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INHIBITION OF BOTH PEROXIDASE AND LACCASE BY DESFERAL (DESFERRIOXAMINE MESYLATE)

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Key Word Index—Desferal; inhibition; catecholase activity; laccase activity; peroxidase activity; phenol oxidation.

Abstract—The effect of desferal (desferrioxamine mesylate), a transition metal chelator with a stability constant for iron of 10^{31}, on the catalytic activity of the hemoprotein peroxidase, and on the catalytic activities of the cuproproteins, laccase and catechol oxidase, was studied. The results showed that desferal is an inhibitor of peroxidase and laccase activities but not of catechol oxidase activity, the inhibitory properties being strongly dependent on the phenolic substrate used to measure enzymatic activities. Thus, the inhibitory effect of desferal was not dependent on the nature of the prosthetic group. However, its use as an inhibitor of phenol-oxidizing enzymes, such as peroxidase and laccase, is promising since desferal seems to deactivate phenoxy radicals formed by the action of these enzymes. A mechanism to explain the inhibitory effect of desferal on these phenol-oxidizing enzymes, based on the reaction of desferal with phenoxy radicals, is proposed.

INTRODUCTION

Desferrioxamine mesylate is a powerful iron chelator obtained from *Streptomyces pilosus*, which is commercially available as desferal. Desferal chelates iron into a catalytically inactive form with a stability constant of 10^{31} and thus inhibits $OH^{\cdot}$ formation by the Haber–Weiss reaction [1]. It is currently used clinically to treat human diseases involving iron-overload [2]. Apart from iron, desferal can bind several other transition metals, although with much lower stability constants [3]. Thus, desferal is a powerful inhibitor of plant peroxidase [3], myeloperoxidase [2], eosinophil peroxidase [2], and prostaglandin-H synthase [4], all of which are hemoproteins.

Over recent years, the use of selective inhibitors, or of selective substrates, appears to be necessary in order to distinguish phenol-oxidizing enzymes [5, 6]. In the literature, it is often difficult to distinguish copper-containing enzymes such as phenol oxidases (catecholase and laccase) from iron-containing enzymes such as peroxidase, when they are assayed through spectrophotometric, zymographic or cytochemical probes [7–10]. A similar problem arises when comparing catecholase and laccase activities [11]. In this paper, we report the unusual properties of desferal as an inhibitor of phenol-oxidizing enzymes. In fact, desferal appears to be an inhibitor of both peroxidase and laccase but not of catecholase activities. This inhibition of laccase by desferal is revealed for the first time. A mechanism to explain the inhibitory effect of desferal on phenol-oxidizing enzymes (laccase and peroxidase), based on the reaction of desferal with phenoxy radicals, is proposed.

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RESULTS AND DISCUSSION

Desferal is a powerful inhibitor of the oxidation of guaiacol by horseradish peroxidase, about 100% of the activity being inhibited by 10 mM desferal (Fig. 1A). Over the concentration range tested, the inhibition of guaiacol oxidation by peroxidase is mainly manifested as a reduction in the steady-state oxidation rate, with no effect of desferal on the lag time of the reaction being apparent (Fig. 1B).

Like peroxidase, desferal appears also to be an inhibitor of laccase activity when this is assayed with *p*-hydroquinone. Such inhibition of laccase by desferal appears to be significant starting from a concentration of 20 mM (Fig. 2). At 40 mM, desferal totally blocks the oxidation of *p*-hydroquinone by laccase (Fig. 2). The inhibition is mainly manifested as a reduction in the steady-state oxidation rate with no effect of desferal on the lag time of the reaction being noted.

Unlike peroxidase (a hemoprotein) and laccase (a cuproprotein), desferal was not an inhibitor of catechol oxidase (also a cuproprotein) when this was assayed

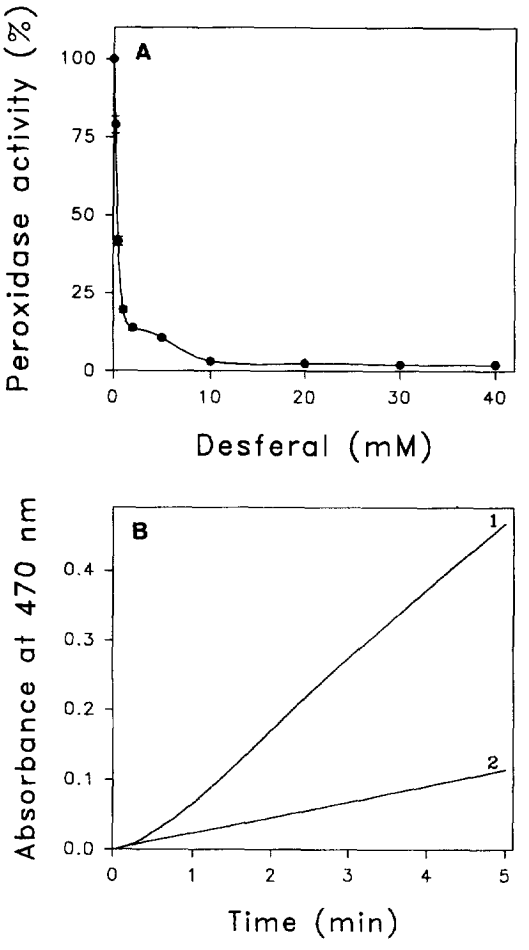

Fig. 1. (A) Effect of desferal concentration on peroxidase activity assayed with guaiacol. (B) Time-course of the *A* at 470 nm during the oxidation of guaiacol by peroxidase in the absence (1) and the presence (2) of 1 mM desferal ($n = 3$).

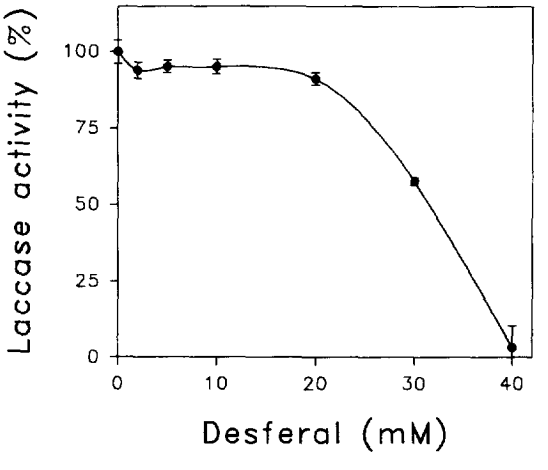

Fig. 2. Effect of desferal concentration on laccase activity assayed with *p*-hydroquinone ($n = 3$).

with *o*-catechol (Fig. 3). In fact, desferal appears to be a powerful activator of catechol oxidase activity in the range of the assayed concentrations (Fig. 3). These

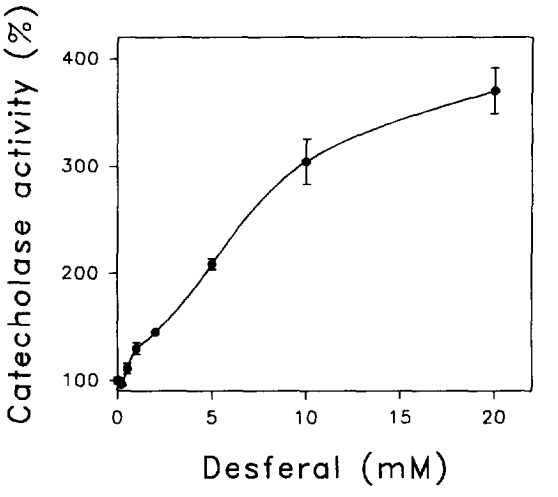

Fig. 3. Effect of desferal concentration on catechol oxidase activity assayed with *o*-catechol ($n = 3$).

results suggest that desferal may be considered as a selective inhibitor of laccase and peroxidase activities and may thus be used to discriminate laccase and peroxidase from catechol oxidase activities.

For a long time, the inhibition by desferal of peroxidase-mediated oxidations has been explained by its properties as a transition metal chelator, which removes the iron from the prosthetic group of peroxidases. Such properties would also explain desferal's inhibition of laccase, and the necessity for higher desferal concentrations (Fig. 2) when comparing it with peroxidase (Fig. 1), since desferal would also bind copper but with much lower stability constants (see [3]). If this is true, the ability of desferal to inhibit phenolic oxidation by both laccases and peroxidases should be independent of the nature of the phenolic oxidized, and only dependent on the nature of the prosthetic group of the enzyme.

In order to check this, the inhibition by desferal of the oxidation of several phenols, putative physiological substrates of both laccases and peroxidases, was tested. Coniferyl alcohol (a precursor of lignin biosynthesis) and resveratrol (a grapevine phytoalexin) are two well known substrates of both peroxidase and laccase [12–16]. Dihydrocapsaicin (a major pungent compound of hot pepper) is a well known substrate of peroxidases [17], and its oxidation by laccase is shown for the first time (Fig. 4).

When the inhibition effect of desferal was tested on these phenolic oxidations catalysed by both peroxidase and laccase, the surprising result (Table 1) was that desferal inhibits the oxidation of each one of these phenols to about the same extent, independently of the nature of the enzyme (Table 1). In fact, at 10 mM, the inhibitory effect of desferal on both peroxidase and laccase was almost identical for the same phenolic, varying from 84–98% for dihydrocapsaicin to 17–23% for coniferyl alcohol (Table 1). These results show that the chelating properties of desferal are not responsible for the inhibition.

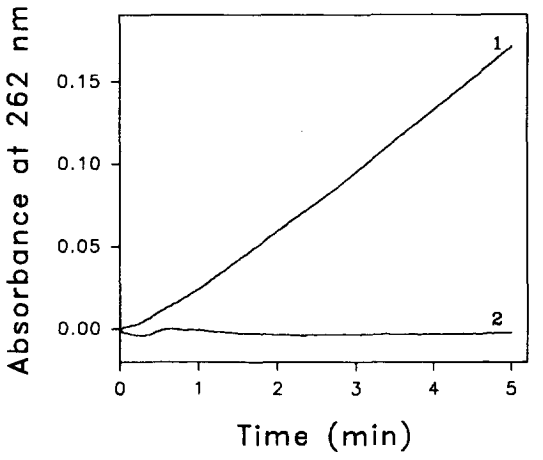

Fig. 4. Time-course of the oxidation of dihydrocapsaicin by laccase (1). Control in the absence of enzyme (2).

Similar results were found when using non-physiological substrates such as 4-methoxy-α-naphthol and aminofluorene for laccases and peroxidases, although in the case of the amine aminofluorene, the inhibition by desferal of the laccase-mediated oxidation was somewhat greater than in the case of peroxidase, where a weak inhibition was observed (Table 2). Other substrates used, such as phenol red and leuco crystal violet, were only substrates of peroxidase-mediated oxidations (Table 2), and may thus be used to discriminate between peroxidase and laccase activities.

These results point to the need for an alternative mechanism to explain the inhibition of both peroxidase and laccase-mediated oxidations by desferal. In fact, Morehouse *et al.* [3] have reported that desferal may be oxidized by peroxidase to yield a nitroxide free radical. This property of desferal to act as a substrate of peroxidase could explain the inhibition by desferal of the peroxidase-mediated phenolic oxidation since it would act as a competitive inhibitor of the enzyme. However, this would not explain the inhibition of laccase by desferal, nor the fact that its efficacy as an inhibitor depends more on the substrate employed than on the oxidizing enzyme (Tables 1 and 2). In fact, the inhibition by desferal of phenolic oxidations catalysed by both laccase and peroxidase can easily be explained by assuming that the phenolic radicals formed by the action of these enzymes on substrates react with desferal in accordance with the following reaction mechanism:

$$\mathrm{PhOH} \xrightarrow[\text{peroxidase}]{\text{laccase}} \mathrm{PhO^{\bullet}} \qquad (1)$$

$$\mathrm{PhO^{\bullet}} + \mathrm{D} \longrightarrow \mathrm{PhOH} + \mathrm{D^{\bullet}} \qquad (2)$$

where $\mathrm{PhO^{\bullet}}$ is the radical phenoxy of any phenolic oxidized by either laccase or peroxidase, D is desferal, and $\mathrm{D^{\bullet}}$ is the nitroxide free radical. The reaction (2) would compete with the deactivation reaction of the phenoxy radical:

$$\mathrm{PhO^{\bullet}} \longrightarrow \text{products} \qquad (3)$$

This reaction mechanism would explain why inhibition by desferal is more dependent on the substrate assayed than on the oxidizing enzyme, since the inhibition would be determined by the reactivity of desferal with phenoxy radicals, according to eqn (2).

The use of desferal as specific inhibitor of the hemoprotein peroxidase should be discarded. However, its use as inhibitor of phenol-oxidizing enzymes, such as peroxidase and laccase, is promising, since desferal appears to deactivate phenoxy radicals, the main products of these phenolic oxidations.

Table 1. Effect of 10 mM desferal on the oxidation of coniferyl alcohol, dihydrocapsaicin and resveratrol by both peroxidase and laccase ($n = 2$–3). Inhibition (in percentage) of the oxidation by desferal is shown in parentheses

	Peroxidase (AU $\mathrm{min^{-1}}$)		Laccase (AU $\mathrm{min^{-1}}$)	
Substrate	Control	Desferal	Control	Desferal
Coniferyl alcohol	0.183	0.151 (17%)	0.073	0.056 (23%)
Dihydrocapsaicin	0.050	0.001 (98%)	0.038	0.006 (84%)
Resveratrol	0.139	0.081 (42%)	0.163	0.113 (31%)

Table 2. Effect of 10 mM desferal on the oxidation of 4-methoxy-α-naphthol, diaminofluorene, phenol red and leuco crystal violet by both peroxidase and laccase ($n = 2$–3). Inhibition (in percentage) of the oxidation by desferal is shown in parentheses

	Peroxidase (AU $\mathrm{min^{-1}}$)		Laccase (AU $\mathrm{min^{-1}}$)	
Substrate	Control	Desferal	Control	Desferal
Methoxy-α-naphthol	0.057	0.052 (9%)	0.027	0.026 (4%)
Diaminofluorene	0.425	0.411 (3%)	0.118	0.074 (37%)
Phenol red	0.010	0.000 (100%)	0.000	
Crystal violet	0.166	0.047 (71%)	0.000	

EXPERIMENTAL

Chemicals. Desferrioxamine mesylate (desferal), coniferyl alcohol, dihydrocapsaicin, phenol red, diaminofluorene and leuco crystal violet were obtained from Sigma. Guaiacol was from Merck, *o*-Catechol and *p*-hydroquinone from BDH Chemicals and 4-methoxy-α-naphthol from Fluka. *trans*-Resveratrol [(*E*)-3′,4,5′-trihydroxystilbene] was synthesized by a Wittig reaction linking 3,5-dimethoxybenzyltriphenylphosphine bromide and *p*-anisaldehyde through a styrene double bond utilizing the general concept proposed in ref. [18] but with significant modifications (M. Morales, J. Alcántara, A. A. Calderón, A. Ros Barceló, and J. M. Zapata, unpublished results). Methylated precursors were used to protect the OH groups, which were removed by boron tribromide. The Wittig products were a mixture of (*E*) and (*Z*) isomers. The (*E*)/(*Z*) mixture was isomerized to the pure (*E*) isomer by heating with a catalytic amount of diphenyl disulphide in tetrahydrofuran. The structure of *trans*-resveratrol was confirmed by IR, ^{1}H NMR ($CDCl_3$, CD_3COCD_3, 300 MHz), ^{13}C NMR ($CDCl_3$, CD_3COCD_3, 75 MHz) and MS, the data being in accordance with those previously reported in ref. [18]. Confirmation of *trans* conformation was deduced from their UV-spectral characteristics (λ_{max} = 307 nm), IR absorption peak at 965 (w) cm^{-1} (*trans* form of the double bond); ^{1}H NMR ($CDCl_3$, CD_3COCD_3, 300 MHz): δ 6.79–6.84 (*m*, 3H, $H_3 + H_a/H_b$) and 6.97 (*d*, 1H, J = 16.5 Hz, H_a/H_b) and ^{13}C NMR ($CDCl_3$, CD_3COCD_3, 75 MHz): δ 125.07 (C_a/C_b) and 127.38 (C_a/C_b).

All the other chemicals used in this work were obtained from various commercial suppliers and were of the highest purity available.

Enzymes. Horseradish peroxidase (type IX) (donor: hydrogen peroxide oxidoreductase, EC 1.11.1.7), tyrosinase (catechol oxidase, *o*-benzenediol: oxygen oxidoreductase, EC 1.14.18.1) and laccase (*p*-benzenediol: oxygen oxidoreductase, EC 1.10.3.2) were obtained from Sigma.

Spectrophotometric assays. Unless otherwise noted, the spectrophotometric assay for the oxidation of compounds by peroxidase, tyrosinase and laccase activities was performed at 25° in a reaction medium containing 0.1 M Tris–acetate buffer, pH 5. In the case of peroxidase, the reaction medium also contained 0.05 mM H_2O_2. The concn and wavelength at which oxidation was tested for each compound as: guaiacol (5 mM, 470 nm), catechol (25 mM, 390 nm), hydroquinone (7.5 mM, 250 nm), coniferyl alcohol (0.1 mM, 260 nm), dihydrocapsaicin (0.1 mM, 262 nm), resveratrol (0.05 mM, 305 nm), 4-methoxy-α-naphthol (0.1 mM, 593 nm), diaminofluorene (0.1 mM, 600 nm), phenol red (0.05 mM, 610 nm) and leuco crystal violet (0.05 mM, 590 nm). In all cases, the reaction was initiated by the addition of enzyme.

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