Plant Physiol. (1971) 48, 232–233

Short Communication

Effect of Scopoletin on Two Anodic Isoperoxidases Isolated from Tobacco Tissue Culture W-38[1]

Received for publication March 17, 1971

PHILLIP SCHAFER, SIMON H. WENDER, AND EDDIE C. SMITH
Department of Chemistry, Biochemistry Division, University of Oklahoma, Norman, Oklahoma 73069

The oxidation of indole-3-acetic acid is one of the several catalytic actions exhibited by peroxidase preparations from plants (3, 15). Further, this IAA oxidase activity has been reported to be both inhibited and stimulated by scopoletin (6-methoxy-7-hydroxycoumarin) (1, 13). More recently, Imbert and Wilson (6) found that scopoletin inhibited IAA oxidase activity at high concentrations (12.5–250 nmole/ml), but stimulated activity at low concentrations (0.25–10 nmole/ml).

In that the existence of multiple forms of peroxidase in plants is now well documented (14), we have sought to clarify the various reports concerning how scopoletin modifies peroxidase activities by studying the effect of this compound on individual isoperoxidases isolated from tobacco tissue W-38 grown in culture. Although various, excellent physiological studies, such as those by Galston and coworkers (4), have been made on the isoperoxidases of tobacco, the detailed enzymic properties (*i.e.*, substrate specificity, effector studies, kinetics, and physical properties) of the individual isoperoxidases have not been reported for this plant. A few studies, however, have been conducted in other species (2, 10, 11). The present communication is a preliminary report on the separation and characterization of two anodic isoperoxidases of tobacco and on the effect that scopoletin has on each of these two partially purified isoperoxidases.

METHODS AND MATERIALS

W-38 tobacco callus tissue supported on filter paper as described by Heller (5) was grown at the surface of liquid Linsmaier-Skoog medium (9). Dense callus tissue approximately 8 mm in diameter by 2 mm thick cut from 5-week-old stock culture was used as the inoculum. While receiving continuous subdued reflected light, the inoculum was allowed to grow for 35 days at room temperature.

Enzyme extracts were prepared in 50 mM tris-maleate buffer, pH 7.0. Sufficient tissue to yield 5 to 7 g fresh weight was crushed in a chilled mortar. Suspensions, prepared by adding 2 ml of 50 mM tris-maleate buffer, pH 7.0, per g fresh weight of tissue, were homogenized at 4 C for 6 min in a Sorvall Omnimixer set at 8000 rpm. The homogenates were filtered through cheesecloth, and the resulting filtrates were centrifuged for 20 min at 27,000*g* at 4 C. The resulting supernatants were stored at 4 C and were used for isoelectric focusing and polyacrylamide electrophoresis.

Using the method of Vesterberg and Svensson (17), isoelectric focusing was accomplished on a LKB 440 ml isoelectric focusing column. Runs were made at 4 C for 48 hr (pH 3–10 gradient) and 64 hr (pH 3–6 gradient) at a potential of 450 to 600 v. After isoelectric focusing, the contents of the column were fractionated into 4-ml volumes, and the pH of all fractions having significant peroxidase activity was determined so that the isoelectric point of each peroxidase peak could be ascertained.

Furthermore, the fractions with peroxidase activity were subjected to polyacrylamide gel electrophoresis analysis. The method of Ornstein and Davis (12) was used for polyacrylamide-gel electrophoresis using a Buchler polyanalyst disk electrophoresis apparatus. Peroxidase bands were visualized by placing the gel for 2 hr in a mixture of two parts 1% guaiacol in pH 7.0 buffer, two parts 50 mM tris-maleate buffer, pH 7.0, and one part 0.5% H_2O_2.

Enzyme assays were run by a procedure based on that of Lance (7). The final reaction mixture was 3 ml and contained 13 mM guaiacol, 5 mM H_2O_2 and 40 mM tris-maleate buffer, pH 7.0. The reaction was initiated by the addition of the enzyme preparation and the absorbance at 470 nm was measured at 1-min intervals in a Hitachi-Perkin Elmer Model 139 spectrophotometer. Scopoletin in tris-maleate buffer was added at various concentrations in studies on the effect of scopoletin on peroxidase activity.

pH profiles were determined by measuring activity in range of 40 mM tris-maleate buffers. Michaelis constants were determined by the method of Lineweaver and Burk (8).

RESULTS AND DISCUSSION

When the cells of tobacco callus tissue culture W-38 are broken and then subjected to polyacrylamide gel electrophoresis at pH 9.3, we have found that four bands of activity appear after application of guaiacol and H_2O_2 to the gel. These four bands of activity appear to coincide in relative electrophoretic mobility to the four anodic isoperoxidases recently reported by Stafford and Galston (16). We will, therefore, use their nomenclature to distinguish the anodic isoperoxidases that we have separated from W-38 tobacco tissue. A schematic representation of a typical electrophoresis run in our laboratory is shown in Figure 1.

In attempts to separate the anodic isoperoxidases, we have subjected cell-free preparations of the isoperoxidases to isoelectric focusing with gradients ranging from pH 3 to 10 and pH 3 to 6. Isoelectric focusing, using either gradient, yields two separated peaks of approximately the same peroxidase activity (guaiacol assay) with one peak at pH 3.96 and another at pH 4.79. Polyacrylamide gel electrophoretic analysis demon-

[1] This research was supported by the National Science Foundation Grant GB-14585.

strated that the pH 4.79 band of peroxidase activity contains only band A_1 (Fig. 1). Furthermore, analysis of the fractions in the peroxidase activity (guaiacol assay) band centered at pH 3.96 indicates that fractions between pH 4.00 and 4.05 contain band A_3 as the only peroxidase active enzyme, while those fractions between pH 3.93 and 4.00 have increasing contamination of band A_3 (Fig. 1). Band A_4 was not detected by isoelectric focusing. Thus, we have developed a method for the separation of two individual peroxidase "isoenzymes". This initial separation has allowed us to do preliminary studies on these two isoperoxidases.

Table I summarizes the comparison of the properties of two anodic isoperoxidases. The two isoperoxidases differ in all the parameters tested. Band A_3 has a higher affinity for guaiacol than band A_1 as is reflected by the Km.

The finding that band A_3 is stimulated by scopoletin while band A_1 is unaffected is especially interesting. Figure 2 shows the effect of various concentrations of scopoletin in the assay mixture on the activity (guaiacol-H_2O_2).

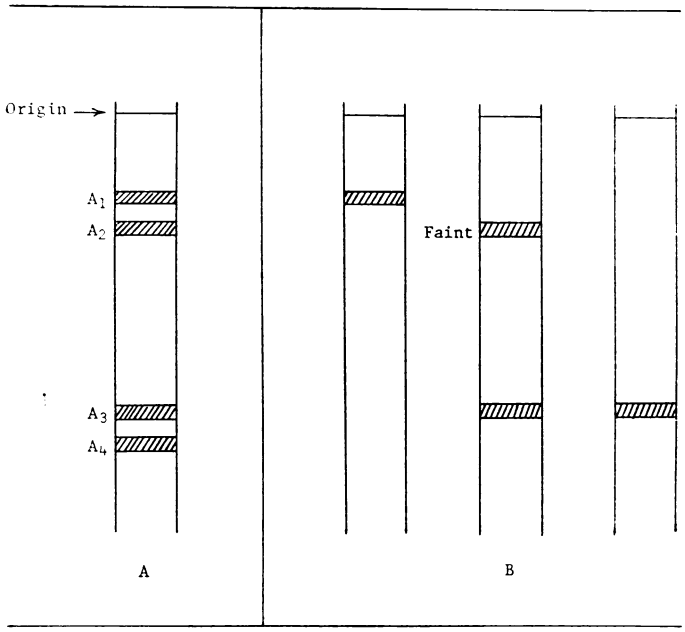

FIG. 1. Anodic peroxidases from tobacco callus tissue culture W-38. Visualization was accomplished by reaction with guaiacol and H_2O_2 after polyacrylamide gel electrophoresis at pH 9.3. A: Extracts of the tobacco tissue; B: fractions 56, 84, and 90 collected from isoelectric focusing with a gradient from pH 3 to 6.

Table I. *Comparison of Properties of the Two Isoperoxidases Separated by Isoelectric Focusing*

	Band A_1	Band A_3
Isoelectric point		
pH 3–6 run	4.79 ± 0.1	3.96 ± 0.03
pH 3–10 run	(4.8 – 5.1)	(3.8 – 4.1)
pH Optimum	6.0 ± 0.3	6.6 ± 0.2
Km for guaiacol[1]	9.1 mM	4.5 mM
Maximum staining on polyacrylamide gel	2 hr	15 min
Stimulation of peroxidase activity by 0.33 mM scopoletin	none	65%–75%

[1] Determined at pH 7.0.

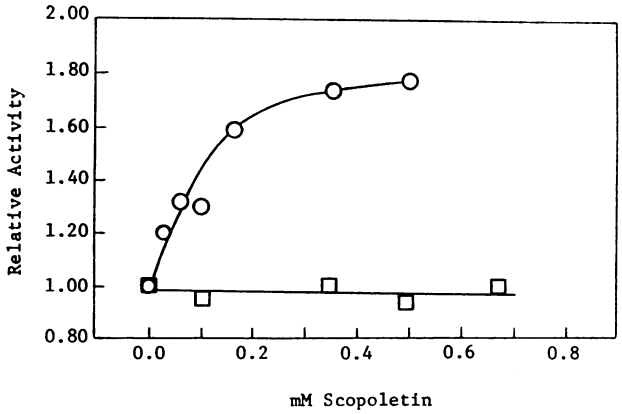

FIG. 2. Effect of scopoletin on isoperoxidases A_1 and A_3 from tobacco callus tissue culture W-38. □: Band A_1; ○: band A_3.

This present work with individual isoperoxidases shows that the effect of scopoletin on peroxidase activity can be different for individual isoperoxidases. The amounts of scopoletin used for our highest stimulatory effect would be inhibitory in the system of Imbert and Wilson (6). Thus, the report of Imbert and Wilson may reflect a cumulative effect on the different isoperoxidases having IAA oxidase activity.

LITERATURE CITED

1. ANDREAE, W. A. 1952. Effect of scopoletin on indoleacetic acid metabolism. Nature 170: 83–84.
2. EVANS, J. J. 1970. Spectral similarities and kinetic differences for two tomato plant peroxidase isoenzymes. Plant Physiol. 45: 66–69.
3. GALSTON, A. W., J. BONNER, AND R. S. BAKER. 1953. Flavoprotein and peroxidase as components of the indoleacetic acid oxidase system of peas. Arch. Biochem. Biophys. 122: 481–487.
4. GALSTON, A. W., S. LAVEE, AND B. Z. SIEGEL. 1968. The induction and repression of peroxidase isozymes by 3-indoleacetic acid. *In:* F. Wightman and G. Setterfield, eds., Biochemistry and Physiology of Plant Growth Substances. The Runge Press, Ottawa, Canada. pp. 455–472.
5. HELLER, R. 1965. Some aspects of the inorganic nutrition of plant tissue cultures. *In:* P. R. White and A. R. Grove, eds., Proceedings of an International Conference on Plant Tissue Culture. McCutchan Publishing Corporation, Berkeley, Calif. pp. 1–18.
6. IMBERT, M. P. AND L. A. WILSON. 1970. Stimulatory and inhibitory effects of scopoletin on IAA oxidase preparations from sweet potato. Phytochemistry 9: 1787–1794.
7. LANCE, C. 1955. Sur la détermination de l'activité peroxydase des extraits bruts de tissus végétaux. Rev. Gen. Bot. 62: 609–621.
8. LINEWEAVER, H. AND D. BURK. 1934. The determination of enzyme dissociation constants. J. Amer. Chem. Soc. 56: 658–666.
9. LINSMAIER, E. M. AND F. SKOOG. 1965. Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18: 100–127.
10. MCCUNE, D. C. 1961. Multiple peroxidases in corn. Ann. N. Y. Acad. Sci. 94: 723–730.
11. MACNICOL, P. K. 1966. Peroxidases of the Alaska pea (*Pisum sativum* L.). Enzymic properties and distribution within the plant. Arch. Biochem. Biophys. 117: 347–356.
12. ORNSTEIN, O. AND B. J. DAVIS. 1962. Disc electrophoresis, Part II. Distillation Products Industries. Rochester, New York.
13. SCHAEFFER, G. W., J. G. BUTA, AND F. SHARPE. 1967. Scopoletin and polyphenol-induced lag in peroxidase catalyzed oxidation of indole-3-acetic acid. Physiol. Plant. 20: 342–347.
14. SHANNON, L. M. 1968. Plant isoenzymes. Annu. Rev. Plant Physiol. 19: 187–210.
15. SIEGEL, B. Z. AND A. W. GALSTON. 1967. Indoleacetic acid oxidase activity of apoperoxidase. Science 157: 1557–1559.
16. STAFFORD, H. A. AND A. W. GALSTON. 1970. Ontogeny and hormonal control of polyphenoloxidase isoenzymes in tobacco pith. Plant Physiol. 46: 763–767.
17. VESTERBERG, O. AND H. SVENSSON. 1966. Isoelectric fractionation, analysis, and characterization of ampholytes in natural pH gradients. Acta Chem. Scand. 20: 820–834.