



Hydrogen peroxide-independent generation of superoxide by plant peroxidase: hypotheses and supportive data employing ferrous ion as a model stimulus

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When plants are threaten by microbial attacks or treated with elicitors, alkalization of extracellular space is often induced and thus pH-dependent extracellular peroxidase-mediated oxidative burst reportedly takes place, especially at the site of microbial challenge. However, direct stimulus involved in activation of peroxidase-catalyzed oxidative burst has not been identified to date. Here, we would like to propose a likely role for free ferrous ion in reduction of ferric native peroxidase into ferrous enzyme intermediate which readily produces superoxide anion *via* mechanism involving Compound III, especially under alkaline condition, thus, possibly contributing to the plant defense mechanism. Through spectroscopic and chemiluminescence (CL) analyses of reactions catalyzed by horseradish peroxidase (HRP), the present study proposed that plant peroxidase-catalyzed production of superoxide anion can be stimulated in the absence of conventional peroxidase substrates but in the presence of free ferrous ion.

Keywords: alkalization, auxin, Compound III, oxidative burst, peroxidase, superoxide

INTRODUCTION

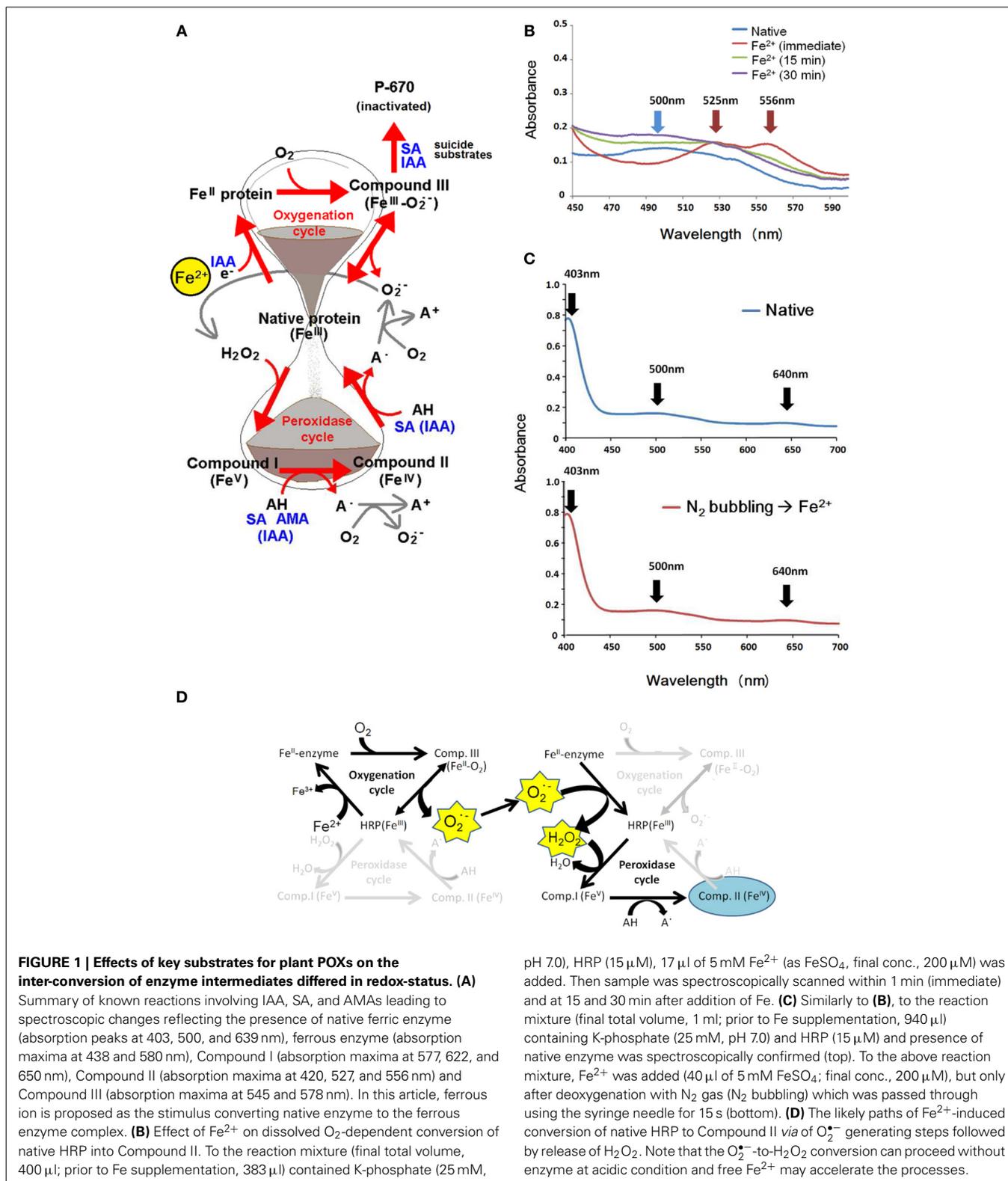
In plants, two major mechanisms leading to the production of reactive oxygen species (ROS) involving either NADPH oxidases or peroxidases (POXs) have been proposed (Yoshioka et al., 2008). Events of plant defense against pathogenic microorganisms, represented by plant cellular perception of microbial molecules contributing to so-called microbe-associated molecular patterns (MAMPs) such as bacterial flagellin, referred to as pattern-triggered immunity, reportedly trigger a rapid and transient accumulation of ROS (O'Brien et al., 2012a). In *Arabidopsis*, molecular evidence for involvement of two identified cell wall POXs, namely, PRX33 and PRX34, in MAMPs-responsive apoplastic ROS generation has been reported (Bindschedler et al., 2006; O'Brien et al., 2012a,b).

In fact, plants are rich sources of enzymes involved in production and removal of ROS (Yoshioka et al., 2008). A Swiss group of POX research specialists metaphorically described that plant enzymes belonging to POXs (EC 1.11.1.7) display more functions than a “Swiss army knife” (Passardi et al., 2005). As suggested, highly diversified roles of plant POXs including regulation of hydrogen peroxide (H_2O_2) level, oxidation of various substrates, generation of ROS coupled to oxidation of aromatic monoamines (AMAs) such as phenylethylamine (Kawano et al., 2000a,b) and phenolics such as salicylic acid (SA) (Kawano et al., 2004; Kawano and Bouteau, 2013) in living plants have been documented to date. By using a variety of electron (e^-)-donating substrates and H_2O_2 , the common e^- acceptor, plant POXs achieve a great deal of oxidation reactions essential for the functions of living cells (Kawano, 2003a). Through production of certain POX isoforms at specific timing and localization, thus by properly and precisely

making use of a variety of plant POX functions, the growing plants can respond to and combat a wide variety of stressful challenges with biotic or abiotic nature (Penel, 2000; Hiraga et al., 2001).

As intensively discussed in the plant research community, oxidation of phenolics is one of the key functions of POXs (Passardi et al., 2005). It is widely accepted that, in the presence of H_2O_2 , plant POXs can catalyze the generation of superoxide anion radical ($O_2^{\bullet-}$) upon oxidation of substrates, chiefly phenolics (Kawano, 2003a; Yoshioka et al., 2008). Lower half of **Figure 1A** summarizes the mode of $O_2^{\bullet-}$ production *via* e^- acceptor-dependently initiated conventional POX cycle enabling various substrates (AH) such as SA and AMAs. Apart from such H_2O_2 -requiring reaction, plant POXs are also capable of $O_2^{\bullet-}$ generation *via* e^- donor-dependently initiated and oxygen-requiring cycle involving few known substrates such as indole-3-acetic acid (IAA), the principal form of natural auxin in higher plants (Gazarian and Lagrimini, 1998; Savitsky et al., 1999; Kawano et al., 2001). Therefore, the $O_2^{\bullet-}$ -generating reactions catalyzed by plant POXs can be dissected into (i) the H_2O_2 -dependent POX cycle and (ii) H_2O_2 -independent oxygenation cycle as illustrated in **Figure 1A**. This model dissecting two distinct cycles initiated by interaction of native POX with e^- acceptor or e^- donor, is sometimes referred to as the hourglass model due to its shape (Kawano, 2003a; Takayama et al., 2012; Kawano and Bouteau, 2013).

We view here that the role of IAA in POX-catalyzed generation of $O_2^{\bullet-}$ is one of effective e^- donors converting native enzyme into ferrous intermediate in the oxygenation cycle (**Figure 1A**, upper half). Idea on the IAA-dependent reduction of native plant POX



to ferrous enzyme intermediate has been proposed by Smith et al. (1982). The series of reactions triggered by IAA further proceeds under the atmospheric condition rich in O_2 , therefore, the ferrous complex might be short-lived and readily converted to O_2 -bound

form of enzyme intermediate known as Compound III (CIII) in which the state of heme iron can be described as O_2 -heme- Fe^{II} or $\text{O}_2^{\bullet-}$ -heme- Fe^{III} (Kawano et al., 2002a). Then, gradual decay of this complex into native enzyme at heme- Fe^{III} state accompanies

the release of $O_2^{\bullet-}$ (Figure 1A) as confirmed with IAA-stimulated horseradish peroxidase (HRP) using $O_2^{\bullet-}$ -specific chemiluminescence (CL) probe, *Cypridina* luciferin analog (CLA) (Kawano et al., 2001).

Assuming that the hypothetical model mechanism proposed in Figure 1A 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, eventually leading to a robust and long-lasting burst of $O_2^{\bullet-}$ production. After testing a wide range of chemicals, we observed that free ferrous ion (Fe^{2+}) acts as a novel inducer of $O_2^{\bullet-}$ production in aid of plant POX, possibly by behaving as an effective e^- donor for Fe^{III} -to- Fe^{II} conversion of heme in a model POX, HRP. The aim of the present article is to share our novel finding on the Fe-driven $O_2^{\bullet-}$ production mechanism involving HRP.

MATERIALS AND METHODS

CHEMICALS

Purified HRP was purchased from Sigma (St. Louis, MO., USA), and used without further purification. CLA (2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one), a chemiluminescent probe for $O_2^{\bullet-}$, was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Luminol, SA, IAA, metals, and other chemicals except for enzyme were purchased 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

Concentration of HRP was determined spectroscopically by measuring the concentration of heme ($\epsilon_{403\text{ nm}} = 102\text{ mM}^{-1}\cdot\text{cm}^{-1}$) (Gazaryan et al., 1996). Changes in absorption spectra of HRP 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. Compounds II (CII) and CIII derived from native HRP (7.5 μM) were determined spectroscopically.

CHEMILUMINESCENCE (CL) ANALYSIS

Generation of H_2O_2 and $O_2^{\bullet-}$ in the HRP reaction mixture were monitored with H_2O_2 -specific CL of luminol and $O_2^{\bullet-}$ -specific CL of CLA using a luminometer (Luminescensor PSN AB-2200-R, Atto Corp., Tokyo, Japan) and expressed as relative luminescence units (rlu) as previously described for HRP-catalyzed generation of ROS (Kawano et al., 2001).

RESULTS

PRELIMINARY SPECTROSCOPIC ANALYSES

Basically, formation of CII from native ferric POX in the conventional peroxidase cycle requires the presence of H_2O_2 , as we have previously observed that addition of excess H_2O_2 to HRP reaction mixture readily results in transient formation of Compound I (CI) followed by increase in CII without supplementation of any additional molecules known as POX substrate such as phenolics

or amines (Kawano et al., 2002c,d). However, we observed that addition of Fe^{2+} to HRP resulted in accumulation of CII without addition of exogenous H_2O_2 (Figure 1B), suggesting that H_2O_2 is formed after a series of reactions involving redox changes in POX/ Fe^{2+} system. Interestingly, conversion of native HRP to CII by Fe^{2+} was completely inhibited by deoxygenating treatment such as bubbling with N_2 gas (Figure 1C) and addition of sodium dithionite (data not shown), indicating the involvement of molecular oxygen at least at a certain step in the course of native-to-CI conversion.

IRON-INDUCED GENERATION OF ROS

As expected from the behavior of Fe^{2+} converting the native enzyme to CII, release of H_2O_2 could be detected in Fe^{2+} -added HRP reaction mixture (Figure 2). Therefore, it is tempting to conclude that Fe^{2+} -dependently produced H_2O_2 plays a key role in conversion of native enzyme to CII *via* transient formation of CI.

Similarly to the profile of H_2O_2 , robust production of $O_2^{\bullet-}$ was also observed after addition of Fe^{2+} to HRP (Figure 3). Note that the addition of Fe^{2+} resulted in biphasic increase in CLA-CL, consisting of an immediate short-lasting spike followed by secondary but intense and long-lasting peak depending on the concentration of Fe^{2+} added. As far as we understand, the initial spikes sized similar to the water control are kinds of artifacts

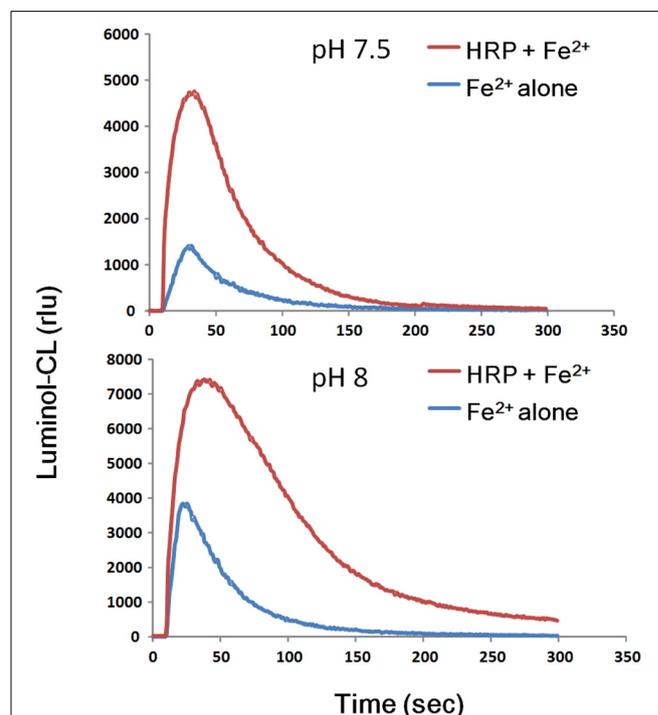
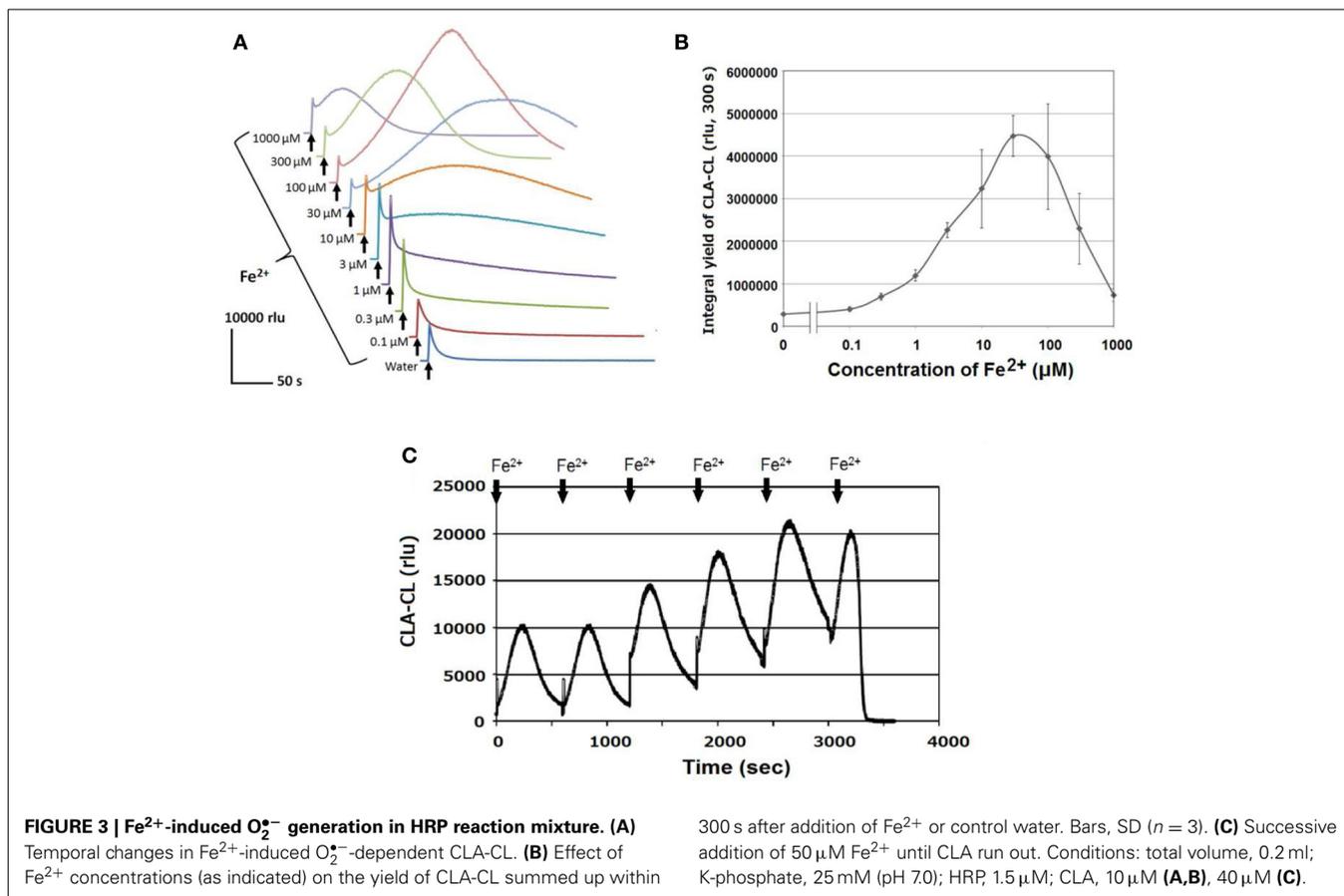


FIGURE 2 | Transient production of H_2O_2 following addition of Fe^{2+} to HRP reaction mixture. Reaction mixture (0.2 ml) contained 25 mM K-phosphate (pH 7.5 or 8.0), 1.5 μM HRP, 10 μM luminol, and 50 μM $FeSO_4$. For comparison, 50 μM $FeSO_4$ was added to the mixture lacking HRP (Fe^{2+} alone). The resultant H_2O_2 might be used for further reactions converting native enzyme to unstable Compound I and eventually to Compound II.



by rapidly injecting the reagent or control water through a syringe which rapidly causes the mixing of media and air (containing oxygen). This type of spikes can be commonly observed for CLA-CL monitoring as described in our previous studies (Monetti et al., 2014). In fact, the increase higher than the level of water control could have been attributed to the action of Fe. Therefore, there would be two modes of oxidative burst induced by ferrous ion, one is rapidly induced by lower range of Fe²⁺ concentrations observed as the short-lasting increase in initial spike of CLA-CL, and another follows the initial spike, gradually attaining much higher peak level of CLA-CL by responding to relatively higher range of Fe²⁺ concentrations (Figure 3A).

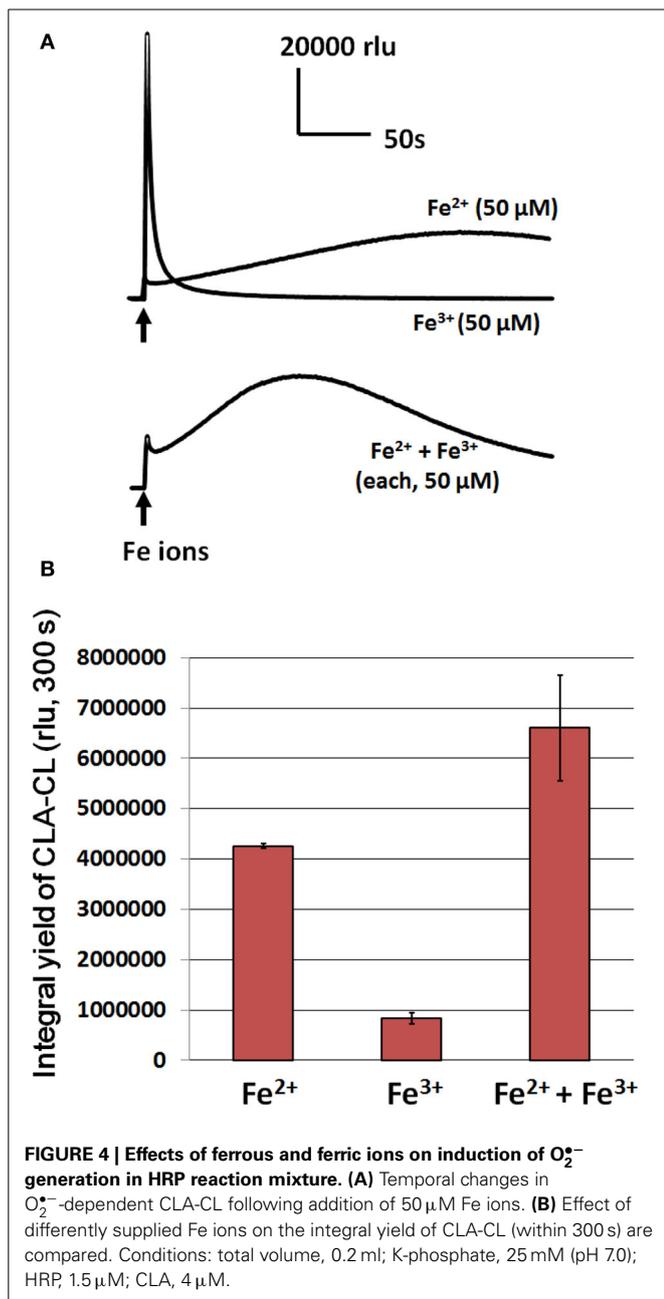
Here, we emphasized the secondary peaks which last for more than some minutes. Interestingly, depending on the concentrations of Fe²⁺ added to HRP, both the extent and duration of oxidative burst largely varied. Furthermore, we observed that successive additions of Fe²⁺ (6 times an hour) repeatedly caused the burst of O₂^{•-} by HRP until the CL probe was completely consumed (Figure 3C), suggesting that the enzyme has capacity for continuous and robust oxidative burst if reducing agents are continuously supplied.

As shown in Figure 4, both ferric and ferrous ions induce the generation of O₂^{•-}. However, the temporal profiles of ferric and ferrous-induced O₂^{•-} generation largely differed, suggesting that the modes of O₂^{•-} generation may also differ (at present, such difference is unknown). The Fe²⁺-induced O₂^{•-} generation last for

5–10 min while the Fe³⁺ induces a short-lived spike of O₂^{•-} generation only. By comparing the yield of CLA-CL (within 300 s), the extent of Fe²⁺-induced O₂^{•-} generation is at 5-fold greater level compared to the Fe³⁺-induced one (Figure 4B). Addition of Fe²⁺/Fe³⁺ mixture induced a compromised pattern of CLA-CL (Figure 4A), with much higher yield of CLA-CL (Figure 4B). These data are indicative of the potential impact of free iron ions both at ferric and ferrous state may stimulate the oxidative burst mediated by plant POX although we focus mostly on the action of ferrous ion in the present study.

INVOLVEMENT OF O₂ IN Fe²⁺-INDUCED HRP REACTION LEADING TO O₂^{•-} GENERATION

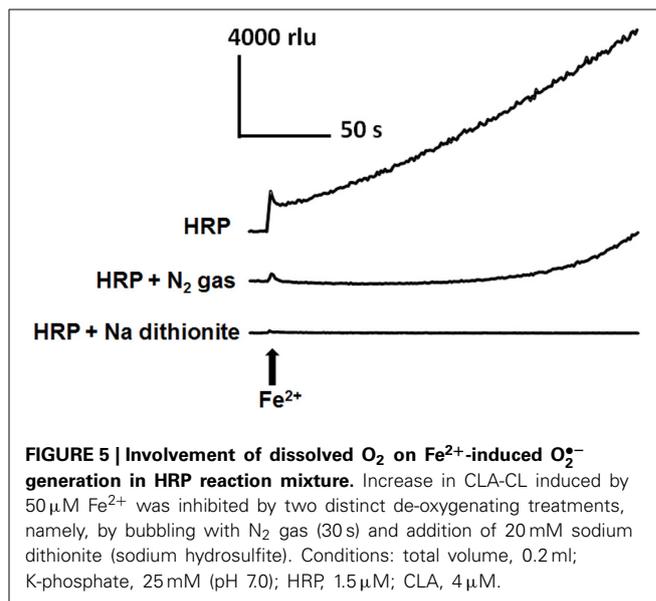
As the involvement of dissolved oxygen was suggested by preliminary spectroscopic monitoring of N₂-sensitive conversion of Fe²⁺-treated native enzyme into CII, we examined the requirement for O₂ in Fe²⁺-induced HRP reaction leading to O₂^{•-} generation (Figure 5). The Fe²⁺-induced increase in CLA-CL was effectively lowered or delayed by removal of dissolved oxygen by N₂ bubbling and inhibited by addition of sodium dithionite which is a convenient reagent that rapidly removes dissolved oxygen. As the air diffuses back into the reaction mixture even after replacement with N₂ gas by bubbling, the state of inhibition due to the lack of O₂ did not last long. On the other hand, chemically performed deoxygenation by Na dithionite resulted in clear-cut inhibition.



Above data are in support of our working hypothesis that, similarly to IAA-responsive mechanism, Fe²⁺-mediated conversion of native POX into ferrous enzyme intermediate further reacts with O₂ to form catalytically inactive CIII which readily dissociates and releases O₂^{•-} and native enzyme (Figure 1D).

EFFECT OF pH ON HRP-CATALYZED OXIDATIVE BURST

Effects of Fe²⁺ (50 μM) on induction of O₂^{•-} generation was compared with two known inducers of POX-mediated oxidative burst, namely, SA and IAA (each 50 μM) under neutral and alkaline pH (pH 7.0 and 8.0; Figure 6). Among three O₂^{•-} inducers, IAA was shown to be most active in induction of the spike of O₂^{•-} while Fe²⁺ was the only chemical active in stimulation of a



gradual and long-lasting mode of O₂^{•-} production. As reported (Kawano and Muto, 2000; Takayama et al., 2012), SA-induced production of O₂^{•-} was not impressive in the absence of initial H₂O₂ supplementation.

Despite of difference in the temporal profiles of induced O₂^{•-} generation, the cumulative yields of O₂^{•-} in response to Fe²⁺, SA, and IAA under neutral condition (pH 7.0) were at similar level (Figure 6C). It is noteworthy that the burst of Fe²⁺-stimulated O₂^{•-} generation drastically increased under alkaline condition (pH 8.0) while SA and IAA showed no significant pH response (Figures 6B,C). Effect of pH on the Fe²⁺-induced O₂^{•-} generation in HRP reaction mixture was further assessed by altering the medium pH between pH 4.26 and 8.95 (Figure 7). In the alkaline range (pH >7.0), the height of CLA-CL was shown to be drastically elevated. However, integral yield of CLA-CL was slightly lowered at highest pH examined as the pattern of O₂^{•-} generation likely becomes spiky and less sustainable as pH elevated.

Above data suggested that HRP H₂O₂-independently catalyzes the production of O₂^{•-} from dissolve oxygen in the presence of ferrous ion. This model is distinct from the previously known mode of ROS production catalyzed by plant POXs. Therefore, in the below section, we wish to review and compare the likely mechanisms.

DISCUSSION

Interestingly, nitric oxygen (NO) is one of known agents that bind and convert the ferric form of hemoproteins such as non-symbiotic hemoglobin from *Arabidopsis* (Perazzolli et al., 2004) into ferrous hemoproteins. In cases of plant POXs such as of soybean, the consequence of exposure of native POX to gaseous NO was accumulation of CII without exogenous supplementation of H₂O₂, suggesting that protein is once converted to ferrous form and eventually converted to and arrested as CII (Takayama et al., 2012). Among the known intermediates of POX and hemoproteins, the intermediate with ferrous heme is the only form with affinity to molecular oxygen; therefore, we should consider the

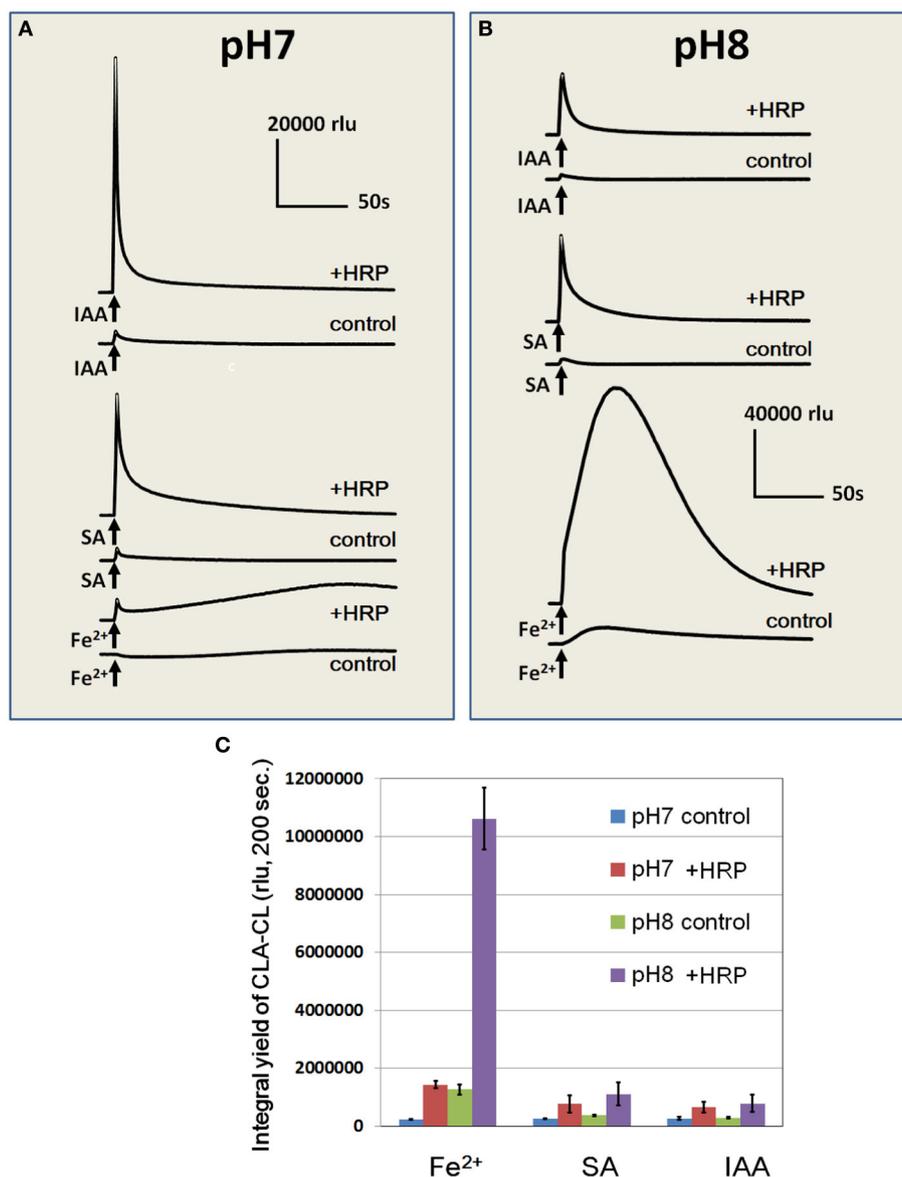


FIGURE 6 | Effects of Fe²⁺, SA, and IAA on O₂^{•-} generation in HRP reaction mixture at pH 7.0 and 8.0. Fe²⁺, SA, or IAA (each 50 μM) was added to control buffer or HRP reaction mixture at pH 7.0 (A) and 8.0 (B).

(C) Integral yields of CLA-CL induced by Fe²⁺, SA, and IAA under different pH were compared. Error bars, SD (n = 3). Conditions: total volume, 0.2 ml; K-phosphate, 25 mM (pH 7.0 or 8.0); HRP, 1.5 μM; CLA, 4 μM.

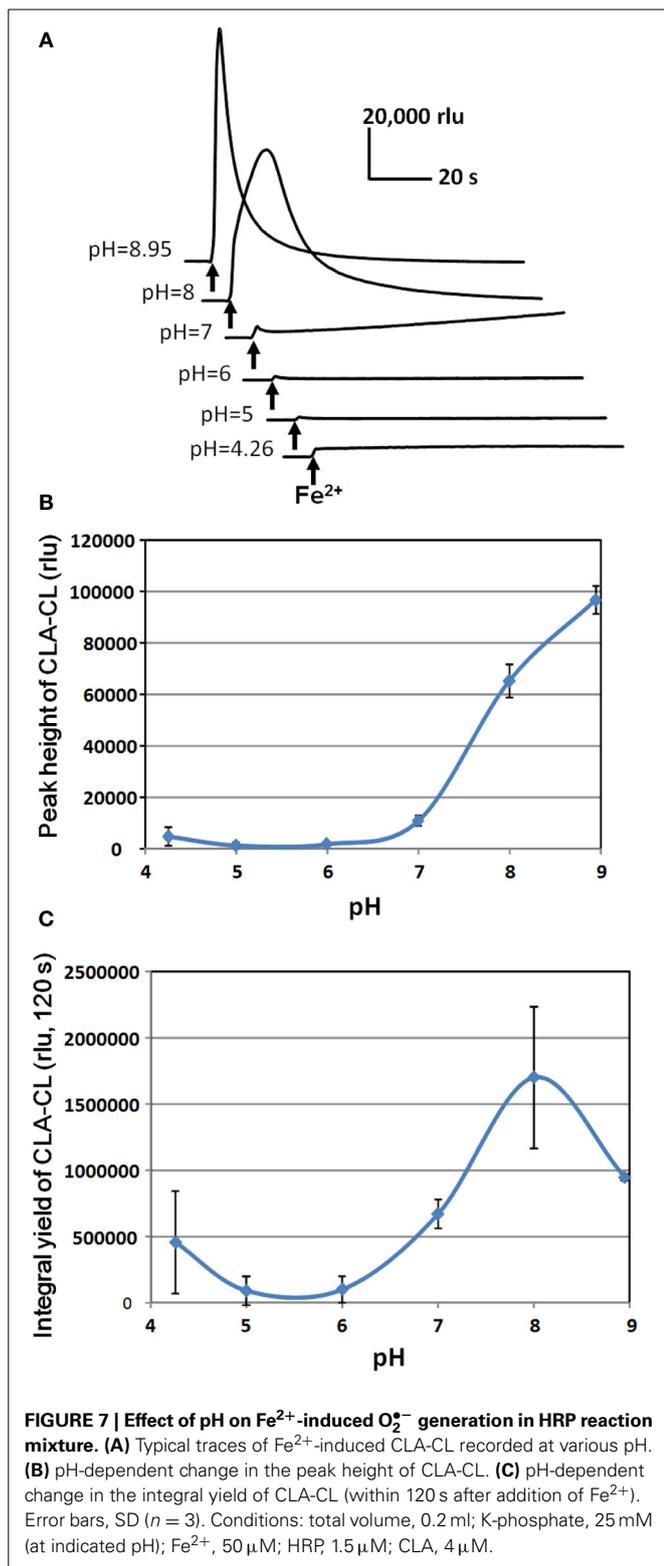
series of steps converting the native POX into ferrous enzyme, and involvement of dissolved oxygen, in order to obtain the CII.

By analogy to NO-responsive events, we attempted to propose a working hypothesis explaining the paths of reactions Fe²⁺-dependently converting the native POX into CII in the absence of exogenous supplementation of H₂O₂ (Figure 1D). Firstly, the Fe²⁺-mediated conversion of native POX into ferrous enzyme intermediate occurs. Then, this intermediate molecule further reacts with O₂ to form catalytically inactive CIII which readily dissociates and releases O₂^{•-} and native enzyme. Eventually, CII can be formed through interaction between the native enzyme and H₂O₂ which is derived from O₂^{•-}. Note that the O₂^{•-}-to-H₂O₂

conversion can proceed in the presence of hemoproteins and non-heme free iron ions as discussed later.

Our working hypothetical model can be divided into two phases. In the first phase, conversion of native enzyme into ferrous intermediate must be caused so that finally resulting in production of O₂^{•-} upon interaction with molecular oxygen (oxygenation cycle). After completing this cycle, the enzyme must go into further cycles if excess of Fe²⁺ is present. In the second phase, supply of H₂O₂ possibly derived from O₂^{•-} must occur in order to fuel the conventional POX cycle.

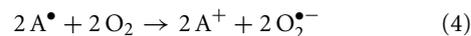
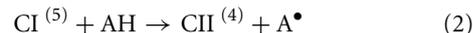
Therefore, we have carried out (a) spectroscopic analysis of the fate of native HRP following the addition of Fe²⁺,



(b) direct measurements of Fe²⁺-induced oxidative burst represented by generation of H₂O₂ and O₂^{•-} and (c) examinations on the involvement of molecular oxygen in (a) and (b).

POX-CATALYZED ROS PRODUCTION INVOLVING HYDROGEN PEROXIDE

The formulae shown below originally proposed for describing the mechanism for SA-dependent generation of O₂^{•-} in plant system (Kawano et al., 1998; Kawano and Muto, 2000) suggest that the byproducts of POX-catalyzed oxidation of phenolics are necessarily involved in generation of O₂^{•-}.



POX N stands for native ferric enzyme. A[•] and A⁺ are free radical species and the two-electron oxidized intermediate product derived from substrate AH (such as phenolics or AMAs), respectively. The formal oxidation states of the heme within the enzyme are indicated by numbers in the small brackets. As above, phenolics form a group of e⁻ donating substrates while H₂O₂ is viewed as the only e⁻ acceptor. Then, phenoxy radical (shown as A[•]) released thereafter may react with molecular oxygen to form O₂^{•-}. Since O₂^{•-} is readily transformed into H₂O₂ in biological systems, a single cycle of AH-oxidizing POX reactions initiated by single unit of H₂O₂ results in yield of two units of O₂^{•-} which is equivalent to two units of H₂O₂, and therefore, by this way, ROS could be amplified (Kawano, 2003a).

In place of phenolics, AMAs could be used as another group of active substrates (Kawano et al., 2000a,b). Pinontoan and his colleagues have shown that aromatic monoamine-dependent oxidative burst can be widely observed not only in plants but also in yeast cells *in vivo* (Pinontoan et al., 2002) and pseudo-POX cycle of human hemoglobin (Kawano et al., 2002b).

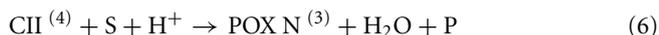
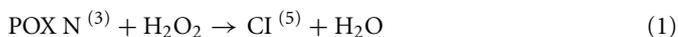
In case of SA oxidation by plant enzymes, analytical data in support of the production of SA radical species (one of A[•]) has been obtained using electron spin resonance spectroscopy by employing a natural spin trapper, ascorbate (Kawano and Muto, 2000). After above studies, the likely structures of the radical and derived cationic intermediate were proposed by Gozzo (2003). In addition, the involvement of CI and CII as the intermediate species required for SA-dependent and AMA-dependent O₂^{•-} generation was spectroscopically confirmed (Kawano et al., 2002c,d).

In plants, the SA-dependently produced O₂^{•-} acts as a chemical signal required for development of defense mechanism against pathogenic microbes (Kawano et al., 1998) and closure of stomata on leaves (Mori et al., 2001; Khokon et al., 2011). In model plant cells, TPC1 calcium-permeable cation channel is a likely target of the SA signal transduction pathway mediated with O₂^{•-} (Lin et al., 2005).

H₂O₂-INDEPENDENT ROS PRODUCTION CATALYZED BY IAA-STIMULATED POXS

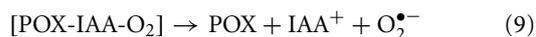
Metabolism of IAA is common interest to many plant biologists. Through oxidation of IAA *via* two different mechanisms, it has been considered that plant POXs are involved in the metabolism of IAA. One mechanism involves the conventional

H₂O₂-dependent pathway and the other requires the incorporation of molecular oxygen (O₂) but not of H₂O₂ (Gazaryan et al., 1996; Savitsky et al., 1999; Kawano et al., 2001). The conventional POX cycle for the oxidation of various substrates coupled to the consumption of H₂O₂ proceeds as follows:



where S and P are the substrate and product of its one-electron oxidation, respectively (Kawano, 2003a).

IAA can be oxidized by a wide variety of plant POXs, as model has been proposed through the study using HRP focusing on the conventional H₂O₂-dependent reactions with no strict substrate specificity (Kawano, 2003a). It is noteworthy that most plant POXs including HRP oxidize IAA also *via* an alternative H₂O₂-independent pathway requiring O₂ (Smith et al., 1982). Reportedly, unlike animal and microbial POXs, most members of plant POXs are considered to behave as highly specific IAA oxygenases by sharing the domains required for binding of auxin (Gazaryan et al., 1996). The proposed H₂O₂-independent cycle for IAA oxidation involves the formation of a ternary complex, enzyme-IAA-dioxygen (Savitsky et al., 1999), finally yielding IAA cation radicals and O₂^{•-} as by-products as follows (Kawano et al., 2001):



where IAA^{•+} stands for IAA cation radicals. As above, plant POXs can catalyze the IAA-dependent generation of O₂^{•-} in the absence of H₂O₂. However, depending on the concentrations of ROS and IAA, plant enzymes are readily inactivated and degraded by forming P-670 pigment which is an irreversibly inactivated form (Kawano et al., 2002a).

Based on the views that formation of enzyme-substrate complexes such as [POX-IAA-O₂] results in release of O₂^{•-} (Kawano et al., 2001), medical application of HRP-labeled antibodies and IAA has been proposed as a novel O₂^{•-}-generating system for cancer cell-targeted and controlled cell death induction, by designing the HRP-conjugated immuno-labeling of cancer-related molecules or expression of recombinant HRP in mammalian cells (Folkes and Wardman, 2001; Folkes et al., 2002; Kawano, 2003b; Dai et al., 2012). Although the IAA-induced O₂^{•-} in HRP reaction mixture is very intense, the IAA-induced oxidative burst likely lasts only for few seconds (Kawano et al., 2001). This is largely due to the fact that IAA behaves as a suicide substrate for plant POXs, in its excess, irreversibly converting the oxygen-dependently formed CIII into inactivated verdohemoprotein (P-670) (Kawano et al., 2002a). In this point of view, induction of robust and long-lasting oxidative burst by application of Fe²⁺ may expand the possible applications of plant POXs for medical purposes.

POSSIBLE ROLES FOR FERROUS POX INTERMEDIATE AND CIII

As reviewed elsewhere, the conventional POX cycle involves the formation of CI in which the localization of second radical could be on the heme or on amino acid residues around the heme pocket depending on the nature of protein species (Kawano, 2003a,b). This variation may largely contribute to determination of the types of reaction catalyzed by hemoproteins. On the other hand, we have previously proposed the hypothetical reactions in the oxygenation cycle of plant POXs (Figure 1A) which can be solely attributed to the chemistry of heme, by analogy to the behaviors of other hemoproteins such as hemoglobin (Kawano et al., 2004).

CIII is the temporally inactive POX intermediate (heme-Fe^{II}-O₂) which is analogous to oxygen-bound hemoglobin which is readily auto-oxidized and decomposed into O₂^{•-} and met-hemoglobin (Arayash, 1999; Kawano et al., 2002b). Note that met-hemoglobin is a ferric protein analogous to the native ferric POX and interestingly, pseudo-peroxidase catalytic activity can be found in met-hemoglobin (Kawano et al., 2002b).

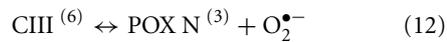
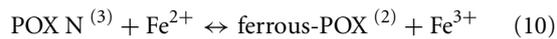
When the heme-oxygen complex in CIII of plant POX dissociates, O₂^{•-} and the native enzyme are released. Many of heme proteins, such as POX from French bean, are capable of generating H₂O₂ (derived from O₂^{•-}) at higher pH by a mechanism that involves the formation of CIII (Bolwell et al., 2002), thus distinct from the conventional POX reaction-mediated oxidative burst as such involving SA (Kawano et al., 1998).

The most likely mechanism considered to form CIII is the direct conversion of ferric proteins into CIII *via* single step in the presence of O₂^{•-}: heme-Fe^{III} + O₂^{•-} → heme-Fe^{II}-O₂ or heme-Fe^{III}-O₂^{•-} (Arayash, 1999). However, this model fails to explain the burst of O₂^{•-} often observed in plant POX model (Bolwell et al., 1998, 2002).

The missing link in the oxygenation cycle of ROS production involving CIII is the mechanism for reduction of native ferric POX (heme-Fe^{III}) to ferrous enzyme (heme-Fe^{II}). It is tempting to hypothesize that pH-dependent CIII-mediated ROS production requires the formation of CIII *via* preceding formation of ferrous enzyme, thereafter allowing spontaneous oxygenation into CIII (heme-Fe^{II} + O₂ → heme-Fe^{II}-O₂) as predicted earlier (Kawano et al., 2004). Therefore, apparently, the formation of ferrous enzyme from native POX would be an important step for supporting the oxidative burst involving CIII.

It is well documented that extracellular alkalization occurs in plant tissues upon attacked by pathogens or treatments with elicitors, eventually allowing the pH-dependent extracellular POX-mediated oxidative burst at the site of microbial challenges (Bolwell et al., 1998). Here, we would like to propose a likely role for free ferrous ion in reduction of ferric POX into ferrous enzyme which readily produces O₂^{•-} *via* mechanism involving CIII, especially under alkaline condition possibly contributing to the plant defense mechanism.

In conclusion, the present study proposed that plant POX-catalyzed production of O₂^{•-} can be stimulated in the absence of conventional POX substrates but in the presence free Fe²⁺ through following reactions (Figure 1D):



We view here that the recorded H_2O_2 could be derived from $\text{O}_2^{\bullet-}$. Recent study provided us a notation that iron peroxide species have been identified as important intermediates in a number of nonheme iron as well as heme-containing enzymes (Namuswe et al., 2008). Therefore, by analogy, we could possibly expect that ferrous intermediate of plant POXs also interact with $\text{O}_2^{\bullet-}$ to yield H_2O_2 in a manner similar to bacterial superoxide reductase. Or, excess of $\text{Fe}^{2+}/\text{Fe}^{3+}$ unbound to enzymes also non-enzymatically catalyses the disproportionation of $\text{O}_2^{\bullet-}$ into H_2O_2 .

Eventually, resultant H_2O_2 may contribute to conversion of native POX to CII via CI as previously demonstrated (Kawano et al., 2002c,d) (Figure 1D). Conversion of native POX to CII in the absence of initial supply of H_2O_2 reportedly occurs by treating soybean POX with NO (Takayama et al., 2012). Since NO converts some heme enzymes such as catalase into CIII (unpublished results), the mechanisms undermined should be similar to the case of Fe^{2+} -induced redox changes in HRP.

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