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Production of Lignin Peroxidase by *Phellinus igniarius* and Cytotoxic Effects of Lignin Hydrolysates Derived from Wood Biomass on Cancer Cells

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Abstract – Over the past several years, research efforts have been directed both at economically producing valuable substances from the wood biomass and at producing lignolytic enzymes at a lower cost. In the present study, we found that *Phellinus igniarius*, the basidiomycetes, secreted lignin peroxidase as a main lignolytic enzyme, which was detected maximum activity at 16th day of culture and showed 37 kDa of molecular mass in identification by activity assay and purification by anion-exchange chromatography. The *Phellinus igniarius*-derived lignin peroxidase hydrolyzed steam-exploded wood (*Quercus mongolica)* powder into small molecules showing cytotoxicity against cancer cells (HepG2 hepatoma, SK-N-SH neuroblastoma, B16 melanoma, MBT-2 bladder cancer). In addition, the enzyme hydrolysates of lignins (ELg) that were extracted from the steam-exploded oak showed more potent cytotoxic effects on the cancer cells than the enzyme hydrolysates of wood biomass (EWp), indicating that the cytotoxic effect of EWp may be due to the enzyme-degraded products of lignin among the lignocellulosics. Furthermore, the cytotoxic effect of ELg on Chang, normal liver cells, was much less potent than that of ELg on HepG2 and B16 cancer cells, indicating that the cytotoxic effect of ELg may be specific for cancer cells. The present results suggest that *Phellinus igniarius* may be a useful resource for the large-scale production of lignin peroxidase and that the lignin peroxidase may be applied for the generation of valuable biodegradation products from wood lignocellulosics for medical use.

Keywords □ lignin peroxidase, *Phellinus igniarius*, wood biomass, cytotoxicity, cancer cell

INTRODUCTION

Wood biomass composed of mainly cellulose, hemicellulose and lignin is the most abundant renewable organic resource. The utilization of wood biomass is based on the fractionation of lignocellulosics to obtain a variety of practical chemicals from the polymeric fractions of the raw materials such as cellulose, hemicelluloses and lignin (Eriksson, 1993; Kirk, 1983; Wyman, 2003). Since lignin, a major component of wood biomass, perturbs the biological and chemical degradation of cellulose, selective degradation of lignin is the important prerequisite for converting wood biomass into useful substances (Lee, 1997). On the other hand, lignin is the most likely used raw material for the production of pulp and paper. Each year, enormous amount of technical lignins are produced at pulp mills worldwide with no better use than burning. However, fractionated lignins have shown the potential medicinal applications such as anti-immunodeficiency virus activity (Ichimura *at al.*, 1999) and anti-influenza activity (Sakagami *et al.*, 1992). Furthermore, lignans, possible breakdown products of lignin, are reported to have antimutagenic (Mikulasova and Kosikova, 2003) and antitumor activity (Gordaliza *et al.*, 2000; 2001).

Lignin degradation, resistant to chemical and enzymatic process, is achieved most efficiently by white-rot basidiomycetes (Kirk, 1983). Although many haem oxidases from microorganisms, plants and animals are able to oxidize phenolic substrates, only lignin peroxidase secreted from many white-rotting fungi has been reported to oxidize high redox potential aromatic compounds including the non-phenolic phenylpropanoid units of lignin (Field *et al.*, 1992). Therefore, lignin peroxidase has been considered as a key enzyme in lignin degradation (Kirk and Farrell, 1987). Over the past several years, research efforts

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have been directed both at economically producing valuable substances stream and at producing ligninolytic enzymes at a lower cost.

In the present study, we have examined whether i) *Phellinus igniarius* is a useful resource for the large-scale production of lignin peroxidase and ii) wood biomass degradation products by lignin peroxidase have cytotoxic effects on various cancer cells.

MATERIALS AND METHODS

Microorganism culture

Phellinus igniarius-26005 (ASI 26005) was obtained from Department of Applied Microbiology in National Institute of Agriculture Science and Technology (Korea). The fungus was grown in 20 ml of medium (10g Malt extract, 4g glucose and 4g Yeast extract in 1 L) for 10 days at 28°C, and then, the entire culture was homogenized. The homogenates were inoculated into the production culture medium in the ratio of 3% (v/v). The composition of culture medium was identical to the growth medium with Tween 80 (0.005% v/v) and veratryl alcohol (3,4-dimethoxybenzyl alcohol, 2 mM), being added at the beginning of the cultivation to stimulate the production of lignolytic enzyme. The culture medium containing the secreted enzymes was fractionated and concentrated by membrane filtration using amicon diaflo system (Diaflo PM 10, MW cut-off; 10,000) at 16^{th} day of culture.

Purification of lignin-degrading enzymes

To obtain partially purified lignin peroxidase, ion exchange chromatography column was used. The fractionated and concentrated culture medium containing the enzyme solutions applied to a column of DEAE-Sepharose CL-6B (Amersham Pharmacia Biotech, UK) that had been equilibrated with running buffer (Na-succinate) and eluted sequentially with same buffer plus 0, 0.1, 0.2, 0.3M NaCl at 4°C.

Enzyme characterization

Lignin peroxidase activity was analyzed spectrophotometrically by the method of Tien and Kirk (1984). One unit was defined as the amount of enzyme that oxidized 1 nM veratryl alcohol in 1 ml of reaction solution (0.8 mM veratryl alcohol, 0.25 mM H_2O_2, 100 mM Na-tartrate buffer, pH 3.0) for 1 min. Manganase peroxidase activity was analyzed spectrophotometrically according to the method of Kofujita *et al.* (1991). One unit was defined as the amount of enzyme that oxidized 1 nmol guaiacol in 1 ml of reaction solution (0.4 mM guaiacol, 0.2 mM $MnSO_4$, 0.1 mM H_2O_2, 50 mM Na-lactate buffer, pH 4.5) for 1 min. Laccase activity was analyzed spectrophotometrically by the method of Kofujita et al. (1991). One unit was defined as the amount of enzyme that oxidized 1 nmol of *o*-phenylendiamine in 1 ml of reaction solution (4 mM *o*-phenylendiamine, 50 mM Na-succinate, pH 4.5) for 1 min. The molecular mass was determined by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using marker proteins (broad range prestained protein marker, Biolabs, MA, USA).

Preparation of fine granules of wood powder

Oak tree (*Quercus mongolica*) as a wood biomass and a lignin source was obtained from university forest in Gyungbuk province and chopped into pieces of 2×2×0.2 cm. The wood pieces were soaked in the steam and exploded using steam explosion equipment at pressure of 25 kgf/cm^2 (220°C) for 6 minutes. The fine granules of steam exploded-wood powder were separated by using 0.42 mm mesh screen before it was enzymatically hydrolyzed.

Extraction of lignins

The fine granules of steam exploded-*Quercus mongolica* powder (1.5 Kg) were treated with 6 liter of 1% sodium hydroxide solution for 1 hr followed by filtration with filter paper (5C , Toyo Co.,Japan). The filtrate was adjusted to pH 5.5 with glacial acetic acid and then concentrated to the half of the original volume by using a vacuum evaporator. The concentrates were mixed with ethanol (twice the volume of the concentrates) and then filtered through the filter paper. The filtered precipitates were air-dried, resuspended into distilled water and then filtered. The whole procedure was repeated twice before the low molecular weight lignin was obtained.

Enzymatic hydrolysis

The 100 g of steam exploded-*Quercus mongolica* powder (Wp) or 125 g of lignins (Lg) extracted from steam-exploded *Quercus mongolica* was reacted with 200 ml of enzyme solution (E) possessing activity of 7,500U at 25°C for 24 hrs, respectively. The hydrolysates were fractionated by filtration (Amicon diaflo system Diaflo PM10 cut off: 10,000) as described in Fig. 1. Each fraction with molecular weight less than 10,00 was designated as EWp or ELg. Also, steam-exploded wood powder treated with the same procedure in the absence of enzyme in reaction solution was fractionated and designated as Wp. Each fraction was tested for cancer cell inhi-

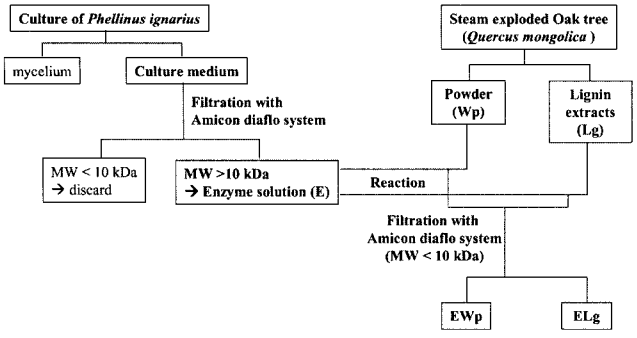

Fig. 1. Procedure for preparation of *Quercus mongolica* hydrolysates by lignin peroxidase secreted from *Phellinus igniarius*.

bition by the MTT assay.

Cell culture and Cell viability assay (MTT assay)

HepG2, MBT-2, B16, SK-N-SH cells were grown at 37°C in a humidified incubator under 5% CO_2/95% air in a MEM supplemented with 10% FBS, 200,000 IU/l penicillin, 200 mg/l of streptomycin and 1 mM sodium pyruvate. Culture medium was replaced every other day. After attaining confluence the cells were subcultured following trypsinization.

Cell viability was assessed by the MTT staining method (van de Loosdrecht *et al.*, 1991). MBT-2 and B16 cancer cells from 4- to 5-day-old cultures were seeded in 24-well plates at the density of 5×10^4 cells/well. The volume of the medium in the wells was 1 ml. In control experiments cells were grown in the same media containing drug-free vehicle. After incubation for 48 hr in the presence or absence of drugs, 100 μl of MTT (5 g MTT/l in H_2O) were added and cells incubated for a further 4 hr. Two hundred microliters of dimethyl sulfoxide (DMSO) were added to each culture and mixed by pipetting to dissolve the reduced MTT crystals. Relative cell viability was obtained by scanning with a microplate reader (Molecular Devices, Menlo Park, CA) with a 540 nm filter.

Data analysis

Data were expressed as mean±standard error of the mean (SEM) and were analyzed using Student's t test for individual comparisons. P values less than 0.05 are considered statistically significant.

RESULTS AND DISCUSSION

Lignin peroxidase production from *Phellinus igniarius*

Since *Phellinus igniarius*, a medicinal mushroom, has been known as one of white-rot fungi, we first examined which

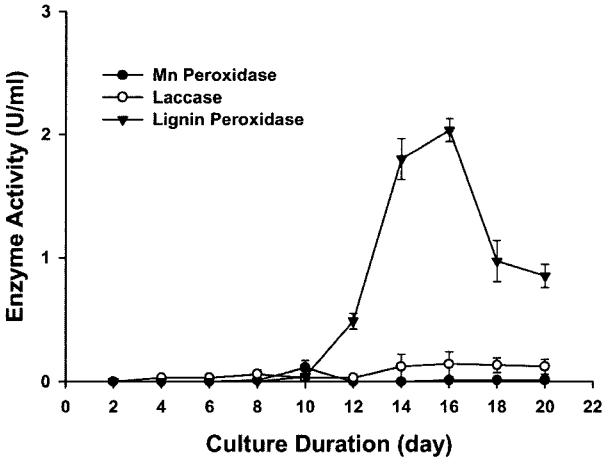

Fig. 2. *Phellinus ignarius* secretes mainly lignin peroxidase and trace amount of laccase and manganese peroxidase into culture medium. The fungus was grown in the medium (10 g Malt extract, 4 g glucose and 4g Yeast extract in 1 L) with Tween 80 (0.005% v/v) and veratryl alcohol (3,4-dimethoxybenzyl alcohol, 2 mM), being added at the beginning of the cultivation to stimulate the production of lignolytic enzyme.

lignolytic enzyme was produced by the fungus. As shown in Fig. 2, the lignin peroxidase activity was detected in culture medium of *Phellinus igniarius* on the 10th day and maximum activity at 16th day of culture. However, the manganese peroxidase and laccase activities were barely detected. Considering that manganese peroxidase and laccase, in general, are more widely distributed among white-rotting fungi than lignin peroxidase (Pelaez *et al.*, 1995), our present result suggests that *Phellinus igniarius* may be a good source for mass production of lignin peroxidase.

In order to further identify the lignin peroxidase we performed a DEAE-Sepharose CL-6B chromatography. The lignin peroxidase activity was detected in the fractions with peak absorbance at 280 nm as shown in Fig 3. In addition, the enzyme activity was detected in the fraction of culture medium separated by membrane filtration which has cut-off pore size with 10,000 molecular weight, suggesting that molecular weight of the enzymes were at least more than 10,000. In fact, the enzyme exhibited a native molecular mass of approximately 37 kDa as determined by SDS-PAGE (Fig. 4), which is very similar to the recent report that a lignin peroxidase from *Phanerochaete sordida* exhibits molecular mass of about 50 kDa (Sugiura *et al.*, 2003).

Cytotoxic activities of lignin peroxidase hydrolysates

To evaluate the usefulness of lignin peroxidase for biodegradation of lignocellulosic waste and its degradation products for

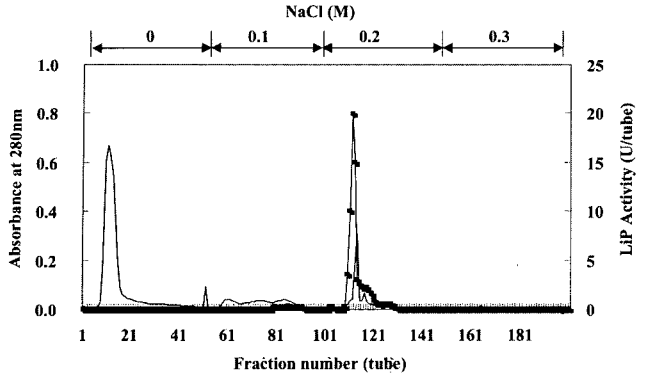

Fig. 3. DEAE-Sepharose CL-6B Chromatography of culture medium of *Phellinus ignarius*. Soild line and solid line with closed circles represent lignin peroxidase (LiP) activity and absorbance at 280 nm, respectively.

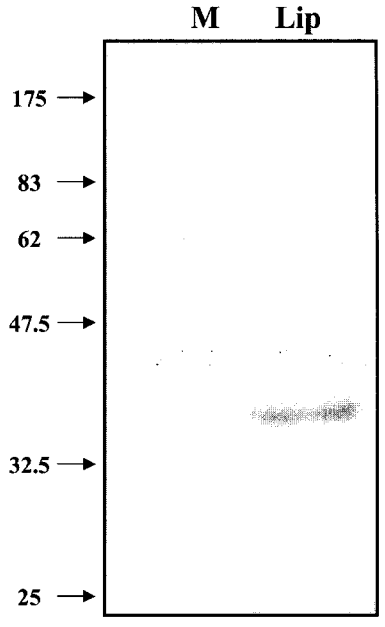

Fig. 4. SDS-PAGE of the partially purified lignin peroxidase from *Phellinus ignarius*. M and Lip represent marker proteins in kDa and partially purified and concentrated lignin peroxidase solution from *Phellinus ignarius*, respectively.

human welfare we tested the cytotoxic activities of the hydrolysates in various cancer cells. First, we examined the effects of the lignin peroxidase-treated hydrolysates of steam-exploded *Quercus mongolica* powder that have molecular weight less than 10,000 (EWp) on B16, MBT-2, HepG2 and SK-N-SH cancer cells. The EWp decreased the viability of the cancer cells in a dose-dependent manner (Fig. 5A). The effect was most obvious in B16 cells. The cytotoxic effect of Ewp on Chang cells, normal hepatic cells, was much weaker than those on MBT-2 and B16 cells.

Next, we further examined whether the cytotoxic effects of wood hydrolysates by the lignin peroxidase were due to the enzyme's full action on lignins among the lignocellulosics that contained in the wood hydrolysates. To verify this, lignins were chemicallly extracted from steam-exploded oak powder, and then, were reacted with lignin peroxidase solution. The enzyme-produced lignin hydrolysates with molecular weight less than 10,000 (ELg) significantly decreased cell viability in the cancer cells, as depicted in Fig. 5B. Even though the dose-response profile was similar, the cytotoxic effect of ELg was more potent than that of EWp. These results imply that biodegradation process by lignin peroxidase is more effective in lignin than in complex wood biomass. Furthermore, the ELg markedly reduced the viability of Chang, normal human liver cells. However, the cytotoxic effect of ELg on Chang cells was much less potent than that of ELg on HepG2 human hepatoma cells

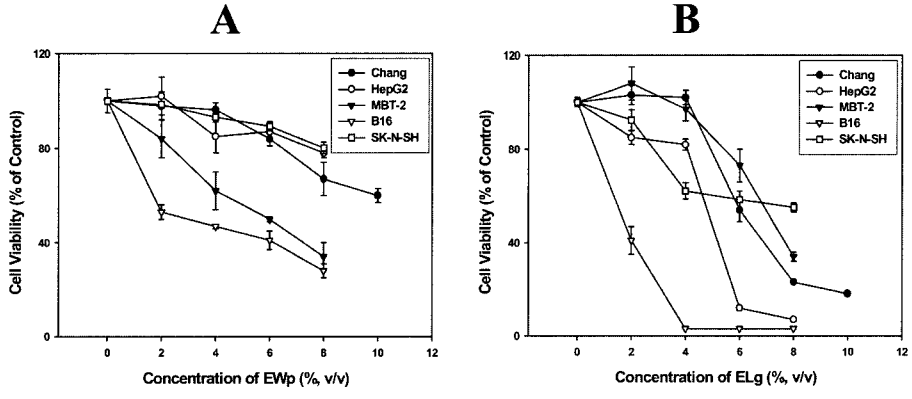

Fig. 5. Cytotoxic effects of lignin peroxidase hydrolysates on various cancer cells. Lignin peroxidase hydrolysates of steam-exploded oak powder (Ewp) (A) and lignin peroxidase hydrolysates of extracted lignin (ELg) (B) were tested. Cell viability was measured by the MTT assay.

or B16 murine melanoma cells, indicating that the effect of ELg may be specific for cancer cells. Even though we did not determine the amount and specific compounds in the degradation products, our results suggest that lignin-degradation products by the enzyme could be a valuable resource for anticancer agent development.

In conclusion, the results suggest that *Phellinus igniarius* may be a useful resource for the large-scale production of lignin peroxidase. In addition, the enzyme appeared to effectively degrade lignin present in wood biomass, resulting in the degraded lignin products that may be valuable for medical use.

ACKNOWLEDGMENTS

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