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EFFECT OF DIFFERENT PHENOLS ON THE NADH-OXIDATION CATALYSED BY A PEROXIDASE FROM LUPIN

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Key Word Index—*Lupinus albus*; Leguminoseae; lupin; NADH-oxidation; peroxidase; phenols.

Abstract—Cell wall-bound peroxidase (EC 1.11.1.7) from lupin (*Lupinus albus*) shows a transition from oxidase to peroxidase activity when it oxidizes NADH. The oxidase phase represents a lag period in the time course of the reaction. This phase is phenol-dependent and responsible for hydrogen peroxide formation. Guaiacol, an assay substrate, and *p*-coumaric, ferulic and sinapic acids, precursors of the cinnamyl alcohols used in the lignification process affect both the length of lag period and the rate of the peroxidase phase of NADH oxidation. The effect of different phenols on the time course of the reaction is related to the efficacy (V_{max}/K_m ratio) of the enzyme when it is acting on them as a peroxidese.

INTRODUCTION

Cell wall peroxidases are probably involved not only in the oxidative polymerization of hydroxylated cinnamyl alcohols [1–6] but also in the biogenesis of the hydrogen peroxide (H_2O_2) needed in the final step of the lignification process [7–11]. Different isoperoxidases might mediate these two processes [12]. Peroxidases involved in both reactions appear to be restricted to lignifying tissues as has been demonstrated by using biochemical and histochemical methods [13, 14].

The H_2O_2 required for the oxidative polymerization of the phenols can be generated *in situ* by the peroxidase catalysed oxidation of the NADH produced by a cell-wall-bound malate dehydrogenase [7, 9, 15], though the precise nature and localization of the NAD-reducing system is still an open question [13]. The oxidation is stimulated by monophenols and Mn^{2+} [9, 12].

Halliwell [10] has proposed that H_2O_2 generation is initiated by reaction of NAD radicals produced in the peroxidative cycle of the peroxidase with molecular oxygen, and that the H_2O_2 required for the oxidation of the ferric form of the enzyme is formed by non-enzymic break-down of NADH at pH 5. According to this author, the stimulation of NADH oxidation caused by phenols is due to the acceleration of the break-down of compound III. Compound III, an enzymatic inactive form of the enzyme in its peroxidative cycle, is formed in the presence of NADH and under aerobic conditions [16]. On the other hand, in the reduction of compound III, H_2O_2 is produced [17].

In the present report, we have studied the effect of different phenols on the NADH oxidation catalysed by a peroxidase which is ionically bound to the cell walls of etiolated hypocotyls of lupin. We have observed the ocurrence of two phases of different rates in the course of NADH oxidation. The first phase is phenol-dependent and is responsible for H_2O_2 formation. The second phase is also phenol-dependent.

The influence of different phenols on the time course of the reaction is related to their efficacy as substrates for the enzyme when it is exhibiting peroxidase activity.

RESULTS AND DISCUSSION

The time courses of NADH oxidation catalysed by peroxidase in the presence as cofactors of different phenolic compounds are shown in Fig. 1. As can be seen two phases of clearly different rates are observed in the presence of *p*-coumaric acid, coniferyl alcohol and ferulic acid.

In order to elucidate the nature of the reactions occurring in the low rate step, we added H_2O_2 (Fig. 2) and catalase (Fig. 3) to the reaction mixtures. The sharp increase of the reaction rate caused by addition of H_2O_2

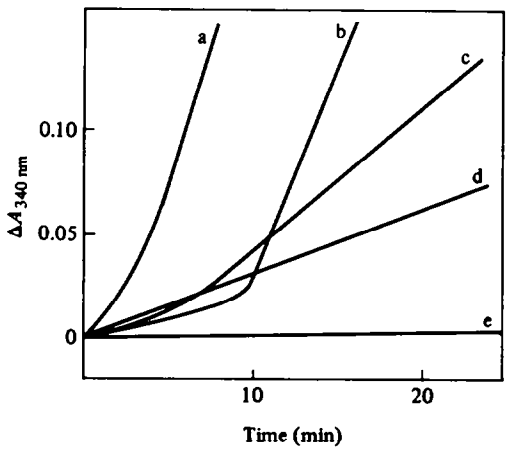

Fig. 1. Time course of NADH oxidation in the presence of different phenols. (a) *p*-Coumaric acid, (b) coniferyl alcohol, (c) ferulic acid, (d) guaiacol and (e) sinapic acid. All the reaction media contain 0.60 U.E. of peroxidase.

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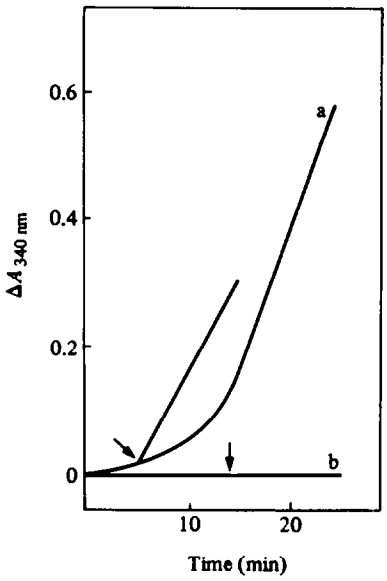


Fig. 2. Effect of H_2O_2 on the time course of NADH oxidation in the presence as cofactors of coniferyl alcohol (a) and sinapic acid (b). At the arrows, 5×10^{-7} M H_2O_2 was added. The reaction media contain 0.60 U.E. of peroxidase. The lag period was also interrupted by H_2O_2 addition when ferulic and *p*-coumaric acids are used as cofactors.

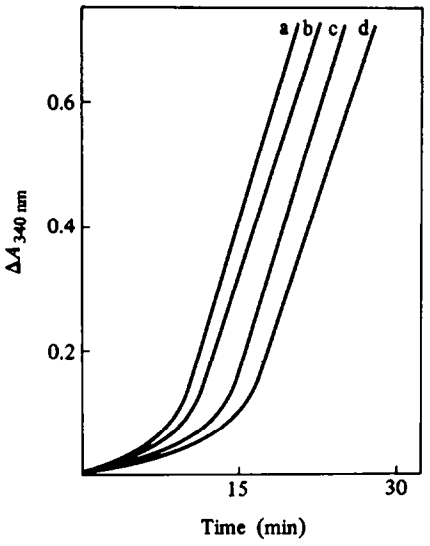
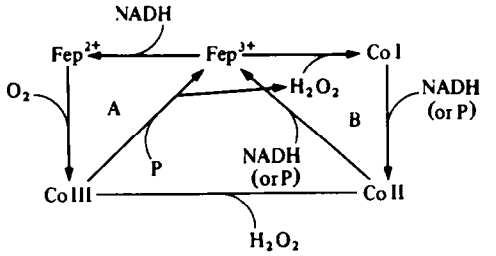


Fig. 3. Effect of catalase on NADH oxidation. (a) No addition, (b), (c) and (d), 3×10^{-3}, 6×10^{-3} and 1.2×10^{-2} catalase units added. All the reaction media contain 0.80 U.E. of peroxidase.

and the lengthening of the lag period caused by addition of catalase suggests that during this phase, H_2O_2 is generated.

In the low rate step, the enzyme is acting according to its oxidase cycle and generating H_2O (see Scheme 1). As the H_2O_2 is accumulating, the enzyme changes to its peroxidative cycle. The lag period, which corresponds to the oxidase phase of the enzyme, is negligible when guaiacol is



Scheme 1. Oxidase (A) and peroxidase (B) catalytic cycles of peroxidase. Drawn from the literature cited in this report. P represents different phenolic compounds.

used as cofactor and tends to infinity when sinapic acid is used (Fig. 1). *p*-Coumaric acid produces as lag period smaller than the ferulic acid.

NADH-oxidation requires the presence of coniferyl alcohol (or other phenols) even in the presence of H_2O_2 (results not shown). This requirement can be explained by supposing that compound II shows a great affinity for H_2O_2, that the resultant compound III formation represents a loss of active forms of the enzyme in its perioxidative cycle and that the return to the ferric form of the enzyme requires the presence of phenols.

The length of the lag period must be the expression of two features: the time taken to generate a suitable amount of H_2O_2 for the oxidation of the ferric enzyme to compound I and the competition between the phenol and the NADH for the enzyme in its peroxidative cycle. Thus phenol oxidation is required in the oxidase phase so that the peroxidase activity predominates. Therefore, it is plausible to think that the better the substrate phenol is when the enzyme acts as peroxidase, and the less its capacity to reduce compound III to the ferric form of the enzyme (reaction responsible of H_2O_2 formation) then the greater will be the lag period produced by the phenol.

In order to prove the different influences of phenols on the course of the reaction, the K_m and V_{max} values of the enzyme were calculated for each phenolic compound in the presence of H_2O_2 (Table 1). The efficacy of guaiacol as a substrate is clearly lower than that of either ferulic acid or *p*-coumaric acids. On the other hand, sinapic acid is not a substrate of the enzyme. Our experimental results are in agreement with the above statements. The lag period caused by guaiacol is lowest and sinapic acid produces one that tends to infinity.

The absence of a lag period when guaiacol is used as cofactor must be due to the fact that it is a poor substrate when the enzyme is acting as a peroxidase, and consequently a bad inhibitor of NADH oxidation. The small amounts of H_2O_2 generated in the medium of the reaction would be enough to support the peroxidase activity of the enzyme since the catalytic cycle of the enzyme autogenerates H_2O_2 by the reaction of NAD radicals with molecular oxygen [10]. When guaiacol is used as a cofactor, the enzyme is working as a peroxidase for short periods of time. The effect of catalase addition on the time course of the reaction (Fig. 4) proves the earlier statement. Ferulic and *p*-coumaric acids, being better substrates of the enzyme are powerful inhibitors of NADH oxidation and so they give rise to long lag periods.

When the high rate phase is reached, the reaction rate is higher in the presence of ferulic and *p*-coumaric acids than

Table 1. K_m and V_{max} values for different phenols

Phenol	K_m (mM)	V_{max} (mM/min × 10^{-2})	V_{max}/K_m (min^{-1} × 10^{-2})
Gualacol (1)	3.33	1.40	0.42
p-Coumaric acid (2)	0.23	3.33	14.50
Ferulic acid (3)	0.20	2.60	13.00
Sinapic acid (4)	—	—	—

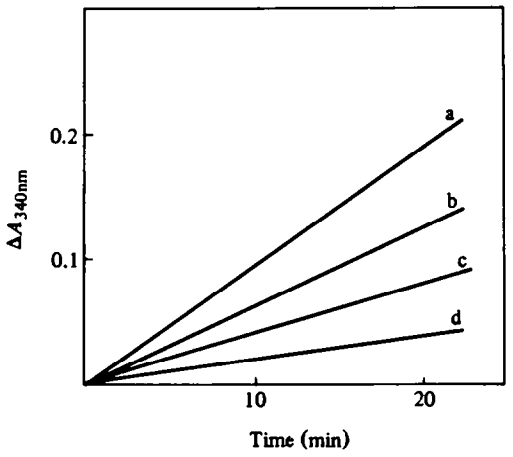


Fig. 4. Effect of catalase on NADH oxidation in the presence of guaiacol. NADH oxidized in the absence of catalase (a) or in the presence of 1.56 U.E. (b), 2.08 U.E. (c) and 3.12 U.E. (d) of catalase in the reaction mixture. The reaction media contain 1.84 U.E. of peroxidase.

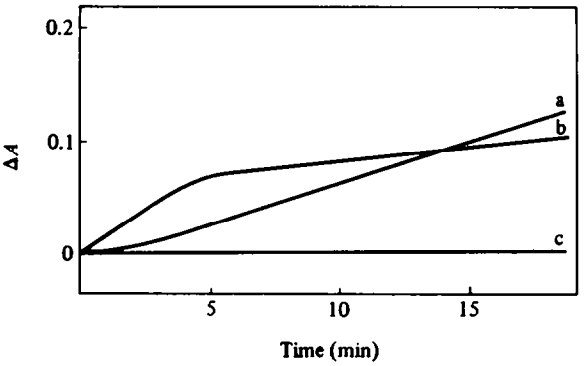


Fig. 5. Time courses of NADH (a) and ferulic acid (b) oxidation. (c) represents the time course of the ferulic acid oxidation in the presence of 7.5×10^{-5} M sinapic acid. The reaction media contain 0.70 U.E. of peroxidase.

in the presence of guaiacol. This result can be explained by assuming that ferulic and *p*-coumaric acids are good substrates in the reduction of compound III to the ferric form of the enzyme as they are of compound I and compound II in the peroxidative cycle (see Table 1). Thus, the concentration of the inactive form of the enzyme (compound III) during the high rate phase would be smaller when ferulic and *p*-coumaric acids are present than in the presence of guaiacol.

On the other hand, there is no NADH oxidation when sinapic acid is present in the reaction medium even in the presence of H_2O_2 (Fig. 2). Sinapic aicd is not a substrate of the enzyme when it is working as a peroxidase and so, the enzyme must oxidise NADH with great effectiveness. The contradictory result of the enzyme not acting as a peroxidase, even in the presence of H_2O_2, is due to the fact that all the enzyme would convert itself into compound III and the sinapic acid would be unable to reduce it to the ferric form.

Since sinapic acid is a structural analogue of ferulic acid, it will inhibit the oxidation of the last compound when ferulic acid is the reductant of compound III. Figure 5 (graph b) shows the course of ferulic acid oxidation when followed by the decrease in absorbance at the isosbestic point (λ = 281.6 nm) of NADH/NAD^+. As can be observed, there is a close relationship between ferulic acid and the NADH oxidations. During the oxidase phase, NADH oxidation is low and the rate of ferulic acid oxidation is high whereas in the peroxidative cycle of NADH, ferulic acid oxidation is slow. The slow rate of phenol oxidation accompanying the peroxidase step proves that the phenol is continously required to restore the ferric form of the enzyme from compound III. Figure 5 (line c) shows the complete inhibition caused by sinapic acid of ferulic acid oxidation, which is required for the NADH oxidation by the enzyme when it is working it its two catalytic cycles.

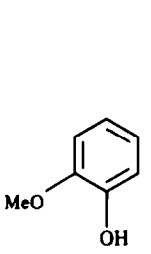
The different behaviour exhibited by phenolic compounds on NADH oxidation catalyzed by peroxidase is in accordance with their structures (1–4). The primary phenol oxidation products are their corresponding phenoxy radicals, which are stabilized by delocalization of the unpaired electron at *o*,*o'* and *p*-positions of the aromatic ring and α-position of the side chain. Thus, the presence of methoxyl groups in both positions *o* to the hydroxyl group in sinapic acid (4) is responsible for its very low efficacy as a substrate with respect to *p*-coumaric (2) and ferulic (3) acids (see formulae in Table 1).

EXPERIMENTAL

Plant material. Periodically irrigated hypocotyls of lupin grown in the dark at 25° are used.

Chemicals. Catalase (EC 1.11.1.6), lactic dehydrogenase (EC 1.1.1.27) and ferulic acid were purchased from Sigma, Chemical Co. St Louis Mo. USA; guaiacol from Merck AG, Darmstadt F.R.G.; *p*-coumaric and sinapic acids and coniferyl alcohol from Fluka AG, Chemische Fabrik Buchs,(Switzerland. All the other chemicals used were analytical grade.

Cell wall preparation. Hypocotyls (6–8 days old, 7–10 cm in length) were ground in a cold mortar with 5×10^{-3} M Tris–HCl,



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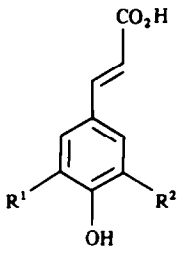

2 $R^1 = R^2 = H$
3 $R^1 = H$, $R^2 = OMe$
4 $R^1 = R^2 = OMe$

pH 7.2, (1:2 wt/v), filtered through cheesecloth and centrifuged for 10 min at 1000 *g*. In order to avoid membrane contamination, the pellet was washed with 5×10^{-2} M Tris–HCl, pH 7.2, containing 1% Triton X-100 (×3) and then with the same buffer (×2) without detergent. The last pellet obtained was considered as wall-fraction.

Enzyme extraction. To isolate ionically bound peroxidase, the cell wall fraction was incubated with 1 M KCl for 3 hr in the cold. After centrifuging at 1000 *g* for 10 min, the supernatant was dialysed overnight against 5×10^{-2} M Tris–HCl, pH 7.2. Disk isoelectrofocusing on 7.5% polyacrylamide gels showed principally two acidic isoperoxidases (pI 4.8 and 5.2). Peroxidase activity on the gels was detected with 10^{-3} M H_2O_2 and saturated benzidine in 10^{-1} M acetate buffer, pH 5.0.

Estimation of NADH-oxidation. The oxidation of NADH was followed at 30° by the fall in absorbance at 340 nm. The standard reaction mixture (final vol = 1 ml) consisted of: 5×10^{-2} M K-Pi buffer, pH 7.5, 10^{-4} M $MnCl_2$, 25×10^{-5} M NADH, 10^{-5} M phenolic compound and enzyme extract. Otherwise, the composition of the reaction media is referred to in the legends figures and Table 1. One U.E. of peroxidase oxidizes 1 μmol of guaiacol per min at pH 7.0 and 30°.

Determination of the isosbestic point of NADH/NAD⁺ The isosbestic point was calculated by repetitive scanning between 275 and 400 nm of a reaction medium (final vol. = 1 ml) containing: 5.10^{-2} M K-Pi buffer, pH 7.5, 33×10^{-5} M sodium pyruvate, 10^{-4} M NADH and 0.1 unit of lactic dehydrogenase.

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