Plant Physiol. (1972) 49, 1012–1018

The Mechanism of the Scopoletin-induced Inhibition of the Peroxidase Catalyzed Degradation of Indole-3-acetate[1]

Received for publication November 1, 1971

J. C. Sirois and R. W. Miller
Chemistry and Biology Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Ontario K1A OC6, Canada

ABSTRACT

The naturally occurring coumarin, scopoletin, has been found to modify horseradish peroxidase rapidly to give a stable, spectroscopically distinguishable form of the enzyme. Peroxidase treated with scopoletin is less active in reactions with molecular oxygen and indole-3-acetic acid. Kinetic data for the degradation of this growth regulator were obtained with a continuously monitored fluorometric procedure. Lineweaver-Burk plots of the reciprocal rate of degradation against the reciprocal substrate concentration were markedly curved in the presence of the inhibitor, scopoletin. Excess indole-3-acetate restored the scopoletin-treated enzyme to a reactive state. In the presence of molecular oxygen, concentrations of indole-3-acetic acid which were at least 10-fold greater than the inhibitor concentration led to the rapid oxidation of the coumarin and converted peroxidase to compound III as expected from previous studies. This form of the enzyme is the catalytically active species in the oxidative degradation of the growth regulator. The kinetically preferential reaction of scopoletin or related coumarins with peroxidase and the suppression of indole-3-acetic acid degradation may provide a possible control mechanism over the oxidative degradation of indole-3-acetate by this plant enzyme.

Naturally occurring coumarins have been known to have marked effects on the growth of plant tissues for some time (1, 2). For example, high concentrations of scopoletin (50 μg/ml) reportedly inhibit, while lower concentrations (1 μg/ml) stimulate the growth of pea root (1). Also, this ubiquitous (3) coumarin has been found to stimulate IAA oxidase preparations of sweet potato roots and etiolated shoots at concentrations below 13 μM while inhibiting the reaction above this concentration (6). Since IAA oxidases extracted from plant cells are inhibited by appropriate concentrations of scopoletin, a possible function has been proposed which involves the control of the catalytic oxidation of the growth regulator. Apparently scopoletin is attacked preferentially by IAA oxidases. The coumarin is reported to be converted to a nonfluorescent compound as a result of this reaction. Evidence has been obtained which indicates that scopoletin may inhibit competitively the oxidation of IAA by partially purified plant oxidases and peroxidases (1, 13). The identification of IAA oxidases as heme enzymes of the peroxidase type rests on comparisons of the properties of the enzymes isolated from plant sources with purified plant peroxidases (1, 10, 12).

Although scopoletin inhibition of IAA oxidation can be overcome by high concentrations of the substrate, the mechanism of the interaction of scopoletin with IAA oxidases (peroxidases) has not been characterized. Indeed, the data of Imbert and Wilson (6) indicate that the inhibition of IAA oxidation by scopoletin is not a case of simple competition between a substrate and an inhibitor for a single site on the enzyme. In the presence of scopoletin, plots of the rate of IAA destruction against time can become sigmoidal, indicating a more complex kinetic effect.

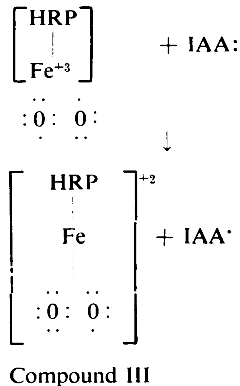
It thus appears that whereas plant peroxidases may play a central role in regulating the IAA levels in plant tissue through oxidative degradation of IAA, an equally important regulatory role might be ascribed to scopoletin through inhibition of the enzyme(s). Indeed, many types of naturally occurring compounds including phenols, catechols, coumarins, manganous salts, peroxides, and compounds possessing unpaired electrons (free radicals) (7, 14) have been found to markedly change the activity of peroxidases towards IAA. However, no clear picture of the *in vivo* control of IAA oxidase activity by such effectors has emerged. The fact that plant peroxidases occur as isoenzymes complicates the assignment of regulatory roles to specific types of effectors. Work with individual isoperoxidases has shown that the effect of scopoletin varies with the isoenzyme (11). The opposing stimulatory and inhibitory effects of the coumarin on IAA degradation may be due in part to specific interactions with the different isoenzymes. Moreover, the report of Imbert and Wilson indicates that the effects of scopoletin on IAA oxidase were dependent on the relative concentrations of IAA, scopoletin, and enzyme (6). For example, a 3-fold decrease in the enzyme concentration changed a 50% inhibition by scopoletin to a 100% stimulation.

The mechanism of the catalytic oxidation of IAA by peroxidases has been widely investigated. The growth regulator is an effective reductant of purified, oxygenated horseradish peroxidase. It is well established that the formation of enzyme intermediates of peroxidase may be followed spectroscopically (4, 7, 8, 14). The identification of the heme-protein products of the reaction between IAA and purified peroxidase as the known peroxidase compounds I and II was proposed by Fox *et al.* (4). These authors did not observe the formation of compound III but postulated that peroxidase first reacts with molecular oxygen to form an oxygenated derivative which then reacts with IAA to give compounds I and II.

However, with the confirmation that peroxidase is readily converted to a single electron reduced compound consisting of

[1] Contribution No. 715 of Chemistry and Biology Research Institute.

the enzyme and the superoxide form of molecular oxygen (compound III), it has become more likely that this enzyme compound primarily is involved in IAA oxidation rather than compounds I and II (7–9, 14). Compound III may be formed directly from the reaction of native peroxidases with oxygen and IAA as shown in diagram I.



Compound III

Diagram I

This species appears to participate actively in the catalytic oxidative degradation of additional molecules of the growth regulator (7, 9, 14). A plausible chain reaction mechanism for the oxidative degradation of IAA involves the relatively slow, single electron reduction of peroxidase and the oxygen molecule to yield compound III and an IAA free radical. The "activated" enzyme (compound III) then rapidly oxidizes a further molecule of unreacted IAA to the free radical intermediate. Chain propagation occurs when the free radical (IAA·) in turn rapidly reduces native peroxidase to compound III in a second rapid reaction (see reactions 1–3, below).

The ultimate product of IAA degradation has been reported to be 3-methylene oxindole (5). The free radical form of IAA and other possible indole intermediates in this reaction have not been well characterized.

Phenolic compounds which may activate or inhibit IAA degradation (depending on relative concentrations of enzyme, modifier, and IAA) were postulated to exert their effect by either enhancing or diminishing the rate of accumulation of compound III depending on the experimental conditions (7). Due to the complex mechanism of the oxidative degradation of IAA, detailed investigations of the interactions of peroxidases with naturally occurring inhibitors or effectors of this reaction are desirable.

The intense fluorescence spectra exhibited by both scopoletin and IAA have allowed us to develop a method for studying fluorometrically the kinetics of degradation of the growth regulator as well as the interaction of the coumarin with electrophoretically homogenous HRP[2] (high spin form). This is possible because the fluorescent characteristics of the two compounds permit independent determinations of the concentration of one in the presence of the other as a function of time. In these studies, scopoletin was inhibitory at all concentrations and spectroscopic evidence for a direct interaction of the inhibitor with peroxidase was obtained.

MATERIALS AND METHODS

Highly purified HRP was obtained from Sigma Chemical Co. The enzyme preparation had a R.Z. value of 3.1 and was electrophoretically homogeneous. Catalytic activity of the enzyme for pyrogallol oxidation was within the limits expected for the pure enzyme. IAA was supplied by Nutritional Biochemicals Corp. One major fluorescent spot was obtained after thin layer chromatography of IAA on silica in a solvent consisting of ammonia, propanol, and water (8:1:1, v/v). Minor fluorescent spots due to breakdown products of IAA accounted for about 2% of the total material applied to the chromatogram. Scopoletin obtained from Sigma was chromatographically pure as determined by fluorescence and ultraviolet absorbance. All solutions of peroxidase, IAA, and scopoletin were prepared with glass distilled water in 50 mM sodium citrate buffer at pH 5 unless otherwise indicated.

Oxygen Determinations. Oxygen concentrations were determined polarographically at 25 C with the Gilson Oxygraph, Model K. The oxidation of IAA could be monitored by following oxygen consumption provided that the initial IAA concentration was over 20 μM. In comparison, fluorometric determinations of IAA in solutions had an *upper* limit of about 50 μM and a lower limit of less than 1 μM.

Spectrophotometry. Difference spectra of peroxidase and scopoletin- or IAA-modified peroxidase were determined with the Perkin-Elmer Model 356 spectrophotometer. Additions of the reactants were made to 3-ml quartz cuvettes in the spectrophotometer with plastic plungers. Spectra were corrected for the small volume changes due to the additions. Rapid changes in the absorbance of peroxidase were determined with a stopped flow spectrophotometer manufactured by the Durrum Instrument Co. Changes in transmittance were recorded from an oscilloscope screen with a Polaroid camera. With this instrument it was possible to monitor changes taking place from about 3 msec after completion of the mixing cycle.

Determination of IAA Oxidase Activity. Cuvettes were prepared with 20 μM IAA in a final volume of 3.0 ml of 50 mM citrate buffer at pH 5.0. After equilibration at 25 C, HRP was added to the mixture with a plastic plunger and the decrease in fluorescence was recorded as a function of time. Enzyme activity was expressed as the initial rate of change in fluorescence units under standardized conditions of excitation energy, band width of the emission monochromator, and sensitivity scale of the spectrofluorometer.

Spectrofluorometry. Fluorescence intensity in solutions and emission and excitation spectra of fluorescent compounds were determined in the Perkin-Elmer MPF-2A scanning spectrofluorometer. The excitation bandwidth was adjusted to 2 nm, while the slit width of the emission monochromator was held constant at 10 nm. IAA exhibited an excitation maximum at 290 nm and a fluorescence emission maximum at 360 nm. Under these conditions IAA gave a linear relationship between molarity and fluorescence units up to a concentration of about 25 μM. Above this level, the relationship departed from linearity as shown in Figure 1. Since only one concentration (50 μM) above 25 μM was used in the detailed kinetic studies, a simple correction was made in the fluorometrically determined initial rate of reaction. Since the molar IAA fluorescence yield was diminished by 13% over the expected value, initial reaction rates (*i.e.*, rates at 50 μM) were multiplied by a factor of 1.1.

With high levels of enzyme it was possible to follow the complete degradation of IAA. The ultimate product of the reaction was less fluorescent but exhibited indole ring excitation and emission spectra qualitatively indistinguishable from those of the starting material. The kinetics of the reaction are discussed further below.

The fluorescence properties of scopoletin were sufficiently different from those of IAA to permit addition of amounts of the coumarin in excess of IAA without significantly changing

[2] Abbreviation: HRP: horseradish peroxidase.

the fluorescence intensity of the latter. The excitation and absorbance maximum for scopoletin was determined to be 360 nm, and the emission maximum occurred at 460 nm. All fluorescence spectra which are mentioned are uncorrected for instrumental or internal parameters and thus the maxima reported represent only the experimentally determined values.

RESULTS

The oxidative degradation of IAA as followed fluorometrically is not a simple conversion of the growth regulator to a single product but apparently involves one or more intermediates (14). The fluorescence characteristics of these intermediates are distinguishable from those of IAA by fluorescence intensity but not by position of the emission maxima. The reaction trace included in Figure 1 represents the time course of the change in fluorescence due to IAA oxidation after addition of 6.7 μM HRP to 30 μM IAA. Initially the fluorescence decreases linearly. However, long before IAA emission is abolished completely the fluorescence of the reaction mixture begins to increase again. This result suggests that IAA is first converted to an unstable, less fluorescent compound, and that this process is followed by the formation of another fluorescent intermediate from the first compound. The equilibrium (final) value of the fluorescence of the fully reacted mixture was 45% of the starting value. The minimum fluorescence level which was attained was around 38% of the starting level. Although the secondary reactions complicate the latter stages of the kinetics of IAA oxidation, they do not affect the initial rate of oxidation of IAA. At zero time neither the nonfluorescent

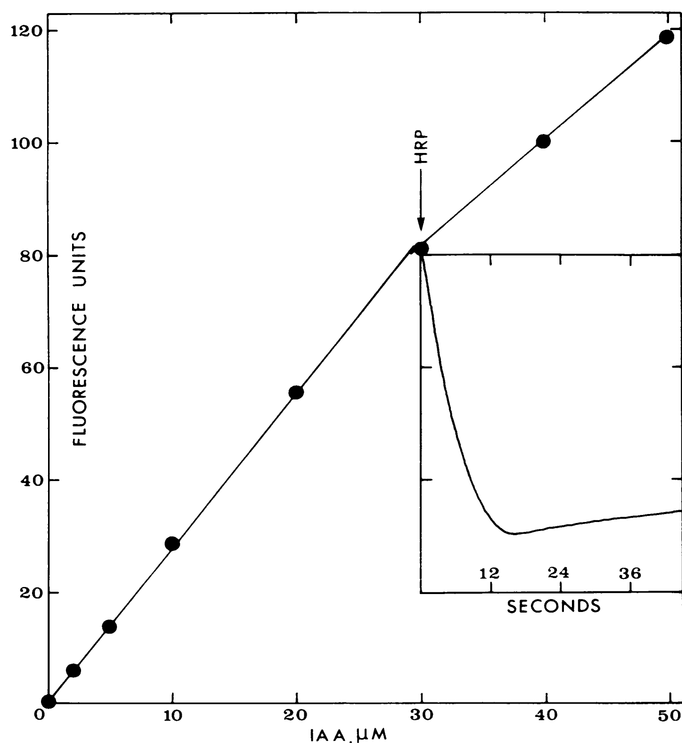


FIG. 1. Relationship between IAA concentration and fluorescence at pH 5. IAA was added to cuvettes containing a final volume of 3.0 ml of 50 mM sodium citrate buffer. Fluorescence was determined at 360 nm at 25 C under conditions where no photodegradation of IAA occurred. HRP (6.7 μM) was added to one sample mixture as indicated in the inset. The changes in fluorescence were recorded as a function of time on the indicated scale to illustrate the initial rate of degradation of IAA and the complex kinetic behavior which occurred near the end of the reaction.

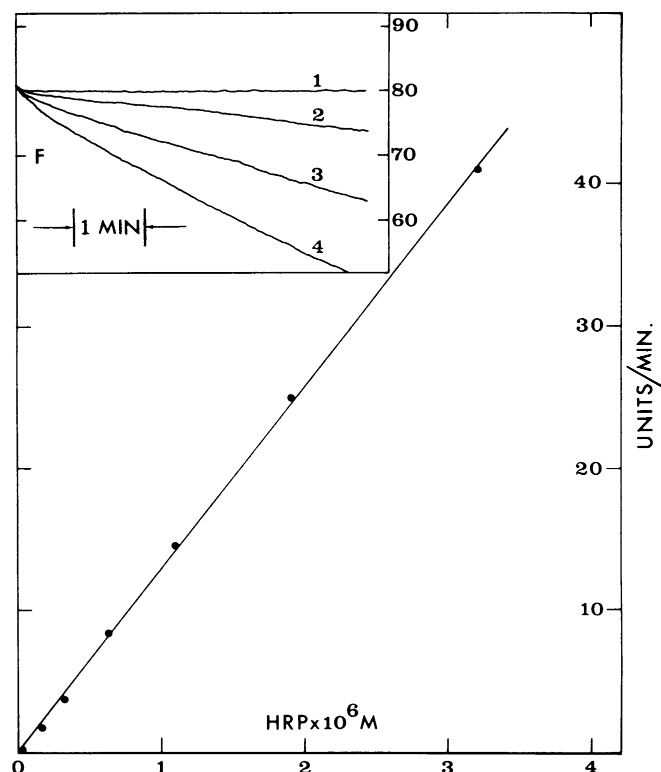


FIG. 2. Linearity of fluorometric assay for IAA oxidase activity of HRP. Cuvettes contained in a final volume of 3.0 ml: sodium citrate buffer, pH 5, 50 mM; IAA, 20 μM; dissolved oxygen, 240 μM. Peroxidase was added to the reaction mixture at zero time on a plastic plunger. The inset in the upper left-hand corner illustrates the rates of reaction at three different enzyme concentrations; curve 1, no HRP; curve 2, 0.17 μM HRP; curve 3, 0.33 μM HRP; curve 4, 0.67 μM HRP. The initial slope of the lines was determined after the rate of decrease in fluorescence had become steady.

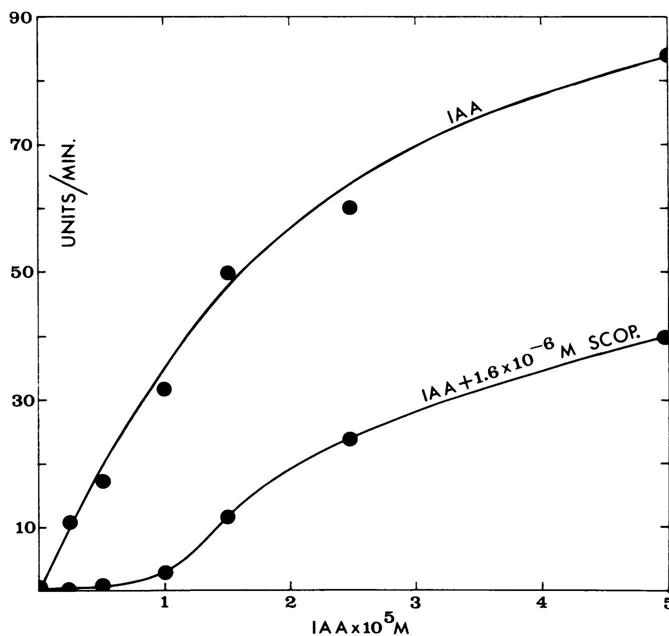


FIG. 3. Inhibition of IAA oxidase activity by scopoletin. The variation of IAA oxidase activity of 1.6 μM HRP with IAA concentration was determined fluorometrically at 25 C. The lower curve was obtained when a stoichiometric amount of scopoletin was added to the cuvette before HRP. Reactions were started by addition of the enzyme.

intermediate nor the fluorescent product is present, and the slope of the trace is a direct measure of the enzyme activity. When IAA degradation is determined fluorometrically, because of the presence of the secondary reactions, it is essential to determine the initial rate of the reaction.

Figure 2 shows that the initial rate of IAA degradation is a linear function of peroxidase concentration under the experimental conditions. The starting IAA concentration was 20 μM; this is close to the experimentally determined Km ($S_{0.5}$) value for this substrate. The substrate concentration which was employed is near optimal for the fluorometric determination of IAA oxidase activities, because above this level the fluorescence of IAA becomes a nonlinear function of concentration as can be seen from Figure 1. Below 5 μM IAA, the enzyme is far from saturation with substrate, and the response of the initial rate to enzyme concentration is nonlinear. The upper left-hand insert of Figure 2 shows typical reaction records at three different enzyme levels. In the absence of peroxidase (curve 1), there is no detectable photochemical degradation of IAA caused by the exciting radiation at 290 nm. At larger excitation slit widths (10 nm), significant nonenzymatic degradation occurred.

As mentioned, all concentrations of scopoletin which were tested were inhibitory with purified high spin HRP. Plots of reaction velocity against IAA concentration were rectangular hyperbolas in the absence of scopoletin. This result implies that the system is first order with respect to the substrate. However, when the inhibitor was added, dependence of rate on IAA concentration became markedly sigmoidal even at the lower scopoletin concentrations. Indeed, practically no reaction took place until IAA was added in 4- to 5-fold excess over the inhibitor as shown in Figure 3.

When kinetic data were obtained over a wide range of substrate and inhibitor concentrations, Lineweaver-Burk plots were curved upward as in Figure 4. The inhibition of IAA oxidation by scopoletin is competitive in the sense that it is overcome at high substrate concentration. The extrapolated maximum velocity for all the curves appears to be the same, indicating that the effect of the inhibitor is exerted primarily on IAA binding and not on the catalytic efficiency of the active site of the enzyme. The expanded scale section in the upper left-hand corner of Figure 4 shows that the curvature of the plots is much less marked at high substrate concentration. At low IAA concentrations, the reaction is vastly slower than would be expected from a simple equilibrium competition between two ligands for binding at a single active site. This result suggests that the inhibitor must alter the rate of reaction by changing the amount of substrate which is bound. This effect could result from prevention of part of the enzyme from reacting with IAA or from a change in the binding affinity of all of the enzyme for IAA. It should be noted that scopoletin and IAA were added to reaction mixtures first and that the degradation of IAA was initiated by addition of 0.33 μM peroxidase. Thus scopoletin appeared to preferentially react with the en-

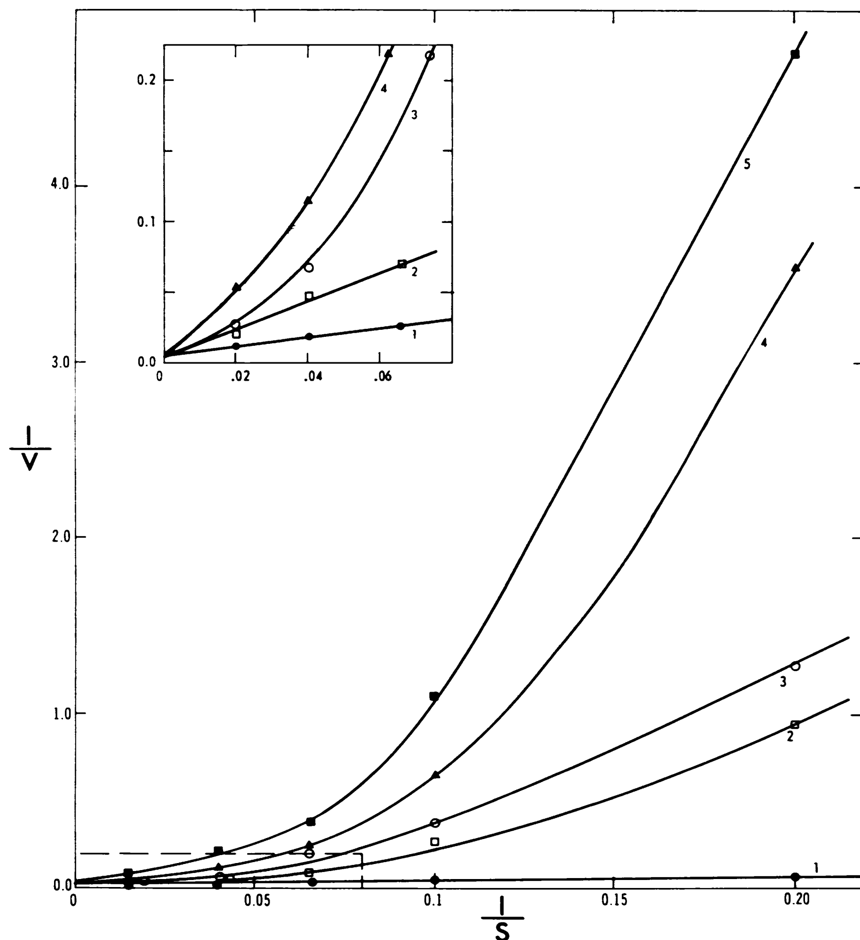


FIG. 4. Lineweaver-Burk plots of the reciprocal rate of IAA degradation against reciprocal IAA concentration. Reaction mixtures were prepared with 0.33 μM HRP in 3.0 ml of 50 mM citrate buffer at pH 5.0. Scopoletin was added as an aqueous solution as follows: curve 1, no scopoletin; curve 2, 1.6 μM; curve 3, 3.2 μM; curve 4, 4 μM; curve 5, 8 μM. The upper left-hand inset shows the data which were obtained over a much smaller range of IAA concentrations. The range is indicated by the area inside the dashed line in the lower left-hand portion of the figure.

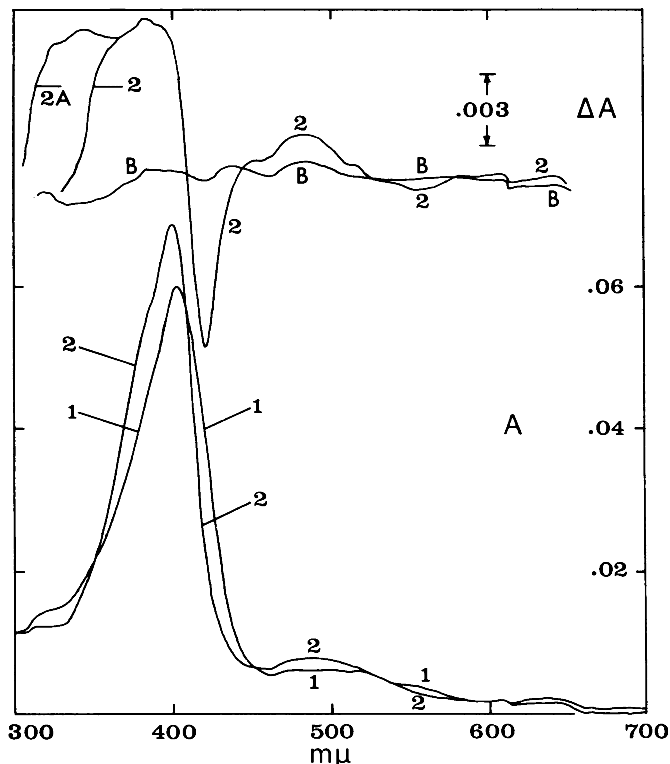


FIG. 5. Absorbance changes in peroxidase spectrum induced by scopoletin. Stoichiometric amounts (1 μM) of HRP and scopoletin were mixed together in 3.0 ml of 50 mM sodium citrate buffer at pH 5.5. The absolute spectra of 1 μM HRP (curve 1) and scopoletin-modified HRP (curve 2, lower) were determined at 25 C. Absorbance values are indicated on the right-hand vertical axis. The difference spectrum (curve 2 minus curve 1) was also recorded (curve 2, upper). Curve B was obtained with buffer in both the sample and reference cuvettes. This curve represents the baseline of the instrument on the same sensitivity scale (0.03 absorbance units full scale) which was used for the difference spectrum. Difference curve 2A was obtained when the scopoletin concentration was increased to 2 μM.

zyme, since the rate of IAA oxidation was steady initially and did not diminish unexpectedly as the reaction progressed. The spectroscopic studies discussed below confirm this indication.

Figure 5 summarizes the changes in the spectrum of 1 μM peroxidase which occur rapidly when 1 μM scopoletin is added at pH 5.5. Because of the high usable sensitivity of the spectrophotometer which was employed, it was possible to obtain reproducibly both absolute and difference spectra of the enzyme down to 310 nm at this concentration. The absolute spectra (curve 1, 2, lower part of Fig. 5) show that the Soret absorbance band of peroxidase is intensified and shifted to lower wavelengths, while absorbance at 422 nm undergoes a sharp drop on reaction with scopoletin. The difference spectrum (curve 2 minus curve 1) at the top of the figure illustrates the magnitude and direction of these changes. With a 2-fold excess of scopoletin over peroxidase, the ultraviolet absorbance of excess scopoletin appears in the region 300 to 320 nm as shown by curve 2A, but no further change in the peroxidase spectrum is observed. The reaction between scopoletin and peroxidase at 1 μM concentrations was accompanied by decrease in the fluorescence of the coumarin to approximately 80% of original value.

In order to determine if this reaction involved catalytic oxidation of the coumarin, 10 μM HRP and 100 μM scopoletin were mixed in a polarographic cell containing 50 mM citrate buffer at pH 5 and 240 μM oxygen. No uptake of molecular oxygen was observed under these conditions, indicating that the reaction was not catalytic and did not utilize dissolved oxygen.

Since the enzyme-coumarin interaction was too rapid to study by conventional means, the reactants were mixed in the stopped flow apparatus, and the resulting spectroscopic changes were recorded at 400, 420, and 430 nm. Figure 6 shows that with 0.1 mM scopoletin and 10 μM peroxidase, the decrease in absorbance near 420 and 430 nm and the increase in absorbance in the region 385 to 405 nm take place coincidentally and are therefore due to the same process. At these concentrations the reaction is complete in 150 msec. At 10-fold lower concentrations of enzyme and scopoletin, the reaction is slower, 4 to 5 sec being required for the maximum absorbance changes. It is clear that the reaction between scopoletin and 1 μM HRP is more rapid than the oxidation of IAA which is catalyzed at the same enzyme concentration. The half time of the reactions at the higher pair of concentrations was determined to be 50 msec, while the half time at the lower levels was 2 sec. The magnitudes of these absorbance changes were not strictly proportional to enzyme concentration. For example, with 0.1 mM HRP the absorbance increase at 400 nm was 0.048 per cm, while the calculated decreases at 420 nm and 430 nm were 0.063 and 0.039 per cm, respectively. With 10 μM HRP the changes were per centimeter: 400 nm, 0.016; 420 nm, 0.024. While changes of this magnitude were easily measured quantitatively with the available instrumentation, the lower enzyme concentration gave a greater percentage of change in the overall peroxidase spectrum.

The modified peroxidase spectrum did not return spontaneously to the spectrum of the native enzyme when the experiment of Figure 5 was carried out at room temperature. Moreover, thin layer chromatography of the reaction mixture showed a partial disappearance of the fluorescent- and ultraviolet-absorbing spot of scopoletin. These facts indicate that scopoletin modified the chromophoric structure of peroxidase. The enzyme remains inactive at concentrations of IAA not greatly exceeding the inhibitor concentration. Of considerable significance was the finding that addition of a 10- to 50-fold excess of IAA over scopoletin to the modified 1 μM HRP causes an immediate oxidation of scopoletin and conversion of the enzyme to compound III which then rapidly degrades all of the added IAA in about 3 min. The spectroscopic processes accompanying the conversion of HRP to compound III are readily identifiable and were described in a previous report (7). These processes are characterized by decreased absorbance at 400 nm and increased absorbance in the region of 420 nm. When the added IAA was completely degraded, HRP returned to a form having an absorbance spectrum nearly the same as that of the native enzyme. Differences which persist after IAA treatment are due to the conversion of a part of the enzyme to another form known as P-670 (14).

If IAA is added in 10-fold excess to the native enzyme, the immediate addition of scopoletin to the reacting mixture causes no spectral shift. Indeed, evidence has been obtained which indicates that in the presence of excess IAA, HRP catalytically oxidizes scopoletin, although this reaction does not affect the initial rates of IAA oxidation by the inhibited enzyme. The spectral species characteristic of the scopoletin-treated enzyme does not appear under these conditions. Since larger quantities of scopoletin may be oxidized catalytically in this way, attempts to characterize the coumarin oxidation products will be continued.

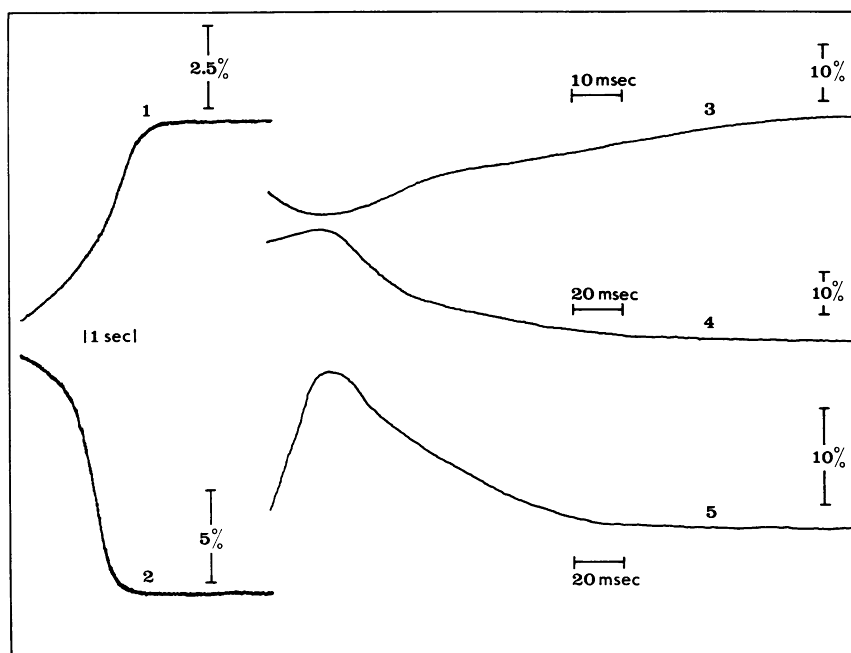


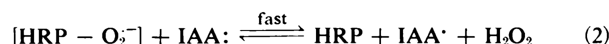
FIG. 6. Kinetics of the reaction of scopoletin with HRP. The absorbance changes due to the reaction of scopoletin with HRP were recorded from an oscilloscope screen after mixing HRP and scopoletin at 25 C. Two sets of reactant concentrations were used. Curves 1 and 2, respectively, indicate the absorbance changes at 400 nm and 420 nm when 10 μM scopoletin and 10 μM HRP are reacted. The time scale for these curves is shown by the 1-sec marks. At 10-fold higher concentrations (0.1 μM scopoletin, 10 μM HRP), curves 3, 4, and 5 were obtained at 400, 420, and 430 nm respectively. Separate time scales are shown for each curve. The transmittance scale was inverted so that an increase in absorbance caused the trace to move upward while a decrease in absorbance caused a downward movement. The initial fall or rise in transmittance which is recorded on the fast time scales for each reaction is due to mixing of the reagents which required about 10 to 15 msec. The oscilloscope trace was triggered at the beginning of the mixing process. The magnitude of transmittance changes are expressed as percent T for each trace. The transmittance expansion varied for each experiment, and the absorbance changes of curves 3, 4, and 5 are actually larger than those of curves 1 and 2 as indicated.

CONCLUSION

The results which were obtained with highly purified high spin HRP were less ambiguous than previous reports, since only inhibition by scopoletin was observed under the experimental conditions. It is possible that other highly purified plant peroxidases will yield unambiguous results which may differ from these reported here due to fundamental differences in reactivity of different isoperoxidases. Clearly, additional investigation in this area is required to elucidate the mechanisms of control of IAA degradation by peroxidase and the interaction of coumarins with the enzyme.

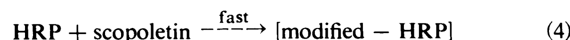
The kinetic data of Figure 4 indicate that scopoletin inhibition of IAA oxidation is not linearly competitive. Results such as these are often obtained with allosteric enzymes which show cooperative effects due to subunit interactions. Since purified HRP is not known to be an oligomeric, allosteric enzyme, a kinetic mechanism which accounts for the results may be postulated. The spectroscopic experiments are consistent with a view that scopoletin reacts rapidly with 1 μM HRP to form a stable, modified enzyme. In the presence of excess IAA this form apparently may revert to native HRP and an unidentified product of coumarin degradation which is nonfluorescent. However, this process is not an equilibrium displacement of the inhibitor by the substrate because such a situation must yield straight Lineweaver-Burk plots. The effect of the inhibitor may be to prevent binding of IAA and the subsequent conversion of the enzyme to compound III which is a compound consisting of HRP and an oxygen molecule having an additional electron. This enzyme compound is the active form which allows establishment of a rapid free radical chain reaction in which the growth regulator is degraded (7, 14). Reactions 1 through 3 illustrate this mechanism.

$$HRP + IAA: + O_2 \xrightarrow{slow} [HRP - O_2^{\cdot -}] + IAA^{\cdot} \quad (1)$$

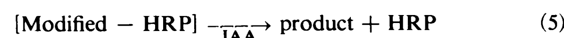


$$[HRP - O_2^{\cdot -}] + IAA: \underset{}{\overset{fast}{\rightleftharpoons}} HRP + IAA^{\cdot} + H_2O_2 \quad (2)$$

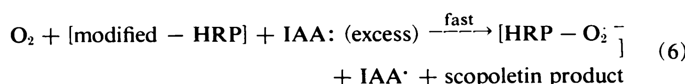
$$HRP + IAA^{\cdot} + O_2 \overset{fast}{\rightleftharpoons} [HRP - O_2^{\cdot -}] + IAA(ox) \quad (3)$$



$$HRP + scopoletin \xrightarrow{fast} [modified - HRP] \quad (4)$$



$$[Modified - HRP] \xrightarrow[IAA]{} product + HRP \quad (5)$$



$$O_2 + [modified - HRP] + IAA: (excess) \xrightarrow{fast} [HRP - O_2^{\cdot -}] + IAA^{\cdot} + scopoletin\ product \quad (6)$$

The rates of reaction 1 and 4 are known for certain sets of conditions as reported here and previously (7). With 1 μM HRP and 10 μM reactant, reaction 4 is much more rapid than reaction 1. Also, conversion of the scopoletin-modified enzyme back to HRP is a very slow reaction in the absence of IAA. However, this conversion is rapid (<1 sec) in the presence of a 10-fold excess of IAA. The mechanism, while most probably incomplete in detail, provides a rationale for the control of IAA oxidase activity by scopoletin. On the basis of the data, modification of the tertiary structure of the enzyme by the ligand (scopoletin) cannot be excluded since cooperative effects reportedly can alter binding affinities in single chain proteins (7).

LITERATURE CITED

1. Andreae, W. A. 1952. Effect of scopoletin on indoleacetic acid metabolism. Nature 170: 83–84.
2. Best, R. J. 1936. Studies on a fluorescent substance present in plants. Aust. J. Exp. Biol. Med. Sci. 14: 199–213.
3. Best, R. J. 1944. Studies on a fluorescent substance present in plants. 2. Isolation of the substance in a pure state and its identification as 6-methoxy-7-hydroxy 1:2 benzo-pyrone. Aust. J. Exp. Biol. Med. Sci. 23: 251–55.
4. Fox, L. R., W. K. Purves, and H. I. Nakada. 1965. The role of horseradish peroxidase in indole-3-acetic acid oxidation. Biochemistry 4: 2754–2763.
5. Hinman, R. L., C. Bauman, and J. Lang. 1961. The conversion of indole-3-acetic acid and 3-methyleneoxindole in the presence of peroxidase. Biochem. Biophys. Res. Commun. 4: 250–254.
6. Imbert, M. P. and L. A. Wilson. 1970. Stimulatory and inhibitory effects of scopoletin on IAA oxidase preparations from sweet potato. Phytochemistry 9: 1787–1794.
7. Kolb, D. and G. Weber. 1972. Cooperative binding due to negative interactions of two liglands in single chain proteins. Federation Proc. 31: 423.
8. Miller, R. W. and E. V. Parups. 1971. The effect of 2.2-diphenyl-1-picryl-hydrazyl and p-cresol on the oxidative degradation of indole-3-acetate. Arch. Biochem. Biophys. 143: 276–285.
9. Peisach, J., W. E. Blumberg, B. A. Wittenberg, and J. B. Wittenberg. 1968. The electronic structure of protoheme proteins. III. J. Biol. Chem. 243: 1871–1880.
10. Ricard, J. and J. Nari. 1967. The formation and reactivity of peroxidase compound. III. Biochim. Biophys. Acta 132: 321–329.
11. Ray, P. M. 1962. Destruction of indoleacetic acid. Arch. Biochem. Biophys. 96: 199–209.
12. Schafer, P., S. H. Wender, and E. C. Smith. 1971. Effect of scopoletin on two anodic isoperoxidases isolated from tobacco tissue culture W-38. Plant Physiol. 48: 232–233.
13. Tang, Y. W. and J. Bonner. 1947. The enzymatic inactivation of indoleacetic acid. 1. Some characteristics of the enzyme contained in pea seedlings. Arch. Biochem. 13: 11–25.
14. Waygood, E. R., A. Oaks, and G. A. MacLachlan. 1956. The enzymatically catalyzed oxidation of indoleacetic acid. Can. J. Bot. 34: 905–926.
15. Yamazaki, I., K. Yokota, and R. Nakajima. 1965. A mechanism and model of peroxidase-oxidase reaction. *In:* T. E. King, H. S. Mason and M. Morrison, eds., Oxidases and Related Redox Systems, Vol. I. Wiley, New York. pp. 485–513.