Involvement of an Extracellular H$_2$O$_2$-Dependent Ligninolytic Activity of the White Rot Fungus *Pleurotus ostreatus* in the Decolorization of Remazol Brilliant Blue R

B. R. M. VYAS and H. P. MOLITORIS*

Botanical Institute, University of Regensburg, D-93041 Regensburg, Germany

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During solid-state fermentation of wheat straw, a natural lignocellulosic substrate, the white rot fungus *Pleurotus ostreatus* produced an extracellular H$_2$O$_2$-requiring Remazol brilliant blue R (RBBR)-decolorizing enzymatic activity along with manganese peroxidase, manganese-independent peroxidase, and phenol oxidase activities. The presence of RBBR was not essential for the production of RBBR-decolorizing enzymatic activity by *P. ostreatus*, because this activity was also produced in the absence of RBBR. This RBBR-decolorizing enzymatic activity in crude enzyme preparations of 14- and 20-day-old cultures exhibited an apparent $K_m$ for RBBR of 31 and 52 µM, respectively. The RBBR-decolorizing enzyme activity was maximal in the pH range 3.5 to 4.0. This activity was independent of manganese, and veratryl alcohol had no influence on it. Manganese peroxidase of *P. ostreatus* did not decolorize RBBR. This H$_2$O$_2$-dependent RBBR-decolorizing enzymatic activity behaved like an oxygenase possessing a catalytic metal center, perhaps heme, because it was inhibited by Na$_2$S$_2$O$_5$, NaCN, NaN$_3$, and depletion of dissolved oxygen. Na$_2$S$_2$O$_5$ brought an early end to the reaction without interfering with the initial reaction rate of RBBR oxygenase. The activity was also inhibited by cysteine. Concentrations of H$_2$O$_2$ higher than 154 µM were observed to be inhibitory as well. Decolorization of RBBR by *P. ostreatus* is an oxidative process.

Basidiomycetous white rot fungi make up the only group of microorganisms known to completely degrade and mineralize lignin (56). Ligninolytic cultures of the white rot fungi studied secrete manganese peroxidase (MnP), lignin peroxidase (LP), laccase, and H$_2$O$_2$-generating enzymes in various combinations (20, 33). Purified MnP and LP have the ability to catalyze partial depolymerization of lignin in vitro (18, 55). These enzymatic activities have also been implicated in the degradation of a variety of xenobiotic compounds (2, 9, 17).

Most of the studies on the production of ligninolytic enzymes have used only few species of white rot fungi. Therefore, a number of researchers have stressed the need to investigate the ligninolytic enzyme systems of other white rot fungi (6, 9, 33, 35, 52). For this purpose, several screening methods involving dyes such as Poly B-411, Poly R-478, and Remazol brilliant blue R (RBBR) have been reported (12, 13, 36, 43). Recently, Pasti and Crawford (36) observed a correlation between RBBR decolorization and ligninolytic ability of microorganisms. Ollikka et al. (34) demonstrated decolorization of RBBR by crude LP activity of the white rot fungus *Phanerochaete chrysosporium*.

Dyes constitute an essential part of our civilization, but they cause environmental pollution from the effluents of dyestuff industries. Dyes are usually aromatic and heterocyclic compounds and are often recalcitrant, some of them being toxic and even carcinogenic. It is therefore important to develop methods for the decolorization and degradation of dyes in industrial effluents. Ligninolytic cultures of several white rot fungi have been reported to degrade and decolorize various dyes (12, 15, 34, 37, 41, 52). Involvement of MnP and LP has been demonstrated in the degradation pathway of some of the dyes (15, 42). However, white rot basidiomycetes are expected to differ in their ability and capacity to degrade dyestuffs on the basis of qualitative and quantitative differences in the production of these enzymes. Pasti-Grigsby et al. (37) showed a way of producing environment-friendly dyes. They demonstrated that introduction of lignin substructures into certain dyes rendered them more susceptible to degradation by ligninolytic enzymes of white rot fungi and by other microorganisms. Such dyes may also provide the means to differentiate between lignin-specific peroxidative enzymatic activities (37).

RBBR is an industrially important dye and is used frequently as a starting material in the production of polymeric dyes. RBBR, an anthracene derivative, represents an important class of often toxic and recalcitrant organopollutants. It structurally resembles certain polycyclic aromatic compounds which are substrates of ligninolytic peroxidases (17, 32, 38). Therefore, we tested a number of basidiomycetous fungi for their ligninolytic activity by using RBBR as an indicator substrate. We observed that the *Pleurotus* species investigated decolorized RBBR (49), although they do not have the ability to produce LP (27, 39). In our report, we present the results of our investigations of ligninolytic RBBR-decolorizing enzymatic activity produced by *Pleurotus ostreatus* during solid-state fermentation of wheat straw, a natural lignocellulosic substrate.

MATERIALS AND METHODS

**Chemicals.** RBBR, 3-methyl-2-benzothiazolinone hydrazone hydrochloride, and 3-dimethylaminobenzoic acid were purchased from Sigma, St. Louis, Mo.

**Microorganism.** *P. ostreatus* 3004, a strain of edible cultivated mushroom, was maintained on malt agar slants at 5°C. Transfers were made on malt agar plates, and the strain was cultivated at 28°C.

**Inoculum preparation.** Seven agar discs punched from cultures growing on malt agar plates were used to inoculate 500-ml Erlenmeyer flasks containing 80 ml of malt extract medium (45). These flasks were incubated at 28°C on a reciprocal shaker (92 strokes · min$^{-1}$, 3.7-cm amplitude) for 7 to 10 days. The entire contents of the flasks were homogenized in a Waring blender (three times at low speed for 20 s) and diluted with 100 ml of sterile distilled water before being used as the inoculum.

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*Corresponding author. Phone: 49-941-943-3131. Fax: 49-941-943-3106. Electronic mail address: Hans-Peter.Molitoris@biologie.Uni-Regensburg.de.*
TABLE 1. Extracellular ligninolytic enzymatic activities produced by *P. ostreatus* during solid-state fermentation of wheat straw

<table>
<thead>
<tr>
<th>Incubation time (days)</th>
<th>Ligninolytic enzymatic activity (U/g [dry wt] of straw)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MnP</td>
</tr>
<tr>
<td>7</td>
<td>0.275</td>
</tr>
<tr>
<td>14</td>
<td>0.809</td>
</tr>
<tr>
<td>20</td>
<td>0.156</td>
</tr>
</tbody>
</table>

* —, not detectable.

Production and preparation of extracellular enzyme extract. Solid-state fermentation of wheat straw, a natural lignocellulosic substrate, was used for the production of RBBR-decolorizing enzyme activity. It was carried out by the method of Vyas et al. (50). The contents of a set of five to seven flasks were harvested (after 7, 14, and 20 days) and immediately processed for the preparation of extracellular enzyme extract as described previously (50). Total proteins in the enzyme extract were precipitated with ammonium sulfate (80% saturation). Precipitates separated upon centrifugation were dissolved in a minimum amount of 0.1 M phosphate buffer (pH 6.5) and desalted by passage through Sephadex G-25 PD-10 columns (Pharmacia, Uppsala, Sweden). The desalted sample was concentrated further by ultrafiltration with a PM-10 membrane (Amicon) with a 10-kDa cut-off limit. The clear supernatant obtained upon centrifuging the concentrate (in an Eppendorf 3200 centrifuge at 5°C for 3 min), referred to as enzyme sample (preparation), was used for various biochemical analyses as discussed below.

Denaturation of crude enzyme preparation. Heat-denatured enzyme used in some reactions was prepared by incubating 500 to 1,000 μl of crude enzyme preparation in Eppendorf vials in boiling-water bath for 2 to 3 min.

Biochemical analyses. MnP, manganese-independent peroxidase (MIP), and laccase activities were determined as described previously (50) by spectrophotometric measurement of oxidation of MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride) plus DMAB (3-dimethylaminobenzoic acid) as chromogen. LP activity was estimated spectrophotometrically by the method of Tien and Kirk (44), following oxidation of veratryl alcohol to veratraldehyde. Veratryl alcohol oxidase (VAO) was determined by spectrophotometric measurement of formation of veratraldehyde in an H2O2-independent oxidation of veratryl alcohol at pH 4.5 and 3.0. One unit of enzyme activity is defined as the amount of activity that will produce 1 μmol of the product per min upon oxidation of the substrates in the above reaction mixtures (RMs).

RESULTS

*P. ostreatus* produced extracellular RBBR-decolorizing enzymatic activity along with MnP, MIP, and laccase (Table 1) during solid-state fermentation of unamended wheat straw in the absence of RBBR under the experimental conditions described above. LP and VAO activities were not detectable.

RBBR decolorization by the concentrated crude extract of extracellular enzymes prepared from *P. ostreatus*-infested wheat straw occurred only when H2O2 was added to the RM (Fig. 1A). In the absence of native enzyme sample, addition of H2O2 had no influence on the RBBR decolorization reaction. RBBR decolorization did not occur in the RMs containing heat-denatured enzyme sample and H2O2, showing that H2O2 alone does not decolorize (Fig. 1B). Moreover, addition of native enzyme sample to the RM containing heat-denatured enzyme preparation in Eppendorf vials in boiling-water bath for 2 to 3 min.

![FIG. 1. RBBR-decolorizing enzyme activity produced by *P. ostreatus* during solid-substrate fermentation of wheat straw. (A) Dependence on H2O2; (B) control with heat-killed enzyme; (C) effect of addition of native enzyme preparation to the complete test system containing heat-denatured enzyme.](http://aem.asm.org/.../14/2/fig1.jpg)
enzymesampleand H2O2initiated RBBR decolorization (Fig. 1C).

Visible spectra of the RM recorded at regular time intervals showed that RBBR is undergoing degradation and that the decrease in \(A_{595}\) is not due to physical removal (e.g., precipitation). This fact was highlighted when the changes in visible spectra of the RM at various points of the decolorization reaction (Fig. 2A and B) were compared with those of aqueous solutions containing different concentrations of RBBR (Fig. 2C).

RBBR decolorization reaction proceeded initially at a uniform rate, but as the reaction progressed further, it tended to slow, perhaps because of the exhaustion of RBBR (Fig. 1) since limiting amounts of H2O2 did not affect the initial reaction rate (as discussed below). When a small amount of enzyme sample was used, the reaction proceeded at a lower rate and the color of the RM changed from blue to pink to yellow to colorless. Moreover, by adding only limiting amount of H2O2, the reaction can be made to stop at any particular stage of the decolorization reaction and can be reinitiated again by adding a fresh aliquot of H2O2 till the RM becomes colorless (Fig. 2A). These color changes were H2O2 dependent and are perhaps brought about by the same enzymatic activity. Similar color changes were also observed in the agar medium containing RBBR during the growth of P. ostreatus (49). Complete decolorization of RBBR is therefore not a single-step reaction.

When H2O2 was present at less than 132 \(\mu\)M, the reaction proceeded normally (as in Fig. 1A) but stopped sooner and was followed by a transient increase in \(A_{595}\) (Fig. 2A). Thus, in the case of exhaustion or limitation of H2O2, a temporary reverse reaction occurred and the color change appeared to take place in the opposite direction, i.e., from yellow to pink or pink to purple.

**Influence of the amount of enzyme sample and RBBR concentration.** The RBBR decolorization reaction rate increased proportionally with the increasing amount of enzyme sample, resulting in a quicker completion of the reaction. RBBR-decolorizing enzyme activity exhibited Michaelian kinetics. RBBR decolorization rates increased with increases in RBBR concentration up to 75 to 100.5 \(\mu\)M. Further increases in RBBR concentration had no influence on the initial decolorization rate, but the reaction did not reach completion as the primary substrate H2O2 became limiting. H2O2-dependent RBBR-decolorizing enzymatic activity showed an apparent \(K_m\) of 31 and 52 \(\mu\)M for RBBR in enzyme sample preparations on days 14 and 20, respectively.

**Influence of H2O2.** RBBR-decolorizing enzymatic activity is strictly H2O2 dependent (described above). With increasing amounts of H2O2 up to 132 to 154 \(\mu\)M in the RM, RBBR decolorization lasted longer. H2O2 concentrations in this range were optimal for RBBR-decolorizing enzyme activity (Fig. 3A). This indicated that H2O2 serves as the primary substrate. H2O2 concentrations higher than 154 \(\mu\)M inhibited decolorizing activity. The inhibitory influence of H2O2 increased with increases in H2O2 concentration in the RM (Fig. 3A). Simultaneous increases in RBBR and H2O2 concentrations in the RM did not change this behavior (Fig. 3B). To bypass the problem of H2O2 limitation and also inhibition of RBBR-decolorizing enzymatic activity by excess H2O2, the glucose oxidase-glucose system could be used to generate H2O2 in the RM.

**Relationship between apparent H2O2 consumption and RBBR decolorization.** As evidenced in Table 2, RBBR-decolorizing enzymatic activity consumed 1 mol of H2O2 in decolorizing 1 mol of RBBR during the initial (linear) phase of the
reaction. However, as the reaction progressed, with the decrease in reaction rates, the apparent H₂O₂ requirement to decolorize 1 mol RBBR increased, as evidenced from the ratio of RBBR decolorized to H₂O₂ consumed (Table 2). This provided additional evidence to support our observation that the degradation of the products of RBBR decolorization is also H₂O₂ dependent, indicating that RBBR decolorization occurs in multistep H₂O₂-dependent reactions. Thus, with time, an increase in the apparent H₂O₂ requirement for the decolorization of 1 mol of RBBR during the reaction was observed.

Influence of pH. The RBBR decolorization reaction catalyzed by crude enzyme preparations from day 14 and day 20 samples proceeded optimally in the pH range of 3.5 to 4.0 (Fig. 4). With a 14-day enzyme preparation, reaction rates decreased rapidly below pH 3.5 while some enzymatic activity was still retained at pH 5.5. Reaction was not perceptible at either pH 2.5 or 6.5. The crude enzyme preparation from day 20 exhibited a slightly different activity profile over the pH range 3.0 to 4.0 from that of the day 14 enzyme preparation.

Influence of Mn²⁺ (as MnSO₄). The RBBR decolorization reaction by the above-described H₂O₂-requiring enzyme activity occurred in the absence of exogenously added Mn²⁺. The enzyme sample used in RBBR decolorization had been desalted and was therefore expected to be free of Mn²⁺. A 100 μM concentration of Mn²⁺ in the RM completely inhibited the reaction. When various lower concentrations of Mn²⁺ were used in the standard RM, RBBR decolorization occurred without any change in the initial reaction rate but terminated sooner with increasing Mn²⁺ concentration (Fig. 5). The inhibitory influence of Mn²⁺ on RBBR-decolorizing enzyme activity at pH 3.5 was less profound than that at pH 4.0 (Fig. 6). MnP activity was observed to be 40% higher at pH 4.0 than at pH

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>Amt of H₂O₂ consumed (nmol)</th>
<th>Amt of RBBR decolorized (nmol)</th>
<th>RBBR-decolorizing enzyme activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.314</td>
</tr>
<tr>
<td>1.0</td>
<td>44</td>
<td>45.80</td>
<td>1.040</td>
</tr>
<tr>
<td>2.0</td>
<td>88</td>
<td>85.02</td>
<td>0.966</td>
</tr>
<tr>
<td>4.5</td>
<td>176</td>
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<td>0.813</td>
</tr>
<tr>
<td>6.0</td>
<td>220</td>
<td>173.64</td>
<td>0.789</td>
</tr>
<tr>
<td>9.0</td>
<td>264</td>
<td>185.73</td>
<td>0.703</td>
</tr>
</tbody>
</table>

Table 2. Relationship between RBBR decolorization and H₂O₂ consumption during the course of the RBBR decolorization reaction catalyzed by crude enzyme prepared from wheat straw-decaying cultures of *P. ostreatus*.

Fig. 3. Influence of various concentrations of H₂O₂ (22, 44, 88, 110, 132, 154, 176, 220, 330, and 440 μM for curves 1 through 10, respectively, at 100.5 μM RBBR [A] and 88, 132, 176, 220, 330, and 440 μM for curves 1 through 6, respectively at 150.75 μM RBBR [B]) on the decolorization reaction.

Fig. 4. Influence of pH on RBBR-decolorizing enzymatic activity produced on days 14 and 20 by *P. ostreatus* in a solid-state fermentation of wheat straw.
3.5. Concentrations of Mn\textsuperscript{II} higher than 7.5 \(\mu\text{M}\) were observed to be inhibitory to the initial reaction rate of RBBR-decolorizing enzymatic activity in the enzyme preparation from day 14 but not in that from day 20 (Fig. 5 and 6). Mn\textsuperscript{II} activity is higher on day 14 than day 20 (Table 1), as was the inhibitory influence of Mn\textsuperscript{II} on the RBBR-decolorizing enzyme activity of the day 14 enzyme preparation.

**Influence of veratryl alcohol.** Addition of veratryl alcohol did not exert any influence on the decolorizing activity. RBBR decolorization occurred equally well in the absence and presence of veratryl alcohol.

**Influence of inhibitors acting on oxidative enzymes.** (i) Sodium metabisulfite. Sodium metabisulfite, which inhibits enzymatic reactions requiring oxygen, did not have any influence on the initial reaction rate of RBBR decolorization but brought about an early end to the reaction (Fig. 7). With increasing concentrations of Na\textsubscript{2}S\textsubscript{2}O\textsubscript{5}, the reaction stopped sooner and did not reach completion.

(ii) Cysteine. Cysteine, a classical inhibitor of phenol oxidase-type activities, also exhibited an inhibitory influence on RBBR-decolorizing activity (Fig. 8A). The inhibitory influence increased with increasing concentrations of cysteine in the RM. Cysteine retarded the reaction rate in the initial stages, and then, with time, the reaction rate gradually picked up before slowing once again, resulting in a sigmoid curve (Fig. 8B).

(iii) Sodium azide. RBBR-decolorizing activity was inhibited by sodium azide, a classic inhibitor of oxidative enzyme reactions (Fig. 9). With increasing concentrations of sodium azide, the reaction needed longer to reach completion. Higher concentrations stopped the reaction before it reached completion.

(iv) Sodium cyanide. The metal-binding agent sodium cyanide is a classic peroxidase inhibitor. It exhibited an inhibitory influence on the RBBR-decolorizing activity similar to that of sodium azide (Fig. 9). The extent of inhibition of RBBR-decolorizing enzymatic activity was observed to be a function of sodium cyanide concentration in the RM.

Table 3 gives a comparative account of inhibition of MIP and RBBR-decolorizing enzymatic activity by various inhibitors. The initial reaction rate of MIP activity was observed to be inhibited by Na\textsubscript{2}S\textsubscript{2}O\textsubscript{5}, unlike that of RBBR-decolorizing activity.
While RBBR-decolorizing activity was more sensitive to NaN₃, MIP activity was more sensitive to NaCN. Cysteine inhibited both these activities almost equally well; MIP activity did not show a sigmoid pattern in its reaction curve.

**RBBR-decolorizing activity under conditions of oxygen depletion.** RMs were flushed with argon to displace dissolved oxygen from the RM and the headspace of the cuvettes. The anaerobic conditions thus achieved retarded the initial reac-

**DISCUSSION**

Several investigators (e.g., Highley [21]) who used the cellulose-RBBR test (40) for detecting cellulase activity observed that certain fungi decolorized RBBR with or without its release from the cellulose-RBBR complex. Pasti and Crawford (36) observed a correlation between RBBR decolorization and the ligninolytic ability of microorganisms. Recently, Thorne (43) proposed the use of cellulose-RBBR complex for detecting both cellulolytic and ligninolytic activities based on the release and decolorization of RBBR. The phenomenon of RBBR decolorization has been attributed to the LP activity of another white rot fungus, *Phanerochaete chrysosporium* (34). Decolorization of other dyes such as Poly B-411 and Poly...
TABLE 3. Inhibition of MIP and RBBR oxygenase activity by various inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc (µM)</th>
<th>% Inhibition of MIP (day 14)</th>
<th>RBBR oxygenase Day 14</th>
<th>Day 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaN₃</td>
<td>2.5</td>
<td>14</td>
<td>32</td>
<td>49</td>
</tr>
<tr>
<td>NaN₂SO₃</td>
<td>20.2</td>
<td>29</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NaCN</td>
<td>40.4</td>
<td>100</td>
<td>34</td>
<td>41</td>
</tr>
<tr>
<td>Cysteine</td>
<td>37.5</td>
<td>68</td>
<td>75</td>
<td>74</td>
</tr>
</tbody>
</table>

* Percent inhibition of the initial reaction rate.

R-478 has also been used as an indicator and measure of ligninolytic activity (9, 12).

This prompted us to use cellulose-RBBR and xylan-RBBR complexes to screen basidiomycetes for their ligninolytic activity. *P. ostreatus* decolorized RBBR before its release (49), suggesting preferential degradation of lignin. *Pleurotus* species decolorized RBBR (49) even though they do not produce LP (27, 39).

Against this background, we resorted to solid-state fermentation of wheat straw by *P. ostreatus* to investigate the ligninolytic RBBR-decolorizing enzymatic activity for several reasons: (i) if ligninolytic enzymes are involved in RBBR decolorization, they would be expected to be produced by *P. ostreatus* during its growth on wheat straw; (ii) this method permitted us to show that synthesis of RBBR-decolorizing enzymes by *P. ostreatus* is independent of the presence of RBBR; and (iii) we were able to avoid the time-consuming process of medium optimization for the production of RBBR-decolorizing enzyme activity.

During its growth on wheat straw, *P. ostreatus* produced ligninolytic activities of MnP, MIP, and laccase. It also produced concomitantly and in significant quantities an extracellular H₂O₂-dependent enzyme activity that catalyzed the decolorization of RBBR. LP and VAO activities were not detectable. This enzymatic activity, which was produced in the absence of RBBR, completely decolorized RBBR in vitro in a multistep reaction. The RBBR-decolorizing enzymatic activity exhibited an apparent *Kₘ* of 31 µM (day 14) and 53 µM (day 20) for RBBR. While lower concentrations of H₂O₂ (<132 µM) were insufficient for the reaction to reach completion, higher concentrations (>154 µM) were inhibitory to the RBBR-decolorizing activity. Similar observations of the inhibitory influence of excess H₂O₂ have been cited for other oxidases (46).

The pH requirement for optimal activity of the RBBR-decolorizing enzyme activity of *P. ostreatus* is on the acidic side, similar to that of ligninolytic peroxidases MnP and LP. The RBBR-decolorizing enzyme activity showed optimal pH in the range of 3.5 to 4.0. MnP and LP have optimal enzymatic activity at pH 4.5 and 2.5, respectively (28, 44). Like the RBBR-decolorizing enzymatic activity of *P. ostreatus*, crude LP of *Phanerochaete chrysosporium* decolorized RBBR optimally in the pH range 3.5 to 4.0 (34). RBBR-decolorizing enzymatic activity has a broad working pH range.

MnIII is a substrate, redox coupler, and mediator of MnP (11, 53, 54). Therefore, the activity of MnP is strictly dependent on MnIII. The fact that RBBR-decolorizing enzyme activity described here is independent of MnIII and was observed to be inhibited by MnIII leads us to conclude that it is different from MnP and that the MnP activity (of *P. ostreatus*) is not involved in the decolorization of RBBR.

The fact that MnIII did not influence the initial reaction rate, in combination with the observation that addition of MnIII resulted in an earlier termination of the RBBR decolorization reaction, led to the supposition that the presence of MnP (H₂O₂ and MnII requiring) in the enzyme preparation might be setting up appropriate conditions for competition between the two oxidative enzymatic activities for the common substrate H₂O₂. Experimental evidence verifying our supposition came from two different sources. One line of evidence was obtained by analyzing the influence of parameters affecting MnP activity, such as pH, on RBBR-decolorizing activity in the presence of various amounts of MnIII. There is virtually no change in RBBR-decolorizing activity in the pH range 3.5 to 4.0. However, MnP activity decreased by 40% at pH 3.5, which permitted RBBR-decolorizing activity to last longer at pH 3.5 than at pH 4.0, furnishing evidence of the competition between the two enzymatic activities for H₂O₂. Another line of evidence was obtained from the extent of inhibitory influence of MnIII at different MnP activity levels. MnP activity was observed to be much higher on day 14 than on day 20, as was the inhibitory influence of MnIII on RBBR-decolorizing activity of day 14 and day 20 enzyme samples. Thus, the inhibitory influence of MnIII on RBBR-decolorizing enzymatic activity was due to the competition offered by MnP for the common substrate H₂O₂.

The dependence of the enzymatic decolorization of RBBR on H₂O₂ and the inhibition of RBBR-decolorizing enzyme activity by cysteine, NaN₃, and NaCN, which are known to inhibit oxidative enzyme reactions, indicated that RBBR decolorization by *P. ostreatus* is indeed an oxidative process. Inhibition of RBBR oxidation under conditions of oxygen depletion showed that molecular oxygen is essential for the oxidation of RBBR in this enzymatic reaction. On the other hand, sodium metabisulfite terminated the reaction without interfering with the initial reaction rates. This provided addi-
tional evidence for the necessity for molecular oxygen in the RBBR oxidation. Dependence on H₂O₂, inhibition by higher H₂O₂ concentrations, and inhibition by cyanide of RBBR-oxidizing enzymatic activity suggested not only the presence of a catalytic metal center such as heme in the enzyme protein but also the possibility that this catalytic metal center plays a role in RBBR-oxidation by this enzyme. Our results led us to conclude that an H₂O₂-requiring oxygenase, perhaps heme containing, is involved in the decolorization of RBBR by P. ostreatus.

The differences in sensitivity to various inhibitors, apparent Kᵣ for RBBR, and activity profiles over the pH range 3.0 to 4.0 of RBBR-decolorizing enzyme activity in the enzyme preparations from days 14 and 20 suggest that the enzyme proteins in the two preparations possess different catalytic properties. It is very likely that this enzyme activity is also represented by isoenzymes similar to other ligninolytic enzymatic activities of white rot fungi (8, 22, 47).

Ligninolytic cultures of certain white rot fungi have been shown to produce pyranose 2-oxidase (5, 50), glyoxal oxidase (25), and aryl alcohol oxidase (39) as major H₂O₂-generating enzymes which furnish H₂O₂, required by some ligninolytic enzymatic activities. P. ostreatus produces extracellular pyranose 2-oxidase activity during its growth on wheat straw (51) and has been shown to produce VAO, another H₂O₂-generating enzymatic activity (7, 39), which was not detectable under our experimental conditions. These activities could provide H₂O₂, which is also required by the RBBR oxygenase activity of P. ostreatus.

LP, which is able to decolorize RBBR (34), also catalyzes oxygenation reactions and was therefore originally described as an H₂O₂-requiring oxygenase (14, 44). LP isoenzymes have been shown to decolorize some other dyes in the presence or absence of veratryl alcohol (34), a mediator of several LP-catalyzed reactions (19). Moreover, LP isoenzymes differ in their potential to decolorize other dyes and in their specificities for dyes used as substrates (34). However, information pertaining to the oxygen requirement during RBBR decolorization by LP and the ability to use RBBR as its substrate is missing, depriving us of any chance to compare the catalytic oxidation of RBBR by the RBBR oxygenase activity of P. ostreatus with that by the LP of Phanerochaete chrysosporium.

The nonspecific nature of oxidation of lignin by white rot fungi led to the supposition that activated oxygen species might have a role in this process (16), and the ensuing research provided evidence for the production of such activated oxygen species (10, 30). However, following the discovery of nonspecific LP and MnP enzymes in ligninolytic cultures of Phanerochaete chrysosporium, the role of activated oxygen species in liginolysis was viewed skeptically, even though some evidence remained. Recently, Backa et al. (3) provided evidence for the production of hydroxyl radicals by Phanerochaete chrysosporium and several brown rot fungi during their growth on wood or cellulose. Barr et al. (4) proposed an LP-catalyzed mechanism for the production of hydroxyl radicals in ligninolytic cultures of Phanerochaete chrysosporium. P. ostreatus also has been shown to cleave α-keto-γ-methylbutyric acid to ethylene at the onset of and during lignin mineralization in a solid-state fermentation, indicating production and involvement of hydroxyl radicals in lignin degradation (23, 24). Moreover, the amount of ethylene produced was similar in the presence and absence of manganese in the medium, and significant degradation and mineralization of lignin also occurred under conditions of manganese deficiency (24). This led Kerem et al. (23, 24) to suggest that during liginolysis by P. ostreatus that lacks LP activity, enzymatic activity other than those of MnP and LP is involved in the production of free radicals by this fungus.

Therefore, the ability of RBBR oxygenase of P. ostreatus to produce hydroxyl radicals should be investigated further.

P. ostreatus, which oxidizes RBBR, also oxidized anthracene to anthraquinone (48), another LP substrate. RBBR (1-amino-9,10-dihydro-9,10-dioxo-4-[3-[(2-sulfooxethyl)sulfonyl]phenyl)amino]-2-anthracensulfonic acid (disodium salt)) is an anthracene derivative and structurally resembles other LP substrates such as anthracene, 2-hydroxy-1,4-naphthoquinone, and anthracenediethanol (17, 32, 38). It is therefore very likely that *Pleurotus* species which do not produce veratryl alcohol-oxidizing peroxidases (27, 39) also produce enzymes similar to LP in their ability to oxidize other lignin substructures such as RBBR, i.e., these *Pleurotus* species therefore being able to produce lignin oxigenases (peroxidases) with different specificities, as has been suggested by Nerud et al. (33).

Ligninolytic cultures of several white rot fungi have been reported to produce peroxidases different from MnP and LP; these are sometimes referred to as MIP (33, 52). There is little information pertaining to the role of this enzymatic activity in the degradation of lignin (7, 29, 31) or xenobiotic compounds (1, 42). P. ostreatus also produced MIP activity along with other ligninolytic activities under our experimental conditions. The MIP and RBBR-oxidizing enzymatic activities responded differently to the inhibitors used, except cyanide, to which the two activities were equally sensitive but differed in the mode of inhibition. These data, however, do not permit us to draw any definite conclusions about whether these two activities are associated with the same enzyme protein.

H₂O₂-dependent RBBR oxygenase of *P. ostreatus* is different from the oxygenase system of *Trametes versicolor* (26), which oxidized in an H₂O₂-independent reaction a large number of di- and trisubstituted benzene rings containing at least one hydroxy group, as well as oxidizing lignin. Thus, the RBBR-decolorizing enzymatic activity of *P. ostreatus* described in this report appears to be a hitherto undescribed ligninolytic enzymatic activity, since it is different from the known ligninolytic enzymatic activities of MnP, LP, and ferulic acid oxygenase of white rot fungi. However, several questions that have arisen as a consequence of our studies must be addressed before any sensible comparison between RBBR oxygenase of *P. ostreatus* and other ligninolytic enzymes (LP, MIP, and ferulic acid oxygenase) of the white rot fungi can be made.

It is therefore important to investigate the role of RBBR oxygenase in the degradation of lignin and xenobiotics. Isolation and biochemical characterization of RBBR oxygenase from *P. ostreatus* are being planned in our laboratory. Moreover, RBBR, which resembles anthraquinone, may provide us with an opportunity to investigate whether the RBBR oxygenase of *P. ostreatus* participates in the anthraquinone degradation pathway followed by white rot fungi.

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