Note

Spectroscopic Evidence in Support of Horseradish Peroxidase Compound II-catalyzed Oxidation of Salicylic Acid but Not of Phenylethylamine

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Salicylic acid and phenylethylamine are putative substrates for naturally occurring reactions for generation of reactive oxygen species, which are catalyzed by plant peroxidases. Here, we used commercially available highly purified horseradish peroxidase-C (HRP-C) as a model enzyme for spectroscopic analysis, and obtained data suggesting that the Compound II form of HRP-C does not utilize phenylethylamine as substrate. In contrast, addition of salicylic acid to Compound II resulted in rapid conversion of Compound II to the native form.

Key words: Compound II; horseradish peroxidase; reactive oxygen species; phenylethylamine; salicylic acid

The peroxidase cycle is well known for oxidation of various substrates coupled to consumption of H₂O₂ by a complex mechanism as follows. In the presence of H₂O₂, the native form of enzymes (FeIII) is converted to Compound I (O = FeIV+ porphyrin radicals). Compound I catalyzes the oxidation of the substrate by converting itself into Compound II (O = FeIV). Compound II also catalyzes the oxidation of substrates while converting itself into the native form and the peroxidase cycle is completed.

It has been shown that peroxidase is involved in production of reactive oxygen species (ROS) during plant defense induction against pathogen attacks. In addition, salicylic acid (SA) known as a plant defense inducer, has shown to be a good substrate for ROS-generating peroxidase reaction in tobacco cell suspension culture. Phenylethylamine (PEA) is another putative substrate for naturally occurring ROS-generating peroxidase reactions in plants. PEA is one of the most abundant aromatic monoamines in tobacco plants and other plant tissues. In tobacco cell suspension culture, PEA-treatment induces the production of H₂O₂ and superoxide as oxidative signals that are involved in the defense mechanism against microorganisms and animal predators.

We have previously demonstrated that extracellularly localized peroxidase in tobacco cell suspension culture catalyzes the monovalent oxidation of PEA, which results in production of PEA-derived free radicals. Then the resultant PEA-derived free radicals enter a series of reactions that yield various members of ROS such as superoxide, H₂O₂, and hydroxy radicals. We have shown that this reaction can be initiated in the presence of PEA and trace of H₂O₂, and the resultant PEA free radicals may mediate the production of superoxide in the presence of dissolved oxygen. We have used a commercially available highly purified horseradish peroxidase (HRP-C) as a model system and confirmed that HRP-C also catalyzes the formation of PEA free radicals, and consequently superoxide is generated in the presence of H₂O₂ as the initiator of the reaction and dissolved oxygen as a source of superoxide. However, it has not been clearly shown which form of HRP-C actually catalyzes the oxidation of PEA, and leads to the production of superoxide.

In this paper, we report on evidence that Compound II accumulates in the HRP-C reaction mixture containing H₂O₂ and PEA. This indicates that Compound II is not able to catalyze the oxidation of PEA.
PEA was obtained from Wako Pure Chemical Co. Ltd. (Osaka, Japan). HRP-C [Type VI; RZ (\(A_{403\,\text{nm}}/A_{275\,\text{nm}} = 3.0\))] and other chemicals were purchased from Sigma (St. Louis, MO., USA). HRP-C was used without further purification. The concentration of HRP-C was measured spectroscopically (\(e_{403\,\text{nm}} = 102\,\text{mM}^{-1}\cdot\text{cm}^{-1}\)).

Absorption spectra of HRP-C dissolved in 20 mM K-phosphate (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 a 1-cm light path. The absorption spectrum of HRP-C was spectroscopically monitored and the data indicated the presence of native enzyme in the reaction mixture (Figs. 1(A), 1(B)).

In the reaction mixture containing 100 \(\mu\text{M}\) \(\text{H}_2\text{O}_2\), 7.5 \(\mu\text{M}\) HRP-C and 100 \(\mu\text{M}\) PEA, accumulation of Compound II was clearly shown. The presence of Compound II was shown by its characteristic absorption spectra showing absorption maxima at 420 (data not shown), 527 and 556 nm (Fig. 1(C)). Accumulation of Compound II in the presence of PEA indicates that Compound II is not able to oxidize PEA and thus the peroxidase cycle cannot be completed unless other reducing agents that reduce the Compound II back to the native form are added.

SA is known to be a good substrate for the ROS-generating peroxidase reaction. When 100 \(\mu\text{M}\) SA was added to the HRP-C arrested at Compound II in the presence of \(\text{H}_2\text{O}_2\) and PEA, the absorption doublet (at 527 and 556 nm) characteristic of the Compound II state was rapidly lowered, and then absorption spectra similar to native enzyme were obtained (Fig. 1(D)). This indicates that the Compound II is readily reduced back to its native form in the presence of SA.

Table 1 summarizes the effects of \(\text{H}_2\text{O}_2\), SA, and PEA on the state of HRP-C (7.5 \(\mu\text{M}\)). When added alone, SA and PEA had no effect on the state of HRP-C. In the presence of low concentrations of \(\text{H}_2\text{O}_2\) ranging from 5 to 20 \(\mu\text{M}\) (Table 1 shows the results for 10 \(\mu\text{M}\)), the presence of Compound II was shown by its characteristic absorption spectra with absorption maxima at 577, 622, and 650 nm when measured immediately after addition of \(\text{H}_2\text{O}_2\) to the reaction mixture containing 7.5 \(\mu\text{M}\) HRP-C. However, at the same time, the concomitant presence of absorption doublet at 527 and 556 nm, characteristic to Compound II, was also observed. The Compound I-specific absorption maxima became weaker and undetectable within 3 min after addition of \(\text{H}_2\text{O}_2\) (5–20 \(\mu\text{M}\)), and in turn the Compound II-specific absorption maxima became more intense. At this range of \(\text{H}_2\text{O}_2\) concentrations (5–20 \(\mu\text{M}\); up to ca. 3 molar equivalents), the spectra obtained were indicative of Compound II formation but always accompanied by a broad non-specific increase in absorption between 520 and 580 nm, probably indicative of a transition state between Compound I and Compound II. Thus, the precise kinetics of Compound I formation and its conversion to Compound II could not be analyzed. Therefore, both Compounds I and II are listed as the final states of enzyme after addition of 10 \(\mu\text{M}\) \(\text{H}_2\text{O}_2\) in Table 1.

It has been known that Compound I, formed from a variety of peroxidases such as HRP and microbial lignin peroxidase in the presence of \(\text{H}_2\text{O}_2\), can be readily self-converted to Compound II without addition of electron-donating substrates such as phenolics. Due to the unstable nature of Compound I, we could not prepare the enzyme arrested at Compound I, which may be useful for the study of substrate oxidation.

As presented in Table 1, the concentration of \(\text{H}_2\text{O}_2\) principally governs the oxidation state of HRP-C. With an increasing concentration of \(\text{H}_2\text{O}_2\), the oxidation state of HRP-C shifts from native enzyme to Compounds I, II, and III. In this series of HRP-C reactions, kinetic analysis for the role of \(\text{H}_2\text{O}_2\) is not simple.

In the presence of moderate \(\text{H}_2\text{O}_2\) (100 \(\mu\text{M}\)), the state of enzyme was shown to be in the relatively stable state, Compound II (Table 1). The presence of Compound II can be observed for more than 15 minutes without any decay of the spectral signals. This concentration of \(\text{H}_2\text{O}_2\) is ca. 13 molar equivalents to HRP-C, which is similar to that used for the study of PEA-dependent superoxide generation by
As described above, PEA was unable to reduce compound II back to native enzyme at any concentration ranging from 10 μM to 1 mM (Table 1 shows the typical results at 10 μM, 100 μM, and 1 mM).

Following addition of 100 μM H₂O₂, addition of 100 μM and higher concentrations of SA to the reaction mixture, resulted in rapid conversion of Compound II to the native enzyme (Table 1). Addition of 100 μM SA following treatments with 100 μM H₂O₂ and 100 μM PEA, also resulted in reproduction of native enzyme (Fig. 1, Table 1).

In addition, the effect of excess H₂O₂ (500 μM) on the state of HRP-C was examined (Table 1). As we have previously reported, an excess of H₂O₂ induces the formation of the temporary inactive enzyme known as Compound III. Addition of SA (100 μM) to the reaction mixture pretreated with 500 μM H₂O₂, resulted in irreversible inactivation of the enzyme characterized by the formation of verdohemoprotein which has an absorption maximum at 670 nm. However, PEA showed no effect on the irreversible inactivation of the enzyme.

Although both SA and PEA are shown to act as superoxide anion-generating substrates of HRP-C and various plant peroxidases, this study showed that only SA is a possible electron-donating substrate for Compound II. While SA targets Compound III and inactivates it by converting it to verdohemoprotein, PEA showed no effect on the state of Compound III. Compound III is often considered as a source of superoxide. Especially in the reaction mixture containing indole-3-acetic acid, Compound III is re-converted to native enzyme and bound oxygen is released as superoxide. Since addition of PEA to Compound III resulted in formation of neither verdohemoprotein nor native enzyme, Compound III is not the target of PEA action during PEA-dependent superoxide generation by HRP-C.

In conclusion, Compound II form of HRP-C does not utilize PEA as a substrate. It is likely that the catalytic form responsible for the oxidation of PEA required for production of ROS is Compound I but not Compound II, thus the peroxidase cycle is incomplete and Compound II accumulates. This may explain why the PEA-induced ROS production in HRP-C reaction mixture is only rapid and transient. However, in the peroxidase-dependent reaction in the live tobacco cell suspension culture, PEA-induced ROS production can last relatively longer than that in the in vitro HRP-C reaction, probably due to the presence of natural reducing agents that reduce the Compound II back to its native form, enabling the enzyme to re-enter the peroxidase cycle.

This work may provide clues to understand the complex mechanisms and multiple roles for peroxidase-catalyzed reactions including consumption and production of ROS in biological systems.

References
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