TO THE MECHANISM OF HORSERADISH PEROXIDASE-MEDIATED DEGRADATION OF A RECALCITRANT DYE REMAZOL BRILLIANT BLUE R

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The *in vitro* enzymatic metabolism of a recalcitrant dye Remazol Brilliant Blue R (RBBR) was investigated using horseradish peroxidase (HRP). At optimum pH (4.5), the apparent Michaelis constant (K_M) value for the oxidation of RBBR catalyzed by HRP is 14.8 µmol l^{-1}. HRP-mediated conversion of RBBR proceeds *via* a conventional peroxidase reaction, by a sequential one-electron oxidation of two molecules of RBBR by the peroxidase Compounds I and II. The oxidation is inhibited by radical trapping agents (nicotinamide adenine dinucleotide reduced (NADH), ascorbate, glutathione). This confirms that the peroxidase-mediated oxidation of RBBR proceeds *via* radical mechanism. Gel permeation profile of the RBBR oxidation products shows that the pattern of molecular weight distribution was shifted to the higher molecular weight region indicating formation of RBBR oligomers. In addition to HRP, the RBBR dye is also oxidized by another peroxidase, the mammalian lactoperoxidase.

Keywords: Recalcitrant organopollutants; Biodegradation; Peroxidases; Enzyme reaction mechanism; Metabolism; Anthracenes; Quinones; Sulfonates; Dyes.

Remazol Brilliant Blue R (C.I. 61200, Reactive Blue 19, RBBR) (**1**) is an industrially important dye frequently used as a starting material in the production of polymeric dyes[1,2]. This dye, an anthracene derivative, represents an important class of recalcitrant organopollutants causing environmental pollution from the effluents of dyestuff industries. Concentrations higher than 40 ppm of RBBR and other Remazol reactive dyes have been observed in washwaters of textile manufacturing sites in Europe ending in waste streams[3]. It is therefore important to develop methods for degradation of this persistent dye in industrial effluents. Traditional technologies, such as activated *C*-adsorption, chemical coagulation, and reverse osmosis, have been used to treat textile wastewaters but they only transfer the contaminant from the wastewater to a solid waste[3]. Microorganisms including sev-

eral white rot fungi[4–8] in cultures, as well as plant cell cultures[9] have been reported to degrade and decolorize various dyes, including RBBR (ref.[4]). These organisms might therefore be useful for biotechnological applications such as decontamination of RBBR in wastewaters.
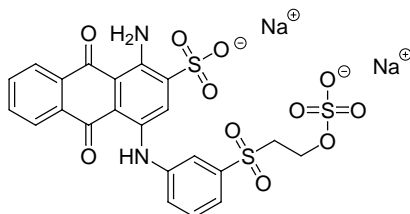
Ramazol Brilliant Blue R

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The question which of the microbial and plant enzymes are responsible for degradation of RBBR has not been fully answered yet. Attempts to identify such enzymes in white rot fungus *Pleurotus ostreatus* were carried out by Vyas and Molitoris[1] and Shin *et al.*[2]. Two oxidative enzymes were determined to be capable of degrading the RBBR dye in this species[1,2]. One of these enzymes is the extracellular peroxidase oxidizing also a wide range of xenobiotics including other artificial dyes and phenolic compounds[2,10]. The other enzyme, which has not been characterized precisely as yet, seems to be a heme-containing oxygenase requiring hydrogen peroxide for its activity[1]. Kinetic characteristics of RBBR oxidation catalyzed by this enzyme exhibit strong similarities with those of other peroxidases[1]. Hence, the possibility that the enzyme is also a peroxidase cannot be excluded. While the lignin peroxidase and veratryl alcohol peroxidase activities of this enzyme were not detectable, the phenomenon of RBBR decolorization by another white rot fungus *Phanerochaete chrysosporium* has been attributed to the lignin peroxidase activity[7]. The discrepancies described in the studies[1,2,6] have not been explained yet. Moreover, the oxidation of RBBR by pure peroxidases, with a well-resolved mechanism of oxidation for other substrates (*e.g.*, horseradish peroxidase, HRP), has also not been studied so far. However, such a study might help to understand the exact character of the enzymes converting RBBR in fungi and plants.

In this report, we investigate capabilities of two model peroxidases, plant horseradish peroxidase and mammalian lactoperoxidase, to convert RBBR.

EXPERIMENTAL

Chemicals

Chemicals were obtained from the following sources: horseradish peroxidase type VIA, lactoperoxidase, catalase, superoxide dismutase, guaiacol (2-methoxyphenol) and RBBR from Sigma Chemical Co.; L-ascorbic acid from Merck; NADH and glutathione (reduced) from Roche; 2-methoxyaniline (*o*-anisidine) from Fluka; Sephadex G-25 from Pharmacia Fine Chemicals. Other chemicals were supplied by Lachema. All chemicals were reagent grade purity or better.

Analytical Methods

The assay mixture for the conversion of RBBR by peroxidases contained, in 2 ml, 0.2–20.0 µg of horseradish peroxidase or lactoperoxidase, 0.0025–0.4 mM H_2O_2, 100 mM sodium acetate buffer (for pH 3.5, 4.0, 4.5, 5.0 or 5.5) or 100 mM sodium phosphate buffer (for pH 6.5, 7.5 or 8.5) and 0.005–0.15 mM RBBR. The reaction was started by addition of hydrogen peroxide, and the disappearance of RBBR with time was followed at 595 nm at 20 °C using a Hewlett–Packard 8453 diode array spectrophotometer. The molar absorption coefficient of RBBR at 595 nm (ref.[3]) was 5 820 mol^{-1} l cm^{-1}. Inhibition of RBBR conversion by radical scavengers was studied in the same reaction mixture with addition of 0.001–1.0 mM NADH or ascorbate or glutathione dissolved in 100 mM sodium acetate buffer (pH 5.0). The effect of catalase and superoxide dismutase was also investigated in the same reaction mixture, but 0.01–100 µg of catalase or 70 µg of superoxide dismutase was added.

Kinetic analyses were carried out using the non-linear least-squares method described by Cleland[11.].

The assay mixture for the conversion of RBBR by the Fe^{3+}–H_2O_2 or Fe^{2+}–H_2O_2 systems contained, in 2 ml, 0.1 mM RBBR, 0.1–10.0 mM $Fe(NO_3)_3$ or $(NH_4)_2Fe(SO_4)_2$, 0.1–10.0 mM H_2O_2 and 100 mM glycine–HCl buffer (pH 2.6). The reaction was started by addition of hydrogen peroxide, and the disappearance of RBBR with time was followed as described above.

The products of RBBR oxidation with HRP present in the reaction medium were separated by gel permeation chromatography on a Sephadex G-25 column (1.5 × 6.0 cm) by elution with 50 mM sodium phosphate buffer (pH 7.5, flow rate 0.5 ml min^{-1}).

RESULTS

In the presence of hydrogen peroxide, both model peroxidases (HRP and lactoperoxidase) catalyzed conversion of RBBR leading to its decolorization (Fig. 1). The UV-VIS spectra of the reaction mixture recorded at regular time intervals showed that RBBR is undergoing degradation which is accompanied by a decrease in absorbance at 595 nm and diminishing the absorption minimum at 450 nm (Fig. 1). No conversion of RBBR was observed when any of the components was omitted from the reaction mixture. Lactoperoxidase was less efficient in catalyzing this reaction than HRP (Fig. 1). The lower efficiency of lactoperoxidase was, however, not specific for RBBR, we also observed lower activity of this peroxidase with other substrates (*i.e.*

guaiacol, *o*-anisidine) (results not shown). Only the more efficient peroxidase (HRP) was used in further experiments presented below.

The rates of RBBR conversion by HRP were measured at pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.5, 7.5 and 8.5. The highest velocity of the reaction was found at approximately pH 4.5 (Fig. 2). The kinetics of the two-substrate reaction of RBBR oxidation with HRP in the presence of hydrogen peroxide was examined. The conversion of RBBR was measured in the reaction medium which contained peroxidase, hydrogen peroxide and various concentrations of
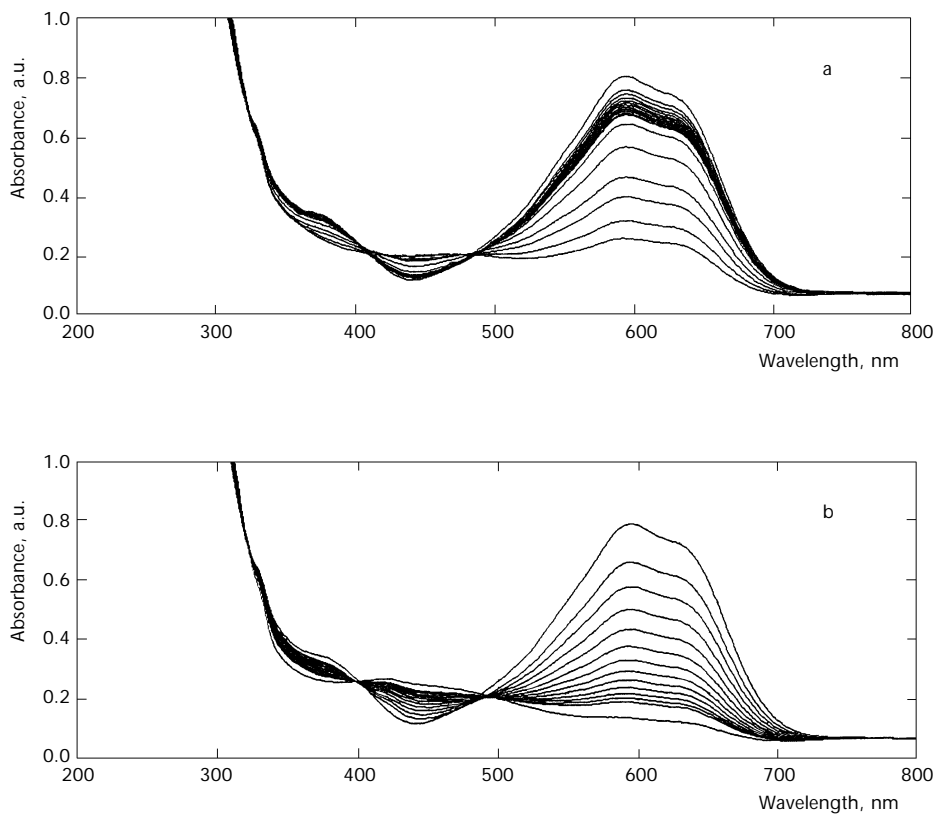

FIG. 1
Absorption spectra in the course of the reaction of RBBR with lactoperoxidase (a, reaction time 60 min) or HRP (b, reaction time 5 min). Experimental conditions: 0.1 M sodium acetate buffer (pH 5.0), 10 μg of HRP or lactoperoxidase, 0.1 mM H_2O_2, 0.1 mM RBBR (for details see Experimental)

RBBR. The reaction followed the hyperbolic kinetics (Fig. 3). Under the conditions used, the apparent Michaelis constant (K_M) value determined for RBBR was the lowest at pH 4.5 (Table I). Besides the dependence of the reac-
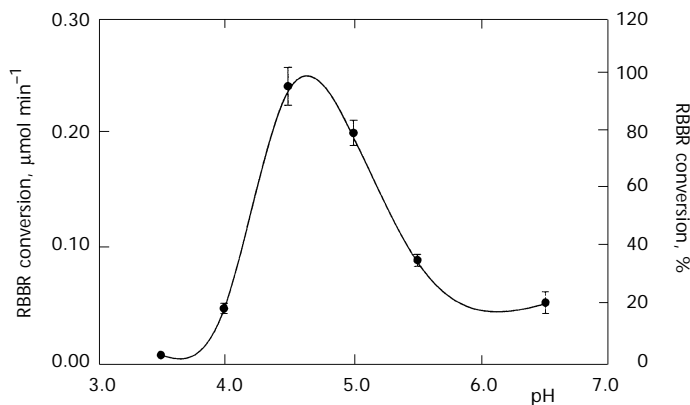

FIG. 2
Dependence of RBBR conversion in the reaction with horseradish peroxidase on pH. Experimental conditions: 10 µg of HRP, 0.1 mM RBBR and 0.1 mM H_2O_2 (for details see Experimental)
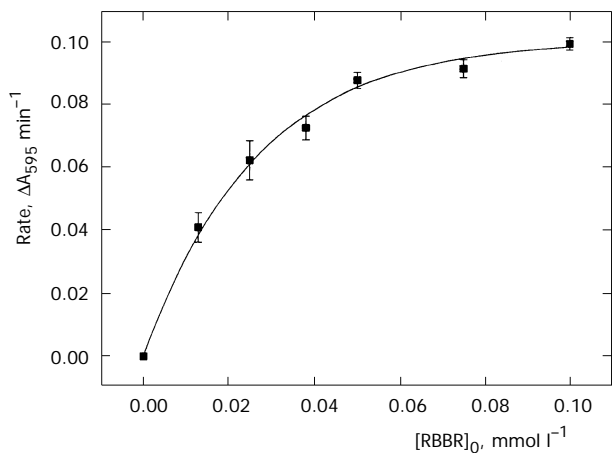

FIG. 3
Dependence of the rate of RBBR conversion in the reaction with horseradish peroxidase on the substrate concentration. Experimental conditions: 0.1 M sodium acetate buffer (pH 4.5), 0.1 µg of HRP, 0.013–0.10 mM RBBR, 0.1 mM H_2O_2 (for details see Experimental)

tion on RBBR, the reaction is also strictly dependent on the concentration of the other substrate of the enzyme, hydrogen peroxide and on the concentration of peroxidase. Increasing amounts of HRP in the reaction mixture resulted in an increase in the rate of RBBR conversion (data not shown). The initial reaction rates are the same for a wide range of H_2O_2 concentrations (Fig. 4). Therefore, the apparent K_M value for H_2O_2 could not be determined, due to this atypical dependence of the reaction rate on the H_2O_2 concentration. Moreover, in the presence of limiting amounts of hydrogen peroxide, the reaction can stop at any particular stage of the decolorization process (Fig. 4). However, this phenomenon is neither caused by inactivation of HRP by hydrogen peroxide nor by reaction with end-products as often seen under some reaction conditions for several

TABLE I
The values of Michaelis constant (K_M) for RBBR oxidation with horseradish peroxidase[a]

pH	4.0	4.5	5.0	5.5	6.5
K_M, μmol l^{-1} [b]	42.0 ± 4.9	14.8 ± 1.3	39.0 ± 3.5	56.4 ± 5.5	103.4 ± 9.3

[a] Experimental conditions are as described in Experimental (2.0, 0.2 and 20 μg of HRP were used at pH 4.0, 4.5–5.5 and 6.5, respectively). [b] The values are the means ± standard errors (in most cases, from five parallel experiments).
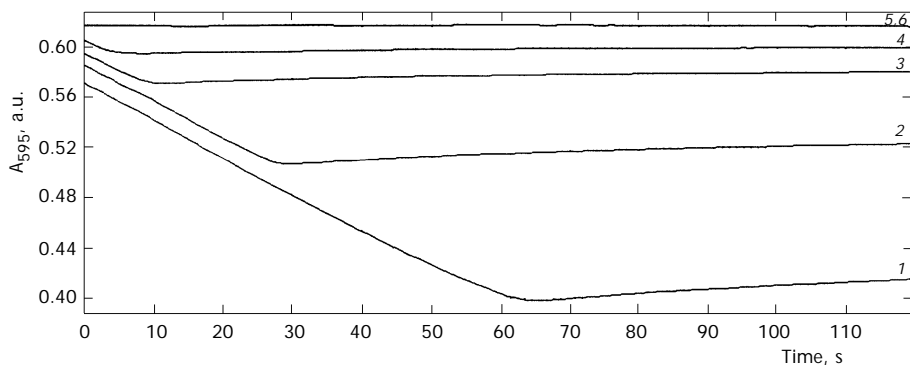

FIG. 4
Dependence of RBBR conversion in the reaction with horseradish peroxidase on H_2O_2 concentration. Experimental conditions: 0.1 M sodium phosphate buffer (pH 6.5), 10 μg of HRP, 0.10 mM RBBR and hydrogen peroxide concentrations (in mmol l^{-1}) 0.02 (*1*), 0.01 (*2*), 0.005 (*3*), 0.0025 (*4*), 0 (*5*) or with 100 μg of catalase (*6*)

peroxidases[12], because the HRP-mediated RBBR decolorization can be reinitiated by addition of a fresh aliquot of hydrogen peroxide (results not shown). Indeed, it is also clearly seen from results shown in Fig. 5 that suicide inactivation of HRP with peroxide or products is not significant. This may be explained either by the great excess of HRP present in the reaction medium or as follows: (i) RBBR is an excellent substrate of HRP consuming the total amount of hydrogen peroxide, which is usually responsible for destroying the peroxidase activity[12–16] and (ii) RBBR is converted to products that do not modify the enzyme molecule and do not inhibit its activity. No RBBR conversion was detected when the enzyme or hydrogen peroxide was omitted and also when catalase was added to the reaction mixture (Fig. 4).

As shown in Fig. 5, the extent of RBBR conversion increased with an increase in hydrogen peroxide concentrations up to the RBBR : H_2O_2 molar ratio approaching 2.0 and then reached a plateau. It is evident that such a molar ratio of substrates (RBBR, H_2O_2) is sufficient to convert RBBR completely (Fig. 5). Therefore, it can be used to estimate the mechanism of the peroxidase-catalyzed reaction. This ratio of substrates indicates that the HRP-mediated conversion of RBBR proceeds *via* a conventional peroxidase reaction, namely by a sequential one-electron oxidation of two molecules of RBBR with peroxidase Compounds I and II (Scheme 1).
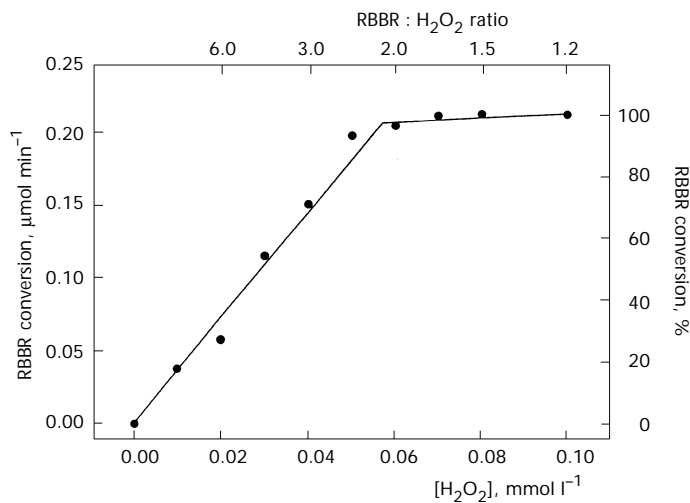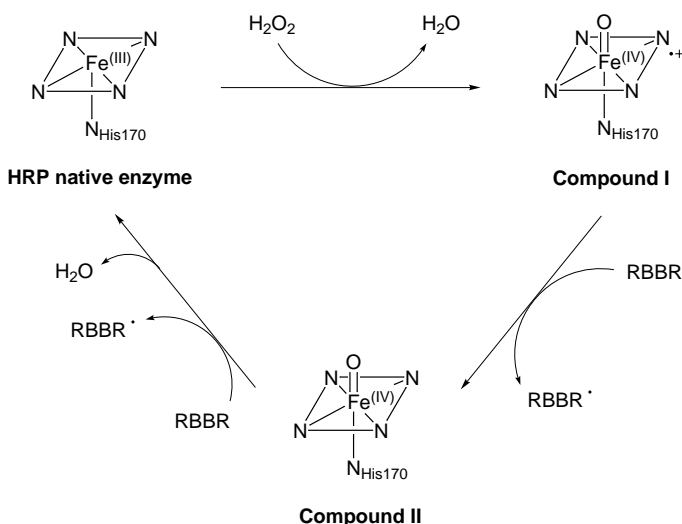

FIG. 5

Dependence of RBBR conversion in the reaction with horseradish peroxidase on the RBBR : H_2O_2 molar ratio. Experimental conditions: 0.1 M sodium acetate buffer (pH 4.5), 20 µg of HRP, 0.12 mM RBBR and H_2O_2 concentrations as shown

SCHEME 1

Indeed, this conclusion was confirmed by the effect of radical scavengers on the reaction. The RBBR conversion is strongly inhibited by three physiological donors, NADH, glutathione and L-ascorbic acid, which are known to be effective radical scavengers[12] (Table II). These findings strongly support

TABLE II
Effect of radical scavengers on oxidation of RBBR with horseradish peroxidase[a]

Scavenger	Concentration mmol l^{-1}	RBBR Conversion[b] μmol min^{-1} per μg of HRP
None		0.193 ± 0.015
L-Ascorbic acid	0.01	0.020 ± 0.002
	0.10	[c]
NADH	0.01	0.101 ± 0.012
	0.10	[c]
Glutathione	0.01	0.041 ± 0.003
	0.10	[c]

[a] Experimental conditions are described in Experimental (pH 5.0). [b] The values are the means ± standard errors (in most cases, from five parallel experiments). [c] Not detectable.

the assumption that radicals are involved in the reaction mechanism. It has already been found[13–15] that none of these scavengers is a substrate of HRP under the conditions used in this work. Thus, the scavengers do not act as competitors with RBBR for binding to the active centre of HRP. The effect of scavengers is therefore based on reduction of the radicals formed from RBBR back to the parent dye.

The RBBR radicals can undergo a variety of reactions depending on circumstances. Usually, the radicals of peroxidase-mediated reaction are further converted in a non-enzymatic reaction to dehydrogenated substrates, oligomers or polymers[16]. To estimate whether RBBR radicals produce oligomeric metabolites, we utilized gel permeation chromatography. The gel permeation profile for the enzymatically treated RBBR is shown in Fig. 6. The absorbance in the low-molecular-weight region (elution volume around 100 ml) corresponding to RBBR (eluted as two peaks due to presence of two ionized forms of this dye at pH 7.5) diminished and a new peak arose in the high-molecular-weight region (in the void volume of the column).

We tested whether HRP can also catalyze conversion of RBBR by the so-called oxidase–oxygenase activity of the enzyme[17,18], that is, employing
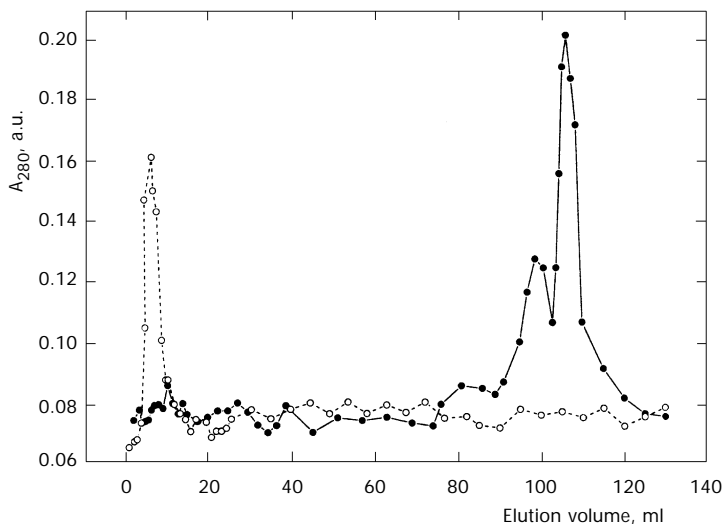

FIG. 6

Gel permeation chromatography on Sephadex G-25 of products of the peroxidase-catalyzed conversion of RBBR. Elution profile of the reaction mixture containing 0.2 mM RBBR, 0.4 mM hydrogen peroxide, 20 µg of HRP in 0.1 mM sodium phosphate buffer (pH 6.5) (○) and the of same reaction mixture, but without H_2O_2 (●)

a superoxide anion radical for the reaction. As this reaction utilizes the superoxide anion radical, we examined whether the RBBR radical(s), generated in the peroxidase-mediated reaction, is (are) able to activate O_2 to superoxide anion radical. Because no inhibition of the RBBR oxidation by HRP was observed when superoxide dismutase was added to the reaction mixture, we may conclude that superoxide is not involved in the reaction mechanism. This indicated that RBBR radical(s) is (are) not able to reduce O_2.

Recently, Herrera and coworkers[3] described photochemical decolorization of RBBR in the presence of Fe^{3+} and H_2O_2. We studied the effect of different concentrations of the Fe^{3+}–H_2O_2 or Fe^{2+}–H_2O_2 systems on this reaction. The RBBR conversion was detected only with concentrations of Fe^{3+}–H_2O_2 or Fe^{2+}–H_2O_2 by two orders of magnitude higher than that of RBBR. Moreover, the reaction occurred only if the reaction mixture was acidic (pH 2.6). When Fe^{3+} or Fe^{2+} ions were used at the concentrations corresponding to their amount present in HRP (0.22 nmol of Fe in 10 μg of HRP), no RBBR conversion occurred, even in large excess of hydrogen peroxide (10 mmol l^{-1}). These results confirmed that the HRP-dependent RBBR conversion is an enzyme-mediated reaction.

DISCUSSION

The present paper clearly shows that pure plant and mammalian peroxidases convert an industrial pollutant, a persistent dye, Remazol Brilliant Blue R. Using plant peroxidase (HRP) as a model enzyme, we partially characterized the mechanism of the RBBR decolorization reaction. It proceeds *via* a conventional one-electron peroxidation mechanism (Scheme 1). We could not identify the structure of any RBBR oxidation products formed by the action of peroxidase. Nevertheless, the results shown in the paper indicate that the radicals formed from RBBR might react to form oligomeric metabolite(s), which is (are) colorless.

We additionally tested, whether HRP might oxidize RBBR also by another reaction mechanism, by the so-called oxidase activity of the enzyme. It is known that HRP (and/or other peroxidases) can, in some cases, utilize O_2 acting apparently as oxidase–oxygenase[17,18]. This is, however, not a true oxidase reaction, as it is known that no direct electron transfer occurs from peroxidases to O_2. Radicals generated by peroxidases from some substrates are able to activate O_2 non-enzymatically, reducing O_2 to superoxide[17,18]. The superoxide anion radical can be subsequently protonated and reduced to H_2O_2. Hydrogen peroxide thereafter acts in the clasic peroxidase reac-

tion, promoting the oxidation of substrate. RBBR radical(s) formed by the action of HRP do not possess sufficient reactivity (redox potential) to reduce molecular oxygen.

The mechanism of HRP-dependent conversion of RBBR determined in our study differs from that described[1] for the reaction catalyzed by one of the enzymes of *Pleurotus ostreatus*. Although the RBBR decolorization reaction by the enzyme of *Pleurotus ostreatus* exhibits several typical features of the peroxidase reaction, it requires, in addition, the presence of molecular oxygen. Vyas and Molitoris showed[1] that oxygen depletion retarded the initial rate of RBBR conversion by 57% compared to normal (aerobic) conditions. Therefore, these authors postulated that the enzyme is an H_2O_2-dependent oxygenase[1].

The kinetics of RBBR conversion by action of HRP strongly resembles those described for another enzyme of the above mentioned fungal species *Pleurotus ostreatus*, the extracellular peroxidase. This indicates that the peroxidase expressed by this fungus and excreted from the fungal cells, acts as a classic plant peroxidase, HRP. This is an interesting feature important for evaluation of efficiencies of microbial and plant organisms to degrade RBBR or other organopollutants. It is known that, in addition to RBBR oxidation, HRP and the peroxidase of *Pleurotus ostreatus* oxidize also many other chemicals including aromatic amines[2,12,19–21], phenolic compounds[12,18,20,22–24], azo dyes[13,15,25–30] and other synthetic dyes[2,19,20]. The peroxidase of *Pleurotus ostreatus* is an efficient enzyme possessing also ligninolytic activity[31]. These published data and the results presented here strongly suggest that not only these two peroxidases, but also plants and microorganisms rich in peroxidases acting by the same reaction mechanism (conventional peroxidase mechanism), might be employed in biotechnological applications. However, the transfer of scientific knowledge about organopollutant biodegradation into practical applications remains a major challenge for future studies. Specially designed bioreactors with specific microbial or plant populations rich in peroxidases should be constructed.

CONCLUSIONS

The results presented in this paper demonstrate capability of the plant peroxidase, HRP, to convert a recalcitrant dye, Remazol Brillant Blue R, which is a contaminant of textile wastewaters. Analyses of our data demonstrate that HRP oxidizes RBBR *via* a conventional peroxidase reaction, by a sequential one-electron oxidation, being very efficient enzyme in this reaction. In addition to HRP, mammalian lactoperoxidase and peroxidase of

fungal cells (*Pleurotus ostreatus*)[2] oxidize RBBR, too. We assume that organisms rich in peroxidases having similar enzyme properties to the above mentioned peroxidases might be able to degrade the studied dye and might be utilized in bioremediation technologies. Characterization of the structure of RBBR products formed in the peroxidation reactions as well as evaluation whether (and how) these products might influence the physiological processes in organisms including human, is the aim of our future work.

REFERENCES

1. Vyas B. R. M., Molitoris H. P.: *Appl. Environ. Microbiol.* **1995**, *61*, 3919.
2. Shin K.-S., Oh I.-K., Kim C.-J.: *Appl. Environ. Microbiol.* **1997**, *63*, 1744.
3. Herrera F., Kiwi J., Lopez A., Nadtochenko V.: *Environ. Sci. Technol.* **1999**, *33*, 3145.
4. Glenn J. K., Gold M. H.: *Appl. Environ. Microbiol.* **1983**, *45*, 1741.
5. Goszczynski S., Pasczynski A., Pasti-Grygsby M. B., Crawford R. L., Crawford D. L.: *J. Bacteriol.* **1994**, *176*, 1339.
6. Ollikka P., Alhonmaki K., Lepparen V.-M., Glumoff T., Raijola T., Suominen I.: *Appl. Environ. Microbiol.* **1993**, *59*, 4010.
7. Spadaro J. T., Gold M. H., Renganathan V.: *Appl. Environ. Microbiol.* **1992**, *58*, 2397.
8. Waldner R., Leisola M. S. A., Fiechter A.: *Appl. Environ. Microbiol.* **1988**, *29*, 400.
9. Kučerová P., Poláchová L., Macek T., Burkhard J., Tříska J., Macková M.: *Int. Biodeterior. Biodegrad.* **1998**, *42*, 250.
10. Shin K.-S, Kim C.-J.: *Biotechnol. Lett.* **1998**, *20*, 569.
11. Cleland W. W.: *Methods Enzymol.* **1983**, *63*, 103.
12. O'Brien P. J.: *Free Radical Biol. Med.* **1988**, *4*, 169.
13. Stiborová M., Frei E., Schmeiser H. H., Wiessler M., Hradec J.: *Carcinogenesis* **1990**, *11*, 1843.
14. Stiborová M., Schmeiser H. H., Frei E.: *Collect. Czech. Chem. Commun.* **1998**, *63*, 857.
15. Stiborová M., Schmeiser H. H., Frei E.: *Cancer Lett.* **1999**, *146*, 53.
16. Dunford H. B.: *Heme Peroxidases*. Wiley-VCH, New York 1999.
17. Penner-Hahn J. E., Eble K. S., McMurry T. J., Renner M., Balch A. L., Groves J. T., Dawson J. H., Hodgson K. O.: *J. Am. Chem. Soc.* **1986**, *108*, 7819.
18. Stiborová M., Mikšanová M., Martínek V., Frei E.: *Collect. Czech. Chem. Commun.* **2000**, *65*, 297.
19. Yamazoe Y., Zenser T. V., Miller D. W., Kadlubar F. F.: *Carcinogenesis* **1988**, *9*, 1635.
20. Eling T. E., Thompson D. C., Foureman G. L., Curtis J. F., Hughes M.: *Annu. Rev. Pharmacol. Toxicol.* **1990**, *30*, 1.
21. Thompson D. C., Eling T. E.: *Chem. Res. Toxicol.* **1991**, *4*, 474.
22. Dec J., Bollag J. M.: *Biotechnol. Bioeng.* **1994**, *44*, 1132.
23. Nicell J. A., Bewtra K., Biswas N., Taylor E.: *Water Res.* **1993**, *27*, 1629.
24. Klibanov A. M., Tu T. M., Scott K. P.: *Science* (*Washington, D. C.*) **1983**, *221*, 259.

25. Stiborová M., Asfaw B., Anzenbacher P.: *FEBS Lett.* **1988**, *232, 387*.
26. Stiborová M., Frei E., Schmeiser H. H., Wiessler M.: *Carcinogenesis* **1992**, *13*, 1221.
27. Stiborová M., Frei E., Schmeiser H. H., Wiessler M., Hradec J.: *Carcinogenesis* **1992**, *13*, 1657.
28. Stiborová M., Frei E., Schmeiser H. H., Wiessler M., Hradec J.: *Cancer Lett.* **1993**, *68*, 43.
29. Stiborová M., Frei E., Schmeiser H. H.: *Collect. Czech. Chem. Commun.* **1997**, *62, 971*.
30. Stiborová M., Schmeiser H. H., Breuer A., Frei E.: *Collect. Czech. Chem. Commun.* **1999**, *64, 1335*.
31. Shin K.-S., Lee Y.-J.: *Biotechnol. Lett.* **1999**, *20, 585*.