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## Scavenging of H<sub>2</sub>O<sub>2</sub> and Production of Oxygen by Horseradish Peroxidase<sup>1</sup>

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Peroxidases catalyze many reactions, the most common being the utilization of H<sub>2</sub>O<sub>2</sub> to oxidize numerous substrates (peroxidative mode). Peroxidases have also been proposed to produce H<sub>2</sub>O<sub>2</sub> via utilization of NAD(P)H, thus providing oxidant either for the first step of lignification or for the “oxidative burst” associated with plant–pathogen interactions. The current study with horseradish peroxidase characterizes a third type of peroxidase activity that mimics the action of catalase; molecular oxygen is produced at the expense of H<sub>2</sub>O<sub>2</sub> in the absence of other reactants. The oxygen production and H<sub>2</sub>O<sub>2</sub>-scavenging activities had temperature coefficients, *Q*<sub>10</sub>, of nearly 3 and 2, which is consistent with enzymatic reactions. Both activities were inhibited by autoclaving the enzyme and both activities had fairly broad pH optima in the neutral-to-alkaline region. The apparent *K*<sub>m</sub> values for the oxygen production and H<sub>2</sub>O<sub>2</sub>-scavenging reactions were near 1.0 mM H<sub>2</sub>O<sub>2</sub>. Irreversible inactivation of horseradish peroxidase by exposure to high concentrations of H<sub>2</sub>O<sub>2</sub> coincided with the formation of an absorbance peak at 670 nm. Addition of superoxide dismutase (SOD) to reaction mixtures accelerated the reaction, suggesting that superoxide intermediates were involved. It appears that horseradish peroxidase is capable of using H<sub>2</sub>O<sub>2</sub> both as an oxidant and as a reductant. A model is proposed and the relevance of the mechanism in plant–bacterial systems is discussed.

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**Key Words:** catalase; catalatic; hydrogen peroxide; peroxidase; reactive oxygen; superoxide.

The production of reactive oxygen species (ROS)<sup>3</sup> by plant cell suspensions during plant–pathogen interactions is well established (1–5). However, it is difficult to accurately measure and characterize this “oxidative burst” due to numerous enzymatic and nonenzymatic mechanisms that can impact ROS production, accumulation, and scavenging (6, 7). The peroxidase enzyme plays a key role in oxidative metabolism and is widely distributed in cells and in the cellular apoplast. In cell walls, peroxidases utilize H<sub>2</sub>O<sub>2</sub> in the oxidative dehydrogenation of phenolics to form lignin precursors (8, 9). In mitochondria, ascorbate peroxidase, using ascorbate as a reductant, acts to prevent accumulation of H<sub>2</sub>O<sub>2</sub> and leakage into the cytosol (10, 11). During plant–pathogen interactions, peroxidases have been reported to utilize NAD(P)H to produce ROS, mimicking NADPH oxidase activity (12–14).

While trying to better characterize the reactions of peroxidase in our model system, we found that the enzyme was able to scavenge H<sub>2</sub>O<sub>2</sub> with the concurrent production of molecular oxygen. No other reactant was necessary. While this property has been noted in myeloperoxidase and lactoperoxidase (15–17), it has not been characterized under conditions relevant to biological systems. In a related study, Barr and Aust (18) characterized a different mechanism of peroxidase that required an exogenous source of cation radicals to produce oxygen from H<sub>2</sub>O<sub>2</sub> (18). While the overall process of producing oxygen from H<sub>2</sub>O<sub>2</sub> appears similar to the two-step catalatic mechanism of catalase, the peroxidase mechanism has multiple steps and is not catalatic. This pseudocatalatic scavenging of H<sub>2</sub>O<sub>2</sub> could conceivably play a role in tissue or cells if normal reduc-

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<sup>3</sup> Abbreviations used: ROS, reactive oxygen species; LDC, luminol-dependent chemiluminescence; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; O<sub>2</sub><sup>•−</sup>, superoxide; HRPOX, horseradish peroxidase; SOD, superoxide dismutase; DETAPAC, diethylenetriaminepentaacetic acid.

tants, such as phenolics, are depleted. Such conditions could exist during periods of oxidative stress. When bacterial cells are introduced into suspensions of tobacco cells, there are periods when H<sub>2</sub>O<sub>2</sub> can be detected. Considering the efficiency of the cell wall and cytoplasmic peroxidases to utilize H<sub>2</sub>O<sub>2</sub> and oxidize reductants, the accumulation and detection of H<sub>2</sub>O<sub>2</sub> indicate that the pool of reductant has been depleted. Under these conditions, the pseudocatalatic mechanism may play a role in scavenging H<sub>2</sub>O<sub>2</sub>.

The objective of this paper is to characterize this pseudocatalatic activity of horseradish peroxidase (HRPOX) under *in vitro* conditions that are more relevant to plant-pathogen interactions.

## METHODS

**Chemicals.** All chemicals were purchased from Sigma Chemical Co. unless noted otherwise. The horseradish peroxidase enzyme (Sigma P-8415) was an affinity-purified, essentially salt-free grade with an activity of 250–300 U per milligram of solid. This unit (U) is defined by Sigma as the amount of enzyme that forms 18  $\mu$ M purpurogallin from pyrogallol in 1 min at 25°C. Since several different preparations of this enzyme were used during the course of this study, we used this same unit rather than milligrams to help standardize the amount of enzyme activity used in different experiments. All other chemicals were of analytical grade. Catalase (Sigma C-100) and superoxide dismutase (Sigma S-2515) were highly purified preparations.

**Oxygen production measurement.** Oxygen production was measured using a multielectrode system developed in our laboratory with the assistance of Microelectrodes Inc. (Londonderry, NH) as previously described (19). This technique provides an accurate comparison of oxygen production or uptake rates between different treatments; however, due to delays in the response of the electrode and the higher concentration of H<sub>2</sub>O<sub>2</sub> routinely used, 1 mM, the numeric value may not be as precise as the scavenging technique. Data acquisition was carried out using hardware and Labview software obtained from National Instruments Corp. (Austin, TX). Simultaneous measurements were performed using oxygen electrodes inserted into 50-ml beakers containing HRPOX and H<sub>2</sub>O<sub>2</sub> in 20 ml of 50 mM phosphate buffer, pH 6.0. The beakers containing the enzyme in buffer were placed in a shaking water bath at 27°C and 170 rpm. After a steady state was established, H<sub>2</sub>O<sub>2</sub> was added and the changes in oxygen concentration of the solutions were determined. All experiments were carried out at least three times, with all treatments duplicated in each experiment. Occasionally when there was a defect with an electrode, the data from that treatment were discarded and the experiment was repeated. Preliminary experiments to determine temperature effects on oxygen evolution were performed using 1-ml reaction volumes maintained at either 20 or 30°C in a circulating-water bath. For these experiments the electrodes were calibrated at each temperature used. Enzyme autoclaved for 15 min was used as a control.

**Peroxidase activity assay.** The effect of H<sub>2</sub>O<sub>2</sub> on peroxidase activity was assayed using a modified guaiacol assay (20). A 10- $\mu$ l sample of HRPOX (1 U/ml) was added to 990  $\mu$ l of varying concentrations of H<sub>2</sub>O<sub>2</sub> in 50 mM NaPO<sub>4</sub> buffer, pH 6.0, and incubated at 27°C for varying time intervals. Fifty microliters of this solution was diluted in a 1-ml reaction mixture containing 0.4 mM guaiacol and 0.4 mM H<sub>2</sub>O<sub>2</sub> in 50 mM NaPO<sub>4</sub> buffer, pH 6.0. Increased absorbance at 470 nm was monitored for 3 min at 0.5-s intervals on a Beckman Model DU-650 spectrophotometer.

Absorbance scans of HRPOX (50 U/ml) in the presence of 1 mM

H<sub>2</sub>O<sub>2</sub> or 10 mM H<sub>2</sub>O<sub>2</sub> in 50 mM NaPO<sub>4</sub> buffer, pH 6.0, were performed. Scans from 400 to 700 nm were recorded every 2 min after addition of the H<sub>2</sub>O<sub>2</sub>.

**H<sub>2</sub>O<sub>2</sub> concentration and scavenging assays.** H<sub>2</sub>O<sub>2</sub> scavenging and concentration were routinely monitored using the luminol-dependent chemiluminescence (LDC) assay as previously described (21). For both assays, samples (0.4 ml) were periodically transferred to cuvettes and placed in the sample carriage of an LKB 1251 luminometer. All reagents were automatically dispensed into the cuvettes in the sample chamber while being monitored for chemiluminescence.

For the temperature experiments, H<sub>2</sub>O<sub>2</sub> concentration was monitored by spectrophotometric absorbance at 240 nm (molar extinction coefficient = 39.4 M<sup>-1</sup> cm<sup>-1</sup>) in a temperature-regulated chamber of a Beckman Model DU-650 spectrophotometer.

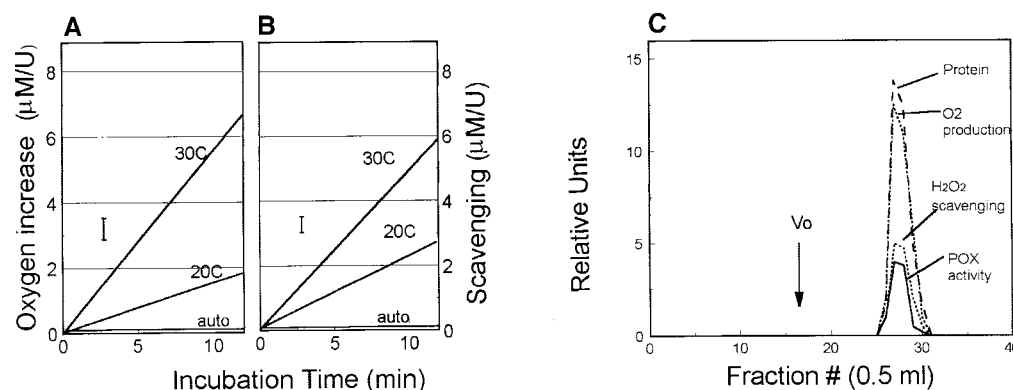
**FPLC analysis of HRPOX.** Horseradish peroxidase preparations were checked for contamination with catalase using FPLC. Two milligrams of HRPOX in 200  $\mu$ l of buffer, 50 mM NaPO<sub>4</sub>, pH 7.0, was loaded onto a Superdex-75 column (Pharmacia, Piscataway, NJ) run at 1 ml/min with the same buffer and monitored for UV absorbance at 280 nm. Fractions were collected and assayed for peroxidase activity, oxygen production, and H<sub>2</sub>O<sub>2</sub> scavenging as described above. Protein content was determined using the micro-BCA protein assay from Pierce (Rockford, IL).

## RESULTS

Preliminary investigations with an oxygen electrode revealed that oxygen production occurred when only HRPOX and H<sub>2</sub>O<sub>2</sub> were present in Mes or NaPO<sub>4</sub> buffer at concentrations of 1 or 50 mM. The data reported here were carried out with 50 mM NaPO<sub>4</sub> since this buffer is more often used in peroxidase studies. No oxygen evolution occurred in controls containing H<sub>2</sub>O<sub>2</sub> in buffer alone, ruling out possible bacterial contamination on electrode surfaces.

To help determine whether the HRPOX was catalyzing this reaction enzymatically, we estimated the temperature coefficient,  $Q_{10}$ , of the reactions (Figs. 1A and 1B). This is the factor by which the rate of a reaction increases by raising the temperature 10°C. Most enzyme reactions have a  $Q_{10}$  value in the range of 2 (22). The  $Q_{10}$  values for oxygen production and H<sub>2</sub>O<sub>2</sub> scavenging were 3.1 and 2.2, respectively, consistent with an enzymatic process being involved. Additionally, both reactions were completely inhibited when the HRPOX was autoclaved for 20 min prior to assay (Figs. 1A and 1B). The addition of 100  $\mu$ M DETAPAC, a metal chelator, to the reaction mixture did not inhibit oxygen production activity, verifying that the reaction was not due to redox reactions with heavy metal contaminants.

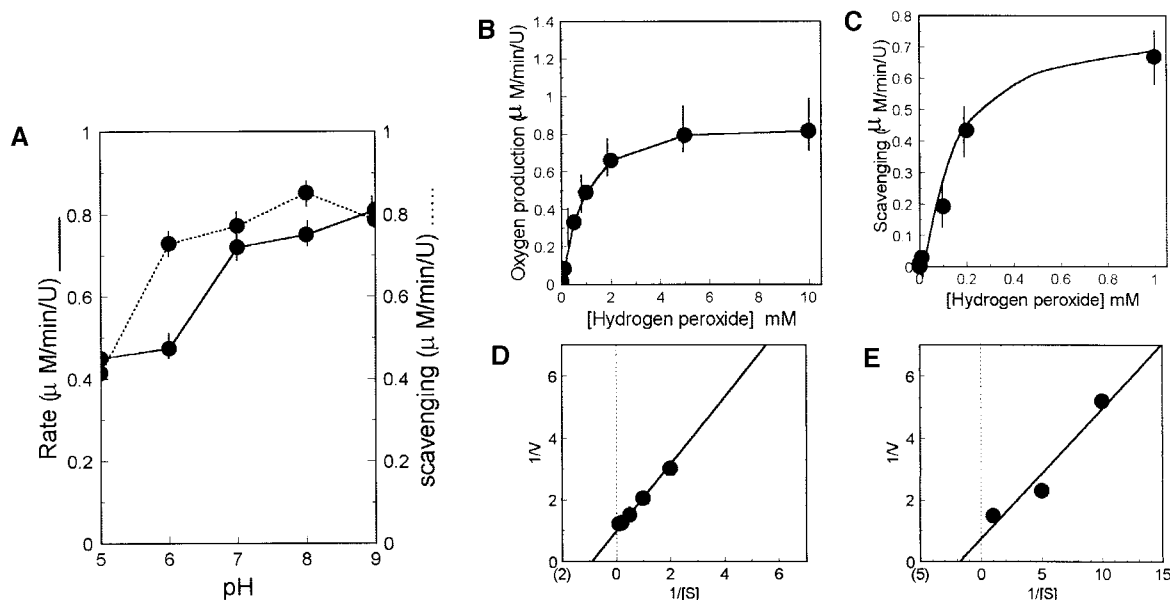
Gel filtration on a Superdex-75 column was carried out to analyze the HRPOX (MW ~ 44 kDa) for possible contamination with trace amounts of catalase (MW ~ 240 kDa), which would elute in the void volume. Analysis of 0.5-ml fractions from the column found that the oxygen production, H<sub>2</sub>O<sub>2</sub> scavenging, and peroxidase activity all coeluted with a single protein peak (Fig. 1C). No contaminating peaks were found.



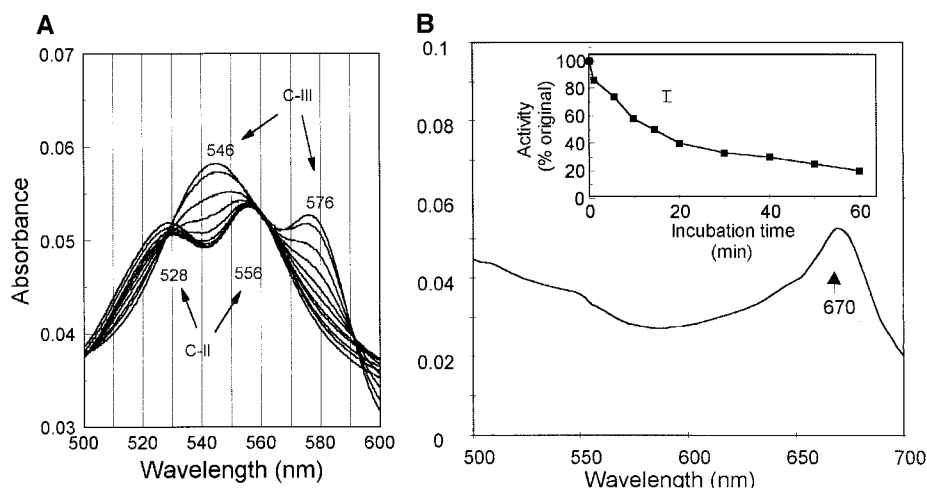
**FIG. 1.** Enzymatic nature of the pseudocatalytic mechanism of HRPOX. Effect of temperature on oxygen production (A) and H<sub>2</sub>O<sub>2</sub> scavenging (B) in reactions containing HRPOX and H<sub>2</sub>O<sub>2</sub>. Reaction mixtures contained 5 mM H<sub>2</sub>O<sub>2</sub> in 50 mM phosphate buffer, pH 6. Oxygen production was measured with an oxygen electrode using a 1-ml enclosed chamber with 3 U/ml HRPOX. Scavenging was estimated by spectrophotometry at 240 nm with 20 U/ml HRPOX. Temperatures of the reactions were maintained using a circulating-water bath. Autoclaved HRPOX showed no activity in either assay (---). A standard error bar is shown. (C) Gel filtration elution of HRPOX. Fractions were assayed for protein, HRPOX activity, oxygen production, and H<sub>2</sub>O<sub>2</sub>-scavenging activity as described under Methods. The assay results are expressed as relative units.

Both oxygen production and H<sub>2</sub>O<sub>2</sub>-scavenging activity were favored by alkaline pH (Fig. 2A). The rate of oxygen production by the HRPOX/H<sub>2</sub>O<sub>2</sub> reaction gradually increased 2-fold as pH increased from 6 to 9. The rate of H<sub>2</sub>O<sub>2</sub> scavenging nearly doubled from pH 5 to 6 and was relatively unchanged as pH increased from 6 to 9. Subsequent experiments were carried out at pH 6.0 to approximate physiological conditions.

The effects of varying initial concentrations of H<sub>2</sub>O<sub>2</sub> on both oxygen evolution and H<sub>2</sub>O<sub>2</sub> scavenging were measured. Oxygen evolution rates increased with H<sub>2</sub>O<sub>2</sub> concentration and appeared to saturate around 5.0 mM (Fig. 2B) with an apparent  $K_m$  of about 1.1 mM as determined by a double-reciprocal plot (Fig. 2D). The rate of H<sub>2</sub>O<sub>2</sub> scavenging monitored by LDC also increased with H<sub>2</sub>O<sub>2</sub> concentration and appeared to sat-



**FIG. 2.** Characterization of the pseudocatalytic mechanism of HRPOX. (A) Effect of pH on oxygen production and H<sub>2</sub>O<sub>2</sub> scavenging in reactions containing HRPOX and H<sub>2</sub>O<sub>2</sub>. Oxygen production (solid line) was measured with oxygen electrodes in 20-ml beakers containing 1 mM H<sub>2</sub>O<sub>2</sub> and 5 U/ml HRPOX buffered with 50 mM NaPO<sub>4</sub>; H<sub>2</sub>O<sub>2</sub> scavenging (dotted line) was estimated by LDC in reaction mixtures containing 1 mM H<sub>2</sub>O<sub>2</sub> and 7 U/ml HRPOX in 50 mM NaPO<sub>4</sub>. (B, C) Effect of hydrogen peroxide concentration on oxygen production (B) and H<sub>2</sub>O<sub>2</sub> scavenging (C) in reactions containing HRPOX and varying concentrations of H<sub>2</sub>O<sub>2</sub> in 50 mM NaPO<sub>4</sub>, pH 6.0. (D, E) Double-reciprocal plots of oxygen production (D) and H<sub>2</sub>O<sub>2</sub> scavenging (E).



**FIG. 3.** Absorbance scan of HRPOX incubated with  $\text{H}_2\text{O}_2$ . HRPOX (50 U/ml) was incubated with (A) 1 mM  $\text{H}_2\text{O}_2$  or (B) 10 mM  $\text{H}_2\text{O}_2$  in 50 mM  $\text{NaPO}_4$  buffer, pH 6.0. Absorbance was scanned from 400 to 700 nm. (A) A time sequence follows the change from compound III (C-III) to compound II (C-II). The scans shown in the figure are about 2 min apart. (B) Spectrophotometric scan of HRPOX after 90-min exposure to 10 mM  $\text{H}_2\text{O}_2$ . (Insert) Decrease in HRPOX activity caused by incubation with 10 mM  $\text{H}_2\text{O}_2$ .

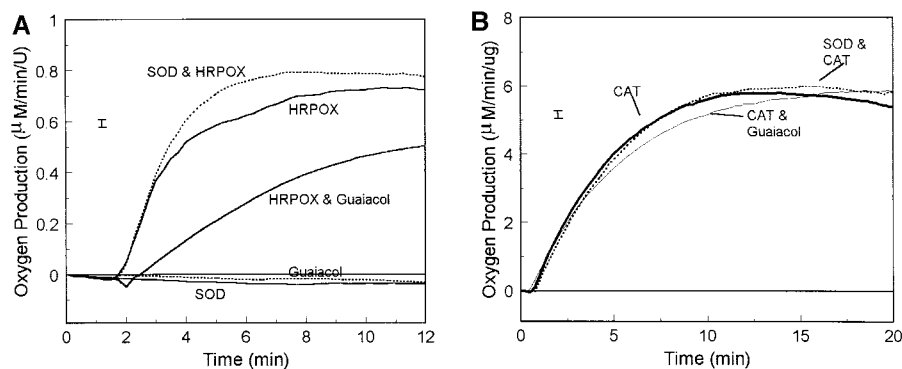
urate near 0.4 mM (Fig. 2C) with an apparent  $K_m$  of about 0.6 mM (Fig. 2E).

To gain further insight into which of the various redox forms of HRPOX might be involved in these processes, reaction mixtures were scanned repeatedly in a spectrophotometer in the presence of 1.0 mM  $\text{H}_2\text{O}_2$  (Fig. 3A). At the beginning of the reaction, compound III (546, 576 nm) was found to be the predominant form. However, as the  $\text{H}_2\text{O}_2$  concentration decreased, compound II (528, 556 nm) became the prevalent form (Fig. 3A).

At high  $\text{H}_2\text{O}_2$  concentrations, around 10 mM, only compound III was observed. Immediate addition of excess guaiacol, which rapidly reduces the  $\text{H}_2\text{O}_2$  levels via the normal peroxidative mechanism, converted HRPOX back to ground state (404 nm). Conversely, continued exposure to 10 mM  $\text{H}_2\text{O}_2$  resulted in the

development of a new peak at 670 nm (Fig. 3B). This peak appeared to be associated with an irreversibly inactivated form of the enzyme, as addition of excess guaiacol did not result in the restoration of ground-state HRPOX. After exposure to 10 mM  $\text{H}_2\text{O}_2$  for 1 h, HRPOX activity decreased by 80% (Fig. 3B, insert) and oxygen production activity decreased by 90%.

The addition of SOD to reaction mixtures of HRPOX and 1 mM  $\text{H}_2\text{O}_2$  increased the rate of oxygen production noticeably, indicating the presence of superoxide and the possibility that oxygen might be formed from either the dismutation of superoxide or its oxidation by HRPOX (Fig. 4A). The addition of excess guaiacol significantly decreased oxygen production, which would be expected if guaiacol was competing with  $\text{H}_2\text{O}_2$  or superoxide as an electron donor. Addition of SOD to reaction mixtures of catalase and  $\text{H}_2\text{O}_2$  had little effect



**FIG. 4.** Effect of SOD and excess guaiacol on oxygen production by horseradish peroxidase (HRPOX) (A) and catalase (CAT) (B). Oxygen production was measured by oxygen electrodes in 20-ml beakers containing 1 mM  $\text{H}_2\text{O}_2$  and either 3 U/ml HRPOX (A) or 5  $\mu\text{g}$  (2.5–6.25 U/ml) of catalase. Beakers received 3.0 U/ml SOD, 10 mM guaiacol, or no additions. Controls include 1 mM  $\text{H}_2\text{O}_2$  plus either SOD or guaiacol.

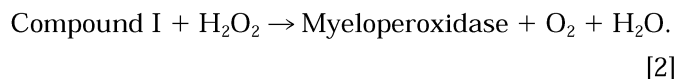
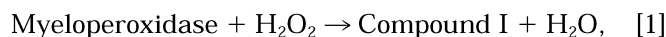
under similar conditions (Fig. 4B). Although addition of guaiacol appeared to have a slight inhibitory effect on the catalase reaction, it was not of the magnitude observed in the peroxidase reaction.

## DISCUSSION

Peroxidases could potentially play a variety of roles in ROS metabolism in plants. Cell wall peroxidases have previously been proposed as a source of the pathogen-elicited ROS "burst" (13) and peroxidases as a group are well established as  $\text{H}_2\text{O}_2$  scavengers in the presence of phenolic precursors of lignin or reductants such as ascorbate (8). The current study suggests that HRPOX is capable of scavenging  $\text{H}_2\text{O}_2$  in the absence of additional reductants, a condition that could conceivably exist in cells or tissues during periods of oxidative stress.

HRPOX was shown to initiate oxygen production at the expense of  $\text{H}_2\text{O}_2$ . This "pseudocatalytic" mechanism was distinguishable from catalase in the following ways. (1) The  $K_m$  values for oxygen evolution and  $\text{H}_2\text{O}_2$  scavenging catalyzed by HRPOX were 1.1 and 0.4 mM, respectively (Figs. 2B and 2C). This is far lower than the reported  $K_m$  of catalase of 0.047–1.1 M (23). (2) Unlike true catalytic activity, the pseudocatalytic mechanism appears to involve  $\text{O}_2^-$  in that addition of SOD enhanced the activity (Figs. 4A and 4B). (3) The addition of guaiacol, a competing electron donor, inhibited oxygen production by HRPOX but not by catalase (Figs. 4A and 4B).

There have been a few reports of the production of molecular oxygen from reaction mixtures of  $\text{H}_2\text{O}_2$  and myeloperoxidase from leukocytes. Iwamoto *et al.* (15) suggested that myeloperoxidase decomposes low levels of  $\text{H}_2\text{O}_2$  in a manner similar to that of catalase (Eqs. [1] and [2]):



However, more recently, Marquez *et al.* (16) reexamined this mechanism with rapid-scan techniques and found that the "catalase-like" pathway described above did not appear to be the dominant pathway. Instead, they suggested that  $\text{H}_2\text{O}_2$  was oxidized by compound I, generating compound II and  $\text{O}_2^-$  (Eq. [3]). As superoxide increases, it may also participate in the conversion of compound I to compound II (Eq. [4]) as well as in the reduction of compound II to native enzyme, yielding molecular oxygen (Eq. [5]), as proposed for lactoperoxidase (17):



Our findings were, for the most part, similar to those reported in the studies with myeloperoxidase mentioned above when the  $\text{H}_2\text{O}_2$  concentration was less than 1 mM. However, at higher levels of  $\text{H}_2\text{O}_2$ , we found that compound III was the more prominent form of HRPOX present (Fig. 3A). Compound III is a further oxidized form of HRPOX, which may be produced by several routes (24):



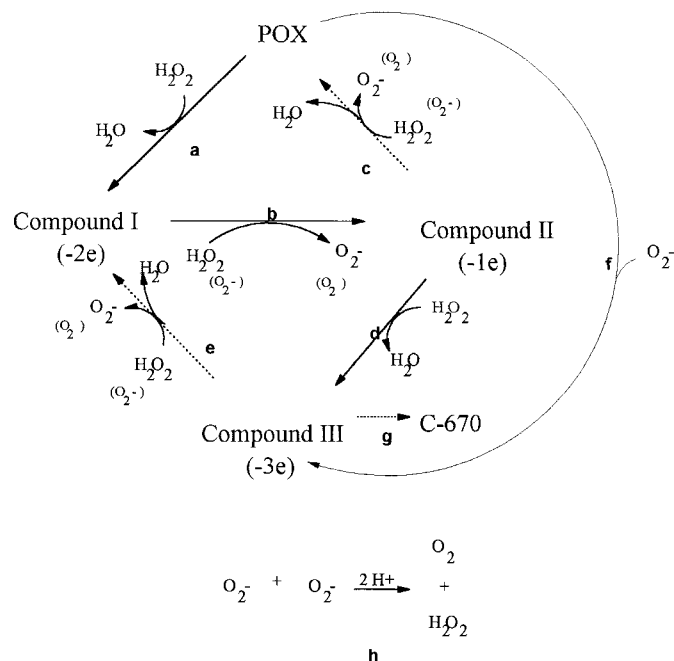
In the present study, it is feasible that both mechanisms may be active in the conversion of HRPOX to compound III. Kobayashi and Hayashi (25) demonstrated that compound III can be reduced to compound I through a one-electron reduction with a reductant (RH):



In concentrations  $>1$  mM  $\text{H}_2\text{O}_2$ , compound III was the predominant redox form of HRPOX (Fig. 3A), indicating that  $\text{H}_2\text{O}_2$  is not an ideal reductant for this reaction. However, a phenol such as guaiacol appears to be an ideal reductant for compound III since almost immediately after its addition, native HRPOX became the predominant form (26).

Based on the studies above, we have suggested a scheme (Fig. 5) similar to that proposed by Saikumar (26) for peroxidase and oxidase reactions. Hydrogen peroxide is required for oxidation of native HRPOX to compound I (reaction a). In the absence of more suitable substrates,  $\text{H}_2\text{O}_2$  readily serves as reductant for compound I, producing compound II and  $\text{O}_2^-$  (reaction b). Conversion back to native enzyme (reaction c) appears to be rate limiting, in that compound II predominates when levels of  $\text{H}_2\text{O}_2$  are in the range of 300  $\mu\text{M}$   $\text{H}_2\text{O}_2$  (Fig. 3A). At higher levels of  $\text{H}_2\text{O}_2$ , compound III appears. It is feasible that  $\text{H}_2\text{O}_2$  at high concentrations acts as an oxidant (reaction d).

As superoxide concentrations increase, from reaction b, c, or e, dismutation can occur (reaction h). As  $\text{H}_2\text{O}_2$  levels decrease and  $\text{O}_2^-$  increases, other mechanisms might be involved (reaction f). Reaction e, like reaction c, is rate limiting when only  $\text{H}_2\text{O}_2$ , or perhaps superoxide, is available as oxidant. Phenols such as guaiacol



**FIG. 5.** Proposed pathways for H<sub>2</sub>O<sub>2</sub> scavenging and oxygen production by peroxidase.

are ideal electron donors for reactions b, c, and e. Therefore, when guaiacol is added to reaction mixtures of HRPOX and H<sub>2</sub>O<sub>2</sub>, the predominant form of HRPOX whether compound II or III quickly reverts to native HRPOX. Oxygen production occurs from dismutation of superoxide (reaction h) and/or its oxidation by reactions b, c, and e. At higher pH, reaction h would be slow due to electrostatic repulsion. Therefore the increased activity observed in Fig. 2A would result from reactions b, c, and e in this model. Reaction g appears to occur slowly and irreversibly at H<sub>2</sub>O<sub>2</sub> concentrations >1 mM, producing the inactive form of the enzyme with an absorbance peak at 670 nm. This is similar to the heme bleaching reported for manganese peroxidase in the presence of excess H<sub>2</sub>O<sub>2</sub> (27).

This study demonstrates that HRPOX is capable of scavenging H<sub>2</sub>O<sub>2</sub> at concentrations that may occur in plant-pathogen interactions and certainly occur under *in vitro* conditions used in many studies (1–7). In a single reaction mixture peroxidase may exist in as many as five different redox states, each simultaneously catalyzing different reactions. It is important to be aware that these different reactions can occur and that in a complex biological system a certain amount of uncertainty exists as to the exact role or mechanisms of peroxidase enzymes. It is likely that several, sometimes competing, reactions may be taking place and that the outcome will depend on subtle changes in pH or fluctuations in various oxidants and reductants.

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