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ORIGINAL ARTICLE

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Glyoxal oxidase supplies hydrogen peroxide at hyphal tips and on hyphal wall to manganese peroxidase of white-rot fungus *Phanerochaete crassa* WD1694

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Abstract Peroxidase activity staining localized at hyphal tips of white-rot fungus *Phanerochaete crassa* WD1694 that was cultivated in a shaken liquid culture containing unbleached kraft pulp was investigated. Manganese peroxidase was detected in culture solution, washing solution of mycelium, and mycelial extract. Glyoxal oxidase was detected only in mycelial extract and was not detected in culture solution. Addition of hydrogen peroxide generated peroxidase activity staining in the culture solution. Addition of catalase resulted in no staining in the culture of *P. crassa* WD1694, and the addition of methylglyoxal resulted in marked peroxidase activity staining at hyphal tips and on hyphal wall. In an optimized culture, glyoxal oxidase was produced in culture solution. Although the production of glyoxal oxidase and manganese peroxidase had a positive correlation, the secretion and the peak of glyoxal oxidase was observed 3 and 2 days later than those of manganese peroxidase. The N-terminal sequence of purified glyoxal oxidase had very high homology with that of *P. chrysosporium*. These results elucidated the hydrogen peroxide supply system in lignin biodegradation by white-rot fungi, i.e., while remaining on the hyphal cell wall, glyoxal oxidase provides hydrogen peroxide to manganese peroxidase that had diffused into the culture solution beforehand.

Key words Glyoxal oxidase · Lignin · *Phanerochaete crassa* · Manganese peroxidase · Hyphal tip

Introduction

Lignin is a major component of wood cell wall; further, after cellulose and hemicellulose, it is the most abundant component of plant biomass.[1] Unlike many biopolymers that have linear structures with uniformly cross-linked unit molecules, lignin has a three-dimensional structure comprising one to three types of randomly connected phenylpropane units; therefore, the biodegradation of lignin is difficult.[2] White-rot fungi are the only organisms that can degrade and metabolize this recalcitrant material.[3,4]

The degradation of high-molecular-weight lignin is the most important process in a series of steps in the biodegradation of wood, during which this molecule is converted to carbon dioxide. Although low-molecular-weight aromatic compounds produced by the degradation of high-molecular-weight lignin can be decomposed by microorganisms other than white-rot fungi, the degradation of high-molecular-weight lignin in wood is achieved exclusively by white-rot fungi.[5] In vitro mechanisms of the degradation of model compounds of low-molecular-weight lignin dimers and trimers by lignin-degrading enzymes have been established at the molecular level; however, the degradation of high-molecular-weight lignin to low-molecular-weight lignin during the early stages of lignin degradation remains to be clarified.[6,7]

White-rot fungi degrade lignin by the extracellular secretion of lignin-degrading enzymes such as lignin peroxidase (LiP) and manganese peroxidase (MnP). LiP and MnP are similar in the sense that they have wide substrate specificity and high oxidizing potential that enables them to degrade various aromatic compounds.[8–13] These peroxidases are considered to be involved in the early stages of lignin biodegradation, which targets the high-molecular-weight lignin in wood.

The in vivo distribution of LiP and MnP has been determined using immunoelectron microscopy.[14–17] These enzymes are unable to function independently and require hydrogen peroxide for their activity. Therefore, the site at which peroxidase reactions occur (i.e., the site of lignin biodegradation) cannot be solely identified by determining the location of the enzymes themselves.

We investigated peroxidase activity using white-rot fungus *Phanerochaete crassa* WD1694 to characterize the reactions of lignin-degrading peroxidases, which occur during the early stage of lignin biodegradation. We observed

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that staining for peroxidase activity occurred at the hyphal tips of the fungus, and not in the culture filtrate into which the lignin-degrading peroxidases are secreted. Careful examination of the peroxidase reaction at the hyphal tips of WD1694 clearly established that this reaction is attributable to MnP; subsequently, we measured the reaction rate of MnP at this site.[18] Moreover, we reported that the peroxidase reaction occurs at the hyphal tips not because these enzymes are concentrated by slime, but because the peroxidase reaction occurs spontaneously.[19] To elucidate the mechanism underlying the peroxidase reaction in the hyphal tips, we sought to locate and analyze the oxidases that produce hydrogen peroxide, which plays a key role in this reaction.

Materials and methods

Fungal strain

The white rot fungus *Phanerochaete crassa* WD1694 [MAFF420737, *Phanerochaete crassa* (Lev.) Burdsall] was obtained from the culture collection of the Forestry and Forest Products Research Institute.[20]

Pulp and reagents

Unbleached kraft pulp (UKP) was obtained from the Yatsushiro mill of Nippon Paper Industries (Yatsushiro, Japan) and was washed with distilled water before use. A solution of BM Blue POD, Precipitating (3,3′-5,5′-tetramethylbenzidine; TMBZ), a chromogenic substrate for precipitating immunoassays for peroxidase activity, was purchased from Roche Diagnostics (Mannheim, Germany). 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was from Nacalai Tesque (Kyoto, Japan). Horseradish peroxidase type II was from Sigma-Aldrich (St. Louis, MO, USA)

Cultivation conditions

Inoculum of *P. crassa* WD1694 was prepared as described previously.[20] 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 flask was incubated on a rotary shaker at 34°C and 100 rpm during each test day. For production of glyoxal oxidase, 100 ml of culture was placed in a 300-ml Erlenmeyer flask containing 60 mg of KH_2PO_4, 40 mg of K_2HPO_4, 50 mg of $MgSO_4$, 10 mg of $(NH_4)_2HPO_2$, 10 mg of yeast extract, 1 g of glucose, 15 mg of $MnSO_4$, and 0.05% Tween 80. The flask was inoculated with 10 ml of inoculum and incubated as described above.

Staining

Staining for peroxidase activity was carried out by adding TMBZ to mycelial pellets taken from the culture at 20% concentration as described previously.[18]

Microscopic analysis

Samples were observed with a Nikon Eclipse TE 2000-U (Nikon Instruments, Tochigi, Japan).

Enzyme activity

MnP activity was measured with ABTS or $MnSO_4$ as substrate by a modified method of Gold and Glenn.[21] Peroxidase activity was measured by following the increase in absorbance at 414 nm with ABTS as a substrate. The reaction mixture (total volume 500 µl) contained 10 mM acetate buffer (pH 4.0), 100 µl of sample, 40 mg/l ABTS, and 20 µM hydrogen peroxide. Oxidase activities were measured by peroxidase-coupled assay with ABTS as the peroxidase substrate.[22] The reaction mixture (total volume 500 µl) contained 10 mM phosphate buffer (pH 6.0), 70 mM oxidase substrate, 100 µl of sample, 40 mg/l ABTS, and 10 µg of horseradish peroxidase. One unit of enzyme activity was defined as the amount of enzyme that oxidized 1 µmol of substrate per minute.

Purification

All purification procedures were conducted at 4°C. The culture was harvested after cultivation for 4 days and filtered. The culture filtrate was adsorbed on an SP-Sepharose column (GE Healthcare UK, Buckinghamshire, England) and further extracted with 0.5 M NaCl in 10 mM phosphate buffer (pH 6.0). Eluate was desalted and loaded on a DEAE-Sepharose column (1×5 cm) equilibrated with 10 mM phosphate buffer (pH 6.0) and eluted with 10 mM acetate buffer, pH 5.6. The resulting protein solution was desalted and concentrated by ultrafiltration.

Electrophoresis

Isoelectric focusing and SDS-PAGE were conducted using a Multiphor II electrophoresis system (GE Healthcare).

Results

P. crassa WD1694 generates an extracellular peroxidase reaction at hyphal tips. Because the peroxidase reaction requires peroxidase, substrate, and hydrogen peroxide, occurrence of the reaction means the existence of all three elements. The distribution of these three elements should explain why the peroxidase reaction occurs at hyphal tips and not in the culture liquids. Enzymes required in the peroxidase reaction at hyphal tips were investigated to clarify the mechanism of localization of peroxidase reaction at hyphal tips.

Previously, we reported that MnP was the main peroxidase causing peroxidase reaction at hyphal tips of *P. crassa* WD1694 cultivated in a UKP culture.[18] MnP activities from different fractions of *P. crassa* WD1694 cultivated in the

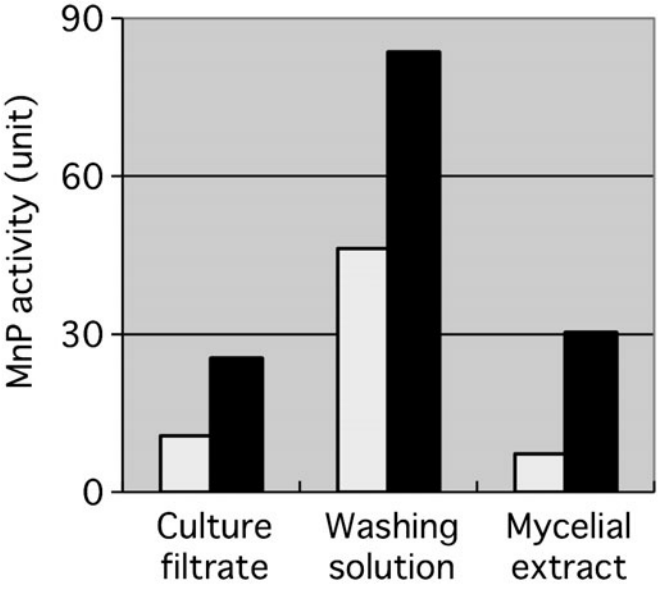


Fig. 1. Manganese peroxidase (*MnP*) activity from different fractions of *Phanerochaete crassa* WD1694 culture. Mycelial pellets were collected by filtration with nylon cloth, thoroughly washed with 10 mM phosphate buffer (pH 6.2), and further extracted gently with 0.1% Tween 80 in the buffer at 4°C. The culture filtrate, washing solution, and extract were collected, dialyzed, and concentrated, respectively. Manganese peroxidase was assayed with 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (*white bar*) or with $MnSO_4$ (*black bar*) as substrate. Manganese peroxidase activity was detected in all the fractions described above

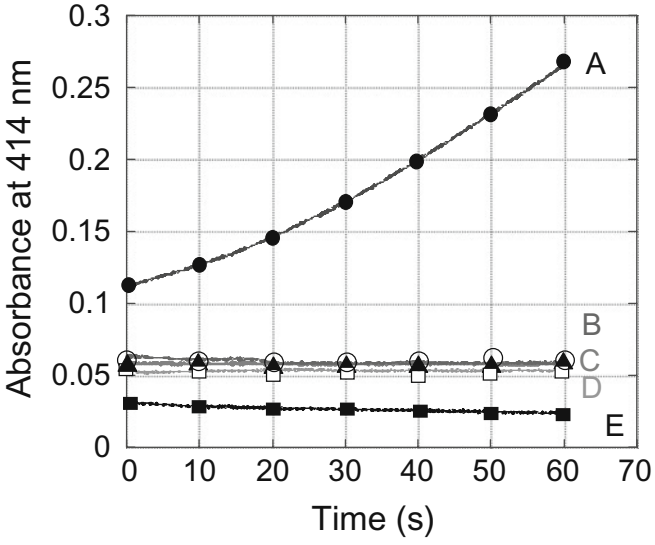


Fig. 2. Measurement of oxidase activities from *P. crassa* WD1694. *Closed circle*, glyoxal oxidase from mycelial extract (*A*); *open circle*, glyoxal oxidase from culture filtrate (*B*); *open square*, glucose oxidase from mycelial extract (*D*); *closed square*, glucose oxidase from culture filtrate (*E*); *closed triangle*, control (*C*). Glyoxal oxidase activity was detected only in mycelial extract and not in the culture liquid. Glucose oxidase was not detected in mycelial extract or culture liquids

UKP culture are compared in Fig. 1. MnP activity was detected from all of these fractions, i.e., culture filtrate, washing solution of mycelial pellets, and extract of mycelial pellets. These results showed that MnP is not the reason of localization of peroxidase reaction at hyphal tips of *P. crassa* WD1694.

Because MnP was not the reason for localization of peroxidase reaction at hyphal tips, we investigated whether generation of hydrogen peroxide was the reason for localization of peroxidase reaction at hyphal tips of *P. crassa* WD1694. In white-rot fungi, oxidases are considered to generate and supply hydrogen peroxide to the ligninolytic peroxidase reaction. Glucose oxidase, glyoxal oxidase (GLX), aryl alcohol oxidase, and methanol oxidase have been reported as sources of hydrogen peroxide in ligninolytic reaction of white-rot fungi.[22–28] Oxidases that could supply hydrogen peroxide to the peroxidase reaction at hyphal tips of *P. crassa* WD1694 were examined (Fig. 2). GLX activity was detected in extract of mycelial pellets of *P. crassa* WD1694 only, and was not detected in culture filtrate. Glucose oxidase was not detected in any fraction. Aryl alcohol oxidase and methanol oxidase were not detected (data not shown). These results suggested that GLX might be the cause of localization of peroxidase reaction at hyphal tips of *P. crassa* WD1694.

Based on the hypothesis that the localized peroxidase reaction at hyphal tips of *P. crassa* WD1694 is caused by the distribution of GLX, the peroxidase activity staining of *P. crassa* WD1694 with addition of substrates of the oxidases was observed and is compared in Fig. 3. When methylglyoxal was added to the mycelial pellets of *P. crassa* WD1694, peroxidase activity staining on the mycelial pellets arose in 30 min, although peroxidase activity staining of the mycelial pellets without addition of methylglyoxal took more than 1 h. Addition of other oxidase substrates, such as glucose, methanol, and 4-methoxybenzyl alcohol did not show any effect on peroxidase activity staining of the mycelial pellets. These results also supported the hypothesis that GLX caused localized peroxidase reaction at hyphal tips of *P. crassa* WD1694.

With these results, we investigated whether hydrogen peroxide generation by GLX was the conclusive reason for the peroxidase activity staining at hyphal tips of *P. crassa* WD1694. The results of peroxidase activity staining with addition of catalase, hydrogen peroxide, or methyl glyoxal are shown in Fig. 4. In the control, which contained TMBZ and an aliquot of culture including mycelial pellets, peroxidase activity staining at the hyphal tips appeared after incubation for 1 h. Addition of excess catalase that consumes hydrogen peroxide resulted in no staining on the mycelial pellets of *P. crassa* WD1694. Addition of hydrogen peroxide to the reaction mixture generated blue deposits of the peroxidase product in the culture solution. The result was consistent with distribution of MnP in the culture filtrate. Addition of methylglyoxal gave the same result as the standard condition that showed marked peroxidase activity staining at hyphal tips and on hyphal wall. The peroxidase reaction extended 200 μm from hyphal tips on the hyphal wall. These results confirmed that the peroxidase activity staining was confined to hyphal tips and hyphal wall because of hydrogen peroxide generation by GLX at hyphal tips and hyphal wall.

Because production of GLX from *P. crassa* WD1694 was very poor in the UKP culture, the culture conditions for GLX production were studied for further analysis of GLX. It has been reported that GLX expression is coordinate with LiP or MnP in *Phanerochaete chrysosporium*.[29] Therefore, the effect of addition of Tween 80 and the concentration of manganese, well known factors that affect the production of MnP, was studied to discover their effect on production of GLX. The results showed that GLX production increased with increasing production of MnP (Figs. 5, 6). The optimized cultivation condition for GLX production was the same as the cultivation condition optimized for MnP production of *P. crassa* WD1694.[30]

Production of GLX and MnP in the optimized culture was studied. Time-course analysis of GLX and MnP activities revealed that the peak and the secretion of the two enzymes did not occur at the same time (Fig. 7). The peak in GLX activity was observed 2 days after the peak in MnP activity.

Four-day-old culture, which showed the highest activity of GLX, was collected and used for purification of GLX. The purified GLX showed one band on SDS-PAGE at a

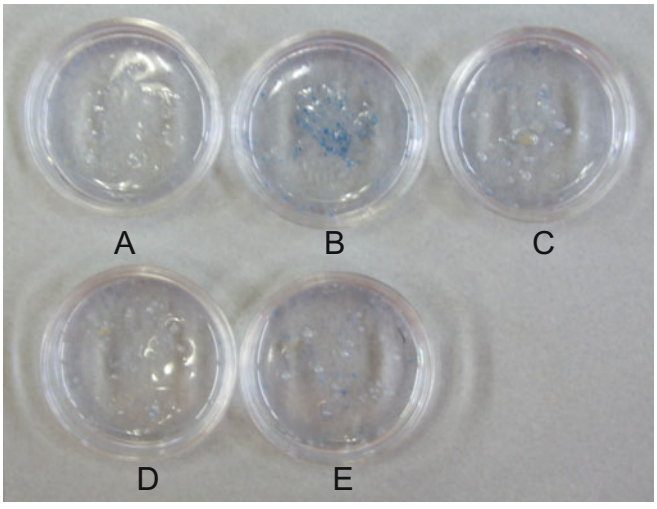


Fig. 3. Peroxidase activity staining of *P. crassa* WD1694 with addition of substrates of oxidases. *A*, control; *B*, methylglyoxal; *C*, glucose; *D,* methanol; *E*, 4-methoxybenzyl alcohol. Addition of methylglyoxal caused peroxidase activity staining on the fungal body of *P. crassa* WD1694 after 30 min (B), although addition of glucose, methanol, or 4-methoxybenzyl alcohol did not affect peroxidase activity staining of *P. crassa* WD1694 (C, D, E)

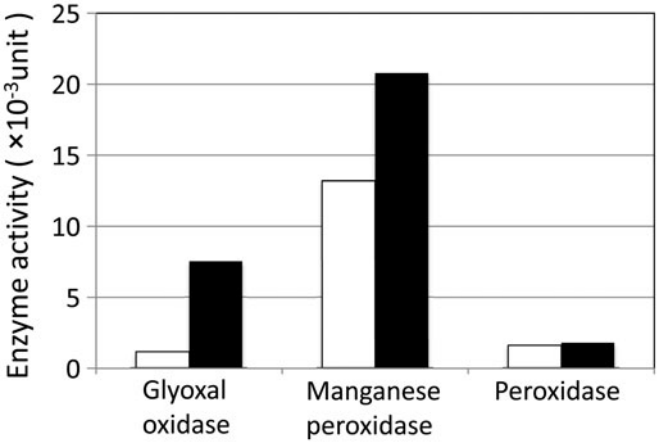


Fig. 5. Effect of Tween 80 on production of glyoxal oxidase and manganese peroxidase. *Black bar*, with addition of Tween 80; *white bar*, without addition of Tween 80

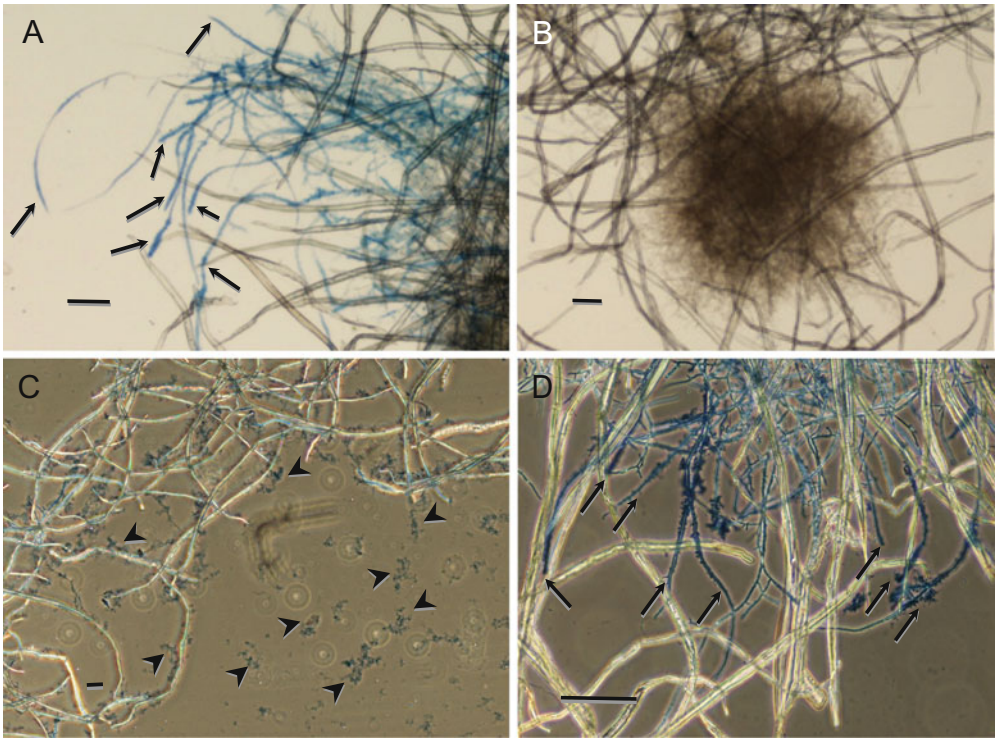


Fig. 4. Glyoxal oxidase resulted in peroxidase activity staining at hyphal tips and on hyphal walls. An aliquot of culture including mycelial pellets were put in a plastic plate and peroxidase activity was stained with 3,3′-5,5′-tetramethylbenzidine (TMBZ). **A** Control; **B** excess of catalase was added before addition of TMBZ; **C** 40 μM hydrogen peroxide was added after addition of TMBZ; **D** 35 mM methylglyoxal was added after addition of TMBZ. *Arrows*, peroxidase activity staining at hyphal tips and on hyphal wall; *arrowheads*, blue deposits of the peroxidase product; *bars* 100 μm

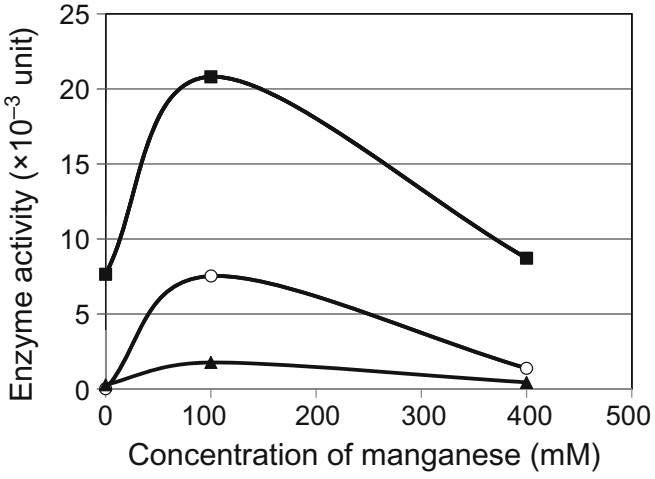


Fig. 6. Effect of manganese concentration on production of glyoxal oxidase and manganese peroxidase. *P. crassa* WD1694 was cultivated for 4 days. *Closed square*, manganese peroxidase; *open circle*, glyoxal oxidase; *closed triangle*, peroxidase

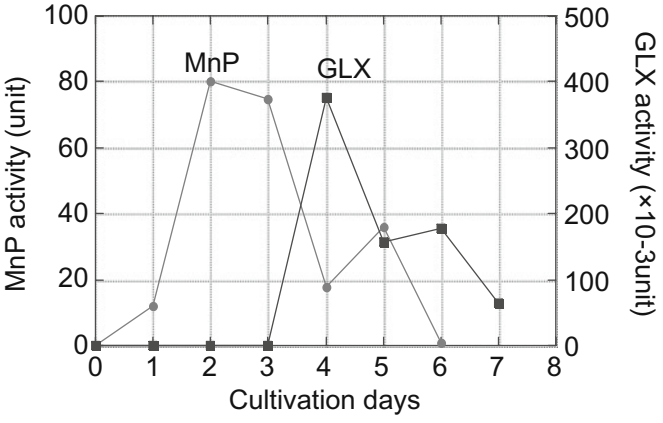


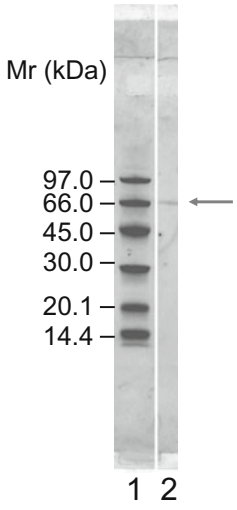
Fig. 7. Time course analysis of glyoxal oxidase (GLX) and manganese peroxidase from *P. crassa* WD1694

molecular weight of 66 kDa (Fig. 8). The analytical result of N-terminal protein sequences was obtained as Ala-Pro-Gly-Trp-Lys-Phe-Thr-Leu-Gln-Pro; the obtained sequence has very high homology with that of *P. chrysosporium*.[29] Previously we reported that N-terminal sequences of MnP from *P. crassa* WD1694 had very high homology with those from *P. chrysosporium*.[30] These results showed that enzymes for lignin biodegradation of *P. crassa* WD1694 are similar to those of *P. chrysosporium*.

Discussion

To elucidate the early stage of the lignin biodegradation process by white-rot fungi, we analyzed peroxidase activity at the hyphal tips of white-rot fungus *P. crassa* WD1694 and we found that MnP and GLX were involved in the peroxidase reaction at the hyphal tips. The increase and decrease of MnP and GLX in *P. crassa* WD1694 were positively correlated when the culture conditions were changed to enhance the production of GLX. It has been reported that GLX is the only oxidase that is secreted extracellularly and the expression of GLX is coordinate with LiP and MnP in *P. chrysosporium*.[22,29] These results showed that GLX is the most important enzyme producing hydrogen peroxide that is supplied to MnP in the ligninolytic peroxidase reaction of *P. crassa* WD1694.

Fig. 8. SDS-PAGE of purified glyoxal oxidase from *P. crassa* WD1694. *Lane 1*, marker; *lane 2*, glyoxal oxidase



The analysis of enzymes involved in the peroxidase reaction at hyphal tips of *P. crassa* WD1694 revealed that MnP was distributed extensively both in the culture fluids and on the fungal body; however, GLX was detected from extraction of mycelial pellets of *P. crassa* WD1694 only. Detailed analysis of peroxidase staining of *P. crassa* WD1694 showed that although MnP was localized both in culture liquid and on the fungal body, GLX worker only at hyphal tips and on hyphal cell wall. When culture contained Tween 80 and manganese sulfate for enhancing production of GLX, GLX was detected in culture liquid; however, the secretion and the peak of GLX activity were detected 3 and 2 days later, respectively, than those of MnP activity. All these results showed that the physiological regulation involved MnP and GLX. At first, MnP is secreted from the tips of hyphae and then diffuses extracellularly. Thereafter, GLX is secreted at the hyphal tips and flows along the cell walls of the hyphae and starts to provide hydrogen peroxide as soon as it emerges from the tips to the outside of the fungal body. Consequently, GLX provides hydrogen peroxide for MnP before it can diffuse, i.e., GLX remains on the cell walls of the hyphae where it has been secreted. As a result, staining for peroxidase activity in WD1694 leads to staining at the hyphal tips. This fact is in accordance with the bulk-flow hypothesis suggested for filamentous fungi as a mechanism of protein secretion.[31,32] According to this hypothesis proteins are secreted from the hyphal tips and they flow along the outside of cell walls; subsequently, the proteins are secreted from the cell walls rather than from the hyphal tips.[31,32] Cai et al. reported that the endoglucanase and β-glucosidase of *Volvariella volvacea* remain on the outside of cell walls at a distance of 60–70 μm from the hyphal tips.[33]

The results from our peroxidase staining indicate that the distribution of GLX in WD1694 extends as far as 200 μm from the hyphal tips.

These results provide a detailed picture of a previously unknown process by which GLX yields hydrogen peroxide. Moreover, these results provide the first visual evidence for the hydrogen peroxide supply system in lignin biodegradation by white-rot fungi.

Although the amount of production of MnP and GLX had a positive correlation, MnP and GLX had a time lag between the secretion and maximum production. The positive correlation of MnP and GLX suggested that these two enzymes are controlled together. Therefore, the time lag between MnP and GLX might be also controlled this way out of necessity. As a hypothesis, the time lag may be a consequence of a shift in the timing of production and transportation of MnP and GLX to avoid their synergistic action in fungal cells. Both MnP and GLX are strong oxidizing enzymes having wide substrate specificity, and synergistic action of the two enzymes results in a strong oxidation reaction that can degrade lignin. If these two enzymes were to exist in the same place simultaneously in fungal cells, there would be a high risk that the organism itself would be oxidized. In another words, oxidative enzymes for lignin biodegradation might be regulated as a part of an active oxygen-controlling system of the organism. The order of secretion, in which MnP is secreted prior to the secretion of GLX, has the following advantage. Due to the difference in molecular weight between MnP and hydrogen peroxide, hydrogen peroxide can "catch up" with the diffused MnP. In contrast, the diffusion of MnP is unable to "catch up" with the diffusion of hydrogen peroxide. Therefore, to make the reaction as efficient as possible without wasting the produced enzymes, MnP is secreted before GLX, and the latter produces the hydrogen peroxide.

Our results showed the physiological mechanism of lignin biodegradation by white-rot fungi. The strong oxidation reaction at hyphal tips and on fungal cell wall results in strong oxidation on the surface of wood cell walls, because fungi glow when attached to woody cell walls. The mechanism is consistent with the morphological mechanism of white rot, i.e., a progressive thinning of the wood cell wall by white-rot fungi originating from the lumen.[34]

In this study, we suggested that the strong ligninolytic peroxidase reaction is restricted to an area on the surface of hyphae. However, our results do not deny the diffusion of oxidative radicals that are generally considered to be the lignin biodegradation mechanism of white-rot fungi. Peroxidase activity staining showed that addition of methylglyoxal shortened the time required for peroxidase active staining, suggesting that the initiation of GLX reaction was dependent on the substrates. In our previous report, we showed that an active species that oxidizes a peroxidase substrate is first produced at the tips of the hyphae, and then occurs in the slime via diffusion when slime exists around the hyphae. Initiation of oxidative reaction in lignin biodegradation might be triggered by association of GLX and substrates of GLX. If substrates of GLX exist far from hyphae, then the reaction also occurs far from hyphae.

Our results demonstrated the hydrogen peroxide supply system in lignin biodegradation by white-rot fungi and the mechanism of action of the peroxidase reaction. The results indicate the possibility that the lignin biodegradation system of white-rot fungi extends beyond the simple production and secretion system for oxidizing enzymes and is controlled from within the fungus body as part of the active oxygen-controlling system. Thus, these findings present us with a new challenge for elucidating the mechanism of lignin biodegradation by white-rot fungi.

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