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INDOLEACETIC ACID INHIBITION OF A PHENYLALANINE AMMONIA-LYASE PREPARATION FROM SUSPENSION CULTURES OF WR-132 TOBACCO*

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Abstract—The effect of indoleacetic acid, 2,4-dichlorophenoxyacetic acid, tryptophan, scopoletin, scopolin, and related compounds on the activity of a phenylalanine ammonia-lyase preparation from tobacco tissue WR-132 grown in suspension culture has been investigated Under the experimental conditions used, IAA and its probable plant precursor trytophan, were found to inhibit the enzyme strongly Scopoletin showed slightly lesser inhibition The synthetic auxin, 2,4-D, produced little effect on the enzyme except at the higher concentration used Scopolin, kinetin, gibberellic acid, and β-indole-3-propionic acid showed no effect The concentration of β-mercaptoethanol used in each assay was found to affect the values obtained for the percentage inhibition of phenylalanine ammonia-lyase by IAA The possible physiological significance of these inhibitions is discussed.

INTRODUCTION

THE PHENYLALANINE ammonia-lyase from potato tuber has been characterized in considerable detail.[1–3] Recently, O'Neal and Keller[4] have reported on the partial purification and on some properties of the phenylalanine ammonia-lyase from leaves of burley tobacco plants The importance of this enzyme in tobacco plants lies not only in its role in lignin production, but also in its reported participation in the synthesis of soluble phenolic compounds which may comprise 2–8% of the dry weight of tobacco.[4] Wender *et al.* have reported an accumulation of some of these phenolics, most commonly scopoletin (6 methoxy-7-hydroxycoumarin) and its 7-glucoside, scopolin, in tobacco plants under various stress conditions, such as nitrogen deficiency,[5] boron deficiency,[6] X-irradiation,[7] spraying with 2,4-dichlorophenoxyacetic acid[8] (2,4-D), etc The effect of increased scopoletin on indoleacetic acid (IAA) oxidase has been reported by several groups of workers.[9–12] In our laboratory, we have also been studying the effect of scopoletin, scopolin, IAA, and closely related compounds on the activity of a phenylalanine ammonia-lyase preparation from

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[1] E A. HAVIR and K R HANSON, *Biochem* **7**, 1896 (1968)
[2] E A HAVIR and K R HANSON, *Biochem* **7**, 1904 (1968)
[3] K R HANSON and E A HAVIR, *Arch Biochem Biophys* **141**, 1 (1970)
[4] D O'NEAL and C J KELLER, *Phytochem* **9**, 1373 (1970)
[5] M ARMSTRONG, L M ROHRBAUGH, E L RICE and S H WENDER, *Phytochem* **9**, 945 (1970)
[6] R WATANABE, W J MCILRATH, J SKOK, W CHORNEY and S H WENDER, *Arch Biochem.* **94**, 241 (1961)
[7] D E KOEPPE, L M ROHRBAUGH, E L RICE and S H WENDER, *Radiation Bot* **10**, 261 (1970)
[8] L J DIETERMAN, C Y LIN, L M ROHRBAUGH, V THIESFELD and S H WENDER, *Anal Biochem* **9**, 139 (1964)
[9] W A ANDREAE, *Nature, Lond* **170**, 83 (1952)
[10] L SEQUEIRA, *Phytopath* **54**, 1078 (1964)
[11] G W SCHAEFFER, J G BUTA and F SHARPE, *Physiol Plant* **20**, 342 (1967)
[12] M P IMBERT and L A WILSON, *Phytochem.* **9**, 1787 (1970)

tobacco tissue (WR-132) grown in suspension culture. This tobacco tissue was chosen for study, because we have been unable—in contrast to whole tobacco plants—to find perceptible amounts of scopolin, scopoletin, or lignin in this tissue under its usual growth conditions in suspension cultures This paper reports our finding that under the experimental conditions used, IAA and its probable plant precursor tryptophan, strongly inhibit the enzyme. The synthetic auxin 2,4-D has little effect on the enzyme except at the higher concentration used. Kinetin, gibberellic acid, and β-indole-3-propionic acid showed no effect

RESULTS

The pH optimum of the phenylalanine ammonia-lyase preparation from tobacco tissue WR-132 grown in suspension culture was found to be approximately 8 8, and the initial velocity was directly proportional to the amount of enzyme added Michaelis–Menten kinetics were observed, and the Michaelis constant was 3×10^{-5} M

Since the biosynthesis of lignin[13] and of phenolic compounds[14] may be affected *in vivo* by varying concentrations of auxin (IAA or synthetic 2,4-D) and kinetin, these compounds and related ones were studied for their effect on cell-free preparations of phenylalanine ammonia-lyase. The results, which are included in Table 1, demonstrate that IAA and tryptophan strongly inhibit the enzyme The synthetic auxin 2,4-D has little effect on the

Table 1 Effect of some growth regulators, related compounds, and scopolin and scopoletin on L-phenylalanine ammonia-lyase activity

Compound added	Concentration (mM)	Substrate concentration (mM)	Relative activity (%)
Indole-3-acetic acid	3	1	39
Indole-3-acetic acid	1	0 1	53
Tryptophan	3	0 1	38
β-indole-3-propionic acid	3	0 1	106
Indole	3	0 1	84
Indole	1	0 1	105
Kinetin	*	1	103
Gibberellic acid	3	1	103
2,4-D	3	0 1	84
2,4-D	1	0 1	101
Scopoletin	0 6	0 1	81
Scopoletin	0 6	0 03	69
Scopoletin	0 15	0 03	77
Scopolin	1	0 1	102
Scopolin	0 6	0 03	100

The radioactive assay was used as described in Experimental
* Saturated solution

enzyme except at the higher concentration Kinetin, gibberellic acid, and indole-3-propionic acid showed no perceptible effect By assaying the enzyme at various phenylalanine concentrations at constant inhibitor concentration, it could be demonstrated[15] that both tryptophan and IAA inhibit competitively.

[13] S A Brown, Lignins *Ann Rev Plant Physiol* **17**, 223 (1966)
[14] J A Sargent and F Skoog, *Plant Physiol* **35**, 934 (1960)
[15] H Lineweaver and D Burk, *J Am Chem Soc* **56**, 568 (1934)

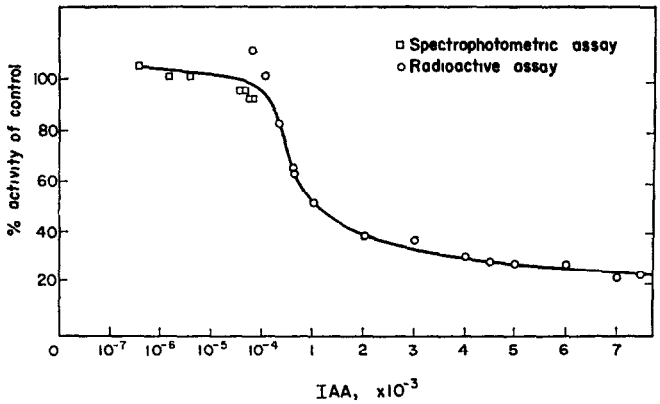

FIG 1

In order to determine the relationship between IAA concentration and the activity of phenylalanine ammonia-lyase, IAA was varied while phenylalanine concentration was kept constant at 0·1 mM. Figure 1 demonstrates that 50% inhibition of the enzyme occurs at 1 2 mM Furthermore, preincubation before assay of phenylalanine ammonia-lyase preparations with IAA for time periods up to 6 hr yields the same inhibition patterns as shown in Table 1 Dialysis of the preincubated enzyme preparations against 0·05 M sodium borate (pH 8 8) overnight restored 91% of the control enzyme activity.

TABLE 2 ANTAGONIST EFFECT OF β-MERCAPTOETHANOL AND IAA ON THE INHIBITION OF PHENYLALANINE AMMONIA-LYASE

Concentration of β-mercaptoethanol (mM)	Concentration of IAA (mM)	Relative activity of enzyme (%)
30	6	56
	3	44
	1	42
	0 1	32
	0	29
10	6	28
	3	46
	1	72
	0 1	79
	0	80
5	6	49
	3	75
	1	83
	0	100
0 66	Control	100

The radioactive assay was used as described in the text. The substrate concentration was 1 mM

The concentration of β-mercaptoethanol used in each assay affected the percentage inhibition of phenylalanine ammonia-lyase by IAA Table 2 illustrates the antagonistic effect that increasing concentrations of β-mercaptoethanol have on the IAA inhibition of the enzyme Also shown in Table 2 is the inhibition by β-mercaptoethanol alone It could be demonstrated by the Lineweaver-Burk analysis[15] that the β-mercaptoethanol inhibition was competitive Since phenylalanine ammonia-lyase from other sources, for example barley,[16] appears to be a sulfhydryl enzyme and β-mercaptoethanol inhibits a sulfhydryl enzyme, we examined the inhibition of our phenylalanine ammonia-lyase preparation from suspension cultures of tobacco by other sulfhydryl reagents, including cysteine, glutathione, and *N*-ethylmaleimide In every case, we found that the enzyme from suspension tobacco cultures was inhibited, similar to the enzyme from the leaves of tobacco, as reported by O'Neal and Keller [4] We have also found that inhibition by β-mercaptoethanol could be relieved by dialysis

In addition to *trans*-cinnamic acid, which has been reported by others[2,4,17] to inhibit the various phenylalanine ammonia-lyase preparations they obtained, we investigated, as possible feedback inhibitors of phenylalanine ammonia-lyase, the effect of various possible intermediates in lignin biosynthesis and of some of the phenolic compounds which accumulate in tobacco under stress conditions Similar to the results reported by O'Neal and Keller,[4] we observed inhibition by scopoletin, but no inhibition by caffeic acid, *p*-coumaric acid, and ferulic acid (although O'Neal and Keller report slight inhibition for ferulic acid) We investigated not only the effect of scopoletin at various concentrations, but also the effect of its glucoside scopolin on the enzyme The results included in Table 1 show that as the substrate concentration is decreased, the inhibition by scopoletin is enhanced, but that under no conditions attempted does scopolin inhibit

DISCUSSION

In general, it would seem that phenylalanine ammonia-lyase from suspension tobacco cultures is somewhat similar to the enzyme from burley tobacco leaves, even though the former tissue apparently does not produce detectable amounts of lignin, scopolin, and scopoletin under the usual suspension culture growth procedure used It is possible that the differences observed in K_m (3×10^{-5} M as compared to 16×10^{-5} M) and in percentage of inhibition by various compounds tested may be due to the particular sulfhydryl compound and its concentration used in the experiments, rather than to the synthesis of a different kind of phenylalanine ammonia-lyase It is observed from the antagonistic effect that β-mercaptoethanol has on IAA inhibition (Table 2), that variations in concentrations of sulfhydryl reagents may yield different results It is also possible that we are dealing with an enzyme preparation from a source in which one particular phenylalanine ammonia-lyase isozyme predominates, whereas another preparation from another source may contain a different ratio of isozymes[18] to give a different K_m value

The data obtained still do not furnish a definite, clearcut explanation of the *in vivo* inhibition of phenylalanine ammonia-lyase by IAA and by scopoletin. It is tempting, however, to speculate that the scopoletin inhibition, coupled with its inhibition, of glucose-6-phosphate dehydrogenase[19] of the pentose phosphate pathway, is a feedback mechanism

[16] J. KOUKOL and E E CONN, *J Biol Chem* **236**, 2693 (1961)
[17] D C WALTON, *Plant Physiol* **43**, 1120 (1968)
[18] S I AHMED and T SWAIN, *Phytochem* **9**, 2287 (1970), T. SWAIN and C A WILLIAMS, *Phytochem* **9**, 2115 (1970)
[19] S KAJINAMI, S H WENDER and E C SMITH, *Phytochem* **10**, 1501 (1971)

to control the biosynthesis of certain phenolic compounds. An intermediate in the pentose phosphate pathway, namely erythrose-4-phosphate, is a precursor of phenylalanine. It is possible that the glucosylation of scopoletin to produce the non-inhibitory scopolin may be to relieve the inhibition produced by the scopoletin It is even more speculative at present to attempt to propose what the role of IAA is in inhibition of phenylalanine ammonia-lyase It may be that since 1·2 mM IAA is required for 50% inhibition that the inhibition by IAA has no physiological significance except when the IAA concentration rises above a 'threshold level'

The inhibition of the phenylalanine ammonia-lyase by both IAA and scopoletin further supports the likelihood of a probable interrelationship between these two compounds, as has been suggested previously by publications on IAA oxidase and scopoletin. However, in view of the recent work by Swain *et al*,[18] one cannot eliminate the possibility that IAA may inhibit one isozyme of phenylalanine ammonia-lyase and scopoletin another isozyme. It would seem, nonetheless, that if scopoletin is acting as a feedback inhibitor, and if isozymes of phenylalanine ammonia-lyase occur in tobacco tissue WR-132 from suspension cultures, that at least one isozyme of phenylalanine ammonia-lyase is involved in a pathway of soluble phenolic compound biosynthesis.

EXPERIMENTAL

Plant material The plant material used was suspension culture line WR-132 of tobacco tissue (*Nicotiana tabacum* L, var Xanthi), obtained from Dr A C Olson of the USDA, Albany, California The cells were grown in 125 ml erlenmeyer flasks containing 50 ml of growth medium [20] During the 10 day growth period each flask of cells grew from 2 g (weight of inoculant) to about 10 g The flasks of cells were constantly agitated on a reciprocal shaker (95–105 reciprocations/min) The temperature of the growth room was 23° and the light intensity was less than 0 05 lux Since light has been reported to result in increased activity of phenylalanine ammonia-lyase,[21] the light intensity was increased to 0 3 lux 12 to 24 hr before the cells were harvested The brighter light significantly increased the apparent level of phenylalanine ammonia-lyase The cells were collected by suction filtration and thoroughly washed with 0 1 M imidazole buffer at pH 6 5 containing 10 mM β-mercaptoethanol and 4 mM $Na_2S_2O_5$

Enzyme preparation About 40 g of washed cells were mixed with 40 g of glass beads, 25 g of washed Polyclar AT which had been soaked with deionized water overnight, and 80 ml of imidazole buffer (pH 6 5) containing 10 mM β-mercaptoethanol and 4 mM $Na_2S_2O_5$ This mixture was blended for 10 min in a blender (Sorvall Omnimixer) at 5000 rev /min The blended mixture was centrifuged at 34,800 *g* to remove the glass beads. Polyclar AT, and cell debris from the crude enzyme solution

To the supernatant thus obtained, enough solid $(NH_4)_2SO_4$ was slowly added to form a solution that was about 28% saturated with respect to the salt The precipitate formed was removed by centrifugation at 34,800 *g* for 10 min and discarded Solid $(NH_4)_2SO_4$ was slowly added to the remaining supernatant solution to give a solution that was about 58% saturated with respect to the salt The precipitate was collected by centrifugation at 34,800 *g* for 10 min and dissolved in about 5 ml of 0 05 M sodium borate buffer containing 5 mM β-mercaptoethanol and dialyzed about 12 hr in 500 ml of the same buffer The enzyme was dialyzed an additional 12 hr in the same buffer without the β-mercaptoethanol The dialyzed enzyme solution was used for all assays In some experiments, a column packed with either Sephadex G-10 or Sephadex G-25 was used to desalt the enzyme

Enzyme assay, spectrophotometric assay The activity of phenylalanine ammonia-lyase was determined by measurement of the cinnamic acid formed The reaction mixture contained enzyme, various amounts of L-phenylalanine, and 150 μmoles of sodium borate buffer (pH 8 8) in a total volume of 3 0 ml Cinnamic acid formation was followed at 290 nm, 1 μmole of cinnamic acid in 3 ml has an absorbance of 3 0 (1 cm light path) [1] The initial velocity was measured on a Beckman DB-G at 23° every 15 min for 1 hr

Radioactive assay The reaction mixture was the same as that used for the spectrophotometric assay except C^{14}-L-phenylalanine (Amersham/Searle) was used After the mixture was incubated at 23° for 1 hr, the reaction was terminated by the addition of 0 2 ml of 50% trichloroacetic acid, followed immediately by 0 1 ml of 3×10^{-2} M carrier *trans*-cinnamic acid dissolved in 0 05 M NaOH.

[20] E M Linsmaier and F. Skoog, *Physiol Plant.* **18**, 100 (1965).

[21] K. R. Hanson, M Zucker and E Sondheimer, in *Phenolic Compounds and Metabolic Regulation* (edited by B J. Finkle and V C Runeckles), pp 74, 75, Appleton–Century–Crofts, New York (1967).

Benzene (5 0 ml) was added to the acidified mixture, which was vigorously stirred until an emulsion formed After 10–15 min, the reaction mixture was centrifuged to remove the protein from the benzene phase A 3-ml aliquot from this phase was transferred to a scintillation vial, and the benzene was completely removed in a vacuum oven 10 ml of dioxane-scintillation fluid were added, and the sample was counted in a Beckman DPM-100 Scintillation Counter A reaction mixture blank, complete with enzyme and substrate, which had been taken through the entire procedure for each set of assays, was terminated at zero reaction time The radioactivity of the blank was subtracted from the total radioactivity of each sample All radioactive assays were in duplicate and the averages are presented

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Key Word Index—Nicotiana tabaccum, Solanaceae, tobacco, phenylalanine ammonia lyase, indolyl-3-acetic acid