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TOBACCO POLYPHENOLOXIDASES: A SPECIFIC STAINING METHOD INDICATING NON-IDENTITY WITH PEROXIDASES

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Abstract—Specific localization of polyphenoloxidases after electrophoresis on polyacrylamide gels is described. The method consists of using caffeic acid as the substrate and *m*-phenylenediamine as a coupling agent at pH 5·5. Isoenzyme patterns of tobacco polyphenoloxidases showed six bands which may be considered to represent true isozymic forms of the enzyme. These bands occupied positions on the gel different from those of tobacco peroxidase isoenzymes developed with guaiacol. Apparent identity of polyphenoloxidases and peroxidases revealed with other substrates and differentiation of these enzymes are discussed.

INTRODUCTION

THE DIFFERENTIATION of polyphenoloxidases and peroxidases on polyacrylamide gels after electrophoresis of plant extracts is often unsatisfactory. Recently, Sheen and Calvert[1] reported similar band patterns for these two enzymes using benzidine to localize peroxidases and either 3,4-dihydroxyphenylalanine (DOPA) or a mixture of chlorogenic acid and *p*-phenylenediamine (PPD) to develop polyphenoloxidases. These authors therefore suggested that these enzymes might be identical in tobacco plants. While studying the biochemistry of tobacco mosaic virus (TMV)-infected tobacco plants, it became of importance to establish whether increased activities of polyphenoloxidases and peroxidases were brought about by different enzymes or only a single one. Quantitative changes in enzyme activities after TMV infection were followed spectrophotometrically and these changes were compared with the changes in isoenzyme patterns on polyacrylamide gels. Although we could confirm the results obtained by Sheen and Calvert,[1] we have been able to differentiate between the two enzymes by changing the conditions for development after electrophoresis. Under these circumstances, there was a direct relationship between the activity measured spectrophotometrically and the intensity of the bands on the gels.

RESULTS AND DISCUSSION

Figure 1a shows a typical pattern of tobacco polyphenoloxidases if DOPA is used as the substrate at pH 7·0. This pattern strongly resembled that obtained when duplicate gels were examined for peroxidases using benzidine as the hydrogen donor (Fig. 1b). These results are in agreement with the findings of Sheen and Calvert.[1] On the basis of such observations these authors tentatively proposed that these enzymes might be identical. The following observations make it clear that their hypothesis is not tenable.

Infection of tobacco var. 'Samsun NN' with TMV resulted in an initial rise in polyphenoloxidase activity, accompanied by a decrease in peroxidase activity, at two different pH's. Only after symptoms appeared, activities of both enzymes increased (Table 1). This demonstrates the independent behaviour of the two enzyme activities and indicates the

[1] S. J. SHEEN and J. CALVERT, *Plant Physiol.* **44**, 199 (1969).

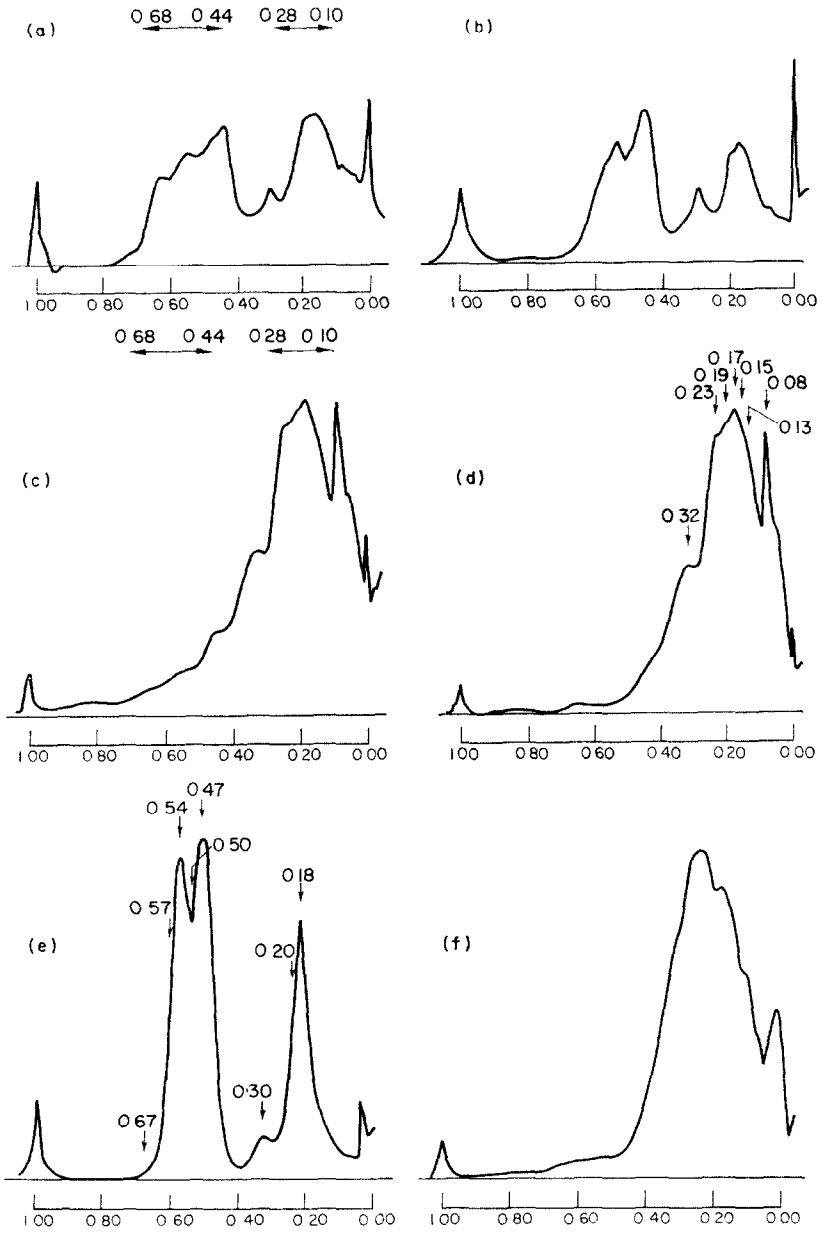

FIG. 1. DENSITOMETER TRACINGS OF ELECTROPHORETIC PATTERNS OF TOBACCO POLYPHENOLOXIDASES, DEVELOPED WITH DOPA (a), CAFFEIC ACID AND MPD AT pH 7·0 (c) AND 5·5 (d); TOBACCO PEROXIDASES, DEVELOPED WITH BENZIDINE (b) AND GUAIACOL (e); AND POTATO PEROXIDASES, DEVELOPED WITH CAFFEIC ACID AND MPD AT pH 5 5 (f).

existence of two separate enzymes. We, therefore, re-examined the procedure of Sheen and Calvert[1] as follows.

DOPA was found to be a suitable hydrogen donor for the demonstration of tobacco peroxidases in the presence of H_2O_2. Moreover, it has been reported that in the oxidation of

TABLE 1. CHANGES IN POLYPHENOLOXIDASE AND PEROXIDASE ACTIVITIES OF TOBACCO VAR. 'SAMSUN NN' AFTER TMV INFECTION

Days after infection	Relative activity (%)			
	Polyphenoloxidase		Peroxidase	
	pH 7·0	pH 5·5	pH 7·0	pH 5·5
0	100	100	100	100
1	165	140	76	85
2	315	170	70	80
3	245	95	123	107
4	404	150	134	280
5	237	286	200	278

The results are taken from two different experiments, at pH 7·0 (phosphate buffer), and at pH 5·5 (phosphate–citrate buffer). Symptoms were apparent 3 days after infection.

DOPA by polyphenoloxidase, H_2O_2 may be produced by side reactions.[2] Hence, the use of DOPA to localize this enzyme on the gels may lead to the simultaneous development of peroxidase bands. Furthermore, DOPA is not a natural constituent of tobacco.[3] As chlorogenic acid is the major phenolic constituent of tobacco leaves,[3] and is a natural substrate of tobacco polyphenoloxidase,[4] specific visualization with this compound seemed more relevant. With this substrate, colour intensity of the bands on the gels would also be expected to be directly proportional to the activities measured spectrophotometrically.

Polyphenoloxidase patterns developed with chlorogenic acid as the substrate were highly unstable, as the green oxidation product was water-soluble. Since chlorogenic acid can be replaced by caffeic acid,[4] this compound was used in further experiments. The orange-brown oxidation product of caffeic acid appeared rather stable, but distributed itself over the protein bands in the region 0·10–0·28 of the gel. To fix the coloured product at the sites of formation, a coupling agent was used. Sheen and Calvert[1] used PPD to fix the oxidation product of chlorogenic acid. In our hands, this compound proved to be unsuitable, because it yielded purple-brown bands on the gels in the absence of caffeic acid. However, *m*-phenylenediamine (MPD) was not oxidized in a control experiment. When this compound was used as a coupling agent, stable, deep brown patterns were revealed (Figs. 1c and 1d). No bands appeared when gels were heated at 80° for 10 min during preincubation, or when 0·1 per cent ascorbic acid—a powerful inhibitor of polyphenoloxidase—was incorporated in the mixture. The intensity of the pattern was roughly proportional to the polyphenoloxidase activity measured in the extract. The pattern did not follow the protein pattern in the region of activity, and appeared to be plant species specific. For comparison, potato polyphenoloxidases are shown in Fig. 1f.

With caffeic acid as the substrate and MPD as a coupling agent, the polyphenoloxidase pattern of tobacco developed at pH 7·0 initially resembled the pattern obtained with DOPA. However, it showed a number of bands in the region 0·10–0·28, and lower intensity of the bands in the region of 0·44–0·68. While bands in the region 0·10–0·28 intensified with time, bands in the region 0·44–0·68 faded away (Fig. 1c).

[2] M. THOMAS in *Moderne Methoden der Pflanzenanalyse* IV (edited by K. PEACH and M. V. TRACEY), p. 661, Springer-Verlag, Berlin (1955).
[3] J. TANGUY and M. GALLET, *Compt. Rend.* **D 269**, 589 (1969).
[4] R. A. CLAYTON, *Arch. Biochem. Biophys.* **81**, 404 (1959).

TABLE 2. THE EFFECT OF pH ON POLYPHENOLOXIDASE ACTIVITY

pH*	Activity†
4·2	0·222
5·0	0·406
5·5	0·570
6 1	0·399
7·0	0·342
8 0	0·111

* Phosphate–citrate buffer.

† The activity is expressed as the decrease in absorptivity at 330 nm/min with chlorogenic acid as the substrate

Spectrophotometric measurements revealed that tobacco polyphenoloxidase had maximal activity at pH 5·5 (Table 2). If DOPA was used as the substrate, the higher polyphenoloxidase activity was not reflected in an increase in colour intensity of the bands on the gels. As compared to pH 7 0, at pH 5·5 the pattern showed a small increase in the bands in the region 0·10–0·28 and a pronounced decrease in bands 0·44–0·68. On the other hand, peroxidase activity and enzyme pattern, developed with benzidine as the hydrogen donor, were relatively unaffected by pH. These observations again seem to exclude the possibility that polyphenoloxidase and peroxidase activity are due to the same enzyme.

If the mixture of caffeic acid and MPD was used to develop polyphenoloxidases at pH 5·5, only the bands in the region 0·10–0·28 were shown (Fig. 1d). Hence, as compared to pH 7·0, at pH 5·5 bands in the region 0·44–0·68 disappeared or were greatly diminished when caffeic acid or DOPA were used as the substrate, respectively. As in this region a number of highly active peroxidase isoenzymes are present, it was considered that these bands were produced possibly by peroxidase action. Therefore, 20 μg of catalase/ml incubation solution were added, to prevent action of any H_2O_2 produced in the gel during incubation. At pH 7·0 the resulting pattern then was identical with that at pH 5·5, although it took somewhat longer for the bands to develop the same intensity. The pattern developed at pH 5·5 was not affected by catalase treatment. It seems therefore that the pattern obtained at the latter pH reflects the true polyphenoloxidases, while trace amounts of H_2O_2 cause the appearance of bands in the peroxidase region 0·44–0·68 at pH 7·0. The difference at pH 5·5 and 7·0 may be due to the fact either that H_2O_2 is not produced at pH 5·5, or that it is rapidly destroyed by tobacco catalase. This enzyme is present in the gels in the polyphenoloxidase region and is most active at this pH, since numerous oxygen bubbles are produced in the gel after incubation in H_2O_2 at this pH.

The polyphenoloxidase region consisted of at least six bands at positions 0·13, 0·15, 0·17, 0·19, 0·23 and 0·32. In some cases, band 0·32 was greatly reduced and a band could be distinguished at position 0·26. In addition, a band at position 0·08 was always present. Since this band coincided with the major protein component which is considered to represent fraction I protein,[5] and is stained in almost every enzyme localization reaction, its function as a polyphenoloxidase seems doubtful.

[5] V. MÁCKO, G. R. HONOLD and M. A. STAHMANN, *Phytochem.* **6**, 465 (1967).

Guaiacol is recommended as a hydrogen donor for the localization of peroxidases,[6] as it is not oxidized by polyphenoloxidases. In our experiments, patterns obtained with guaiacol appeared less complex than those revealed by benzidine in the presence of H_2O_2. As benzidine is not a substrate for polyphenoloxidases either, this observation seems to reflect a difference in co-substrate specificity of the separate isozymes. The possibility also exists that benzidine is not completely specific for peroxidases. For comparisons of the positions of tobacco polyphenoloxidases and peroxidases on the gels, guaiacol seemed more suitable, because the bands in the polyphenoloxidase region were more clearly defined when using this compound.

Tobacco peroxidases, developed with guaiacol, are shown in Fig. 1e. Although minor bands were fully separated on the gels, they were revealed only as slight shoulders in the densitometer tracings. Eight peroxidases could be distinguished at positions 0·18, 0·20, 0·30, 0·47, 0·50, 0·54, 0·57 and 0·67. By comparing the positions of peroxidases and polyphenoloxidases on duplicate gels in a number of experiments, it was confirmed that the two peroxidases at positions 0·18 and 0·20 and the two polyphenoloxidases at positions 0·17 and 0·19 were different.

EXPERIMENTAL

Plants of *Nicotiana tabacum* L. var. 'Samsun NN' were grown from seed in a growth chamber at 18–20°. 11–13 weeks after sowing, leaves were harvested and soluble proteins were extracted as described before.[7] For electrophoresis centrifuged extracts were used either immediately after preparation or after storage under N_2 for 1 or 2 days. Electrophoresis in gels containing 7·5% polyacrylamide was performed using the discontinuous system of Davis[8] as described previously.[7] Duplicate gels were stained for protein, or examined for polyphenoloxidase or peroxidase activity. The resulting patterns were recorded by densitometry, using a Photovolt model 520-A densitometer with reduced slit width, equipped with a Varicord 43 linear/log recorder. The positions of the bands on the gel were expressed by their respective R_f values, taking the distance travelled by bromophenolblue as 1·00. The latter position was marked by puncturing the gels with a needle wetted with indian ink immediately after the run.

Gels were stained for protein using a saturated solution of amidoblack in 5% TCA.[7] Polyphenoloxidases were visualized with DOPA[9] or caffeic acid as follows. In the case of DOPA, gels were equilibrated with 0·1 M phosphate buffer pH 7·0 for 30 min and then incubated for 1 hr in 0·01 M DOPA in the same buffer under vigorous aeration. Black bands indicated sites of polyphenoloxidase activity. Without aeration, it took much longer for the bands to appear; this resulted in less intense and more diffuse patterns. With caffeic acid, gels were preincubated in phosphate-citrate buffer containing 0·1% MPD for 30 min, followed by an incubation in 0·01 M caffeic acid in the same solution under aeration for 15–60 min. For incubation at pH 7·0, caffeic acid was independently adjusted to this pH with 1 N KOH.

Peroxidases were revealed by the method of Mácko *et al.*[5] using benzidine or guaiacol as the hydrogen donor. For incubation at pH 5·5, gels were preincubated in the presence of phosphate-citrate buffer pH 5·5.

Activity measurements in solution were performed spectrophotometrically. 10 ml of centrifuged extracts were dialysed overnight against two changes of phosphate–citrate buffer, and subsequently diluted to 50 ml with buffer. Polyphenoloxidase activity was determined at 330 nm with chlorogenic acid as the substrate, according to Van Kammen and Brouwer.[10] Peroxidase activity was measured at 420 nm using guaiacol as the hydrogen donor, essentially after Tomiyama and Stahmann.[11]

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