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PROPERTIES OF PEROXIDASES FROM TOBACCO CELL SUSPENSION CULTURE*

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

Abstract—An enzyme preparation from suspension cultured tobacco cells oxidized IAA only in the presence of added cofactors, Mn^{2+} and 2,4-dichlorophenol, and showed two pH optima for the oxidation at pH 4·5 and 5·5. Effects of various phenolic compounds and metal ions on IAA oxidase activity were examined. The properties of seven peroxidase fractions separated by column chromatography on DEAE-cellulose and CM-Sephadex, were compared. The peroxidases were different in relative activity toward *o*-dianisidine and guaiacol. All the peroxidases catalyzed IAA oxidation in the presence of added cofactors. The pH optima for guaiacol peroxidation were very similar among the seven isozymes, but the optima for IAA oxidation were different. The anionic and neutral fractions showed pH optima near pH 5·5, but the cationic isozymes showed optima near pH 4·5. With guaiacol as hydrogen donor, an anionic peroxidase (A-1) and a cationic peroxidase (C-4) were very different in H_2O_2 concentration requirements for their activity. Peroxidase A-1 was active at a wide range of H_2O_2 concentrations, while peroxidase C-4 showed a more restricted H_2O_2 requirement. Gel filtration and polyacrylamide gel studies indicated that the three cationic peroxidases have the same molecular weight.

INTRODUCTION

In a previous report [1], we demonstrated the existence of multiple forms of peroxidase from tobacco cells in suspension culture. Although the physiological reasons for the existence of the multiple forms have not yet been established, knowledge of the differences in biochemical properties may be essential to understand the physiological role of peroxidase isozymes in plants. These isozymes are known to differ markedly in biochemical properties [2–6].

Although various physiological studies have been made on the IAA oxidase and peroxidase of tobacco [7, 8], the detailed properties of the enzyme activities have not yet been reported for this plant. This paper describes the catalytic properties of the peroxidases for IAA oxidation and peroxidative reactions.

* Part 2 in the series "Peroxidases from Cell Suspension Culture".

RESULTS

Properties of IAA oxidase activity of crude extract

The enzyme preparation was made from a homogenate in the presence of insoluble PVP and subsequent purification by gel filtration on Sephadex G-25. The pH optimum for peroxidase with guaiacol as hydrogen donor was near pH 5·5. On the other hand, the enzyme preparation showed two pH optima for IAA oxidation near pH 4·5 and 5·5. The enzyme preparation showed an absolute dependence on added Mn^{2+} and 2,4-dichlorophenol (DCP) for IAA oxidation.

DCP is commonly used as phenolic cofactor for the activity, but the phenolic cofactor requirement was satisfied by both monohydroxy and *m*-dihydroxy phenols, although the latter compounds were less effective (Table 1).

Effects of the compounds on the activity in the presence of added cofactors, Mn^{2+} and DCP, are

Table 1. Effects of various phenolics on the activity of IAA oxidase

Compound added	Cofactor activity (% activity)	Inhibitor activity (% inhibition)
None	0	0
2,4-Dichlorophenol	100	
p-Coumaric acid	84	0
Tyrosine	18	0
2,3-Dihydroxybenzoic acid	0	100
2,4-Dihydroxybenzoic acid	10	0
2,5-Dihydroxybenzoic acid	0	100
2,6-Dihydroxybenzoic acid	12	0
o-Hydroxybenzoic acid	0	
m-Hydroxybenzoic acid	4	
p-Hydroxybenzoic acid	22	

For cofactor activity measurements, the compounds listed were added at a concentration of 0·1 mM instead of 2,4-dichlorophenol. The cofactor activities were expressed relative to that obtained with 2,4-dichlorophenol. For inhibitor activity measurements the compounds listed were added at concentration of 0·01 mM to the standard system. The inhibitor activities were expressed relative to that obtained without the compounds.

also shown in Table 1. Among the dihydroxybenzoic acids, *o*- and *p*-dihydroxy derivatives caused inhibition, whereas *m*-dihydroxy compounds did not affect the activity. Naturally occurring *o*-dihydroxy-phenols such as quercetin, chlorogenic acid and caffeic acid also inhibited the activity. The phenolic inhibitors are generally considered to act as radical trapping agents. There was a lag period prior to the oxidation of IAA even in the absence of the inhibitors. The inhibitors introduce a longer lag period prior to the reaction, but do not significantly affect the reaction rate at steady state.

The enzyme preparation made from homogenate in the absence of insoluble PVP and sodium ascorbate showed a much longer lag period, but the reaction rate at steady state was not appreciably changed. Dialysis of the preparation or addition of soluble PVP (0·1%) to the reaction mixture reduced the lag period.

Mn^{2+} was also required for the activity. The activity increased with increasing concentration of Mn^{2+} up to 10^{-2} M. The increase in concentration of Mn^{2+} from 0·01–1 mM caused 6-fold decrease in the lag period and 3-fold increase in the reaction rate. Various divalent cations, Cu^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+}, Fe^{2+} and Mg^{2+}, were ineffective in their ability to substitute for Mn^{2+} at a concentration of 10^{-4} M. The effects of the metal ions on the activity in the presence of added cofactors were also examined at a concentration of 10^{-4} M. Among them, Cu^{2+} showed 95% inhibition of the activity, but other metal ions had no effect. The addition of Cu^{2+} in the range from 10^{-5} to 10^{-3} M resulted in 45–100% inhibition, while it stimulated the activity at concentrations from 10^{-6} to 10^{-7} M. The addition of Cu^{2+} did not alter the lag period, but either increased or decreased the reaction rate depending on its concentration.

Properties of peroxidase fractions separated by ion exchange chromatography

When the crude enzyme preparation from the tobacco cells in suspension culture was subjected to ion-exchange chromatography on DEAE-cellulose and CM-Sephadex [1], the peroxidase activity was separated into seven fractions: N; not retained by either column; A-1, A-2 (DEAE-cellulose), C-1, C-2, C-3 and C-4 (CM-Sephadex); retained by either column and eluted in that order from the columns by the NaCl linear gradient. The seven peak fractions obtained by the chromatographic procedures were respectively dialyzed against 0·01 M Pi buffer, pH 5·5, and were used for the present study. The anionic peroxidases comprise at most 10% of the total activity.

Table 2 lists the properties of the seven peroxidases. Each of the peroxidases showed activity for guaiacol as hydrogen donor over a broad pH range, and the pH optima for all of the seven peroxidases were near pH 5·5. There were some differences among the seven peroxidases in their relative peroxidase activity toward two different hydrogen donors. The *o*-dianisidine–guaiacol activity ratios ranged from 0·47 to 1·3, and fraction A-2 was the most active toward *o*-dianisidine among the seven. The anionic peroxidases possessed higher activity ratios than the cationic peroxidases.

Each of the peroxidases catalyzed IAA oxidation in the presence of added cofactors, Mn^{2+} and 2,4-dichlorophenol. As shown in Table 2, the pH optima for IAA oxidation of peroxidases A-1, A-2 and N were near pH 5·5, but peroxidases C-1, C-2, C-3 and C-4 showed maximum activity near pH 4·5. The IAA oxidase/guaiacol peroxidase activity ratios at optimal pH ranged from 2·3 to 8·3.

The seven peroxidase fractions were analyzed by polyacrylamide disc gel electrophoresis (Table

Table 2. Properties of peroxidase fractions separated by ion-exchange chromatography

Fraction	Components*	pH optimum Peroxidase (guaiacol)	pH optimum Oxidase (IAA)	Peroxidase† (*o*-dianisidine) Peroxidase (guaiacol)‡	Oxidase (IAA)§ Peroxidase (guaiacol)
A-2	a-1, a-3, a-4	5·5	5·5	1·28	8·0
A-1	a-2	5·5	5·5	0·65	6·7
N	a-2, a-3	5·5	5·5	0·84	3·7
C-1	a-2, c-1	5·5	4·5	0·53	6·1
C-2	c-1, c-2	5·5	4·5	0·47	2·3
C-3	c-1, c-2, c-3	5·5	4·5	0·53	6·8
C-4	c-1	5·5	4·5	0·63	8·3

* Each fraction was analyzed by polyacrylamide gel electrophoresis in anionic (a-1 ~ a-4) and cationic (c-1 ~ c-3) systems. The isozymes on gels were numbered from that having minimum electrophoretic mobilities.
† E 460 nm per min.
‡ E 470 nm per min.
§ E 530 nm per 30 min.; IAA oxidase activity was assayed at optimum pH.

2). Fractions A-1 and C-4 were freed from the other peroxidase isozymes. The other five fractions were resolved into several components by electrophoresis. The electrophoretic mobilities of the peroxidases on gels were identical before and after ion-exchange chromatography, so that denaturing is unlikely to have occurred during purification.

The two peroxidases, a cationic peroxidase (C-4) and an anionic peroxidase (A-1), which lacked catalase activity, were further studied. As shown in Table 2, peroxidases A-1 and C-4 had the same pH optimum with guaiacol as hydrogen donor, but had very different H_2O_2 concentration requirements for their maximum activity (Fig. 1). A-1 was active at a wide range of H_2O_2 concentrations, and retained more than 80% of its maximum activity at H_2O_2 concentrations of 0·2–20 mM. On the other hand, C-4 was more restricted in its H_2O_2 requirement, and showed more than 80% of its maximum activity only when the concentration of H_2O_2 was in the range from 5 to 6 mM. C-4 showed its maximum activity at a H_2O_2 concentration of 6·5 mM, which was more than twice that required for A-1, and C-4 had only about 20% of its maximum activity at the H_2O_2 concentration optimum for A-1. As shown in Table 2, A-1 had a pH optimum for IAA oxidation at pH 5·5 and C-4 at pH 4·5. The IAA oxidase activity of A-1 decreased about 55% when run at the optimum pH for C-4, and was completely inactive at pH 4, while C-4 was still 90% active. C-4 retained only about 30% of its activity at the optimum pH for A-1.

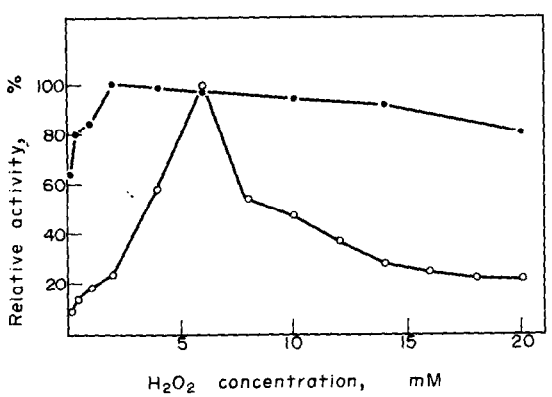

Fig. 1. Effect of H_2O_2 concentration on peroxidases A-1 and C-4 with guaiacol substrate. The assay system consisted of 100 mM acetate buffer, pH 5·5, 2 mM guaiacol, enzyme and H_2O_2 indicated: peroxidase A-1; ● peroxidase C-4 ○.

The effects of various concentrations of Mn^{2+} on the IAA oxidase activities of the two peroxidases were also examined. A-1 was stimulated to a larger extent than C-4 by the addition of 10^{-2} M Mn^{2+}. When the concentration of Mn^{2+} was reduced to 10^{-5} M, the activity of A-1 was not so reduced, while in the case of C-4, it resulted in a 3-fold decrease in activity.

The cationic peroxidase preparation, separated by CM-Sephadex chromatography, on disc gel electrophoresis separated into isozymes C-1, C-2 and C-3. The same preparation was subjected to Sephadex G-100 column, equilibrated with 0·05 M Pi buffer, pH 5·5. The enzyme activity was eluted as a single symmetrical peak. Estimation

of the MW of the peak obtained by the gel filtration gave a value of about 50000. No association-dissociation relationships seemed to exist for the cationic peroxidase isozymes. Using the polyacrylamide gel electrophoresis technique of Hedrik and Smith [9], three cationic peroxidases were shown to have identical mws, so that they only differ in net charge.

EXPERIMENTAL

Plant material. Tobacco cells (*Nicotiana tabacum* cultivar. Hicks 2) were grown in suspension as described previously [1].

Enzyme preparation. Cells were homogenized and the homogenate was centrifuged as previously described [1]. The supernatant was subjected to gel filtration on Sephadex G-25 equilibrated with 0·01 M Pi buffer, pH 5·5. The effluent was used as crude enzyme preparation. Peroxidase isozymes were separated and designated as described previously [1], using column chromatography on DEAE-cellulose and CM-Sephadex. Peaks of the activity were dialyzed against 0·01 M Pi buffer, pH 5·5 and analyzed by polyacrylamide disc electrophoresis as previously described [1].

Enzyme assays. IAA oxidase and guaiacol peroxidase activities were assayed as previously described [1]. Peroxidase activity with *o*-dianisidine as substrate was assayed spectrophotometrically at 460 nm. The reaction mixture contained 0·1 M acetate buffer, pH 5·5, 0·8 mM *o*-dianididine, 0·6 mM H_2O_2 and the enzyme. Catalase activity was assayed spectrophotometrically at 230 nm [10].

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