawdust cultivation. The system presented here for assaying phenol-oxidizing enzymes under liquid culture conditions could therefore provide a practical screening method for examining isozymes of value in mushroom cultivation, particularly since the assay system targets the wood-degrading ability and the genomic characteristics of the genes involved in lignin-degradation.

4. Conclusions

When cultivated on sawdust-based media, the white-rot basidiomycete *Lentinula edodes* frequently produces the lignin-degrading enzymes MnP and Lcc. In this study, MnP produced by *L. edodes* was induced in a liquid culture supplemented with a sawdust extract of *Castanopsis cuspidata*. Lcc activity was induced by the addition of 2 mM $CuSO_4 \cdot 5H_2O$ into the same media 7 days after initial inoculation. In addition to employing native-PAGE and sequential enzymatic staining to detect the MnP and Lcc secreted by *L. edodes*, we also compared the expression of intra- and extracellular MnP isozymes. To distinguish between the phenol-oxidizing enzymes after native-PAGE, the gel was sequentially stained using an improved enzymatic staining solution (referred to as LccS+EDTA). In addition to containing 0.1 mM acetate buffer (pH 4.0) for Lcc detection, the staining solution contained 1.8 mM *o*-dianisidine as the substrate and 130 mM EDTA to eliminate Mn^{2+} contamination. Subsequently, 0.1 mM H_2O_2 was added to the LccS+EDTA for Per detection (PerS+EDTA), and 0.1 mM $MnSO_4 \cdot 5H_2O$ was added to the PerS, without EDTA, for MnP detection (MnPS). The two extracellular isozyme bands, MnP-e (52) and MnP-e (57), detected in culture medium under MnP-induced conditions, were both identified as manganese peroxidase (LeMnP2). Similarity, the bands Lcc-e (61) and Lcc-e (67), which were detected under Lcc-

induced conditions, were both identified as laccase (Lcc1) by Q-TOF mass spectrometry. Four major, intracellular, MnP isozyme bands were detected in mycelial extracts obtained from *L. edodes* cultured under MnP-induced conditions. Of these isozyme bands, two exhibited the same mobilities as extracellular MnP isozymes, while the other two bands, MnP-i (63) and MnP-i (66), were strictly intracellular. The intracellular MnP isozymes were expressed during the initial stage of culture, either several days before, or coincident with, the expression of the extracellular MnP isozymes. Compared to intracellular MnP isozymes, the extracellular MnP isozymes maintained relatively high activities for up to 40 days of culture. While glycosidase treatment of crude enzyme solutions prior to electrophoresis had no effect on the activities of the extracellular MnP isozymes, such treatment completely inactivated the two strictly intracellular MnP isozymes, implying that the intracellular isozymes were active as glycosylated proteins. Both of the extracellular MnP isozymes detected in the dikaryon were also detected in monokaryotic progeny, suggesting that although these isozymes may be encoded by different loci, they are not under allelic control. Southern blot analysis revealed that the probe *lemnp2* region hybridized with the four of the monokaryotic strains used, all of which exhibited the same two hybridization signals that were observed in the parent dikaryon. These observations suggest that there are two copies of *lemnp2* in the *L. edodes* haploid genome. Moreover, degradation assays involving the addition of the β-*O*-4 lignin model compound in cultures under MnP- and Lcc-induced conditions suggest that, rather than laccase (Lcc1), manganese peroxidase (LeMnP2) is a critical enzyme for lignin degradation in *L. edodes*.

In response to the crucial role played by basidiomycetous fungi in the carbon cycle by degrading lignocelluloses, considerable effort has focused on the functional genomics related to the enzymatic systems and mechanisms involved in lignin degradation, particularly in a few model fungus species. Nevertheless, fungal succession on dead logs and leaf litter in nature show that complete degradation of lignocelluloses is a commensal and competitive process affected by numerous fungi. The assay system presented here would be practical and convenient, not only as a method of screening isozymes of value in mushroom breeding and cultivation, but also for evaluating the lignin-degrading abilities of fungi and assessing the antagonistic interactions of different strains under experimental conditions.

5. Acknowledgments

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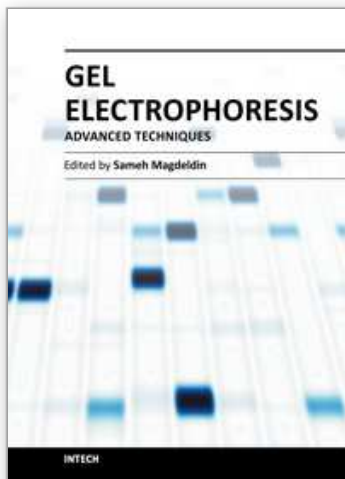
Gel Electrophoresis - Advanced Techniques

Edited by Dr. Sameh Magdeldin

As a basic concept, gel electrophoresis is a biotechnology technique in which macromolecules such as DNA, RNA or protein are fractionated according to their physical properties such as molecular weight or charge. These molecules are forced through a porous gel matrix under electric field enabling uncounted applications and uses. Delivered between your hands, a second book of this Gel electrophoresis series (Gel Electrophoresis- Advanced Techniques) covers a part, but not all, applications of this versatile technique in both medical and life science fields. We try to keep the contents of the book crisp and comprehensive, and hope that it will receive overwhelming interest and deliver benefits and valuable information to the readers.

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