Hydrogen peroxide-independent generation of superoxide catalyzed by soybean peroxidase in response to ferrous ion

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It is well documented that extracellular alkalization occurs in plants under the challenges by pathogenic microbes. This may eventually induce the pH-dependent extracellular peroxidase-mediated oxidative burst at the site of microbial challenges. By employing the purified proteins of horseradish peroxidase as a model, we have recently proposed a likely role for free Fe^{2+} in reduction of ferric enzyme of plant peroxidases into ferrous intermediate and oxygen-bound form of enzyme known as Compound III which may eventually releases superoxide anion radical ($\text{O}_2^{\bullet-}$), especially under alkaline condition, possibly contributing to the plant defense mechanism. In the present study, we employed the purified protein of soybean peroxidase (SBP) as an additional model, and examined the changes in the redox status of enzyme accompanying the generation of $\text{O}_2^{\bullet-}$ in response to Fe^{2+} under alkaline condition.

Introduction

Plant defense mechanisms against pathogens, represented by perception of so-called microbe-associated molecular patterns (MAMPs) reportedly trigger a rapid and transient accumulation of reactive oxygen species (ROS).¹ In higher plants, 2 major mechanisms are known to be responsible for production of ROS, namely one involving NADPH oxidases and one involving peroxidases (POXs).² In *Arabidopsis thaliana*, involvement of 2 cell-wall POXs, namely PRX33 and PRX34 in MAMPs-induced ROS generation in apoplastic space was reported.^{1,3,4}

As in plant responses to MAMPs, the proposed roles for plant POXs cover the homeostasis of hydrogen peroxide (H_2O_2) level, oxidation of various organic substances, and generation of ROS as we have reviewed elsewhere.⁵⁻⁷ The overall actions catalyzed by plant POXs are highly complex and diversified as a group of POX specialists in Switzerland metaphorically described that plant POXs display more functions than a 'Swiss army knife'.⁸ However, we aimed drastic simplification and generalization of the overall reactions catalyzed by plant POXs and we are now proposing that the ROS-generating actions of plant POXs can be dissected into conventional peroxidase cycle requiring the supply of H_2O_2 (Fig. 1A) and oxygenase-like reaction cycle which is H_2O_2 -independent but requires molecular oxygen (O_2) as a key rate-limiting substrate (Fig. 1B). Since this H_2O_2 -independent reaction

cycle releases superoxide anion radical ($\text{O}_2^{\bullet-}$) which is highly reactive against organic molecules by donating or abstracting single electron (e^-) to/from the neighboring molecules, the modes of overall reactions may be described as dioxygenase-like.

ROS producing events, chiefly those producing $\text{O}_2^{\bullet-}$, catalyzed through the conventional H_2O_2 -dependent POX cycle are reportedly coupled to oxidation of aromatic monoamines (AMAs) such as phenylethylamine^{9,10} or key phenolics such as salicylic acid (SA).^{6,7} In the presence of H_2O_2 (the common e^- acceptor) and a variety of e^- -donating substrates, plant POXs may achieve a great deal of oxidation reactions essential for the functions of living cells,⁵ possibly *via* production and localization of certain POX isoforms at specific timing, so that the living plants can respond to and combat a wide variety of stressful challenges with biotic or abiotic nature.^{11,12}

In the absence of H_2O_2 -requiring reactions, plant POXs are still capable of production of ROS ($\text{O}_2^{\bullet-}$ and derived H_2O_2) through the oxygen-requiring cycle involving specific e^- donors such as indole-3-acetic acid (IAA), a natural auxin.¹³⁻¹⁵ The idea on the IAA-dependent reduction of native plant POX to ferrous enzyme intermediate has been proposed by Smith and his colleagues.¹⁶ Thus, our view on the role of IAA is that IAA is one of effective e^- donors converting native enzyme into ferrous intermediate in the oxygenation cycle (Fig. 1A, upper half). The series of reactions triggered by IAA further proceeds under the

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Figure 1. Hourglass model which summarizes the inter-conversions among active and inactive forms of POXs involved in ROS generation. This model emphasizes that 2 distinct cycles are initiated by conversion of native POX with e^- acceptor via conventional peroxidase cycle (A) or e^- donor via oxygenase cycle (B). Model was modified from previously published review articles.⁵⁻⁷

atmospheric condition rich in O_2 , where the ferrous complex might be short-lived and thus readily converted to the O_2 -bound intermediate known as Compound III. It is known that the state of heme iron in Compound III is the O_2 -heme- Fe^{II} or $O_2^{\bullet-}$ -heme- Fe^{III} , which is likely subjected to gradual decay of complex into $O_2^{\bullet-}$ and native enzyme (with heme- Fe^{III}).¹⁷ This process IAA-dependently yielding $O_2^{\bullet-}$ was confirmed in IAA-stimulated horseradish peroxidase (HRP) using *Cypridina* luciferin analog (CLA), a $O_2^{\bullet-}$ -specific chemiluminescence (CL) probe.¹⁵

Assuming that the hypothetical oxygenase cycle model is correct, we should be able to screen or identify some effective e^- donors from a variety of single e^- reducing agents which target the native enzyme to trigger the onset of oxygenation cycle in plant POXs via Fe^{III} -to- Fe^{II} conversion of heme, eventually leading to a robust and long-lasting burst of $O_2^{\bullet-}$ production. We have recently reported a likely mechanism involving free ferrous ion (Fe^{2+}) in reduction of ferric native enzyme of purified HRP into ferrous enzyme intermediate which readily produces $O_2^{\bullet-}$ via formation of Compound III, especially under alkaline condition, thus, possibly contributing to the plant defense mechanism associated with pH changes.¹⁸

Here, we report on the spectroscopic and CL evidences in support of the model for soybean POX (SBP)-catalyzed robust production of $O_2^{\bullet-}$ to be stimulated in the absence of conventional POX substrates but in the presence of free Fe^{2+} .

Materials and Methods

Chemicals

A $O_2^{\bullet-}$ -specific CL probe, *Cypridina* luciferin analog (CLA; 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one) was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Purified soybean peroxidase (SBP) was purchased from Sigma (St. Louis, MO., USA), and used without further purification. IAA, SA, metals, and other chemicals were pur-

Figure 2. H_2O_2 -dependent conversion of native SBP into intermediate forms of enzyme differed in redox-status. (A) Effect of low dose of H_2O_2 (5-fold higher concentration of H_2O_2 over that of enzyme; molar ratio 5:1). (B) Effect of relatively high dose of H_2O_2 (molar ratio 50:1). (C) Effect of excess dose H_2O_2 (molar ratio 100:1). From native ferric enzyme (absorption peaks at 403 and 635 nm), enzyme intermediates such as Compound II (absorption maxima at 420, 527, and 556 nm), Compound III (absorption maxima at 542 and 576 nm), and P-670 (increase in absorption at around 670 nm) were shown to be formed in the presence of H_2O_2 depending on the molar ratio of H_2O_2 over enzyme population.

chased from Wako Pure Chemical Co. (Osaka, Japan). IAA (100 mM) was first dissolved in ethanol and diluted to the desired concentrations with heated water (80°C). Then IAA solution was kept on ice in darkness until used. Final ethanol concentration in the reaction mixture was adjusted to be 0.1 % (v/v).

Spectroscopy

By analogy to the experimental procedure for HRP,¹⁹ spectroscopic determination of SBP concentration was performed based on the measurement of the concentration of heme ($\epsilon_{403\text{nm}} = 102 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). Changes in absorption spectra of SBP in 20 mM K-phosphate buffer (pH 7.0) were recorded on spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) at room temperature with a spectral bandwidth of 1.0 nm in a cuvette with 1-cm light path. Ferrous enzyme, Compounds II and III derived from native SBP (7.5 μM) were determined spectroscopically.

Detection of ROS

Generation of $\text{O}_2^{\bullet-}$ in the SBP reaction mixture were monitored with $\text{O}_2^{\bullet-}$ -specific CLA-CL using a luminometer (Luminiscensor PSN AB-2200-R, Atto Corp., Tokyo, Japan) and expressed as relative luminescence units (rlu) as previously described for HRP-catalyzed generation of ROS.¹⁵

Results and Discussion

Spectroscopic analyses

The preliminary experiments have shown that formation of Compound II from native ferric enzyme of SBP (redox status, Fe^{III}) in the conventional peroxidase cycle requires the presence of H_2O_2 (Fig. 2A) as we have previously observed that addition of excess H_2O_2 to HRP reaction mixture readily results in transient formation of Compound I (redox status, Fe^{V}) followed by rapid increase in Compound II (redox status, Fe^{IV}) without supplementation of any additional molecules known as e^- donating POX substrates such as phenolics or amines.^{17,20} As the molar ratio of H_2O_2 over SBP was elevated, major enzyme intermediate was shown to be Compound III (redox status, $\text{Fe}^{\text{III}}:\text{O}_2$) suggesting that overdose of H_2O_2 without addition of e^- donating POX substrates (required for degradation of H_2O_2), forcibly converted the native form of enzyme into O_2 -bound form of enzyme (Fig. 2B). In other models of heme proteins, such as HRP^{17,20,21} and human hemoglobin,²² similar observations were reported. Compound III was further converted to irreversibly inactivated form of heme proteins known as P-670, if incubated over 1 h with excess dose of H_2O_2 (molar ratio 100:1; Fig. 2C).

In the absence of H_2O_2 , supplementation of single e^- donors to SBP showed different modes of inter-conversion of the enzyme altering the redox status from ferric native form (Fe^{III}) into ferrous form of enzyme (Fe^{II}) (Fig. 3). Ferrous intermediates of various heme proteins show high affinity for molecular oxygen to form the oxygen-bound form of intermediate as represented by O_2 -dependent conversion of deoxy-hemoglobin to oxy-hemoglobin.²³ Reportedly, in the cases of IAA-HRP interaction^{15,21} and Fe^{2+} -HRP interaction,¹⁸ single e^- reduction of native enzyme

Figure 3. Reduction of native SBP into ferrous enzyme by addition of sodium dithionite (sodium hydrosulfite) or free ferrous ion (Fe^{2+}). Enzyme concentration, 15 μM . (A) Effect of 20 mM sodium dithionite. (B) Effect of 100 μM Fe^{2+} . (C) Removal of native enzyme's background spectrum by subtraction of the value in native enzyme (0 time control) from the values recorded for Fe^{2+} -treated sample.

under ambient condition results in formation of ferrous enzyme which is readily converted into Compound III which is eventually dissociated into native enzyme by releasing $\text{O}_2^{\bullet-}$. Sodium dithionite is often used for removal of dissolved O_2 while it is

also known as an effective reducing agent. We expected that SBP inter-conversion could be arrested at ferrous intermediate in the presence of sodium dithionite by allowing single e^- reduction of native ferric SBP while blocking the O_2 -dependent reaction. Sodium dithionite-induced enzyme intermediate showed absorption maximum at 560 nm (Fig. 3A) suggesting that ferrous enzyme was formed.²⁴ With lesser extent, Fe^{2+} -treated sample showed enzyme intermediate with absorption maximum at 560 nm while majority of enzyme were shown to be at native state (Fig. 3B and C). This suggests the progress of Fe^{2+} -dependent formation of ferrous enzyme were followed by further conversion steps back to native enzyme, possibly *via* O_2 -dependent formation of Compound III. If Compound III were formed, release of $O_2^{\bullet-}$ must be detected upon addition of Fe^{2+} to the SBP reaction mixture. As $O_2^{\bullet-}$ is formed, further stimulation of the conventional POX cycle in SBP could be induced as H_2O_2 is readily derived from $O_2^{\bullet-}$ in the presence of Fe ions. In support of above view, there was also a minor increase in absorption at 530 nm in Fe^{2+} -treated SBP, suggesting that minor portion of Compound II is also formed (Fig. 3C).

Iron-induced generation of ROS

As predicted from the spectroscopic data in support of Compound III formation, a robust production of $O_2^{\bullet-}$ was recorded after addition of Fe^{2+} to SBP (Fig. 4). Among three potential inducers of $O_2^{\bullet-}$ (IAA, SA, Fe^{2+}), only Fe^{2+} induced an intense and long-lasting peak of CLA-CL.

Figure 4. Effects of Fe^{2+} , SA and IAA on $O_2^{\bullet-}$ generation in SBP reaction mixture. Fe^{2+} , SA or IAA or water was added to SBP reaction mixture. (A) Temporal changes in $O_2^{\bullet-}$ -dependent CLA-CL. Arrow indicates the timing of chemical addition. (Inset) Long-lasting nature of Fe^{2+} -induced $O_2^{\bullet-}$ production. (B) Integral yields of CLA-CL induced by Fe^{2+} , SA and IAA, which are summed up within 180 s after treatments. Bars, SD ($n = 3$). Conditions: total volume, 0.2 ml; K-phosphate, 25 mM (pH 7.0); SBP, 1.5 μ M; CLA, 10 μ M; reducing agents (Fe^{2+} , IAA, SA), 100 μ M.

Figure 5. Effect of pH on Fe^{2+} -induced $O_2^{\bullet-}$ generation in SBP reaction mixture. (A) Typical traces of Fe^{2+} -induced CLA-CL recorded at various pH. (B) pH-dependent change in the peak height of CLA-CL. (C) pH-dependent change in the integral yield of CLA-CL (within 180 s after addition of Fe^{2+}). Error bars, S. D. ($n = 3$). Conditions: total volume, 0.2 ml; K-phosphate, 25 mM (at indicated pH); Fe^{2+} , 100 μ M; SBP, 1.5 μ M; CLA, 4 μ M.

Effect of pH on SBP-catalyzed oxidative burst

Effects of pH on the Fe^{2+} -induced $O_2^{\bullet-}$ generation was assessed by altering the medium pH between pH 4.5 and 9.0. In

the alkaline range (pH >7.0), the level (peak height) of CLA-CL was shown to be drastically elevated (Fig. 5A and B). However, integral yield of CLA-CL was slightly lowered at highest pH examined as the pattern of $O_2^{\bullet-}$ generation likely becomes spiky and less sustainable as pH was elevated (Fig. 5C). These observations are almost identical to the results obtained with HRP model.¹⁸

Model reactions in SBP

Nowadays, apart from plant biological research purpose, HRP is widely used as biological sensing materials in various areas such as medical diagnosis, biosensors, and nanotechnologies. In addition to HRP, SBP is also widely used as model enzyme applicable to various purposes in various areas. The data shown above suggested that SBP is capable of H_2O_2 -independent catalysis of the production of $O_2^{\bullet-}$ using molecular oxygen in the presence of ferrous ion. This model is distinct from the well-recognized mode of ROS production catalyzed by plant POXs. Based on the results newly obtained with HRP¹⁸ and SBP (present study), we wish to describe the likely mechanisms.

Our previous study showed that SBP can catalyze the generation of $O_2^{\bullet-}$ in the presence of 2 typical model substrates SA and IAA used as the testers for examining the involvement of conventional POX cycle (native Compound I Compound II native) and oxygenase cycle (native ferrous form Compound III native), respectively.²⁵ The series of ferrous-dependently stimulated reactions can be divided into several steps, namely, conversion of native enzyme into ferrous intermediate, O_2 -dependent formation of Compound III, and production of $O_2^{\bullet-}$ upon decay of Compound III into native enzyme. These steps can be summarized as follows.

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Concomitantly, supply of H_2O_2 possibly derived from $O_2^{\bullet-}$ may occur and thus conventional POX cycle is partially initiated.

Effects of several candidate molecules which allow reduction of native POXs into ferrous enzymes have been testified to date.^{18,25} Such molecules include ascorbate, thiols, nitric oxide, and IAA, thus oxygenation of the ferrous enzyme and production of compound III are likely allowed in the presence of these molecules. However, ascorbate, thiols, and nitric oxide actively remove $O_2^{\bullet-}$ released during decomposition of Compound III into native enzyme, thus none or negligible level of $O_2^{\bullet-}$ could be recorded.²⁵

IAA is an effective inducer of H_2O_2 -independent $O_2^{\bullet-}$ generation in POX system,^{15,26} but at the same time it is also consumed by conventional POX cycle initiated by IAA-dependently produced $O_2^{\bullet-}$ (and derived H_2O_2).^{13,14,16,19} Therefore, long-lasting action could not be observed. Obviously, Fe^{2+} is neither consumed by conventional POX cycle nor involved in removal of $O_2^{\bullet-}$. Therefore, Fe^{2+} is an outstanding agent stimulating the POX-catalyzed H_2O_2 -independent $O_2^{\bullet-}$ generation.

Furthermore, it is well documented that extracellular alkalization occurs in plants under the challenges by pathogenic microbes or pathogen-derived elicitors, which eventually induces the pH-dependent extracellular POX-mediated oxidative burst at the site of microbial challenges.^{27,28} We have recently proposed a likely role for free Fe^{2+} in reduction of ferric HRP into ferrous intermediate and Compound III which may further produces $O_2^{\bullet-}$, especially under alkaline condition possibly contributing to the plant defense mechanism.¹⁸ The present work confirmed the similar behavior of SBP in response to Fe^{2+} under alkaline condition.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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