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Production and Purification of Remazol Brilliant Blue R Decolorizing Peroxidase from the Culture Filtrate of *Pleurotus ostreatus*

KWANG-SOO SHIN,[1]* ILL-KYOON OH,[1] AND CHANG-JIN KIM[2]

Department of Microbiology, College of Sciences, Taejon University, Taejon 300-716,[1] and Korea Research Institute of Bioscience and Biotechnology, Taejon 305-600,[2] Republic of Korea

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An extracellular H_2O_2-requiring Remazol brilliant blue R (RBBR) decolorizing enzymatic activity was found in the culture medium of *Pleurotus ostreatus*. The enzymatic activity was maximally obtained in idiophase, and the optimum C/N ratio was 24. High C/N ratios repressed the enzymatic activity, and addition of veratryl alcohol had no effect on the production of enzyme. The enzyme was purified by ammonium sulfate fractionation, Sephacryl S-200 HR chromatography, DEAE Sepharose CL-6B chromatography, and Mono Q chromatography. The purification of RBBR decolorizing peroxidase, as judged by the final specific activity of 6.00 U/mg, was 54.5-fold, with a yield of 9.9%. The molecular mass of the native enzyme determined by gel permeation chromatography was found to be about 73 kDa. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that the enzyme was a monomer with a molecular mass of 71 kDa. The enzyme was optimally active at pH 3.0 to 3.5 and at 25°C. Under standard assay conditions, the apparent K_m values of the enzyme toward RBBR and H_2O_2 were 10.99 and 32.97 μM, respectively. The enzyme had affinity toward various phenolic compounds and artificial dyes, and it was inhibited by $Na_2S_2O_5$, potassium cyanide, NaN_3, and cysteine. The absorption spectrum of the enzyme exhibited maxima at 407, 510, and 640 nm. The addition of H_2O_2 to the enzyme resulted in an absorbance decrease at 407 and 510 nm.

White rot lignin-degrading fungi have been widely studied in order to obtain ligninolytic enzymes useful for biotechnological applications. Although it is still unknown which enzyme is able to degrade lignin in vivo, two groups of peroxidases, lignin peroxidase (LiP) and Mn-dependent peroxidase (MnP), seem to be associated with ligninolytic activity (15). These enzyme systems have been studied extensively in *Phanerochaete chrysosporium* and other white rot fungi, such as *Panus tigrinus* (7), *Panus brevispora* (23), *Trametes versicolor* (4, 10), *Phlebia radiata* (12), *Bjerkandera adusta* (14), and *Phlebia ochraceofulva* (33).

Pleurotus ostreatus produces another type of extracellular peroxidase (PoP) (11) and glucose oxidase as an H_2O_2-generating system (30). PoP catalyzed the H_2O_2-dependent oxidation of a variety of lignin model compounds by one electron to generate reactive intermediates that spontaneously fragment (9, 31). Kinetic and biophysical properties of purified PoP have been determined (11). The enzyme was found to be a glycoprotein of approximately 14 kDa and to consist of two identical subunits. The substrate specificity of PoP was similar to that of MnP in that it could not oxidize nonphenolic compounds, but it was not dependent on manganese for catalytic activity.

A correlation has been demonstrated between lignin degradation and Remazol brilliant blue R (RBBR) decolorization. RBBR is an industrially important dye and resembles certain polycyclic aromatic compounds which are substrates of LiP (8, 20, 24). Vyas and Molitoris (34) reported that the white rot fungus *P. ostreatus* produced an extracellular H_2O_2-requiring RBBR decolorizing enzymatic activity along with MnP, manganese-independent peroxidase, and phenol oxidase activities during solid-state fermentation of wheat straw. They suggested that RBBR decolorizing enzymatic activity appeared to be an undescribed ligninolytic activity and may play an important role in the degradation of lignin and xenobiotics. Recently, we observed H_2O_2-dependent RBBR decolorizing enzymatic activity in the culture filtrate of *P. ostreatus* in chemically defined medium and suggested that another extracellular peroxidase is involved in RBBR decolorization (13). These observations led to studies on the demonstration and purification of the enzyme activity involved in RBBR decolorization.

In this report, we describe the optimum conditions of enzyme production and some properties of the purified enzyme from the culture filtrate of *P. ostreatus.*

MATERIALS AND METHODS

Organism. *P. ostreatus* NFFA 2ml was obtained from the Institute of Microbiology, Seoul National University. The fungus was maintained on malt extract agar plates at 4°C, from which they were transferred to new plates and incubated at 28°C for 6 days before use.

Enzyme production. Incubations were carried out at 28°C by inoculating 100 ml of malt extract broth in shaken 250-ml flasks with *P. ostreatus* mycelia. On day 3 of incubation, the mycelia were harvested, washed with sterile distilled water, suspended, and homogenized. This homogenate was used to inoculate (dry weight of 0.3 g) the medium described by Commanday and Macy (3) and cultured statically at 28°C. To improve peroxidase production, veratryl alcohol was added to the culture broths at a final concentration of 0 to 4.0 mM. To study the effect of the C/N ratio on peroxidase production, the initial glucose and ammonium concentrations were treated differently as follows: 40 mM/100 mM (C/N = 2.4), 40 mM/10 mM (C/N = 24), 40 mM/1.0 mM (C/N = 240), 40 mM/0.1 mM (C/N = 2,400), and 40 mM/0.01 mM (C/N = 24,000).

Chemicals. Sephacryl S-200 HR, DEAE Sepharose CL-6B, RBBR, and M_r markers for gel filtration chromatography were supplied by Sigma. Prepacked Mono Q HR 5/5 and Superose 12 HR 10/30 columns were obtained from Pharmacia and used on a Pharmacia fast protein liquid chromatography (FPLC) system. Standard M_r proteins for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Boehringer Mannheim. All other chemicals used were of the highest quality generally available.

Enzyme assay. RBBR decolorizing peroxidase activity was assayed by measuring the decrease in A_{592}. An aliquot of enzyme solution was incubated in 3 ml of 20 mM sodium acetate buffer (pH 4.0) containing 50 μM RBBR and 0.1 mM H_2O_2 at 25°C. The reaction was initiated by addition of H_2O_2. One unit of

* Corresponding author. Mailing address: Department of Microbiology, College of Sciences, Taejon University, Taejon 300-716, Republic of Korea. Phone: 82 42 280 2439. Fax: 82 42 280 2436.

TABLE 1. Purification of RBBR decolorizing peroxidase from the culture filtrate of *P. ostreatus*

Procedure	Total activity (U)	Total protein (mg)	Sp act (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	36.4	329.4	0.11	100	1
$(NH_4)_2SO_4$ treatment	10.0	28.8	0.35	27.5	3.2
Sephacryl S-200 HR chromatography	9.9	12.8	0.77	27.2	7.0
DEAE Sepharose CL-6B chromatography	5.6	1.7	3.29	15.4	29.9
Mono Q HR 5/5 chromatography	3.6	0.6	6.00	9.9	54.5

enzyme activity was defined as the amount of enzyme required for oxidation of 1 μmol of RBBR per min in the reaction mixtures. A molar extinction coefficient of RBBR (ε_{592} = 6,170 cm^{-1} M^{-1}) was used to compute units of enzyme activity.

Protein and carbohydrate estimation. Protein was determined by a modification of the method of Lowry et al. (19), with bovine serum albumin as a standard protein. The carbohydrate content was determined as described by Dubois et al. (5), using glucose as a standard carbohydrate.

Purification of enzyme. Unless otherwise stated, all procedures were performed at 4°C. The culture fluid was used for enzyme purification after filtration through Whatman no. 1 filter paper. The aqueous solution was centrifuged to remove particles. Solid ammonium sulfate was added to give 60% saturation. The mixture was stirred for 30 min, and the precipitated proteins were removed by centrifugation at 28,000 × *g* for 20 min. The supernatant was adjusted to 80% saturation with solid ammonium sulfate and stirred for 30 min. The precipitated proteins were collected by centrifugation at 28,000 × *g* for 20 min and redissolved in a minimal volume of 20 mM sodium acetate buffer, pH 4.5 (buffer A). The redissolved protein was loaded onto a Sephacryl S-200 HR column (2.5 by 120 cm) equilibrated with buffer A. The enzyme was eluted with the same buffer at a flow rate of 30 ml/h, with collection of 10-ml fractions. These samples were assayed for enzyme activity, and those containing the highest activity were selected. The retained fractions were concentrated in an Amicon stirred cell by using a PM-10 membrane and applied to a DEAE Sepharose CL-6B column (2.8 by 18.5 cm) equilibrated with buffer A. The column was subsequently washed with 200 ml of equilibration buffer; the enzyme fractions were then eluted with a linear concentration gradient of 0 to 0.25 M NaCl in the same buffer at a flow rate of 30 ml/h. The pooled active fractions were further purified by the Pharmacia FPLC system. The enzyme solution was applied to an analytical Mono Q HR 5/5 column (volume, 1 ml) equilibrated in buffer A. After a wash with the same buffer, protein was eluted with a gradient of 0 to 0.15 M NaCl in 25 ml of buffer A. Fractions of 0.5 ml were collected and assayed as described above.

Molecular mass determination. The native M_r of the purified protein was estimated by gel filtration chromatography on a Superose 12 HR 10/30 column (volume of 24 ml) mounted on the Pharmacia FPLC system. Standard proteins of known molecular masses were used to calibrate the column: apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). SDS-PAGE was performed on a 5 to 20% polyacrylamide gel as described by Laemmli (18). As standard molecular mass proteins, β-galactosidase (116.4 kDa), fructose-6-phosphate kinase (85.2 kDa), glutamate dehydrogenase (55.6 kDa), aldolase (39.2 kDa), and triosephosphate isomerase (26.6 kDa) were used. Coomassie brilliant blue G-250 was used for staining as described by Neuhoff et al. (21).

Isoelectric focusing. The isoelectric point of the enzyme was determined on an isoelectric focusing gel with a gradient of pH 2.5 to 5 (125 by 65 mm, 0.4 mm thick; Bio-Rad, Richmond, Calif.) as described previously (11).

Kinetic calculations. Rates of substrate oxidation were determined by spectrophotometry, using molar extinction coefficients of various substrates. The molar extinction coefficients determined in 20 mM sodium acetate buffer (pH 4.0) were 6,170 cm^{-1} M^{-1} at 592 nm for RBBR, 14,500 cm^{-1} M^{-1} at 591 nm for crystal violet, and 186,100 cm^{-1} M^{-1} at 617 nm for malachite green. The extinction coefficients of phenol red, sinapinic acid, syringic acid, and vanillic acid were used as described previously (11). The K_m values were determined by measuring initial velocity. All kinetic studies were performed at least three times, and the kinetic data were fitted to hyperbola by using the Michaelis-Menten equation. The best values were determined by a linear least-squares regression analysis.

RESULTS AND DISCUSSION

Production of enzyme. The production of enzyme was highly dependent on C/N ratios. At high C/N ratios, the level of enzyme activity decreased slowly. In media containing 10 mM ammonium (C/N = 24), a high level of enzyme was produced, reaching a maximum of 1.52 U/mg. In *P. chrysosporium*, LiP activity was maximally obtained in cultures exposed to air when the C/N ratio was 7.47 (28). This finding suggested that the influence of the C/N ratio on the production of RBBR decolorizing peroxidase in *P. ostreatus* was similar to the effect on the production of LiP of *P. chrysosporium*. It was reported that different nitrogen sources can produce different effects on ligninolytic catabolic systems (16, 26). However, there was no

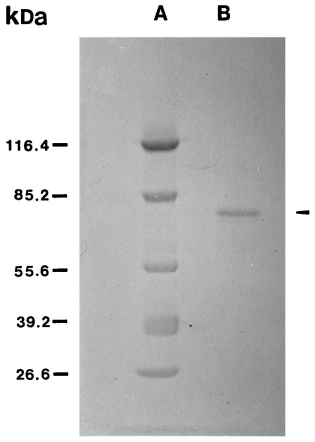

FIG. 1. SDS-PAGE of RBBR decolorizing peroxidase. Lane A, standard molecular mass marker proteins (β-galactosidase [116.4 kDa], fructose-6-phosphate kinase [85.2 kDa], glutamate dehydrogenase [55.6 kDa], aldolase [39.2 kDa], and triosephosphate isomerase [26.6 kDa]); lane B, purified enzyme. Relative mobilities of the standard markers versus common logarithms of their molecular masses were plotted. With the linear regression output, the molecular mass of RBBR decolorizing peroxidase was estimated.

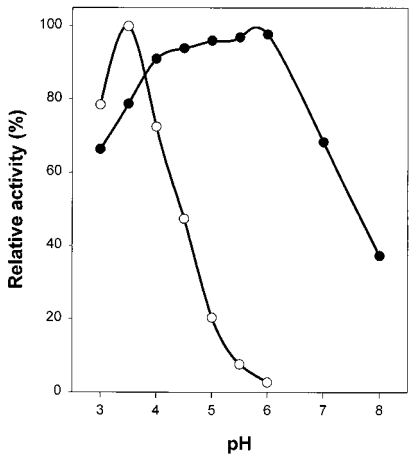

FIG. 2. pH dependence of RBBR decolorizing peroxidase activity. Enzyme activity was measured at various pH values under the standard assay conditions with 0.1 M citrate-phosphate buffer (○). The enzyme was incubated in the same buffer at the indicated pH values at 30°C for 1 h, and the remaining activity was measured (●).

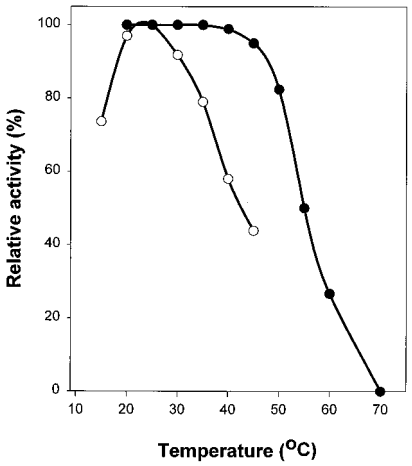

FIG. 3. Effect of temperature on *P. ostreatus* RBBR decolorizing peroxidase activity. Enzyme activity was assayed in 20 mM sodium acetate buffer (pH 4.0) at various temperatures (○). The enzyme was incubated at various temperatures for 1 h, and the remaining activity was measured (●).

significant difference in RBBR decolorization with different nitrogen sources in this fungus (13).

When veratryl alcohol was added to the medium at different concentrations (0 to 4.0 mM), no differences were observed in the production of enzyme. The difference in mycelial growth rate was also negligible. Although *P. ostreatus* and *P. sajor-caju* produced veratryl alcohol oxidases in the ligninolytic cultures (1, 29), veratryl alcohol may not play a central role in the production of ligninolytic enzymes in *P. ostreatus.*

Like other ligninolytic enzyme systems, RBBR decolorizing peroxidase was excreted during the idiophase at optimal conditions. Enzyme activity had reached its maximum level (1.54 U/mg) at day 8, and the level of enzyme activity decreased rapidly after day 9.

Purification of enzyme. The enzyme was purified from the culture filtrate of *P. ostreatus* as summarized in Table 1. The purification of enzyme as judged by the final specific activity of 6.00 U/mg was 54.5-fold, with a recovery of 9.9%.

Molecular mass and carbohydrate content. Purified enzyme was used for gel filtration chromatography. A single peak of RBBR decolorizing peroxidase activity was eluted, and the measured peak elution volume corresponded to a native molecular mass of 73 kDa. When the enzyme was subjected to SDS-PAGE, a single band of 71 kDa was found (Fig. 1). These data indicate that the enzyme is monomeric and differs from the *P. chrysosporium* peroxidases (LiP and MnP) and the dimeric PoP found in the same fungus. The enzyme contains approximately 38% carbohydrate by weight, estimated by the method of Dubois et al. (5). Its carbohydrate content is very high compared to that of LiP from *P. chrysosporium* (13%), which was reported by Tien and Kirk (32), and that of lignase-I (21%) and that of peroxidase-M2 (17%) from *P. chrysosporium* BKM-F-1767, which were reported by Paszczynski et al. (25).

Isoelectric point and N-terminal amino acid sequences. The isoelectric point of the enzyme was determined to be 3.0 by isoelectric focusing. The N-terminal amino acid was not detected by the Edman procedure, indicating that the N terminus of this enzyme may be blocked, as is the case for PoP of *P. ostreatus* (11).

Effects of pH and temperature. The effect of pH on enzyme activity was examined at pH values ranging from 3.0 to 6.0. From the bell-shaped profile shown in Fig. 2, the optimal pH for enzyme activity was estimated to be around 3.5. Enzyme reaction rates decreased rapidly above pH 4.0. The enzyme was stable at 30°C for 1 h in neutral pH ranging from 4.0 to 6.0 (Fig. 2).

The temperature optimum was between 20 and 30°C. The highest rate of enzymatic reaction was observed at 25°C, and rapid inactivation occurred above 35°C. The optimum temperature of the enzyme was lower than that of PoP, which had its optimum activity at 40°C. When the enzyme was incubated at various temperatures for 1 h at pH 4.0, it was found to be stable below 50°C, and 100% of the initial activity was lost at 70°C (Fig. 3).

Kinetic properties. The relationship between enzyme activity and substrate concentration was of the Michaelis-Menten type. The apparent K_m and V_{max} values for RBBR determined from a Lineweaver-Burk plot were estimated to be 10.99 μM and 0.58 μmol min^{-1} mg^{-1} under the standard enzyme assay conditions; the K_m and V_{max} values for H_2O_2 were 32.97 μM and 8.37 μmol min^{-1} mg^{-1}, respectively. Vyas and Molitoris (34) suggested that there were two RBBR decolorizing enzyme proteins which possess different catalytic properties and that the production of enzymes was culture time dependent. The enzymatic activity in crude enzyme preparations of 14- and 20-day-old cultures exhibited apparent K_ms for RBBR of 31 and 52 μM, respectively. These two enzyme preparations also exhibited differences in sensitivity to various inhibitors and activity profiles over the pH range from 3.0 to 4.0. In this respect, our results led us to conclude that an enzyme kineti-

TABLE 2. Substrate specificity of RBBR decolorizing peroxidase of *P. ostreatus*

Substrate	V_{max} (μmol min^{-1} mg^{-1})	K_m (μM)	Relative activity (%)
RBBR	0.58	10.99	100
Sinapinic acid	1.65	20.56	284.5
Crystal violet	1.43	6.04	246.6
Phenol red	0.49	17.41	84.5
Syringic acid	0.32	28.28	55.2
Vanillic acid	0.28	54.40	48.3
Malachite green	0.16	29.44	27.6
Methoxyphenoxy propanediol	NR[a]	NR	0.0
Veratryl alcohol	NR	NR	0.0

[a] NR, not reacted.

TABLE 3. Effects of various chemicals on the activity of RBBR decolorizing peroxidase of *P. ostreatus*

Reagent	Concn	% Inhibition
NaN_3	10 μM	27
	100 μM	95
	1 mM	100
Potassium cyanide	10 μM	5
	100 μM	76
	1 mM	97
$Na_2S_2O_5$	10 μM	0
	100 μM	100
	1 mM	100
L-Cysteine	10 μM	62
	100 μM	94
	1 mM	97

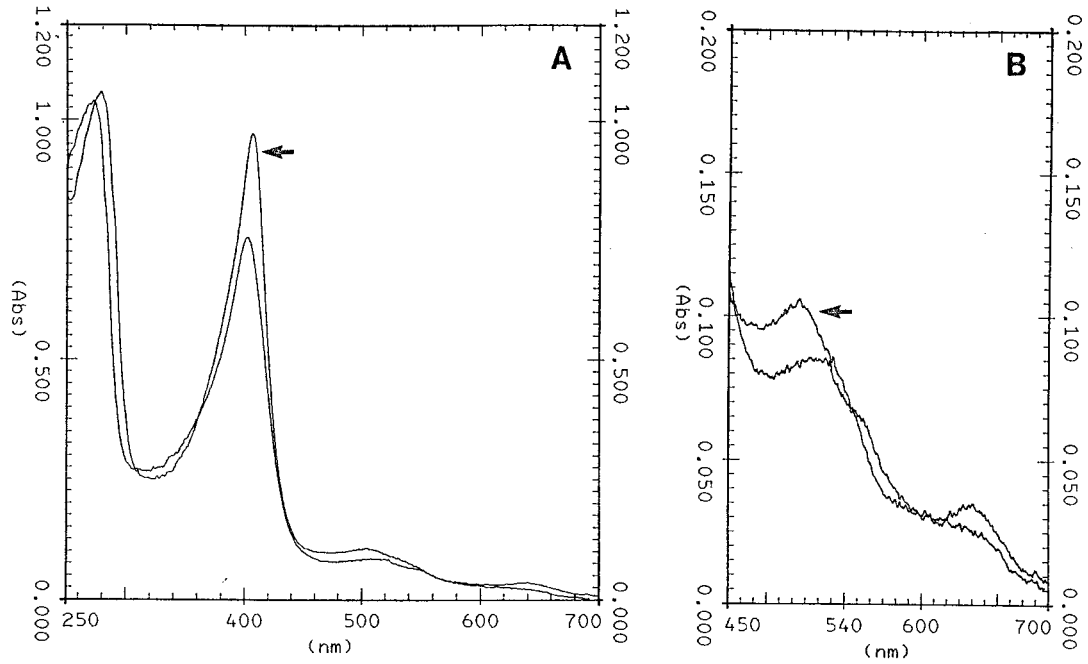

FIG. 4. Absorption (Abs) spectrum of RBBR decolorizing peroxidase of *P. ostreatus*. Purified enzyme was dissolved in 0.1 M sodium acetate buffer, pH 4.5 (A) The oxidized form was obtained by adding 0.1 mM H_2O_2 to the reduced form (arrow). The enlarged spectrum in the wavelength region indicated is shown in panel B.

cally different from those of *P. ostreatus* may be purified in solid-state fermentation of wheat straw.

Substrate specificity. The ability of the enzyme to catalyze oxidation of aromatic compounds and dyes was investigated. The enzyme was able to oxidize several phenolic compounds and artificial dyes in standard assay conditions (Table 2). RBBR, sinapinic acid, and crystal violet are the preferred substrates of the enzyme. The apparent K_ms determined for sinapinic acid and crystal violet were 20.56 and 6.04 μM, respectively. This peroxidase has high affinity toward phenolic compounds containing dimethoxyl and *p*-hydroxyl groups directly attached to the benzene ring, such as sinapinic acid and syringic acid. However, the enzyme does not oxidize veratryl alcohol, which is a well-known substrate of LiP. Also, this peroxidase shows no affinity for nonphenolic compounds, which are readily attacked by LiP. Among the tested dyes, the enzyme shows highest affinity toward the triphenyl methane dye crystal violet. LiP isozymes of *P. chrysosporium* decolorize various dyes in the presence of veratryl alcohol, and the decolorizing ability decreased greatly when veratryl alcohol was omitted from the reaction mixture. In contrast, omission of veratryl alcohol from the reaction mixture had no effect on the ability of the crude LiP to decolorize the dyes. We initially concluded that those apparent contradictions were due to the fact that there was veratryl alcohol left in the crude enzyme (22, 24) but found that even with purified enzyme, the addition of veratryl alcohol did not influence the decolorization of dyes by our enzyme.

These results suggest that the mode of action of this enzyme is different from that of LiP of *P. chrysosporium* (15).

Effects of inhibitors. The effects of several specific peroxidase inhibitors were studied, and the results are shown in Table 3. The enzyme was inhibited by potassium cyanide and NaN_3, known as typical hemoprotein inhibitors. In the presence of 100 μM NaN_3, the enzyme activity was inhibited by 95% of its initial value, and it was completely inhibited in the presence of 1 mM NaN_3. Potassium cyanide exhibited an inhibitory influence on the enzyme activity similar to that of NaN_3. $Na_2S_2O_5$, which inhibits enzymatic reactions requiring oxygen, had no effect on the enzymatic activity at 10 μM. With increasing concentrations of $Na_2S_2O_5$ to 100 μM and 1 mM, the enzyme activity was inhibited completely. Cysteine, which is known to inhibit the oxidative enzyme reaction, also inhibited RBBR decolorizing peroxidase activity.

Spectroscopic properties. The final homogeneous preparation of the enzyme was brownish red in solution, suggesting the presence of the heme group in its active site. The absorption spectrum of the purified enzyme showed maxima at 407, 510, and 640 nm, and the A_{407}/A_{280} ratio reached 0.9. This value was low compared to those of peroxidase from *Coprinus cinereus*, *C. macrorhizus*, and *Arthromyces ramosus* (17), diarylpropane oxygenase of *P. chrysosporium* (6), and PoP of *P. ostreatus* (11), which were 2.5, 4.97, and 1.25, respectively. The molar extinction coefficient of the enzyme was 320,500 cm^{-1} M^{-1} at 407 nm. The addition of H_2O_2 to the enzyme solution resulted in an absorbance decrease at 407 and 510 nm (Fig. 4). This spectral characteristic was identical to that of the protoheme IX group found in *o*-dianisidine peroxidase of *Escherichia coli* B (2) and PoP of *P. ostreatus* (11). The color of RBBR was changed by RBBR decolorizing peroxidase from blue to pink to yellow to colorless. Similar color changes were observed in the agar medium containing RBBR during the growth of *P. ostreatus* (13).

In conclusion, our results showed that this new enzyme is an RBBR decolorizing peroxidase produced by *P. ostreatus*. Moreover, the enzyme may have an important role in the decolorization of xenobiotic dyes.

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