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PARTIAL PURIFICATION AND SOME PROPERTIES OF PHENYLALANINE AMMONIA-LYASE OF TOBACCO (*NICOTIANA TABACUM*)

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Abstract—The enzyme responsible for the first step in the synthesis of phenolic compounds in tobacco, L-phenylalanine ammonia-lyase, was purified approximately 200-fold from leaves of *Nicotiana tabacum* var. Burley 21. The purest preparation was at least 70 per cent homogenous as defined by disc electrophoresis. The optimal pH for the reaction was between 8·0 and 8·6. At pH 7·4, the K_m for the substrate L-phenylalanine was $1{\cdot}6 \times 10^{-4}$ M and at pH 8·55 it was $2{\cdot}2 \times 10^{-4}$ M. D-Phenylalanine, L-tyrosine, *m*-tyrosine, *o*-tyrosine, and dihydroxyphenylalanine were not deaminated by the enzyme. Sulfhydryl compounds caused a significant stimulation of activity, but no cofactor requirements were found. The enzyme was sharply inhibited by *trans*-cinnamic acid, in a competitive manner, with a K_i of $3{\cdot}1 \times 10^{-5}$ M at pH 7·1. Other phenolic inhibitors included *o*-coumaric acid, *o*-tyrosine, and quercetin. It is suggested that *trans*-cinnamic acid may play a role *in vivo* in regulating biosynthesis of phenolic compounds in tobacco.

INTRODUCTION

THE FIRST reaction leading to the eventual biosynthesis of a wide variety of phenolic compounds in higher plants is catalyzed by the enzyme L-phenylalanine ammonia-lyase [EC 4.3.1.5] to yield *trans*-cinnamate and NH_4^+, a reaction similar in several ways to the reactions catalyzed by L-tryosine ammonia-lyase [EC 4.3.1].[1] The *trans*-cinnamate produced by phenylalanine ammonia-lyase gives rise to *p*-coumaric acid, a compound also derived directly from L-tyrosine by the action of tyrosine ammonia-lyase.[1] *p*-Coumaric acid gives rise in turn to other simple phenols used in the synthesis of lignin, soluble polyphenols, and other related compounds in fungi and higher plants.[2–5]

Phenylalanine ammonia-lyase activity has been detected in a large number of vascular plants[6] and in several genera of basidiomycetes.[7] The enzyme has been partially purified from a variety of plants and microorganisms[8–12] and recently has been purified extensively and characterized from potato tuber,[13,14] corn[15] and the basidiomycete *Rhodotorula glutinis*.[16]

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The presence of phenylalanine ammonia-lyase had not been demonstrated previously in tobacco. However, its importance in this plant lies not only in its role in lignin production, but also its participation in the synthesis of soluble phenolic compounds which may comprise 2 or 8 per cent of the dry weight of tobacco.[5, 17, 18]

RESULTS

The flow sheet of the purification procedure is presented in Fig. 1. The quantitative data for the preparation of the enzyme are shown in Table 1. The techniques described here were

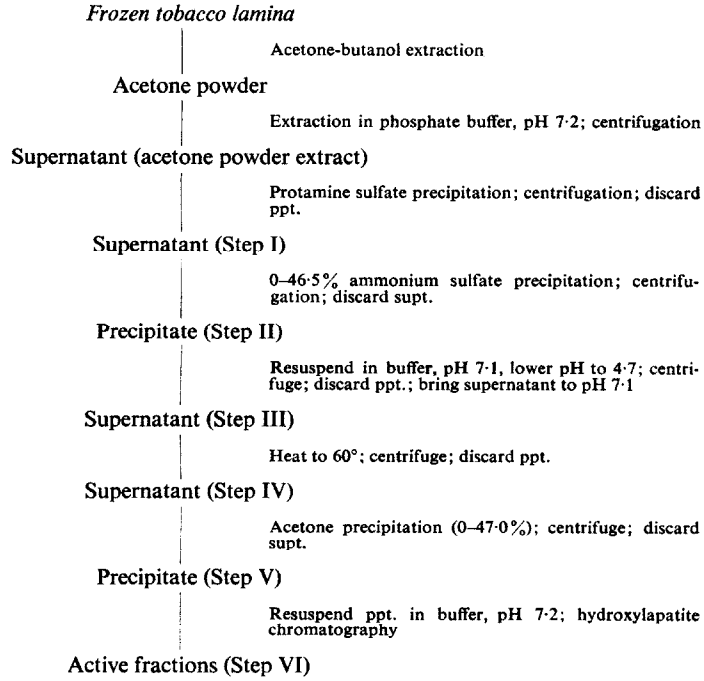

FIG. 1. ABBREVIATED FLOW CHART OF THE ENZYME PURIFICATION.

TABLE 1. SUMMARY OF PURIFICATION PROCEDURE—PHENYLALANINE AMMONIA-LYASE

Description	Total protein (mg)	Total m Units	mU/mg protein	Recovery (%)	Purification
Acetone powder extract	2080	1950	0·94	100	1·0
Protamine sulfate (step I)	1392	—	—	—	—
Ammonium sulfate (step II)	307	1872	6·10	96	6·5
pH (4·70) (step III)	224·8	1609	7·16	82·5	7·6
Heat (60°) (step IV)	112·2	1573	14·02	80·7	15·0
Acetone (step V)	17·4	1204	69·20	62	73·4
Hydroxylapatite (step VI)	3·24	621	191·67	31·9	204

[17] H. E. WRIGHT, in *The Phenolics of Tobacco and Their Significance in Plant Phenolics and Their Industrial Significance* (edited by V. C. RUENCKLES) (1962).
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generally reproducible, although final purification with different lots of enzyme varied between 160–235-fold. Step V was the most critical and variable step, recovery here varying from 70–90 per cent of the units after Step IV, and specific activity varied slightly more. A typical elution pattern is illustrated in Fig. 2. The fractions containing the greatest activity were pooled and stored in crushed ice. Upon storage for several days there appeared a small amount of precipitated protein, which was removed by centrifugation before disc gel electrophoresis.

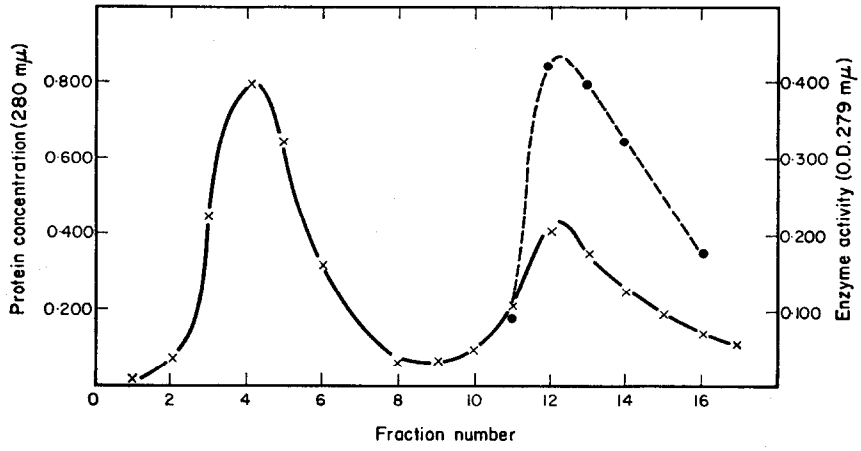

FIG. 2. ELUTION OF PHENYLALANINE AMMONIA-LYASE (o) FROM A 2·5 × 4·2 cm HYDROXYLAPATITE COLUMN WITH pH 7·6 PHOSPHATE BUFFER.

(X) absorbance, O.D. 280 mμ. Molarity fraction 6 = 0·0035 M; fraction 11 = 0·011 M.

Enzyme stability. The purified enzyme (after Step VI) was fairly stable even though the protein concentration was quite low at this stage. After storage for 1 week in crushed ice the enzyme had lost 30 per cent of its initial activity. In a similar preparation, in which 2·5 mg/ml of bovine serum albumin was added, the enzyme lost only 16 per cent of its initial activity in 9 days. Freezing and thawing the enzyme (minus albumin) after the final purification stage resulted in loss of over 75 per cent of the activity. After Steps II through V the enzyme could be frozen and thawed, usually with less than 10 per cent unit loss. The enzyme from Step III was stable at 60° for at least 2 min.

Homogeneity. The enzyme which had been purified approximately 201-fold was over 70 per cent homogenous, as determined by visual examination of the bands following disc gel electrophoresis. There was only one major band corresponding to the enzyme activity, and two very light bands of faster-moving protein. No attempts were made to further purify the enzyme by eliminating these minor components.

pH optimum. The optimal pH range for phenylalanine ammonia-lyase was between 8·0 and 8·6 at 37° in tris-HCl buffer. The enzyme was about 50 per cent as active at pH 7·0 and 10·2. Glycine–NaOH buffer was somewhat inhibitory at a concentration of 0·03 M.

Linearity with time. The reaction was linear for at least 2 hr in pH 8·55 tris-HCl buffer at 37°, when the purified enzyme was used. Step II enzyme gave a linear reaction for at least 90 min.

K_m determination. The K_m values for L-phenylalanine at pH 7·4 (phosphate buffer) and pH 8·55 (tris-HCl buffer) were determined according to Lineweaver and Burk.[19] The respective K_m values were $1{\cdot}6 \times 10^{-4}$ M and $2{\cdot}2 \times 10^{-4}$.

Substrate specificity. Several compounds related in structure to L-phenylalanine were tested to see if they were also deaminated. D-Phenylalanine, L-tyrosine, *m*-tyrosine, *o*-tyrosine and dihydroxyphenylalanine gave no significant activity when incubated in the presence of the purified enzyme, as determined by the fact that no products were produced with absorption peaks between 240 and 370 nm. Unless these compounds require a very different pH for deamination than does L-phenylalanine, it can be concluded that they are not deaminated by the enzyme. A variety of structural analogues were tested to determine if they compete with L-phenylalanine for a binding site, and only a few of these were significantly inhibitory (Tables 2 and 7). Inhibition by D-phenylalanine was probably competetitive in nature, as found by Havir and Hanson using enzyme purified from potato.[14]

TABLE 2. EFFECT OF STRUCTURAL ANALOGUES OF PHENYLALANINE ON ENZYME ACTIVITY

Addition	Concentration of analogue	Concentration of L-phe	Relative activity (%)
Control		$7{\cdot}2 \times 10^{-4}$ M	100
L-Tyrosine	$6{\cdot}0 \times 10^{-4}$ M	$2{\cdot}64 \times 10^{-4}$ M	89
Acetylphenylalanine	$7{\cdot}2 \times 10^{-4}$ M	$7{\cdot}2 \times 10^{-4}$ M	82
D-Phenylalanine	$8{\cdot}0 \times 10^{-4}$ M	$7{\cdot}2 \times 10^{-4}$ M	76
Phenyllactic acid	$2{\cdot}0 \times 10^{-3}$ M	$2{\cdot}0 \times 10^{-3}$ M	93
3-OH tyramine	$1{\cdot}0 \times 10^{-3}$ M	$2{\cdot}0 \times 10^{-3}$ M	91
Dihydroxyphenylalanine	1×10^{-3} M	$2{\cdot}0 \times 10^{-3}$ M	93
Acetyltyrosine	$7{\cdot}2 \times 10^{-4}$ M	$7{\cdot}2 \times 10^{-4}$ M	100
L-Histidine	$1{\cdot}2 \times 10^{-3}$ M	3×10^{-4} M	100
Phenylacetic acid	8×10^{-4} M	$2{\cdot}64 \times 10^{-4}$ M	84
Phenylbutyric acid	8×10^{-4} M	$2{\cdot}64 \times 10^{-4}$ M	83
1-Phenyl-2′butene	8×10^{-4} M	$2{\cdot}64 \times 10^{-4}$ M	100
1-Phenyl-3′butene	8×10^{-4} M	$2{\cdot}64 \times 10^{-4}$ M	104

Assayed 90 min. Dithiothreitol = 2·0 mM, L-phenylalanine concentration as indicated. Assayed at pH 8·55.

Effect of sulfhydryl compounds and sulfhydryl reagents. Using an enzyme preparation which had been stored for 4 days in the presence of 4·0 mM mercaptoethanol and containing $2{\cdot}3 \times 10^{-4}$ M mercaptoethanol during assay, the addition of more mercaptoethanol up to 6·0 mM or dithiothreitol to 2·0 mM had little or no effect on activity. However, after dialysis for 30 hr against 0·02 M pH 7·4 phosphate buffer (250 vol.), the enzyme activity was enhanced by some sulfhydryl compounds, as shown in Table 3. Dithiothreitol gave the greatest stimulation, whereas cysteine was somewhat inhibitory. Iodoacetate and iodacetamide, at concentrations of 5·0 mM, gave only slight inhibition when present during the assay; however, if the enzyme was preincubated at 1° for 35 min in the presence of 5·0 mM iodoacetamide before assay, inhibition was significantly greater. It therefore appears that sulfhydryl groups may play some role in maintaining optimal enzyme activity, although not as important a role as seems to be the case with the barley enzyme,[8] which was more sensitive to sulfhydryl compounds and reagents than this enzyme isolated from other plant sources.

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TABLE 3. EFFECT OF SULFHYDRYL COMPOUNDS AND SULFHYDRYL-ACTIVE REAGENTS ON ENZYME ACTIVITY

Addition	Concentration	Relative activity (%)
Control	—	100
SH-glutathione	0·002 M	123
2-Mercaptoethanol	0·002 M	121
Dithiothreitol	0·0005 M	121
	0·002 M	144
	0·004 M	144
L-Cysteine	0·002 M	90
Iodoacetate	0·005 M	88
Iodoacetamide	0·005 M	89
Iodoacetamide*	0·005 M	74

pH 8·55, 90 min assay. Enzyme dialyzed thoroughly. L-Phenylalanine = 2·5 mM.
* Preincubated at 0° for 35 min with 5 mM iodoacetamide before assay.

Effect of metals and other compounds on enzyme activity. A variety of monovalent and divalent cations and anions were incubated with the enzyme to test their effect on activity. Most low molecular divalent cations were somewhat inhibitory, while heavy metals inhibited significantly (Table 4). Ammonium ions, a product of the reaction catalyzed by the enzyme, were only slightly inhibitory.

TABLE 4. EFFECT OF CERTAIN CATIONS AND ANIONS ON ENZYME ACTIVITY

Addition	Concentration	Relative activity (%)
None	—	100
$MgSO_4$	0·002 M	84
$MnCl_2$	0·002 M	79
$CaCl_2$	0·002 M	100
$ZnCl_2$	0·001 M	38
$CuCl_2$	0·001 M	39
$PbCl_2$	0·0002 M	80
$CoCl_2$ (pH 7·80)	0·003 M	117
$CoCl_2$ (pH 7·80)	0·006 M	90
$CoCl_2$ (pH 7·10)	0·0005 M	112
KF	0·004 M	88
KCl	0·004 M	89
$(NH_4)_2SO_4$	0·002 M	94
NH_4Cl	0·004 M	89

pH of 8·55 (tris-HCl buffer) unless otherwise noted. 2-Mercaptoethanol = 2·5 mM, L-phe = 2·5 mM. Assayed for 90 min.

A variety of other compounds were also tested with respect to their effect on enzyme activity (Table 5). Chelating compounds, such as EDTA and sodium diethyldithiocarbamate, at relatively high concentrations, did not significantly inhibit the enzyme. However, NaCN and $NaBH_4$ were inhibitory. The implications of cyanide and borohydride inhibition are discussed later.

TABLE 5. EFFECT OF CHELATING COMPOUNDS, CARBONYL REAGENTS, COFACTORS AND MISCELLANEOUS COMPOUNDS ON ENZYME ACTIVITY

Addition	Concentration	Relative activity (%)
Control	—	100
EDTA	0·01 M	105
DDC*	0·005 M	85·5
NaCN	0·00125 M	65·5
	0·005 M	57·6
$NaBH_4$	0·00025 M	90
NADH	0·0005 M	91
NADPH	0·0005 M	94
PLP†	0·001 M	100
ATP	0·002 M	97
MgATP	0·002 M	85
IAA‡	0·0075 M	86

Assayed 75 min (37°), pH 8·55. Dithiothreitol = 2·0 mM, L-phe = 2·5 mM.

* DDC = Sodium diethyldithiocarbamate.

† PLP = Pyridoxal phosphate.

‡ IAA = Indole-3′-acetic acid.

Feedback inhibition and non-specific phenol inhibition. Phenylalanine ammonia-lyase catalyzes the first step in the synthesis of lignin, polyphenols, and many other compounds derived from simple phenols, and would thus be a likely point for feedback inhibition or repression by "end-products". What constitutes an "end-product" is difficult to ascertain, for there are a very large number of products derived from cinnamic acid, and some of them, such as lignin and other bound or insoluble products, cannot be expected to act as inhibitors *in vivo*.

TABLE 6. EFFECT OF METABOLITES DERIVED FROM L-PHENYLALANINE OR TYROSINE ON ENZYME ACTIVITY

Addition	Concentration additive	Concentration DL-phe (^{14}C)	Relative activity (%)
Control	—	$3{\cdot}12 \times 10^{-4}$ M	100
trans-Cinnamic acid	$6{\cdot}67 \times 10^{-4}$ M	$3{\cdot}12 \times 10^{-4}$ M	18
p-Coumaric acid	$6{\cdot}0 \times 10^{-4}$ M	$3{\cdot}12 \times 10^{-4}$ M	100
o-Coumaric acid	$6{\cdot}0 \times 10^{-4}$ M	$3{\cdot}12 \times 10^{-4}$ M	27
Caffeic acid	$6{\cdot}0 \times 10^{-4}$ M	$3{\cdot}12 \times 10^{-4}$ M	100
Chlorogenic acid	$8{\cdot}3 \times 10^{-4}$ M	$3{\cdot}12 \times 10^{-4}$ M	100
Sinapic acid	$6{\cdot}0 \times 10^{-4}$ M	$2{\cdot}64 \times 10^{-4}$ M	100
Ferulic acid	6×10^{-4} M	$2{\cdot}64 \times 10^{-4}$ M	89
Scopoletin	5×10^{-4} M	$2{\cdot}64 \times 10^{-4}$ M	77
Benzoic acid	$1{\cdot}2 \times 10^{-3}$ M	$2{\cdot}64 \times 10^{-4}$ M	77
Protocatechuic acid	5×10^{-4} M	$2{\cdot}64 \times 10^{-4}$ M	96
Kaempferol	5×10^{-4} M	$2{\cdot}64 \times 10^{-4}$ M	32
Rutin	6×10^{-4} M	$2{\cdot}64 \times 10^{-4}$ M	89

Assayed using ^{14}C-phenylalanine at 37° for 105 min. Concentration of ^{14}C-DL-phenylalanine as indicated. 2-Mercaptoethanol = 4·0 mM. Run at pH 8·30 (tris-HCl buffer).

Several commercially available polyphenols were tested with respect to possible inhibition of the enzyme. Particular attention was given to those compounds (chlorogenic acid, caffeic acid, rutin, quercetin, kaempferol and scopoletin) which normally occur in tobacco. The results of these experiments are given in Tables 6 and 7. Of the simple phenols, only *trans*-cinnamic acid and *o*-coumaric acid caused a high degree of inhibition at pH 8·3, while closely related compounds such as sinapic acid, ferulic acid, caffeic acid, and *p*-coumaric acid caused little or no inhibition. Slight but significant inhibition was caused by DOPA (dihydroxyphenylalanine), benzoic acid, coumarin, scopoletin, and rutin. On the other hand, the flavonols kaempferol and quercetin were highly inhibitory. At pH 7·1 the inhibition by several of the foregoing compounds was increased, especially for quercetin. The K_i values for *trans*-cinnamate at pH 7·10 and 8·30 were $3{\cdot}1 \times 10^{-5}$ M and $4{\cdot}6 \times 10^{-5}$ M, respectively.

TABLE 7. ADDITIONAL STUDIES ON INHIBITION BY PHENOLS AND RELATED PRODUCTS, CONTINUED FROM TABLE 6

Addition	pH	Concentration of additive	Concentration of DL-phe (^{14}C)	Relative activity (%)
Control	8·3	—	$2{\cdot}64 \times 10^{-4}$ M	100
Coumarin	8·3	6×10^{-4} M	$2{\cdot}64 \times 10^{-4}$ M	81
Quercetin	8·3	2×10^{-4} M	$2{\cdot}64 \times 10^{-4}$ M	44
Control	7·1	—	$2{\cdot}64 \times 10^{-4}$ M	100
trans-Cinnamate	7·1	2×10^{-4} M	$2{\cdot}64 \times 10^{-4}$ M	14
trans-Cinnamaldehyde	7·1	2×10^{-4} M	$2{\cdot}64 \times 10^{-4}$ M	100
Cinnamyl alcohol	7·1	2×10^{-4} M	$2{\cdot}64 \times 10^{-4}$ M	100
Scopoletin	7·1	6×10^{-4} M	$2{\cdot}64 \times 10^{-4}$ M	50
Rutin	7·1	6×10^{-4} M	$2{\cdot}64 \times 10^{-4}$ M	90
Quercetin	7·1	$6{\cdot}67 \times 10^{-5}$ M	$5{\cdot}0 \times 10^{-4}$ M	5
Control	8·3	—	$2{\cdot}64 \times 10^{-4}$ M	100
trans-Cinnamide	8·3	8×10^{-4} M	$2{\cdot}64 \times 10^{-4}$ M	100
o-Tyrosine	8·3	6×10^{-4} M	$2{\cdot}64 \times 10^{-4}$ M	6
		6×10^{-4} M	$1{\cdot}50 \times 10^{-4}$ M	2·6
m-Tyrosine	8·3	6×10^{-4} M	$2{\cdot}64 \times 10^{-4}$ M	92
o-Coumaric acid	8·3	6×10^{-4} M	$1{\cdot}50 \times 10^{-4}$ M	3·5

Run for 105 min. pH as indicated. 2-Mercaptoethanol = 2·0 mM. Assayed using ^{14}C-DL-phenylalanine.

Structural analogues of *trans*-cinnamic acid, including cinnamaldehyde, cinnamyl alcohol, cinnamide, and the non-polar derivatives 1-phenyl-2-butene and 1-phenyl-3-butene, were not inhibitory.

DISCUSSION

Many of the general properties of tobacco phenylalanine ammonia-lyase are rather similar to those of this enzyme from other higher plants, although there are some significant differences. The K_m for the tobacco enzyme was approximately $1{\cdot}6 \times 10^{-4}$ M at pH 8·55, which is considerably less than that for the barley enzyme ($1{\cdot}7 \times 10^{-3}$ M).[8] The value was similar to that of the sweet potato root enzeme, $1{\cdot}01 \times 10^{-4}$ M.[9] Such differences in affinity may have evolved from differences in the phenylalanine concentration of different organisms, although other possibilities exist.

As is the case in the potato tuber[14] and *Rhodotorula*[16] enzymes, the tobacco enzyme is sensitive to inhibition by NaCN and $NaBH_4$, both carbonyl reagents, although the sensitivity to these inhibitors varies rather widely between these organisms. Both Havir and Hanson[14] and Hodgins[16] explained the inhibition by NaCN and $NaBH_4$ by assuming that the enzyme mechanism required the participation of a carbonyl group, and this is probably also the case with the tobacco enzyme.

Phenylalanine ammonia-lyase from tobacco is inhibited by *trans*-cinnamic acid (and *o*-coumaric acid), but a wide variety of other phenols caused little inhibition in a competitive sense. There is a good possibility that the synthesis of cinnamate is regulated *in vivo* by feedback inhibition by cinnamate, in view of its low K_i value. At pH 8·3–8·55, cinnamate is bound about five times as tightly as the substrate, L-phenylalanine, and it is not unlikely that at times cinnamate may accumulate to a level where it causes significant inhibition. This, of course, depends on the *in vivo* concentration of cinnamate at the enzyme site, and not on the cellular level. Whether the reaction catalyzed by phenylalanine ammonia-lyase is sometimes the limiting one in the synthesis of phenolic compounds is probable, but other steps may be equally important in this respect. Recently, Russell and Conn,[20] working with pea seedlings, found that the enzyme-synthesizing *p*-coumaric acid from cinnamate is strongly inhibited by *p*-coumaric acid. Cinnamic acid also inhibited phenylalanine ammonia-lyase from potato[14] and bean,[21] although the enzyme from the latter organism was not inhibited nearly as much by cinnamic acid as was the tobacco enzyme.

The significance of *o*-coumaric acid inhibition is difficult to ascertain. This compound has been detected in three out of eighty species in Leguminosae[22] but may be of more common occurrence, for it may be cyclized to coumarin during isolation.[23] *o*-Coumaric acid is believed to give rise to coumarin.[24, 25] Whether it is a normal metabolite in tobacco and ever accumulates in sufficient amounts to cause significant inhibition of phenylalanine ammonia-lyase is unknown.

Scopoletin, a coumarin constituent of tobacco, was significantly inhibitory at pH 7·1. Quercetin and kaempferol, both flavonols occurring in tobacco,[17] were very potent inhibitors of the enzyme; in fact, quercetin was much more inhibitory than any heavy metal, carbonyl reagent, or sulfhydryl group reagent tested. It would be of interest to discover the specific nature of quercetin (or kaempferol) inhibition, such as the identity of the amino acid residue(s) it complexes with. Quercetin was also found to inhibit magnesium, sodium, and potassium-activated adenosine triphosphatase of beef heart.[26] It is noteworthy that caffeic acid, which is very abundant in tobacco in the form of its depside, chlorogenic acid, is not inhibitory to the enzyme (Table 6) although it is structurally similar to the end product, *trans*-cinnamic acid.

The enzyme shows very high specificity with respect to substrate and competitive inhibitors. For example, hydroxylation of phenylalanine in the *para*-or-*meta* position produces substrates with very low affinity for the enzyme, while *o*-tyrosine is a potent inhibitor, as is *o*-coumaric acid. In general, compounds containing a *para*-hydroxyl group caused little or no inhibition. This includes caffeic acid, sinapic acid, and ferulic acid. It is interesting to note that although *o*-tyrosine is bound tightly, it is not deaminated to yield *o*-coumaric acid. A

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free α-amino group is not an absolute requirement for binding, for phenylacetic, phenylbutyric, and phenylpyruvic acids and acetyl phenylalanine did cause some inhibition, although these compounds were not bound with the affinity that L-phenylalanine was. The presence of a terminal carboxyl group appears necessary for binding, for the *trans*-cinnamate analogues *trans*-cinnamaldehyde, cinnamyl alcohol, and cinnamide caused no inhibition, nor did 1-phenyl-2′-butene and 1-phenyl-3′-butene.

There were no cofactor requirements discovered for the enzyme; in fact, the most logical cofactor, pyridoxal phosphate, caused no activity increase, and u.v. examination of the purified protein gave no peak corresponding to a pyridoxal phosphate-enzyme complex. Of all the metal ions tested, only cobalt gave a slight degree of stimulation.

The enzyme tyrosine ammonia-lyase, which is also of potential importance in the synthesis of phenolic compounds, was present in very low amounts in the several varieties of tobacco tissue tested. Its specific activity was generally less than 5% of the activity of phenylalanine ammonia-lyase. This agrees with findings in other dicotyledonous plants.[6]

EXPERIMENTAL

Source of Plant Materia

Although the enzyme was detected in several varieties of tobacco of widely varying age, preparations reported in this paper were obtained from *Nicotiana tabacum* L. var. Burley 21 obtained during July and August from locally grown field plants between 11 and 15 weeks after transplanting. In general, extracts obtained from upper leaves of older plants had greater specific activity than that found in leaves of younger plants, so only the leaves from the upper 1/4 to 1/3 of the plant were collected. Harvested leaves were frozen in crushed dry ice and were later stored at −90° for intervals ranging from 2 days to several weeks. The midribs were removed prior to preparation of the acetone powders.

Enzyme Assay

The enzyme activity was assayed by measuring the amount of *trans*-cinnamic acid produced. This was done by two methods.

I. The standard reaction mixture (2·5 ml) contained L-phenylalanine (5·0 μmoles), tris-HCl buffer, pH 8·55 (100 μmoles), 2-mercaptoethanol (10·0 μmoles) and the enzyme. A blank was employed in which phenylalanine was absent until the reaction had been ended. The reaction mixture was incubated for 60 min at 37° in Pyrex Sorvall centrifuge tubes, without agitation. The reaction was terminated by the addition of 0·20 ml of 1·0 N trichloracetic acid, and the volume adjusted to 3·0 ml with water. After standing at 37° for 15–25 min the tubes were centrifuged for 20 min at 28,000 *g* to remove the precipitated protein. The supernatants were then decanted and retained and the cinnamic acid produced was assayed by measuring absorption at 279 nm against a control in which phenylalanine was not added until after the reaction was terminated. This is the wave-length of maximal absorption of this compound at low pH. When enzyme which had been purified through at least step four was assayed, protein content was so low that centrifugation was not required after terminating the reaction.

This method of assay gave good reproducibility when used with acetone powders after $(NH_4)_2SO_4$ precipitation. However, when green leaves were extracted in buffer, or the crude extract from acetone powders was assayed directly, reproducibility was generally poor. This was due to the presence, even in acetone powders, of compounds (probably phenolic in nature) which strongly absorbed in the same spectral region as *trans*-cinnamic acid. Furthermore, when crude extracts (from acetone powders) were assayed without phenylalanine vs. a zero-time blank, the absorbance at 240–320 nm increased appreciably due to metabolism of endogenous substrates present. Perhaps this reflects oxidation of phenols to quinones catalyzed by phenol oxidases, enzymes which are abundant in tobacco.

II. The second method of assay, used for certain studies on properties of the enzyme, employed DL-phenylalanine-3-^{14}C (specific activity 1·3 μc/mM). After incubation at 37° for 30–60 min, 0·50 ml of 0·10% unlabeled *trans*-cinnamic acid was added, followed immediately by 0·20 ml of 1·0 N trichloroacetic acid. To this mixture 6·0 ml of toluene was added, and the tubes were stoppered and shaken vigorously. After standing for several minutes the tubes were centrifuged at 4000 *g* for 8 min, and 4·0 ml of the upper (toluene) layer was drawn off with a syringe and added to a scintillation vial containing 10 ml of the PPO-POPOP* toluene counting mixture,

* 2,5-Diphenyloxazole and *p*-bis[2-(5-phenyloxazolyl)]-benzene.

and counted at 6° in a Packard Automatic Tri-Carb Liquid Scintillation Spectrometer (Model 4322). A reaction mixture without enzyme was used as a blank, and this value was subtracted from those with enzyme. The counting efficiency was approximately 76 per cent.

A unit of phenylalanine ammonia-lyase is defined as the amount of enzyme catalyzing the synthesis of 1·0 μM *trans*-cinnamic acid per min under the conditions employed.

Analytical Methods

Protein concentration was determined by the procedure of Lowry *et al.*,[27] using a bovine serum albumin standard. The u.v. 280:260 nm ratio of the tobacco enzyme preparations was never above 1·10, and usually about 0·90–1·00. Using a freshly prepared extract from acetone powder, the protein concentration as determined by the Lowry procedure was approximately the same as that obtained from micro-Kjeldahl analysis.

Identification of Cinnamic Acid

Trans-cinnamic acid was identified as the product of the reaction by comparing the u.v. spectrum of the product of the reaction with that of a commercial preparation of authentic *trans*-cinnamic acid. Further identification was provided by chromatography and co-chromatography on thin-layer MN polyamide chromatography plates (Brinkmann Instruments, Inc.), using methanol as the solvent.

Disc electrophoresis was performed on polyacrylamide gel using gel 7·5 per cent cross-linked. The sample was applied in 0·6 ml aliquots and electrophoresis was performed at pH 8·6. A duplicate gel sample was not stained and was sectioned to correlate enzyme activity with the protein bands on the stained gel. The 4-mm sections were incubated for 3 hr at 37°, and the recovery of activity was about 35 per cent.

Purification Procedure

200–300-g lots of frozen tobacco leaves were placed in a Waring Blendor (Model B-4). Unless otherwise noted, all operations were conducted at 2–5°. The frozen leaves were extracted with 8–10 vol. of redistilled acetone and *n*-butanol (9:1 v/v) pre-cooled in dry ice to −40° to −50°, and containing 0·07 M 2-mercaptoethanol. The leaves were extracted for 60–90 sec at maximum speed, and the extract was filtered through Whatman filter paper in a Buchner funnel. The filter cake was resuspended in 8 vol. of −40° to −50° acetone containing 0·056 M mercaptoethanol, homogenized 60 sec and filtered. The filter cake was washed with 2 vol. of acetone, and the light-green cake was spread evenly over filter paper and the lumps broken up and allowed to dry for 2–3 hr at room temperature. The powder was stored at −90°, and lost less than 15 per cent of its initial activity after 3 months.

Step I. Extraction and protamine sulfate treatment. 13 g of the acetone–butanol powder were extracted with 150 ml 0·025 M phosphate (Na^+) buffer, pH 7·2, for 15 min with slow stirring. The buffer contained 0·025 M 2-mercaptoethanol and 1·0 mM EDTA. The suspension was centrifuged at 22,000 *g* for 12 min at 0° and the supernatant was filtered through cheesecloth. Protamine sulfate, in a solution of 4·0 mg/ml, was slowly added with stirring until the ratio of protein to protamine sulfate was approximately 10:1. The cloudy suspension was stirred for 10–15 min and centrifuged for 5 min at 22,000 *g*.

Step II. $(NH_4)_2SO_4$ fractionation. To the above solution saturated $(NH_4)_2SO_4$ (adjusted to pH 7·2–7·4 with NH_4OH) was added, with stirring, to a final concentration of 46·5% saturation and stirred slowly for 15 min. The precipitate was sedimented by centrifugation (15 min, 22,000 *g*) and the supernatant carefully decanted. The precipitate was dissolved in 30 ml of 0·01 M phosphate buffer, pH 7·10, containing 8·0 mM mercaptoethanol and 2·0 mM L-phenylalanine.

Step III. pH precipitation. The solution above was immersed in a beaker of ice water and the pH was lowered by dropwise addition of cold 0·25 M acetic acid, with stirring, to 4·70 (at 0–1°). The pH was measured on a Model 22 Radiometer. The solution was immediately centrifuged at 22,000 *g* for 5 min at −4°. The supernatant was immediately brought to pH 7·1 by the dropwise addition of 0·8 M tris.

Step IV. Heat treatment. The somewhat cloudy solution was placed in a 250 ml Erlenmeyer flask and placed in a water bath at 63–64°. The solution was gently swirled and when the temperature had reached 58° it was kept in the bath for 2 min more, after which it was rapidly cooled by swirling the flask in a large volume of ice water and centrifuged at 22,000 *g* for 10 min. The supernatant was frozen at −90° overnight.

Step V. Acetone precipitation. To the above solution redistilled acetone (−29°) was slowly added, with stirring, to 47·0% saturation. The solution was placed in crushed ice for several minutes after which time the temperature was approximately 2–3°. Following centrifugation at 22,000 *g* for 5 min (at −6°), the pellet was suspended in 10 ml of 0·01 M phosphate buffer, pH 7·2, containing 8·0 mM mercaptoethanol. The pellet was thoroughly suspended with a glass stirring rod and the solution was centrifuged a second time, discarding the pellet.

Step VI. Hydroxylapatite chromatography. The solution was applied to a 2·5 × 4·2 cm column of hydroxylapatite, equilibrated in $1{\cdot}25 \times 10^{-4}$ M phosphate buffer, pH 6·8. After the solution had reached bed level, the sides of the column were washed with an additional 8 ml of 0·01 M phosphate buffer, pH 7·6, containing 6 mM

[27] O. H. LOWRY, A. L. ROSEBROUGH and R. J. RANDALL, *J. Biol. Chem.* 193 (1951).

mercaptoethanol. The enzyme was then eluted with 60 ml of 0·030 M pH 7·6 phosphate buffer containing 6 mM mercaptoethanol. The flow rate was about 50–55 ml/hr and 3-ml fractions were collected with an LKB automatic fraction collector.

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