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ORIGINAL ARTICLE

Mariko Takano · Hisashi Abe · Noriko Hayashi

Extracellular peroxidase activity at the hyphal tips of the white-rot fungus *Phanerochaete crassa* WD1694

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Abstract The white-rot fungus *Phanerochaete crassa* WD1694 was cultivated and peroxidase activity staining was performed to determine the sites at which the extracellular peroxidase reaction actually occurs in vivo. Although the ligninolytic peroxidases were found in the culture filtrates, the culture medium did not show a color reaction. However, a particularly strong color reaction was observed on the hyphal tips. Visible spectra and absorbance of the staining were analyzed by microspectrophotometry, and the catalytic rates of the peroxidase reaction at the hyphal tips were calculated. The estimated catalytic rate of the peroxidase reaction at the hyphal tips peaked at 794 μM/min, expressed as the consumption rate of H_2O_2, on day 3 of the cultivation. Analysis of the extracellular enzyme eluted with 0.1% Tween 80 from the mycelium revealed that manganese peroxidase accounted for 89% of all the peroxidase activity measured. The results clearly showed the existence of the concentrated manganese peroxidase reaction around the hyphal tips of the organism.

Key words White-rot fungi · *Phanerochaete crassa* · Microspectrophotometry · Manganese peroxidase · Hyphal tips

Introduction

Lignin, together with cellulose and hemicellulose, is one of the major components of the frameworks of plants, and its content in wood reaches 25%–30%.[1] Although most polymers derived from organisms have a linear chain structure in which unit molecules are linked by the same types of bonds, lignin is a polymer without a single repeating unit, and, moreover, contains bonds in a random three-dimensional arrangement.[2] Therefore, it is very difficult for lignin to be degraded by common organisms. White-rot fungi are organisms that can degrade this persistent polymer very efficiently, and there has been much research interest in the mechanism of how this is done.[3,4]

Studies of the biodegradation of lignin by white-rot fungi have made significant progress since the two enzymes, lignin peroxidase (LiP) and manganese peroxidase (MnP), were found.[5–7] The structures, gene sequences, and catalytic mechanisms of both peroxidase enzymes have been investigated in detail.[8–10] Furthermore, the mechanisms by which various bonds between the aromatic nuclei of lignin are cleaved have been revealed in vitro using model lignin compounds.[11,12] The intracellular and extracellular localizations of LiP and MnP in living organisms have been revealed by immunoelectron microscopy.[13–16]

However, there is a wide gap between what we know about the cleavage mechanisms at a molecular level and the actual decay process of woods. Because the reaction of the peroxidases requires the enzyme itself, the substrate, and hydrogen peroxide, we cannot specify the site of reaction on the basis of localization of the peroxidase enzyme alone.

To determine the sites at which the peroxidase reaction actually occurs in vivo, the white-rot fungus *Phanerochaete crassa* WD1694 was cultivated and subjected to peroxidase activity staining. The culture medium did not show a color reaction, but a particularly strong color reaction was observed on the surfaces of the mycelium. We used microspectrophotometry to measure the absorbance at these stained sites on the mycelium, and the in situ peroxidase activity was estimated.

Materials and methods

Fungal strain

The white-rot fungus *Phanerochaete crassa* WD1694 [MAFF420737, *Phanerochaete crassa* (Lev.) Burdsall] was

M. Takano (✉) · H. Abe · N. Hayashi
Forestry and Forest Products Research Institute, PO Box 16
Tsukuba Norin Kenkyu Danchi-nai, Tsukuba 305-8687, Japan
Tel. +81-29-873-3211 ext. 456; Fax +81-29-873-3797
e-mail: marin@ffpri.affrc.go.jp

previously selected for biological bleaching of unbleached hardwood kraft pulp (UKP), and was obtained from the culture collection of Forestry and Forest Products Research Institute.[17]

Pulp and reagents

UKP was obtained from a mill of Nippon Paper Industries and was washed with distilled water before use. BM Blue POD substrate solution, precipitating 3,3′, 5, 5′-tetramethylbenzidine (TMBZ) was purchased from Roche Diagnostics (Germany). Horseradish peroxidase type II was obtained from Sigma (USA). All other reagents were special grade.

Culture conditions

Inoculum of *P. crassa* WD1694 was prepared as described previously.[17] Each of the 300-ml Erlenmeyer flasks containing 250 mg of UKP was sterilized at 121°C for 20 min, and 10 ml of the inoculum and 50 ml of sterilized distilled water were added. The flasks were incubated on a rotary shaker at 30°C and 100 rpm for each test day. The culture medium was used because it represented conditions that were more similar to the natural decay process than that for high production of MnP, which included Tween 80. The mycelial pellets produced in the shaken culture were small enough to be used for microscopic analysis without sectioning.

Staining

An aliquot of the culture including the mycelial pellets was taken from the culture and put in a microtube. For staining of peroxidase activity, TMBZ was added to the microtube at a concentration of 20% and incubated at 26°C until blue staining was observed.

Microspectrophotometry

Immediately after blue staining was observed, the stained mycelial pellet was placed on a slide glass, covered, and the absorption was measured at 600 nm with a Carl Zeiss UEM UV-FL microspectrophotometer. The measurement was conducted for 38 spots for 2 to 4 cultivation days.

Light microscopy

The stained mycelial pellets were observed with a Nikon Optiphot microscope.

Enzyme activity

All of the preparation procedure was conducted at 4°C. Mycelial pellets were collected by filtration with nylon cloth and thoroughly washed with 10 mM phosphate buffer (pH 6.2), and further extracted gently with 0.1% Tween 80 in the buffer. The culture filtrate, washing solution, and extract were each collected, dialyzed, and concentrated. Measurement of MnP activity and isoelectric focusing was conducted as described previously.[18–20]

Results

The shake culture of *Phanerochaete crassa* WD1694 produced many spherical mycelia that were about 1.0–1.5 mm in diameter, and the apices of many hyphae protruding outward from the spheres were stained by TMBZ (Fig. 1). The staining formed the shape of a string, with the hypha as the axis. The string was about 100 μm long on the first day of cultivation and it elongated to 200–300 μm by the second and third days.

Calculation theory of the catalytic rate at the stained hyphal tips

When enzyme activity is calculated as the amount of product per unit time with the absorbance of the product, the values of reaction time, molar absorbance coefficient of the product, and light path length are required in addition to the absorbance. Therefore, we calculated the catalytic rate by fixing the above values as follows.

Light path length

In most cases the staining of the hyphal tip was symmetrical relative to the axis of the hypha; therefore, we estimated that staining occurred in a cylindrical or conical shape, with the hypha as the axis (Fig. 2). Figure 3 is a schematic diagram of the staining of the apical tip of the hypha. We measured the width of the stained part assuming that it represented the light path length of each measurement spot.

Absorbance

Staining of the hyphal tips was analyzed by microspectrophotometry. The visible light spectrum of the measurement spot showed a maximum absorbance at a wavelength of 600 nm (Fig. 4). The spectrum almost coincided with the spectrum of the oxidation product of TMBZ by horseradish peroxidase performed in a test tube. The actual absorbance values of the measurement spots at 600 nm were obtained by the microspectrophotometer.

Reaction time

Figure 5 shows the change of absorbance at a wavelength of 600 nm and the same measurement over time. Peroxidase activity staining was started at time 0, and the first microspectrophotometric measurement was performed immediately after the samples had been prepared. The absorbance

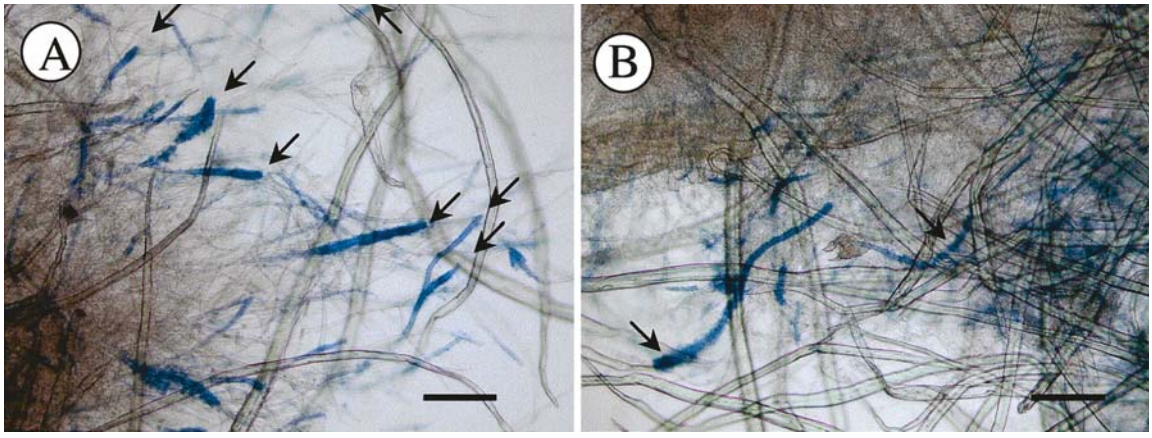

Fig. 1A,B. Staining of peroxidase reactions of *Phanerochaete crassa* WD1694. **A** Cultivated for 1 day. Apical staining was clearly observed (*arrows*). **B** Cultivated for 3 days. Elongations of apical staining were observed (*arrows*). *Bars* 100 μm

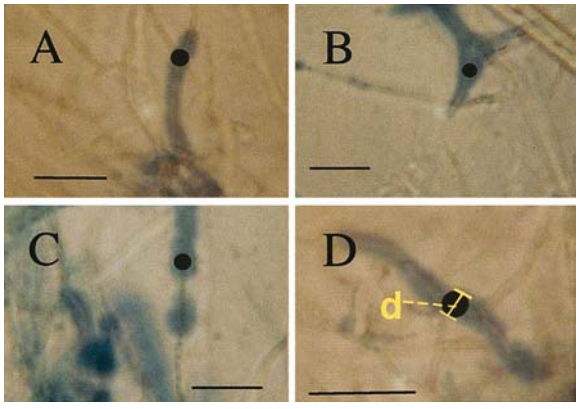

Fig. 2. **A–C** Photographs of staining on the hyphae used for microspectrophotometry. **D** An example of the catalytic rate calculation of the measurement spot. The catalytic rate (1135 μM/min) of the extracellular peroxidase was obtained with Eq. 3 and data, $A = 0.593$, $t = 50$ min, $d = 6.81 \times 10^{-4}$ cm. *Black circles* are measurement spots. Diameters of the spots were 6.25 μm. *Bars* 25 μm

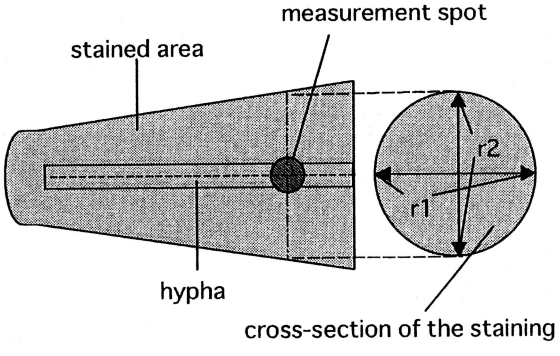

Fig. 3. Schematic diagram of staining of a hyphal tip observed by microspectrophotometry. The length of *r*1 corresponds to the light path length, and the length of *r*2 corresponds to the width of the stained area

increased significantly at the first measurement but did not change much after the second measurement. On the basis of the result, we made a simple assumption that the reaction time was equal to the time between the start of staining and

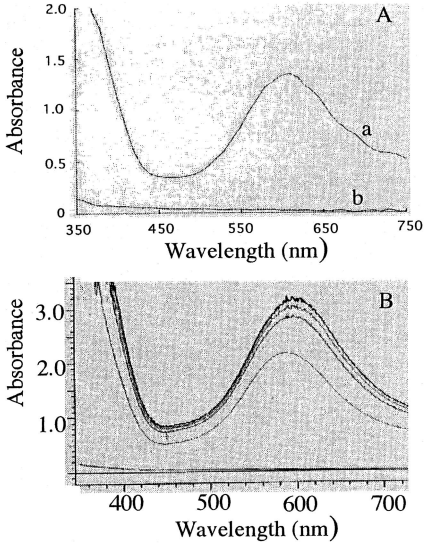

Fig. 4A,B. Absorbance spectra of staining at a hyphal tip and an oxidation product of 3,3′,5,5′-tetramethylbenzidine (TMBZ). **A** Spectra of staining at the hyphal tip (*a*) and the unstained area (*b*) on the hyphae were measured by microspectrophotometry. **B** Spectra of the product of TMBZ oxidized by horseradish peroxidase. Time-dependent increases of the absorption were recorded

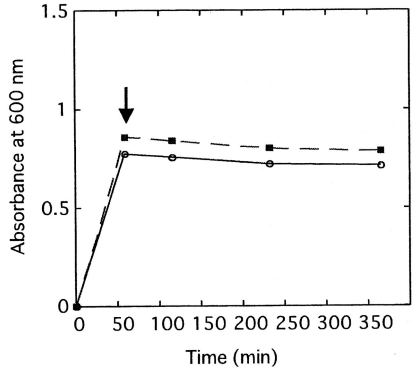

Fig. 5. Change in absorbance over time at one spot on the stained area of the hyphae. *Arrow* indicates the time when the samples were placed on a slide glass and covered with a coverslip. *Closed squares* and *open circles* correspond to each measurement spot

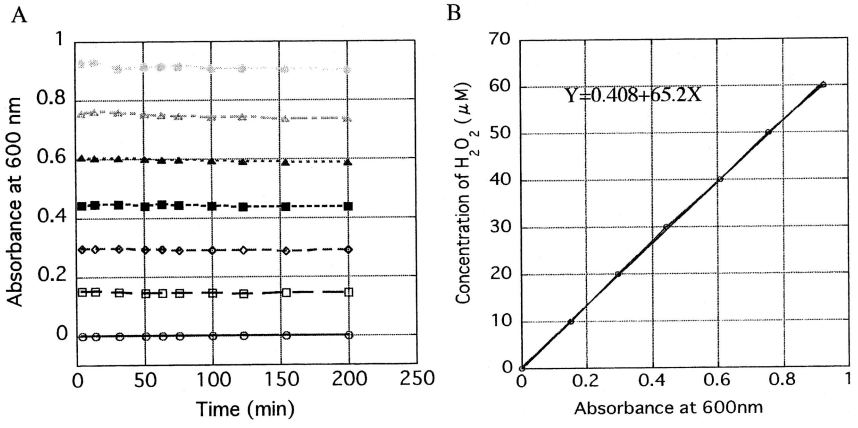

Fig. 6A,B. Relationships between concentration of H_2O_2 and absorbance at 600 nm in oxidation of TMBZ. **A** TMBZ (20%) was oxidized by horseradish peroxidase (1.56×10^{-5} unit/l) at 26°C in the presence of H_2O_2 at different concentrations. Concentrations of H_2O_2: *closed circles*, 60 μM; *open triangles*, 50 μM; *closed triangles*, 40 μM; *closed squares*, 30 μM; *open diamonds*, 20 μM; *open squares*, 10 μM; *open circles*, 0 μM. **B** Calibration curve of H_2O_2 concentration versus absorbance at 600 nm in oxidation of TMBZ. The calibration curve is expressed by the equation in the graph

the end of sample preparation and applied this assumption to the measurements that followed.

Calibration curve

We were unable to measure the molar absorption coefficient directly because the oxidation product of TMBZ was a polymerized substance of unknown molecular weight. Therefore, instead of converting absorbance into the concentration of the product using the molar absorption coefficient, we made a calibration curve to convert the absorbance into the concentration of hydrogen peroxide consumed (Fig. 6). As is described later, laccase and catalase, which also oxidize TMBZ, were present in trace amounts in the extracted enzyme of *P. crassa* WD1694. The calibration curve showed a linear relationship expressed by the following equation:

$$Y = 0.408 + 65.2X \quad (1)$$

where Y is the concentration of hydrogen peroxide (μM), and X is the absorbance at 600 nm (light path length 1 cm). Given that $X = A/d$, where A is the absorbance as measured by microspectrophotometry and d is the light path length, Eq. 1 becomes:

$$Y = 0.408 + 65.2A/d \quad (2)$$

The concentration of hydrogen peroxide consumed was obtained from Eq. 2 and was used for calculation of the catalytic rate.

Calculation of the catalytic rate

From Eq. 2, the catalytic rate can be obtained by Eq. 3.

$$Y/t = \left(0.408 + 65.2A/d\right)/t \quad (3)$$

An example of the catalytic rate calculation of the measurement spot is shown in Fig. 2D. Similarly, we calculated the catalytic rate of the peroxidase reaction at each spot.

Figure 7 compares the calculated catalytic rate of the stained area of the hyphal tips and the peroxidase activity of the culture filtrate. Because the peroxidase activity of the hyphal tips was clearly stained only after 2 to 4 days of cultivation, only the catalytic rate for this period is shown in the graph. The staining on the hyphae of 5-day-old culture was vague and could not be used to determine the catalytic rate. The cultures older than 7 days were not stained. Peroxidase activity at the tips of the hyphae was 723 μM/min at day 2, 794 μM/min at day 3, and 390 μM/min at day 4 (Fig. 7). These values were significantly higher than the activity of the culture filtrate during the entire period of cultivation.

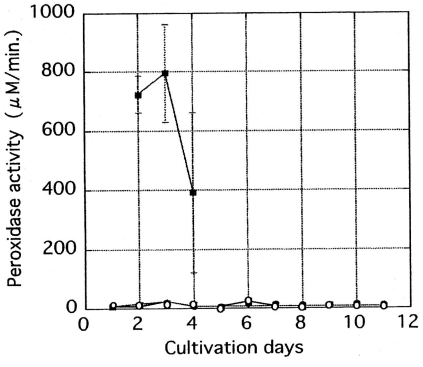

Fig. 7. Time courses of peroxidase activities detected at the hyphal tips and in the culture filtrate of *P. crassa* WD1694. The peroxidase activities are defined as concentration change of each substrate at the hyphal tips or in the culture filtrate. *Closed squares*, peroxidase activities measured as H_2O_2 consumptions at the stained apical tips. The values of 2, 3, and 4 cultivation days are averages of 8, 12, and 18 measurement spots, respectively. *Closed circles*, peroxidase activities in the culture filtrate. The reaction mixture contained 2,2′-azino-bis (3-ethyglbenzthiazoline-6-sulfonic acid) (ABTS; 0.073 mM), H_2O_2 (0.02 mM), and 100 μl of culture filtrate in 50 mM malonate buffer (pH 3.5, 500 μl). *Open circles*, manganese peroxidase activities in the culture filtrate. The reaction mixture contained $MnSO_4$ (5 mM), H_2O_2 (0.02 mM), and 100 μl of culture filtrate in 50 mM malonate buffer (pH 3.5, 500 μl). The values of closed and open circles are averages of three measurements. *Bars* indicate standard deviations

Analysis of enzyme activity

The presence of oxidation activity of Mn (II) in the enzyme extracted from *P. crassa* WD1694 was confirmed by the

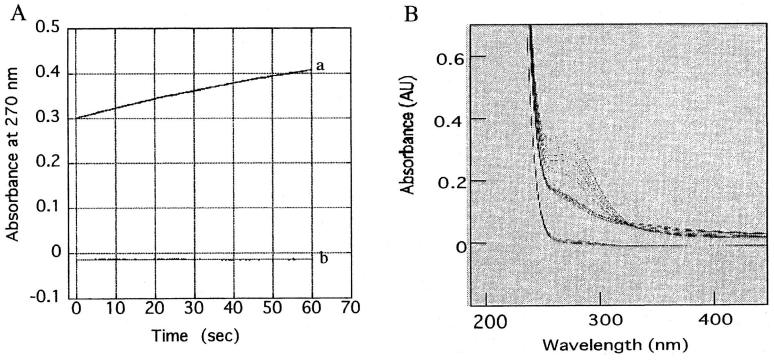

Fig. 8A,B. Manganese peroxidase activities of the enzyme extracted from *P. crassa* WD1694. **A** Peroxidase activities measured with $MnSO_4$: *a*, the reaction mixture contained the enzyme extracted from *P. crassa* WD1694 (100 μl), 5 mM $MnSO_4$, 0.02 mM H_2O_2, in 50 mM malonate buffer (pH 3.5); *b*, the reaction mixture was the same as in *a* but without the enzyme. **B** Production of Mn (III)-malonate from the oxidation of $MnSO_4$ by the enzyme extracted from *P. crassa* WD1694

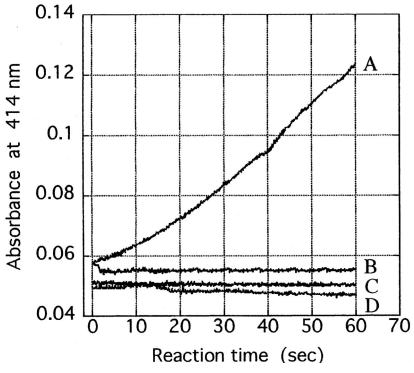

Fig. 9. Measurement of peroxidase activity of the enzyme extracted from *P. crassa* WD1694. *A*, the reaction mixture contained ABTS (0.073 mM), the enzyme extracted from *P. crassa* WD1694 (50 μl), $MnSO_4$ (5 mM), H_2O_2 (0.02 mM), in 50 mM malonate buffer (pH 3.5). *B*, the reaction mixture was the same as *A* without $MnSO_4$. *C*, the reaction mixture was the same as *A* without $MnSO_4$ and H_2O_2. *D*, the reaction mixture contained ABTS (0.073 mM), $MnSO_4$ (5 mM), H_2O_2 (0.02 mM), in 50 mM malonate buffer (pH 3.5)

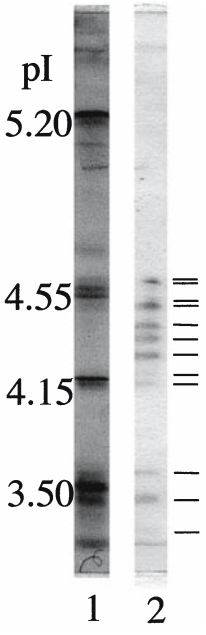

Fig. 10. Analysis of the enzyme extracted from *P. crassa* WD1694 by isoelectric focusing and peroxidase activity staining. Peroxidase activity staining was conducted with 2 mM β-naphtol, 2 mM 3-amino-9-ethylcarbazole, and 200 μM H_2O_2 as substrates. *Lane 1*, pI markers; *lane 2*, peroxidase activity staining. *Bars*, peroxidase bands. The four bands near pI 4.55 are MnPs previously reported

increase of absorbance at 270 nm, which was caused by the Mn (III)-malonate complex produced by oxidation of Mn (II) (Fig. 8).

Figure 9 shows measurement of the peroxidase activity of the enzyme extracted from *P. crassa* WD1694. Subtraction of peroxidase activity with $MnSO_4$ (absorbance increase 0.138/min) and without $MnSO_4$ (absorbance increase 0.0148/min) revealed that manganese-dependent peroxidase activity accounted for 89% of all the peroxidase activity measured. Oxidation activities without addition of H_2O_2 that corresponded to activities of laccase or catalase were at the trace level. LiP activity was not detected (data not shown). The results for the washing solution were similar to those of the dissolved solution. Peroxidase activity was not detected in the culture filtrate, but weak peroxidase activity was detected when the culture filtrate was concentrated. Manganese-dependent peroxidase activity accounted for 96% of all the peroxidase activity in the culture filtrate.

Figure 10 shows the results of isoelectric focusing electrophoresis of the enzyme extracted from *P. crassa* WD1694. Twelve peroxidase bands were confirmed that included MnPs previously reported.[18]

Discussion

We performed peroxidase activity staining to visualize extracellular peroxidase reaction in the culture of *Phanerochaete crassa* WD1694. Although the ligninolytic peroxidases were found in the culture filtrates, the culture medium did not show a color reaction. However, a particularly strong color reaction was observed on the surfaces of the fungal bodies (Fig. 1).

Usually, only the total peroxidase activity in the whole medium can be determined by conventional methods, because culture filtrates or medium extracts are used to measure the peroxidase activity. However, if the peroxidase reaction occurs locally in the culture, the reaction rates at the actual reaction sites will be significantly different from those obtained from whole-culture filtrates or medium extracts. We estimated the in situ peroxidase activity at the tips of the hyphae by microspectrophotometry. This is the first report of the measurement of enzyme activity at the tips of the hyphae, although some reports have demonstrated localization of enzymes in this location.[21–26]

The catalytic rates of the peroxidase reaction at the hyphal tips were calculated on the basis of the strength of the stain. The principle of the calculation was the same as that used to measure enzyme activities with a spectrophotometer. The differences between the current measurement and the conventional one are: (1) it was necessary to obtain the light path length of the measurement point, and (2) we obtained the amount of substrate transformed not directly from the concentration of the product but indirectly from the concentration of hydrogen peroxide consumed.

In calculating the catalytic rate of the peroxidase reaction at the hyphal tips, we used approximations for the light path length and reaction time (Figs. 3, 5). These approximations make it possible to obtain values that are lower than the true values. This is because: (1) the light path length chosen represented the longest possible length on the model, and (2) usually the initial reaction rate is the fastest, because of the consumption of substrate as the reaction proceeds. Furthermore, the culture conditions in the present study did not allow especially high production of MnP, because the culture medium did not contain Tween 80 and manganese. Also, because the medium was a shake culture consisting of only water and UKP, we anticipated that enzymes would diffuse throughout it with ease. Despite these conditions, the catalytic rate of the peroxidase reaction at the hyphal tips was markedly higher than we had anticipated.

In this study, the estimated catalytic rate of the peroxidase reaction at the hyphal tips peaked at 794 μM/min on day 3, and 89% of this corresponded to MnP (Figs. 7, 9). Thus, the catalytic rate of the MnP reaction at the tips of the hyphae was calculated to be 710 μM/min. This reaction rate was much higher than 0.204 and 6.3, the values obtained under the experimental conditions of Hammel and Wariishi, respectively.[27–28] In these reports, degradation of lignin polymer to low molecular weight products occurred in vitro by LiP and MnP activity when the hydrogen peroxide concentration in the reaction solution was limited to quite a low level, i.e., when the rate of peroxidase catalysis was limited. In contrast, when the rate of peroxidase catalysis was high, repolymerization occurred in similar experiments in vitro.[29]

To date, studies of the mechanism of wood decay by white-rot fungi have been performed on the basis of the premise that the enzymes diffuse into the wood. This assumption is consistent with low catalytic rate being more advantageous for making lignin into low molecular weight products than reaction at high catalytic rate. However, it is also obvious that diffusion is disadvantageous for enzymatic reactions that require many factors (such as the MnP reaction, which requires Mn, a chelator of manganese ions, and hydrogen peroxide, as well as the substrate), because the reaction rate is proportional to the concentrations of the individual components.

We have shown that MnP catalyzes the reaction in a concentrated condition, at least for a while. The color reaction was observed for 3 days or more, and we also confirmed that the sites of the color reaction at the hyphal tips became elongated as the culture days passed. The result indicates that MnP of *P. crassa* WD1694 does not simply diffuse from the tips of the hyphae. Instead, as one step in the dynamic movement from inside the cells to the culture filtrate, MnP might be present on the cell walls at the hyphal tips and may also catalyze the reaction. Several extracellular enzymes of fungi, including LiP, have been reported to concentrate at the hyphal tips, so localization at the tips is considered to be one step in the process of secretion of the extracellular enzymes of fungi.[21–26] If this is the case, the result does not contradict the known phenomenon of MnP secretion into culture filtrates.

It is notable that there is a layer outside the hyphae known by various names such as a hyphal sheath or slimes.[30,31] The color reaction that we observed at the hyphal tips corresponded closely to the location of the hyphal sheath in white-rot fungi, as reported by Palmer et al.[32,33] The hyphal sheath is considered to be a polysaccharide layer consisting mainly of β-glucan and is supposed to concentrate and retain the extracellular enzymes of the fungi.[34–36] Localization of MnP and LiP in association with slime materials surrounding hyphae has been confirmed immunologically.[16] MnP activity at the apical tips might be supported by these extracellular materials.

From the results of the experiments in vitro, peroxidase reaction at the tips would cause repolymerization.[29] However, we did observe a very interesting related phenomenon. In the standard curve experiment, the oxidation products aggregated and were precipitated out with a hydrogen peroxide concentration of 100 μM or more in the buffer. In contrast, an almost uniformly blue color reaction was observed at the stained tips of the hyphae (Fig. 1). If the extracellular materials in the fungus support MnP, they may also prevent the aggregation and repolymerization of peroxidase oxidation products.

We demonstrated that the MnP activity of *P. crassa* WD1694 was localized at the tips of the hyphae and catalyzed the reaction. Although the results do not deny the conventional assumption that lignin degradation is caused by diffusion of ligninolytic enzymes into the cell wall of woods, the results clearly showed the existence of the concentrated peroxidase reaction outside the organism. Whether the extracellular materials control the localization of the extracellular enzymatic reaction is unclear, at least reactions under these localized conditions might be different from those that occur under free diffusion conditions. Localization of enzymes that produce H_2O_2 also has the possibility to affect the extracellular peroxidase reaction. More analysis of the extracellular peroxidase reaction under the localized condition in vivo is required. Our study of the relationship between the extracellular peroxidase reaction and extracellular materials of the fungus is now in progress.

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