Degradation Characteristics of Ligninsulfonate by Laccase and Mn-peroxidase[*1]

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Laccase와 Mn-peroxidase 에 의한 Ligninsulfonate의 분해 특성[*1]

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要 約

리그닌 모델화합물은 리그닌 분해효소에 의해 생분해되는 과정에서 해중합 또는 중합되는 현상이 나타남으로써, 리그닌분해효소가 진정으로 리그닌의 분해에 관여하는지 의문이 제기되고 있다. 본 연구는 구름 버섯과 표고버섯으로부터 단리한 laccase와 Mn-peroxidase(MnP)가 고분자 리그닌모델화합물로서의 ligninsulfonates(LS)을 분해시키는 특성을 이해하고자 시도되었다. 특히 pH의 변화와 糖의 첨가에 따른 LS의 분자량의 변화를 관찰하였다. laccase의 경우 pH 6.0에서는 LS의 중합현상이 나타났으나 pH 4.5에서는 고분자분획의 LS가 현저히 분해됐다. glucose의 첨가시 이같은 현상은 두드러졌다. MnP 역시 pH 4.5에서 LS가 분해되었으며, 특히 배양액에 cellobiose를 첨가했을 때 현저하게 나타났다. 이상의 결과로부터, 리그닌 분해효소에 의한 LS의 분해는 효소의 종류와 반응조건에 따라 달라짐을 확인할 수 있었다. 흥미롭게도 LS의 분해에 따른 반응액의 色度(color index)의 변화는 관찰되지 않았다.

ABSTRACT

To understand whether ligninolytic enzyme catalyze polymerization or depolymerization of the high molecular weight (HMW) lignin, the action of laccase and Mn-peroxidase (MnP) towards commercial ligninsulfonates (LS) was examined in various conditions of pH and cosubstrates. Polymerization occurred when LS was incubated with laccase at pH 6.0. In contrast, the high molecular weight portions were significantly reduced at pH 4.5, especially when glucose was added. When LS was treated with MnP at pH 4.5, compounds of low molecular weight were produced.

In particular, when cellobiose was added to Mn-P reaction mixture, the breakdown of LS was observed. In conclusion, degradation of LS by laccase and MnP occurred primarily at pH 4.5 where-

as polymerization of LS was dominant at pH 6.0. Color index, however, was not greatly changed in the degradation mixtures of LS.

1. INTRODUCTION

Ligninolytic enzymes are believed to take part in the transformation of lignin and are currently being examined for the degradation of high molecular weight(HMW) lignins such as lignin sulfonates(LS), kraft lignin and other lignin derivatives. When compared to those of low molecular model compounds, degradation mechanism of macromolecular lignins are not fully understood. It has long been known that laccase and lignin peroxidase(LiP) catalyze the polymerization of lignin model compounds rather than gradation of lignin(Hammerl; *et al*, 1986; Hüttermann *et al*, 1977; Leonowicz *et al*, 1985). On the other hand, the depolymerization of lignin by laccase and other ligninolytic enzymes are also well documented(Bergbaur *et al*, 1990; Clayton & Srinivasan, 1981; Haider & Trojanowski, 1981; Ritter *et al*, 1990).

Following work was undertaken to understand whether ligninolytic enzymes caused the HMW lignin to polymerize or depolymerize under the various conditions of pH and cosubstrates. We examined the activity of laccase and Mn-dependent peroxidase(MnP) towards commercial ligninsulfonates(LS) as HMW lignin model compounds.

2. MATERIALS & METHODS

Commercial sodium LS(Tokyo Chemical Co.) was used in this work without further purification. Laccase(mol. wt. 62,000 dalton) and MnP (mol. wt. 45,000 dalton) fractions were harvested from *Trametes versicolor* and *Lentinus edodes* respectively described elsewhere(Bae *et al*, 1993). Briefly extracellular enzymes isolat-

ed from the liquid culture media grown for 8 weeks in stationary culture conditions were precipitated with ammonium sulfate and concentrated with an ultrafiltration, subsequently fractioned by ion-exchange chromatography by using DEAE Sephadex A-25 and gel-filtration using Sephadex G-75. SDS-PAGE showed only one band in each enzyme fraction.

Molecular weight distribution of LS was determined using gel filtration on Sephadex LH-60column(1.6×60cm). Three ml of LS (5mg/ml) treated with laccase and MnP for 7 days at 26 ℃ in pH 4.5 and 6.0 was placed on the column previously equilbrated with 0.1M phosphate buffer. The flow rate was adjusted to 2ml/min using 0.1M phosphate buffer as eluant and 4ml fractions collected were measured at 280nm. One percent of glucose and cellobiose as cosubstrate was added to LS reaction mixture to examine the effect of carbohydrates on the degradation of LS. Molecular weights were estimated with standard markers. Decolorization of LS by laccase and MnP was also determined by the change of color unit by absorbance at 460 nm.

3. RESULTS & DISCUSSION

3.1 Degradation pattern of LS by laccase

The fractionation of untreated LS on Sephadex LH-60 column shows a bimodal molecular weight distribution; HMW and low molecular weight(LMW) fractions(Fig.1). The general pattern of molecular mass distribution in the present work was in agreement with the results from various LS, describing that the molecular weight of the LS showed two fractions in high-
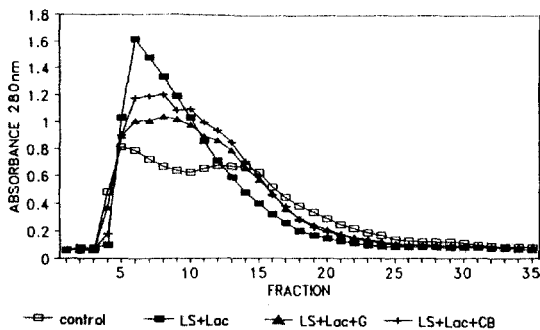
Fig. 1. Polymerization of ligninsulfonates(LS)-(5g/l, w/v) by laccase after 7 days of incubation at pH 6.0. Column material: Sephadex LH-60 eluted with 0.1M phosphate buffer (pH 6.0).
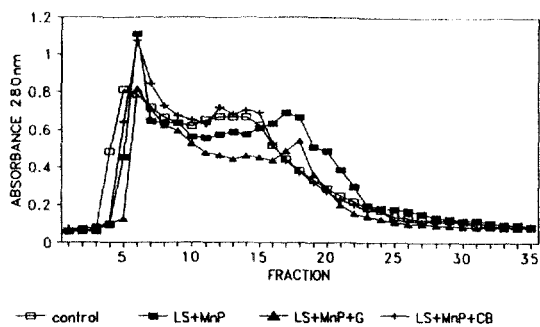Notes: G : glucose, CB : cellobiose, Lac : laccase

Fig. 3. Gel permeation chromatography of LS after incubation with MnP isolated from *Lentinus edodes* at pH 6.0. Column material was same as in Fig. 1.
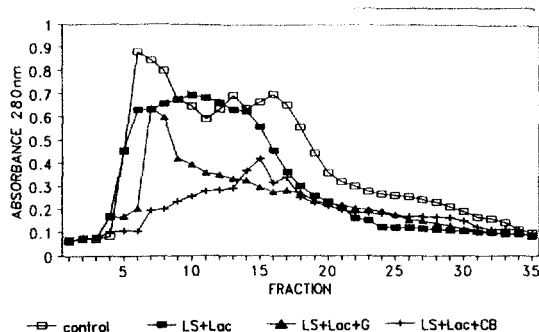Notes: G : glucose, CB : cellobiose Lac : laccase

Fig. 2. Elution pattern of LS treated with laccase at pH 4.5. Fractionation on Sephadex LH-60 was same as in Fig. 1.
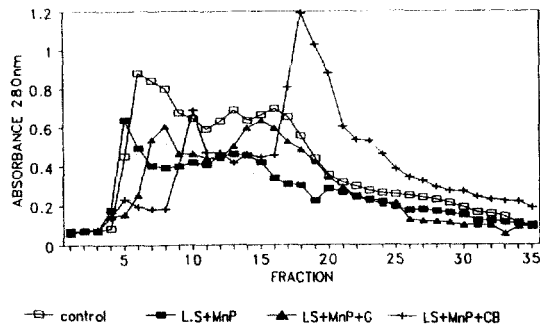Notes: G : glucose, CB : cellobiose, Lac : laccase

Fig. 4. Transformation of LS by MnP incubated at pH 4.5. Column conditions were same as in Fig. 1.
Notes: G : glucose, CB : cellobiose, Lac : laccase

(M.W.:ca.6,000) and low-molecular weight (M.W.:ca.2,000) region(Hüttrmann, 1977).

Gel permeation chromatography(GPC) of LS after incubation with laccase for 7 days at pH 6.0 showed only one pronounced peak in the high molecular region (M.W. : ca 7,600) of GPC with the disappearance of low molecular fractions (Fig. 1). Formation of HMW fraction can be explained by the polymerization of LS, resulting from the partial conversion of LMW fraction of LS into higher products. When glucose or cellobiose was added to laccase, LS also showed a significantly higher proportion of HMW lignin than the control. Fig. 1 clearly

indicates that polymerization of LS was not hindered by the addition of carbohydrate as cosubstrates in the reaction mixture at pH 6.0.

In contrast, LS incubated with laccase showed a different pattern of molecular mass distribution at pH 4.5. The intermediate molecular mass(fraction 8-15) appeared, suggesting that LS underwent a slight degradation. In particular, when laccase and glucose were incubated at pH 4.5, significant reduction in the molecular mass distribution of LS was evident in the high molecular mass range. When cellobiose was added at the same pH, an appreciable breakdown of the low molecular mass in LS was also

noticed (Fig. 2).

3.2 Degradation pattern of LS by MnP.

After 7 days of incubation with MnP at pH 6.0, the profile of molecular mass distribution of LS incubated with MnP at pH 6.0 maintained the bimodal pattern as shown in the control. It is interesting to note that all the profiles of molecular mass distribution of LS treated with MnP appeared bimodal patterns (Fig. 3, 4), while those treated with laccase showed the monodisperse ones (Fig. 1, 2), suggesting the different degradation modes of LS depending upon the enzymes.

The LS treated with MnP at pH 6.0 did not show any significant degradation in comparision with the control. When cellobiose was added at pH 6.0, the profile of LS was nearly same as the control except a little increased peak in the high molecular region. In contrast, LS incubated with MnP at pH 4.5 showed the alterations in the molecular mass distribution (Fig. 4).

An appreciable reduction in molecular mass was revealed when LS was treated only with MnP at pH 4.5, without any addition of sugars in reaction mixtures. When cellobiose was added in the reaction mixture, the displacement of the second peak towards the lower molecular region was noticed. However, only slight reduction of molecular mass of LS appeared when MnP was incubated with glucose at pH 4.5. Although there was an indication of LS-degradation by MnP, the extent of the degradation of LS by MnP at pH 4.5 was not so significantly enhanced as those appearing in laccase+glucose at the same condition of pH.

3.3 Change of color units in LS reaction mixtures

The change of color units in the LS reaction mixture after incubation with ligninolytic enzymes was examined. Table 1 shows the increase of color index in almost all the LS culture media after incubation with laccase. In con-

Table. 1. Color change in LS after incubation with Laccase and MnP.

(Unit : Absorbance at 460nm)

Reaction mixture \ pH	pH 6.0	pH 4.5
LS	0.3438	0.3438
LS+Lac	0.5972	0.6293
LS+Lac+glucose	0.6102	N.D.
LS+Lac+cellobiose	0.6214	N.D.
LS+MnP	0.3759	0.3272
LS+MnP+ glucose	0.3039	0.3173
LS+MnP+cellobiose	0.3632	0.3556

Notes : Lac : laccase,
  N.D. : not determined due to high absor-bance values.

trast, the change of color index in the reaction mixtures treated with MnP was not significant when compared to the laccase-cultured media. The slight decrease of coloring was found in the culture medium incubated with MnP and glucose.

4. CONCLUSION

LS as a by-product in the acidic sulfite process are water soluble, but resistant to microbial degradation. Hence, these lignin derivatives are a serious threat to the aquatic environment (Gahin & Krause, 1977). Biological treatment of the waste products as an alternative is of interest in these days. White-rot fungi are the most promising organisms for the degradation of HMW lignin such as LS, kraft lignin etc. However, the arguments on the degradation of lignins by ligninolytic enzymes from white-rot fungi remains unresolved. Some workers reported the polymerization of substrates, others depolymerization (Clayton & Srinivsan, 1981: Hiroi *et al*, 1976: Ishihara, 1976: Kern, 1983: Kirk *et al*, 1978: Lee *et al*, 1993: Leonowicz, 1985: Ritter *et al*, 1990: Szklarz & Leonowicz, 1986).

Ishihara(1976, 1983) found that degradation of syringic acid by laccase was pH-dependent. He observed that polymerization in pH 6.0 was

dominant whereas depolymerization at pH 4.5. Our work was comparable with that of Ishihara in that the double function of laccase was also observed in the degradation process of HMW lignin compounds. At pH 4.5, depolymerization of LS by laccase was dominant over polymerization. In contrast, polymerization was favored at pH 6.0. Similar results were also appeared in MnP.

Kirk *et al* (1978) have shown that the pH of the culture-medium is critical for lignin degradation, with an optimum at pH 4.5. Ishihara(1983a,b) observed that polymerization occurred at non-optimum pH of the laccase in which radical formation was slow. Conversely when the incubation occurred at the optimal pH of laccase where radical concentration was high, the coupling reaction between the radicals and the hydroxyl radical (· OH) occured rapidly, resulting in the depolymerization of lignin model compounds. Similar mechanism would be expected in the degradation process of LS by MnP.

Our work also showed that LS could be degraded in the absence of lignin peroxidase(LiP). Archibald(1992) reported recently that LiP activity was not obligatory for lignin depolymerization *in vivo*.

Carbohydrates are known to be obligate for the co-oxidation of lignin and have a great influence upon the transformation of LS. Sklarze and Leonowicz(1986) reported that *in vitro* lignin depolymerization by the laccase was increased in the presence of glucose/glucose oxidase system since the phenoxy redicals formed by laccase were reduced by the glucose oxidase and their repolymerization thus avoided. Hiroi *et al* (1976) suggested that LS was predominantly polymerized with glucose while depolymerization was favored with cellulose. In contrast, Kern(1983a,b) found that depolymerization of LS was not necessarily connected with carbohydrate additives. Our work showed that depolymerization of LS was different depending upon carbohydrate sources: glucose was much effective for the depolymerization of LS when used with laccase, while cellobiose was effective with MnP. Further studies are needed to determine the effect of various kinds of carbohydrates on the degradation of lignin polymer.

Present work showed that almost all the color units in reaction mixtures increased. Simultaneous reduction of color units was not observed even in the degraded reaction mixtures. Lee *et al* (1993) observed that a small amount of glucose (0.5%) contributed to the decolorization of bleaching effluent by white rot fungus IZU-154. The effect of carbohydrates as cosubstrate on the reduction of coloring in the LS was, however, not found in the present study. Our work suggests that the increase of color index was not merely due to the polymerization of LS. The enhancement of color units would be ascribed to the formation of chromophoric compounds during the breakdown reaction of LS.

Our experiment showed that various parameters seem to be responsible for the degradation of lignin polymers such as fungal strains, kinds of enzymes(Morohoshi, 1991), substrates, cosubstrates and culture conditions(Bergbauer *et al*, 1990; Ishihara, 1983; Kirk *et al*, 1978). Present work suggests that the environment of ligninolytic enzymes also played the important role in the degradation of HMW lignin and that the depolymerization of LS was greatly enhanced by partially controlling the environment of lignin-degrading enzymes.

ACKNOWLEDGEMENT

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