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SCOPOLETIN: A SUBSTRATE FOR AN ISOPEROXIDASE FROM *NICOTIANA TABACUM* TISSUE CULTURE W-38*

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Abstract—Scopoletin was found to be a substrate for a single anodic isoperoxidase isolated from tobacco callus tissue W-38. Isolation of this peroxidase was accomplished using DEAE-cellulose chromatography. This isoperoxidase catalyzed the destruction of scopoletin in the presence of H_2O_2 only. An enzyme assay for the scopoletin reaction was developed. The pH optimum of the enzyme was 5·5 and the apparent K_ms for scopoletin and H_2O_2 were 0·6 and 0·9 mM respectively.

INTRODUCTION

SINCE Andreae[1] first demonstrated in 1952 that crude indoleacetic acid (IAA) oxidase preparations from potato tissue could destroy scopoletin (6-methoxy-7-hydroxycoumarin), there have been several reports implicating scopoletin in the physiological action of peroxidases. Imbert and Wilson[2] have reported that, depending on the concentration, scopoletin either inhibits or stimulates IAA oxidase preparations from sweet potato.

More recently, Sirois and Miller[3] have implicated scopoletin in the control of the oxidation of IAA by horseradish peroxidase. They indicated that scopoletin was oxidized by their peroxidase preparation. They did not, however, maintain that their enzyme preparation contained only a single isoperoxidase and the existence of multiple forms of peroxidase is now well documented.[4] Schafer *et al.*[5] have shown that the effect of scopoletin on peroxidase activity can be different for two individual isoperoxidases. These workers found a stimulatory effect of scopoletin on the guaiacol oxidizing activity of one isolated isoperoxidase (A_3) and none on another isoperoxidase (A_1), both obtained from tobacco tissue W-38 grown in culture. Continuing studies on isoperoxidase A_3 in this laboratory have now indicated that scopoletin is actually a substrate for this isoperoxidase, which we have tentatively named "scopoletin peroxidase".† That scopoletin, a physiologically significant

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† This terminology is to imply only a certain specificity for scopoletin as a substrate and is not meant to imply that this is the only substrate altered by this enzyme or the only enzyme capable of altering scopoletin.

[1] ANDREAE, W. A. (1952) *Nature* **170**, 83.
[2] IMBERT, M. P. and WILSON, L. A. (1970) *Phytochemistry* **9**, 1787.
[3] SIROIS, J. C. and MILLER, R. W. (1972) *Plant Physiol.* **49**, 1012.
[4] SHANNON, L. M. (1968) *Ann. Rev. Plant Physiol.* **19**, 187.
[5] SCHAFER, P., WENDER, S. H. and SMITH, E. C. (1971) *Plant Physiol.* **48**, 232.

compound, is a substrate has been verified by several independent methods which include the appearance of a new substance as detected by TLC and difference absorption spectroscopy.

RESULTS AND DISCUSSION

Separation of a single isoperoxidase from all the other isoperoxidases of tobacco callus tissue culture W-38 was accomplished using DEAE-cellulose chromatography. Based on its electrophoretic mobility on polyacrylamide gels, this isoperoxidase is apparently equivalent to the isoperoxidase designated A_3 by Stafford and Galston.[6]

This isoperoxidase when added to a mixture of scopoletin and H_2O_2 initiates a rapid formation of a yellow product. Comparison of the absorption spectra of the initial assay mixture and of the assay mixture after the addition of the enzyme confirms the formation of a new substance absorbing at 450 nm. Since scopoletin absorbs negligibly at 450 nm, this observation served as the basis for the assay procedure described in the Experimental. Furthermore, decreased absorbance in the range 340–400 nm verifies the conversion of scopoletin into this product as do observations using TLC. In addition, scopoletin is highly fluorescent and fluorescence diminishes upon reaction. These data verify that scopoletin is converted to a product and this isoperoxidase (A_3) thus has the capability of serving as a "scopoletin peroxidase".

The effect of pH on initial velocity was determined at 1·25 mM scopoletin and 10 mM H_2O_2. Maximum activity appears at pH 5·5.

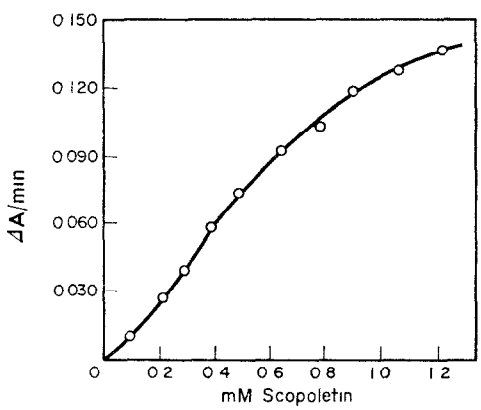

FIG. 1. EFFECT OF SCOPOLETIN CONCENTRATION ON THE ACTIVITY OF SCOPOLETIN PEROXIDASE. ASSAYS WERE RUN AT 10 mM H_2O_2 IN 40 mM SODIUM CITRATE BUFFER (pH 5·5).

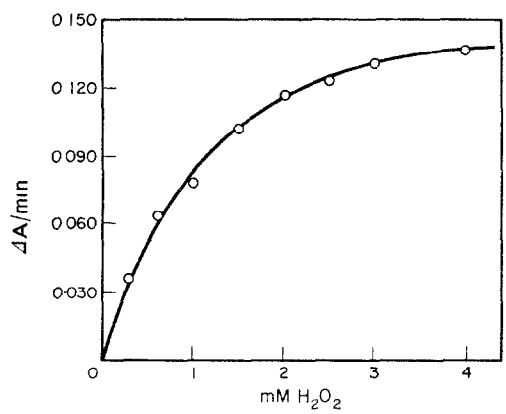

FIG. 2. EFFECT OF H_2O_2 CONCENTRATION ON THE ACTIVITY OF SCOPOLETIN PEROXIDASE ASSAYS WERE RUN AT 1·25 mM SCOPOLETIN IN 40 mM SODIUM CITRATE BUFFER (pH 5 5).

Using the assay based on a new absorption peak at 450 nm, we found that the initial velocity of this isoperoxidase depends on scopoletin concentration (Fig. 1).* The shape of the curve is not the expected rectangular hyperbola, and, thus, the enzyme does not display Michaelis–Menten kinetics. Apparent K_ms have been determined using a modified Lineweaver–Burk plot.[7] The K_m for scopoletin is 0·6 mM compared to 3 mM for guaiacol. This

* The solubility of scopoletin sets the upper limit of scopoletin concentration in Fig. 2.

[6] STAFFORD, H. A. and GALSTON, A. W. (1970) *Plant Physiol* **46,** 763
[7] LINEWEAVER, H. and BURK, D. (1934) *J. Am. Chem. Soc.* **56,** 658.

much lower apparent K_m for scopoletin is indicative that it binds more readily to the enzyme than guaiacol. Figure 2 shows the velocity versus substrate concentration curve at varying H_2O_2 concentrations. The K_m for H_2O_2 is 0·9 mM.

Since scopoletin is a substrate for an isolated isoperoxidase, it is suggested that it could be a physiological substrate and that this enzyme may act *in vivo* as a "scopoletin peroxidase". If this hypothesis is correct, then not all other isoperoxidases should have this same ability to oxidize scopoletin. Four other isoperoxidases (2 cathodic and 2 anodic) have been isolated from tobacco tissue in our laboratory. Under conditions yielding maximum activity for isoperoxidase A_3, no scopoletin oxidation is observed with any of the four other isoperoxidases (unpublished results).

Schafer *et al.*[5] reported the stimulation of guaiacol oxidizing ability by scopoletin on isoperoxidase A_3 but no stimulation by scopoletin on isoperoxidase A_1. No stimulation is observed with the cathodic isoperoxidases mentioned above. Since the product of the scopoletin oxidation reaction absorbs at 470 nm as does the guaiacol oxidation product, the stimulation of the guaiacol reaction could be due to an activator effect of scopoletin or to a substrate effect. It is now generally considered that enzymes having an S-shaped dependence on velocity (see Fig. 1) are allosteric in nature.[8] In either case, all data point to the fact that isoperoxidase A_3 should be considered allosteric. The possibility of a regulatory role does thus exist and warrants investigation.

Most investigators of peroxidases have utilized synthetic substrates such as guaiacol or synthetic electron acceptors such as dichlorophenol in the IAA oxidase reaction and in the study of the effect of physiological compounds on it. Our investigations indicate a need to analyze more closely these effects. The apparent diversity of isoperoxidase activities suggests that future investigations of peroxidases should include consideration of individual isoperoxidase activities. Attempts to determine and utilize possible physiological substrates must also be strongly encouraged.

EXPERIMENTAL

Chemicals were obtained commercially; scopoletin was recrystallized from MeOH before use.

Tobacco tissue culture W-38. The W-38 tissue was grown on the revised medium (RM-1964) of Linsmaier and Skoog[9] with 2 mg/l. of IAA, 200 μg/l. of kinetin and an increased amount of thiamine hydrochloride (1 mg/l.). The tissue was grown at *ca.* 25°. Three pieces of tissue which were about 5 × 5 × 3 mm were placed in an Erlenmeyer flask containing 50 ml of the above medium. The pieces of tissue were cut from tissue 5- to 6-weeks-old. The transferring was done in a laminar flow hood (Agnew-Higgins model M-142) using standard sterile technique.

Preparation of enzyme. 40 g (fr. wt) tobacco tissue plus 25 g of Polyclar AT in 100 ml of 0·05 M phosphate buffer (pH 6·0) were homogenized for 8 min in a Sorvall Omnimixer set at 8000 rpm. The homogenate was then centrifuged for 20 min at 27 000 *g*. The resulting supernatant was desalted on a Sephadex G25 column eluted with 0 005 M phosphate buffer (pH 6·0). The eluant was concentrated in a Zeineh microconcentrator. The concentrated sample was dialyzed for 24 hr against 400 vol. 0 005 M phosphate buffer (pH 6·0) and then applied to a DEAE-cellulose column (pre-equilibrated with 0 005 M phosphate buffer). Elution was step-wise up to 0 06 M phosphate buffer (pH 6·0). All preparative procedures and storage were at 4°. Peaks of activity were concentrated and analyzed by polyacrylamide gel electrophoresis using a Buchler Polyanalyst Disc Electrophoresis apparatus[10] and by starch gel electrophoresis using a modified Smithies apparatus.[11] Peroxidase bands were visualized by placing gels in a mixture of 2 parts 1% guaiacol, 2 parts 0·05 M phosphate buffer (pH 7 0) and 1 part 0·5% H_2O_2.

[8] KOSHLAND, D. E., JR. (1970) *The Enzymes* **1**, 341.
[9] LINSMAIER, E. M. and SKOOG, F. (1965) *Physiol Plant.* **18**, 100.
[10] ORSTEIN, O. and DAVIS, B. J. (1962) *Disc Electrophoresis—II*, Distillation Products Industries, Rochester, New York.
[11] BREWER, JR., G. (1970) *An Introduction to Isozyme Techniques*, Academic Press, New York.

Enzyme assays. A typical assay contained 1·25 mM scopoletin, 10 mM H_2O_2 and 0·04 M citrate buffer (pH 5·5). The reaction was initiated by the addition of enzyme and the absorbance (A) at 450 nm was measured at 30 sec intervals. Linear rates were obtained. No observable oxidation of scopoletin occurred in the absence of enzyme over the time interval of the assay.

TLC. Plates of Avicel SF were developed with methylisobutylketone–HCO_2H–H_2O (14:3:2) for separation of scopoletin products; detection was in UV light (366 nm).

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