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# Indoleacetic Acid Oxidase: A Dual Catalytic Enzyme?

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## ABSTRACT

**The isolation of a unique enzyme capable of oxidizing indoleacetic acid, but devoid of peroxidase activity, has been reported for preparations from tobacco roots and commercial horseradish peroxidase. Experiments were made to verify these results using enzyme obtained from *Betula* leaves and commercial horseradish peroxidase. Both indoleacetic acid oxidase and guaiacol peroxidase activity appeared at 2.5 elution volumes from sulfoethyl-Sephadex. These results were obtained with both sources of enzyme. In no case was a separate peak of indoleacetic acid oxidase activity obtained at 5.4 elution volumes as reported for the tobacco enzyme using the same chromatographic system. Both types of activity, from both sources of enzyme, also eluted together during gel filtration. Successful column chromatography of *Betula* enzyme was dependent upon previous purification by membrane ultrafiltration. These results indicate indoleacetic acid oxidase activity and guaiacol peroxidase activity are dual catalytic functions of a single enzyme.**

The molecular "residence" of IAA oxidase activity is not known precisely. And this imprecision in our knowledge about IAA oxidase activity is a hindrance toward further understanding about the regulation of IAA levels in living plants.

Up to now, three hypotheses, with varying amounts of substantiating evidence, have been reported in the scientific literature. The first considers that the two types of activity (*i.e.*, IAA oxidase and peroxidase) are present on separable and distinct enzymes; the second considers that the two types of activity are resident on one enzyme (peroxidase) but with two active centers; and the third calls attention to the fact of peroxidase isoenzymes where one member of the family of isoenzymes may be the primary residence of IAA activity. These are examined briefly.

The idea of separate enzymes was reported by Sequeira and Mineo (8). They had noted that fresh preparations (tobacco roots) lost IAA oxidase activity after several weeks in storage, whereas peroxidase activity was unchanged. Further, they found that thermal inactivation points and pH optima were different. Attempts to separate the two types of activity on columns of silica gel, carboxymethyl cellulose, diethylaminoethyl cellulose, and diethylaminoethyl Sephadex failed, but with SE[1]-Sephadex and 0.1 M eluting buffer they reported a major IAA oxidase peak (at 5.4 elution volumes) with little or no peroxidase activity from both tobacco root extracts and commercial HRPO.

The belief that both types of activity reside with one enzyme (*i.e.*, peroxidase) is more widely held. Evidence offered in support of this belief is mainly that both types of enzyme activity remain together through various stages of purification (7, 10), and also there is evidence that thermal inactivation is the same for both (7). The work of Siegel and Galston (9) suggests that the dual catalytic functions of peroxidase may result from two active sites on the enzyme. By separating the apoenzyme from its heme prosthetic group with acidified acetone, they found that apoenzyme alone would oxidize IAA, but was devoid of peroxidase activity. However, partial restoration of peroxidase activity occurred with recombination of heme and apoenzyme. They concluded that apoenzyme possesses the IAA oxidase function, and that a heme-protein attachment is needed for the peroxidase function. The IAA oxidase activity of apoenzyme, from horseradish peroxidase, prepared in similar fashion has been found in the author's laboratory also. However, Ku *et al.* (5) have recently found that horseradish peroxidase cleaved into heme and apoenzyme by acid butanone is nearly devoid of IAA oxidase activity.

In most studies, the *total* oxidase and/or peroxidase activity has been measured without consideration of the multiple forms of an enzyme (*i.e.*, isoenzymes). Macnicol (6) has separated four isoenzymes (three cationic and one neutral) from Alaska peas. He found that all of the isoenzymes could catalyze the peroxidation of guaiacol and the oxidation of IAA, but the $C_3$ cationic species had an IAA oxidase-guaiacol peroxidase ratio that was 10-fold higher than the next most active species.

In this paper, the purification of IAA oxidase from *Betula alleghaniensis* leaves is reported. The purpose of the experiments was to determine (*a*) if IAA oxidase activity could be separated from guaiacol peroxidase activity, or (*b*) if both types of activity were common to a single enzyme. Results obtained with *Betula* enzyme were compared to results obtained with commercial HRPO.

The data obtained from these experiments do not support the contention of Sequeira and Mineo (8) that a separate peak of IAA oxidase activity, devoid of peroxidase activity, can be separated at 5.4 elution volumes on a column of SE-Sephadex. On the contrary, both types of activity moved together during membrane ultrafiltration, gel filtration, and gel ion exchange chromatography. Results obtained with the *Betula* enzyme agreed with those obtained with commercial HRPO. It was concluded that both types of activity reside on one enzyme.

[1] Abbreviations: SE: sulfoethyl; HRPO: commercial horseradish peroxidase (Nutritional Biochemicals Co., Cleveland, Ohio). Mention of a particular chemical or product should not be taken as endorsement by the Forest Service or the United States Department of Agriculture.

## MATERIALS AND METHODS

**Crude Enzyme Preparation.** Leaves were taken from 10-week-old *Betula* seedlings, cut into small pieces, extracted with 50 mM acetate buffer, pH 5.2, and centrifuged at 20,000*g* for 20 min. All crude extracts were treated with hydrated PVP before making assays for activity or conducting further purification steps.

**Membrane Ultrafiltration.** Routinely, 50 ml of crude extract, treated with polyvinylpyrrolidone, were added to an Amicon (Amicon Corp., Lexington, Mass.) ultrafiltration cell above an XM-100A membrane (retains molecules above 100,000 mol wt). Filtration was carried out under pressure (18 p.s.i. $N_2$) with magnetic stirring. Filtrate was collected in 10-ml fractions (ice bath) at a flow rate of 1 ml/10 min. Cold extracting buffer was used to replenish liquid volume in the ultrafilter cell as filtration proceeded.

The XM-100A filtrate fractions were concentrated on a UM-10 membrane (retains molecules above 10,000 mol wt) under 40 p.s.i. $N_2$ at a flow rate of 1 ml/5 min. The retained fraction from this second ultrafiltration was used for further purification.

**Column Chromatography.** Gel filtration with Bio-Gel P-100 (100–200 mesh) was carried out in the cold (4 C) in columns 2.5 × 28.0 cm. Flow rate was usually around 10 ml/hr, and 5-ml fractions were collected. Cold extracting buffer (50 mM acetate, pH 5.2) was used to equilibrate the gels and to elute the protein fractions.

Ion exchange chromatography on porous gel was carried out at 4 C with SE-Sephadex C-50, medium grade, packed in 2.5- × 28.0-cm columns. Cold, 0.1 M phosphate buffer, pH 5.4, was used for equilibration and elution of protein. Flow rates were maintained at about 8 ml/hr, and 5-ml fractions were collected.

Effluent from both types of columns was monitored at 280 nm for protein estimation.

**IAA Oxidase Assays.** Oxygen uptake was measured with a Clark type oxygen cathode (Gilson Medical Electronics, Middleton, Wisc.) and recorded. Reaction mixtures for *Betula* enzyme consisted of: enzyme plus distilled water to equal 0.6 ml; 0.4 ml of 0.2 M acetate buffer, pH 3.6; 0.1 ml of 9 mM *p*-coumaric acid; 0.1 ml of 4 mM $H_2O_2$; and 0.2 ml of 1.5 mM IAA. IAA oxidase activity in HRPO was measured similarly, but *p*-coumaric acid and $H_2O_2$ were omitted from the reaction mixture, and enzyme plus distilled water equaled 0.8 ml. In both cases, total volume was 1.4 ml. All assays were made at 35 C. Control mixtures without enzyme were inactive.

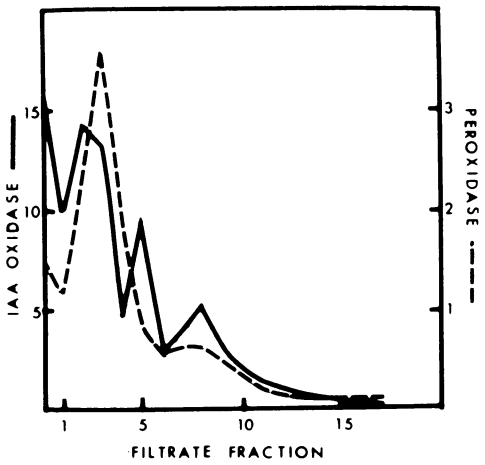


FIG. 1. Membrane ultrafiltration of crude enzyme extracted from *Betula* leaves. The first point on the Y-axis represents the activities measured in the crude extract before filtration was begun. Assays were conducted as in "Materials and Methods" except that an additional 0.1 ml of 4 mM $H_2O_2$ was required to measure full IAA oxidase activity. (-----: $\Delta A_{470\ nm}/20$ min × $10^1$; ——: $\mu$g $O_2$/min)

**Peroxidase Assays.** Peroxidase activity was measured by change in absorbance at 470 nm. Basic composition of the reaction mixtures for *Betula* enzyme and HRPO consisted of: 3.8 ml of 0.2 M acetate buffer, pH 3.6; 0.2 ml of enzyme; 0.1 ml of 20 mM guaiacol; and 1 ml of 40 mM $H_2O_2$. Total volume was 5.1 ml. For some column chromatography experiments, the amount of enzyme was increased and the amount of buffer decreased by an equal amount to maintain the same total volume. Reactions were carried out at 35 C.

## RESULTS

Initial attempts to reduce the nonenzymatic protein of crude extracts by using small pore Sephadex gels were unsuccessful. Protein was smeared across the entire elution diagram, and enzymatic activity (barely measurable) was eluted in different places in replicated trials. Both Sephadex G-25 and G-50 gave highly variable results.

It was postulated that the viscous material present in crude extracts was responsible for preventing the classical chromatographic separations that are usually possible with these gels. Consequently, a typical desalting experiment, as described by Flodin (3), was conducted with NaCl dissolved in crude extracts. Salt and protein did not elute in well-separated peaks. Both components eluted together across the entire elution diagram.

**Ultrafiltration.** Gross fractionation of protein in crude extracts was finally achieved by high pressure ultrafiltration through microporous membranes. The initial filtration retained molecules greater than 100,000 mol wt (such as polyphenoloxidase and catalase) and filtered molecules of lower molecular weight. In addition, the troublesome viscous material was retained above the membrane.

IAA oxidase activity and guaiacol peroxidase activity (assumed to be around 40,000 mol wt) were both present in all filtrate fractions (Fig. 1). The bulk of enzyme activity was collected in the first five fractions, and total filtration of activity was nearly achieved after 15 fractions; though a small amount of activity, both oxidase and peroxidase, was still measurable in the retained fraction. In the data given here (Fig. 1) and in other data (not shown), several of the filtrate fractions showed enzyme activity (oxidase and/or peroxidase) in excess of the activity measured in crude extracts before filtration. In order to measure the maximum velocity of IAA oxidase activity in these filtrate fractions, an additional 0.4 $\mu$mole $H_2O_2$ was required. This indicated that large quantities of phenol inhibitor were moving with the enzyme during filtration.

Both types of enzyme activity in these XM-100A filtrates were successfully concentrated on a UM-10 membrane, which retained all molecules above 10,000 molecular weight. Tests of the UM-10 filtrates showed that smaller size proteins were passing, but both types of enzyme activity were retained by this membrane. The concentrated enzyme had a watery consistency and a faint yellow color. Since only about one-tenth the amount of this purified enzyme was needed to give the same initial velocity of IAA oxidase activity as in crude extracts, it appeared that a 10-fold increase in purification had been achieved.

**Gel Filtration.** The partly purified and concentrated *Betula* enzyme obtained with ultrafiltration was used in all column chromatography experiments. Commercially purified HRPO was also chromatographed on the same columns and in identical systems.

Bio-Gel P-100 fractionates over the range 5,000 to 100,000 molecular weight, and is estimated (by the manufacturer) to pass molecules of 40,000 mol wt (calibrated on egg albumin) at 1.42 elution volumes. Both IAA oxidase activity and guaiacol peroxidase activity from *Betula* leaves appeared at approximately 1.6 elution volumes (Fig. 2) in well defined peaks. Major peaks of protein appeared before and after this point. Commercial HRPO produced major peaks of oxidase and peroxidase activity at 1.5 elution volumes (Fig. 3). The major protein peak of HRPO eluted in the same volume as the enzyme activity.

Since each type of activity was measured by a different method and expressed in different units, it is not possible to say that any particular fraction has more or less of one particular type of activity than another. The results obtained in these experiments were quite reproducible.

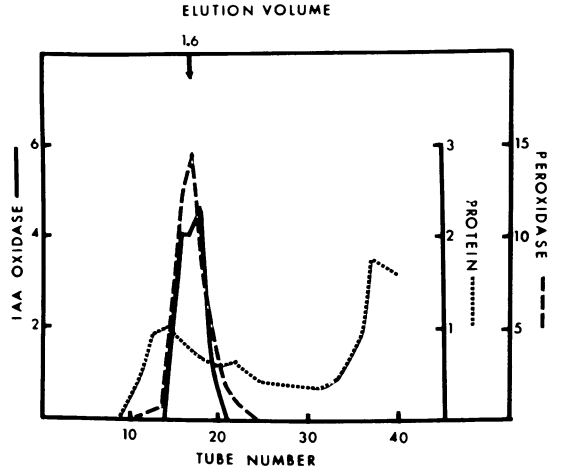


FIG. 2. Gel filtration of *Betula* enzyme on Bio-Gel P-100. The 2-ml sample applied to the column was partly purified and concentrated by membrane ultrafiltration. Column characteristics as in "Materials and Methods" except that actual flow rate was 10.2 ml/hr and an additional 0.1 ml of 4 mM $H_2O_2$ was used in IAA oxidase assays. (-----: $\Delta A_{470\ nm}$/20 min × $10^2$; ·····: $A_{280\ nm}$ × 10; — —: μg $O_2$/min)

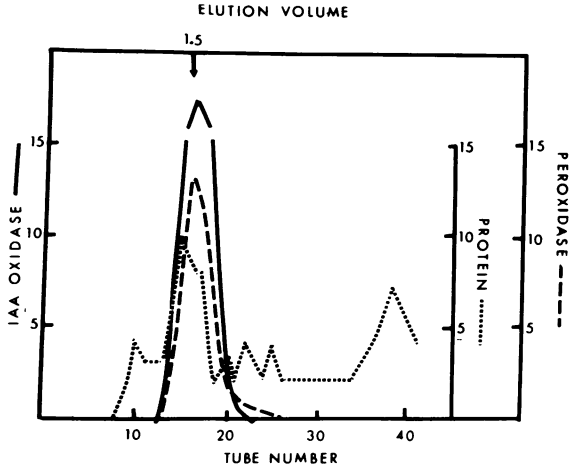


FIG. 3. Gel filtration of commercial horseradish peroxidase on Bio-Gel P-100. Column characteristics as in "Materials and Methods" except that actual flow rate was 13.5 ml/hr and 2 mg of enzyme was applied to the column in 2 ml of eluting buffer. (-----: $\Delta A_{470\ nm}$/20 min × 10, ·····: $A_{280\ nm}$ relative scale)

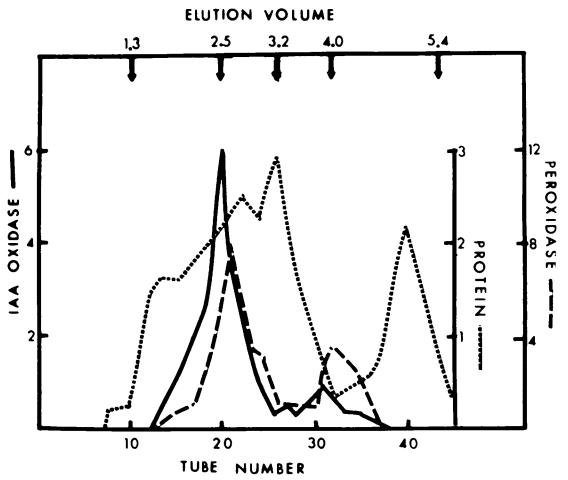


FIG. 4. Ion exchange chromatography of *Betula* enzyme on SE-Sephadex. The 2-ml sample applied to the column was partly purified and concentrated by membrane ultrafiltration. Column characteristics as in "Materials and Methods." (-----: $\Delta A_{470\ nm}$/20 min × $10^2$; ·····: $A_{280\ nm}$ × 10; — —: μg $O_2$/min)

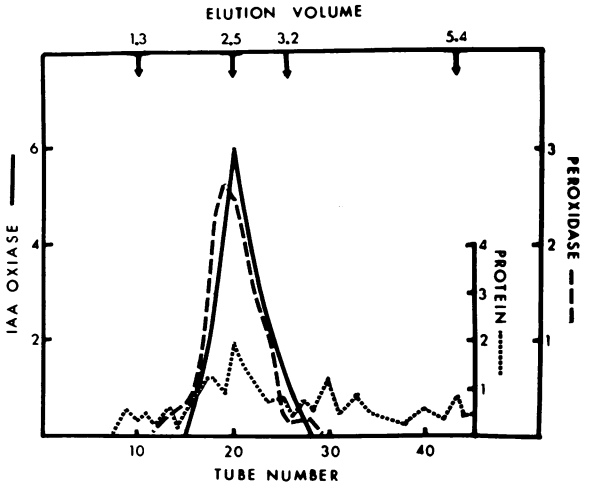


FIG. 5. Ion exchange chromatography of commercial horseradish peroxidase on SE-Sephadex. Column characteristics as in "Materials and Methods" except that actual flow rate was 8.8 ml/hr and 1 mg of enzyme was applied in 2 ml of eluting buffer. IAA oxidase assay was made with 0.2 M phosphate buffer, pH 5.7, and peroxidase assay was made at pH 4.0. (-----: $\Delta A_{470\ nm}$/30 min × 10; ·····: $A_{280\ nm}$ × $10^2$; — —: μg $O_2$/min × 10)

**Ion Exchange Chromatography.** *Betula* enzyme run through a column of SE-Sephadex with 0.1 M phosphate buffer showed four protein peaks at about 1.7, 2.8, 3.2, and 5.0 elution volumes. IAA oxidase and guaiacol peroxidase were not associated with any of these protein peaks, but eluted instead at 2.5 elution volumes (main peaks) and 4.0 elution volumes (much smaller secondary peaks) (Fig. 4). No activity of either type appeared at 5.4 elution volumes.

Commercial HRPO, run in like manner, showed a single peak of both oxidase and peroxidase activity at 2.5 elution volumes, which was also the major elution peak of protein. Again no activity of either type was found at 5.4 elution volumes (Fig. 5). These results were reproducible for both *Betula* enzyme and HRPO.

## DISCUSSION

Direct column chromatography of crude birch extracts was ineffective due to the presence of viscous materials. Active ammonium sulfate fractions also included this viscous material and did not lead to improved chromatographic results. Polyvinylpyrrolidone removed a large amount of interfering phenolic compounds, but membrane ultrafiltration was the only effective means of separating the oxidase-peroxidase enzyme from the troublesome viscous material. In addition, this technique removed larger enzymes, such as polyphenoloxidase and catalase, from the purified filtrates.

Assay of the filtrate fractions showed that much more activity is present in the crude extracts than initial assays would indicate. Possibly the oxidase-peroxidase enzyme may be in some inactive conformation in the crude extracts, but the high levels of inhibitor that move with the enzyme during purification suggest that enzyme activity may be blocked according to the ligand exclusion theory recently proposed by Fisher *et al.* (2). They suggested that steric exclusion occurs through interactions between multifunctional ligands, which bind to common and topologically distinct subsites. The requirement for additional $H_2O_2$ in IAA oxidase assays throughout several purification procedures suggests that some inhibitory ligand remains attached to the enzyme. The promoter ligand (*p*-coumaric acid) for IAA oxidase is apparently not bound to the enzyme, because more of this compound is required as purification proceeds.

The results from chromatographic separation, based on difference in size (gel filtration) and net charge (ion exchange chromatography), give clear evidence that IAA oxidase activity and guaiacol peroxidase activity are dual catalytic functions of a single enzyme. Using the same system as Sequeira and Mineo (8), I could not detect a separate peak of IAA oxidase activity at 5.4 elution volumes for either the *Betula* enzyme or the commercial horseradish enzyme. The main peak of dual enzyme activity appeared at 2.5 elution volumes for both sources of the enzyme, and these results were reproducible. The appearance of a dual activity peak at 4.0 elution volumes indicates there is an isoenzyme in *Betula* leaves, but this secondary peak did not appear with horseradish enzyme, probably because some isoenzymes are lost during preparation of commercial HRPO as reported by Kasinsky and Hackett (4).

Dual catalytic enzymes are not uncommon in plants. Widely recognized among such enzymes is the plant phenolase, which has dual capabilities of hydroxylation and oxidation of phenolic compounds (1).

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