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Shinichi Yoshida · Akinobu Chatani · Yoichi Honda
Takashi Watanabe · Masaaki Kuwahara

Reaction of manganese peroxidase of *Bjerkandera adusta* with synthetic lignin in acetone solution

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Abstract The reaction of manganese peroxidase (MnP) of the white-rot fungus *Bjerkandera adusta* with synthetic lignin (dehydrogenation polymer, DHP) in acetone medium was investigated. Gel-permeation chromatography of the DHP treated by MnP demonstrated depolymerization of syringyl DHP in the reaction mixture containing 70% acetone; moreover, concomitant repolymerization occurred to give highly polymerized products. Guaiacyl DHP was only repolymerized by MnP in the same acetone solution without giving degradation products. Addition of ascorbic acid to reaction mixtures containing acetone resulted in preferential depolymerization of syringyl DHP.

Key words Lignin biodegradation · Manganese peroxidase · *Bjerkandera adusta* · Acetone

Introduction

Lignin is a complex, optically inactive, random phenylpropanoid polymer that constitutes 20%–30% of woody plants.[1] Although lignin is the most abundant aromatic polymer on earth, the production of useful products from lignin has been hindered by its structural complexity. White-rot fungi are the best lignin degraders among all known microorganisms, and they secrete extracellular ligninolytic enzymes including laccase, manganese peroxidase (MnP), and lignin peroxidase (LiP).[2] LiP catalyzes the H_2O_2-dependent oxidation of lignin model compounds, halogenated phenolic compounds, polycyclic aromatic compounds, and other aromatic compounds by a one-electron oxidation mechanism followed by a series of nonenzymatic reactions that yield various degradation products.[2,3] MnP catalyzes the H_2O_2-dependent oxidation of phenolic lignin model compounds.[2] Laccase is known to oxidize various phenolic compounds and lignin[4] via the phenoxy radical formed by one-electron oxidation.

Many studies have been performed on the incubation of lignin preparations with ligninolytic enzymes in water-rich media.[5–11] Lignin is known to be difficult to dissolve in water, so development of an enzyme reaction system using organic solvents as the reaction medium was necessary for the degradation and transformation of lignin. Our interests are focused on whether lignin is degraded by ligninolytic enzymes in the reaction mixture containing highly concentrated organic solvents. Degradation of lignin in mixtures of organic solvents and buffers using different peroxidases remains to be investigated in detail. For example, Dordick et al. used organic solvent for depolymerization of dehydrogenation polymer (DHP), milled wood lignin (MWL), and kraft lignin by horseradish peroxidase.[12] Lewis et al. could not duplicate these promising results.[13] On the other hand, Hammel et al. reported that addition of organic solvents capable of dispersing the polymer to the reaction medium is important for ligninolysis by enzymes.[9] LiP has been shown to be able to catalyze the oxidation of 3,3′-dimethoxybenzidine[14] and polycyclic aromatic hydrocarbons[15] in reaction mixtures containing organic solvents. The effects of these solvents on the reactivity of LiP was also investigated.[16] Furthermore, chemical modification of LiP increased the oxidation rate of aromatic compounds in the reaction mixture containing 70% water-miscible organic solvents.[17,18] The reaction of ligninolytic enzymes with lignin in highly concentrated organic solvents remains to be investigated. In this study, the activity of the MnP of *Bjerkandera adusta* in organic solvents was investigated, and the depolymerization of DHP by the MnP of *B. adusta* in organic solvents is discussed.

S. Yoshida[1] · A. Chatani · Y. Honda · T. Watanabe · M. Kuwahara (✉)
Wood Research Institute, Kyoto University, Uji 611-0011, Japan
Tel. +81-774-38-3640; Fax +81-774-39-3643
e-mail: mkuwahar@kuwri.kyoto-u.ac.jp

Present address:
[1] Industrial Technology Institute, Tottori Prefectural Government, Tottori 680-0902, Japan
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Materials and methods

Preparation of enzyme

Bjerkandera adusta (K-2679) was grown statically at 30°C in 200-ml Erlenmeyer flasks with 20 ml of culture medium containing 2% glucose, 0.5% polypeptone, 0.2% yeast extract, 0.1% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ as described by Kimura et al.[19] MnP activity was determined by the method of Kofujita et al.[20] One unit of activity was defined as a 1.0/min increase in absorbance at 465 nm. Protein was determined by the method of Bradford.[21] MnP was purified from 13-day-old cultures by the method of Kirk et al.[22] on a DEAE-Sepharose CL-6B and a Pharmacia Mono-Q column (10/10).

Isoelectric focusing was carried out with a polyacrylamide gel containing Servalyt (pH 2–4, Serva) using the Multiphor II (Pharmacia). Polyacrylamide gel was prepared from 10 ml of aqueous solution containing 4.8% acrylamide, 0.15% *N,N'*-methylene-bis(acrylamide), 7.2% urea, 3.0% glycerol, 10% Servalyt, 0.1% *N,N,N',N'*-tetramethylenediamine, and 0.16% ammonium persulfate. The pI of purified MnP was estimated to be 2.7 using a standard proteins (Low pI Kit, pI 2.8–6.5; Pharmacia). The molecular weight was estimated to be 45000 by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Measurement of MnP activity in organic solvents

In water-miscible organic solvents, the activity was determined spectrophotometrically using reaction mixtures containing 14 mM guaiacol or 2,6-dimethoxyphenol as the substrate, 50 mM lactate buffer (pH 4.5), 0.2 mM $MnSO_4$, 0.25 mM H_2O_2, 70% organic solvent, and the enzyme solution in a final volume of 1 ml. Reactions were started by adding H_2O_2 and were quantified by monitoring the initial rate of increase in absorbance at 465 nm (substrate: guaiacol) or 469 nm (substrate: 2,6-dimethoxyphenol). One unit of activity in organic solvent was defined as an increase in absorbance of 1.0/min at 465 or 469 nm.

Incubation of MnP with synthetic lignin in organic solvents

Coniferyl and sinapyl alcohols were synthesized by the method of Freudenberg and Hübner.[23] Guaiacyl-type DHP (c-DHP) was prepared with horseradish peroxidase (HRP)/H_2O_2 from coniferyl alcohol by the method of gradual monomer addition (zutropfverfahren).[24] Syrigyl-type DHP (s-DHP) was prepared from sinapyl alcohol by the dialysis tube method.[25]

In vitro reactions were carried out at 37°C in 2.0 ml of reaction mixture containing 70% organic solvent: 50 mM Na lactate (pH 4.5), 13.6 μg MnP, 2.0 mg DHP (added as a stock solution in 200 µl of methylcellosolve solution), 10% methylcellosolve (added with DHP as the stock solution), 0.2 mM $MnSO_4$, 0.1 mM H_2O_2, and 60% acetone. Additional MnP and H_2O_2 were added at various intervals. After incubation for the desired period, the reaction mixtures were evaporated to dryness under high vacuum.

Gel permeation chromatography

Samples were dissolved in 200 µl *N,N*-dimethylformamide (DMF), and insoluble material was removed by centrifugation at 4000 rpm for 15 min at room temperature. The supernatant (195 µl) was applied to a Sephadex LH-60 column (1.5 × 41.0 cm) equilibrated in DMF containing 0.1 M LiCl. The column was eluted with the same solvent (flow rate 22.8 ml/h), and 1.5-ml fractions were collected. Absorbance of fractions at 280 nm was measured. The column was calibrated with polystyrene standard (MW 9000, 2500, and 761) and syringaldehyde (MW 182).

Results and discussion

Activity of MnP of *Bjerkandera adusta* in organic solvents

Manganese peroxidase activity in water-miscible organic solvents was measured using guaiacol and 2,6-dimethoxyphenol as guaiacyl- and syringyl-type model substrates, respectively. Lignin has been reported to be completely or partially soluble in the solvents used in this study.[26] The absorption spectrum of the solutions oxidized by MnP in 70% organic solvents used in this study was similar to that in water (data not shown). In addition, 4 mM guaiacol and 2,6-dimethoxyphenol were oxidized by MnP in aqueous buffer solution, and an aliquot of the reaction mixture was diluted with organic solvent to a final concentration of 70%. For the control, the aliquot was diluted with water. The absorbance of the solution diluted with organic solvent was similar to that with water (data not shown), which indicates that the addition of organic solvents does not affect the extinction coefficient of the oxidation products by MnP. Therefore, the activities in 70% organic solvents were uncorrected.

As summarized in Table 1, MnP oxidized both substrates in 70% water-miscible organic solvents including acetonitrile, acetone, dioxane, ethylene glycol, and methylcellosolve. Guaiacol was not oxidized by MnP in reaction mixtures containing 70% ethanol and methanol, but 2,6-dimethoxyphenol was slightly oxidized in both solutions. DMF and dimethylsulfoxide (DMSO) completely inhibited the oxidation of both substrates by MnP. In the presence of 70% organic solvent, the residual activities on 2,6-dimethoxyphenol oxidation by MnP were higher than those on guaiacol oxidation. The reason for this is still not understood, and further investigations are now in progress.

Manganese peroxidase activity retained 77% and 73% of the original activity in reaction mixtures containing 70% acetonitrile and acetone, respectively, so these substances appear to be effective solvents for the reaction of MnP with lignin. To investigate the stability of MnP in both solvents, the increases in absorbance due to oxidation of both sub-

Table 1. Activity of MnP of *Bjerkandera adusta* in organic solvents

Solvent	Relative activity (%)	
	Guaiacol	2,6-Dimethoxyphenol
Water[a]	100	100
Acetonitrile 70%	35	77
Acetone 70%	26	73
Dioxane 70%	20	23
Ethylene glycol 70%	17	31
Methylcellosolve 70%	11	16
Ethanol 70%	0	20
Methanol 70%	0	1
DMF 70%	0	0
DMSO 70%	0	0

DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; MnP, manganese peroxidase.
[a]In water the specific activity of MnP for guaiacol and 2,6-dimethoxyphenol was 231 and 1065 U/mg, respectively

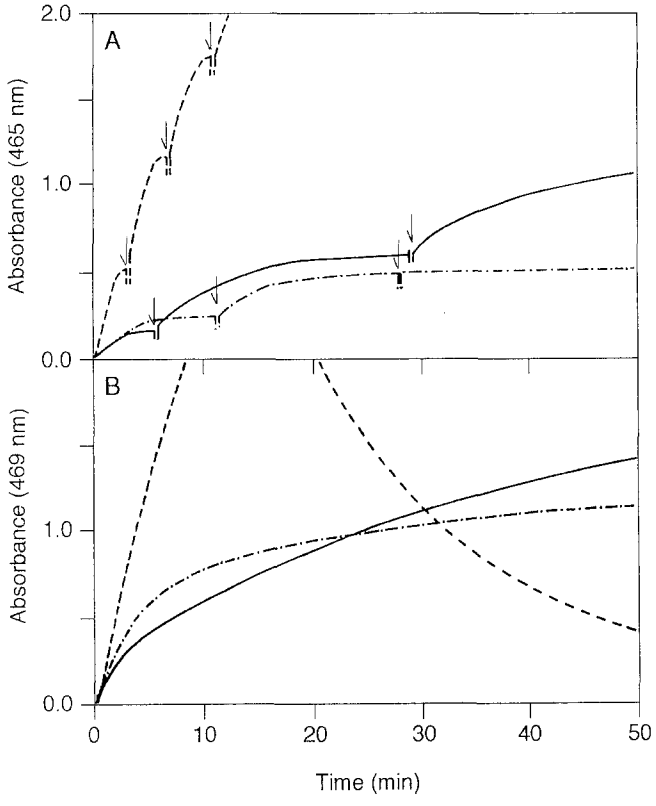

Fig. 1. Oxidation of phenolics by manganese peroxidase (MnP) of *Bjerkandera adusta* in aqueous 70% acetonitrile and acetone media. Reaction was initiated as described in Materials and Methods. *Arrows* show the points at which 5 µl of 50 mM H_2O_2 was added to the solutions. The oxidation of guaiacol (**A**) and 2,6-dimethoxyphenol (**B**) was carried out using reaction mixtures containing 70% acetone (*solid lines*) and acetonitrile (*dash-dot lines*). Oxidation of both substrates was also carried out in the absence of co-solvents (*dashed lines*). All reaction mixtures contained 0.85 µg MnP

strates were monitored for 50 min. As shown in Fig. 1, increases in absorbance were observed for both substrates for 50 min in reaction mixtures containing 70% acetone. The initial oxidation rates in 70% acetonitrile solution were higher than those in acetone, but the oxidation rates decreased with time after 10 min. In addition, the oxidation of guaiacol by MnP was restarted by further addition of H_2O_2, suggesting that MnP was not disturbed in 70% acetone solution. The increase in absorbance for both substrates were higher in the absence than in the presence of acetone and acetonitrile (Fig. 1). After 20 min, decreases in absorbance were observed when 2,6-dimethoxyphenol was used as the substrate (Fig. 1B). Further investigations on these decreases in absorbance are now in progress.

Addition of organic solvents to the reaction mixture decreased the MnP activity, and the solubility of lignin was reported to increase. In addition, a low oxidation rate of substrate in organic solvents is expected to lead to depolymerization of lignin, because high transient concentration of lignin phenoxy radicals in the reaction mixture leads to polymerization of lignin.[9] Oxidation of substrates by MnP continued for a longer time in the reaction mixture containing 70% acetone than in acetonitrile, as shown in Fig. 1. Therefore, acetone was used in the following depolymerization experiments.

Oxidation of synthetic lignin by MnP of *B. adusta* in organic solvents

The c- and s-DHPs were incubated with MnP for 4 h in reaction mixtures containing 60% acetone and 10% methylcellosolve added with DHP as a stock solution. Additional MnP and H_2O_2 were added at 1-h intervals. Control experiments were carried out with omission of H_2O_2 (data not shown) or MnP. A gel permeation chromatogram of c-DHP is shown in Fig. 2A. The molecular weight of c-DHP increased, and no depolymerization products were observed. No reaction was observed in control experiments. In contrast, s-DHP was depolymerized by MnP in reaction mixtures containing acetone, and polymerized compounds were detected (Fig. 2B). The depolymerized fraction of s-DHP had a molecular weight of 180. Depolymerized products were checked by thin-layer chromatography using *n*-hexane/ethyl acetate (2:1) as the developing solvents. Syringaldehyde was detected by ultraviolet (UV) irradiation (data not shown), suggesting that C_α-C_β cleavage of s-DHP was induced by MnP.[8] Depolymerization also occurred when Mn(III) was used instead of MnP/H_2O_2 in the solution (data not shown). The difference in the behavior of MnP against c- and s-DHPs is thought to be due to their chemical substructures. c-DHP contains a greater proportion of unsubstituted aromatic 5-positions than s-DHP[27] and consequently would be more susceptible to further polymerization or repolymerization.[7,8]

Previous studies have demonstrated the repolymerization of DHP by ligninolytic enzymes in vitro.[7–9,28] Although MnP of *Phanerochaete chrysosporium*, like LiP, both polymerized and depolymerized lignin in vitro, MnP induced more polymerization and less depolymerization than LiP.[9] In this study, repolymerization of c- and s-DHPs by MnP was also observed. Depolymerization is probably initiated by oxidation of the free phenolic group to form a phenoxy radical, as previously shown for the degradation of

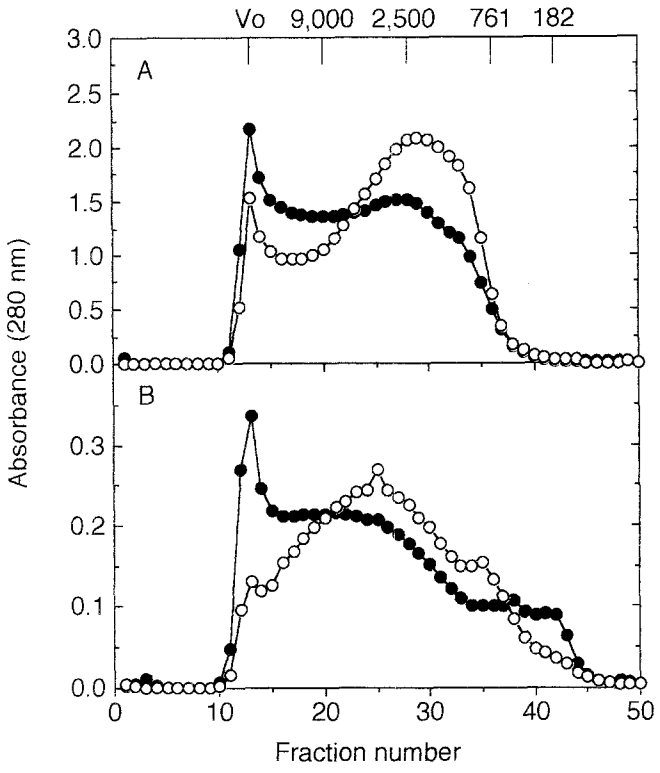

Fig. 2. Gel permeation chromatograms of dehydrogenation polymers (DHPs) incubated with MnP of *B. adusta* in reaction mixtures containing both 60% acetone and 10% methylcellosolve. Incubation of DHPs prepared from coniferyl (**A**) and sinapyl alcohols (**B**) was initiated as described in Materials and Methods and continued with further addition of the same amounts of MnP and H_2O_2 at 1-h intervals for a total reaction time of 4 h. These incubated DHPs were analyzed as described in Materials and Methods. *Closed circles*, complete reaction; *open circles*; MnP was omitted. *Vo*, void volume

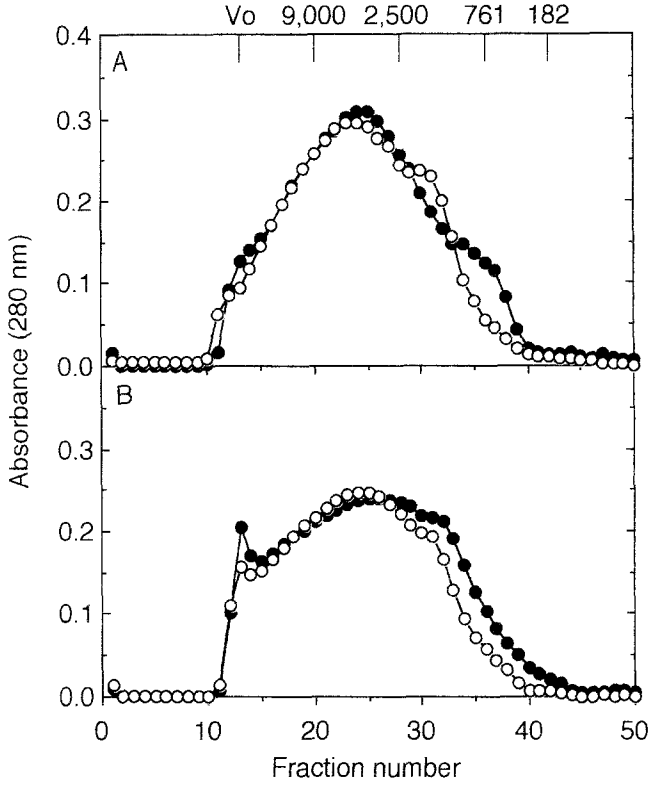

Fig. 3. Gel-permeation chromatograms of syringyl (s)-DHP incubated with MnP of *B. adusta* in the reaction mixture containing both 60% acetone and 10% methylcellosolve under various conditions. (**A**) Incubation of s-DHP was initiated as described in Materials and Methods and continued with further addition of the same amounts of MnP and H_2O_2 at 2.5 and 19.0 h of incubation. H_2O_2 was also added after 5.5 and 26.0 h of incubation. Incubation was carried out for a total reaction time of 28 h. (**B**) In the presence of 10 mM ascorbic acid, incubation of s-DHP was initiated as described in Materials and Methods and continued with further addition of the same amounts of MnP and H_2O_2 at 1-h intervals for a total reaction time of 4 h. These incubated DHPs were analyzed as described in Materials and Methods. *Closed circles*, complete reaction; *open circles*; MnP was omitted

diarylpropane model compounds and phenolic arylglycerol-β-aryl ether by MnP.[29,30] The high transient concentration of lignin phenoxy radicals is thought to facilitate repolymerization rather than depolymerization as described by Hammel et al.[9] Therefore, to retain low concentrations of the phenoxy radicals, additional enzyme and H_2O_2 were added to the reaction mixture at extended intervals for a total reaction time of 28 h. Incubation of s-DHP with MnP was initiated with 13.6 μg MnP plus 0.1 mM H_2O_2 and continued with further addition of the same amounts of MnP and H_2O_2 at 2.5 and 19.0 h of incubation. H_2O_2 was also added after 5.5 and 26.0 h of incubation. The molecular weight of s-DHP decreased using this reaction system, suggesting that polymerization of lignin was suppressed (Fig. 3A). The difference of the profiles between control experiments in Figs. 2 and 3 is attributable to the differences of the reaction conditions, including incubation time, and additive concentrations. In addition, these facts suggested that s-DHP was modified to some extent by H_2O_2 alone (data not shown).

Radical concentrations can be decreased by addition of radical scavengers, such as ascorbic acid, tocopherols, and certain phenols.[31] Oxidation of 2,6-dimethoxyphenol by MnP was decreased to 1/1470 of the original activity in 70% acetone by addition of 10 mM ascorbic acid (data not shown). Therefore, s-DHP was incubated with MnP in aqueous 70% acetone medium containing 10 mM ascorbic acid. As shown in Fig. 3B, repolymerization was suppressed by the addition of ascorbic acid. These reaction conditions were thus effective for the depolymerization of DHP. The function of ascorbic acid in the depolymerization of DHP is poorly understood. MnP is known to oxidize Mn(II) to Mn(III), which acts as a mediator and further oxidizes phenolic compounds.[32] Ascorbic acid is thought to reduce Mn(III) to Mn(II), which results in a decrease in the production rate for phenoxy radicals. Phenoxy radicals are also thought to be directly reduced to phenolic compounds. Further investigations on the mechanism are now in progress.

In reaction mixtures containing high concentrations of lignin without co-solvents, dispersal of the polymer is thought to be poor, as discussed by Hammel et al.[9] In previous studies, reactions were conducted in the presence of low concentrations of organic solvents (<40%).[7-10] The influ-

ence of dispersion of lignin on enzymatic degradability was also investigated.[33] Addition of 70% organic solvents to the reaction mixture can be expected to improve the dispersion of lignin. The effects of organic solvents on dispersion of lignin are poorly understood and remain to be investigated. This subject is now being studied in detail. In addition, the ratio of polymerized compounds to degraded products in the reaction mixture containing 70% acetone were higher than that in water-rich medium.[8] The decrease of lignin phenoxy radicals by the addition of ascorbic acid is thought to lead to the depolymerization of s-DHP.

Conclusions

The MnP of *B. adusta* oxidized DHP in a reaction mixture containing 70% acetone in the presence of H_2O_2. Gel permeation chromatography showed that the molecular weight of c-DHP increased, whereas that of s-DHP decreased, with polymerized compounds present. The repolymerization of s-DHP was suppressed by the addition of H_2O_2 and MnP for extended intervals. Similarly, addition of ascorbic acid to reaction mixtures containing acetone resulted in preferential depolymerization of s-DHP.

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