Hydrogen Peroxide-mediated Oxidation of Indole-3-acetic Acid by Tomato Peroxidase and Molecular Oxygen

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ABSTRACT

The oxidation of indole-3-acetic acid by anionic tomato peroxidase was found to be negligible unless reaction mixtures were supplemented with H₂O₂. The addition of H₂O₂ to reaction mixtures initiated a period of rapid indole-3-acetic acid oxidation and O₂ uptake; this phase ended and O₂ uptake fell to a low level when the H₂O₂ was exhausted. The stoichiometry of the reaction, which is highly dependent on enzyme concentration and pH, suggests that H₂O₂ initiates a sequence of reactions in which indole-3-acetic acid is oxidized.

EXPERIMENTAL

Many plant peroxidases catalyze the oxidation of IAA by molecular O₂ in the presence of a phenol and manganous ion (2, 6, 10, 14, 16). The reaction is generally believed to proceed autocatalytically in the sense that certain active intermediates are formed and accumulate; a distinct lag period is sometimes seen in the initiation of the reaction. A role for H₂O₂ in this reaction is suggested by the observations that H₂O₂ reduces or eliminates the lag period (14, 17) or even stimulates the rate of O₂ uptake in other cases (9, 17). A role for H₂O₂ is further supported by the observed inhibition of the IAA oxidase reaction by catalase in some instances (16).

We have previously reported that tomato anionic peroxidase lacked the capacity to oxidize IAA in the presence of DCP and manganous ion as activators, while commercially purified HRP oxidized IAA effectively under the same conditions without a significant lag period (11). The inability of the tomato peroxidase to carry out the oxidation of IAA was then attributed to the low reactivity of the compound II form of the enzyme as compared to HRP compound II. In this respect, we generally agreed with the theory of Yamazaki and Yamazaki (24) on the involvement of the compound II in both the initiation and propagation steps of the IAA oxidase reaction.

It is the aim of this paper to document the ability of tomato anionic peroxidase to catalyze the IAA oxidase reaction, the oxidation of IAA by molecular oxygen, provided that H₂O₂ is supplied to the reaction mixture (12). The stoichiometry of H₂O₂, O₂, and IAA utilization was investigated, as well as the effect of H₂O₂ on the rate of the reaction.

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4 Abbreviations: HRP: horseradish peroxidase; DCP: 2,4-dichlorophenol.

MATERIALS AND METHODS

Enzyme Source and Estimation. Tomato anionic peroxidase was extracted from tomato (Lycopersicon esculentum var. tropic) pericarp tissue and purified to about 85% homogeneity as previously described (11). Enzyme concentrations were estimated from the 403 nm A of solutions, using a millimolar extinction coefficient of 107 for the enzyme (11). A spectra from 400 nm to 600 nm wavelength were run on a Perkin-Elmer 124 spectrophotometer and associated chart recorder and used to determine if the enzyme was present as ferric enzyme, ferrous enzyme, compound I, compound II, or compound III (11).

IAA Oxidase Reaction. O₂ uptake measurements were made with a Gilson 20 oxygraph. Reaction mixtures totaled 1.6 ml volume and contained the following unless otherwise specified: 1.87 μM IAA, 750 μM DCP, 9.38 μM citrate buffer (pH 4.0), with H₂O₂ and enzyme concentrations as specified for each experiment. When reactions were carried out at pH values other than 4.0, citrate buffer was replaced with citrate-phosphate buffers made by mixing 0.1 M Na₂HPO₄ and 0.05 M citric acid. Reactions were carried out at 21°C and both the rate of change of O₂ concentration (μM/min) and the total change in O₂ concentration (μM) were determined from the oxygraph tracings.

Total IAA utilization was determined by measuring residual IAA colorimetrically using the Cole-Hopkins reaction. Additions of 0.4 ml of 10% glyoxylic acid and 4.0 ml of 70% H₂SO₄ were made to each 1.6-ml reaction mixture. The samples were allowed to stand at room temperature for 30 min and the 445 nm A was determined. A small correction was applied due to the formation of a chromogenic reaction product (15).

Spectrophotometric Studies. Tomato peroxidase, 4 mMol, dissolved in 1.5 ml of 0.05 M phosphate buffer (pH 6.5), was placed in a cuvette with a 1-cm light path. The peroxidase was converted to compound I by the addition of 10 μl of 12 mM H₂O₂ solution. The IAA oxidase reaction was initiated by the addition of 20 μl of 5 mM IAA solution. A changes at 250 nm (destruction of IAA) as well as 404 nm (ferric peroxidase) and 418 nm (compound II) were followed with a Perkin-Elmer 124 recording spectrophotometer. The spectrum of the enzyme at various stages of the reaction was also recorded.

Reported Results. The data reported in each case are the results of a single typical experiment. Each experiment was repeated in whole or in part several times.

RESULTS

O₂ Uptake Studies. Figure 1 demonstrates the effect of the addition of small amounts of H₂O₂ to reaction mixtures containing tomato anionic peroxidase, IAA, and DCP. The H₂O₂ addition resulted in a brief period of rapid O₂ uptake, after which the rate fell to a low level. A second addition of H₂O₂ resulted in a second...
brief period of $O_2$ uptake. Thus, $O_2$ uptake seemed to proceed only as long as $H_2O_2$ was available.

The effect of varying initial $H_2O_2$ concentration on the rate of $O_2$ uptake is seen in Figure 2A. The rate of $O_2$ uptake was essentially zero in the absence of $H_2O_2$ but increased linearly with $H_2O_2$ concentration up to about 6 $\mu M$. The rate of $O_2$ uptake did not increase noticeably once the $H_2O_2$ concentration reached 11 $\mu M$. Both the total amount of $O_2$ taken up and the total amount of IAA oxidized after the addition of $H_2O_2$ were shown to be proportional to the amount of $H_2O_2$ added, up to 30 $\mu M$, as seen in Figure 2B. Above 30 $\mu M$ complete utilization of the IAA in the reaction mixture was achieved. Analysis of the data in Figure 2B indicates that under these assay conditions, the utilization of 1 molecule of $H_2O_2$ could initiate the utilization of at least 4 molecules of $O_2$.

With $H_2O_2$ concentrations as high as 1.65 mM in reaction mixtures otherwise identical to those for Figure 2, the rate of $O_2$ uptake was undiminished, but the total amount of $O_2$ uptake decreased markedly. The IAA/$O_2$ ratio (ratio of IAA utilized to $O_2$ utilized) increased from 1.15 for 66 $\mu M$ $H_2O_2$ to 1.55 for 1.65 mM $H_2O_2$. This observation is not attributed to enzyme inactivation, but will be discussed later.

Figures 3 and 4 indicate the effects of varying enzyme concentration on the rate of $O_2$ uptake and on IAA utilization and total $O_2$ uptake. The data in Figure 3 were generated using 0.055 mM $H_2O_2$. The rate of $O_2$ uptake (Fig. 3A) increased with increasing enzyme concentration over the range tested, although the relationship is not quite linear. Figure 3B indicates that the $H_2O_2$ concentration used was sufficient for total consumption of the IAA, but also indicates that with increasing enzyme concentrations, the fraction of IAA oxidized by $O_2$ became smaller. When 22 $\mu M$ $H_2O_2$ was substituted, as shown in Figure 4A, the rate of $O_2$ uptake was proportional to the enzyme concentration only at low enzyme concentrations. Figure 4B indicates that the amount of $H_2O_2$ used was not sufficient to accomplish total oxidation of the IAA available at high enzyme concentrations, but that as the concentration of enzyme was decreased there was an increase both in total IAA oxidation and in $O_2$ uptake. The decrease in the IAA/$O_2$ ratio with increasing enzyme concentration in both Figures 3 and 4 is indicative of utilization of fewer molecules of $O_2$ per molecule of $H_2O_2$ at increasing enzyme concentrations. Calculations based on the data from Figure 4, A and B, indicate that increasing the enzyme concentration from 0.16 $\mu M$ to 0.70 $\mu M$ increased the rate of $O_2$ uptake 20% but increased the rate of IAA destruction 73%.

The effects of pH on the rate of $O_2$ uptake and on total $O_2$ uptake during the oxidation of IAA are seen in Figure 5. The rate
of O₂ uptake is seen to increase as the pH is lowered from 7 to 3, with no distinct optimum having been observed above pH 3. H₂O₂ was the factor limiting total O₂ uptake in the range of pH 7 to pH 5, and the total amount of O₂ utilized per μmol of H₂O₂ added increased with decreasing pH. This is reflected in a shift in the IAA/O₂ ratio, which decreased with decreasing pH. This effect is seen even at pH 4 and pH 3 where complete IAA utilization occurs.

Table I shows the effect of DCP concentration on the rate of the oxidase reaction. Characteristically, low concentrations of DCP stimulate the reaction while higher concentrations are inhibitory. Manganese ion will also affect the rate of IAA destruction to some extent, but whether activation or inhibition is observed depends on the concentration of manganese ion and the concentrations of the IAA and the enzyme.

Spectrophotometric Studies. Spectrophotometric studies were performed as described under “Materials and Methods.” When IAA was added to ferric enzyme (H₂O₂ omitted), there was no O₂ uptake, no IAA destruction, and no change in the A spectrum of the peroxidase. When H₂O₂ was added to the ferric form of the enzyme, the enzyme was converted to compound I. With a subsequent addition of IAA, there was a period of O₂ uptake paralleled by concomitant destruction of IAA. During this period, the enzyme existed as a mixture of ferric enzyme and compound II. With exhaustion of the H₂O₂, IAA oxidation ceased, and the enzyme reverted to its ferric form. No accumulation of compound III was detected during the course of the reaction. It should be noted that in this study DCP was omitted from the reaction mixtures.

Inactivation of Tomato Anionic Peroxidase. Tomato anionic peroxidase can be inactivated during IAA oxidation in the presence of H₂O₂ at pH 4 or lower, as reported for HRP (24). The data in Table II, obtained at pH 4 with low enzyme concentrations, indicate that the use of high H₂O₂ concentrations (110 and 330 μM) results in decreased total O₂ uptake and decreased total IAA oxidation. This is attributed to enzyme inactivation since a second addition of peroxidase reinitiated the oxidase reaction, while further additions of H₂O₂ or IAA were without effect. Moreover, oxygraph tracings obtained with 66 and 82 μM H₂O₂ were generally linear during the course of IAA oxidation, while those for 110 and 666 μM H₂O₂ showed a marked decrease in rate of O₂ uptake during the course of the reaction. Complications due to enzyme inactivation were avoided in the present study by using ratios of peroxidase to H₂O₂ of 1 × 10⁻⁴ or greater.
DISCUSSION

The rate of oxidation of IAA by tomato anionic peroxidase has been shown to be controlled by the availability of H$_2$O$_2$. A similar dependence on H$_2$O$_2$ has been shown for both the IAA oxidase-peroxidase complex of yellow birch and HRP, tested under appropriate conditions (9), although the catalytic capability of HRP seems to differ markedly from both the tomato (11) and yellow birch enzymes (9). The data presented here are similar to those of Ray (17) obtained with a fungal peroxidase, in that the rate of IAA oxidation fell to a negligible level once the H$_2$O$_2$ in the reaction mixtures had been depleted. Ray (17) emphasized that the peroxide only served to increase an already significant rate of IAA oxidation by his enzyme preparation.

Although the complete mechanism of IAA oxidation by peroxidases is still unknown, the involvement of endogenous H$_2$O$_2$ in the initiation and propagation of the reaction has been proposed by several investigators (3, 22, 23). Yamazaki and Yamazaki (24) only recently rejected the idea of the involvement of H$_2$O$_2$ in the autocatalytic oxidation of IAA by HRP based on observations that catalase cannot influence the initiation or propagation of this reaction. However, in order to explain the autocatalytic nature of IAA oxidation, they replaced the H$_2$O$_2$ with the peroxide of IAA and other radical intermediates in their proposed mechanism. In this respect, HRP and tomato peroxidase differ in the catalysis of IAA oxidation even though the same products have been observed in both cases (Gingrich and Brooks, unpublished results). Therefore, a variation of Yamazaki’s mechanism must be sought for the tomato peroxidase-catalyzed reaction.

When tomato peroxidase catalyzes the oxidation of IAA, the fact that H$_2$O$_2$ is not only required for the initiation of the reaction but also is consumed in order to maintain a supply of active intermediates also indicates that these intermediates are generated mainly from the decomposition of the peroxidase-H$_2$O$_2$ complexes (compounds I and II). A further reaction of these intermediates with molecular oxygen (8, 13, 17) is responsible for the O$_2$ uptake. The sequence of these events is depicted in the following set of reactions:

1. **Compound I + IAA → Compound II + IAA**
2. **Compound II + IAA → Ferriperoxidase + IAA**
3. **IAA + O$_2$ → IAA-O$_2$**
4. **IAA-O$_2$ + IAA → IAA-O$_2$H + IAA**
5. **IAA-O$_2$H → Products**

The rapid increase of the IAA/O$_2$ ratio observed when the enzyme concentrations are increased can be explained according to the above scheme. As the enzyme concentration increases, the number of independent radical chains (reactions 3 and 4) increases and therefore the steady-state concentration of the free radicals is expected to increase. This will speed up the O$_2$ uptake, but at the same time the length of these radical chains will be reduced as chain termination reactions will become more probable. An analogous argument might be used to explain the shift in IAA/O$_2$ ratios with changing pH.

The above scheme for the oxidation of IAA by tomato peroxidase is an oversimplification and is not designed to reflect all of the details of this complicated reaction. Free radicals such as IAA-O$_2$ could induce the formation of the oxyferriperoxidase form (compound III) of the tomato enzyme in a manner similar to that proposed for HRP (24). However, the involvement of compound III in the oxidation of IAA by the tomato peroxidase is not expected to be extensive mainly because the reaction of this complex with IAA results in the regeneration of H$_2$O$_2$ (25) according to reaction 6.

1. **Compound III + IAA → Ferriperoxidase + IAA + H$_2$O$_2$**
2. **Compound III + IAA-O$_2$ → Ferriperoxidase + IAA-O$_2$ + O$_2$**

This reaction not only competes with reaction 6 for compound III when IAA-O$_2$ has reached maximum levels but also serves as a chain termination reaction. From a physiological standpoint, the H$_2$O$_2$-mediated oxidation of IAA by the tomato fruit peroxidase suggests that the destruction of IAA in tissue could be controlled by the availability of H$_2$O$_2$ or other peroxidases. This view was first expressed by Siegel and Galston (19) and extended to cover the role of auxin protectors by Stonier and Yang (20). A very strong relationship between inhibition of pear ripening by auxins, the accumulation of H$_2$O$_2$ in ripening pears, and the oxidative destruction of IAA in pear ripening has also been pointed out by Frenkel and his co-workers (1, 4, 5). The same situation may hold for other fruits (5, 21) including tomatoes.

LITERATURE CITED

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