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Participation of Mn(II) in the catalysis of laccase, manganese peroxidase and lignin peroxidase from *Phlebia radiata*

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Abstract

Oxidation capacities of laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) from *Phlebia radiata* were compared using non-phenolic (veratryl alcohol and ABTS) and phenolic (syringaldazine, vanillalacetone and Phenol red) compounds as reducing substrates. The effect of Mn(II) on enzyme reactions was also studied. Highest specific activities were recorded with laccase in the oxidation of phenolic compounds or ABTS and irrespective of Mn(II) concentration. LiP and MnP oxidized all these substrates but only the catalysis of MnP was dependent upon Mn(II). Only LiP clearly oxidized veratryl alcohol. However, Mn(II) interfered with this reaction by repressing veratraldehyde formation. These results point to multiple participation of manganese ions, either as a reducing (Mn(II)) or oxidizing (Mn(III)) agent in the enzymatic reactions.

Key words: Lignin degradation; White-rot fungus; Enzymatic oxidation; Peroxidase; Laccase; Manganese(II)

1. Introduction

The white-rot fungus *Phlebia radiata* possesses an efficient ligninolytic system in which all the three types of known lignin-modifying extracellular oxidoreductases, namely lignin peroxidase (ligninase; LiP), manganese-dependent peroxidase (manganese peroxidase; MnP) and a phenol oxidase of the laccase type, are expressed [1,2]. *P. radiata* differs from *Phanerochaete chrysosporium*, the lignin-degrading system of which has been studied intensively for the past ten years, since the latter fungus produces only lignin and manganese peroxidases but no laccase [3,4]. The array of ligninolytic enzymes generated by *P. radiata* – one laccase, two MnPs and three LiPs [2,5] – may explain why this fungus selectively and efficiently degrades various lignins to CO_2 [6], and causes a rapid decolourization of Kraft bleach plant effluent [7].

The three LiP isozymes from *P. radiata*, designated L1, L2 and L3, are glycosylated hemoproteins [5] which exhibit a high degree of sequence homology with the LiPs from *P. chrysosporium* [8]. All three LiP isozymes catalyse the H_2O_2-dependent oxidation of veratryl alcohol and β-O-4 dimeric lignin model compounds. Furthermore, the main *P. radiata* LiP isozyme L3 is kinetically similar to *P. chrysosporium* LiP H8 [9]. The characterized *P. radiata* MnP also resembles biochemically the *P. chrysosporium* MnPs [10]. However, *P. radiata* laccase appears to be different from the typical blue copper laccases [11] and very little is known about the kinetics and oxidation capacities of either the laccase or the MnPs from *P. radiata*.

In this study, the catalytic properties of isolated laccase, MnP and LiP L3 from *P. radiata* have been investigated. Several aromatic compounds commonly used as enzyme substrates were introduced as reductants to select a specific assay for each enzyme and to compare their redox capacities. Also, the effect of Mn(II) on enzyme reactions was studied due to the known oxidation of Mn(II) to Mn(III) by MnP [12,13] and the possible function of chelated Mn(III) as a diffusible oxidant between the fungus and lignin polymer upon degradation.

2. Materials and methods

2.1. Fungus and cultivation

The ligninolytic enzymes of the white-rot fungus *Phlebia radiata* Fr. strain 79 (ATCC 64658) [6] were produced by growing the fungus immobilized on polypropylene carrier in 2 liter bioreactors [7] on low-nitrogen (2.0 mM N) ADMS medium [6] containing 56 mM glucose, 0.05% (w/v) Tween 80, the basal low level of manganese (2 μM $MnSO_4$) and 1.0 mM veratric (3,4-dimethoxybenzoic) acid or veratryl (3,4-dimethoxybenzyl) alcohol. LiP, MnP and laccase activities were followed [1] and the culture medium was harvested at the peak of each enzyme production.

2.2. Purification of enzymes

Harvested culture supernatants were frozen (−20°C) and then thawed, filtrated (Whatman GF-C glass filters) and concentrated 10-fold (10 kDa cut-off Omega casette, Filtron). Extracellular proteins were separated by FPLC anion-exchange chromatography at pH 5.5 on Sepharose Q (Pharmacia Biotechnology) as described previously [2,9]. Enzyme activities of protein fractions were analyzed and the peak fractions showing only laccase, MnP or LiP activity were pooled and concentrated (10 kDa cut-off Microsep concentrators, Filtron). The purity of enzyme preparations was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and visualized by silver staining [14]. Isoelectric points of the proteins were determined by analytical isoelectric focusing (IEF) on acrylamide gels (pH gradient 2.5–5) [14]. Protein was determined by the method of Bradford [15] using a Bio-Rad kit. Enzyme preparations showing only

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Abbreviations: LiP, lignin peroxidase; MnP, manganese peroxidase; ABTS, 2,2′-azino-di-(3-ethylbenz-thiazoline-6-sulfonate).

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one protein band under SDS-PAGE and no side activities were used in further experiments.

2.3. Substrate oxidation experiments

Oxidation of syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine; Aldrich-Chemie), ABTS (2,2′-azino-di-[3-ethylbenz-thiazoline-6-sulfonate], diammonium salt, Boehringer Mannheim), veratryl alcohol (3,4-dimethoxybenzyl alcohol; Aldrich-Chemie) and vanillalacetone (4-[4-hydroxy-3-methoxyphenyl]-3-buten-2-one) (Fig. 1) by purified enzyme preparations were determined in 20 mM sodium lactate buffer, pH 4.5. Veratryl alcohol was distilled before use and vanillalacetone was kindly obtained from Assoc. Prof. Gösta Brunow (Department of Chemistry, University of Helsinki, Finland). Lactate, pH 4.5, was selected since lactic acid is a good chelator for manganese ions [16,17] and all the enzymes tested are active at this pH (see section 3). Reaction mixtures (1 ml) contained buffer, 50 μl enzyme, a constant concentration of the reducing substrate (25–500 μM), various concentrations of Mn(II) as $MnSO_4 \cdot H2O$ (p.a., Merck), and 100 μM of H_2O_2 (Suprapur, Merck) except in laccase assays where H_2O_2 was omitted. In addition, Phenol red assay [1,22] was used to examine the MnP activity. Oxidation rates were determined by Shimadzu 160A spectrophotometer at 30°C using the appropriate absorbance wavelength. Syringaldazine oxidation was monitored at 525 nm ($\varepsilon = 6.5 \times 10^4$ $M^{-1} \cdot cm^{-1}$) [18], ABTS oxidation to a coloured radical cation [19] at 436 nm ($\varepsilon = 2.9 \times 10^4$ $M^{-1} \cdot cm^{-1}$) [5], oxidation of veratryl alcohol to veratraldehyde at 310 nm ($\varepsilon = 9.3 \times 10^3$ $M^{-1} \cdot cm^{-1}$) [20] and vanillalacetone oxidation at 336 nm ($\varepsilon = 1.8 \times 10^4$ $M^{-1} \cdot cm^{-1}$) [21]. Controls lacked either enzyme or H_2O_2. In the case of veratryl alcohol oxidation, veratraldehyde formation was checked by high performance liquid chromatography (HPLC) using the methods previously described [1].

2.4. Determination of pH optima

The pH optimum of LiP L3 was determined using the veratryl alcohol oxidation assay [20] in 20 mM sodium tartrate buffer. The effect of pH on laccase and MnP activities was determined in 0.1 M citrate-phosphate and 20 mM sodium lactate buffers, respectively, using syringaldazine as substrate [18]. In the case of LiP and MnP, 100 μM H_2O_2 was added to reaction mixtures to inititate the peroxidase cycle.

3. Results

3.1. Enzyme properties

The molecular masses and isoelectric points of the several extracellular enzymes purified from *P. radiata* are listed in Table 1 using previously adopted nomenclature [2]. In the subsequent studies, laccase 1 and the main LiP and MnP isozymes, i.e. LiP L3 and MnP, were used.

3.2. Effect of pH on enzyme activities

The effect of pH on the catalytic activity of LiP L3, MnP and laccase in the oxidation of specific substrates (see below) is shown in Fig. 2. LiP L3 was clearly different from laccase and MnP because of its very acidic pH optimum (at pH 2.5). In addition, the rate of veratryl alcohol oxidation by LiP L3 showed a linear increase with decreasing pH within the range pH 2.5–4.5, similar to that reported for LiP (H8) from *P. chrysosporium* [22]. The pH vs. activity curves of laccase and MnP resembled each other in having broad optima ranging between pH 4.5 and 5.5. However, the specific activity of MnP when syringaldazine served as substrate, was approximately 15% of the value for laccase (Fig. 2). Moreover, LiP exhibited only 1/5 of its maximum activity at pH 4.5, which was the optimum pH for both laccase and MnP. Catalytically, laccase was clearly the most active enzyme at this pH. However, since all the enzymes retained some catalytic activity at pH 4.5, this pH was selected for further experiments.

Table 1
Molecular masses and isoelectric points of the ligninolytic enzymes of *Phlebia radiata*

	M (kDa)	p*I*
LiP L1	40	4.1
LiP L2	45	3.9
LiP L3	44	3.2
MnP	48	3.7
MnPx	47	4.7
Laccase 1	64	3.5

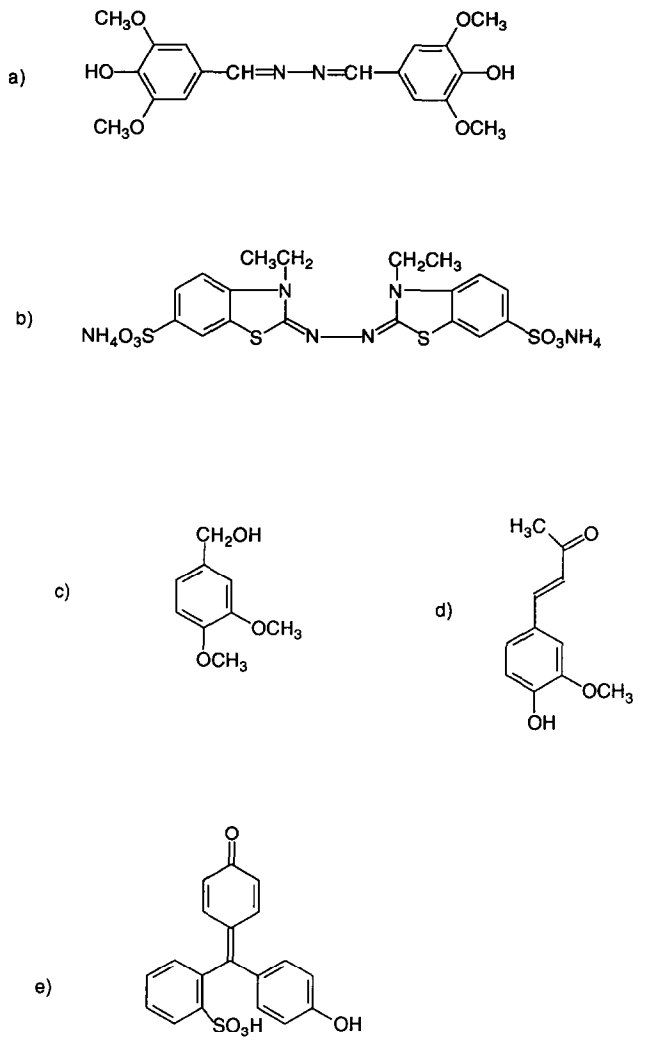

Fig. 1. Chemical structures of the compounds used as reducing substrates in the enzyme reactions: (a) syringaldazine; (b) ABTS as diammonium salt; (c) veratryl alcohol; (d) vanillalacetone; and (e) Phenol red.

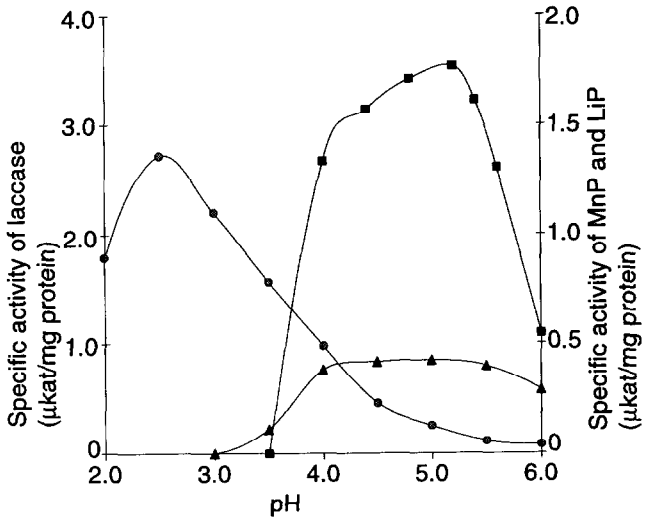

Fig. 2. Effect of pH on the specific catalytic activities of the laccase (■), manganese peroxidase (isozyme MnP) (▲), and lignin peroxidase (isozyme L3) (●) from *Phlebia radiata*.

3.3. Laccase

The redox capacities of purified LiP L3, laccase and MnP were compared using four common 'specific' substrates, both phenols and non-phenols (Fig. 1). The effect of Mn(II) on the enzyme reactions was also investigated and the results are shown in Fig. 3. Highest specific activities were obtained with laccase and oxidation rates were 10–100-fold higher than those observed with MnP or LiP, except in the case of veratryl alcohol which was not a substrate for laccase (Fig. 3c). Manganese ions did not participate in the oxidation of syringaldazine and ABTS by laccase (Fig. 3a,b), since increasing Mn(II) levels did not markedly alter reaction rates and small oscillations in enzyme activity may fall within the range of experimental error. However, the rate of oxidation of vanillalacetone decreased with increasing concentrations of Mn(II) up to 1.0 mM and, when the concentration of Mn(II) was raised to 3.0 mM, the oxidation rate returned to the initial level (Fig. 3d).

3.4. Lignin peroxidase

Although syringaldazine, ABTS and vanillalacetone were oxidized by all the enzymes, albeit with different specificities (Fig. 3a,b,d), the effects of increasing Mn(II) concentrations on the catalytic activity of each enzyme varied. Lactate buffer (20 mM, pH 4.5) was used in these experiments to ensure that manganese ions participated in the enzymatic reactions as the lactate chelate [12,16]. In the presence of H_2O_2, LiP oxidized all the substrates although reaction rates were comparatively low with ABTS and the phenols (Fig. 3). The specific activity of LiP was highest when veratryl alcohol served as the substrate (Fig. 3c) and formation of veratraldehyde as end-product was confirmed by HPLC. However, high manganese concentrations (over 0.5 mM) repressed veratraldehyde formation (Fig. 3c). With other reductants,

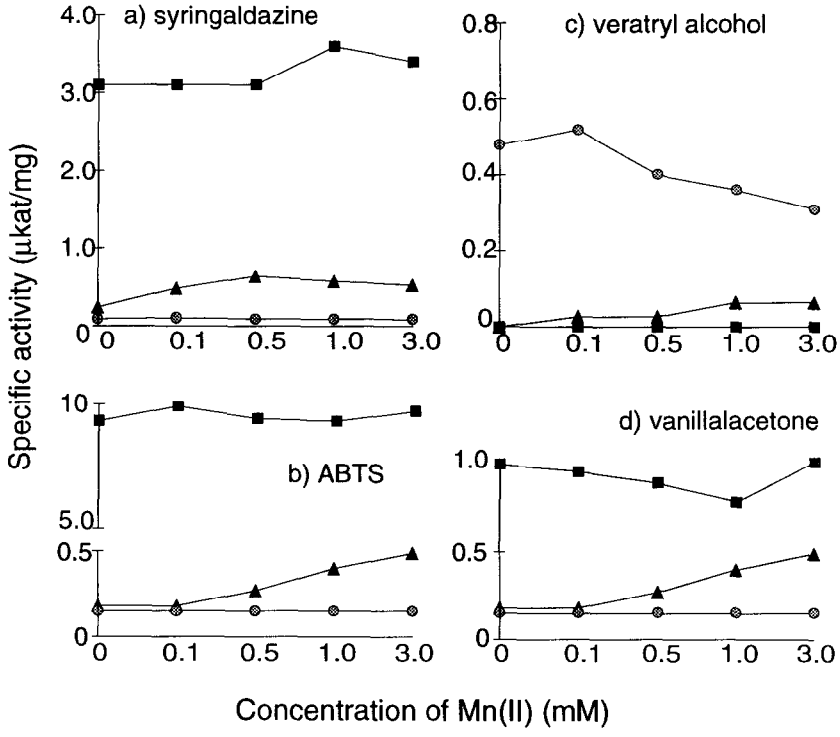

Fig. 3. Oxidation of aromatic substrate compounds: (a) syringaldazine; (b) ABTS; (c) veratryl alcohol; and (d) vanillalacetone, by laccase (■), manganese peroxidase (MnP) (▲), and lignin peroxidase (LiP L3) (●), from *Phlebia radiata* under various concentrations of Mn(II). The reactions were carried out in 20 mM lactate buffer (pH 4.5). For more details, see section 2.

manganese ions did not affect or interfere with LiP catalysis, since reaction rates remained constant over the range of Mn(II) used (Fig. 3a,b,d).

3.5. Manganese peroxidase

Only the catalytic activity of MnP was clearly accelerated by increasing levels of Mn(II). However, the optimum level differed depending on the reducing substrate: in reaction mixtures measuring the oxidation of syringaldazine, the system was saturated when the level of Mn(II) exceeded 0.5 mM (Fig. 3a) whereas in oxidations of ABTS and vanillalacetone, reaction rates increased with increasing concentrations of Mn(II) (Fig. 3b,d). However, reaction rates were lower than those observed with syringaldazine. The optimum Mn(II) concentration obtained, 0.5 mM (Fig. 3a), may reflect a depletion of the reductant, because due to its poor solubility in water, syringaldazine was present at concentrations of only 25 μM in the reaction mixtures.

The rate of oxidation of phenol red by both MnP isozymes was enhanced when the concentration of Mn(II) was increased (Fig. 4). This effect was also observed in MnP-catalysed oxidation of ABTS and vanillalacetone (Fig. 3b,d). In reaction mixtures containing MnP, Mn(II), veratryl alcohol and H_2O_2, slow increase in absorbance at 310 nm was observed (Fig. 3c), possibly indicating veratraldehyde formation. The same effect was observed with Mn(III) lactate, prepared by mixing Mn(III) acetate and lactate buffer [12,16,23]. However, in contrast to LiP oxidation, no veratraldehyde was detected as end-product in either MnP/Mn(II)/H_2O_2/veratryl alcohol or Mn(III) lactate/H_2O_2/veratryl alcohol systems by subsequent HPLC-analysis (data not shown).

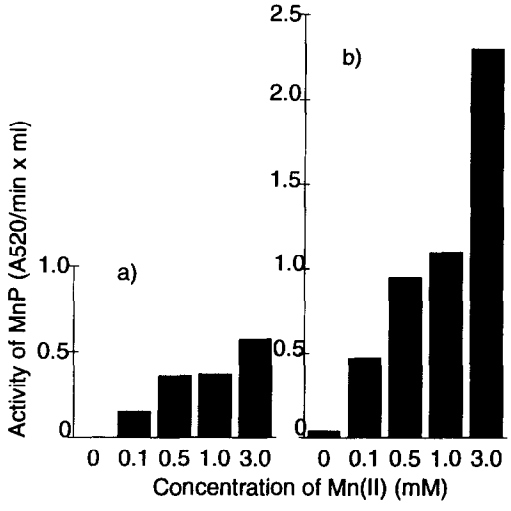

Fig. 4. Comparison of the catalytic activities of the two MnP isozymes, MnPx (a) and MnP (b), of *Phlebia radiata* in the H_2O_2-dependent oxidation of Phenol red under conditions of various concentrations of Mn(II). The reaction rates were determined from the increase in absorbance at 520 nm [1]. The amount of enzyme in 1 ml reaction mixtures was 0.1 mM in both cases.

4. Discussion

4.1. Oxidation capacities of the enzymes

Highest reaction rates and specific catalytic activities were recorded with laccase, when the phenolic compounds, syringaldazine and vanillalacetone, were used as substrates for the ligninolytic enzymes. However, even higher laccase specific activities were obtained in the oxidation of the non-phenolic compound, ABTS, which is a well-known substrate for peroxidases [19]. These data imply that laccase serves as an important oxidant for aromatic rings substituted with electron withdrawing groups such as free phenolic hydroxyls or sulfonate groups in lignins or related compounds. Mn(II) ions did not affect laccase-catalysed oxidation of syringaldazine or ABTS.

In contrast, slow decrease in the laccase-catalysed oxidation of vanillalacetone was observed with increasing Mn(II) concentrations up to 1.0 mM, indicating participation of manganese ions in the reaction. It has been reported recently that laccase may oxidize Mn(II) to Mn(III) in the presence of phenolic compounds [24], thus resembling MnP functionally and releasing Mn(III) chelates to act as oxidizing species. However, our data indicate that with preferred reducing substrates such as syringaldazine and ABTS, coupled oxidation of Mn(II) by laccase may not be involved.

Furthermore, although laccase showed the highest specific activity on most of the substrate compounds tested, no veratryl alcohol oxidation was observed even in the presence of Mn(II). This is compatible with the poor oxidizing ability of laccase towards non-phenolic aromatic compounds of high redox potential, such as methoxybenzenes [25].

The extent of manganese ion participation in the enzymatic oxidations varied with respect to the enzyme investigated. Only the catalytic activity of MnP was clearly enhanced by increasing the concentration of Mn(II). This is in agreement with published data [12,13,21] and indicated that, in our experiments, enzyme-generated Mn(III) ions [12,13] were appropriately chelated by lactic acid [16] and thereby sufficiently stabilized to initiate further oxidation of the aromatic compounds. Mn(III) is a powerful oxidizing agent with a standard reduction potential of 1.51 V [16]. However, the properties of the reducing substrate affected the degree to which manganese ions participated in these reactions. Syringaldazine was a good substrate but the oxidation system was saturated at 0.5 mM concentrations of Mn(II).

On the other hand, the initially low rates of oxidation of ABTS and vanillalacetone, as well as the rate of Phenol red oxidation by MnP, were improved by increasing levels of Mn(II) up to 3.0 mM. Syringaldazine, ABTS and vanillalacetone are common substrates for MnP [12,21]. However, as our results show, not one of these compounds is specific for MnP. On the contrary, all are

readily oxidized by laccase and also by LiP. When using these substrates in MnP assays, appropriate controls (without Mn(II), without H_2O_2), should be introduced in order to exclude other enzyme activities.

4.2. Effect of Mn(II) on veratryl alcohol oxidation

Being a non-phenolic compound with a relatively high redox potential, veratryl alcohol is a substrate for LiP [22] but not for MnP [12,21]. However, in reaction mixtures containing MnP, H_2O_2, veratryl alcohol and Mn(II), very slow increase in absorbance at 310 nm was detected indicating veratryl alcohol oxidation to veratraldehyde. Although Mn(III)-lactate caused also a slow increase in absorbance at 310 nm, HPLC-analysis did not show any veratraldehyde formation. This indicates that chelated Mn(III) was not able to oxidize veratryl alcohol directly, which is in contrast with previous data [26,27] but in accordance with the inability of Mn(III) to oxidize methoxybenzenes of higher redox potential than 1.2 V [28].

No veratraldehyde formation was detected in reaction mixtures containing MnP and H_2O_2, but without added Mn(II), thereby eliminating the possibility of the enzyme preparate containing LiP as a contaminant. The slow increase in absorbance observed at 310 nm with MnP/Mn(II)/H_2O_2/veratryl alcohol may have been due to Mn(III) lactate absorbing at this wavelength [12]. MnP is reported to oxidize veratryl alcohol and other non-phenolic compounds in the presence of Mn(II), H_2O_2, and a thiol reductant, such as glutathione [26,29]. The reaction proceeds via the formation of a thiyl radical by Mn(III) chelate, and the thiyl radical acts as an oxidant for non-phenols [29]. Since no thiol reductant was added in reaction mixtures, the indirect oxidation of veratryl alcohol by MnP most probably did not occur in the present study.

In contrast, the LiP-catalysed oxidation of veratryl alcohol was influenced by Mn(II), and high Mn(II) concentrations markedly repressed veratraldehyde formation. Our results conflict with previous data reported for LiP under corresponding experimental conditions [17], i.e. using lactic acid as a chelator for manganese ions, which showed that addition of 0.1 mM Mn(II) significantly increased the amount of veratraldehyde formed both at pH 3.0 and 4.5. It was proposed [17] that the observed increase was due to the scavenging of superoxide anions by Mn(II) lactate, which would then generate H_2O_2 thereby leading to an overproduction of veratraldehyde by LiP [17].

Our results are more consistent with those of Popp et al. [23] who also found that less veratraldehyde is formed in LiP-catalysed oxidations of veratryl alcohol in the presence of Mn(II) and a chelator, either malonate or oxalate. The authors [23] observed that Mn(III) was generated and oxygen was consumed in the reaction. We propose that the Mn(II) lactate complex is involved in the LiP-catalysed oxidation cycle of veratryl alcohol but, instead of scavenging superoxide anions [17,23], reduces the veratryl alcohol radical cation – the first intermediate formed after one-electron oxidation [30] – back to veratryl alcohol (Fig. 5). Thus, less veratraldehyde is formed as end-product.

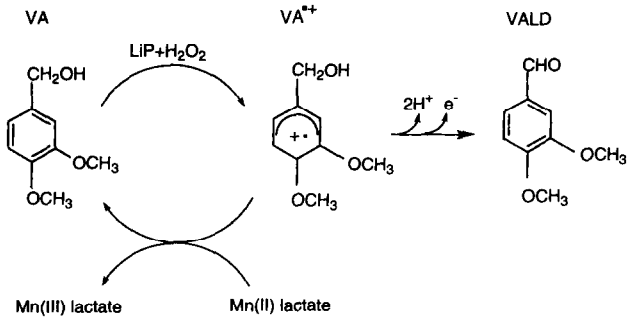

Fig. 5. Proposed mechanism for the interference caused by Mn(II) in the LiP-catalysed oxidation of veratryl alcohol.

In addition, it has been observed that increasing the amount of LiP used in the reaction mixtures leads to exceeding amounts of veratraldehyde and Mn(III) produced in respect to the equivalent of H_2O_2 added [23]. LiP concentrations used in our experiments were kept constantly low (0.2 μM). Furthermore, reactions were carried out without agitation, which may have reduced oxygen incorporation and the subsequent formation of superoxide and excess H_2O_2, again explaining the decrease in veratraldehyde formation in LiP-catalysed oxidation of veratryl alcohol in the presence of Mn(II).

These data imply multiple roles of participation for manganese ions in enzymatic oxidation of lignin-related compounds. Depending on enzyme specificity and chemical character of the reducing substrates, manganese ions may act as reductants (Mn(II)) or oxidants (Mn(III)) in the presence of suitable chelator. This phenomenon requires to be taken into account when different enzyme activities are measured from fungal culture liquor. We noticed in the case of *Phlebia tremellosa* that especially the existence of extracellular MnP protein should be confirmed by other means, e.g. protein purification [31]. Also, the concerted action of the ligninolytic enzymes tested, laccase, MnP and LiP, together with manganese ions, should be investigated in order to elucidate the enzymatic and non-enzymatic mechanisms of lignin breakdown by white-rot fungi.

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