Oxidation of coniferyl alcohol by cell wall peroxidases at the expense of indole-3-acetic acid and O_2

A model for the lignification of plant cell walls in the absence of H_2O_2

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The oxidation of coniferyl alcohol (CA), a lignin precursor, by cell wall peroxidases may take place at the expense of indole-3-acetic acid (IAA) and O_2, and in the absence of H_2O_2. The peroxidase-catalyzed oxidation of CA shows an optimum at an IAA concentration of 0.33 mM, while higher IAA concentrations are inhibitory. The observation that the oxidation of CA by cell wall peroxidase at the expense of IAA and O_2 is inhibited by genistein, a putative endogenous inhibitor of lignification in lupin hypocotyls, supports the view that the H_2O_2-generating system coexists with cell wall peroxidase activities involved in lignification, and that it takes place at the expense of IAA and O_2.

Cell wall peroxidase; Coniferyl alcohol; Indole-3-acetic acid; Genistein; Lignification; *Lupinus albus*

1. INTRODUCTION

Peroxidase (EC 1.11.1.7) located in plant cell walls are involved not only in the oxidative polymerization of hydroxylated cinnamyl alcohols to lignins [1], but probably also in the biogenesis of the H_2O_2 needed for initiating this cell wall compartimentalized enzymatic reaction [2]. Thus, peroxidases involved in both phenol-oxidizing and H_2O_2-generating reactions appear to be localized in cell walls of both lignifying and non-lignifying tissues [3].

In this context, it has been proposed that the H_2O_2 required in this oxidative pathway for the oxidative polymerization of phenols may be generated in muro through the peroxidase-catalyzed oxidation of extracellular NADH [4], although our present knowledge suggests that there is no evidence for export of NADH to the plant cell wall.

Apostol et al. [5] proposed that this H_2O_2 could be generated by a trans-plasmamembrane reductase which employs cytosolic NADH and, coupled to an outside-bound plasmamembrane oxidase, reduces extracellular O_2 to H_2O_2. However, this does not agree with the cytochemical observation which suggests that the H_2O_2-generating system coexists with peroxidase activities in the outer part of the radial epidermal cell wall far from the plasmalemma [3].

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Abbreviations: CA, coniferyl alcohol; 2,4-DCP, 2,4-dichlorophenol; IAA, indole-3-acetic acid

Based on previous studies of the catalase-sensitive aerobic oxidation of IAA by peroxidase located in cell walls [6], we have proposed a model [7] to explain the oxidative coupling of cell wall phenolic moieties, in the absence of H_2O_2, and at the expense of IAA and O_2, through an oxidase-peroxidase cycle of the cell wall-localized enzyme. In this cycle, the H_2O_2 necessary for initiating the peroxidase cycle of the enzyme, is generated in an oxidase cycle dependent of IAA.

In this study, we show that CA, a substrate of lignification, can be oxidized by cell wall peroxidases at the expense of IAA and O_2, and we conclude that, since peroxidase is a constitutive enzyme of the plant cell wall, only the export of cinnamyl alcohols and the availability of IAA, might be per se the outbursts for initiating the enzymatic process which leads to the lignification of the plant cell wall.

2. MATERIALS AND METHODS

2.1. *Materials*

Cell wall-bound peroxidases were extracted from exponentially growing lupin (*Lupinus albus* cv. multolupa) hypocotyls as already described [8]. This enzymatic fraction has been previously characterized by disk-isoelectrofocusing on 4.0–9.0 pH gradients, and contains mainly an acidic isoenzyme A_2 of pI 5.2 and, in a lesser proportion, a second acidic isoenzyme A_1 of pI 4.7 [6].

Genistein (5,7,4′-trihydroxyisoflavone) was prepared from biochanin A (5,7-dihydroxy-4′-methoxyisoflavone) by hydrolysis of the latter under reflux for 1 h in 48% (v/v) HBr in anhyd. AcOH, and crystallized from AcOH/H_2O (10:5, v/v). It was immediately purified by preparative TLC [9], and characterized by UV and MS data [10].

2,4-Dichlorophenol (2,4-DCP) was obtained from BDH (Poole, UK), and was purified by distillation prior to use, this being free of

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contamination of the corresponding quinone. Indole-3-acetic acid (IAA) and biochanin A were purchased from Sigma Chemical Co. (St. Louis, MO). Coniferyl alcohol (CA), H_2O_2 and $MnCl_2$ from Merck (Darmstadt, FRG). All the other chemicals used in this work were reagent grade, generally supplied by Merck (Darmstadt, FRG).

2.2. *UV difference spectra*

Spectrophotometric measurements were performed with a Hitachi 150-20 spectrophotometer at 25°C. All the reactions were carried out in 50 mM Tris-HCl buffer, pH 7.5, and were started by the addition of 2.0 nkat of cell wall peroxidase. The additional composition of each reaction media is described in the legend figures. All spectra of the reaction media were scanned against a reference cuvette containing the complete media minus enzyme, every 60 s, from 315 nm at a rate of 400 nm · min^{-1}.

3. RESULTS AND DISCUSSION

A set of difference UV spectra for both the peroxidative oxidation of CA and the aerobic oxidation of IAA by cell wall peroxidase is shown in Fig. 1A,B. These illustrate that while the oxidation of CA is accompanied by a strong decrease in the absorbance at 260 nm of the reaction media, together with other minor spectral changes, the oxidation of IAA is accompanied by a strong increase in the absorbance at 245 nm. The latter fact indicates that oxindoles are mainly formed as products of the IAA oxidation by these acidic isoperoxidases, a fact that has been previously established [11] on the basis of the isolation and characterization of the reaction products.

These differences between the values of the λ_{min} (=260 nm) in the case of CA, and λ_{max} (=245 nm) in the case of IAA, for the oxidation products, were used by us to detect simultaneously the oxidation of CA and IAA in reaction media carried out in the absence of H_2O_2.

A set of consecutive difference UV spectra for such a reaction medium is shown in Fig. 2A, which illustrates

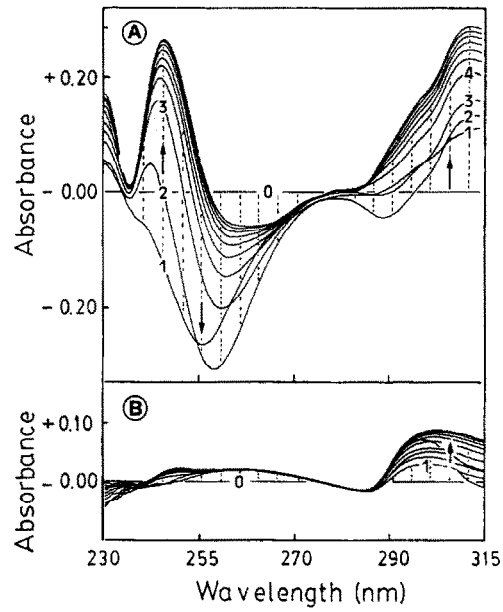

Fig. 2. Repeated scan of UV difference spectra of an oxidative medium composed of (A) 0.33 mM IAA, 0.1 mM CA, 0.1 mM 2,4-DCP, 0.1 mM $MnCl_2$, cell wall peroxidase and 1.6 μM H_2O_2, and (B) the reaction media described in (A) containing additionally 80 μM genistein. Otherwise as for Fig. 1.

complex spectral changes in the 235–275 nm range of the spectrum. These changes are characterized, firstly, by a strong decrease in the absorbance at 260 nm during the two first min of the reaction, due to the oxidation of CA in the reaction media, and, secondly, by an increase in absorbance at 245 nm during the last 8 min of the reaction, which is clearly associated to the formation of oxindoles as products of the oxidation of IAA by these cell wall peroxidases. Because of the complexity of the reaction media, which contain 3 absorbing and oxidizable species (IAA, CA, and 2,4-DCP), the spectral

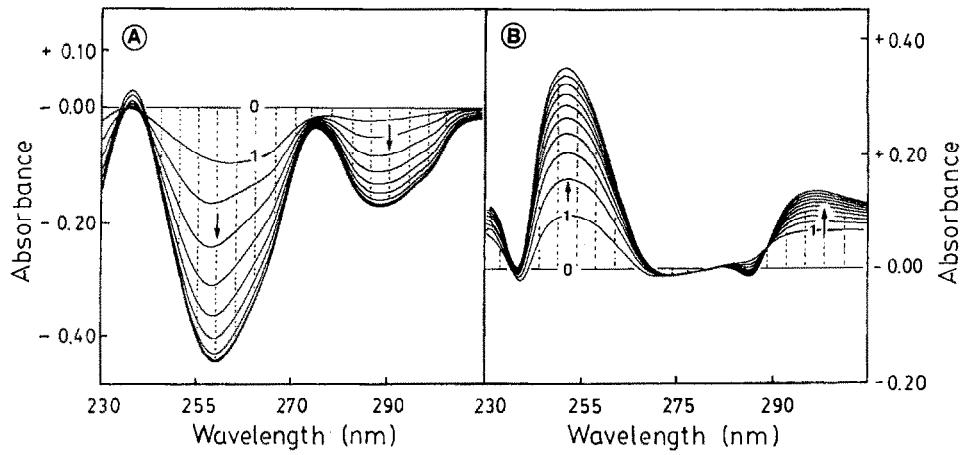

Fig. 1. Repeated scan of UV difference spectra of an oxidative medium containing (A) 0.1 mM CA, cell wall peroxidase, and 0.40 mM H_2O_2, and (B) 0.33 mM IAA, cell wall peroxidase, 0.1 mM $MnCl_2$ and 0.1 mM 2,4-DCP. The time course follows the direction of the arrow. Line 0: before the addition of enzyme; line 1: 1 min after the addition of enzyme.

changes occurring for wavelengths greater than 285 nm were difficult to analyze, and remain uninterpreted.

The presence of CA in an oxidative reaction medium containing IAA is accompanied by an increase in the lag period of the enzymatic reaction. This also occurs when NADH is used instead of IAA as the reductor for initiating the oxidase cycle of the cell wall localized enzyme [12]. The effect of different phenols on the lag period of the oxidase-peroxidase reaction, which is broken by the addition of traces of H_2O_2 [12], is related to the efficacy (V_{max}/K_m ratio) of the enzyme acting through the peroxidase cycle [12]. For this reason, it was necessary to add traces of H_2O_2 (1.6 μM) to a reaction medium containing IAA and CA for the reaction to take place at a rate permitting the spectral changes to be detected at short times (10 min, see Fig. 2A). However, doubling or halving the H_2O_2 concentration did not significantly affect the extension of these spectral changes (data not shown). Likewise, trace amounts of organic hydroperoxides may be generated during the reaction with O_2 of the skatole carbon-centered free radical intermediate of the IAA-oxidation [13,14].

The apparent discrepancy between the temporal sequence of the spectral changes (oxidation of CA is detected previous to the oxidation of IAA) may be due to the fact that the formation of oxindoles as products of the IAA oxidation takes place through a series of several non-enzymatic reactions which result from the decay of the skatole carbon-centered free radical intermediate [15,16]. Thus, since the non-enzymatic reactions, which result in the formation of oxindoles, compete with the enzyme for the H_2O_2 generated [15], and since the H_2O_2 is more efficiently consumed in the peroxidase cycle than by the chemical reaction with the free radical indol intermediate [15], it cannot be discounted that the spectral changes due to the appearance of oxindoles in the reaction media follow the spectral changes due to the oxidation of CA, the latter being mainly due to the formation of the first radical phenol intermediate [17].

Genistein, a putative endogenous inhibitor of the lignification in lupin hypocotyls [18], was also inhibitor of the peroxidase-catalyzed co-oxidation of IAA and CA (Fig. 2B).

The effect of IAA concentration on these spectral changes was complex. Optimum IAA concentration was 0.33 mM (Fig. 2A), while a higher concentration (0.66 mM) inhibited these spectral changes appreciably (Fig. 3A). Lower concentrations of IAA (0.16 mM) also resulted in a reduction of these spectral changes (Fig. 3B) while, in the total absence of IAA, the spectral changes in the 235–275 nm range in the reaction media were negligible (Fig. 3C). This dual effect of IAA on the spectral changes may be due to the fact that at higher concentrations IAA competes with CA as substrate in the peroxidase cycle of the enzyme. This conclusion is supported by the observation that the inhibition of the CA oxidation (estimated at 65% on the basis of the decrease in absorbance at 260 nm) was greater than the inhibition observed for the oxidation of IAA (estimated at 25% on the basis of the increase in absorbance at 245 nm), when spectral changes in reaction media containing 0.66 mM IAA (Fig. 3A) were compared with those obtained in the presence of 0.33 mM IAA (Fig. 2A).

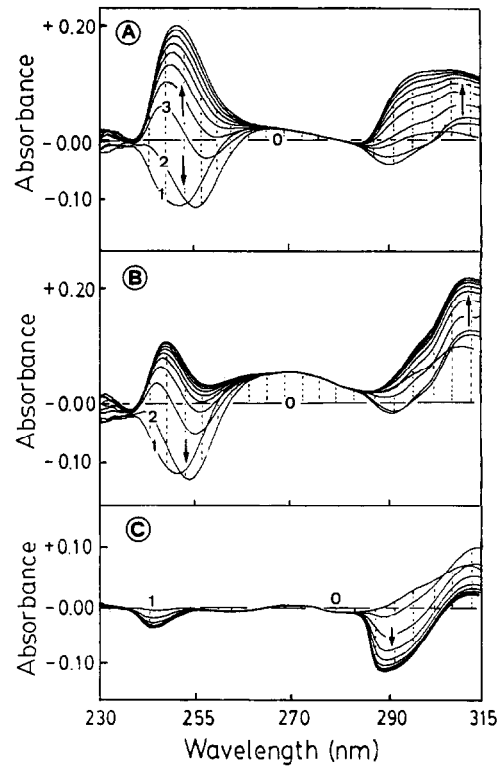

Fig. 3. Repeated scan of UV difference spectra of an oxidative medium composed of (A) 0.66 mM IAA, (B) 0.16 mM IAA, and (C) in the absence of IAA, containing additionally 0.1 mM CA, 0.1 mM $MnCl_2$, 0.1 mM 2,4-DCP, 1.6 μM H_2O_2, and cell wall peroxidase. Otherwise as for Fig. 1.

It can be concluded then that the oxidation of CA by cell wall peroxidases may take place at the expense of IAA and O_2. In this case, an independent H_2O_2-generating system is not absolutely necessary for the lignification of plant cell walls, since IAA, which is transported from cell to cell through the extracellular spaces [19], might be the reductor that initiates the oxidase-peroxidase cycle of the wall-localized enzyme. Likewise, IAA at higher concentrations may act as an inhibitor of CA oxidation, maintaining the juvenile (non-lignified) state of the plant cell, as is in accordance with its plant growth regulating effects.

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