Partial Purification and Kinetics of Indoleacetic Acid Oxidase from Tobacco Roots[1, 2]

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Summary. Extracts from roots of *Nicotiana tabacum* L var. Bottom Special contain oxidative enzymes capable of rapid degradation of indoleacetic acid (IAA) in the presence of Mn^{2+} and 2, 4-dichlorophenol. Purification of IAA oxidase was attempted by means of ammonium sulfate fractionation and elution through a column of SE-Sephadex. Two distinct fractions, both causing rapid oxidation of IAA in the absence of H_2O_2, were obtained. One fraction exhibited high peroxidase activity when guaiacol was used as the electron donor; the other did not oxidase guaiacol. Both enzyme fractions caused similar changes in the UV spectrum of IAA; absorption at 280 mμ was reduced, while major absorption peaks appeared at 254 and 247 mμ. The kinetics of IAA oxidation by both fractions were followed by measuring the increase in absorption at 247 mμ. The peroxidase-containing fraction showed no lag or a slight lag which could be eliminated by addition of H_2O_2 (3 μmoles/ml). The peroxidase-free fraction showed a longer lag, but addition of similar amounts of H_2O_2 inhibited the rate of IAA oxidation and did not remove the lag. With purified preparations, IAA oxidation was stimulated only at low concentrations of H_2O_2 (0.03 μmole/ml). A comparison of K_m values for IAA oxidation by the peroxidase-containing and peroxidase-free fractions suggests that tobacco roots contain an IAA oxidase which may have higher affinity for IAA and may be more specific than the general peroxidase system previously described from other plant sources. A similar oxidase is present in commercial preparations of horseradish peroxidase. It is suggested that oxidation of IAA by horseradish peroxidase may be due to a more specific component.

The existence of a unique enzyme or combination of enzymes capable of degradation of IAA has been questioned in the literature (5). This view has been supported by the fact that horseradish peroxidase, in the presence of certain cofactors (Mn^{2+}, phenols), catalyzes the reaction without addition of H_2O_2 (3, 6, 7). For this reason, horseradish peroxidase has been used by numerous workers as a model system for oxidation of IAA. Thus IAA oxidase could be considered merely a peroxidase which acts in the presence of certain cofactors which serve as physiological sources of peroxide for oxidation of IAA.

Generally oxidation of IAA by IAA oxidase is characterized by a marked lag or induction period. Some workers (23) consider that the initiation of the reaction, during the induction phase, depends upon traces of H_2O_2 in the system. Since H_2O_2, when added to the system, will remove the lag period, it would appear that H_2O_2 either is or is converted into a reaction intermediate essential in an autocatalytic cyclical reaction (15). IAA oxidase therefore behaves as a typical peroxidase, but apparently is linked to an aerobic system which produces H_2O_2 as a result of the reaction of free radical intermediates with O_2 (23). Further evidence for the peroxidase nature of the enzyme was obtained by Stutz (21), who failed to separate IAA oxidase from peroxidase in preparations from *Lupinus* by means of starch block electrophoresis.

In attempts to correlate changes in IAA oxidase activity in diseased tobacco tissues (19) with the accumulation of phenolic inhibitors, it was noted that the enzyme system from tobacco roots followed unusual kinetics. Highly active preparations did not exhibit the induction period typical of IAA oxidase from other sources (17). It was felt that these results could be due to the simultaneous action of IAA oxidase and a separate peroxidase in the system. It was also noted that fresh extracts from tobacco roots contained both IAA oxidase and peroxidase activities, but the ability to oxidize IAA disappeared after the preparations were lyophilized or stored at $-10°$ for several weeks, whereas the activity on polyphenols remained unaffected. The thermal inactivation points and the pH optima for both oxidations were different. Whereas these observations were not inconsistent with the view that a single enzyme with 2 separate centers for substrate attachment was involved, it seemed more

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likely that 2 separate enzymes were present in the preparations. The purpose of these investigations was to determine whether or not enzymes catalyzing oxidation of IAA could be separated from general peroxidases present in tobacco roots. Preliminary results (20) suggested that these activities could be separated by means of standard fractionation procedures.

Materials and Methods

Preparation of Crude IAA Oxidase. Roots of tobacco plants (*Nicotiana tabacum* L. var. Bottom Special) grown for 1 month in sand culture under Sylvania Gro-Lux lights providing 700 ft-c were collected, immediately frozen in dry ice, and ground to a fine powder at dry ice temperature. The powder was suspended in cold 0.02 M KH_2PO_4 (pH 4.7) in the proportion of 10 g of tissue to 50 ml phosphate solution and the mixture was stirred for 1.5 hours at 4°. The mixture was centrifuged at 18,000 g and solid $(NH_4)_2SO_4$ was added to the supernatant fluid to 35 % saturation. The precipitate that collected overnight at 4° was removed by centrifugation as above and discarded. The supernatant was brought to 70 % $(NH_4)_2SO_4$ saturation and the precipitate that collected overnight at 4° was recovered by centrifugation and resuspended in 0.02 M KH_2PO_4 at one-fortieth of the original volume. The solution was dialyzed overnight against several changes of 2000 ml phosphate solution. This dialyzed solution, which contained both IAA oxidase and peroxidase activities, will be referred to as crude enzyme.

Chromatography. Separation of IAA oxidase and peroxidase was accomplished by elution of the crude enzyme through a column of SE-Sephadex at room temperature (20–24°). SE-Sephadex C-50 (medium grade) was first washed with 0.5 N NaOH, then with distilled water, then with 0.5 N HCl, and again with distilled water, before equilibrating with the elution buffer. In most experiments the elution buffer was 0.1 M phosphate buffer at pH 5.4. The Sephadex column had an elution volume of approximately 27 ml and was loaded with 3 to 6 ml of crude enzyme containing approximately 0.2 mg protein/ml. One ml of crude enzyme contained protein extracted from 11 g (fr wt) of tobacco roots. Buffer flow through the column was adjusted to 6 ml/hr, and 3 ml fractions were collected by means of an automatic fraction collector, and were stored at 4°. Elution of protein through the column was monitored by measuring absorbance at 280 mμ with a Beckman Model DB spectrophotometer. For accurate determinations of protein in individual fractions the method of Lowry, et al. (12) was followed.

Peroxidase Assay. Peroxidase activity in fractions of preparations eluted through the Sephadex column was determined by a modification of the procedure of Chance and Maehly (1). The reaction mixture contained 0.1 ml of 0.02 M guaiacol, 1.0 ml of 0.38 M H_2O_2, 3.8 ml 0.02 M KH_2PO_4 (pH 4.7), and 0.1 ml of enzyme. The increase in absorbance at 470 mμ, due to a colored product of the reaction, was followed with a Bausch and Lomb Spectronic 20 colorimeter for 6 minutes after the reaction was initiated.

IAA Oxidase Assay. IAA oxidase activity in fractions collected after elution of crude enzyme through the Sephadex column was determined both by colorimetric and spectrophotometric methods. The colorimetric method allowed a rapid, convenient assay of large numbers of samples, but was not entirely reliable due to precipitation of the colored product of the reaction by certain types of proteins and inhibition of color formation by nonspecific inhibitors (13). The reaction mixture consisted of 0.25 ml 1 mM 2,4-dichlorophenol, 1.00 ml of a mixture of 1 mM IAA and 0.5 mM $MnCl_2 \cdot H_2O$, 3.25 ml 0.02 M KH_2PO_4 at pH 4.7, and 0.50 ml of enzyme. The mixture was shaken in a water bath for 2 hours at 30° and then 1.0 ml of Salkowski reagent (22) was added. The mixture was shaken for an additional 3 hours and absorbance at 525 mμ was determined with a Bausch and Lomb Spectronic 20 colorimeter. To determine rates of IAA destruction, Salkowski reagent was added to samples of a reaction mixture at 10-minute intervals during the 2-hour incubation period and residual IAA measured as above.

The kinetics of IAA destruction in fractions containing high IAA oxidase activity were determined spectrophotometrically. A modification of the procedure suggested by Ray (14) was used. The reaction mixture contained 0.125 ml 1 mM 2,4-dichlorophenol, 0.375 ml of a mixture of 1 mM IAA and 0.5 mM $MnCl_2 \cdot H_2O$, 1.750 ml of 0.02 M KH_2PO_4 at pH 4.7, and 0.250 ml of enzyme sample. The cofactors, buffer, substrate, and enzyme were incubated separately at 34°, before mixing in a quartz cuvette. Changes in the absorption spectrum of IAA in the range 310-220 mμ were followed with a Beckman Model DB spectrophotometer by continuous scanning during a 20-minute incubation period. In some experiments, only the increase in absorbance at either 247 or 254 mμ was determined. The temperature of the sample compartment in the spectrophotometer remained constant at 34° during the entire incubation period.

In experiments designed to determine the effect of substrate concentration on the activity of various enzyme preparations, final concentrations of IAA ranged from 0.10 mM to 0.45 mM. In these experiments the proportion of IAA to Mn^{2+} remained constant, but the concentration of DCP in the reaction mixture was not altered.

Results

Separation of Peroxidase and IAA Oxidase. Initial attempts to separate these enzymes by column chromatography, employing a variety of column

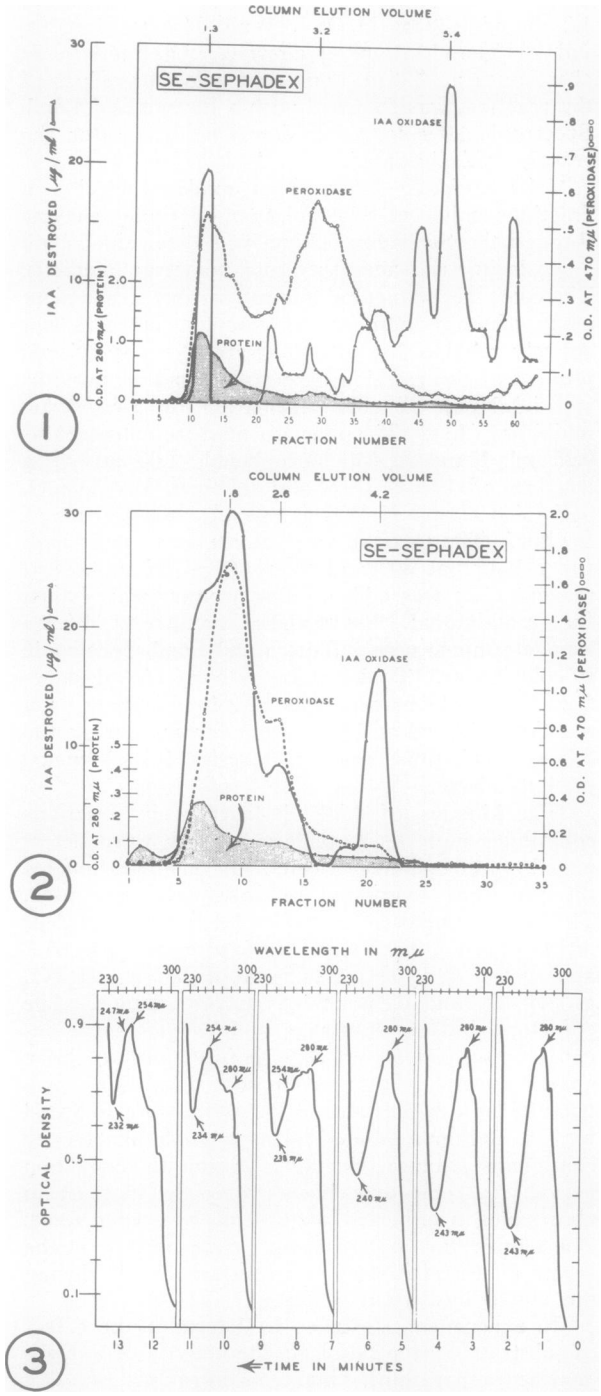


FIG. 1. (top) Chromatography of crude IAA oxidase from tobacco roots eluted through a column of SE-Sephadex. Six ml of crude enzyme [35 to 70% $(NH_4)_2SO_4$ precipitate] corresponding to 66 g tissue were placed on top of the column and were eluted with 0.1 M phosphate buffer. Column elution volume was 9 ml; flow rate was adjusted to 6 ml/hr and 3 ml fractions were collected by means of an automatic fraction collector. The relative protein content in each fraction was monitored by measuring absorbance at 280 mμ. IAA oxidase and peroxidase activities in each fraction were determined colorimetrically as indicated in the text.

FIG. 2. (middle) Rechromatography of the major protein peak obtained after elution of crude IAA oxidase through a column of SE-Sephadex. Fractions at 1.2 to 1.4 elution volumes (as in fig 1) were collected, reduced to 4 ml and placed at the top of a column of SE-Sephadex. Chromatography as described in figure 1. IAA oxidase and peroxidase activities were determined colorimetrically as indicated in the text.

FIG. 3. (bottom) Changes in the ultraviolet absorption spectrum of IAA following addition at zero time of crude IAA oxidase from tobacco roots. Reaction mixture as indicated in the text. The spectrum was scanned from 310 to 220 mμ at approximately 2.25-minute intervals for 14 minutes.

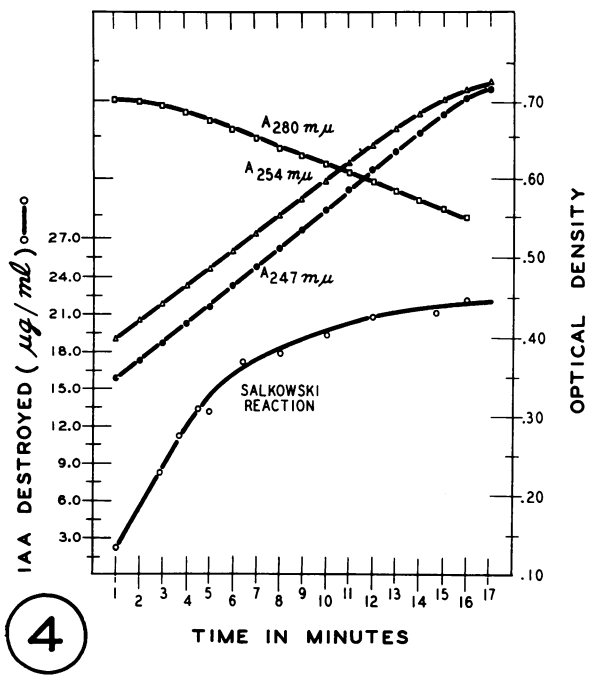
materials such as DEAE cellulose, carboxy-methyl cellulose, silica gel, DEAE-Sephadex, and others, failed. Peroxidase and IAA oxidase activities showed coincident or nearly coincident peaks after elution of crude enzyme preparations through columns of these materials. Manipulation of factors such as pH, buffer type and molarity, salt gradients, etc., did not result in separation of the 2 enzymes. However, when crude enzyme was eluted with phosphate buffer of relatively high molarity (0.1 M) from a column of SE-Sephadex C-50, it was possible to separate IAA oxidase from peroxidase. The typical elution pattern consisted of: A) a high protein peak at 1.3 elution volumes, containing high activity of both IAA oxidase and peroxidase, B) a major peroxidase peak at 3.2 elution volumes, generally with low IAA oxidase activity, and C) a major IAA oxidase peak at 5.4 elution volumes with very low or no peroxidase activity (fig 1). Fractionation through SE-Sephadex also revealed a number of minor peaks of both IAA oxidase and peroxidase. The separation of enzymes into the 3-peak pattern described above was highly reproducible when fresh enzyme preparations were used. However, resolution could be altered significantly by ageing the crude enzyme. Storage of crude enzyme at 4° for 1 week resulted in appreciable loss in activity and poor chromatographic separation. Good resolution was obtained only with fresh preparations. The storage problem could not be obviated by freezing or lyophilizing the preparations, since these treatments destroyed IAA oxidase activity but did not affect peroxidase activity.

The aggregated nature of the proteins in the 1.3 elution volume peak could be easily demonstrated by rechromatography. When this fraction was re-eluted through SE-Sephadex with 0.1 M phosphate buffer (pH 5.4), an IAA oxidase peak separated from the major peroxidase-IAA oxidase peak which was associated with the initial protein peak that eluted rapidly from the column (fig 2). Although the general pattern of elution was similar to that obtained with crude enzyme, peaks of peroxidase and IAA oxidase appeared more rapidly than with crude enzyme. The reasons for the

change in elution pattern are not clear, but it is possible that IAA oxidase may be constituted by a series of isozymes of widely different physical properties.

Our results suggested that crystalline horseradish peroxidase, which contains at least nine different components separable by starch gel electrophoresis (9), could also contain an IAA oxidase separable from other peroxidases. Crystalline horseradish peroxidases, purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, (200 units/mg) and from Calbiochem, Los Angeles, California (RZ approx. 0.6), were fractionated by SE-Sephadex chromatography as described under Methods. In each instance, the pattern of elution was similar to that described for our crude enzyme preparations from tobacco. These results suggested that our preparations from tobacco roots, as well as commercial horseradish peroxidases, contain an enzyme capable of rapidly degrading IAA and separable from peroxidases active on polyphenols. The IAA oxidase from both sources was strictly aerobic; no destruction of IAA was obtained when reaction mixtures were placed under an atmosphere of N_2.

Kinetics of IAA Oxidation. Comparisons of the kinetics of IAA oxidation by preparations containing IAA oxidase, and IAA oxidase together with peroxidase, were made. The spectrophotometric method provided a simple, convenient method of determining rates of IAA oxidation. When IAA was oxidized by crude enzyme preparations from tobacco, there were significant changes in the UV absorption spectrum of this substance. The most significant changes involved a decrease in absorption at 280 mμ, increases in absorption at 254 and 247 mμ, and gradual disappearance of a trough at 243 mμ which shifted to 232 mμ (fig 3). Of the many complex changes in the UV spectrum of IAA that occurred during the initial 5 minutes of the reaction, only a few showed linearity when IAA was disappearing at a steady rate. The changes at 280 mμ, for instance, were linear only after the first 4 minutes of the reaction (fig 4). A comparison of rates of disappearance of IAA from the solution (measured colorimetrically) with spectrum changes showed that the increases in absorbance at 247 and 254 mμ were linear when IAA destruction was also linear (fig 4). However, with crude preparations, the change in absorbance at these wavelengths was not proportional to the change in concentration of IAA. It may be inferred that these changes in absorbance do not correspond to appearance of the first product of the reaction. Furthermore, absorbance at 247 and 254 mμ continued to increase at a linear rate for almost 12 minutes after the crude-enzyme reaction was initiated, whereas colorimetric measurements indicated a considerable diminution in rate of disappearance of IAA after 5 minutes. When measured colori-



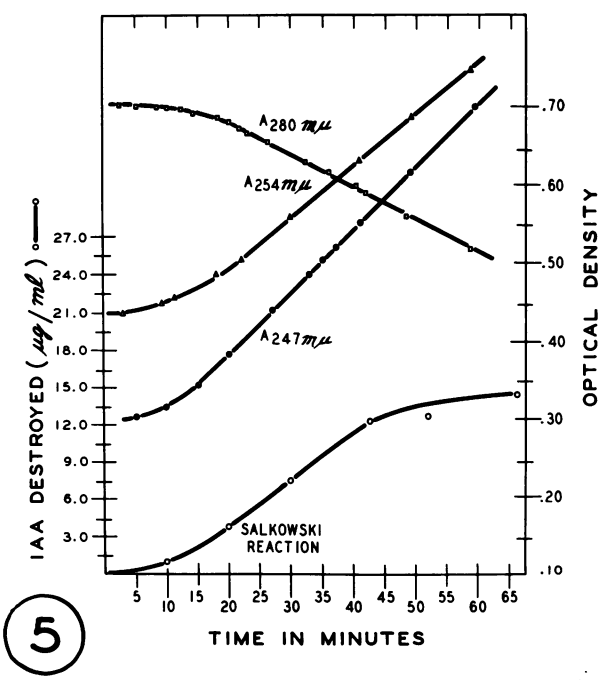
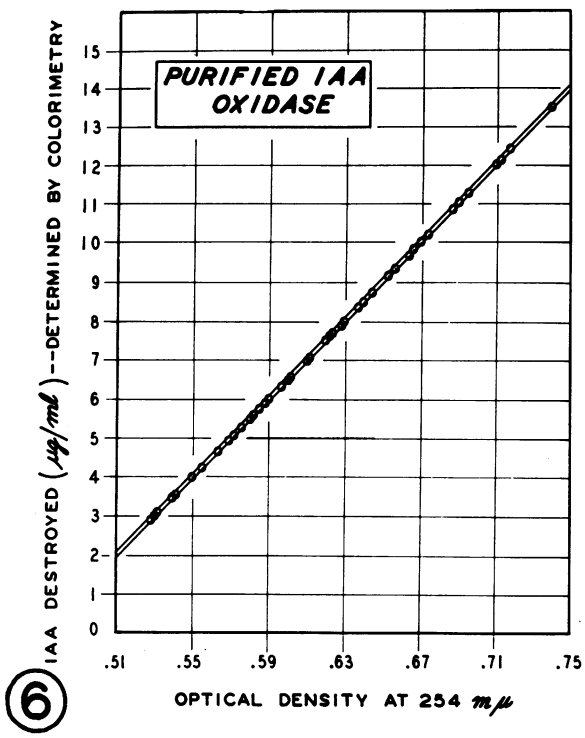
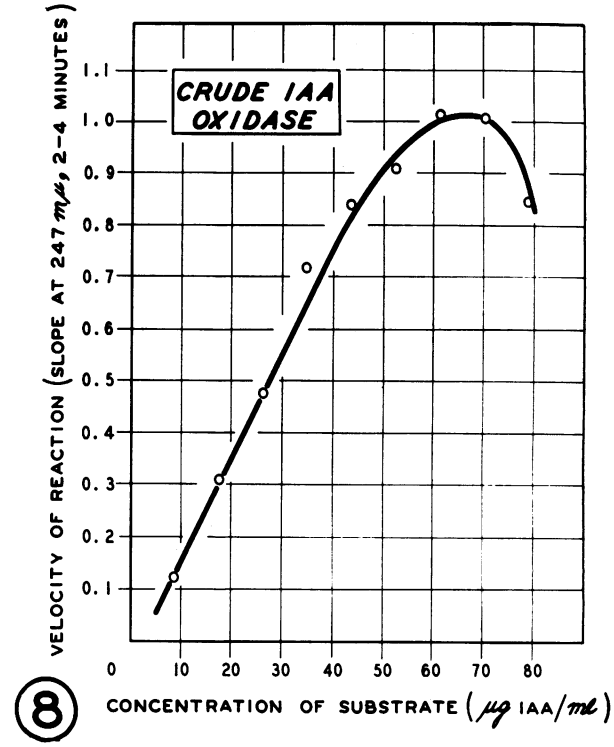
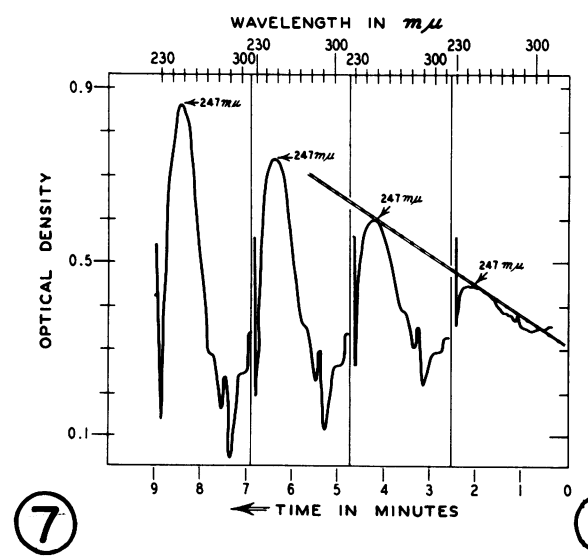


FIG. 4. (left) Comparison of kinetics of IAA oxidation by crude IAA oxidase from tobacco roots and changes in absorption spectrum of IAA at 280, 254, and 247 mμ. Rates of IAA oxidation were determined colorimetrically by means of the Salkowski reaction. Spectral changes were determined in a Beckman Model DB spectrophotometer. Reaction mixtures as indicated in the text.

FIG. 5 (right) Comparison of kinetics of IAA oxidation by purified IAA oxidase from tobacco roots and changes in absorption spectrum of IAA at 280, 254, and 247 mμ. Methods as indicated under figure 4 and in the text.







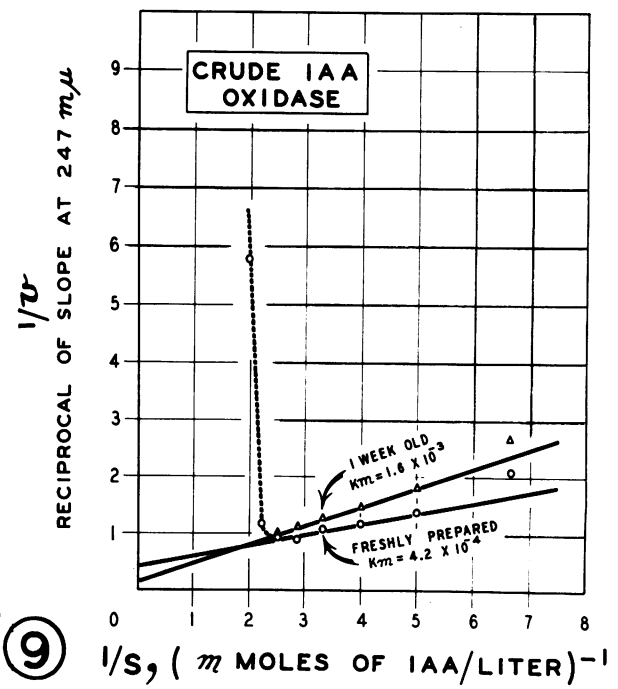


FIG. 6. (top, left) Relationship between amount of IAA destroyed by purified IAA oxidase from tobacco roots, determined by the Salkowski reaction, and changes in absorbance by IAA at 254 mμ during steady state kinetics. Reaction mixtures as indicated in the text.

FIG. 7. (bottom, left) Net change in the absorption spectrum of IAA caused by oxidation by crude IAA oxidase from tobacco roots. Both reference and sample cuvettes of a Beckman Model DB spectrophotometer contained: 0.125 ml 1 × 10^{-3} M 2,4-dichlorophenol, 0.375 ml of a mixture of 1 × 10^{-3} M IAA and 5 × 10^{-4} M $MnCl_2 \cdot H_2O$, and 1.750 ml of 0.02 M KH_2PO_4 at pH 4.7. In addition, the reference cuvette received 0.250 ml of heat-inactivated crude enzyme. At zero time, 0.250 ml of crude enzyme was added to the sample cuvette.

metrically, the apparent decrease in IAA destruction may have been due in part to an increase in 1 of the products of the reaction which reacted strongly with Salkowski reagent. Extrapolation of the initial rate determined by colorimetry suggests that the reaction would have been completed in approximately 10 minutes, whereas absorbance at 247 and 254 mμ continued to change even after no further change in IAA content could have occurred. Changes in the UV spectrum of IAA are very complex and probably represent a number of unstable products of the reaction (6, 14). There is no clear choice as to changes at specific wavelengths that would be representative of the kinetics of the reaction catalyzed by crude preparations.

Specific spectral shifts of IAA caused by crude and purified preparations of IAA oxidase were similar, but the kinetics of the reactions with these 2 preparations differed in 2 important respects. First, the purified preparations invariably showed a relatively long, 10- to 15-minute lag or induction period (fig 5). Crude preparations or fractions containing high peroxidase activity showed no lag or a very short, 1- to 2-minute lag period. Second, after steady state kinetics were obtained with purified preparations, the increases in absorption at 254 and 247 mμ were proportional to the changes in concentration of IAA in the solution (fig 5). Thus, the presence of less substrate-specific peroxidases alters the kinetics of IAA oxidation very significantly and introduces serious questions as to the usefulness of model systems for oxidation of IAA based on commercial peroxidase preparations. At both 254 and 247 mμ, excellent correlations were obtained between increase in absorbance and amounts of IAA destroyed within the range of 3 to 13.5 μg IAA/ml of reaction mixture; that for 254 mμ is illustrated in figure 6.

Although increases in absorbance at either 254 or 247 mμ could be used to advantage in the determination of rates of IAA destruction with purified preparations, the absorbance peak at 247 mμ provided the wavelength of choice. When IAA was placed in both sample and reference cuvettes of a double-beam spectrophotometer and enzyme was added only to the sample cuvette, the net change in the spectrum of IAA caused by oxidation could be determined. A single broad peak at 247 mμ appeared (fig 7), indicating that the difference between extinction coefficients of IAA and of products of the reaction was greater at this wavelength than at 254 mμ. For this reason the kinetics of the reaction were determined by recording the increases in absorbance at 247 mμ.

Because of the differences in lag period between preparations that contained both IAA oxidase and peroxidase, and those that contained only IAA oxidase, data on kinetics of IAA oxidation were obtained during the first 2 to 4 minutes at steady state. Using this method the effect of substrate concentration was determined for both crude and partially purified IAA oxidase preparations. Both preparations showed approximately linear responses between 0.05 and 0.35 μmole IAA/ml of reaction mixture and both showed rapid, marked inhibition at substrate levels above 0.40 μmole/ml (fig 8). This marked substrate inhibition made it difficult to prepare reciprocal plots for calculation of the Michaelis constant (K_m) by the method of Lineweaver and Burk (10), because the left-hand portion of the plot curved upwards as it approached the y axis. Also, at substrate concentrations below 0.2 μmole/ml, the right-hand portion of reciprocal plots invariably curved upwards. However, such plots indicated an approximate straight line between substrate concentrations of 0.2 and 0.4 μmole/ml (fig 9). By extrapolation to the y axis, it was possible to obtain approximate K_m values. These values varied considerably depending on age of the preparations. Fresh crude preparations gave values between 0.42 and 0.45 mM; preparations that had been allowed to stand at 4° for 1 week gave values of approximately 1.6 mM. Purified preparations containing no peroxidase activity gave values ranging from 0.06 to 0.10 mM; similar preparations gave values of 0.13 mM when allowed to stand for several days.

Specific Activity of IAA Oxidase. Since the change in absorbance at 247 or 254 mμ was correlated with disappearance of IAA from solutions containing purified IAA oxidase, it was possible to convert the slope (Δ OD/Δt) at steady state to μg of IAA destroyed/(min × ml) as shown in figure 10. One unit of IAA oxidase was defined as that which will destroy 1 μg IAA/(mg protein × min). Calculations of specific activities of a crude preparation and of various fractions obtained after elution through a column of SE-Sephadex are shown in table I. The elution pattern in this particular experiment was similar to that shown in figure 1. Purification of IAA oxidase resulted in a 450-fold increase in specific activity. This value is only approximate, since the method of determining specific activities was not directly applicable

FIG. 8. (top, right) Effect of substrate concentration on rate of IAA oxidation by crude IAA oxidase from tobacco roots. Velocity of the reaction is expressed in terms of the slope (Δ OD × $10/\Delta t_{min}$) at 247 mμ during steady state kinetics. Reaction mixtures as indicated in the text.

FIG. 9. (bottom, right) Lineweaver-Burk reciprocal plot illustrating inhibition by excess substrate on activity of crude IAA oxidase from tobacco roots and method of determining the value of 1/V for calculation of K_m constants The plot also depicts alteration in K_m values due to ageing of the preparation for one week at 4°. Reaction mixtures as indicated in the text. Velocity of the reaction expressed as the slope (Δ OD × $10/\Delta t_{min}$) at 247 mμ during steady state kinetics.

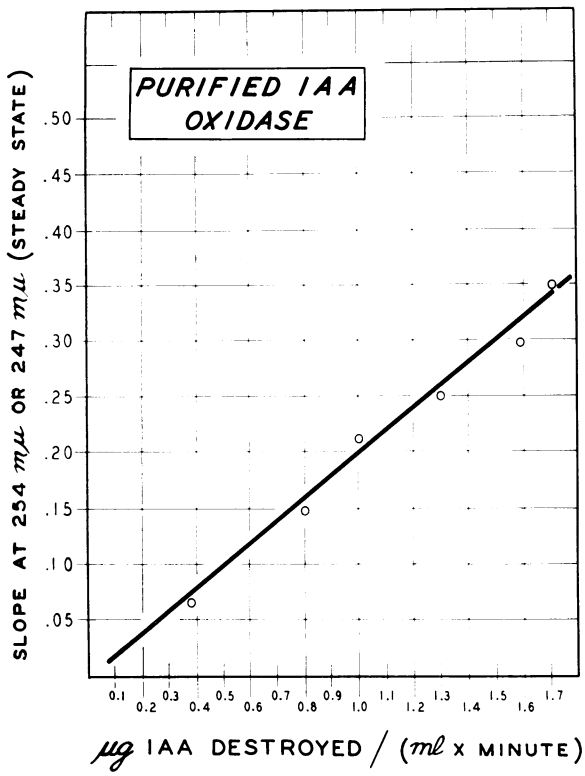


FIG. 10. Relationship between rates of IAA oxidation by purified IAA oxidase from tobacco roots, determined by the Salkowski reaction, and slope ($\triangle$ OD $\times$ 10/$\triangle t_{min}$) at 247 or 254 mμ during steady state kinetics Reaction mixtures as indicated in the text.

to crude preparations, because of problems in the kinetics of the system described previously.

Effect of Hydrogen Peroxide. Addition of catalytic amounts of H_2O_2 affected preparations containing both peroxidase and IAA oxidase, and those containing only IAA oxidase, very differently. With fractions containing both enzyme activities, addition of 3 μmoles H_2O_2/ml caused a rapid increase in rate during the first minute of the reaction, thereby eliminating the short induction period characteristic of preparations containing peroxidase. After this short period of very rapid degradation of IAA, the reaction proceeded at approximately

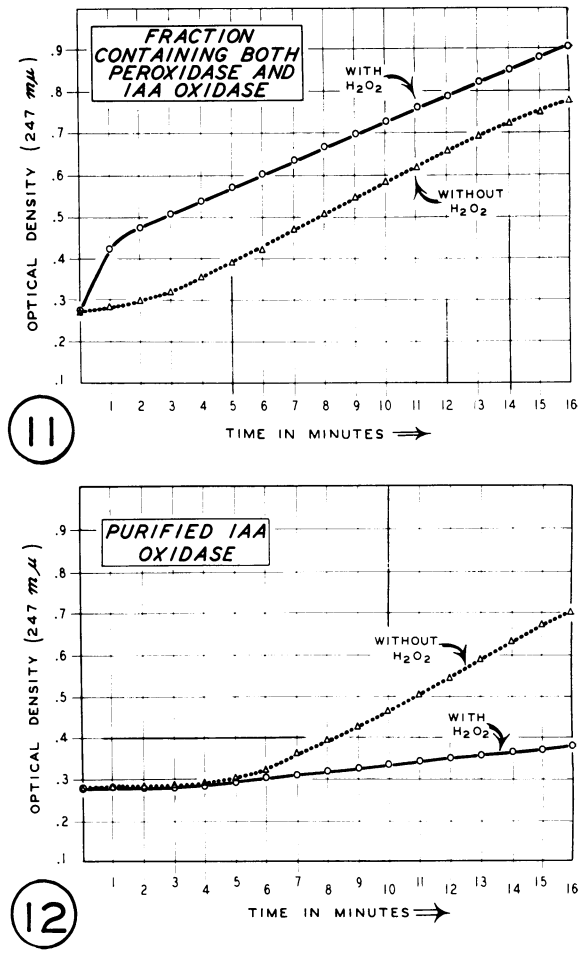


FIG. 11. (top) Effect of hydrogen peroxide (3 μmoles/ml) on kinetics of IAA oxidation by a fraction (No. 7, fig 2) containing both peroxidase and IAA oxidase activities. Reaction mixtures as indicated in the text, except for addition of 0.02 ml 0.4 M H_2O_2 at zero time to the reaction illustrated by the curve (○ — ○) in the upper portion of the graph.

FIG. 12. (bottom) Effect of hydrogen peroxide (3 μmoles/ml) on kinetics of IAA oxidation by purified IAA oxidase containing no peroxidase activity (fraction No. 21, fig 2). Reaction mixtures as indicated in the text, except for addition of 0.02 ml 0.4 M H_2O_2 at zero time to the reaction illustrated by the curve (○ — ○) in the lower portion of the graph.

Table I. *Kinetics and Specific Activities of Various Fractions Obtained by Elution of Crude IAA Oxidase from Tobacco Roots through a Column of SE-Sephadex*

Fraction no.	Column elution volumes	μg protein/ml	Slope at steady state**	μg IAA destr. ml × min	Units*
Crude	...	200.0	0.50	2.47	12
18	2.0	11.3	0.58	2.88	256
22	2.5	2.9	0.51	2.50	870
38	4.2	0.5	0.51	2.53	5060
50	5.6	0.2	0.22	1.08	5400

* One unit IAA oxidase activity = 1 μg IAA destroyed/(mg protein × min).
** Calculated from increase in absorbance at 247 mμ during steady state kinetics ($\triangle$ OD × 10/$\triangle t_{min}$).

the same rate as in preparations without added H_2O_2 (fig 11). When partially purified IAA oxidase was used, addition of similar amounts of H_2O_2 resulted in marked inhibition of IAA destruction and the induction period was unaltered (fig 12). These results indicated that IAA oxidase is extremely sensitive to H_2O_2 and suggested that inhibition could result from complex III formation (1) or from nonspecific attack by H_2O_2 on S-H bonds in the protein molecule. That complex III may be involved is suggested by the fact that if the H_2O_2 concentration was reduced to 0.03 μmole/ml, slight stimulation of the reaction, rather than inhibition, was obtained. The slopes at 247 mμ during steady state were 0.65 before addition and 0.75 after addition of 0.03 μmole H_2O_2. Even at this stimulatory level, however, H_2O_2 did not reduce the induction period.

The extreme sensitivity of the purified IAA oxidase to H_2O_2 suggested that the lack of guaiacol oxidation by this enzyme, in the presence of H_2O_2, could be due to complex III formation rather than to its inability to oxidize polyphenols. Because peroxidase assays were carried out at concentrations of H_2O_2 that inhibit IAA oxidation, a similar effect could exist in the case of guaiacol. However, a reduction in concentration of H_2O_2 from 3 μmoles/ml to 0.003 μmole/ml did not induce guaiacol oxidation by purified IAA oxidase. No oxidation of guaiacol occurred in the absence of H_2O_2. Similar results were obtained when pyrogallol (0.02 M) was used as the electron donor. It was concluded, therefore, that complex III formation was not the factor responsible for the low level of polyphenol oxidation exhibited by purified IAA oxidase.

Discussion

The experiments described in this paper have shown that tobacco roots contain an oxidative enzyme with higher affinity for IAA than that exhibited by peroxidases capable of oxidizing guaiacol and pyrogallol. This enzyme is more specific in its action and may represent a true IAA oxidase. The fact that a similar enzyme can be recovered from commercial, crystalline horseradish peroxidase suggests that IAA oxidase may be primarily responsible for oxidation of IAA in systems in which horseradish peroxidase has been used as the catalyst (3, 6, 7, 23). Although purified IAA oxidase was rapidly inhibited by usual catalytic amounts of H_2O_2, the promotive effects of peroxide at extremely low concentrations would indicate that this enzyme can act peroxidatically on IAA. The difficulties we encountered, and those reported by others (21), in attempts to separate IAA oxidase from peroxidase suggest that the 2 enzymes are intimately associated and may exist as aggregates. Elution of crude tobacco peroxidase through a column of SE-Sephadex suggested that our preparations, as well as those available commercially, consist of a series of enzymes with fairly well defined substrate specificities. Although Klapper and Hackett (9) reported no difference in catalytic properties among multiple components of horseradish peroxidase, other workers (8) have reported differences in the substrate specificities of various components of horseradish peroxidase. Also, Lockhart (11) obtained an effective peroxidase from *Xanthium* leaves which was incapable of destroying IAA even in the presence of DCP. Thus present evidence suggests that true IAA oxidase may consist of a component, or components, of a highly heterogenous mixture of plant peroxidases. Differences in the relative amounts of these components may have considerable effect on the enzymatic activities of unfractionated peroxidase preparations.

Because crude or peroxidase-contaminated preparations of IAA oxidase showed significantly different kinetics from those of purified fractions, it seems likely that interactions of peroxidase with phenolic cofactors of IAA oxidase may result in complexes which affect the rate of IAA oxidation. A 3-phase kinetic pattern, typical of IAA oxidase from other sources (17) was obtained only with purified IAA oxidase preparations which contained low or no peroxidase activity. In crude preparations, the presence of high peroxidase activity reduced the induction phase sharply. The 2 types of reaction appear to differ kinetically. With purified IAA oxidase the reaction was autocatalytic. The induction period probably represents the gradual increase in concentrations of reaction intermediates upon which the rate of IAA oxidation depends (18). The induction period became shorter as the activity of peroxidase increased, and with crude preparations the reaction did not appear to be autocatalytic. The interaction of IAA and peroxidase, therefore, resulted in secondary complications on the induction period and steady state rate. The relative contributions of each enzyme to the observed kinetics could not be assessed.

Purified IAA oxidase obtained from tobacco roots or from fractionated horseradish peroxidase exhibited kinetics which were similar to those described by Ray (14, 18) for the IAA oxidase from *Omphalia flavida*. However, the fungal enzyme exhibited peroxidase activity on pyrogallol, and Ray (16) considered that both peroxidase and IAA oxidase activities were due to 1 enzyme, since both activities were parallel during thermal inactivation and purification of the enzyme. Unlike our preparations, the *Omphalia* enzyme was not inactivated by relatively high concentrations of H_2O_2. Thus it seems that tobacco IAA oxidase has catalytic properties that differ significantly from those of the *Omphalia* enzyme.

The changes in the UV spectrum of IAA during enzymatic oxidation by the tobacco oxidase were surprisingly similar to those reported by Hinman and Frost (6) for horseradish peroxidase and by Ray (14) for the *Omphalia* enzyme. With all these

enzymes, increases in absorption at approximately 254 and 247 mμ concurrent with IAA oxidation have been reported. From our results it appears that the main increase in absorption occurs at 247 mμ, although it is likely that the changes at both 247 and 254 mμ correspond to the same product. Hinman and Lang (7) consider that the product of the peroxidase catalyzed oxidation of IAA is 3-methyleneoxindole, which has absorption peaks at 247 and 253 mμ. Although this reaction may not be entirely applicable to our enzyme preparations, it is clear that increases in absorbance at 254 or 247 mμ may correspond to the oxindole product and not to a first product of the reaction, since absorbance at these wavelengths continued to change even after no further change in IAA content could be detected by the Salkowski reaction (7, 14). In our enzyme preparations, analysis of the spectral changes is complicated by interference in the colorimetric determination of IAA by Salkowski positive products of the reaction. It is evident, however, that the destruction of IAA results in the formation of a number of unstable intermediates leading to a product that absorbs strongly at 254 and 247 mμ. Although these spectral changes were correlated with rates of IAA disappearance at steady state (with purified IAA oxidase), and these changes could be used to determine rates of the reaction, simple extrapolation in figure 5 indicates that the spectral changes did not level off at the time that substrate would have become limiting if the reaction had been carried to completion.

It is interesting to note that reciprocal plots calculated from rates obtained at steady state gave straight lines only within a relatively narrow range of substrate concentrations. The fact that a curved reciprocal plot rather than a straight line was obtained at concentrations below 0.2 μmole/ml may be of considerable significance. Curves of this type may arise from the fact that the active ES complex contains two substrate molecules (2). At relatively high substrate concentrations (0.2 to 0.4 μmole/ml) the curve may approximate a straight line, giving an apparent Michaelis constant, because the dissociation constant for the second molecule may be small in relation to other K's. The kinetics are further complicated by the fact that rate inhibition was obtained at high substrate concentrations, suggesting that 2 substrate molecules bind to the same active site (4). Sufficient data are not available at present to allow an evaluation of the various alternative explanations for nonlinear plots.

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