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Extracellular Reducing Enzyme Produced during Biobleaching of Hardwood Kraft Pulp by White-Rot Fungi*1

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白色腐朽菌を用いたバイオブリーチング時に産生される菌体外還元系酵素*1

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Phanerochaete chrysosporium 及びカワラタケを用いて低窒素-高炭素（LN-HC）及び高窒素-高炭素（HN-HC）条件下で広葉樹未晒クラフトパルプ（UKP）の固体培養を行い，バイオブリーチングにおける還元系酵素の役割を検討した。*P. chrysosporium* を用いたバイオブリーチングにおいて，MnP と還元系酵素の産生時期が異なっており，両者が相補的に作用してリグニン生分解を行っている可能性が考えられた。しかしながら，*P. chrysosporium* 及びカワラタケを用いた処理において白色度上昇と累積 MnP 活性との関係は，LN-HC 及び HN-HC の両培養条件下で同一直線を示し相関が認められたのに対し，還元系酵素の場合にはこのような相関は認められず，累積の還元系酵素活性と UKP 中のセルロース減少との間に相関があった。以上の結果から，還元系酵素は UKP 中の残留リグニンの分解には関与しておらず，セルロース分解に関与していると推察された。

The role of reducing enzyme in the biobleaching of hardwood unbleached kraft pulp (UKP) by *Phanerochaete chrysosporium* Burds. and *Trametes versicolor* (L.: Fr.) Pilât in the solid-state fermentation system with low-nitrogen and high-carbon (LN-HC) and high-nitrogen and high-carbon (HN-HC) culture media was investigated. The profiles of manganese peroxidase (MnP) and reducing enzyme productions during the biobleaching using *P. chrysosporium* were very different from each other, suggesting the complementary function of both enzymes for the lignin degradation. Although a positive relationship between cumulative MnP activity and brightness increase was observed in the treatment with *P. chrysosporium* and *T. versicolor* under both LN-HC and HN-HC conditions, no positive correlation was observed in the case of the cumulative reducing enzyme activity. On the other hand, a positive correlation between cumulative reducing enzyme activity and the degradation of cellulose in UKP was observed. These results suggest that reducing enzyme may not be involved in the degradation of residual lignin in UKP and is related to the degradation of cellulose.

Keywords: white-rot fungi, biobleaching, reducing enzyme, lignin degradation, cellulose degradation.

1. INTRODUCTION

The white-rot fungi, including *Phanerochaete chrysosporium* Burds. and *Trametes versicolor* (L.: Fr.) Pilât, are known as the most effective lignin-

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degrading microorganisms.[1-3] It has been suggested that laccase, manganese peroxidase (MnP), and lignin peroxidase (LiP) produced by the white-rot fungi are involved in the oxidative breakdown of lignin. Hammel and Moen reported that crude LiP preparations catalyze at least the partial depolymerization of synthetic syringyl/guaiacyl lignin.[4] Wariishi *et al.* showed that MnP catalyzes the partial depolymerization of four different synthetic lignin preparations.[5] However, *in vitro*, the synthetic lignin is polymerized by LiP and MnP rather than it is depolymerized.[5,6] On the other hand, polymerization of lignin is not prominent *in vivo*,[7-9] indicating that the white-rot fungi may have an ability that prevents polymerization of lignin and phenolic products by these oxidizing enzymes. Kirk and Farrell proposed that phenols are oxidized rapidly past the phenoxy radical step or that the radicals are reduced back to the phenols by the enzyme that prevents polymerization.[10] Westermark and Eriksson suggested that phenoxy radicals might be reduced back to phenols by the enzyme cellobiose: quinone oxidoreductase (CBQase)[11] which catalyzes the oxidation of cellobiose with simultaneous reduction of a quinone.[12] Ander *et al.* reported that the polymerization of kraft lignin by LiP is decreased in the presence of CBQase.[13] However, Kirk and Farrell have reported that CBQase does not prevent polymerization of phenols by LiP or by horseradish peroxidase.[10]

In a previous paper,[14] we reported the investigation of the role of oxidizing enzymes, MnP, LiP, and laccase, in biobleaching, and showed that MnP is the most important enzyme in brightening and delignification of hardwood unbleached kraft pulp (UKP) by *P. chrysosporium* and *T. versicolor*. In this paper, to clarify the role of reducing enzymes, we examine the relationship between the brightening of UKP and the cumulative activity of reducing enzyme produced by *P. chrysosporium* and *T. versicolor* in the solid-state fermentation system with two different culture media.

2. MATERIALS AND METHODS

2.1 Microorganisms and biobleaching of kraft pulp

P. chrysosporium ME-446 and *T.* (*Coriolus*) *versicolor* IFO-30340 were used in this study. Biobleaching of hardwood UKP (brightness, 29%; kappa no., 15.5) in the solid-state fermentation system with low nitrogen-high carbon (LN-HC) and high nitrogen-high carbon (HN-HC) culture media was performed as described in a previous paper.[14]

2.2 Enzyme assays

In the assays of MnP and reducing enzyme activities, 0.1 g (as bone dry weight) of the fungus-treated pulp was added to 50 ml of the reaction mixtures containing substrates, the mixtures were homogenized by a high-speed mixer (HM-5SA, NRK, Japan) for 30 sec at 10,000 rpm, and the enzyme activities were determined as follows: MnP activity was determined by the method described previously.[14] Reducing enzyme activity was measured at 30°C by monitoring the reduction of 2,6-dichlorophenol-indophenol (sodium salt) at 600 nm.[15] The reaction mixture contained 50 μM 2,6-dichlorophenol-indophenol (sodium salt), 100 μM cellobiose in 20 mM phosphate buffer (pH 6.0). One unit of activity is defined as the amount of enzyme that changes the absorbance by 0.1 per min, and enzyme activity was expressed in units per gram of treated pulp. Data are means of triplicate analyses.

2.3 Pulp properties and determination of cellulose content in the UKP

After incubation with fungi, pulp samples were washed with water, and pulp sheets were prepared with a Buchner funnel (diameter, 11 mm) and then air dried. Brightness was determined with a colorimeter (model CR-300; Minolta, Tokyo, Japan). The values determined with the colorimeter were multiplied by a coefficient to adjust them to ISO brightness values. The kappa number is defined as the amount (in milliliters) of a 0.1 N $KMnO_4$ solution consumed by 1 g of moisture-free pulp under standard conditions (Standard T 236 of the Technical Association of the Pulp and Paper Industry, Atlanta, Ga.).

Cellulose content was obtained by the following equation: cellulose=dry weight of UKP$\times$(1$-$kappa number$\times$0.15/100).

3. RESULTS AND DISCUSSION

The hardwood UKP was inoculated with *P. chrysosporium* and *T. versicolor* in the solid-state fermentation system with LN-HC and HN-HC culture media and incubated for six days. After incubation, the manganese peroxidase (MnP) activity, the reduc-

ing enzyme activity, and the pulp brightness were determined. In our reducing enzyme assay system, 2, 6-dichlorophenol-indophenol and cellobiose were used as the substrates which have been reported for the assay of cellobiose : quinone oxidoreductase (CBQase) and/or cellobiose oxidase (CBO) activity.[15]

Figure 1 shows the changes in MnP activity, reducing enzyme activity, and brightness observed during the treatment with *P. chrysosporium*. The profiles of MnP and reducing enzyme productions during the biobleaching were very different from each other. As we showed previously MnP is involved in the brightening of UKP,[14] the different profiles of both enzymes suggested that the phenoxy radicals produced by MnP might be reduced back to the phenols by the reducing enzyme as proposed by Kirk and Farrell,[10] and reducing enzymes may interact with oxidizing enzyme(s) such as MnP in the lignin biodegradation.

Figure 2 shows the time courses of the brightness increase of UKP and cumulative reducing enzyme activity during treatment with *P. chrysosporium* under LN-HC and HN-HC culture conditions. Although the brightness increase under an LN-HC condition was greater than that under an HN-HC condition, the activity of reducing enzyme was much more under the latter. In *P. chrysosporium*, lignin is degraded only during secondary metabolism,[16,17] which is triggered by a limitation of an essential nutrient such as nitrogen. This was consistent with the result that a greater brightness increase was obtained with an LN-HC condition. On the other hand, the reducing enzyme was produced extensively under an HN-HC condition in which sufficient nitrogen was supplied and showed a smaller brightness increase.

Previously,[14] a linear relationship was observed between the brightness increase and the cumulative activity of MnP produced by *P. chrysosporium* and *T. versicolor* in the solid-state fermentation system with different culture media, and the similar relationship was obtained in this study (Fig. 3A). However, any positive correlation was not observed in the case of cumulative reducing enzyme activity (Fig. 3B). These results indicate that reducing enzyme may not be involved in the brightening of UKP.

As mentioned above, the reducing enzyme activity was assayed with 2,6-dichlorophenol-indophenol and cellobiose; therefore, this activity might be due to CBQase and/or CBO. Renganathan *et al.* reported that CBQase and CBO bind strongly to microcrystal-

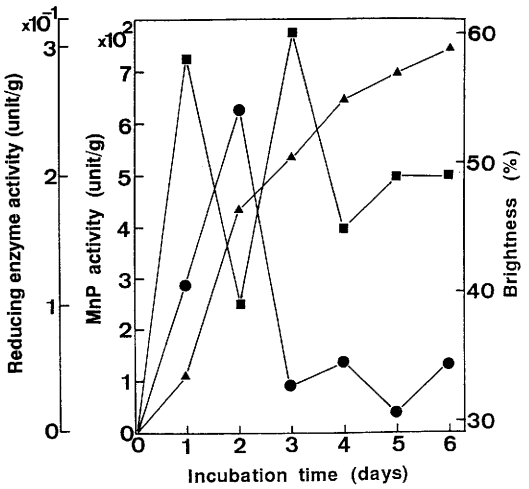

Fig. 1. Changes in the MnP activity, reducing enzyme activity, and brightness of UKP during treatment with *P. chrysosporium* in the solid-state fermentation system with LN-HC culture medium.
Legend: ●: MnP; ■: reducing enzyme; ▲: brightness.

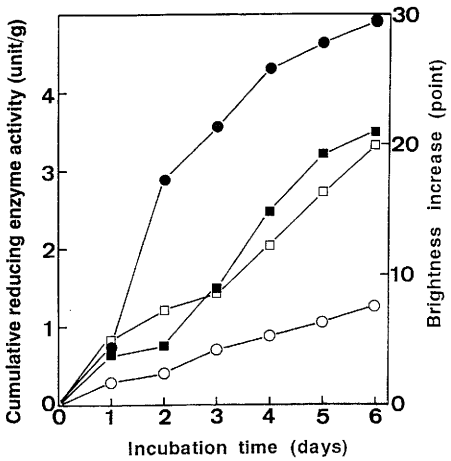

Fig. 2. Time courses of the brightness increase of UKP and cumulative reducing enzyme activity during treatment with *P. chrysosporium* in the solid-state fermentation system with LN-HC and HN-HC culture media.
Legend: ●: brightness, LN-HC; ■: brightness, HN-HC; ○: reducing enzyme, LN-HC; □: reducing enzyme, HN-HC.

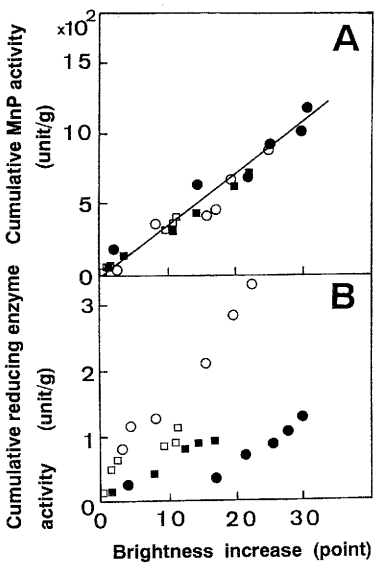

Fig. 3. Relationship between the cumulative enzyme activity and brightness increase in the solid-state fermentation system.
Legend: ●: *P. chrysosporium*, LN-HC; ○: *P. chrysosporium*, HN-HC; ■: *T. versicolor*, LN-HC; □: *T. versicolor*, HN-HC. (A) Cumulative MnP activity. (B) Cumulative reducing enzyme activity.

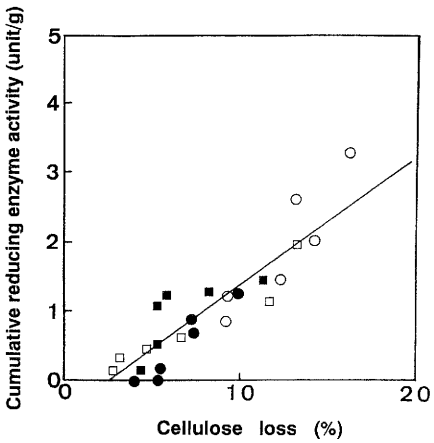

Fig. 4. Relationship between the cumulative reducing enzyme activity and cellulose loss in the solid-state fermentation system.
Legend: ●: *P. chrysosporium*, LN-HC; ○: *P. chrysosporium*, HN-HC; ■: *T. versicolor*, LN-HC; □: *T. versicolor*, HN-HC.

line cellulose, and that these enzymes may be involved in the cellulose degradation process.[15] Bao *et al.* showed that CBO enhances crystalline cellulose degradation by cellulase.[18] Therefore, the relationships between the cellulose degradation and cumulative reducing enzyme activities during treatment with *P. chrysosporium* and with *T. versicolor* in the solid-state fermentation system with LN-HC and HN-HC culture media were examined. A linear relationship was observed between the cellulose loss and cumulative activity of reducing enzyme produced by the two fungi (Fig. 4), indicating that the reducing enzyme may not be involved in the lignin degradation but in the cellulose degradation of UKP. This was coincident with the result that the fungal reducing ability which was detected by the colorization of tetrazorium salts is appeared in the primary metabolism and does not correlate directly with the degradation of lignin in secondary metabolism.[19]

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