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RELATIONSHIPS BETWEEN PEROXIDASE, IAA OXIDASE AND POLYPHENOL OXIDASE*

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; cell tissue culture; peroxidase; IAA oxidase; polyphenol oxidase.

Abstract—Peroxidase, IAA oxidase and polyphenol oxidase activities were detected in tobacco cells in suspension culture. Using gel filtration, ion-exchange chromatography and disc gel electrophoresis, it was concluded that IAA oxidase activity is entirely due to peroxidases. Peroxidase and polyphenol oxidase zymograms using anionic gel separation were also similar, but the occurrence of true polyphenol oxidases, distinct from peroxidase, was demonstrated by cationic disc gel electrophoresis and gel filtration.

INTRODUCTION

Multiple forms of peroxidase are known to exist in various plants, and many catalytic functions have been ascribed to the enzyme, although its physiological role is still obscure. The ability of peroxidase to catalyse the oxidation of indole-3-acetic acid (IAA) has been of particular interest. It is generally accepted that peroxidase is responsible for some IAA oxidase activity, but it has not yet been clearly established whether all such activity can be ascribed to peroxidase, since the occurrence of a specific non-peroxidative IAA oxidase has been reported [1, 2]. In addition, peroxidase has been suggested to possess polyphenol oxidase (PPO) activity [3], and the pattern of PPO isozymes in tobacco tissues was reported to be similar to that of peroxidase isozymes [4]. However, there is also evidence of the non-identity of PPO and peroxidase zymograms from extracts of tobacco leaves [5]. There have also been several reports about the existence of true PPO in tobacco plants.

It would be of interest, therefore, to know whether IAA-oxidase and PPO activities are directly related to peroxidase in a single tissue. Recently it has been reported that patterns of peroxidase isozymes in tobacco callus cultures are affected by hormonal conditions in the medium [6, 7] suggesting that this enzyme plays an important role in growth regulation of tobacco cells in the culture system. In the present investigation, tobacco cells in suspension culture were used, since this system provides more homogeneous cells than callus tissue, and enzyme preparations from the suspension cells contain peroxidase of high specific activity. Although the release of peroxidase by such cells into the medium has been reported by various workers [8–10], little is known about the activity of the peroxidases in the cells.

The purpose of the present report is to clarify whether enzymes catalyzing the oxidation of IAA or polyphenols are separable from peroxidase.

RESULTS

Gel filtration on Sephadex G-100

The crude enzyme preparation from the cells of tobacco suspension culture was analyzed by gel filtration on Sephadex G-100 (Fig. 1). The main peak of PPO was clearly separated from those of peroxidase and IAA oxidase although some PPO activity was also associated with the peroxidase peaks. Peroxidase was eluted in two closely associated peaks, which coincided exactly with the peaks of IAA oxidase activity.

* Part I in the series "Peroxidase From Tobacco Cell Suspension Cultures".

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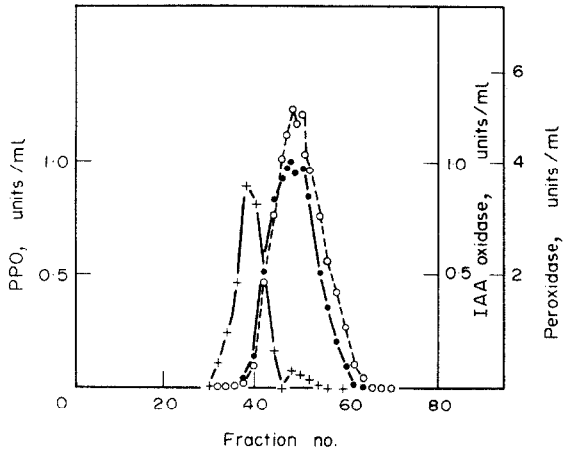

Fig. 1. Gel filtration of the crude enzyme preparation on Sephadex G-100. The crude enzyme preparation (5 ml) was applied to a column of Sephadex G-100 (2·5 × 80 cm) equilibrated with the 0·1 M phosphate buffer, pH 5·5. The column was eluted with the same buffer and 5 ml fractions were collected. ○ Peroxidase; ● IAA oxidase; + polyphenol oxidase.

Ion-exchange column chromatography

DEAE-cellulose column chromatography of the crude enzyme preparation is shown in Fig. 2. Two components were retained by the column, and the elution profile of peroxidase and IAA oxidase again coincided. The first fraction not adsorbed by the column, was concentrated and dialyzed, and subjected to a CM-Sephadex C-50 column chromatography. Five components were separated and again the activities of IAA oxidase and peroxidase showed coincident peaks after elution through the column (Fig. 3).

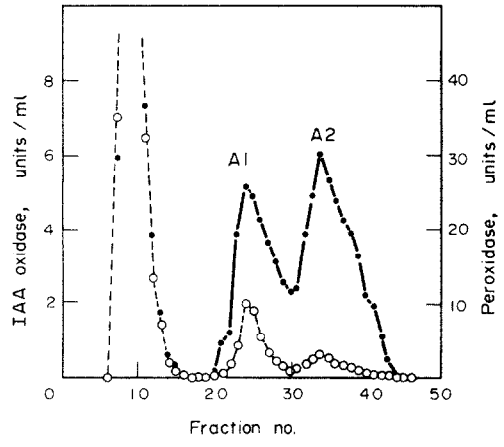

Fig. 2. Elution profile of the crude enzyme preparation on a DEAE-cellulose column. The crude enzyme preparation (10 ml) was adsorbed onto a DEAE-cellulose column (2·1 × 15 cm) equilibrated with 0·01 M Tris–HCl buffer, pH 7·5. The column was washed with 75 ml of the buffer, and then elution was carried out with a linear gradient of 0–0·8 M NaCl in the buffer (200 ml). Fractions of 5 ml each were collected. ○ Peroxidase; ● IAA oxidase.

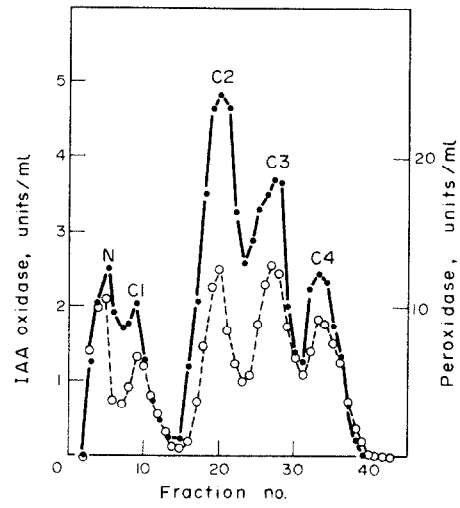

Fig. 3. Elution pattern of the cationic fraction on a CM-Sephadex C-50 column. The fraction that was not adsorbed by the DEAE-cellulose column (cationic fraction) was concentrated and dialyzed against 0·05 M phosphate buffer, pH 6·5. The dialyzate (5 ml) was adsorbed onto a column of CM-Sephadex C-50 column (1·5 × 11 cm). The column was washed with 50 ml of the buffer, and then elution was carried out with 200 ml of a 0–0·4 M NaCl linear gradient in the buffer. Fractions of 5 ml each were collected. ○ Peroxidase; ● IAA oxidase.

Disc electrophoresis

A suitably diluted crude enzyme preparation was applied to disc gel electrophoresis. When

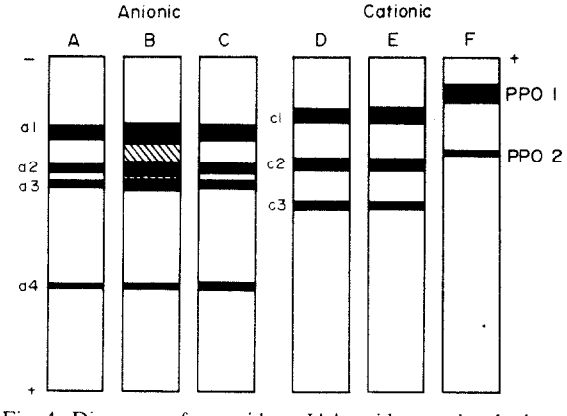

Fig. 4. Diagrams of peroxidase, IAA oxidase and polyphenol oxidase isozymes separated by electrophoresis on acrylamide gels at pH 8·4 (A, B and C) and pH 4·5 (D, E and F). Anionic and cationic separations were conducted in 7% acrylamide gels. (A and D) peroxidase; (B and E) IAA oxidase; (C and F) polyphenol oxidase.

guaiacol was used to stain for peroxidase isozymes on gels, four anionic and three cationic isozymes were detected (Fig. 4, A and D). Four anionic and three cationic IAA oxidase isozymes were also detected by using *p*-*N*,*N*′-dimethylaminocinnamaldehyde (Fig. 4, B and E) and, as can be seen, the electrophoretic mobilities of the IAA oxidase isozymes were identical with those of peroxidase isozymes. Electrophoresis performed using different concentrations (6, 7 and 7·5%) of acrylamide gels gave the same results.

PPO was detected using catechol as substrate. Four anionic and two cationic isozyme bands were prepared (Fig. 4, C and F). Chlorogenic acid was also tested as substrate, but the same results were obtained. The two cationic PPO isozymes had different mobilities from peroxidases, but those of the anionic PPO isozymes were identical with those of peroxidase isozymes. Electrophoresis in different concentrations of acrylamide gels and dual staining of PPO and peroxidase, further supported that the fact that the two cationic PPO isozymes are different from those of the peroxidases, and that the anionic PPO isozymes have identical mobilities with those of peroxidase isozymes. The two PPO isozymes, PPO-1 and PPO-2 (Fig. 4), could oxidize both chlorogenic acid and catechol in the staining technique used. Cationic peroxidase isozymes did not exhibit PPO activities under the same staining conditions.

DISCUSSION

IAA oxidase and peroxidase activities gave coincident peaks by either gel filtration or ion-exchange column chromatography on DEAE-cellulose and CM-Sephadex. Furthermore, electrophoresis on acrylamide gels gave the same number of isozymes with identical mobilities regardless of the concentration of the gels used.

These results indicate that IAA oxidase activity is related to oxidase function of peroxidase, consistent with other reports [10, 11]. Since it is well established that some purified peroxidases catalyze IAA oxidation in which molecular oxygen serves as electron acceptor [3, 12], it is reasonable to conclude that the IAA oxidase activity in the extracts from tobacco cells in suspension culture is all due to peroxidase. Our data also indicate that all peroxidase isozymes in the extracts possess IAA oxidase activity.

Several workers have speculated that peroxidase isozymes also exhibit PPO activities [10]. A similarity between zymograms of these enzymes developed from the extracts of tobacco leaves have been reported [4]. From the present results, this identity is true as far as anionic isozymes were concerned. However, the cationic disc gel electrophoresis and gel filtration on Sephadex G-100 demonstrated that the main PPO activity is separable from peroxidases in tobacco.

Two PPO isozymes, chlorogenic acid oxidase and catecholase, have been postulated in tobacco leaves [13], and it was reported that partially purified chlorogenic acid oxidase did not catalyze the oxidation of catechol. However, two PPO isozymes in our preparation oxidized both chlorogenic acid and catechol equally.

The cationic peroxidases on gels did not form bands showing PPO activity under the same staining condition, which may be considered to demonstrate different functions between the cationic and anionic peroxidase isozymes. The results reported here indicate the existence of multiple forms of peroxidase with different properties. Further characterization of these peroxidases will be reported in a subsequent paper.

EXPERIMENTAL

Material. A homogeneous cell suspension culture of tobacco was used during present studies. The culture was derived from seedlings (*N. tabacum* cv. Hicks 2). The cells have been reproducibly subcultured for 1 yr. The suspension culture was grown in the basal medium of Linsmaier and Skoog [14] with 1 mg thiamine–HCl, 100 mg myo-inositol and 0·2 mg 2,4-dichlorophenoxy acetic acid/l. After cultivation for 6 days, cells were harvested and kept frozen until enzyme extraction.

Enzyme assays. For peroxidase activity determination, guaiacol was used as hydrogen donor. The standard reaction mixture contained, in a final vol. of 3·0 ml, 100 mM acetate buffer pH 5·5, 0·2 mM guaiacol, 2 mM H_2O_2 and a suitable amount of enzyme. The activity was assayed spectrophotometrically at 470 nm. One unit of enzyme activity was defined as the amount which produced a change in absorbance of 1·0/min. For IAA oxidase activity determination, the standard reaction mixture contained, in a final vol. of 1·0 ml, 0·04 M phosphate buffer, pH 5·5, 0·2 mM IAA, 0·1 mM 2,4-dichlorophenol, 0·1 mM $MnCl_2$ and a suitable amount of enzyme. The reaction was carried out at 30°. After 30 min 2·0 ml of modified Salkowski reagent [15] was added, and the absorbance was read at 530 nm 30 min later. One enzyme unit was defined as a change in absorbance of 1·0 in 30 min. For PPO activity determination, catechol was used as substrate. The standard reaction mixture contained in a final vol. of 3·0 ml, 100 mM phosphate buffer, pH 6·5, 1·0 mM catechol and a suitable amount of enzyme. The activity was assayed spectrophotometrically at 420 nm. One enzyme unit was defined as a change in absorbance of 1·0 in 30 min.

Enzyme preparation. Frozen cells (30 g) were homogenized in 60 ml of 0·01 M phosphate buffer, pH 7·5, containing 0·2 M KCl, 0·005 M EDTA, 0·1 M sodium ascorbate and 15 g of purified insoluble polyvinylpyrrolidone. The homogenate was centrifuged at 25000 *g* for 20 min. Solid $(NH_4)_2SO_4$ was added to the supernatant to give 90% satn. The solution was stirred for 30 min, and the ppt recovered and dissolved in 0·01 M Tris–HCl buffer, pH 7·5, and dialyzed against the same buffer overnight (crude enzyme preparation).

Gel filtration column chromatography. Gel filtration was on a column of Sephadex G-100 equilibrated with 0·1 M phosphate buffer, pH 5·5.

Ion-exchange chromatography. The crude enzyme preparation was adsorbed onto a column of DEAE-cellulose, previously equilibrated with 0·01 M Tris–HCl buffer, pH 7·5. The column was washed with 75 ml of the buffer and elution was carried out with a linear gradient of 0–0·8 M NaCl in 200 ml of the buffer. The fraction that was not adsorbed by the column was conc to 5 ml by the use of collodion bag and dialyzed against 0·05 M phosphate buffer, pH 6·5, overnight. The dialyzate was adsorbed onto a column of CM-Sephadex C-50, previously equilibrated with 0·05 M phosphate buffer, pH 6·5. The column was washed with 50 ml of the buffer and then elution was carried out with a linear gradient of 0–0·4 M NaCl in 200 ml of the buffer.

Electrophoresis. The polyacrylamide disc gel electrophoresis for anionic [16] and cationic [17] separations was carried out as described. The staining mixture for peroxidase contained 5 mM guaiacol, 5 mM H_2O_2, and 0·05 M phosphate buffer, pH 5·5. IAA oxidase was detected by incubating the gel in a mixture containing 1 mM IAA, 0·1 mM 2,4-dichlorophenol and 0·5 mM $MnCl_2$ in 0·1 M phosphate buffer, pH 5·5, and then adding *p*-*N*,*N*′-dimethylaminocinnamaldehyde [18, 19]. PPO was stained using catechol or chlorogenic acid as substrate. The gels were incubated for 1 hr in 5 mM catechol or chlorogenic acid in 0·1 M phosphate buffer, pH 6·5. After staining gels were scanned, and then mobilities calculated.

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