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Bleaching of Kraft Pulp with Lignin - Degrading Enzymes

Koich HARAZONO, Ryuichrto KONDO, AND Kokki SAKAI

Department of Forest Products, Faculty of Agriculture,
Kyushu University, Fukuoka 812-81, Japan

Abstract.

An unbleached hardwood kraft pulp was bleached in vitro with partially purified manganese peroxidase (MnP) from the fungus *Phanerochaete sordida* YK-624 without the addition of $MnSO_4$ in the presence of oxalate, malonate or gluconate known as manganese chelator. When the pulp was treated without the addition of $MnSO_4$, the pulp brightness increased by about 10 points in the presence of 2 mM oxalate, but the brightness did not significantly inerease in the presence of 50 mM malonate. Residual MnP activity decreased faster during the bleaching with MnP without $MnSO_4$ in the presence of malonate than in the presence of oxalate. Oxalate reduced MnO_2 which already existed in the pulp or was produced from Mn^{2+} by oxidation with MnP and thus supplied Mn^{2+} to the MnP system. Thus, bleaching of hardwood kraft pulp with MnP, using manganese originally existing in the pulp, became possible in the presence of oxalate, a good manganese chelator and reducing reagent.

Properties of partially purified MnPs from liquid cultures of white rot fungi, *Ganoderma* sp. YK-505, *Phanerochaete sordida* YK-624 and *Phanerochaete chrysosporium* were compared. MnP from *Ganoderma* sp. YK-505 was superior to MnPs from *P. sordida* YK-624 and *P. chrysosporium* in stabilities against high temperature and high concentration of H_2O_2. The MnP from *Ganoderma* sp. YK-505 differed in pH-activity profile from other MnPs. These data suggest that MnP from *Ganoderma* sp. YK-505 has different structure from those of other fungi. Bleaching of hardwood kraft pulp using the MnP from *Ganoderma* sp. YK-505 is now in progress.

1. INTRODUCTION.

General concern about the environmental impact of chlorine bleaching effluents has led to a trend toward elementary chlorine free (ECF) or totally chlorine free (TCF) bleaching methods. Biobleaching with white rot fungi has been studied by some workers (1-6) to establish chlorine-free bleaching processes. However, it is concerned that the biobleaching with fungi is rather slow compared with chemical bleaching, and that the cellulose in the pulp is also attacked by polysaccharidase.

Thus, direct application of the ligninolytic enzymes involved in bleaching with fungi, lignin peroxidase (LiP) (7), manganese peroxidase (MnP) (8,9) and laccase (10), has been attempted. Recently, some reports have shown that MnP plays an important role in the bleaching of unbleached

hardwood kraft pulp by white rot fungi(9, 11-13). MnP is a heme-containing enzyme and oxidizes Mn^{2+} to Mn^{3+}, which chelated with an organic acid , in turn oxidizes phenolic substrates, including lignin model compounds, dehydropolymerizate and high-molecular-weight chlorolignin (14-17). Therefore, in vitro bleaching of the kraft pulp was performed with MnP from the fungus *Phanerochaete sordida* YK-624 (8), which was isolated from decayed wood obtained from a forest and exhibited remarkable bleaching ability with the pulp (11, 13). Treatment of the pulp with partially purified MnP in the presence of Tween 80 and with continuous addition of H_2O_2 resulted in a pulp brightness increase by about 10 points and a kappa number decrease by about 6 points (8).

However, there are some differences between in vivo bleaching with fungi and in vitro one with MnP with respect to treatment conditions. Major differences between both bleaching systems were the use of additional $MnSO_4$ and high concentrations of malonate as a Mn^{3+}-chelator during in vitro bleaching with MnP. The manganese in the pulp must have been used by the MnP catalytic system in the living fungi. Some organic acids which chelate and stabilize Mn^{3+} generated by MnP in aqueous solution play important roles in the MnP system. In this connection it was reported that millimolar concentrations of oxalate are produced by various fungi such as *P. chrysosporium* and *T. versicolor* (15, 18-20). Oxalate is also known to have reductive activity. In the present paper, we show the successful bleaching of kraft pulp with MnP without addition of $MnSO_4$ by using oxalate as an effective Mn^{3+}-chelating and reductive agent.

Many ligninolytic fungi produce MnP in various cultures such as synthetic liquid and solid-state media. For the ubiquity of MnP among various fungi, a MnP isozyme which has a different function from well-known MnP isozymes from *P. chrysosporium* (23-25) may be present. Enzymological and kinetic studies of the isozyme could be useful for understanding catalytic characteristics and improving stability of peroxidases. In a previous study (11), a relationship between pulp brightness increases and MnP activities was observed during the bleaching with almost all fungi which markedly brightened hardwood kraft pulp. However, bleaching with a fungus *Ganoderma* sp. YK-505 resulted in more extensive brightening of the pulp than expected from its MnP activity. Therefore, we expect that MnP from the fungus YK-505 have different properties from those of other white rot fungi. Properties of partially purified MnPs from liquid cultures of three white rot fungi, the fungus strain YK-505, *P. sordida* YK-624 and *P. chrysosporium* were compared in this study.

2. MATERIALS AND METHODS

2.1. Microorganism. Fungal strains *Phanerochaete sordida* YK-624 (14) (ATCC 90872) and *Ganoderma* sp. YK-505 which were isolated from decayed wood in a forest (11), were used for MnP preparation. *Phanerochaete chrysosporium* ME-446 was also used for MnP preparation. These fungi were maintained on a potato dextrose agar medium (Difco Laboratories).

2.2. Production and preparation of MnP was described earlier (8). Each strain grown on the potato dextrose agar was homogenized in a liquid culture medium with a Waring blender and inoculated into a 500-ml Erlenmeyer flask containing 200 ml of the culture medium at pH 4.5. After 7 days of incubation, the supernatant was separated from the mycelium, dialyzed overnight and subjected to anion-exchange chromatography (DEAE-Sepharose CL-6B; Pharmacia) as previously reported (8). The fraction containing MnP activity was used for pulp treatments.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed. Staining was done with Coomassie blue.

2.3. Enzyme assays. MnP activity was assayed by monitoring the oxidation of 2,6-dimethoxyphenol at 470 nm and expressed as the amount of spectral change (change in absorbance per minute) as previously described (8). The assay was also used to measure the activity of MnP remained in solution and absorbed onto the pulp during bleaching of the pulp with MnP. After pulp treatments, pulp suspensions were filtrated. The pulp was added to the above reaction mixture and the oxidation of 2,6-dimethoxyphenol was monitored. The dry weight of the pulp used for the assay was measured after the reaction. MnP activity was expressed as the amount of change in absorbance per minute and per gram of pulp. To evaluate the effect of organic acids on the oxidation of 2,6-dimethoxyphenol by MnP, 50 mM succinate buffer (pH 4.5) was also used in the assay instead of malonate buffer. Glucose oxidase activity was determined by the peroxidase-coupled production of hydrogen peroxide in the presence of glucose (21).

2.4. Pulp treatments. An unbleached hardwood kraft pulp (brightness, 31.0%, kappa number, 16.7) produced in an industrial pulp mill was used in this study. The pulp was suspended at a consistency of 1% (1 g/100 ml) in 50 mM malonate buffer (pH 4.5) or 50 mM succinate buffer (pH 4.5) containing 0.05% Tween 80 and 100 U of MnP. The suspension was stirred at 30℃. The enzymatic reaction was started and maintained by continuously adding H_2O_2 at a flow rate of 3 ml/h with a peristaltic pump. The reaction of glucose and glucose oxidase (from *Aspergillus niger*; Wako Pure Chemicals Industries, Ltd.) was also used to supply H_2O_2 continuously to the pulp suspension. The suspension in a 100-ml Erlenmeyer flask contained 0.5 g of kraft pulp, 50 U of MnP, 0.05% Tween 80, 25 mM glucose and glucose oxidase in 50 ml of 50 mM succinate buffer (pH 4.5). The flasks were shaken at 150 rpm and 30℃ after they were purged with oxygen for about 30 minutes.

2.5. Pulp properties. After the enzyme bleaching pulp was washed with distilled water. Pulp sheet preparation and determination of the pulp brightness and the kappa number were conducted as described in a precious paper (8).

2.6. Measurement of Manganese Dioxide Reduction. The reaction mixtures (50 ml) contained 5 mM MnO_2 in oxalate, malonate or gluconate buffer (50 mM, pH 4.5) and were stirred at 30℃ in air. Reduction of MnO_2 was measured by monitoring the appearance of Mn^{3+}-complex. The absorptivities of Mn^{3+} complexes were directly measured. $\varepsilon_{500} = 0.29$ and $\varepsilon_{270} = 11.59$ mM^{-1} cm^{-1}

were used for the determination of Mn^{3+}-oxalate and Mn^{3+}-malonate, respectively (22). $\varepsilon_{300} = 2.43$ mM^{-1} cm^{-1} was used for the determination of Mn^{3+}-gluconate.

2.7. Enzyme properties. The effect of temperature on the activity of MnP from each fungus was evaluated by assaying the activity at different temperatures in the range 30-70℃ at pH 4.5 during the reaction and 10 minutes before the reaction. Enzyme solutions (1 U/ml) were also incubated for different periods of time at 30℃, 40℃ or 50℃. After the incubation, MnP activities were measured under the standard assay condition. The effect of H_2O_2 on MnP activity was determined by adding aqueous H_2O_2 in the range 0.2-10 mM. pH-activity profiles were determined for each MnP by using 3 mM guaiacol instead of 2,6-dimethoxyphenol because the latter autooxidized in a pH range higher than 6.0. MnP activities at each pH were assayed by monitoring the oxidation of guaiacol at 470 nm.

3. RESULTS

3.1. Bleaching of kraft pulp with MnP of *Phanerochaete sordida* YK-624

Unbleached hardwood kraft pulp was treated with MnP by adding 1, 3, 5 and 10 mM aqueous H_2O_2 for 24 h. The effect of the addition of $MnSO_4$ on bleaching of the pulp with MnP in the presence of various organic acids was examined by changing the concentration of aqueous H_2O_2 added (Fig. 1). In 50 mM malonate buffer, the brightness increase was observed with the addition of $MnSO_4$, but not without additional $MnSO_4$. When the pulp was treated with continuous addition of 3 and 5 mM aqueous H_2O_2 in the presence of 2 mM oxalate, the brightness increased by about 13 points without the addition of $MnSO_4$. An increase in brightness was observed in the presence of 2 mM gluconate without the addition of $MnSO_4$, although it was less than that in oxalate. Brightening was also observed in 50 mM succinate buffer without the addition of $MnSO_4$ by pulp treatment.

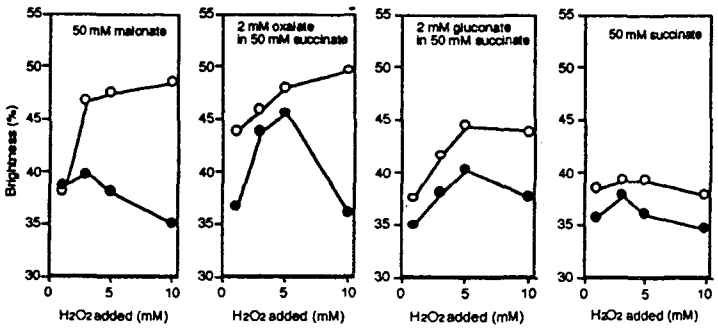

Fig. 1. Effect of the addition of $MnSO_4$ on the brightness of kraft pulp treated with MnP with continuous addition of various concentrations of aqueous H_2O_2 at 3 ml/h for 24 h.
Symbols: ●, no addition of $MnSO_4$; ○, addition of 0.1 mM $MnSO_4$.

Changes in residual MnP activity and pulp brightness during bleaching with MnP without the addition of $MnSO_4$ and in the presence of organic acids are shown in Fig. 2. The low MnP amounts adsorbed onto the pulp were detected early in treatment. The brightness increased progressively in the presence of oxalate and the rate of the decrease in MnP activity was slower than those in the presence

of other organic acids. Unbleached pulp used in this study contained 30.0 mg of manganese per kg. After treatment with MnP without exogenous manganese in the presence of 2 mM oxalate, the pulp contained 2.3 mg of manganese per kg. The decrease in manganese content suggests that MnP uses manganese originally present in the pulp, resulting in brightening.

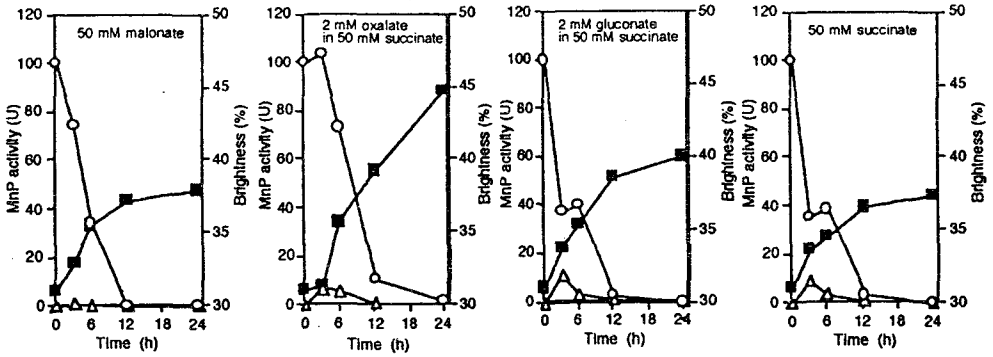

Fig. 2. Changes of residual MnP activity and pulp brightness as a function of time during the bleaching of kraft pulp with MnP without the addition of $MnSO_4$. 5 mM aqueous H_2O_2 was added at a rate of 3 ml/h for 24 h. Symbols: ○, MnP activity in solution; △, MnP activity sorbed on pulp; ■, pulp brightness.

Oxalate was capable of reducing MnO_2 rapidly and complexed with Mn^{3+}, whereas malonate and gluconate reduced MnO_2 slowly (Fig. 3). It could be that oxalate reduces MnO_2 which either existed in the pulp or formed from Mn^{2+} by oxidation with MnP.

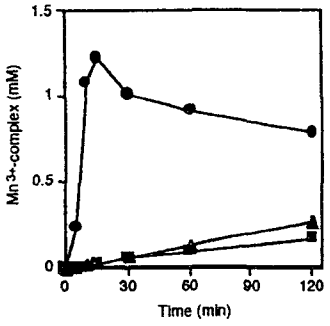

Fig. 3. Change of Mn^{3+}-complex produced by the reduction of insoluble MnO_2 by organic acids as a function of time. Symbols: ●, Mn^{3+}-oxalate at 500 nm; ▲, Mn^{3+}-malonate at 270 nm; ■, Mn^{3+}-gluconate at 300 nm.

It can be concluded that the presence of oxalate, which is a good Mn^{3+}-chelator and MnO_2-reducing agent, supports the bleaching of the kraft pulp with MnP by the effective use of manganese originally present in the pulp.

3.1. Characteriztion of MnP isozyme from *Ganoderma* sp. YK-505

Molecular mass of each MnP fraction from cultures of the fungus strain YK-505, *P. sordida* YK-624 and *P. chrysosporium* was determined by SDS-PAGE. All fractions produced bands near a molecular mass of 43 kDa (data not shown).

Thermal stability of MnPs. Activity of MnP from YK-505 increased with increasing temperature up to 55 ℃, when enzyme reaction runs were done with 2,6-dimethoxyphenol at various temperatures for 3 minutes after incubation at each temperature for 10 minutes (data not shown). Optimum activity was 55℃ for the fungus YK-505. About 50% activity was observed even at 65℃. On the other hand, MnPs from both *P. sordida* YK-624 and *P. chrysosporium* exhibited maximal activities in the temperature range 40-50℃. Their significant activities were not detected at temperatures higher than 50℃.

MnP from each fungus was incubated at 30, 40 and 50℃, and then the remaining activity was measured using standard assay at 37℃ (Fig. 4). All MnPs were stable at 30℃ for 24 h. MnP from YK-505 showed almost the same stability at 40℃, but activities of MnPs from *P. sordida* YK-624 and *P. chrysosporium* decreased to 50% of the initial activity after 12 h. When MnPs were incubated at 50℃, activities of MnPs from *P. sordida* YK-624 and *P. chrysosporium* disappeared immediately. However, MnP from YK-505 lost its activity slowly and the activity was detected even after incubation for 12 h.

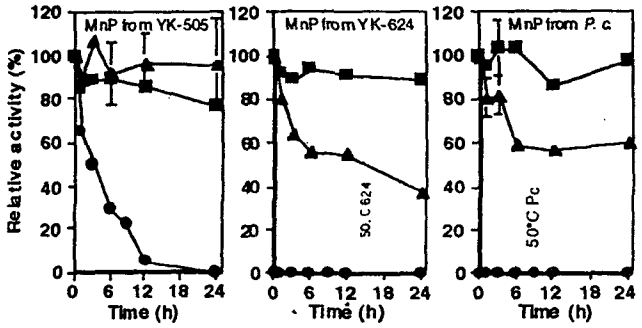

Fig. 4. Thermostability of MnP. Enzyme solutions were incubated at 30 (●), 40 (△) and 50℃ (□).

Stability against H_2O_2. The influence of H_2O_2 concentration on MnP activity is shown in Fig. 5. Although increasing concentrations of H_2O_2 decreased the enzyme activity of all MnPs for the oxidation of 2, 6-dimethoxyphenol, MnP from YK-505 was superior to MnPs from other fungi against high concentration of H_2O_2.

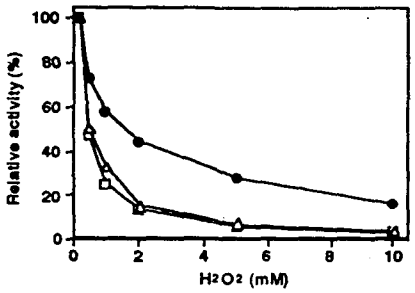

Fig. 5. Effect of H_2O_2 concentration on the activity of MnP from *Ganoderma* sp. YK-505 (●), *P. sordida* YK-624 (△) and *P. chrysosporium* (□). Control activity (100%) was obtained with 0.2 mM H_2O_2.

pH-activity profile. Using guaiacol as a substrate, MnPs of *P. sordida* YK-624 and *P. chrysosporium* showed an optimal pH of 4.0 (Fig. 6), and their activities decreased as the pH was raised. On the other hand, MnP from YK-505 had different pH dependency from other MnPs, and showed an wider optimal pH range between 4.0 and 6.5.

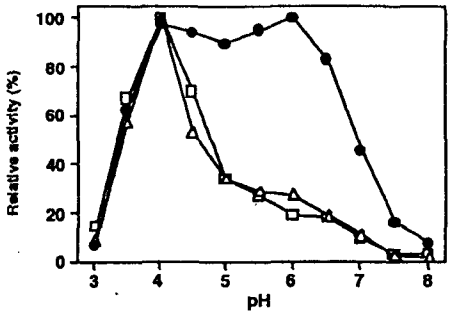

Fig. 6. Effect of pH on the activity of MnP from *Ganoderma* sp. YK-505 (●), *P. sordida* YK-624 (△) and *P. chrysosporium* (□). Reaction mixtures contained 3 mM guaiacol, 0.1 mM $MnSO_4$, 0.2 mM H_2O_2 and 50 mM malonate. Control activity (100%) was obtained at pH 4.0.

Use of MnP from *Ganoderma* sp. YK-505, which has stabilities against high temperature and high concentration of H_2O_2, may improve the bleaching of kraft pulp with MnP. The structural and enzymological studies of the MnP may be useful for industrial applications. We are now studying the properties of the purified ligninolytic enzymes produced by the white rot fungus *Ganoderma* sp. YK-505 in further detail.

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