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TOBACCO ANIONIC PEROXIDASE OVEREXPRESSED IN TRANSGENIC PLANTS: AEROBIC OXIDATION OF INDOLE-3-ACETIC ACID*

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; oxygen uptake; spectral studies; kinetics; reaction mechanism; hydrogen peroxide; activation effect; peroxidase; indoleacetic acid.

Abstract—We have investigated the catalytic properties of the tobacco anionic peroxidase with regards to the oxidation of indole-3-acetic acid (IAA). As judged by oxygen uptake, the homogeneous enzyme was capable of oxidizing IAA in the absence of additional cofactors such as manganese ion, hydrogen peroxide and phenols. Phenolic substrates such as caffeic acid, chlorogenic acids, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt or phenol inhibited the oxidation of IAA. The spectral changes in the course of IAA oxidation allow us to conclude that the oxidation cycle is functioning separately from peroxidation. The addition of trace amounts of hydrogen peroxide to the reaction eliminates a distinctive lag phase in the consumption of oxygen. This activation by hydrogen peroxide is connected with the formation of compound II, which appears to be a key intermediate in the oxidation process. However, there is still no direct evidence regarding the mechanism of initiation of IAA oxidation, though the appearance of the ferrous form has been clearly demonstrated. There is some indirect evidence of IAA hydroperoxide formation in the course of aerobic oxidation of IAA by the tobacco enzyme based on the slight inhibition effect of catalase. The results obtained have been rationalized in the reaction scheme, which proposes the existence of an enzyme–substrate complex and is in agreement with the previous data on IAA oxidation catalysed by horseradish and turnip peroxidases.

INTRODUCTION

Indole-3-acetic acid (IAA)—one of the most studied of the plant growth regulators—has a very complicated catabolism, which has been the subject of intensive investigation. Examination of the products of IAA degradation indicate that peroxidases (EC 1.11.1.7) play a significant role in auxin catabolism through the oxidative decarboxylation of IAA [1]. Our efforts have focused on the characterization of the tobacco anionic peroxidase (TOP) [2, 3]. Specific overexpression or suppression of the TOP in transgenic tobacco plants has resulted in numerous phenotypes, suggesting abnormal auxin metabolism [4, 5]. Phenotypes indicating depressed IAA in plants overexpressing the anionic peroxidase include decreased root branching and closed stomata. Phenotypes indicating elevated IAA levels were seen in plants in which the anionic isoenzyme was specifically suppressed via antisense RNA, e.g. rapid shoot growth, fused leaves and shorter time to flower. To help determine the role of the anionic peroxidase in IAA metabolism, we set out to characterize the kinetic properties of the purified enzyme.

Previous investigations have either focused on the steady-state and transient-state kinetics of purified enzyme, or the characterization of the oxidation products of IAA produced either *in vitro* or *in vivo*. Peroxidases carry out a series of one electron oxidations in the order ferrous enzyme {Fe(II)] → native enzyme [Fe(III)] → Compound II → Compound I → Compound III}. Two pathways for the *in vitro* oxidation of IAA by horseradish peroxidase (HRP) have been proposed [6]. At high enzyme to substrate ratios the conventional peroxidative cycle operates (native enzyme → Compound I → Compound II → native enzyme). At low enzyme to substrate ratios the ferrous enzyme/Compound III shuttle operates (native enzyme → ferrous enzyme → Compound III → ferrous enzyme). Recently, Compound III was detected at high enzyme to substrate ratios using stopped-flow spectrophotometry [7]. This observation creates uncertainty about the role of the ferrous form of HRP in the oxidation of IAA. The unusual catalytic properties of the tobacco enzyme made it possible to clarify some details of the IAA oxidation mechanism. The data obtained allow one to distinguish oxidative and peroxidative reaction mechanisms and to present spectral

*Part 2 in the series 'Tobacco Anionic Peroxidase Overexpressed in Transgenic Plants'. For part 1, see ref. [3].

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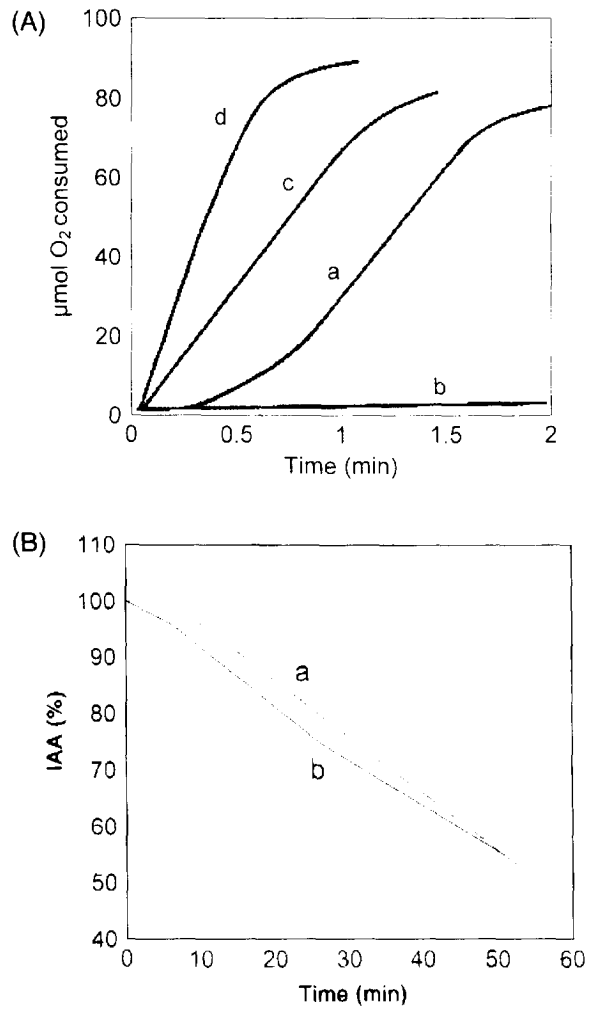


Fig. 1. (A) Time course of oxygen consumption in the course of 0.2 mM IAA oxidation by 0.12 μM peroxidase in 0.05 M Na citrate buffer, pH 4.5 (a), and the effects of 0.3 mM ABTS added before the substrate (b), 10 μM (c) and 100 μM (d) of hydrogen peroxide added simultaneously with the substrate. (B) Time course of 20 μM IAA degradation by 0.03 μM tobacco peroxidase at pH 6.7 in (a) absence of hydrogen peroxide, and (b) presence of 2 μM hydrogen peroxide. IAA degradation rate was determined by the analysis of reaction products by HPLC.

evidence of the ferrous enzyme in the course of IAA oxidation, though its role still has to elucidated. Also, we propose a role for hydrogen peroxide as a stress-induced activator of the tobacco peroxidase, which could potentially result in the rapid metabolism of IAA.

RESULTS AND DISCUSSION

Oxygen uptake studies

The purified tobacco enzyme was active towards IAA without the addition of metal ion or co-factors. Kinetic curves of oxygen uptake had a characteristic sigmoid shape (Fig. 1A, curve a). Oxygen uptake catalysed by the tobacco peroxidase (0.05 μM) followed Michaelis–Menten kinetics in the range 0.2–2 mM IAA. The Michaelis constant for IAA was minimally affected by changes in buffer; however, the maximal reaction rates were pH dependent (Table 1). The calculated turnover number was an order of magnitude lower at pH 6.0 than that at pH 4.5. This indicates the involvement of reaction intermediates whose reactivity significantly depends on pH. For example, IAA cation radicals are known to convert into less reactive indolyl radicals via proton release at pH > 5 [8]. Taking into account that the reactivity of Compound II significantly depends on pH, we could propose its participation in the oxidation reaction. In which case there should be a route leading to its formation, such as the production of IAA hydroperoxide at the initiation step of the reaction.

Oxygen uptake was nearly insensitive to the presence of catalase. Only a 20-fold excess of catalase over peroxidase resulted in a 20–25% inhibition. This indicates that, if the reaction starts via the formation of IAA hydroperoxide using the conventional peroxidase

Table 1. Kinetic parameters of IAA oxidation by tobacco peroxidase (0.5 μM peroxidase)

	Buffer		
	Na acetate	Na citrate	Tris–HCl
pH	4.5	4.5	6.0
K_m IAA (mM)	0.8±0.2	0.65±0.05	0.56±0.2
V_m (μmol O_2 sec^{-1})	1.83	1.83	0.17
Turnover number (sec^{-1})	36.7	36.7	3.3

cycle including Compounds I and II, then hydroperoxide is nearly inaccessible for catalase. Perhaps hydroperoxide is formed inside the active centre or is not a catalase substrate. Taking into account that there is some inhibition effect of catalase, we can suggest the formation of IAA hydroperoxide during the course of the reaction. The existence of a lag-period for oxygen consumption may indicate the formation of Compound II upon the initiation of the reaction.

Effect of peroxidase substrate on IAA oxidation

Peroxidase substrates such as chlorogenic and caffeic acids (1 mM) inhibited oxygen consumption; however, both were oxidized in the presence of IAA. The phenomenon of simultaneous oxidation of IAA and phenolic substrates (*p*-coumaric, ferulic or caffeic acids) by peroxidase was first described in 1964 [9]. More recently, Ferrer *et al.* [10] demonstrated that the oxidation of coniferyl alcohol by cell wall peroxidases is linked to IAA oxidation, and Krylov and Chebotareva [11] described peroxidase-catalysed co-oxidation of IAA and xanthene dyes. The mechanism of this phenomenon; however, remains unclear. One possible explanation considers the interaction of IAA hydroperoxide at the peroxidase active centre.

2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (diammonium salt) (ABTS) added in the course of IAA oxidation also led to the inhibition of oxygen uptake. There was a decrease of the final steady-state oxygen concentration, and the appearance of the ABTS oxidation product (green coloured). ABTS addition before IAA resulted in the complete inhibition of oxygen uptake (Fig. 1A, curve b) and no reaction products were detected. Thus, ABTS addition to peroxidase blocks access of the IAA molecule to the active centre and makes the formation of IAA hydroperoxide impossible.

Phenol, being one of the worst substrates for the tobacco enzyme, had an inhibition effect only at concentrations an order of magnitude higher than that of IAA (Table 2). The inhibition effect could indicate that IAA and phenol compete for one and the same enzyme form.

Hydrogen peroxide effect on IAA oxidation

Hydrogen peroxide stimulated oxygen uptake up to 200% in the range 70–300 μM, which corresponds to 140–600-fold excess of hydrogen peroxide over the enzyme (Table 3). However, hydrogen peroxide affected only the initial rate of the reaction catalysed by peroxidase, in particular, removing the lag-period (Fig. 1A, curves c and d), but not the steady-state oxygen concentration achieved. The kinetic curves of IAA degradation by tobacco peroxidase in the presence and absence of hydrogen peroxide (Fig. 1B) also showed the elimination of the lag-period. Thus, the addition of hydrogen peroxide stimulated both oxygen uptake and IAA degradation, pointing out that IAA was consumed simultaneously with oxygen. The activation effect of hydrogen peroxide on the initial rate of oxygen consumption may be due to a switching on of the peroxidative cycle, or IAA radical production via IAA interaction with hydrogen peroxide in the presence of peroxidase and their further interaction with oxygen. This has been confirmed by anaerobic transient-state kinetic analysis of HRP and the tobacco peroxidase [12]. Further, these anaerobic studies confirm that the tobacco peroxidase Compound II catalyses the oxida-

Table 2. Effect of phenol on oxygen consumption by tobacco peroxidase (0.05 μM peroxidase, 0.5 mM IAA, 0.05 M Na citrate buffer, pH 4.5)

Phenol concentration (mM)	0	1.1	2.2	5.5	11	22
Reaction rate (μmol O_2 sec^{-1})	1.87	1.83	1.60	1.07	0.67	0.47
Reaction rate (%)	100	98	87	57	36	25

Table 3. Effect of hydrogen peroxide on oxygen consumption by tobacco peroxidase (0.05 μM peroxidase, 0.2 mM IAA, Na citrate buffer, pH 4.5)

Hydrogen peroxide concentration (μM)	0	10	20	50	100	300
Reaction rate (μmol O_2 sec^{-1})	1.10	0.67	1.67	1.83	2.40	2.33
Reaction rate (%)	100	106	152	167	202	201

tion of IAA; thus, hydrogen peroxide addition leads to the higher concentration of Compound II and accelerates oxygen consumption [12]. In the absence of hydrogen peroxide the tobacco peroxidase is primarily in the native state. Upon the addition of hydrogen peroxide the enzyme is converted into Compound II, and thus initiates the oxidative cycle which is seen as an accelerated rate of oxygen consumption.

The effect of hydrogen peroxide on HRP-catalysed IAA oxidation was investigated 22 years ago [13] and it was shown that trace amounts of peroxide at $-15°$ accelerated the reaction. The activation effect of hydrogen peroxide on IAA oxidation catalysed by horseradish peroxidase isozyme A was described later by Nakajima and Yamazaki [14]. They also observed the elimination of a lag-period for oxygen uptake in the presence of hydrogen peroxide.

The stimulation of IAA degradation by hydrogen peroxide may have biological significance in that the plant's response to stress and infection is thought to be mediated through hydrogen peroxide [15, 16]. Stress-induced accumulation of hydrogen peroxide could elicit a rapid and localized degradation of apoplastic IAA by directly affecting the reaction kinetics of the anionic peroxidase.

Extracts of transgenic tobacco leaves were unable to catalyse IAA oxidation in the absence of hydrogen peroxide. The rate constant determined for the extract in the presence of 50 μM hydrogen peroxide was nearly the same as that for the purified enzyme without hydrogen peroxide. The K_m for the homogeneous enzyme in the presence of hydrogen peroxide showed only a two-fold increase. Leaf extract contained considerable brown polyphenolic pigments and exhibited phenol oxidase activity, and completely inhibited oxygen uptake when added to purified enzyme. Considering the strong inhibitory effect of chlorogenic and caffeic acids on peroxidase-catalysed IAA oxidation, it is not surprising that the crude tobacco extract would have no observable IAA oxidase activity. The restoration of activity with the addition of hydrogen peroxide could result from the removal of endogenous phenolics through their peroxidase-catalysed polymerization.

Special studies of IAA interaction with tobacco peroxidase

The spectral studies of the reaction reveal the unusual properties of the tobacco peroxidase as compared to HRP (Figs 2 and 3). The ferrous form of the tobacco enzyme was easily detected using high concentrations of enzyme and IAA at acidic pH values (Fig. 2A). There was a lag in the appearance of ferroperoxidase, and the concentration of the ferrous enzyme observed was proportional to the IAA concentration (Fig. 2B). This is consistent with the dependence of ferrous enzyme content on the IAA/enzyme ratio reported previously for HRP and turnip peroxidases [13].

The appearance of the ferrous form corresponded to the apparent anaerobic conditions in the reaction mixture. One can speculate that the ferrous form is produced via radical reactions after the oxygen is consumed, e.g. the IAA cation radical could reduce the enzyme forming an IAA cation. Such a proposal was made in the early investigations of IAA oxidation by HRP under anaerobic conditions created with an ascorbate/ascorbate oxidase system [14]. However, the steady-state data cannot be used to confirm this proposal, and the role of the ferrous form in the process is still in question. The main steady-state form of tobacco peroxidase in the case of IAA oxidation was represented by the native enzyme with a shifted Soret band (to 406 nm)—independent of pH and enzyme and substrate concentrations. We reported previously that the tobacco enzyme reacts with hydrogen peroxide slower than HRP [3]. This gives the spectrum of Compound I in a wide range of hydrogen peroxide concentrations at pH 4–5 and indicates that Compound II of the tobacco enzyme is much more active than Compound I. The observed shift of the Soret band could be the result of some Compound I species present, but it is also possible that this reflects the formation of an enzyme–substrate complex.

At pH 6.0, it was possible to catch the formation of Compound II immediately after mixing IAA with a 1000-fold excess of tobacco peroxidase (Fig. 3A). A minute later, the spectrum returned to that of the native form. The same picture was obtained in 0.1 M K phosphate buffer, pH 7.5 (data not shown). At low concentrations of IAA and peroxidase, only the native form of the enzyme was detected during the course of the reaction. No Compound III species were observed during the course of the reaction at pH 4.5–7.5, which is in contrast to HRP [6, 7]. The addition of IAA to preformed tobacco peroxidase Compound III gave the spectrum of Compound II, whereas in the case of HRP the addition of IAA accelerated the decomposition of Compound III (Fig. 3B) [7]. Moreover, the addition of hydrogen peroxide to the native tobacco peroxidase and IAA resulted only in Compound II formation (Fig. 3C). The Compound II spectrum was observed as long as hydrogen peroxide and oxygen were present in the system.

The formation of Compound II was reported for HRP and turnip peroxidase-catalysed IAA oxidation at $-15°$ by Ricard and Job on the basis of stopped-flow studies [13]. Compound II was regenerated as long as oxygen was present in the reaction medium. Neither ferroperoxidase nor Compound I appeared during the process. The authors wrote that Compound II was mostly obtained by reduction of Compound III. To explain the direct two-electron reduction they proposed binding of two IAA molecules on the enzyme (1), and the reaction cycle (2)–(3) was suggested for the Compound II regeneration:

$$\underset{\text{Compound III}}{\text{HRP-OOH}\cdot} + 2\text{IAA} \rightarrow \underset{\text{Compound II}}{\text{HRP(OH}\cdot)} + 2\text{IAA}\cdot + \text{H}_2\text{O} \quad (1)$$

$$\text{HRP(OH}\cdot) + \text{IAA} \rightarrow \text{HRP-IAA}\cdot + \text{H}_2\text{O} \quad (2)$$

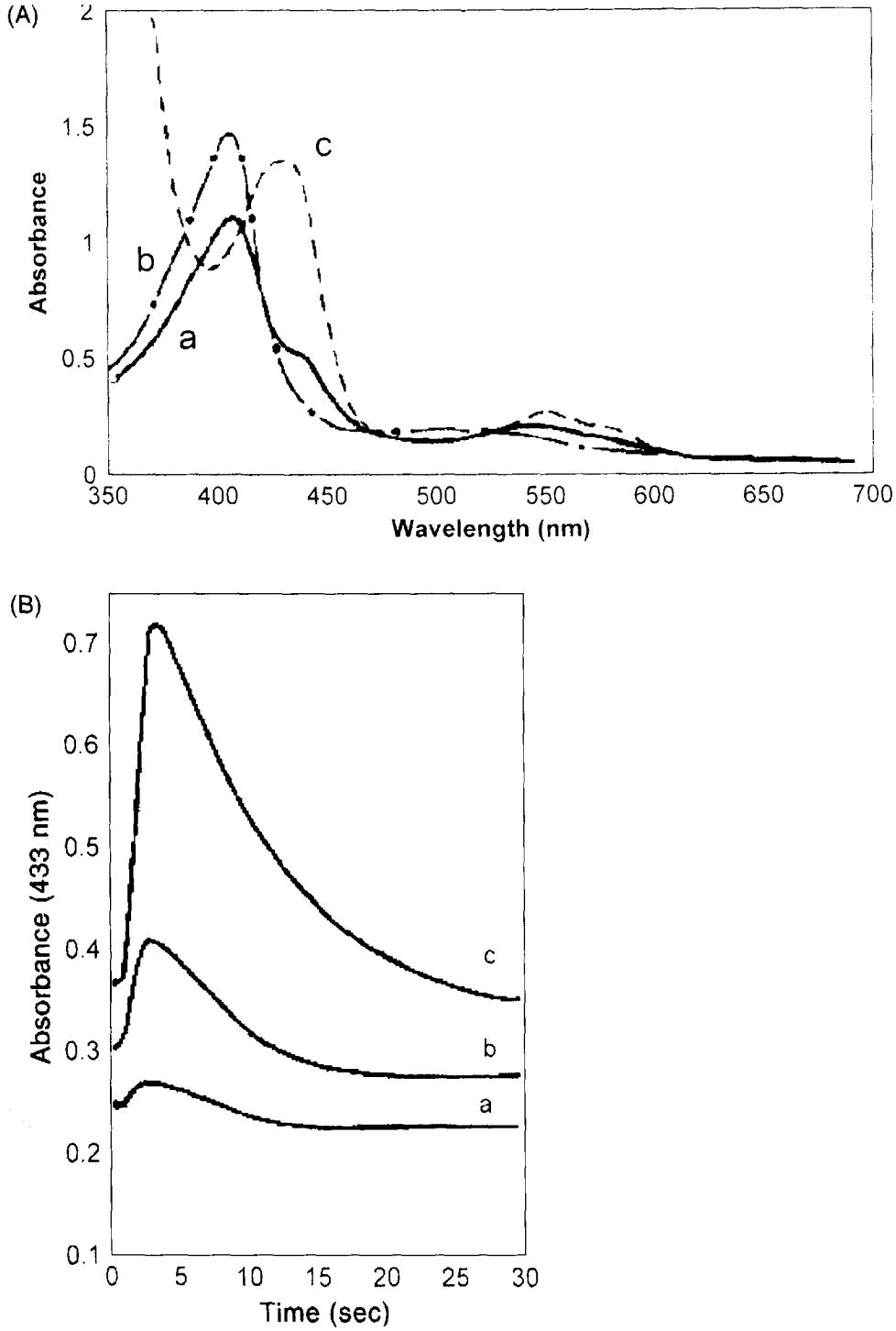


Fig. 2. (A) Appearance of the ferrous enzyme just after the addition of IAA (a) and its disappearance 1 min later (b) (8 μM tobacco peroxidase, 10 mM IAA, 0.05 M Na citrate buffer, pH 4.5). (c) The spectrum of ferrous tobacco peroxidase obtained in the excess of Na dithionite. (B) Time course (433 nm) of the ferrous enzyme appearance during IAA oxidation by tobacco peroxidase (8 μM) at pH 4.5. IAA concentrations used (mM): (a) 1; (b) 2; (c) 5. Delay in recording 2 sec.

Compound II native

$$\text{HRP-IAA}\cdot + O_2 \rightarrow \text{HRP(OH}\cdot) + CO_2 + \text{indole-epoxide} \quad (3)$$

Ten years later the authors observed the formation of Compound II in the course of IAA oxidation with low concentrations of HRP at room temperature, but attributed its formation to the presence of traces of hydrogen peroxide. Prior to this, Compound II formation by HRPs A and C was considered as a proof of the peroxidative cycle in the oxidation of IAA, and the

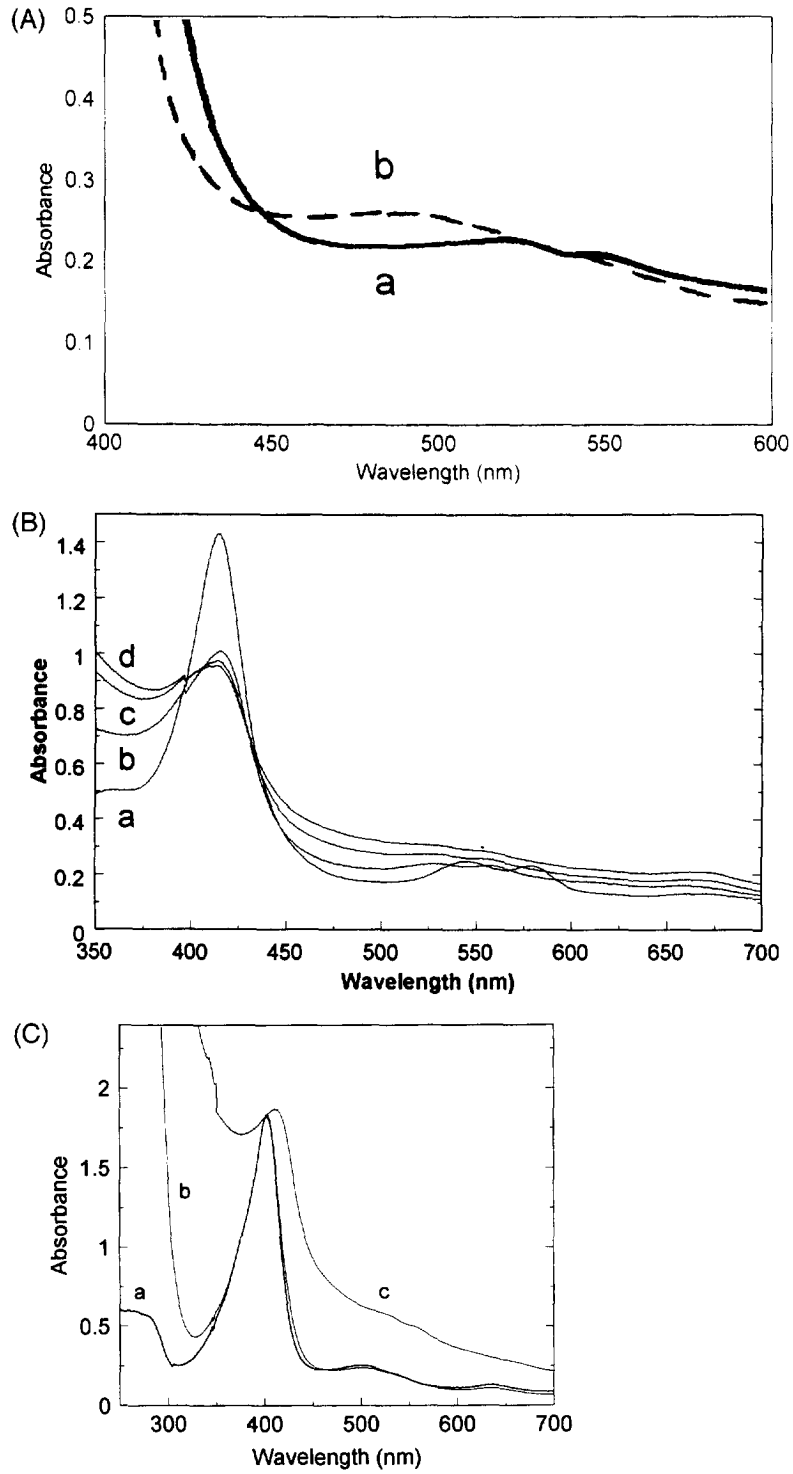


Fig. 3. (A) Appearance of Compound II traces just after 0.5 mM IAA addition to 10 μM peroxidase in 0.1 M Tris–HCl buffer, pH 6.0. (a) Immediate record (3 sec delay) and (b) 1 min later. (B) Spectral changes in the course of 1 mM IAA oxidation by preformed Compound III of 18 μM tobacco peroxidase in 0.1 M Na acetate buffer, pH 5.0. (a) Native enzyme; (b) Compound III spectrum obtained after the addition of 5 mM hydrogen peroxide; (c) Compound II spectrum obtained after the addition of 1 mM IAA; (d) the spectra recorded 20 min after IAA addition. (C) Compound II formation after the addition hydrogen peroxide to the mixture of the native enzyme with IAA in 0.1 M Na acetate solution, pH 6.7. (a) Native enzyme; (b) the spectrum after 1 mM IAA addition; (c) enzyme spectrum after 2 mM hydrogen peroxide addition.

formation of IAA hydroperoxide via IAA radical interaction with oxygen was proposed [14]. In the course of IAA oxidation by HRP, Compound II formation was observed at neutral pH in aerobic stopped-flow studies, thus indicating the participation of Compound II in the reaction [7]. Thus, there is a consensus that Compound II is the main enzyme species responsible for IAA oxidation. However, in our opinion reaction (1) could proceed as a direct oxygen transfer from the active centre to IAA (4). This is more probable than the binding of two IAA molecules on the enzyme.

$$HRP(OOH\cdot) + IAA \rightarrow HRP(OH\cdot) + IAAOH \qquad (4)$$

The direct oxygen transfer in the reaction of sulphooxidation [17] is well known and suggests substrate binding at the haeme distal site in close vicinity to the haeme iron [18]. Thus, it might be that IAA binds at the distal site as well. IAAOH in reaction (4) could correspond to oxindole-3-acetic acid solving the problem of its identification in plants. The reaction (3) proposed [13] also suggests the existence of a real enzyme–substrate complex between the IAA radical bound to the enzyme and oxygen. Oxygen transfer to the second position of the indole ring in reaction (3) can be proposed as well. This is supported by our observation that 2-methyl-IAA is not a substrate for oxidation by molecular oxygen [12]. In this case, we expect the formation of compound (I) instead of indole-epoxide (II) as suggested by Ricard and Job [13]. Hydrolysis of (I) would give rise to oxindole-3-carbinol, which is the main product of IAA degradation by HRP at acidic pH [19]. The data obtained on HRP [6, 7, 13, 14, 19] as well as our results are easily explained with such a model.

Compound II has never been observed as a steady-state enzyme form in the course of interaction with hydrogen peroxide at acidic pH, and is only a transient species in the course of IAA oxidation at neutral pH. Taking into account the high reactivity of the tobacco peroxidase Compound II, its stable appearance in the case of IAA peroxidation has to be explained by 'switching on' the peroxidative cycle (5)–(6), leading to Compound II in addition to reaction (1):

$$TOP + H_2O_2 \rightarrow \underset{\text{Compound I}}{TOP(O\cdot+)} + H_2O \qquad (5)$$

$$TOP(O\cdot+) + IAA \rightarrow TOP(OH\cdot) + IAA\cdot \qquad (6)$$

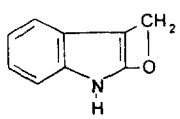
The fact that oxygen is consumed in the course of IAA oxidation, even with a vast excess of hydrogen peroxide over enzyme, shows that the peroxidative cycle is switched on only to promote the oxidative one, e.g. to accelerate the formation of Compound II which is responsible for the oxidation process. Thus, it seems possible that hydrogen peroxide play a physiologically important role promoting the reaction of IAA oxidation.

CONCLUSION

Our work with transgenic tobacco plants with altered peroxidase activity has revealed numerous phenotypes which resemble an 'auxin-like' response [4]. To investigate further the possibility that the tobacco peroxidase is modifying IAA levels, we chose to characterize the kinetic parameters of IAA oxidation using the purified enzyme. It became apparent that the catalytic properties of the tobacco peroxidase are significantly different from those of the horseradish enzyme. This should allow us to obtain new insights into the oxidation of IAA by plant peroxidases.

We conclude from this work that the IAA oxidation cycle is functioning separately from peroxidation. This is in agreement with the conclusion made by Pires de Melo *et al.* for HRP-catalysed IAA oxidation on the basis of chemiluminescent studies [20]. Moreover, the addition of hydrogen peroxide in the course of IAA oxidation switches the peroxidation cycle on only at the initiation step. Compound II seems to be a key intermediate in the oxidation reaction independent of peroxidase origin. No answer is yet available about the mechanistic details of the initiation of IAA oxidation. There is some indirect evidence of IAA hydroperoxide formation in the course of IAA aerobic oxidation by the tobacco enzyme. This is based on the slight inhibition effect of catalase, the appearance of Compound II species at neutral pH values, and the activation effect of hydrogen peroxide. The hydroperoxide appears to be formed inside the active centre and decomposes there, otherwise the effect of catalase would be much stronger. The ferrous form detected in the reaction course could be produced via secondary radical reaction, or it is produced as a step in the reaction. However, the absence of Compound III in the course of IAA oxidation by the tobacco enzyme makes the latter proposal less tenable. Steady-state measurements presented here have been further supported by transient-state kinetic analyses reported by Gazaryan *et al.* [12].

We can also conclude from this work that IAA oxidation can be considered as a physiological important role for the tobacco peroxidase. This enzyme and HRP are able to oxidize IAA without additional substrates or cofactors like manganese salts, hydrogen peroxide and phenols. Also, it is possible the activation



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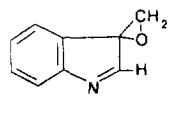

II

of the enzyme by hydrogen peroxide may serve an important biological role in the plants defence response through the acceleration of IAA degradation.

EXPERIMENTAL

Enzyme. TOP was obtained from transgenic *Nicotiana sylvestris* plants as described earlier [3]. The homogeneous prepn characterized by M_r 36 kDa, p*I* 3.5 and RZ 3.4 was used for all the experiments. IAA (Sigma) stock soln was freshly prepd by dissolving in 0.1 M Tris–HCl buffer, pH 8.5, and then diluting with 0.05 M Na citrate buffer, pH 4.5.

Oxygen uptake. The studies were performed using an O_2 electrode fitted with a plastic chamber (Rank Brothers, Cambridge, UK). Kinetic measurements were performed in the IAA concn range 0.2–2 mM and 0.05 μM peroxidase in 0.1 M NaOAc and 0.05 M Na citrate buffer solns, pH 4.5, and 0.1 Tris–HCl buffer, pH 6.0, at 22°. The standard error of oxygen uptake rate determinated from 3 repetitions was <5%. O_2 consumption in absence of enzyme was <0.1% hr^{-1}. K_m and V_m values were obtained from double-reciprocal plots, and turnover numbers were calculated by dividing V_m by the enzyme concn. The effects of catalase in the concn range 0.1–1 μM and H_2O_2 in the concn range 10–300 μM were studied with 0.2 mM IAA and 0.05 μM peroxidase in 50 μM Na citrate buffer, pH 4.5. The effects of peroxidase substrates were studied under the same conditions, but the IAA concn was 0.5 mM. The K_m value for tobacco leaf extracts was calculated from initial rates of O_2 consumption in the presence of 0.05 M H_2O_2.

HPLC studies. IAA degradation was measured by RP-HPLC using a Hewlett Packard HP1090 Liquid Chromatograph with a UV detector (254 nm). The incubation mixt. containing 20 μM IAA and 0.03 μM TOP in presence or absence of 2 μM H_2O_2 in 0.1 M Na acetate soln, pH 6.7, was incubated at 24°. Aliquots were taken and analysed by isocratic elution in MeOH–1% HOAc (2:3) on a Supelcosil LC-18-BD column (150 × 4.6 mm) (Supelco). The total IAA peak area before reaction initiation was taken as 100%. The standard error estimated from 3 repeats of the initial probe was <2% of the magnitude.

Spectral studies. Measurements were performed on a Shimadzu UV160U spectrophotometer at 22°. To detect the ferrous enzyme, an IAA aliquot (100 μl 5–25 mM soln) was added to 400 μl concd enzyme soln (10–20 μM) in 0.05 M Na citrate buffer, pH 4.5, and spectral changes were immediately recorded (3 sec delay after mixing reagents). The steady-state spectra in the course of IAA oxidation were followed in the range 0.1–20 μM enzyme concns. Compound III was performed by addition of H_2O_2 in 300-fold excess over the enzyme in 0.1 M Na acetate buffer, pH 5.0, and incubation of the mixt. for 1 min. Spectral changes were recorded immediately after addition of the IAA aliquot.

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