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CINNAMIC ACID PRODUCTION AS A METHOD OF ASSAY FOR PHENYLALANINE AMMONIA-LYASE IN ACETONE POWDERS OF *PHASEOLUS VULGARIS**

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Abstract—Phenylalanine ammonia-lyase (PAL) activity was demonstrated in extracts of acetone powders of bean hypocotyls. Since it was observed that cinnamic acid was produced with or without added L-phenylalanine, the origin of this cinnamic acid was investigated. Assay of boiled extracts indicated that cinnamic acid production, with or without added substrate, was enzymatic, and it was shown dialyzed extracts produced cinnamic acid only with added substrate. It was found that extracts contained phenylalanine at four times the level necessary to account for cinnamic acid production without added substrate, and the loss of phenylalanine during autolysis was stoichiometrically equivalent to the cinnamic acid produced. Cinnamic acid was not released during acid or alkaline treatment of extracts, but ferulic acid was found from the alkali treatments. No other evidence for the existence of bound forms of cinnamic acid was obtained, and we conclude that measurements of cinnamic acid production is a valid method of assay for PAL in extracts of acetone powders of bean hypocotyls.

INTRODUCTION

AN ACCUMULATION of phenolic compounds at the site of infection is frequently observed in plants inoculated with fungi. The direct conversion of phenylalanine to cinnamic acid, catalyzed by phenylalanine ammonia-lyase (PAL), is a major source of C_6-C_3 intermediates for the synthesis of phenolic compounds in plants.[1] We reported[2] alterations of phenolic content and PAL activity associated with infection of bean seedlings by *Colletotrichum lindemuthianum* (Sacc. & Magn.) Scribner and other fungi. Extracts of acetone powders were used for assays of PAL activity. El-Basyouni and Neish[3] reported that bound forms of cinnamic acid were extracted into cold buffer from acetone powders of wheat and barley shoots, and that significant amounts of cinnamic acid were released by autolysis and by acid or alkaline treatment of the extracts at room temp.

Determinations in crude extracts are often preferable to those in purified preparations when attempting to correlate enzyme activities with physiological or pathological processes. Since the assay of PAL is based on measurement of cinnamic acid production, the release of cinnamic acid from bound forms would constitute a source of error. The release of cinnamic acid from bound forms in extracts of acetone powders of bean hypocotyl tissue was investigated.

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[1] A. C. NEISH, in *Plant Biochemistry* (edited by J. BONNER and J. E. VARNER), p. 581, Academic Press, New York (1965).
[2] J. E. RAHE, J. KUC', CHIEN-MEI CHUANG and E. B. WILLIAMS, *Neth. J. Plant Pathol.* **75**, 58 (1969).
[3] S. Z. EL-BASYOUNI and A. C. NEISH, *Phytochem.* **5**, 683 (1966).

RESULTS

Assay of PAL Activity in Untreated Extracts

PAL was extracted from acetone powders of etiolated bean hypocotyls with solutions of NaCl or borate buffer. Similar levels of activity were obtained by either method; determinations in buffer extracts averaged 9 per cent higher than those in NaCl extracts. Assays were based in cinnamic acid production by extracts with and without added L-phenylalanine. Production of cinnamic acid in assays without substrate approached the levels produced in substrate assays at low levels of PAL activity (Fig. 1).

Trans-cinnamic acid was verified as the product of the PAL reaction in both cases, both chromatographically and by its u.v. spectrum.

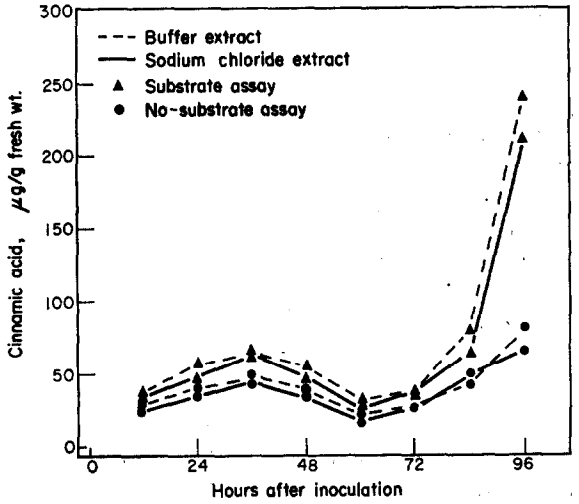

FIG. 1. CINNAMIC ACID PRODUCTION BY EXTRACTS OF ACETONE POWDERS OF "TOPCROP" HYPOCOTYLS INOCULATED WITH THE β RACE OF *Colletotrichum lindemuthianum*. (PLANTS 8 DAYS OLD AT TIME OF INOCULATION.)

Effect of Boiling, Acid and Alkaline Treatment on Cinnamic Acid and Extracts of Acetone Powders

Cinnamic acid from reaction mixtures was detectable by the chromatographic procedure used at amounts equivalent to 1·6–2·7 μg/g fresh wt. Cinnamic acid was not detected chromotographically in NaCl extracts before or after boiling, but was barely detected in both boiled and unboiled buffer extracts. PAL activity of extracts was destroyed by boiling, and additional cinnamic acid was not produced with or without substrate with boiled extracts nor during acid or alkaline treatment at 40° of boiled or unboiled extracts. Commercial cinnamic acid was recovered quantitatively and unchanged after boiling or treatment with acid or alkali at 40°. The results are summarized in Table 1.

Alkaline treatment of extracts at 40° released a deep-blue fluorescing material with an absorption maximum at 345 nm in 0·05 N NaOH. This material and ferulic acid gave corresponding R_f values in three chromatographic solvents, identical color reactions with diazotized sulfanilic acid and diazotized *p*-nitroaniline, and similar u.v. spectra. The data are summarized in Table 2. The amount of a predominantly *trans* isomeric mixture obtained was 13–26 μg/g fresh wt.

TABLE 1. EFFECT OF BOILING, ACID AND ALKALINE TREATMENTS ON APPARENT PRODUCTION OF CINNAMIC ACID IN EXTRACTS OF ACETONE POWDERS

Treatment prior to assay:	Cinnamic acid (μg/g f.w.)*			
	0°		Boiled	
Method of extraction:	Buffer	NaCl	Buffer	NaCl
Method of assay				
Substrate assay	61·2	54·4	10·3‡	2·4‖
No-substrate assay	45·6	37·0	8·4‡	2·8‖
Zero time stop	6·1‡	2·5‖	9·4‡	2·8‖
1 N HCl treatment†	5·9‡	2·8‖	8·4‡	3·3‖
1 N NaOH treatment†	9·8‡,§	7·7§,‖	13·1‡,§	7·7§,‖

* Determined by absorbance at 268 nm.

† At 40° for 1 hr.

‡ Values higher than those for NaCl extracts due to cinnamic acid produced during extraction. Cinnamic acid detected chromatographically and estimated at 1·6–5·4 μg/g fresh wt.

§ Absorbance predominantly due to ferulic acid (13–26 μg/g fresh wt.), as indicated by chromatography.

‖ Cinnamic acid not detected chromatographically.

TABLE 2. PAPER CHROMATOGRAPHIC DATA FOR CINNAMIC ACID AND SOME PHENOLIC DERIVATIVES, AND AN UNKNOWN RELEASED BY ALKALINE HYDROLYSIS* OF EXTRACTS

Compound	R_f†			Color‡		
	S_1	S_2	S_3	D_1	D_2	D_3
Unknown	0·26, 0·60	0·55	0·2–0·80s	dB	P	lG
Caffeic acid	0·25, 0·62	0·46	0·10	bD	lBr	T-Br
Ferulic acid	0·29, 0·63	0·59	0·4–0·80s	dB	P	lG
o-Coumaric acid	0·47	0·77	0·2–0·64s	BW	—	f?
m-Coumaric acid	0·44	0·70	0·0–0·52s	B	Y	Pi-P
p-Coumaric acid	0·41	0·82	0·0–0·49s	A-dP	Pi-P	G
Coumarin	0·70	0·89	not det.	A	—	Pi-P
trans-Cinnamic acid	0·60	0·79	0·86	A	—	—

* At 40° for 1 hr.

† Paper: Whatman No. 1, developed ascending at 23–24°.

Solvents: S_1, 2% acetic acid: S_2, organic phase of *n*-butanol–ethanol–water (4:1:5, v/v); S_3, organic phase of chloroform–methanol–water–formic acid (250:25:24:1, v/v).

Detection: D_1, u.v. radiation (254 mμ); D_2, diazotized sulfanilic acid; D_3, diazotized *p*-nitroaniline.

‡ A, absorbs; B, blue; Br, brown; G, gray; P, purple; Pi, pink; T, tan; W, white; b, bright; d, deep; f, faint; l, light; s, streak.

Cinnamic acid was almost totally destroyed by refluxing in acid or alkali. Absorbance at 268 nm was increased by these treatments but chromatography indicated only trace amounts of cinnamic acid, and the appearance of a diffuse streak of blue-white fluorescence at R_f 0·6–0·8 with the 2 per cent acetic acid solvent.

Dialysis of Extracts of Acetone Powders

PAL activity in NaCl extracts assayed with substrate was reduced 16 per cent and 25 per cent by dialysis against 0·1 M NaCl and deionized water, respectively. Cinnamic acid was not

produced without substrate with dialyzed extracts, but was produced in no-substrate assays of mixtures of equal volumes of dialyzed and boiled portions of the same extract. The data are summarized in Table 3.

TABLE 3. EFFECT OF DIALYSIS AND BOILING ON CINNAMIC ACID PRODUCTION* IN EXTRACTS OF ACETONE POWDERS

	Method of assay			
	Substrate assay		No-substrate assay	
Treatment prior to assay	†	‡	†	‡
Not dialyzed	61·4	73·0	41·7	46·6
Dialyzed	46·1	61·4	1·5	2·9
Boiled	1·4	2·9	2·4	2·6
Dialyzed + boiled (1:1)	25·1	32·4	16·3	21·4

* Determined by absorbance at 268 nm and expressed as μg/g fresh wt. of hypocotyl tissue.
† 0·1 M NaCl extract of acetone powder of 8-day-old "Topcrop" hypocotyls, deionized water used for dialysis.
‡ 0·1 M NaCl extract of acetone powder of 10-day-old "Topcrop" hypocotyls, 0·1 M NaCl used for dialysis.

Amino Acid Analysis of Buffer Extract

The amino acids from a buffer extract were determined before (0° control) and after (40° autolyzate) conditions of no-substrate assay. In addition to the eighteen components

TABLE 4. DIALYZABLE AMINO ACIDS FROM BUFFER EXTRACT OF AN ACETONE POWDER*

	Treatment prior to assay	
Elution peak	0° control	40° autolyzate
Aspartic acid	†	†
Threonine	†	†
Serine	†	†
Glutamic acid	2·46	2·79
Proline	‡	2·60
Glycine	1·05	0·98
Alanine	6·28	5·88
Valine	24·90	‡
Cysteine	0·03	‡
Methionine	1·50	1·47
Isoleucine	8·21	7·82
Leucine	8·27	7·80
Tyrosine	2·78	2·74
Phenylalanine	7·30	5·44
Ammonia	0·49	0·49
Lysine	0·85	0·91
Histidine	9·70	9·35
Arginine	13·75	13·14

* Expressed as μg/mg of acetone powder.
† Concentration too high for quantitative measurement.
‡ Peak obscured.

reported in Table 4, both the 0° control and 40° autolyzate gave nine unknown elution peaks. Cinnamic acid production during autolysis of the buffer extract was 1·67 μg/mg of acetone powder. Endogenous phenylalanine in the dialyzate of the 0° control was four times that required to account for cinnamic acid production, and was reduced 1·86 μg/mg of acetone powder by autolysis (Table 4).

DISCUSSION

Autolytic release of cinnamic acid in buffer or NaCl extracts of acetone powders of bean hypocotyls was demonstrated in the absence of phenylalanine. The amount released approached that produced during substrate assays at low levels of PAL activity, suggesting that an endogenous substrate becomes limiting at high levels of PAL. If the autolytically produced cinnamic acid is released in some manner other than by PAL, the measurement of cinnamic acid production would not be a valid method of assay for the enzyme.

The production of cinnamic acid in the absence of substrate is consistent with the observations of El-Basyouni and Neish[3] concerning release of cinnamic acid from bound forms in buffer extracts of wheat and barley shoots by autolysis at room temp. An alternative explanation to release of cinnamic acid from bound forms is that endogenous L-phenylalanine is being converted to cinnamic acid by PAL.

Boiling destroys both PAL activity and the capacity to autolytically release cinnamic acid, as indicated by the absence of cinnamic acid production during assays of boiled extracts. Dialysis eliminates autolytic release of cinnamic acid but does not destroy PAL activity. When boiled and dialyzed portions of an extract are mixed, cinnamic acid is produced either with substrate or without substrate. This indicates that cinnamic acid production during either assay is enzymatic, the endogenous precursor of autolytically released cinnamic acid is dialyzable and stable to boiling, and suggests the absence of a tightly bound enzyme- or protein-cinnamic acid complex in the extracts. Treatment with acid or alkali at 40° did not release cinnamic acid, suggesting the absence of hydrolyzable bound forms of cinnamic acid.

All PAL activity in a mixture of equal volumes of boiled and dialyzed portions of an extract must come from the dialyzed portion. The PAL activity of such mixtures relative to that of the undialyzed extract should be 0·5 times the fraction of activity remaining after dialysis. After dialysis against deionized water the activity of PAL was 75·2 per cent that of the undialyzed extract. Substrate and no-substrate assays of the undialyzed extract indicated cinnamic acid production of 61·4 and 41·7 μg/g fresh wt., respectively. The calculated levels of cinnamic acid production in the mixture are (0·5 × 0·752) × 61·4 and (0·5 × 0·752) × 41·7, or 23·1 and 15·7 μg/g fresh wt. for substrate and no-substrate assays, respectively. These values are in good agreement with those actually obtained (25·1 and 16·3 μg/g fresh wt.). For the extract dialyzed against 0·1 M NaCl the calculated (30·7 and 19·6 μg/g fresh wt.) and assay values (32·4 and 21·4 μg/g fresh wt.) are in equally good agreement. The correspondence of calculated and assay values indicate that significant amounts of cinnamic acid are not released other than by the action of PAL.

Analyses of the dialyzable amino acids of a buffer extract indicated that four times the amount of phenylalanine necessary to account for autolytically released cinnamic acid was extracted from the acetone powder. Autolytic release of 1·67 μg of cinnamic acid/mg of acetone powder requires 1·85 μg of phenylalanine/mg of acetone powder. The concentration of endogenous phenylalanine in the buffer extract was reduced by 1·86 μg/mg of acetone powder by autolysis.

The small amounts of cinnamic acid detected in buffer extracts apparently are due to the action of PAL during extraction. Extraction with buffer provides an optimum pH for PAL activity (pH 8·8) whereas the enzyme is essentially inactive at the pH of NaCl extraction[4] (pH 5–6). Production of cinnamic acid during buffer extraction partially accounts for the observed differences between NaCl and buffer extraction assays of PAL activity.

Hydrolyzable bound forms of cinnamic acid as reported by El-Basyouni and Neish[3] in low temp. buffer extracts of acetone powders of barley and wheat were not detected in extracts of acetone powders of bean hypocotyls. Alkaline hydrolysis released significant amounts of ferulic acid, which is consistent with the findings of El-Basyouni and Towers[5] concerning alkaline hydrolysis of the ethanol-insoluble fraction of wheat seedlings.

The authors conclude that cinnamic acid production in extracts of acetone powders of bean hypoctyls during substrate or no-substrate assay is the result of the action of PAL on phenylalanine. No evidence for the existence of bound forms of cinnamic acid was obtained. Bound forms, if present, do not constitute a significant source of error to the determination of PAL activity by the methods described in this paper.

EXPERIMENTAL

Assays for PAL Activity

Methods for growing etiolated bean seedlings (*Phaseolus vulgaris* L., var. "Topcrop") and *Colletotrichum lindemuthianum* have been described previously.[2] Acetone powders were prepared by homogenizing the hypocotyls of healthy or infected plants for 2 min at −15° with acetone previously chilled to −15° (10 ml/g fresh wt.). The homogenates were filtered (Whatman No. 5 paper) and rinsed with a second equal volume of chilled acetone. The mats were dried for 1 hr under vacuum at room temp. and stored at −15°.

PAL was extracted by suspending the acetone powder in cold 0·1 M borate buffer, pH 8·8 (0·1 g of powder/6 ml). The suspension was kept at 0° for 1 hr with occasional stirring, filtered through cheese-cloth, and the filtrate centrifuged at 5000 *g* at 0° for 10 min. For the assays, 1·5 ml of the supernatant was added either to 1·0 ml of 0·05 M L-phenylalanine and 2·5 ml of 0·1 M borate buffer, pH 8·8 (substrate assay), or 1·0 ml H_2O (no-substrate assay). Alternatively, the powders were extracted with 0·1 M NaCl; 2·5 ml of 0·16 M borate buffer was used for assay of these extracts. Assays were carried out at 40° for 1 hr and stopped by adding 0·1 ml of 5 N HCl. The acidified mixture was extracted with 7·5 ml of ether. 2·5 ml of the ether were evaporated to dryness and the residue dissolved in 2·5 ml of 0·05 N NaOH. Cinnamic acid was determined by absorbance at 268 nm, with 0·05 N NaOH as reference. A zero time stop reaction served as a control.

Portions of the ether extracts of assay mixtures were evaporated to dryness. The residues were dissolved in ethanol, spotted on Whatman No. 1 chromatography paper, and developed with 2 per cent acetic acid (ascending).

Boiling, Acid and Alkaline Treatments of Cinnamic Acid and Extracts of Acetone Powders

Trans-cinnamic acid (Matheson Coleman & Bell) was dissolved in 0·05 N NaOH, neutralized with 0·2 N HCl, and made 0·1 M in NaCl. Acetone powders were extracted with cold 0·1 M NaCl or buffer, as described. Portions of the extracts and solutions of cinnamic acid in 0·1 M NaCl were kept for 10 min at 100°. The remaining portions were kept at 0°. Boiled and unboiled extracts and solutions of cinnamic acid were made 1 N with respect to HCl or NaOH, kept for 1 hr at 40° or reflux temp., and then neutralized. Boiled and unboiled extracts were assayed by the substrate and no-substrate assay procedures. All treatments were adjusted to 5·0 ml with deionized water, acidified with 0·1 ml of 5 N HCl, and extracted with ether. Cinnamic acid was determined by its absorbance at 268 nm and verified chromatographically.

Two additional chromatographic solvents were used to characterize the ether extracts of the various treatments: 1. the organic phase obtained after shaking 500 ml $CHCl_3$ with 100 ml of methanol–water–formic acid (25:24:1, v/v), and 2, the organic phase of *n*-butanol–ethanol–water (4:1:5, v/v).

Dialysis of Extracts of Acetone Powders

Portions of 0·1 M NaCl extracts of acetone powders of healthy "Topcrop" hypocotyls were dialyzed overnight against three changes (100:1, v/v) of deionized water or 0·1 M NaCl. One-half of the remaining portions

[4] J. KOUKOL and E. E. CONN, *J. Biol. Chem.* **236** 2692 (1961).
[5] S. Z. EL-BASYOUNI and G. H. N. TOWERS, *Can. J. Biochem.* **42**, 203 (1964).

of the extracts were kept at 100° for 10 min and volume corrections were made with deionized water. Except when boiling, all extracts were kept at 0° until assay. Cinnamic acid production by dialyzed, boiled, and unboiled extracts was determined by substrate and no-substrate assays.

Amino Acid Analysis of Buffer Extract

Portions of a 0·1 M borate buffer extract of an acetone powder were treated as shown in Fig. 2. After overnight dialysis against two changes (100:1, v/v) of double-deionized water the dialyzates of each treatment were evaporated to dryness under vacuum at 70°. A Technicon Amino Acid Analyzer with a resin bed of 133 × 6 mm (Technicon Chromobeads-B resin) was used for chromatography. The residues were dissolved in the starting column buffer, applied to the column, and eluted. The instrument was standardized by elution of a standard mixture of seventeen amino acids and ammonia (Technicon) through the column. Autolytic cinnamic acid production was determined by no-substrate assay of a portion of the undialyzed extract.

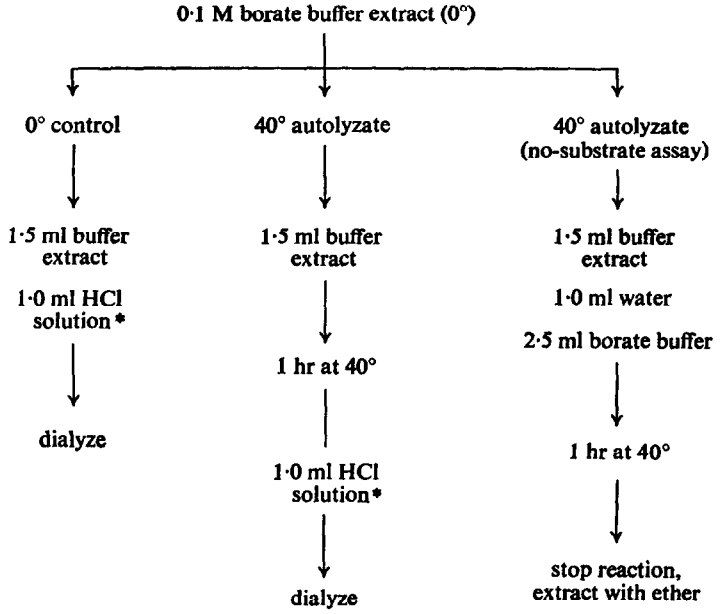

FIG. 2. TREATMENT OF BUFFER EXTRACT OF ACETONE POWDER PRIOR TO AMINO ACID ANALYSIS.

* HCl concentration such that 1·0 ml added to 1·5 ml of 0·1 M borate buffer gives pH of 2·5–3·0.