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STIMULATORY AND INHIBITORY EFFECTS OF SCOPOLETIN ON IAA OXIDASE PREPARATIONS FROM SWEET POTATO

M. P. IMBERT and L. A. WILSON

Department of Biological Sciences, University of the West Indies, St. Augustine, Trinidad, W. Indies

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Abstract—IAA oxidase preparations from sweet potato (*Ipomoea batatas*) roots and etiolated shoots catalysed the disappearance of IAA in the absence of added H_2O_2, manganese and phenolic cofactors. Scopoletin inhibited IAA oxidase activity at high concentrations (12·5–250 nmol/ml) but stimulated activity at low concentrations (0·25–10·00 nmol/ml); these effects were dependant on the concentration of IAA and enzyme. Competitive inhibition of IAA oxidase activity by scopoletin is discussed and the compound is described as the most potent, naturally occurring stimulator of IAA oxidase activity so far reported. A vital role in the *in vivo* regulation of IAA levels in plants is suggested for this compound.

INTRODUCTION

SINCE its classical description by Tang and Bonner,[1] indole acetic acid (IAA) oxidase has been demonstrated in several plant species.[2–4] The enzyme system usually includes a peroxidase acting as an oxidase, since peroxidation in the strict sense, i.e. by hydrogen peroxide in the absence of oxygen, does not occur. Cofactor requirements for different enzyme preparations have been extensively studied,[5–7] but some enzymes are known to oxidize IAA in the absence of added cofactors. Stimulators of the enzyme system include manganous ions, resorcinol, 2,4-dichlorophenol (DCP) and a number of monophenols, many of which are DCP analogues.[7] None of these phenolics are naturally occurring compounds. Many naturally occurring di- and poly-phenols,[8] are, however, known to inhibit IAA oxidase activity.

Closely related coumarin derivatives have been reported to have opposite effects on IAA oxidase activity. Thus, scopoletin[9] has been shown to inhibit, and methylumbelliferone[10] to stimulate, IAA oxidase. Mechanisms suggested to explain the inhibitory effects of scopoletin on IAA oxidase are based on competitive inhibition of proposed components of the enzyme system by this compound.[9,11]

While investigating the IAA oxidase in sweet potato roots and etiolated shoots, enzyme preparations were found to catalyse rapid disappearance of IAA in the absence of added hydrogen peroxide, manganous ions and phenolic cofactors. Scopoletin was found to inhibit

[1] Y. W. TANG and J. BONNER, *Arch. Biochem. Biophys.* **13**, 11 (1957).
[2] Y. W. TANG and J. BONNER, *Am. J. Botany* **35**, 570 (1948).
[3] A. C. WAGENKNECHT and R. H. BURRIS, *Arch. Biochem. Biophys.* **25**, 30 (1950).
[4] W. A. GORTNER and M. J. KENT, *J. Biol. Chem.* **204**, 593 (1953).
[5] P. L. GOLDACRE, A. W. GALSTON and R. L. WEINTRAUB, *Arch. Biochem. Biophys.* **43**, 358 (1953).
[6] P. M. RAY and K. V. THIMANN, *Arch. Biochem. Biophys.* **64**, 175 (1956).
[7] E. R. WAYGOOD, A. OAKS and G. A. MACLACHLAN, *Can. J. Botany* **34**, 54 (1956).
[8] R. S. RABIN and R. M. KLEIN, *Arch. Biochem. Biophys.* **70**, 11 (1958).
[9] W. A. ANDREAE, *Nature* **170**, 83 (1952).
[10] W. A. ANDREAE and S. R. ANDREAE, *Can. J. Botany* **31**, 425 (1953).
[11] E. R. WAYGOOD, A. OAKS and G. A. MACLACHLAN, *Can. J. Botany* **34**, 905 (1956).

IAA oxidase activity at high concentrations and to stimulate enzyme activity at low concentrations. In the present paper, effects of a range of scopoletin and IAA concentrations on the activity of the sweet potato enzyme are described.

RESULTS

Active IAA oxidase preparations were obtained using an acetone precipitation procedure. Individual preparations were found to have widely different IAA oxidase activities, but the enzyme in phosphate–citrate buffer, pH 6·8, could be stored in the frozen state for several weeks without serious loss of enzyme activity. Induced photo-oxidation of IAA by scopoletin in controls was negligible. Although the experiments described here were carried out on sweet potato root preparations, similar results were obtained with etiolated shoot enzyme preparations. All enzyme preparations used were shown to have considerable peroxidase activity when measured by the rate of pyrogallol oxidation, up to 222 μmol pyrogallol peroxidized/min/ml enzyme.

Inhibition of Sweet Potato IAA *Oxidase by Scopoletin*

A typical curve of IAA oxidase activity[12] of the sweet potato root enzyme preparation is shown in Fig. 1. Rates of IAA disappearance of the order of 5·0 nmol IAA/min/ml enzyme were obtained in the absence of added hydrogen peroxide, manganese and phenolic cofactors. The inhibitory effect of scopoletin is also shown in Fig. 1. The initial 80 per cent inhibition of enzyme activity (after 30 min) declines to approximately 50 per cent inhibition after 180 min. Higher scopoletin concentrations (240 nmol/ml) completely inhibited IAA oxidase activity and concentrations as low as 7·7 nmol/ml were found to give 50 per cent inhibition with other reaction mixtures.

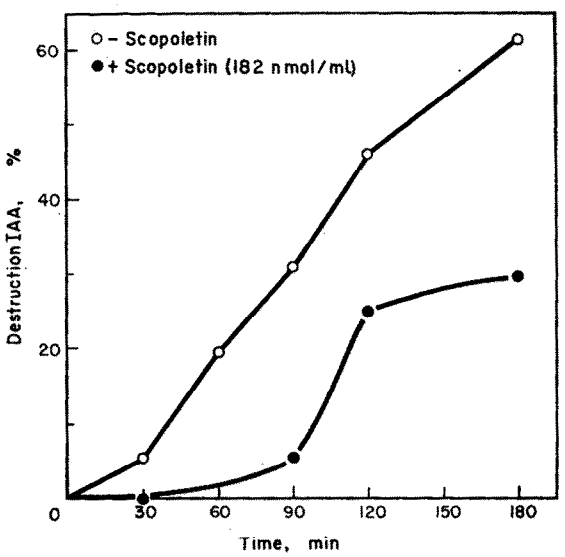

FIG. 1. REACTION MIXTURE: IAA (0·96 μmol); BUFFER (pH 6·8, 200 μmol); SCOPOLETIN (1 μmol); ENZYME (1·5 ml); IN TOTAL 5·5 ml.

[12] S. A. GORDON and R. P. WEBER, *Plant Physiol.* **26**, 192 (1951).

Stimulation of Sweet Potato IAA *Oxidase Activity by Scopoletin*

Andreae and Andreae[10] reported that scopoletin always inhibited IAA oxidase activity. Rabin and Klein[8] found that scopoletin inhibition of enzyme activity was pH dependent in etiolated pea seedling preparations, there being no inhibitory effects in the presence of 52 nmol/ml scopoletin at pH 5·5–5·6. Experiments were therefore carried out to investigate the effect of low concentrations of scopoletin (up to 5 nmol/ml) on sweet potato IAA oxidase activity. In these experiments, low scopoletin concentrations were found to stimulate IAA oxidase activity of both root and etiolated shoot enzyme preparations. (Fig. 2) The greatest stimulation of enzyme activity (325% over controls) was obtained at 1·25 nmol/ml scopo-

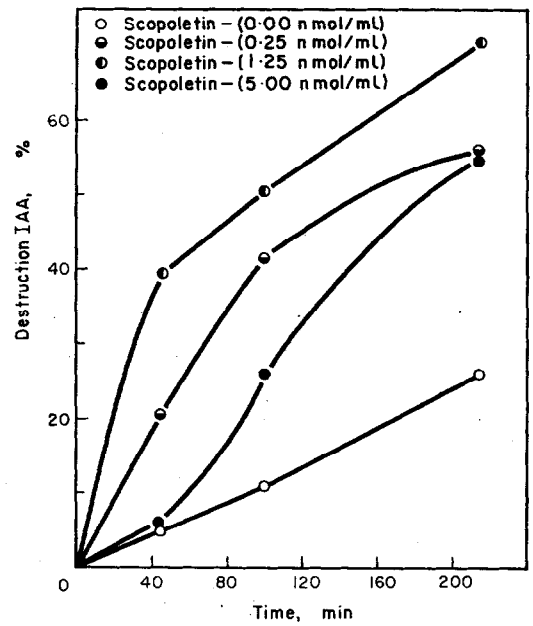

FIG. 2. REACTION MIXTURE: IAA (1 μmol); BUFFER (pH 6·8, 200 μmol); SCOPOLETIN (DILUTED as SHOWN); ENZYME (1·0 ml); IN TOTAL 4·0 ml.

letin, indicating that there might be an optimal concentration for scopoletin-induced stimulation of IAA oxidase activity. The level of stimulation obtained indicated that scopoletin was more effective than other recorded stimulators of IAA oxidase activity, e.g. 2,4-dichlorophenol, resorcinol and methylumbelliferone.

Concentrations of scopoletin ranging from 0·25 to 25 nmol/ml were used in a further series of reaction mixtures (Fig. 3). Peak stimulation of enzyme activity occurred at 5·0 nmol/ml scopoletin. Stimulation of enzyme activity decreased rapidly at concentrations above this concentration and the curve (Fig. 3) indicated that at a concentration of approximately 130 nmol/ml scopoletin, neither stimulation nor inhibition occurred.

The comparatively low percentage stimulation obtained in this experiment, compared with those shown in Fig. 2, might be accounted for by the initially high IAA oxidase activity of the enzyme preparation used in this experiment (see Fig. 3).

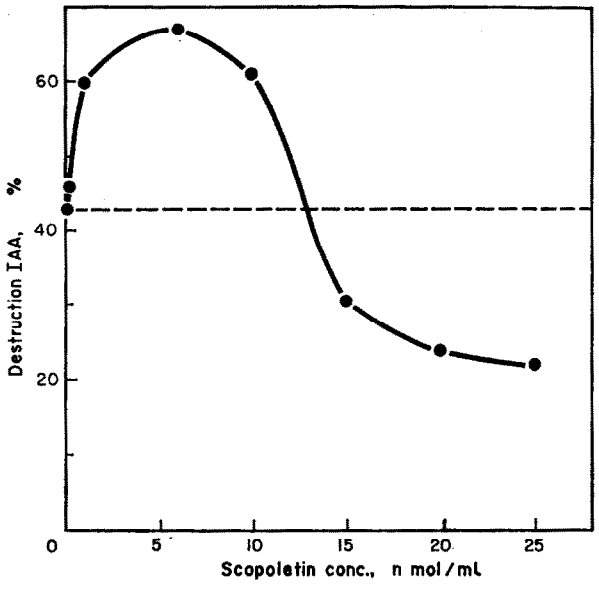

FIG. 3. REACTION MIXTURE: IAA (1 μmol); BUFFER (pH 6·8, 200 μmol); SCOPOLETIN (DILUTED AS SHOWN); ENZYME (1·0 ml) IN TOTAL 4·0 ml; REACTION TIME—150 min.

Reversal of Inhibitory and Stimulatory Effects of Scopoletin on Sweet Potato IAA *Oxidase*

It may be deduced from the results already described that the inhibitory and stimulatory effects of scopoletin on IAA oxidase activity were dependant on the relative concentrations of IAA, scopoletin, and enzyme. To test this hypothesis, the effects of IAA and enzyme concentration on oxidase activity in the presence of a fixed amount of scopoletin were examined. Increasing IAA concentration from 103 to 308 nmol/ml (Fig. 4) in the presence of

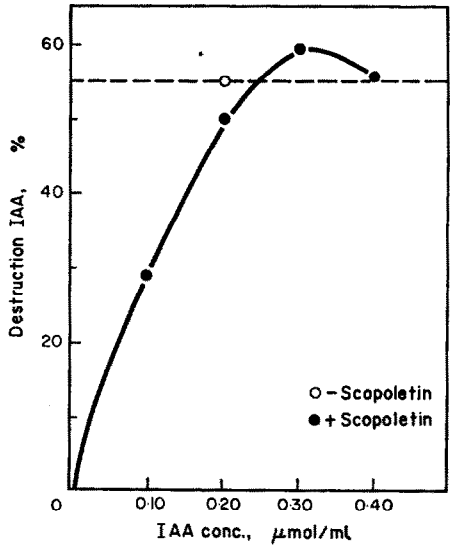

FIG. 4. REACTION MIXTURE: IAA (0·67 μmol); BUFFER (pH 6·8, 100 μmol); SCOPOLETIN (0·05 μmol); ENZYME (1·0 ml) IN TOTAL 6·5 ml; REACTION TIME—180 MIN.

inhibitory amounts of scopoletin was found to reverse a 49 per cent inhibition of IAA oxidase activity to an 8 per cent stimulation of enzyme activity. Percentage inhibition and stimulation of IAA oxidase activity were measured in relation to a scopoletin-free control containing 205 nmol/ml IAA or 50% of the highest IAA concentration used. The 57 per cent change in enzyme activity effected by a 3-fold increase in IAA concentration might have been greater if activity were measured with reference to a control containing 103 nmol/ml IAA, i.e. the concentration at which scopoletin-induced inhibition of IAA oxidase activity was highest.

Conversely, decreasing enzyme concentration from 100% to 25% reversed a 52 per cent scopoletin-induced inhibition of IAA oxidase activity with the original enzyme preparation, to a 100 per cent stimulation, when the enzyme preparation was diluted 3-fold (Fig. 5). Again, percentage inhibition and stimulation of enzyme activity were measured with reference to the activity of a scopoletin-free control, containing enzyme diluted to 50% of the original enzyme preparation.

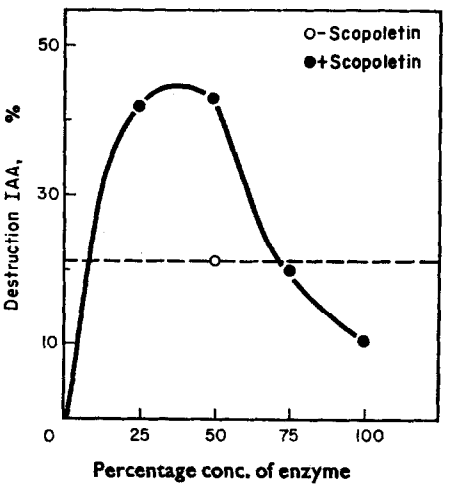

FIG. 5. REACTION MIXTURE: IAA (0·67 μmol) IN BUFFER (pH 6·8, 200 μmol); SCOPOLETIN (0·2 μmol); ENZYME (4·0 ml); IN TOTAL 6·2 ml; REACTION TIME—120 min.

DISCUSSION

Enzyme preparations from sweet potato roots and etiolated shoots have been found to show IAA oxidase activity in the absence of added hydrogen peroxide and cofactors.[13] All enzyme preparations, here described, were shown to have considerable peroxidase activity. Although special precautions were not taken to remove manganous ions and phenols from sweet potato enzyme preparations, their method of preparation should have reduced these cofactors to low levels. Tests for phenols in enzyme preparations, including chromatography were negative and the use of phosphate–citrate buffer (pH 6·8) in reaction mixtures probably resulted in the chelation of already low levels of manganous ions.[14] Participation of endogenously produced hydrogen peroxide in the enzymatic destruction of IAA by the sweet potato enzyme is not excluded, however, since IAA itself has been shown to be peroxigenic in the course of IAA destruction by IAA oxidase.[15]

[13] S. AKITA, F. YAMAMOTO, M. ONO, M. KUSUHARA, M. KOBAYASHI and S. OKEMOTO, *Bull. Chugoku Agri. Exptl. Sta.* **8**, 75 (1962).
[14] R. H. KENTEN, *Biochem. J.* **59**, 110 (1955).
[15] S. M. SIEGEL and A. W. GALSTON, *Arch. Biochem. Biophys.* **54**, 102 (1955).

The previously demonstrated inhibitory effects of high scopoletin concentrations (2·5–25 ppm)[9] on IAA oxidase activity were confirmed with the sweet potato enzyme. Waygood *et al.*[11] explained competitive inhibitory effects of scopoletin on IAA oxidase activity, on the basis of competition for manganic ions. The suggested chelation of manganese in our preparations seemed to exclude this explanation. Andreae[9] accounted for the inhibitory effects of scopoletin by competition for hydrogen peroxide, known to be involved in the oxidation of both IAA and scopoletin[10] by IAA oxidase preparations. The decrease in percentage inhibition of IAA oxidase activity with time (Fig. 1) might, therefore, be explained either by reduction in scopoletin levels due to scopoletin oxidation and/or to increased availability of endogenously produced hydrogen peroxide as the reaction proceeded. Neither of these factors were, however, studied in sweet potato IAA oxidase preparations. Nevertheless, uncertainty of the exact role of hydrogen peroxide in IAA oxidase activity and the demonstration of stimulatory and inhibitory effects of scopoletin within narrow concentration limits (Fig. 3) make it difficult to accept "hydrogen peroxide competition" as an unequivocal explanation of the action of scopoletin on IAA oxidase activity.

Ray[16] suggested that strong inhibition of IAA oxidase activity in a purified *Omphalia* enzyme preparation by pyrogallol was due to the accumulation of a form of enzyme incapable of oxidizing both substrates, rather than competition for hydrogen peroxide. Inhibitory effects of small amounts of IAA on peroxidation of pyrogallol by the same enzyme were interpreted in terms of a suppression of peroxidative activity by IAA. Inhibition of IAA oxidase activity by scopoletin has not been conclusively shown to be H_2O_2-dependent rather than H_2O_2-stimulated. In fact, a considerable fraction of the H_2O_2-stimulated scopoletin oxidase activity of IAA oxidase preparations reported by Andreae and Andreae[10] occurred in the absence of added hydrogen peroxide. An important difference between pyrogallol and scopoletin inhibition of IAA oxidase activity seemed to be that, unlike pyrogallol peroxidation,[16] scopoletin oxidation[10] is apparently not inhibited by IAA. However, the stimulation of scopoletin oxidase activity by IAA and putrescine[10] does not necessarily mean that hydrogen peroxide is the effective causal agent in the competitive inhibition of IAA oxidase by scopoletin. It is suggested that hydrogen peroxide enhances rather than causes this competitive inhibition. The demonstrated reversibility of inhibitory and stimulatory effects of scopoletin on IAA oxidase activity in response to small changes of relative concentrations of IAA, enzyme and scopoletin (Figs. 3–5) seemed to suggest that inhibitory effects of scopoletin were due to competition between IAA and scopoletin for enzyme, rather than to competition for hydrogen peroxide. This possibility was also mentioned by Andreae.[9] The role of hydrogen peroxide in this competition in the light of recently proposed mechanisms of IAA oxidase action[17,18] is yet to be determined.

Explanation of the inhibitory effects of scopoletin in terms of direct competition between IAA and scopoletin for enzyme allows for the explanation of its stimulatory effects as an IAA oxidase cofactor. Low concentrations of competitive inhibitors are known to stimulate growth and enzyme activity.[19] Similar stimulation of oxyhaemoglobin formation at low carbon monoxide concentrations has been known for a long time. Quesnel[20] proposed a mechanism for explaining the stimulatory action of carbon monoxide, based on calculations

[16] P. M. RAY, *Arch. Biochem. Biophys.* **87**, 19 (1960).
[17] P. M. RAY, *Arch. Biochem. Biophys.* **96**, 199 (1962).
[18] I. YAMAZAKI and L. H. PIETTE, *Biochim. Biophys. Acta* **77**, 47 (1963).
[19] D. W. WOOLLEY, *A Study of Antimetabolites*, John Wiley, New York (1952).
[20] V. C. J. QUESNEL, *Exptl. Cell Res.* **10**, 575 (1956).

which indicated a sigmoid rather than a hyperbolic oxygen dissociation curve, for the relationship between oxygen pressure and percentage oxyhaemoglobin. It was suggested that at low carbon monoxide concentrations, oxyhaemoglobin formation was stimulated by a "co-operative effect" between substrate and inhibitor molecules. Peroxidase, like haemoglobin, is an iron-porphyrin containing conjugated protein. Apparently genuine light-reversible carbon monoxide inhibition of IAA oxidase has also been recently demonstrated by Ray,[16] suggesting the existence of ferroperoxidase. It is proposed, therefore, that scopoletin-induced stimulation of IAA oxidase activity might take place by a mechanism analogous to that outlined for the stimulation of oxyhaemoglobin formation, by low carbon monoxide concentrations. Rapid increase in IAA oxidase activity at low scopoletin concentrations to peak stimulation and equally rapid decrease in enzyme activity thereafter to inhibitory levels of activity, with increasing scopoletin concentration (Fig. 3) were compatible with the model proposed by Quesnel.

Scopoletin is known to be present especially in the roots of many plant species.[21-23] Externally supplied scopoletin has been shown to function both as a stimulator[9] and inhibitor[24,25] of root growth, depending on the concentration used. Endogenously produced scopoletin has also been reported to be involved in the growth and differentiation of tobacco callus.[26] It has now been demonstrated that scopoletin is the most potent, naturally occurring stimulator of IAA oxidase activity so far described, being effective at concentrations as low as 0·25 nmol/ml. Together with its inhibitory effects at higher concentrations and its capacity for promoting stimulation or inhibition of IAA oxidase activity within narrow concentration limits (Fig. 3), the scopoletin molecule is well suited to play a major role in the *in vivo* regulation of IAA levels, through its versatile effects on IAA oxidase activity.

EXPERIMENTAL

Materials

Roots were obtained by growing sweet potato stem cuttings (Cv. 049) in water culture in the greenhouse, using standard techniques.[27] Etiolated shoots were produced by germinating sweet potato tubers in the dark at room temp. (*ca.* 30°).

Enzyme Preparation

Roots and etiolated shoots were harvested and rapidly weighed in dim light and stored in black polyethylene bags at −10°. IAA oxidase preparations were made by the acetone precipitation procedure.[28] Acetone precipitates were suspended in 0·2 M phosphate–citrate buffer (pH 6·8) for 24 hr at 5° and centrifuged at 6000 *g* for 10 min. The resulting clear supernatant was stored at −10° and used as the enzyme preparation.

Assay of IAA *Oxidase Activity*

IAA oxidase activity was assayed by determining residual IAA using the modified Salkowski reagent.[12] Assays were carried out at 30° with shaking and were carried out in duplicate. Enzyme activity is expressed as percentage IAA destroyed, using as reference an enzyme-free or boiled enzyme controls. Mixtures for individual assays are given under results.

Assay of Peroxidase Activity

Peroxidase was determined by measuring purpurogallin formation colorimetrically, in the presence of pyrogallol and hydrogen peroxide.[15]

[21] R. H. GOODWIN and F. KAVANAGH, *Bull. Torrey Botan. Club* **76**, 255 (1949).
[22] R. H. GOODWIN and F. POLLOCK, *Am. J. Botany* **41**, 516 (1954).
[23] S. HOUSLEY and W. C. TAYLOR, *J. Exptl. Botany* **9**, 458 (1958).
[24] B. M. POLLOCK, R. H. GOODWIN and S. GREEN, *Am. J. Botany* **41**, 516 (1954).
[25] A. M. MAYER and M. EVENARI, *J. Exptl. Botany* **3**, 246 (1952).
[26] J. A. SARGEANT and F. SKOOG, *Plant Physiol.* **35**, 934 (1960).
[27] E. J. HEWITT, *Sand and Water Culture Methods Used in The Study of Plant Nutrition.* Tech. Commun. No. 22. Comm. Bur. Hort. and Plantation Crops. East Malling (1966).
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