

H₂O₂ generation during the auto-oxidation of coniferyl alcohol drives the oxidase activity of a highly conserved class III peroxidase involved in lignin biosynthesis

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Abstract Characterization of lignified *Zinnia elegans* hypocotyls by both alkaline nitrobenzene oxidation and thioacidolysis reveals that coniferyl alcohol units are mainly found as part of 4-*O*-linked end groups and aryl-glycerol- β -aryl ether (β -*O*-4) structures. *Z. elegans* hypocotyls also contain a basic peroxidase (EC 1.11.1.7) capable of oxidizing coniferyl alcohol in the absence of H₂O₂. Results showed that the oxidase activity of the *Z. elegans* basic peroxidase is stimulated by superoxide dismutase, and inhibited by catalase and anaerobic conditions. Results also showed that the oxidase activity of this peroxidase is due to an evolutionarily gained optimal adaptation of the enzyme to the μ M H₂O₂ concentrations generated during the auto-oxidation of coniferyl alcohol, the stoichiometry of the chemical reaction (mol coniferyl alcohol auto-oxidized/mol H₂O₂ formed) being 0.496. These results therefore suggest that the H₂O₂ generated during the auto-oxidation of coniferyl alcohol is the main factor that drives the unusual oxidase activity of this highly conserved lignin-synthesizing class III peroxidase.
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Key words: Auto-oxidation; Coniferyl alcohol; H₂O₂ generation; Oxidase reaction; Peroxidase

1. Introduction

Lignins are three-dimensional phenolic heteropolymers covalently associated with polysaccharides in plant cell walls [1]. They are mainly localized in the impermeable water transport conduits of the xylem and other supporting tissues, and result from the oxidative polymerization of three cinnamyl (*p*-coumaryl, coniferyl and sinapyl) alcohols in a reaction that can be mediated by both peroxidases and H₂O₂-independent oxidases [2–4], leading to an optically inactive hydrophobic heteropolymer [5]. The process of sealing plant cell walls through lignin deposition is known as lignification, and provides mechanical strength to the stems, protecting cellulose fibers from chemical and biological degradation [6]. Plant cell wall lignification is one of the main restrictive factors in the use and recycling of plant biomass [6].

Zinnia elegans is a seasonal-cycle flowering plant belonging to the Asteraceae family (considered to be one of the most evolved families among dicotyledon angiosperms) and is fre-

quently used as a model for lignification studies [7]. The cell wall of lignifying *Z. elegans* hypocotyls contains a basic peroxidase of high *pI*, which has been characterized as a high-spin heme class III peroxidase (EC 1.11.1.7) [8]. This strongly basic peroxidase has been localized in the lignin-forming xylem from *Z. elegans* hypocotyls by means of competitive inhibitor-dissected histochemistry [9], and it is considered a marker of xylem element differentiation in *Z. elegans* cell cultures [10].

A characteristic of this basic peroxidase is that it shows coniferyl alcohol oxidase activity [8], i.e. an oxidase activity in the absence of H₂O₂. This special catalytic property is not shown by other plant peroxidases, such as acidic peroxidases, which need a redox mediator to manifest such activity [11]. Furthermore, this basic peroxidase shows a particular affinity for cinnamyl alcohols with *k_M* values in the μ M range, and some specificity for syringyl-type phenols [12]. It is worth noting that the affinity of this strongly basic peroxidase for cinnamyl alcohols and aldehydes is similar to that shown by the preceding enzymes in the lignin biosynthetic pathway (microsomal 5-hydroxylases and cinnamyl alcohol dehydrogenase), which also use cinnamyl alcohols and aldehydes as substrates [12], indicating that the one-way highway of lignin macromolecule construction has no metabolic ‘potholes’ into which the lignin building blocks might accumulate. This fact suggests a high degree of metabolic plasticity for this basic peroxidase, which has been widely conserved during the evolution of vascular plants [13], making it one of the driving forces in the evolution of plant lignin heterogeneity.

In this report, we study the nature of the oxidase activity of this basic peroxidase. The results illustrate that this basic peroxidase shows a high affinity for H₂O₂, a property which allows an optimal adaptation to the H₂O₂ generated during the auto-oxidation of coniferyl alcohol, which is the main factor that drives the unusual oxidase activity of this highly conserved lignin-synthesizing class III peroxidase.

2. Materials and methods

2.1. Plant material

Seeds of *Z. elegans* were purchased from W.R. van der Schoot (Hillegom, The Netherlands). Seedlings were grown for 26 days in a greenhouse under daylight conditions at 25°C [8]. Fully grown (lignifying) hypocotyls were used for these studies.

2.2. Isolation of cell walls, alkaline nitrobenzene oxidation and thioacidolysis

Lignifying cell walls were prepared by a Triton X-100 washing

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procedure [9]. Alkaline nitrobenzene oxidation and HPLC analysis of the solubilized products was performed as described [14]. Quantitation of *p*-hydroxybenzaldehyde, vanillin and syringaldehyde was performed at 280 nm using the corresponding standards. Thioacidolysis of lignifying cell walls, which solubilizes the β -O-4 lignin core [15], and GC-MS analyses were carried out using a Hewlett Packard 5890 Series II gas chromatograph, an HP 5972 mass spectrometer and an HP5 (30 m $\times$ 0.25 mm I.D.) column. Mass spectra were recorded at 70 eV. Quantification of chromatographic peaks was performed from the total ionic current (TIC) chromatograms.

2.3. Isolation of the *Z. elegans* basic peroxidase

The *Z. elegans* basic peroxidase was purified from intercellular washing fluids of lignifying hypocotyls according to [16]. The protein was characterized as pure by SDS-PAGE [8].

2.4. Enzymatic oxidation of coniferyl alcohol

The oxidation of coniferyl alcohol by the *Z. elegans* basic peroxidase was assayed spectrophotometrically at 25°C, in a reaction medium containing 50 mM Tris-acetate buffer (pH 5.0) and variable H₂O₂ and coniferyl alcohol concentrations using a $\Delta\epsilon_{260}$ for the reaction of 9.75 mM⁻¹ cm⁻¹ [12]. The reactions were initiated by the addition of 0.175 μ g protein. Anaerobic conditions were created by passing a N₂ stream for 15 min through a previously evacuated reaction medium in an anaerobic glove box.

2.5. Auto-oxidation of coniferyl alcohol and determination of H₂O₂

The auto-oxidation of coniferyl alcohol was followed spectrophotometrically at 260 nm and 25°C in a air-saturated reaction medium containing 50 mM Tris-acetate buffer (pH 5.0) and 50 μ M coniferyl alcohol. Coniferyl alcohol auto-oxidation was quantified using the above described extinction coefficient. The H₂O₂ evolved during the auto-oxidation of coniferyl alcohol was measured by the xylenol orange method [17], using a calibration curve in the 0.1–5.0 μ M H₂O₂ range performed in the presence of 50 μ M coniferyl alcohol.

2.6. Electrophoretic analysis and Western blot

Isoelectric focusing (IEF) in non-equilibrium conditions was performed on 5% (w/v) polyacrylamide gels in 3.5–10.5 pH gradients using a MiniProtean II (Bio-Rad) electrophoresis kit [18]. Protein migration was allowed at 200 V for 90 min at 4°C using cytochrome *c* as a migration marker. Non-denaturing SDS-PAGE was performed on 10% (w/v) polyacrylamide gels using a MiniProtean II electrophoresis kit and a pH 8.8 electrophoresis buffer composed of 192 mM glycine and 25 mM Tris containing 0.1% SDS. SDS-PAGE was performed at 100 V for 20 min followed by 150 V for 70 min at 4°C. Western blot analyses were performed using anti-horseradish peroxidase (anti-HRP) rabbit IgGs as primary antibodies and HRP-conjugated goat anti-rabbit IgGs as secondary antibodies. Endogenous peroxidase electroblotted onto Immobilon-P polyvinylidene difluoride membranes (0.2 μ m) was inactivated by heating (100°C for 5 min). Peroxidase activity was stained with 4-methoxy- α -naphthol [18].

2.7. Chemicals

Coniferyl alcohol, xylenol orange, HRP (type IX), anti-HRP rabbit IgGs (P7899), HRP-conjugated goat anti-rabbit IgGs (A9169), superoxide dismutase (SOD) and catalase were purchased from Sigma (Madrid, Spain). The rest of the chemicals were obtained from various suppliers and were of the highest purity available.

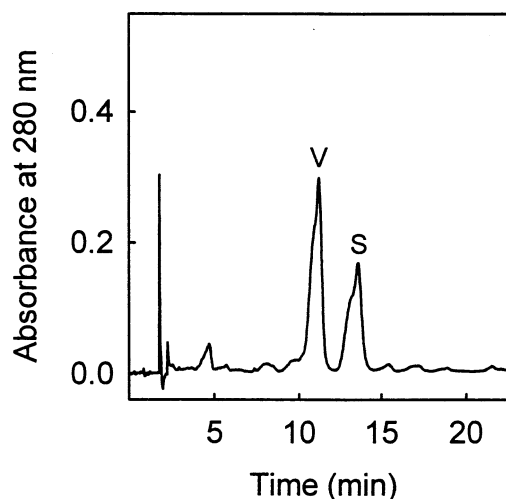


Fig. 1. HPLC profile of alkaline nitrobenzene oxidation products obtained from lignifying cell walls of *Z. elegans* hypocotyls. V, vanillin; S, syringaldehyde.

3. Results and discussion

3.1. Coniferyl alcohol units in lignins isolated from *Z. elegans* hypocotyls

Characterization of the lignins synthesized by *Z. elegans* hypocotyls by alkaline nitrobenzene oxidation (Fig. 1) reveals that they were composed of guaiacyl (G) and syringyl (S) units in a G/S ratio of 41/59. In lignins isolated from *Z. elegans* hypocotyls, *p*-hydroxybenzyl (H) units derived from *p*-coumaryl alcohol were absent. To ascertain the nature of the bonds in which coniferyl alcohol was involved, cell walls isolated from lignifying *Z. elegans* hypocotyls were subjected to thioacidolysis, and the recovered thioethylated monomers were analyzed by GC-MS. The results showed the presence of thioethylated monomers derived from 4-*O*-linked coniferyl alcohol end groups (3.82% TIC, Fig. 2A,B) and of the thioethylated (*erythro*- and *threo*-isomers) monomers arising from aryl-glycerol- β -aryl ether structures derived from coniferyl alcohol (37.67% TIC, Fig. 2C).

In addition to these thioethylated monomers arising from the cleavage of the linear β -O-4 lignin fraction, thioacidolysis also revealed the presence of significant amounts of stilbene structures (C₆C₂C₆) derived from the β - β cross-coupling of coniferyl alcohol (2.15% TIC), and trace amounts (<0.2% TIC) of C₆C₂ enol ether structures derived from the β -5 cross-coupling of coniferyl alcohol [19].

Table 1

Oxidation rates of coniferyl alcohol (100 μ M) by the *Z. elegans* basic peroxidase

Reaction medium	nmol substrate oxidized min ⁻¹ g ⁻¹ FW	
	–H ₂ O ₂	+H ₂ O ₂ (100 μ M)
Coniferyl alcohol (aerobic conditions)	3.8 $\pm$ 1.1	143.4 $\pm$ 6.3
Coniferyl alcohol+catalase (200 U ml ⁻¹)	1.1 $\pm$ 0.9	ND ^a
Coniferyl alcohol+SOD (200 U ml ⁻¹)	6.5 $\pm$ 1.5	ND
Coniferyl alcohol (anaerobic conditions)	0.0 $\pm$ 0.0	ND

^aNot determined.

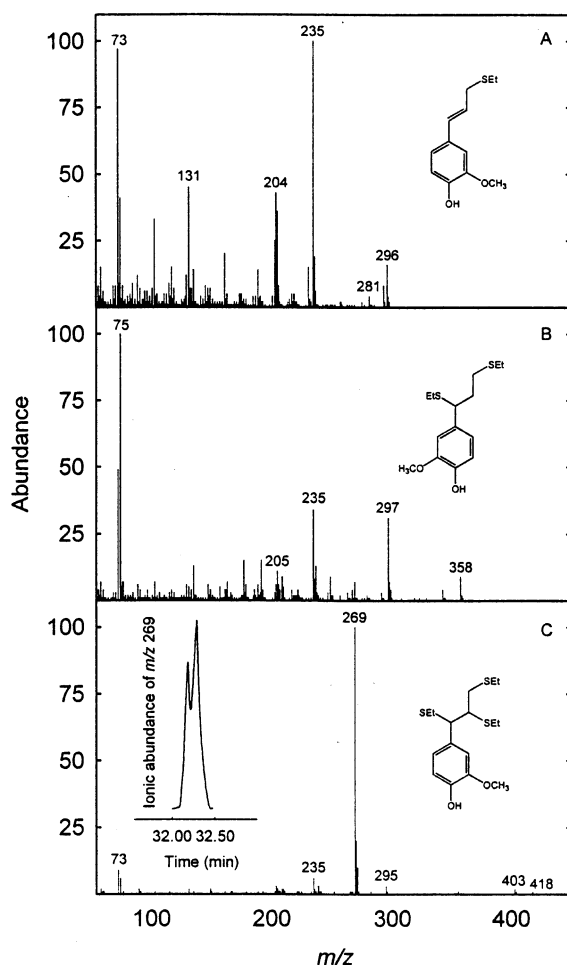


Fig. 2. Mass spectra of the trimethylsilyl derivatives of the thioethylated monomers arising from the *O*-4 coniferyl alcohol end group (A,B) and the β -*O*-4 coniferyl alcohol (C) obtained from GC-MS analyses. Chromatographic profile (C, inset) obtained for the ion of *m/z* 269 illustrating the presence of both the *erythro*- and *threo*-isomers of the thioethylated monomers arising from the β -*O*-4 coniferyl alcohol, which were identified as such by the mass spectrum illustrated in C.

3.2. Oxidation of coniferyl alcohol by the basic peroxidase isolated from lignifying *Z. elegans* hypocotyls

From the above results, it may be concluded that coniferyl alcohol is a ubiquitous constituent of *Z. elegans* hypocotyl lignins, where it may be found with several types of cross-linkages (*O*-4, β -*O*-4, β - β , and β -5 bonds). A peroxidase capable of oxidizing coniferyl alcohol is also present in lignifying *Z. elegans* hypocotyls. This basic peroxidase oxidizes coniferyl alcohol following a Michaelis–Menten type kinetics (Fig. 3), which fits the equation

$$v^{\text{RH}} = \frac{2Ek_1[\text{H}_2\text{O}_2][\text{RH}]}{(k_1/k_3)[\text{H}_2\text{O}_2] + [\text{RH}]} \quad (1)$$

where RH is coniferyl alcohol. This basic peroxidase possesses antigenic epitopes which are recognized by antibodies directed against HRP (Fig. 4A). Antibody-recognized epitopes were surface-exposed by the native enzyme form resolved by IEF (Fig. 4B) and by a partial SDS-denatured enzyme form resolved by anionic PAGE (Fig. 4C). A noticeable property of this basic peroxidase is that it is capable of oxidizing coniferyl alcohol in the absence of H_2O_2 , the oxidase reaction being stimulated by SOD, and inhibited by catalase and anaerobic conditions (Table 1).

The oxidase activity of plant peroxidases may be explained by two distinct mechanisms. One mechanism [20] assumes that the ferric form of peroxidase (Fe^{III}) is reduced by the substrate (R) to yield the ferrous form of the enzyme (Fe^{II}) [$\text{Fe}^{\text{III}} + \text{R} \rightarrow \text{Fe}^{\text{II}} + \text{R}^+$], which further reacts with O_2 to yield compound III [$\text{Fe}^{\text{II}} + \text{O}_2 \rightarrow \text{CoIII}$]. CoIII decomposes into Fe^{III} and O_2^- [$\text{CoIII} \rightleftharpoons \text{Fe}^{\text{III}} + \text{O}_2^-$] and, finally, O_2^- dismutates [$2 \text{O}_2^- + 2 \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$] to generate the H_2O_2 which is used by peroxidases to initiate the normal peroxidatic reaction. However, this mechanism is unlikely to occur during the oxidase reaction of the *Z. elegans* basic peroxidase, since coniferyl alcohol was unable to reduce the ferric form of the enzyme when this was checked by measuring the subsequent CoIII formation [21], an observation that is in accordance with the high redox potential of this cinnamyl alcohol [22]. The second mechanism [12] assumes that the oxidase activity of peroxidases may be due to a residual peroxidase activity

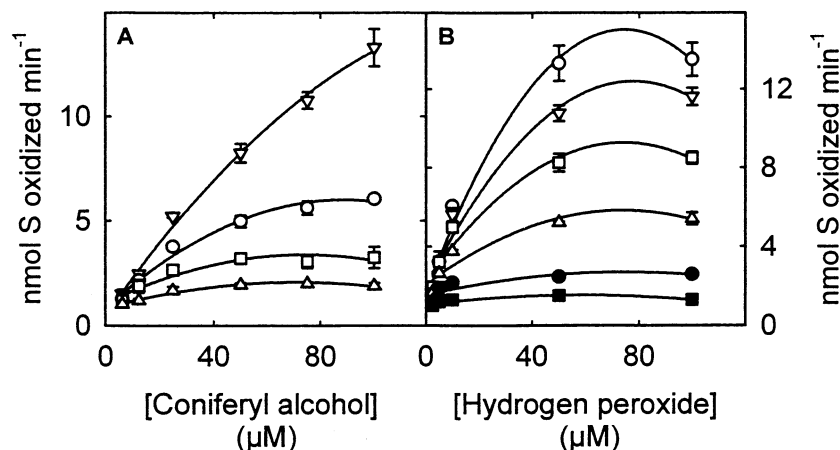


Fig. 3. Dependence of the coniferyl alcohol oxidation rate by the *Z. elegans* basic peroxidase on coniferyl alcohol (A) and H_2O_2 (B) concentration. In A, the reaction medium contained 2.5 (Δ), 5 ($\square$), 10 ($\circ$) or 50 μM (∇) H_2O_2 . In B, the reaction medium contained 6.25 ($\blacksquare$), 12.5 ($\bullet$), 25 (Δ), 50 ($\square$), 75 (∇) or 100 μM ($\circ$) coniferyl alcohol.

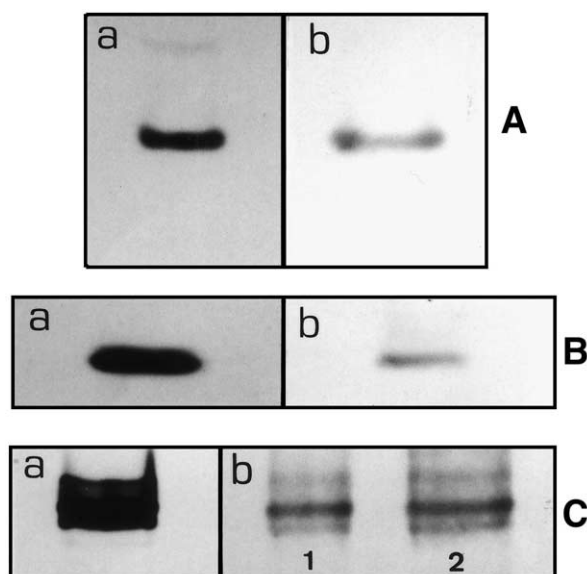


Fig. 4. Enzymatic activity assays (a) and simultaneous Western blot analyses (b) of HRP (A, 1.0 μg protein) and *Z. elegans* peroxidase (B, 2.0 μg protein, and C, 0.8 [1] and 8.0 [2] μg protein), separated by IEF in 3.5–10 pH gradients (A,B) and SDS-PAGE (C).

that is switched on by the traces of H_2O_2 formed during a slow kinetic phase, characterized by the auto-oxidation of the anion derived from coniferyl alcohol:



where R^- is the phenoxyl anion of coniferyl alcohol and $\text{R}^\bullet$ its corresponding phenoxyl radical, and which yields, as balance, a net H_2O_2 production:



This H_2O_2 formed at trace level could initiate the normal catalytic cycle of the enzyme. Eqs. 2–4 could explain the par-

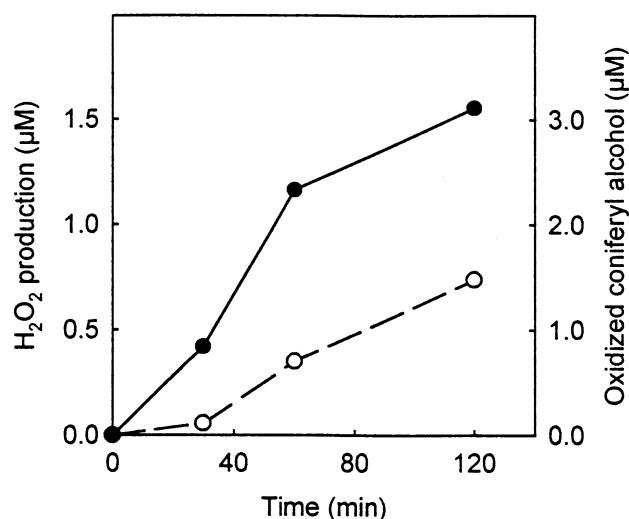


Fig. 5. Time course of the coniferyl alcohol oxidation (●) and the simultaneous H_2O_2 production (○) during the auto-oxidation of this cinnamyl alcohol at pH 5.0. Values are means of six independent replicates.

tial sensitivity of the oxidase reactions of *Z. elegans* peroxidase to both SOD, catalase and anaerobic conditions (Table 1). Furthermore, Eqs. 2–4 could also explain why the oxidase activity of the *Z. elegans* basic peroxidase is directly related to its capacity to use H_2O_2 , as was deduced when the oxidation rates of cinnamyl alcohols and aldehydes in the absence of H_2O_2 (which follow the order: sinapyl alcohol > coniferyl alcohol > coniferyl aldehyde) were compared with its efficacy ($V_{\text{max}}^{\text{H}_2\text{O}_2}/K_M^{\text{H}_2\text{O}_2}$ values) to use H_2O_2 (which follows the same order: sinapyl alcohol > coniferyl alcohol > coniferyl aldehyde) [12].

3.3. Nature of the oxidase reaction of *Z. elegans* peroxidase

To test the mechanism described in Eqs. 2–4, an aerated coniferyl alcohol solution was aged for 2 h at 25°C. Results showed that during this time, coniferyl alcohol solutions were unstable, and the decay of coniferyl alcohol, as monitored by the appearance of its oxidized forms, was concomitant with H_2O_2 generation (Fig. 5). The auto-oxidation of coniferyl alcohol thus generated H_2O_2 concentrations in the μM range,

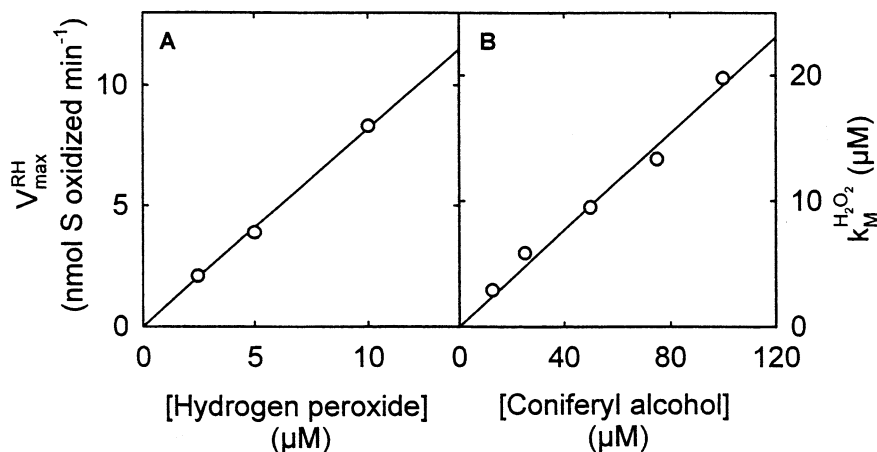


Fig. 6. Plot of $V_{\text{max}}^{\text{RH}}$ values against H_2O_2 concentration (A) and plot of $K_M^{\text{H}_2\text{O}_2}$ values against coniferyl alcohol concentration for the oxidation of coniferyl alcohol by the *Z. elegans* basic peroxidase.

the stoichiometry of the reaction (mol coniferyl alcohol auto-oxidized/mol H_2O_2 formed) being 0.496, i.e. about 2 mol of coniferyl alcohol were oxidized per mol of H_2O_2 formed, a stoichiometry that is in accordance with the mass balance described in Eq. 5. Auto-oxidation of lignin-type compounds containing guaiacyl groups, such as that present in coniferyl alcohol, produces electron spin resonance signals which can be attributed to 4-*O*-phenoxy radicals [23], lending support to the spontaneous nature of Eq. 3. At this point, it is of interest to note that the radicals generated during the auto-oxidation of coniferyl alcohol couple in an similar pattern to the radicals formed by enzymatic oxidation of coniferyl alcohol, the lignins formed being similar although not identical to enzymatically synthesized lignins [24].

That H_2O_2 is formed during the auto-oxidation of coniferyl alcohol is clear from the results shown in Fig. 5, but it may be wondered whether these μM H_2O_2 concentrations are sufficient to trigger the peroxidatic activity of the *Z. elegans* basic peroxidase. To answer this question, V_{max} values for the oxidation of coniferyl alcohol ($V_{\text{max}}^{\text{RH}}$) were plotted vs. H_2O_2 concentration (Fig. 6A). The results showed that μM H_2O_2 concentrations give noticeable values of $V_{\text{max}}^{\text{RH}}$. $V_{\text{max}}^{\text{RH}}$ in peroxidase-catalyzed reactions follows a linear dependence, $V_{\text{max}}^{\text{RH}} = 2 E k_1 [\text{H}_2\text{O}_2]$ [12], which fits well the data represented in Fig. 6A. The reason for this special sensitivity of the *Z. elegans* basic peroxidase to μM H_2O_2 concentrations resides in the strong affinity of the enzyme for H_2O_2 , which shows k_{M} values in the μM range (Fig. 6B). The k_{M} for H_2O_2 ($k_{\text{M}}^{\text{H}_2\text{O}_2}$) in peroxidase-catalyzed reactions follows a linear dependence, $k_{\text{M}}^{\text{H}_2\text{O}_2} = (k_3/k_1) [\text{RH}]$ [12], which fits well the data represented in Fig. 6B.

In conclusion, these results demonstrate that the *Z. elegans* basic peroxidase has a high affinity for H_2O_2 , and that the μM H_2O_2 concentrations generated during the auto-oxidation of coniferyl alcohol are therefore the main factor driving the unusual oxidase activity of this highly conserved lignin-synthesizing class III peroxidase.

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