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N-Demethylation of Methylene Blue by Lignin Peroxidase from *Phanerochaete chrysosporium*

Stoichiometric Relation for H_2O_2 Consumption

Viridiana S. Ferreira,[1] Denise B. Magalhães,[1] Sérgio H. Kling,[2] José G. da Silva Jr.,[1] and Elba P. S. Bon*,[1]

[1]Instituto de Química, Universidade Federal do Rio de Janeiro, CT, Bloco A, Ilha do Fundão, Rio de Janeiro, RJ, CEP 21949-900, Brazil, E-mail: elba1996@iq.ufrj.br; and [2]Instituto de Química, Universidade do Estado Rio de Janeiro, São Francisco Xavier 524, Rio de Janeiro, RJ, Brazil

Abstract

Phanerochaete chrysosporium lignin peroxidase (LiP) can degrade synthetic dyes such as heterocyclic, azo, and triphenylmethane on its activation by H_2O_2. Analysis of the reaction products indicated that *N*-demethylation reactions are involved in the degradation of crystal violet and methylene blue (MB). We studied LiP oxidation of methylene blue and azure B (AB) in reaction mixtures containing different dye:H_2O_2 stoichiometric relations aiming at the selective formation of *N*-demethylated derivatives. High yields, about 70%, of the mono- and didemethylated derivatives, azure B and azure A, were obtained with the use of 1:1 and 1:2 MB:H_2O_2, respectively. Using azure B as substrate in reaction mixtures containing 1:1 AB:H_2O_2, a yield of 70% was also observed in azure A. Reaction mixtures containing 1:3 MB:H_2O_2 and 1:2 AB:H_2O_2, originated several oxidation products in similar proportions. These results indicated that the process of enzymatic degradation of methylene blue and azure B initiates via $N(CH_3)_2$ oxidation. According to the yields that were obtained for azure B and azure A, this enzymatic route can be used for the synthesis of these dyes since these data compare favorably to the chemical route that has a yield of 35%. The use of a dye:H_2O_2 relation of 1:10 resulted in a decoloration level of about 85%, showing the usefulness of this procedure for wastewater treatment. The reaction products were followed by spectrophotometric analysis within the wavelength of 500–700 nm. The product identifications were performed using a reverse-phase

*Author to whom all correspondence and reprint requests should be addressed.

high-performance liquid chromatography (HPLC) C-18 column and thin-layer chromatography.

Index Entries: *Phanerochaete chrysosporium* lignin peroxidase; stoichiometric dye:H_2O_2 relation; enzymatic *N*-demethylation reactions; methylene blue; azure B.

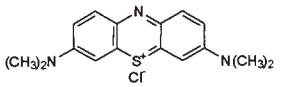
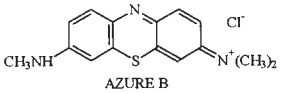
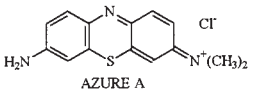
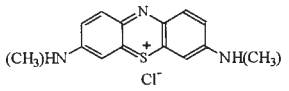
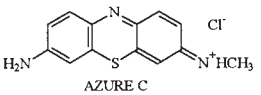
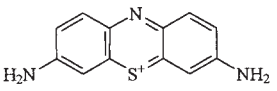
Introduction

The ligninolytic enzymatic system of *Phanerochaete chrysosporium* is able to oxidize various recalcitrant xenobiotics released into the environment as a result of human activity *(1–5)*. Among these compounds are many synthetic dyes such as heterocyclic, azo, and triphenylmethane that are widely used by the textile, photography, and dyestuffs industries. These compounds are resistant to biological wastewater treatment and therefore cause high chemical pollution when discharged into the soil and water *(6–12)*. The degradation of synthetic dye, e.g., orange II, tropacolin O, congo red, azure B (AB), bromophenol blue, methylene blue (MB), methyl green, methyl orange, remazol brilliant blue, toluidine blue, amaranth, and orange G, has been studied using ligninolytic cultures of *P. chrysosporium (6,8)*, the culture supernatant *(10,12)*, or purified lignin peroxidase (LiP) isoforms *(7)*. These data collectively point to the ability of this biocatalyst to oxidize these xenobiotics. This feature conveys to LiP a potential use as an ecofunctional enzyme by playing a role in environmental biocatalysis. Different substrate:H_2O_2 relations have been used on dye enzymatic oxidative reaction mixtures, such that both stoichiometric *(13)* and nonstoichiometric ratios have been reported *(7,10)*. In most cases, product formation has been analyzed qualitatively rather than on the yields of the reaction products in relation to the substrate:H_2O_2 ratio. Thus, although it is well known that crystal violet and methylene blue, which are hexa and tetramethylated, respectively, are degraded by LiP and that fewer methylated derivatives are formed *(10–12)*, the effects of specific reaction conditions such as substrate concentrations on the nature and yields of the products have not been evaluated. Therefore, considering that LiP is both activated by H_2O_2 and inactivated by its excess *(14)*, it would be useful to envisage the qualitative and quantitative pattern of product formation in reaction mixtures showing specific dye:H_2O_2 ratios.

This work studied the formation of demethylated methylene blue and azure B derivatives in reaction mixtures containing 1:1, 1:2, and 1:3 MB:H_2O_2 and 1:1 and 1:2 AB:H_2O_2. In addition, product formation in reaction mixtures containing MB:H_2O_2 ratios of 1:0.1, 1:0.25, 1:0.5, and 1:0.75 were analyzed to evaluate the pattern of product formation under H_2O_2-limiting concentrations.

Table 1 shows the chemical structures of methylene blue that present two $N(CH_3)_2$ groups and its demethylated derivatives—azure B, azure A, azure C—and thionine that have three, two, one, and zero *N*-methyl groups, respectively *(15)*.

Table 1
Chemical Structures of Methylene Blue and Its *N*-Demethylated Derivatives[a]

COMPOUNDS	λ_{MAX} *(15)*	λ_{MAX} *(19)*	MB: H_2O_2 In reaction mixtures	λ_{MAX} **Our work**
 METHYLENE BLUE	668	667	1:0	656-666
 AZURE B	648-655	652	1:1	642-646
 AZURE A  AZURE A (isomeric form)	620-634	638	1:2	626-630
 AZURE C	611-617	611	1:3	Not clear
 THIONINE	596-601	602	---	----

[a]The reported values of λ_{max} (nm) for these compounds and the λ_{max} obtained in this work for the reaction mixtures where they were produced are also compared.

Materials and Methods

Lignin Peroxidase

Enzyme production by *P. chrysosporium* has previously been described *(16)*. The crude enzyme preparation was dialyzed for the removal of residual veratryl alcohol to avoid substrate competition.

Oxidation of Methylene Blue and Azure B by LiP

The reaction mixtures contained 2.2 mL of LiP presenting 140 U/L in terms of veratryl alcohol activity *(17)*, 100 μL of 1 m*M* MB, 600 μL of 0.25 *M* sodium tartarate buffer (pH 4.0), and 100 μL of 1, 2, or 3 m*M* H_2O_2. The final concentration of MB in the reaction mixtures was 33 μ*M*, and the concentrations of H_2O_2 were 33, 66, and 99 μ*M (12)*. Methylene blue was previously purified using the same condition described for HPLC analysis of the reaction products. The same overall reaction conditions were used for azure B oxidation except that the initial concentration of the dye was 1.25 m*M* and that of H_2O_2 was 1.25 or 2.50 m*M*. Thus, the azure B final concentration was 42 μ*M* and that of H_2O_2 was 42 or 84 μ*M*. Reactions were started by adding H_2O_2 at the relevant concentration. In control experiments performed in the absence of LiP or H_2O_2, no dye degradation was observed. Reactions were followed in the visible range, 500–700 nm, until completion using a Hewlett Packard spectrophotometer Diode Array 8452-A (Hewlett Packard, Corvallis, OR).

HPLC Analysis

The products were identified using a reverse-phase C-18 column (7.8 × 300 mm, μbondpack, Waters/Millipore Corporation, Millford, MA). Compounds were eluted with a gradient consisting of 0.1% trifluoroacetic acid (A) and 80% acetonitrile with 0.07% trifluoroacetic acid (B). The gradient was developed starting with 70:30 (A:B), which varied linearly to 20:80 (A:B). A solvent flow rate of 2.0 mL/min was used, and the product elution was monitored at 600 nm *(10,18)*. The reaction product yields were calculated considering the response factor for each derivative at 600 nm.

Thin-Layer Chromatography

Thin-layer chromatography (TLC) was performed using silica gel plates 60G F-254 (E-Merck-AG, Darmstadt, Germany), and a solvent system of 99:1 ethyl alcohol:HCl (v/v) for 10 min *(11)*. In both chromatographic procedures, methylene blue, azure B, azure A, azure C, and thionine (Sigma, St. Louis, MO) were used as standards.

Results and Discussion

Figure 1 shows the spectra of absorbance of the products obtained by the enzymatic oxidation of methylene blue in reaction mixtures containing 1:1, 1:2, and 1:3 MB:H_2O_2. For comparison, the absorbance spectrum of a reaction mixture in which H_2O_2 was substituted by distilled water is included (1:0 MB:H_2O_2). To each stepwise increase in H_2O_2 concentration corresponded a hypsochromic shift and a decrease in absorbance (Table 1). These results suggested the occurrence of consecutive *N*-demethylations in response to the increase in H_2O_2 concentration, because according to the literature the loss of one *N*-methyl group leads to an approximate shift of 10–20 nm *(19)*. Figures 2–5 present the HPLC profiles of the control experi-

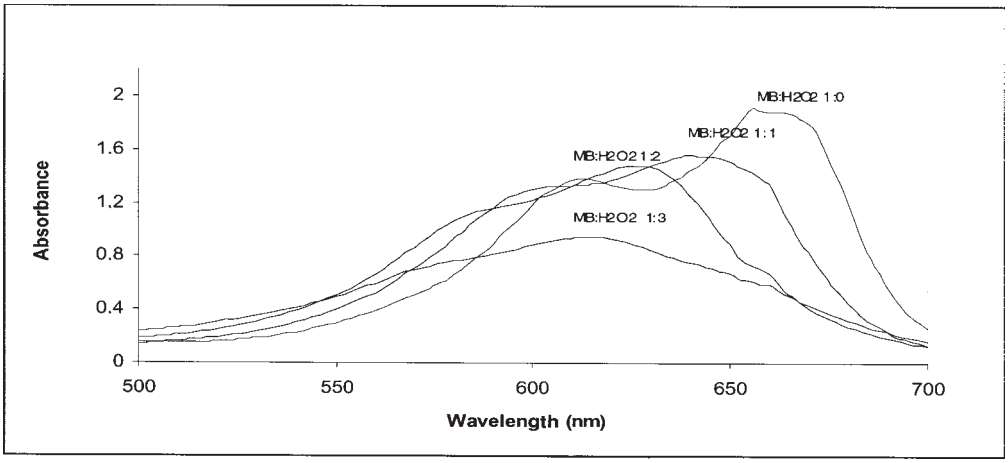


Fig. 1. Spectrophotometric analysis of the reaction products obtained by LiP oxidations, in reaction mixtures containing 1:0 MB:H_2O_2, 1:1 MB:H_2O_2, 1:2 MB:H_2O_2, and 1:3 MB:H_2O_2.

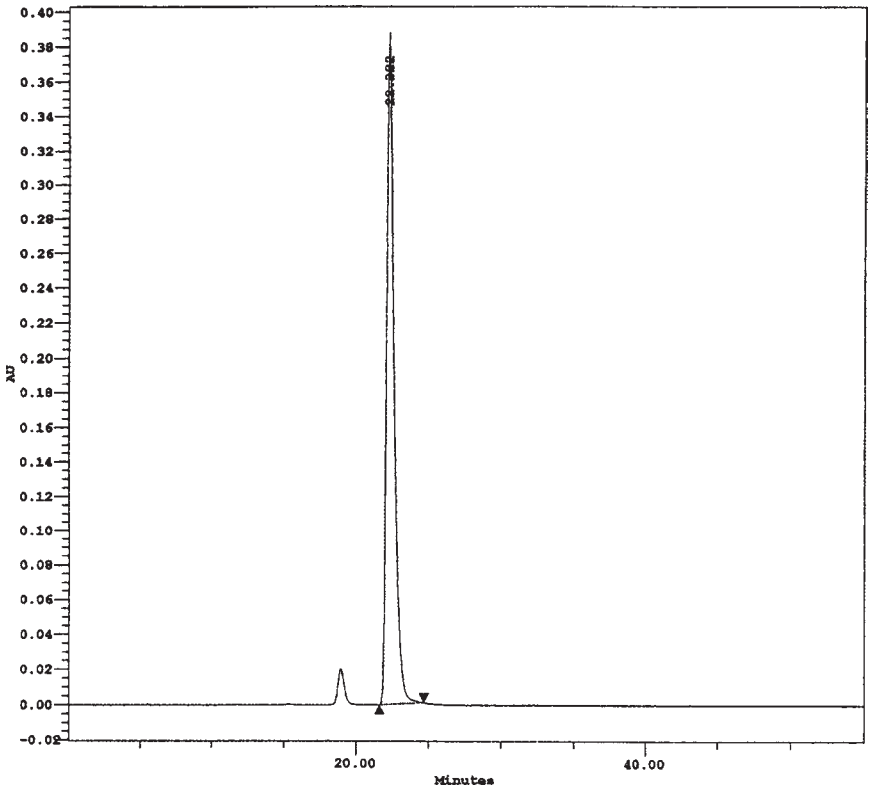


Fig. 2. HPLC profile of the reaction mixture containing 1:0 MB:H_2O_2 (control experiment). The elution of methylene blue (T_r = 22.322 min) confirmed no occurrence of enzymatic oxidation in the absence of H_2O_2.

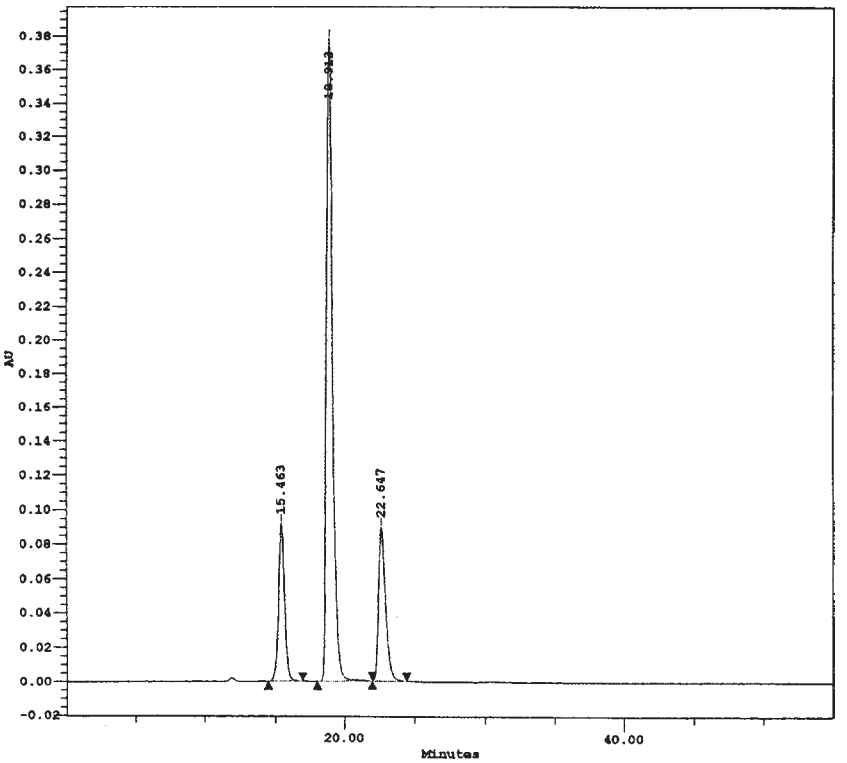

AU
0.38
0.36
0.34
0.32
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
15.463
18.913
22.647
20.00
40.00
Minutes

Fig. 3.

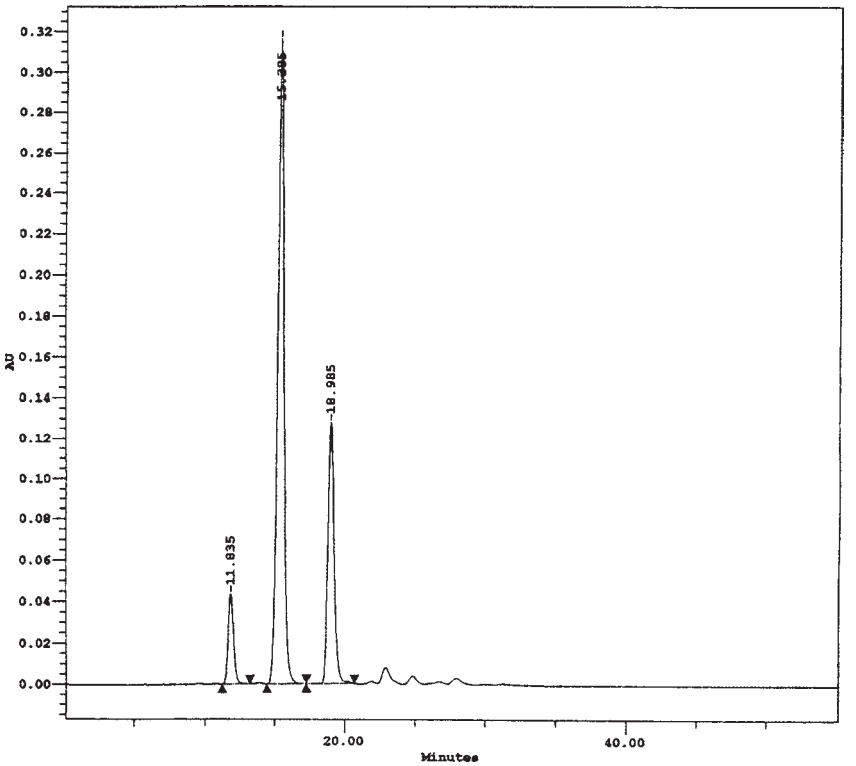

AU
0.32
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
11.835
15.395
18.985
20.00
40.00
Minutes

Fig. 4.

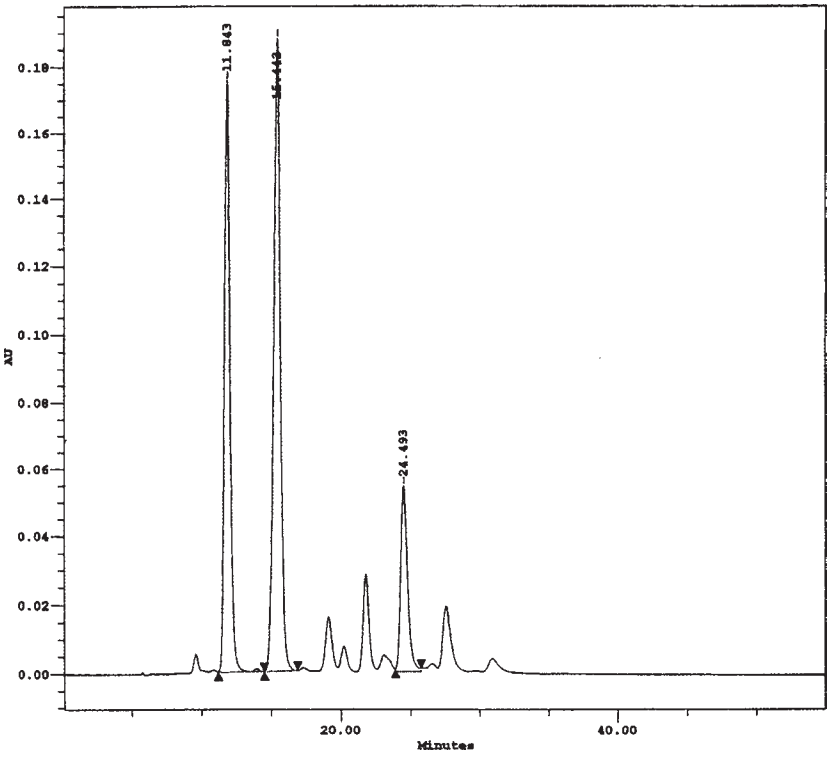


Fig. 5. HPLC elution profile of the products that were formed in reaction mixtures containing 1:3 MB:H_2O_2. The two major peaks correspond to the *N*-demethylated derivations azure A (T_r = 15.443 min) and azure C (T_r = 11.843 min).

ment and that of the reactions products. The use of 1:1 MB:H_2O_2 and 1:2 H_2O_2 resulted in the formation of azure B and azure A, respectively, with yields of about 70% (Figs. 3 and 4). The use of an MB:H_2O_2 relation higher than 1:2 resulted in the formation of several products (Fig. 5), in agreement with the spectrum shown in Fig. 1 for this reaction condition, in which no clear λ_{max} can be identified.

Figures 6–8 present HPLC profiles corresponding to the products of azure B oxidations. According to the results presented in Fig. 6, the absence of H_2O_2 prevented the dye oxidation, as expected. The use of 1:1 AB:H_2O_2 allowed the formation of 70% AA (Fig. 7) and of 1:2 AB:H_2O_2 resulted in an

Fig. 3. *(previous page)* HPLC elution profile of the products formed in reaction mixtures containing 1:1 MB:H_2O_2. The major product formed, with a yield of 70%, was identified as azure B (T_r = 18.913 min). The profile also shows the formation of azure A (T_r = 15.463 min) and the presence of residual amounts of methylene blue (T_r = 22.647 min).

Fig. 4. *(previous page)* HPLC elution profile of the products formed in reaction mixtures containing 1:2 MB:H_2O_2. The major product, formed with a yield of 70%, was identified as azure A (T_r = 15.385 min). The profile also shows the formation of azure C (T_r = 11.835 min) and the presence of residual amounts of azure B (T_r = 18.985 min).

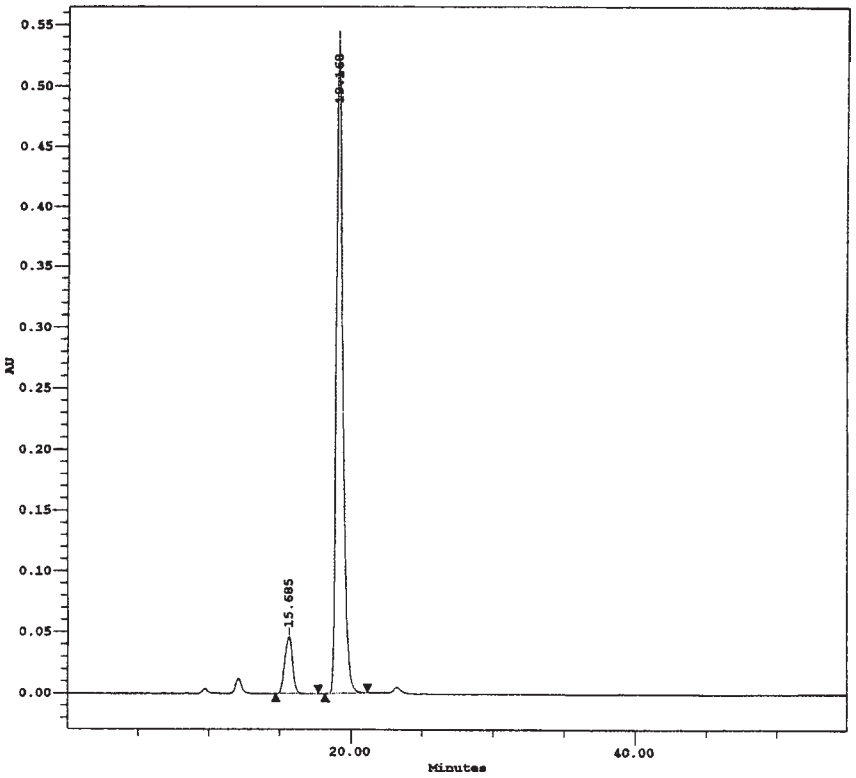

0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
AU
15.685
20.00
40.00
Minutes

Fig. 6.

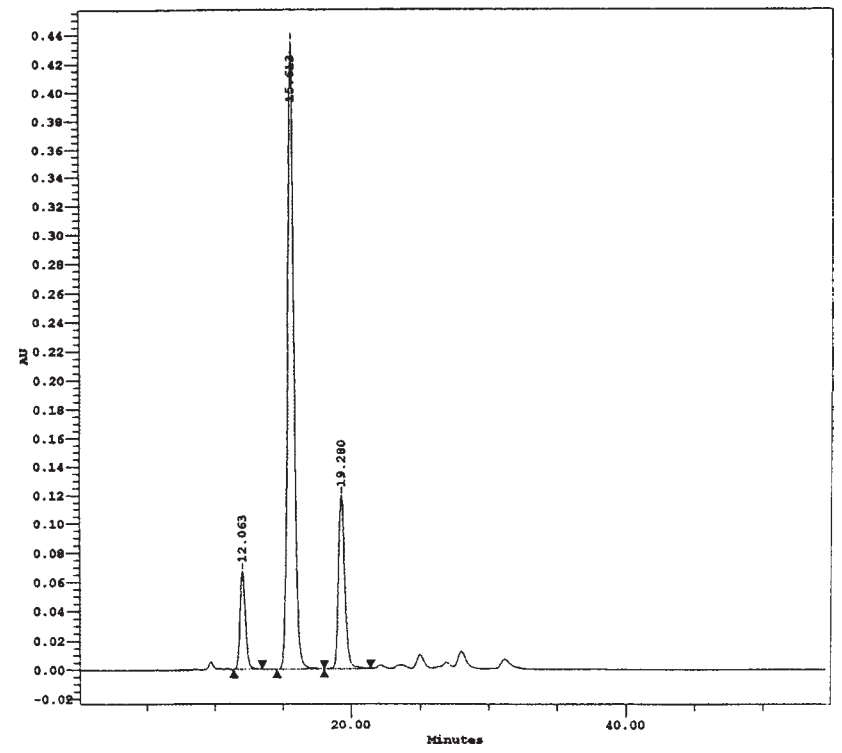

0.44
0.42
0.40
0.38
0.36
0.34
0.32
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
AU
12.063
19.280
20.00
40.00
Minutes

Fig. 7.

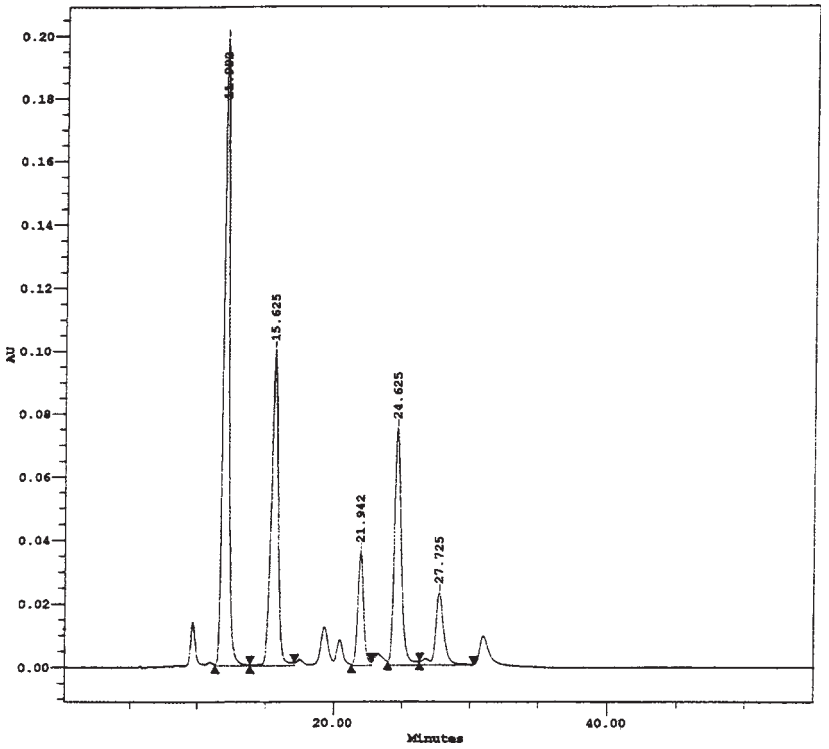


Fig. 8. HPLC elution profile of the products formed in reaction mixtures containing 1:2 AB:H_2O_2. Major peaks correspond to the azure A (T_r = 15.625 min) and azure C (T_r = 11.992 min). Methylene blue (T_r = 21.942 min) is also present.

HPLC profile similar to the one already observed for 1:3 MB:H_2O_2, i.e., the presence of several products (Fig. 8).

Our results indicated the use of the 1:1 MB:H_2O_2 relation resulted in the preferential removal of one methyl group and that of 1:2 resulted in the removal of two methyl groups from the methylene blue molecule. The use of 1:3 MB:H_2O_2 resulted in an absorbance profile without a defined λ_{max} and an intense decrease in the absorptivity that is justified by the formation of several products in low concentration, which included the presence of products with retention times according to the expected *N*-demethylated derivatives. The presence of more than six products suggests the occurrence of modifications on the aromatic rings besides the demethylation reactions. The selective formation of azure B and A and the unspecific

Fig. 6. *(previous page)* HPLC profile of the reaction mixture containing 1:0 AB:H_2O_2 (control experiment). The elution of azure B (T_r = 19.168 min) confirmed no occurrence of enzymatic oxidation in the absence of H_2O_2.

Fig. 7. *(previous page)* HPLC elution profile of the products formed in reaction mixtures containing 1:1 AB:H_2O_2. The major product formed, with a yield of 70%, was identified as azure A (T_r = 15.463 min). The profile also shows the formation of azure C (T_r = 12.063 min) and the presence of residual amounts of azure B (T_r = 19.280 min).

Table 2
Azure B Yields in Reaction Mixtures Containing MB:H_2O_2 Ratios Lower Than 1:1[a]

Substrate	Substrate:H_2O_2 ratio	Products (% area)			
		MB	AB	AA	AC
MB	1:0.10	89.5	10.5	—	—
	1:0.25	74.2	25.7	—	—
	1:0.50	50.4	49.6	—	—
	1:0.75	29.6	63.9	6.8	—

[a]MB, Methylene blue; AB, azure B; AA, azure A; AC, azure C.

Table 3
MB and AB Decoloration Level Obtained
in Reaction Mixtures Containing Dye:H_2O_2 (1:10)[a]

Substrate:H_2O_2 ratio	Substrate consumption (%)	$\Sigma ABS_{initial} - \Sigma ABS_{final}$ (%)
1:10 MB	98.0	85.2
1:10 AB	98.4	90.6

[a]The decrease in MB and AB absorbance at 600 nm was calculated considering the initial HPLC absorbance of MB or AB and the total HPLC absorbance values of the oxidation products. ABS, absorbance.

substrate oxidation when MB:H_2O_2 is higher than 1:2 and AB:H_2O_2 is higher than 1:1 confirms the enzyme preference toward the $N(CH_3)_2$ group oxidation. The results of TLC (data not shown) were in agreement with HPLC analysis in which a major spot appeared on the use of 1:1 and 1:2 MB:H_2O_2 and 1:1 AB:H_2O_2 and a set of spots with low color intensity for MB:H_2O_2 higher than 1:2 and AB:H_2O_2 higher than 1:1.

Table 2 summarizes the pattern of product formation for MB:H_2O_2 ratios lower than 1:1. A close relationship between the H_2O_2 concentration and the amount of formation of dye derivative was obtained, suggesting a 1:1 stoichiometric relation for the enzymatic oxidative reactions. The definition of the dye:H_2O_2 relation is valuable, not only because it allows a better understanding of the role of the H_2O_2 in the LiP activation step, but also because of its practical importance for enzymatic dye decoloration. Moreover, it allows the use of the minimum necessary H_2O_2 concentration, thereby, avoiding enzyme inactivation by an excessive H_2O_2 concentration *(20)*. According to the data presented in Table 3, the use of 1:10 MB:H_2O_2 and 1:10 AB:H_2O_2 resulted in an 85% decrease in absorbance, 85.2% and 90.6%, respectively. The data confirm the possibility of LiP application as an ecofunctional enzyme. Considering potential biotechnological applications, besides environmental biocatalysis, LiP could be used as a catalyst for selective organic synthesis of dyes with high yields, because it is reported that the chemical process for azure B production results in a maximal yield of 35% *(14)*.

Acknowledgments

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