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A fungal auxin antagonist, hypaphorine prevents the indole-3-acetic acid-dependent irreversible inactivation of horseradish peroxidase: inhibition of Compound III-mediated formation of P-670☆

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Abstract

Hypaphorine, an indolic alkaloid from an ectomycorrhizal fungus is a putative antagonist of indole-3-acetic acid (IAA) known to inhibit the effect of IAA in growing roots of *Eucalyptus* seedling. Previously we have used horseradish peroxidase-C (HRP) as a sensitive reporter of IAA-binding to the IAA-binding domain, and reported that hypaphorine specifically inhibits the HRP-catalyzed superoxide generation coupled to oxidation of IAA [Kawano et al., Biochem. Biophys. Res. Commun. 288]. Since binding of IAA to the auxin-binding domain is the key step required for IAA oxidation by HRP, it was assumed that the inhibitory effect of hypaphorine is due to its competitive binding to the auxin-binding domain in HRP. Here, we obtained further evidence in support of our assumption that hypaphorine specifically inhibits binding of IAA to HRP. In this study, HRP arrested at the temporal inactive form known as Compound III was used as a sensitive indicator for binding of IAA to HRP. Addition of IAA to the preformed Compound III resulted in rapid decreases in absorption maxima at 415, 545, and 578 nm characteristic to Compound III, and in turn a rapid increase in absorption maximum at 670 nm representing the formation of P-670, the irreversibly inactivated form of hemoproteins, was induced. In contrast, the IAA-dependent irreversible inactivation of HRP was inhibited in the presence of hypaphorine. In addition, the mode of interaction between IAA and hypaphorine was determined to be competitive inhibition, further confirming that hypaphorine is an IAA antagonist which specifically compete with IAA in binding to the IAA-binding site in plant peroxidases.

Keywords: Compound III; Hypaphorine; IAA; Inactivation; P-670; Peroxidase

Indole-3-acetic acid (IAA), the principal form of auxin in higher plants, is first synthesized within young apical tissues, then conveyed to its basal target tissues by a specialized delivery system termed polar auxin transport [1]. Homeostasis in endogenous IAA level results from strict regulation of biosynthesis and metabolism of IAA, including conjugation, de-conjugation, and catabolism [2–4]. Plant peroxidases (EC 1.11.1.7) including horseradish peroxidase (HRP), but not the non-plant peroxidases, are known to be highly specific IAA oxygenases which oxidize IAA both in the absence and presence of H_2O_2. Thus, plant peroxidases are believed to play an important role in catabolism of IAA. The oxidation reaction of IAA proceeds via two known different mechanisms: a conventional H_2O_2-dependent pathway and one that requires O_2 but not H_2O_2 [5–7]. The peroxidase cycle oxidizing wide variety of substrates including IAA proceeds in the presence of H_2O_2 as follows [8,9]:

$$\mathrm{POX} + \mathrm{H_2O_2} \rightarrow \mathrm{POX\ I} + \mathrm{H_2O} \quad (1)$$

$$\mathrm{POX\ I} + \mathrm{S} \rightarrow \mathrm{POX\ II} + \mathrm{P} \quad (2)$$

$$\mathrm{POX\ II} + \mathrm{S} + \mathrm{H^+} \rightarrow \mathrm{POX} + \mathrm{H_2O} + \mathrm{P} \quad (3)$$

where POX, POX I, and POX II are the native enzymes and its Compounds I and II, respectively; and S and P

☆ *Abbreviations:* HRP, horseradish peroxidase; IAA, indole-3-acetic acid.

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are the substrates and the product of its one-electron oxidation, respectively. IAA can be oxidized by this mechanism but there is no strict substrate specificity in this catalytic cycle. Thus no specific binding of IAA to peroxidases takes place. On the other hand, plant peroxidases can catalyze the oxidation of IAA via H_2O_2-independent pathway requiring molecular dioxygen. It has been reported that plant peroxidases such as anionic tobacco peroxidase, cationic peanut peroxidase, soyabean peroxidase, and the major HRP isoenzyme belonging to the C group, are highly specific IAA oxygenases which possess the domains with structural similarity with all known auxin-binding proteins such as those from *Arabidopsis thaliana*, ABP-1 and ABP-4 from *Maize*, and T85 and T92 from *Nicotiana tabacum* [5–7,10–12]. The reaction cycle for IAA oxidation is initiated by binding of IAA to the enzyme, that followed by binding of O_2, thus a ternary complex, peroxidase-IAA-dioxygen is formed, and then IAA cation radicals and superoxide anion radicals are yielded as follows:

$$\mathrm{POX} + \mathrm{IAA} \leftrightarrow [\mathrm{POX\text{–}IAA}] \quad (4)$$

$$[\mathrm{POX\text{–}IAA}] + \mathrm{O_2} \rightarrow [\mathrm{POX\text{–}IAA\text{–}O_2}] \quad (5)$$

$$[\mathrm{POX\text{–}IAA\text{–}O_2}] \leftrightarrow \mathrm{POX} + \mathrm{IAA}^{\cdot+} + \mathrm{O_2^{\cdot-}} \quad (6)$$

where $IAA^{\cdot+}$ and $O_2^{\cdot-}$ are the IAA cation radicals and superoxide anion radicals, respectively. Thus IAA-dependent generation of superoxide catalyzed by plant peroxidases can be observed in the absence of H_2O_2 [13]. However, peroxidases from non-plant sources are not able to oxidize IAA in the absence of H_2O_2. Specificity of plant peroxidases to IAA may be conferred by the auxin-binding protein-like regions in amino acid sequences at vicinity of the heme pocket, which are missing in non-plant peroxidases [7].

Hypaphorine (tryptophan betaine) has been isolated from a number of medicinal plants such as *Glycyrrhiza* species [14] and *Erythrina* species [15]. As shown in Fig. 1, the structures of IAA and hypaphorine are very similar. In the previous studies from our group, hypaphorine has been determined to be the major indolic compound, present at 1000-fold higher concentrations than IAA, in free-living hyphae of the ectomycorrhizal fungus, *Pisolithus tinctorius* [16] and its synthesis in *P. tinctorius* hyphae can be stimulated by interaction with host plants [17].

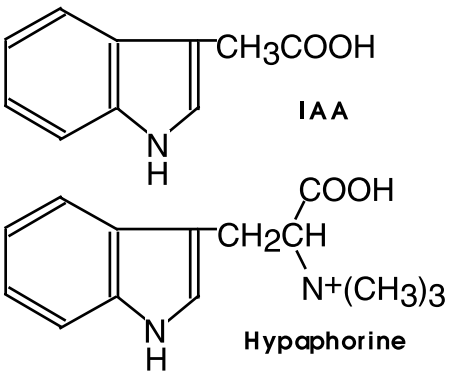

Fig. 1. Structures of IAA and hypaphorine.

It has been shown that hypaphorine secreted from the infecting hyphae of *P. tinctorius* controls the development of root hairs in the host plant, *Eucalyptus* [17], by counteracting the action of endogenous and exogenous IAA [18]. Since hypaphorine inhibited all measurable morphological effects of IAA in *Eucalyptus* seedlings, a putative function of hypaphorine as an anti-auxin which binds to the auxin receptors, playing a role in plant–microbe interactions has been proposed [19]. We have used HRP/superoxide system and demonstrated the antagonistic interaction between hypaphorine and IAA on the purified protein for the first time and competitive mode of interaction was uncovered [13]. It has been clearly shown that hypaphorine inhibits the IAA-dependent generation of superoxide anion radicals by HRP, that occurs in the absence of H_2O_2. This implies the importance of structural similarity between hypaphorine and IAA in competitive binding to the auxin binding domains in HRP.

In this study, we show further evidence in support of our assumption that hypaphorine specifically inhibits the binding of IAA to plant peroxidases, the most abundant auxin-binding proteins in plants. Here, HRP arrested at temporal inactive form known as Compound III was used as a sensitive indicator of IAA binding. Effect of hypaphorine on IAA-dependent conversion of HRP Compound III to the irreversibly inactivated from of HRP was examined. In addition, the mode of interaction between IAA and hypaphorine was determined.

Experimental procedures

Chemicals. Hypaphorine was synthesized from tryptophan according to Romburgh and Barger [20], and quality of the HPLC-purified products was examined with H^1NMR according to Beguiristain et al. [16]. IAA and other chemicals were purchased from Sigma (St. Louis, MO, USA). IAA solution was prepared by first dissolving 100 mM IAA in ethanol and diluted to the desired concentrations with distilled water heated to 80 °C. Then IAA solution was kept on ice in dark until used. Final concentration of ethanol in the reaction mixture was adjusted to 0.1% (v/v).

Enzyme and analyses. HRP [Type VI; RZ ($A_{403\,nm}/A_{275\,nm} = 3.0$)] was purchased from Sigma (St. Louis, MO, USA), and used without further purification. Concentration of HRP was determined spectroscopically ($\varepsilon_{403\,nm} = 102\,mM^{-1}\,cm^{-1}$) [5]. Absorption spectra of HRP in 25 mM potassium phosphate buffer (pH 6.0) were recorded on a Beckman DU-70 spectrophotometer at room temperature with a spectral bandwidth of 1.0 nm in a cuvette with 1-cm light path [5]. Compounds II and III were prepared by adding 5–150 μM H_2O_2 and 0.4–1.0 mM H_2O_2 to the native HRP (10 μM), respectively. To the preformed compound III, hypaphorine and/or IAA was added and well mixed, and then spectral changes were monitored.

Results and discussion

Absorption spectra for native HRP, Compound II, Compound III, and P-670

The conventional peroxidase cycle is well known for oxidation of various substrates coupled to consumption of H_2O_2. In the presence of H_2O_2, the native form of enzyme (Fe^{III}) is converted to Compound I (heme with Fe^{IV}=O plus porphyrin radicals) that catalyzes the oxidation of substrates by converting itself into Compound II (Fe^{IV}=O). Compound II also catalyzes the oxidation of substrates while converting itself into the native form, and then the peroxidase cycle is completed.

Fig. 2 shows the characteristic spectra for different oxidation states of HRP. Typical absorption spectra for native HRP (Fig. 2A), Compound II (Fig. 2B), Compound III (Fig. 2C), and P-670 (Fig. 2D) are shown. Native enzyme has absorption maxima at 403, 500, and 639 nm (the peak at 403 nm is not shown here). Addition of 0.5–1.5 molar equivalent of H_2O_2 to native HRP resulted in formation of Compound I that has characteristic absorption maxima at 404, 550, and 650 nm [21,22] (data not shown). However Compound I is not stable and easily converted to Compound II that has characteristic absorption maxima at 420, 527, and 556 nm [22]. As previously reported [22], addition of 2–15 molar equivalents of H_2O_2 to HRP resulted in accumulation of Compound II. Typical absorption spectrum obtained after addition of 10 molar equivalents of H_2O_2 (addition of 100 μM H_2O_2 to 10 μM HRP) is shown in Fig. 2B. In the presence of 40–100 molar equivalent of H_2O_2, increases in absorption maxima at 415, 545, and 578 nm were observed. This indicates that majority of HRP molecules has been converted to Compound III known as a temporal inactive form [23,24]. Typical absorption spectrum obtained after addition of 30 molar equivalents of H_2O_2 (300 μM) is shown in Fig. 2C. As we have previously reported [21], Compound III of HRP was stable at pH 6.0 and no decay of the absorption maxima was observable within 15 min.

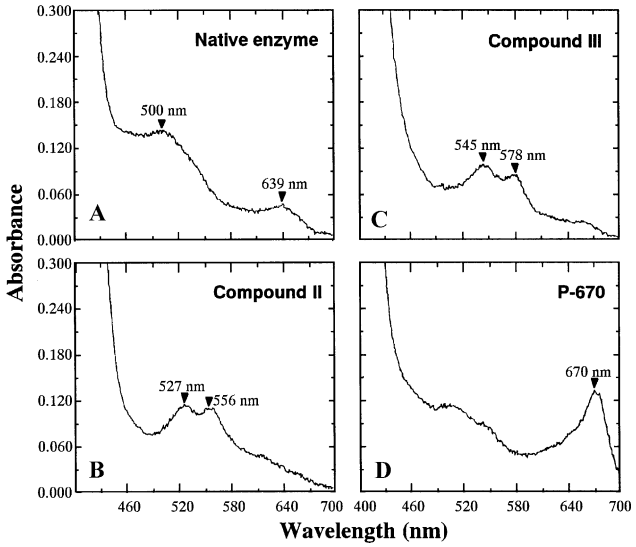

Fig. 2. Typical absorption spectra for native HRP, Compound II, Compound III, and P-670. Absorption spectra were collected for 10 μM HRP-C dissolved in 25 mM K-phosphate buffer (pH 6.0). (A) Typical absorption spectrum for native enzyme is shown. The arrowheads indicate the absorption maxima at 500 and 639 nm. (B) Compound II generated from HRP-C in the presence of 100 μM H_2O_2 (10 molar equivalent to HRP-C). The arrowheads indicate absorption maxima at 527 and 556 nm. (C) Compound III, generated from HRP-C in the presence of 300 μm H_2O_2 (30 molar equivalents to HRP-C). The arrowheads indicate absorption maxima at 545 and 578 nm. For (B) and (C), spectra were obtained immediately after addition of H_2O_2 to native HRP-C. (D) Typical spectrum for IAA-induced P-670. To the preformed Compound III (C), 140 μM IAA was added. Absorption spectrum was recorded 5 min after addition of IAA to the reaction mixture.

When IAA was added to the preformed Compound III, decrease in Compound III specific absorption maxima and increase in absorption maxima around 670 nm were observed. Fig. 2D shows the typical spectrum induced by addition of 140 μM IAA to Compound III which was formed from 10 μM HRP in the presence of 300 μM H_2O_2. Formation of P-670 was observed only when IAA was added to the preformed Compound III.

IAA-dependent conversion of preformed Compound III to irreversibly inactivated protein

Following addition of IAA (140 μM), the P-670-specific absorption (at ca. 500 and 670 nm) increased with time (Fig. 3). On the other hand, the major absorption of heme at 403 nm markedly decreased (Fig. 3A). This indicates the degradation of heme accompanying the irreversible inactivation of the enzyme. The increase in absorption at 670 nm was shown to be slow process reaching the maximal level ca. 10 min after addition of IAA, and the absorption was stably observed 40 min after addition of IAA (Fig. 3C).

Then the effect of IAA concentration on conversion of preformed HRP Compound III to the P-670 pigment was examined (Fig. 4). Up to 200 μM, IAA-induced P-670 absorption linearly increased. High concentrations of IAA (>300 μM) were shown to be inhibitory.

Effect of hypaphorine

Effect of a fungal indolic alkaloid, hypaphorine which has similar structure with IAA (Fig. 1A) on the formation of P-670 pigment was examined. Addition of hypaphorine to the native enzyme induced no detectable change in absorption spectrum of native enzyme (Table 1). Addition of hypaphorine to the preformed Compound II

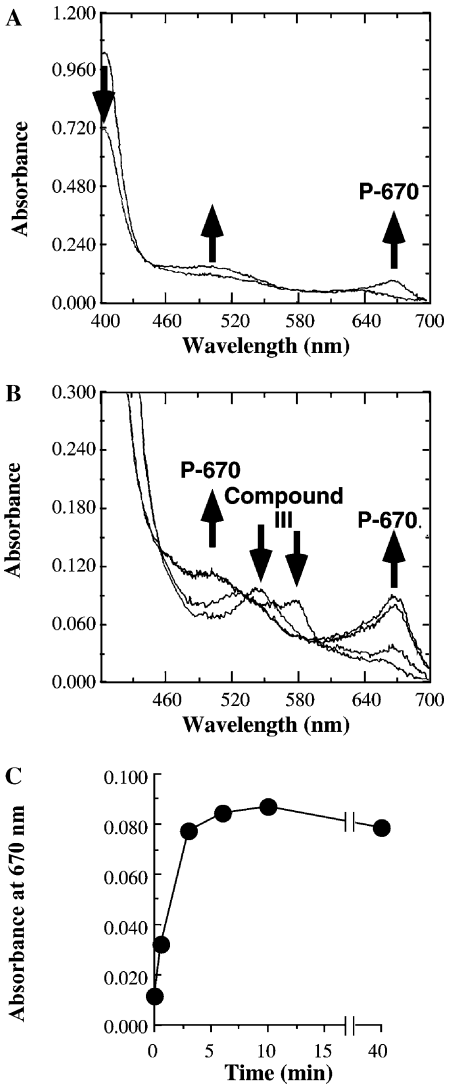

Fig. 3. Kinetic analysis of IAA-dependent conversion of Compound III to P-670: Time-course. (A) Absorption spectra recorded before and 10 min after addition of IAA to the preformed HRP Compound III are shown. Increases in absorption at around 500 and 670 nm, and marked decrease at 403 nm are shown. (B) Increases in P-670-specific absorption maxima at 500 and 670 nm, and decreases in Compound III-specific absorption maxima at 545 and 578 nm are shown. The spectra in (B) were recorded at 0, 0.5, 3, 10 min after addition of IAA to the preformed HRP Compound III. (C) Changes in absorption at 670 nm were plotted as a function of time (min). Data were obtained 0, 0.5, 3, 6, 10, and 40 min after addition of 140 μM IAA to the preformed HRP Compound III.

changed the enzymic absorption spectrum into that of native enzyme (Table 1), indicating that hypaphorine is a good substrate (an electron donor) for conventional peroxidase cycle. Similar results were observed for IAA (data not shown). It is notable that addition of hypaphorine to the preformed Compound III (prepared by addition of 30 molar equivalents of H_2O_2 to HRP) induced no concomitant formation of P-670, thus final state of enzyme remained in Compound III (Table 1). This is one of the major differences from the effect of IAA on HRP's

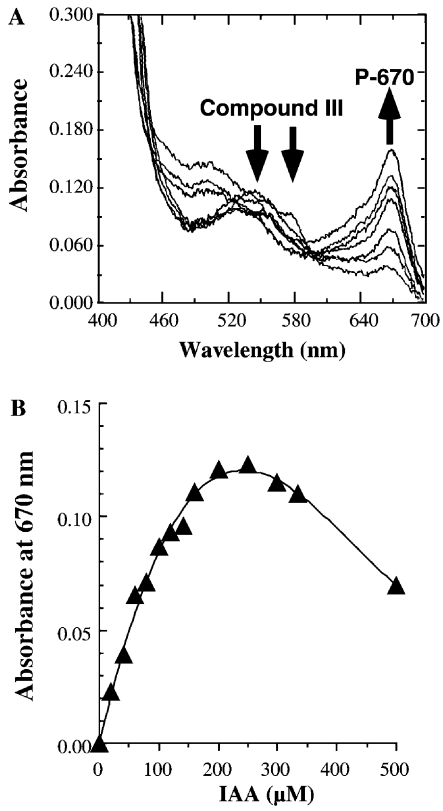

Fig. 4. Effect of IAA concentration on conversion of HRP Compound III to P-670. (A) Absorption spectra recorded 10 min after addition of 0, 20, 40, 60, 80, 100, and 140 μM IAA to the preformed HRP Compound III are shown. (B) Increase in absorption at 670 nm measured 10 min after addition of IAA (30–500 μM) was plotted as a function of IAA concentration.

Table 1
Inhibition of IAA-dependent conversion of preformed Compound III to P-670 by hypaphorine

HRP-C	H_2O_2	Hypaphorine	IAA	Final state of enzyme
+	++	–	–	Compound III
+	–	+	–	Native
+	–	–	+	Native
+	++	+	–	Compound III
+	++	–	+	P-670
+	++	+	+	Compound III
+	+	–	–	Compound II
+	+	+	–	Native

Note. The reaction mixture, total volume of 1 ml, buffered with 25 mM K-phosphate (pH 6.0); initial HPR-C concentration, 10 μM. The reagents 50 μM (+) or 300 μM (++) H_2O_2, 1 mM hypaphorine, and 100 μM IAA were added to the reaction mixture in this order with 30 s of intervals, and the final oxidation state of the enzyme was determined spectroscopically, 5 min after final addition of reagents or control water.

oxidation states. Interestingly, addition of hypaphorine prior to addition of IAA resulted in inhibition of the IAA-induced P-670 formation, thus the final state of enzyme remained in Compound III (Table 1).

Mode of IAA–hypaphorine interaction

Since addition of hypaphorine prior to addition of IAA prevented the IAA-dependent conversion of preformed Compound III to P-670 (Table 1), the mode of hypaphorine action against IAA action on enzyme inactivation (P-670 formation) was examined by the Lineweaver–Burk's double-reciprocal analysis in which the reciprocals of the IAA-induced increase in absorption at 670 nm were plotted against the reciprocals of IAA concentration (Fig. 5). Data points obtained in the presence and absence of 200 μM hypaphorine were co-plotted and the IAA–hypaphorine interaction was graphically analyzed. Two lines obtained in the presence and absence of hypaphorine crossed near the *y*-axis and similar V_{max} values were obtained (absorbance 0.17, control; absorbance 0.18, hypaphorine-treated). The apparent K_m was elevated from 39.9 μM (control) to 70.48 μM (+hypaphorine). This result indicates that hypaphorine inhibits the IAA-dependent P-670 formation via a competitive manner. We therefore propose the following mode of hypaphorine action: hypaphorine specifically binds to IAA-binding domains on HRP (Compound III) protein because of structural similarity between IAA and hypaphorine, and blocks the binding of IAA, thus inhibiting the IAA-dependent inactivation of the enzyme. Present results are similar to our previous demonstration that structural similarity between IAA and hypaphorine results in competitive inhibition by hypaphorine of the IAA-dependent superoxide generation in HRP reaction mixture [13] and of the IAA-dependent retardation of primary root development in *Eucalyptus* seedling [25].

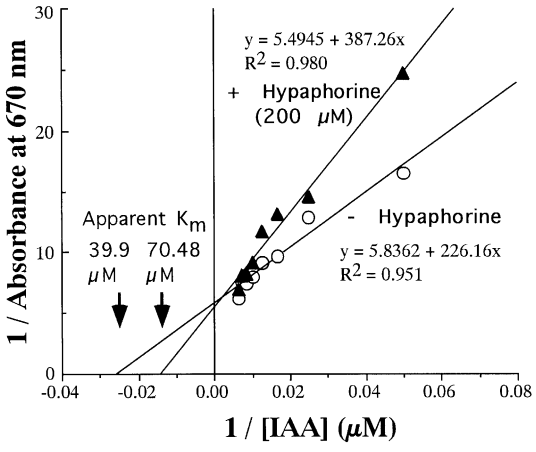

Fig. 5. Kinetic analysis of the hypaphorine inhibition of the IAA-dependent P-670 formation from preformed Compound III. Lineweaver–Burk analysis for assessing the mode of interaction between IAA and hypaphorine in IAA-dependent conversion of HRP Compound III to P-670. Typical results from three replicates were used for graphical analysis with double-reciprocal plotting.

Recently, we have reported that salicylic acid known to be a good substrate for peroxidase-dependent superoxide generation [26,27], has weak effect on conversion of preformed Compound III to P-670 [22]. Hypaphorine showed no inhibitory effect on the salicylic acid-dependent conversion of HRP Compound III to P-670 (unpublished results). This observation is also supporting our view that effect of hypaphorine is specific to IAA.

Role of fungal auxins and related compounds

Not only plants but also microorganisms can synthesize auxins and related compounds [28]. However, the role of auxin biosynthesis by microorganisms is not fully elucidated. A pathogenic fungus of rice, *Pythium aphanidermatum* is known to produce and accumulates IAA in host plants, that is 200 times higher than that in normal plants, and causes serious leaf-bent symptom [29]. Recent studies have confirmed that fungal IAA controls major anatomical features and gene expression in ectomycorrhiza such as that of pine [30,31], spruce [32], and *Eucalyptus* [33,34]. Other indolic compounds released from ectomycorrhizal fungi also play a role in symbiosis development [33,34]. Hypaphorine first determined as one IAA analog [35] is the major indolic compound detectable in free-living hyphae of *P. tinctorius* [16]. It has been shown that host plants *Eucalyptus* stimulates the accumulation of hypaphorine in *P. tinctorius* hyphae during ectomycorrhizal infection, and in turn *P. tinctorius*-derived hypaphorine controls the development of root hairs [17]. Ditengou and Lapeyrie [19] have proposed a model mechanism that hypaphorine counteracts IAA but not synthetic auxins by binding to putative receptors for IAA such as auxin-binding proteins in *Eucalyptus* seedlings.

In this study, we employed HRP as a model plant peroxidase, which represents one class of putative auxin-binding proteins abundant in plant apoplasts, probably mimicking the IAA receptors in binding to IAA. It is likely that hypaphorine prevents or interferes with IAA catabolism catalyzed by host plant peroxidases in ectomycorrhiza. However, consequence of hypaphorine-mediated disturbance in IAA catabolism remains to be elucidated in the future researches.

Conclusion

We have used HRP as a model enzyme to study a plant-specific and IAA-specific biochemical event. Fig. 6 summarizes the HRP-catalyzed overall IAA oxidation reactions and their inhibition by hypaphorine. In the presence of H_2O_2, plant peroxidases catalyze the oxidation of wide-variety of substrates including IAA (Fig. 6A). Hypaphorine is also a good substrate for this

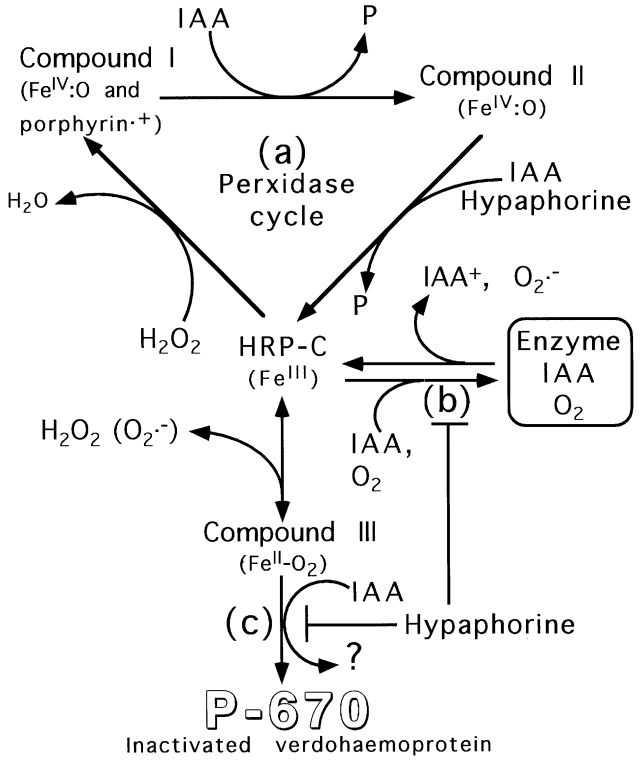

Fig. 6. A model for IAA oxidation catalyzed by various states of HRP. (A) Conventional peroxidase cycle requiring H_2O_2 as an electron acceptor. (B) Superoxide-generating reaction coupled to IAA-specific oxidation requiring molecular oxygen, and its competitive inhibition by hypaphorine. (C) IAA-dependent conversion of preformed HRP Compound III to P-670, and its competitive inhibition by hypaphorine.

reaction, thus no IAA–hypaphorine competition takes place. The first biochemical evidence for hypaphorine–IAA antagomism on plant peroxidases catalyzing IAA-specific oxidation reaction yielding superoxide, was presented in our previous report [13]. This IAA–hypaphorine counteraction is summarized in Fig. 6B. In this study, the effect of IAA on the preformed Compound III was examined (Fig. 6C). Compound III was generated by addition of excess of H_2O_2 (30 molar equivalent) to HRP (Figs. 2–4). Following addition of various concentrations of IAA to the reaction mixture rich in preformed Compound III, absorption spectra were recorded. We observed the spontaneous decay in the characteristic absorption doublet (545 and 578 nm) of Compound III, indicating that IAA spontaneously converted Compound III to other forms of HRP. At the same time, an increase in absorption maximum at 670 nm was observed. It is well known that absorption maximum at 670 nm represents the formation of P-670, an irreversibly inactivated form of hemoproteins so-called verdohemoprotein [36,37].

Here, we presented a spectroscopic method for the detection and kinetic analysis of IAA binding to plant peroxidases, employing HRP arrested at Compound III. It was shown that hypaphorine, a fungal auxin analog, known to act as a putative inhibitor of IAA action in plants, specifically blocks this IAA-dependent HRP inactivation by a competitive manner. This is in support of our proposal that IAA and a fungal alkaloid competitively interact on a protein which shares some structural similarity with auxin-binding proteins.

Acknowledgments

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