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# Polymerization and Depolymerization of Lignins by White-Rot Fungi (I)

- Degradation of Lignosulfonate by Lignin-degrading Fungi -

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# 백색부후균에 의한 리그닌의 중합화와 탈중합화 (제1보)

## - 리그닌분해균에 의한 Lignosulfonate의 분해 -

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## ABSTRACT

백색부후균에 의한 리그닌의 분해양상을 검토하기 위해 리그닌 분해능이 우수하고 laccase 활성이 높은 LKY-7 및 C. versicolor-13 균주와 manganese peroxidase 활성은 비교적 높으나 laccase활성이 전혀 나타나지 않는 LSK-27 균주로 lignosulfonate를 처리하였다. LKY-7 과 C. versicolor-13 균주에서는 lignosulfonate의 중합화 현상이 관찰되었으며, 중합화는 laccase 활성과 비례하는 것으로 나타났다. LSK-27 균주에서는 lignosulfonate의 고분자 영역이 분해되면서 탈중합화가 일어났으며 리그닌 분해 효소로는 manganese peroxidase만 검출되었다. 보조기질로 glucose를 첨가한 결과, LKY-7 균주에서는 laccase 활성이 감소하면서 중합화 현상이 어느정도 감소하였으나 C. versicolor-13 균주는 laccase 활성의 증가와 함께 중합화도 촉진되는 것으로 나타났다. 또한 LSK-27 균주에서도 glucose 첨가에 의해 manganese peroxidase 활성이 증가되면서 lignosulfonate의 중합화가 관찰 되었다. lignosulfonate 중합화에는 laccase 뿐만 아니라 manganese peroxidase도 관여하며 보조기질로서 탄소원의 종류도 영향을 미칠것으로 검토되었다.

*Keywords* : Lignin-degrading fungi, Lignosulfonate, Polymerization, Depolymerization, Cosubstrate, Laccase, Manganese peroxidase.

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## 1. INTRODUCTION

White-rot fungi are the most effective microorganisms in degrading lignin. It has been recognized that white-rot fungi and their ligninolytic enzymes have potential applications in biopulping and biobleaching, as well as in the bioremediation of aromatic pollutants. The process by which fungi degrade lignin is oxidative, probably involving enzymes such as lignin peroxidase, manganese peroxidase, and laccase (Faison *et al.*, 1985; Eriksson *et al.*, 1990). Lignin peroxidase catalyzes the one-electron oxidation of various aromatic compounds, with subsequent formation of aryl cation radicals which are decomposed spontaneously by various pathways (Hammel *et al.*, 1985). Manganese peroxidase catalyzes the oxidation of Mn(Ⅱ) to Mn(Ⅲ), which in turn can oxidize several phenolic substrates (Glen *et al.*, 1985). And laccase reduces dioxygen to two molecules of water and simultaneously performs one-electron oxidation of many aromatic substrates. The range for oxidizing reaction of substrate is fairly broad and includes polyphenols, methoxy-substituted monophenols, aromatic amines, and other easily oxidized aromatic compounds (Thurston, 1994). These phenol oxidizing enzymes secreted by lignin-degrading fungi has been known to catalysis not only the depolymerization but also polymerization of lignin by coupling of the phenoxy radicals produced from oxidation of lignin phenolic group (Haemmerli et al., 1986; Kern *et al.*, 1987). However, the factors governing this alternative capacity are still unclear. Various parameters like presence of cosubstrate, laccase activity, and $H_2O_2$ concentration, seem to be responsible for polymerization reaction (Kirk *et al.*, 1990). Especially, it has been suggested that laccase possesses both polymerization and depolymerization activities on lignin, though the former was predominent(Leonowicz *et al.*, 1985).

We have some high active lignin-degrading fungi screened from decayed wood. Of these lignin-degrading fungi, LKY-7 and *Coriolus versicolor*-13 isolates have high level of laccase activity, on the other hand LSK-27 isolate exhibited high level of manganese peroxidase activity and no laccase activity in glucose-peptone broth. The objective of this series of experiment was to determine the degradation aspect and catalytic actions of these screened fungi on various lignin sources, lignosulfonate, chloro lignin, and kraft lignin, and to investigate the possibility of application of these fungi on treatment of aromatic compounds and pulp and paper mill effluents, and on bleaching of pulp.

In the present work, we examined the pattern of lignosulfonate degradation, polymerization and depolymerization, of these three lignin-degrading fungi and the effect of extracellular ligninolytic enzymes which may play an important role in depolymerization and polymerization of lignosulfonate.

## 2. MATERIALS & METHODS

### 2.1. Microorganisms

LKY-7, *Coriolus versicolor*-13 and LSK-27 isolates having high ligninolytic acvity, which was reported previously (Jung *et al.*, 1995), were used in the present work. LKY-7 and *C. versicolor*-13 isolates have high level of laccase activity, and LSK-27 isolate unable to produce laccase has high level of manganese peroxidase activity to some extent on glucose-peptone broth. These lignin-degrading fungi were maintained on potato-dextrose agar slants.

### 2.2. Treatment of lignosulfonate

Glucose-peptone agar plates (30g of glucose, 10g of peptone, 1.4g of $KH_2PO_4$, 0.5g of $MgSO_4 \cdot 7H_2O$, 2mg of Thiamin-HCl, 20mg of $CuSO_4$, and 18g of agar per liter, pH 5) were inoculated with each fungus and incubated for 7 days at 28℃. Five disks punched from the growing edge of fungal colony were homogenized for 30 sec. with 50ml of glucose-peptone broth medium and then put in a 250ml Erlenmeyer and shaken at 150 rpm. After 7 days incubation at 28 ℃, the mycelium of lignin-degrading fungi was homogenized once again and then 10ml of the culture was taken. The precultured mycelium of lignin-degrading fungi was seperated from glucose-peptone broth medium and aseptically added to a 250ml Erlenmeyer flask containing 50ml of lignosulfonate medium. This medium contained 5g of lignosulfonate (Na salt, Aldrich CO.), as a carbon source, solublized in Czpek's Dox Broth ( 2.0g of $NaNO_3$, 1.0g of $K_2HPO_4$, 0.5g of $MgSO_4 \cdot 7H_2O$, 0.5g of KCl, and 0.01g of $FeSO_4$ per liter ) which was exclude sucrose. The lignosulfonate solubilized in Czpek's Dox Broth supplimented with and without 1% of glucose as a cosubstrate were adjusted to pH 5.0 and sterilized by autoclaving before fungal treatment. The lignosulfonate medium inoculated with each lignin-degrading fungi were then incubated at 28 ℃ for 10 days in shaking incubator (150rpm).

### 2.3. Gel permeation chromatography of lignosulfonate

After incubation for a disignated period, the mycelial pellets were seperated with a Buchner funnel equipped with Whatman NO. 2 filter paper under reduced pressure. The filtrates were used in gel permeation chromatography, determination of color units of lignosulfonate medium, and assay of extracellular ligninolytic enzyme activity. Gel permeation chromatography of lignosulfonate was performed on a sephadex G-75/liquid chromatography system(Econo System, Biorad CO.) with a eluting solvent of 0.7M NaCl (pH 7.0) at a flow rate of 0.35ml/min.

### 2.4. Determinations of color units and ligninolytic enzyme activity

Color unit of the lignosulfonate medium was measured as the decrease in $A_{465}$, and the change of absorbance unit was taken as color unit. Laccase activity was determined by oxidation 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) (Bourbonnais *et al.*, 1995). The assay mixture contained 0.5mM ABTS, 0.1 M

sodium acetate buffer (pH 5.0) and a suitable amount of enzyme. Oxidation of ABTS was monitered by determining the increase in $A_{420}$. Manganese peroxidase activity was determined by measuring the changes in $A_{465}$ with guaiacol as the substrate and subtracting the increase in absorbance caused by laccase activity (Pasczynski *et al*, 1988). The reaction mixture contained 0.1 M sodium tartrate buffer (pH 5.0), 0.1mM guaiacol, 0.1mM $H_2O_2$, and 0.1mM $MnSO_4$. The reaction was initiated by the addition of $H_2O_2$. Enzyme solution boiled for 5 minutes was used in the control.

## 3. RESULTS and DISCUSSION

### 3.1. Gel permiation chromatography of lignosulfonate

Extracellular lignin peroxidase, manganese peroxidase, and laccase produced by lignin-degrading fungi have been known to catalyze the oxidative degradation of lignin source (Eriksson *et al.*, 1990). Especially, this catalytic actions by laccase preferentially polymerize lignin by coupling of the phenoxy radicals produced from oxidation of lignin phenolic group. To evaluate the catalytic action of white-rot fungi, degradation of lignosulfonate was performed with lignin-degrading fungi, LSK-27, LKY-7, and *C. versicolor*-13, under two different nutrient condition : supplimented with and without 1% glucose as a cosubstrate. The oxidizing activity of lignin-degrading fungi on lignosulfonate were measured by determination of the molecular weight distribution changes of lignosulfonate by gel permiation chromatography. The elution profiles of lignosulfonate after 5 days of incubation with lignin-degrading fungi were illustrated Fig. 1. When the lignosulfonate was incubated with LSK-27 isolate in absence of glucose, depolymerization of the high molecular weight portion of lignosulfonate was observed, but with LKY-7 and *C. versicolor*-13 isolates apparent polymerizations were observed. Especially, with LKY-7 isolate the most striking polymerization effect was exhibited, which the low molecular weight portion of lignosulfonate shifted towards high molecular weight. When 1 % of glucose as a cosubstrate was added, the polymerization

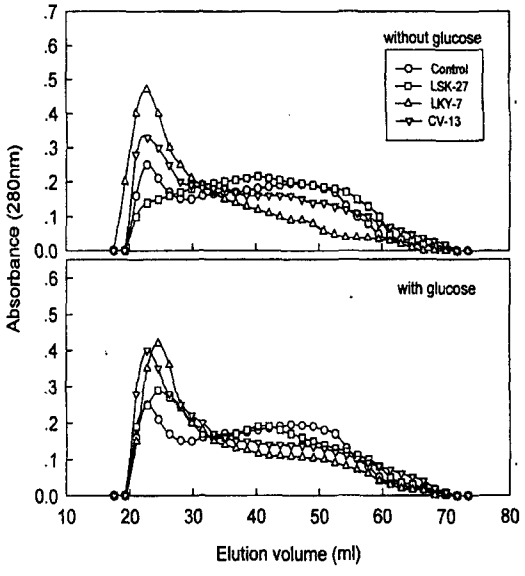


**Fig. 1. Gel permeation chromatography of lignosulfonate (5g/1) by lignin-degrading fungi after 5 days of treatment with and without cosubstrate.**

was observed with LSK-27 isolate, and with *C. versicolor*-13 isolate the polymerization was a little accelerated as compared with that of without glucose. On the other hand, in the case of LKY-7 isolate the polymerization of lignosulfonate was weaken to some extent by addition of glucose. It was showed that these screened lignin-degrading fungi might polymerize lignosulfonate according to culture condition.

### 3.2. Determinations of color units

In cases polymerization and depolymerization of lignin take place, it is accompanied by the color changes in the culture medium (Kirk *et al.*, 1990). This color changes were detected by monitering absorbance unit at $A_{465}$. The changes of color units of lignosulfonate medium supplimented with and without glucose by lignin-degrading fungi treatment were shown in Fig. 2. The color unit of lignosulfonate medium in absence of glucose incubated with LSK-27 isolate decreased distinctively in the early stage of incubation, and then persisted approximately constant with incubation time. That is, it was appeared that LSK-27 isolate achieved decoloration of 50% of lignosulfonate medium without cosubstrate at early stage of incubation. But with LKY-7 and *C. versicolor*-13 isolates, it was showed that the color units increased abruptly for 3 and 5 day incubation. In general, to obtain the high degree of degradation of various lignins and aromatic compounds by lignin-degrading fungi, a suitable cosubstrate such as glucose was required (Kirk *et al.*, 1990). And if added cosubstrate would not be perfectly consumed during the degradation stage, it may be harmful to environment, since the released nutrition increase the BOD and COD of the effluents. On this point, although various factors affected in lignin degradation are

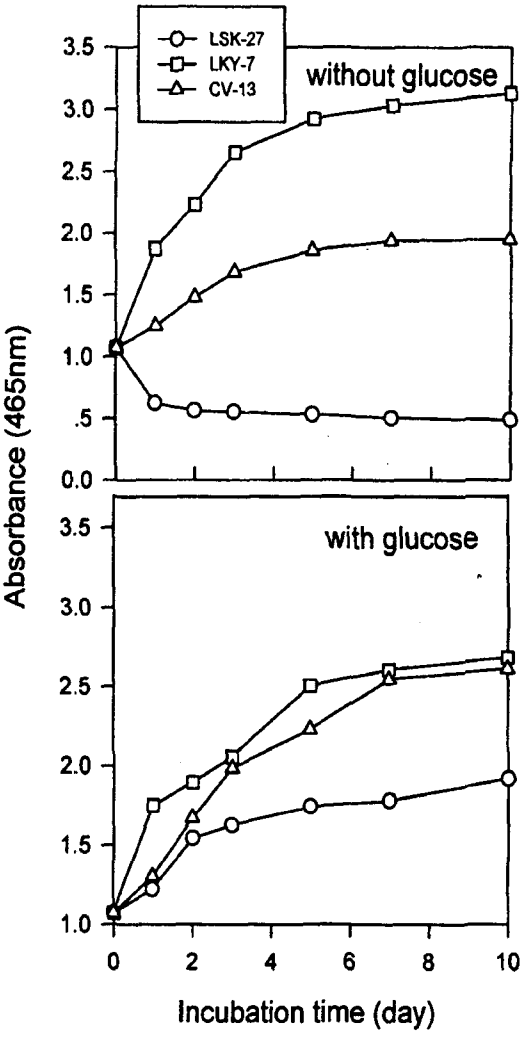


Fig. 2. Color units of lignosulfonate substrate by lignin-degrading fungi treatment with and without cosubstrate(initial absorbance:1.0723).

not investigated yet, LSK-27 isolate was thought to be effective degrader of lignin, and have possible biotechnological application. However, when 1% of glucose as a cosubstrate was added, with LSK-27 isolate the color unit increased according to incubation time, and in the case of C. versicolor-13 isolate the addition of glucose enhanced the increase of color unit. In contrast, with LKY-7 the color units exhibited to be repressed a little by addition of glucose as compared with that of lignosulfonate medium without glucose. These changes of color unit proceeded in a way similar to results of gel permeation chromatography as can be seen in Fig. 1.

### 3 . 3 Extracellular ligninolytic enzyme activity

To investigate the effect of ligninolytic enzyme of these screened isolates on lignosulfonate degradation, extracellular ligninolytic enzyme activities were evaluated periodically. As shown in Fig. 3, LKY-7 and *C. versicolor*-13 isolates secreted mainly laccase and LSK-27 isolate secreted only manganese peroxidase in lignosulfonate medium without glucose. LKY-7 isolate exhibited higher level of laccase activity than *C. versicolor*-13 isolate, which reached a maximum at 5 day incubation and then declined to relatively low levels. In contrast, laccase of *C. versicolor*-13 isolate showed low level activity for 1 to 5 day incubation as compared with LKY-7 isolate, and reached a maximum activity at 7 day and then declined with the incubation time. The levels of manganese peroxidase activity secreted LKY-7 and C. versicolor-13 were appeared to be very low level in the entire incubation time, only detected as a trace after 5 to 7 days incubation. With LSK-27 isolate only the manganese peroxidase activity were secreted at early stage of incubation, which regulated the degradation of lignosulfonate although manganese peroxidase activity overall

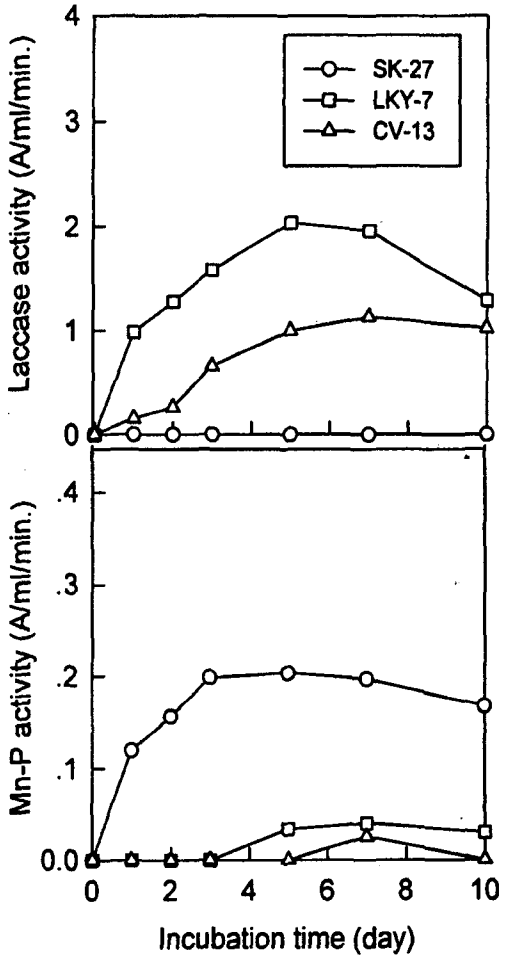


Fig. 3. Extracellular ligninolytic enzyme activities during treatment of lignosulfonate by lignin degrading fungi without glucose.

showed low level. The addition of glucose as a cosubstrate increased the laccase activity of *C. versicolor*-13 and the manganese peroxidase activity of LSK-27 to some extent but decreased the laccase activity of LKY-7 isolate, as shown in Fig. 4. And it had approximately no effect on the manganese peroxidase activity of LKY-7 and *C. versicolor*. Consequently, in the lignosulfonate treatment with and without glucose, manganese peroxidase of LKY-7 and *C. versicolor* isolates was hardly detected during the entire incubation time; that is to say, it were less than 0.1 unit of absorbance. Lignin peroxidase, one of the ligninolytic enzymes, was never detected in entire degradation system of lignosulfonate with these lignin-degrading fungi. In general, polymerization products are observed when laccase oxidizes phenolic substrates. For example, milled wood lignin and soluble lignosulfonate are both polymerizied to some extent by laccase (Leonowicz *et al.*, 1985). However, LSK-27 isolate seemed to have various degradation aspect of lignosufonate or the other lignin sources according to cosubstrates since manganese peroxidase of this isolate exhibited to polymerize the lignosulfonate with glucose. It is not known in this experiments whether the direct cause of polymerization is the isozyme of manganese peroxidase or glucose as a cosubstrate. In the light of this point, the further study should be continued.

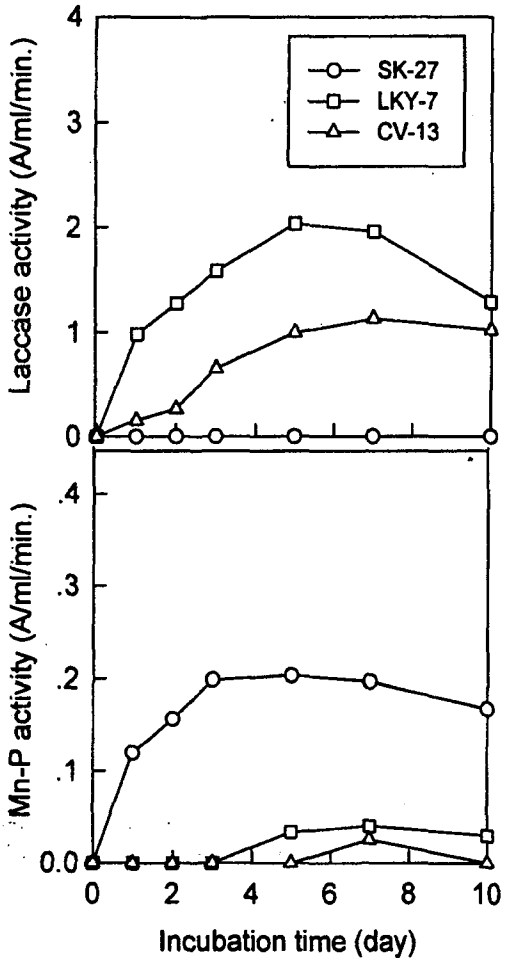


Fig. 4. Extracellular ligninolytic enzyme activities during treatment of lignosulfonate by lignin-degrading fungi with glucose.

## 4. CONCLUSION

To investigate the catalytic action and the characteristics of lignin-degrading fungi in lignin degradation, lignosulfonate solubilized in Czpek's Dox Broth was treated with LKY-7, *C. versicolor*-13, and LSK-27 isolates. LKY-7 and C. versicolor-13 isolates have high level of laccase activity, on the other hand LSK-27 isolate exhibited high level of manganese peroxidase activity and no laccase activity

in glucose-peptone broth.

Treatment with LKY-7 and *C. versicolor*-13 isolates exhibited polymerization of lignosulfonate, which correlated with laccase activity. With LSK-27 isolate depolymerization of lignosulfonate was observed to some extent, and the high molecular weight portion decreased a little. Only manganese peroxidase activity was detected in lignosulfonate medium incubated with LSK-27 isolate. When 1% of glucose as a cosubstrate was added, the polymerization was observed with LSK-27 isolate as manganese peroxidase activity increase, and with *C. versicolor*-13 isolate the polymerization was a little accelerated as compared with that of without glucose. On the other hand, in the case of LKY-7 isolate the polymerization of lignosulfonate and extracellular laccase activity were weaken to some extent by addition of glucose. It was thought that the polymerization of lignosufonate, with these lignin-degrading fungi, might be influenced by not only laccase and manganese peroxidase but also carbon sources as a cosubstrate.

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