

The Metabolism of Aromatic Compounds in Higher Plants

IV. PURIFICATION AND PROPERTIES OF THE PHENYLALANINE DEAMINASE OF *HORDEUM VULGARE**

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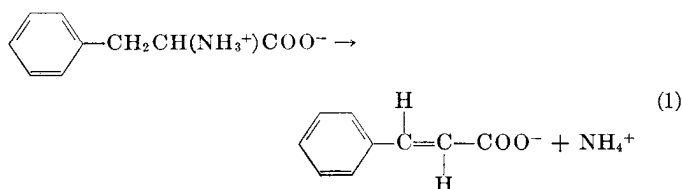
(Received for publication, March 22, 1961)

The biosynthesis of certain benzenoid compounds found in plants, such as the hydroxycinnamic acids, lignin, flavonoids, and coumarins, has recently been actively investigated. In his review (1) on the biosynthesis of these secondary plant products, Neish points out that the results of tracer experiments show the hydroxycinnamic acids to be important precursors in the formation of lignin and other secondary plant products. More specifically, in their studies on lignification in intact plants, Neish *et al.* (2-6) have demonstrated that both L-phenylalanine and cinnamic acid are good precursors of lignin or of phenylpropanoid units that can be incorporated into lignin in monocotyledons and dicotyledons. In addition, they have found that monocotyledons but not dicotyledons can readily convert L-tyrosine to lignin (2, 3, 5).

From the results of experiments in which radioactive L-phenylalanine and cinnamic acid were tested as precursors of lignin (2, 3), Brown *et al.* (6) postulated that L-phenylalanine underwent conversion of cinnamic acid through a sequence of reactions involving transamination, reduction, and dehydration. From other experiments with monocotyledons (2, 3), it appeared that L-tyrosine was converted to *p*-coumaric acid (*p*-hydroxycinnamic acid) by analogous reactions (6). However, Neish recognized that the results of the above experiments did not rule out the formation of cinnamic acid and *p*-coumaric acid by the direct deamination of the corresponding amino acids (1).

In a recent publication on the biosynthesis of chlorogenic acid, Levy and Zucker (7) presented additional evidence for the conversion of L-phenylalanine to cinnamic acid in disks of potato tuber.

The present report describes the isolation, partial purification, and characterization of phenylalanine deaminase, an enzyme from barley (*Hordeum vulgare* L. var. Aravat) that converts L-phenylalanine to cinnamic acid and ammonia (Reaction 1).



* In a preliminary report of this work presented at the meeting of the American Society of Biological Chemists in Atlantic City in April 1961, the enzyme was tentatively referred to as phenylalanase. This study was supported in part by a research grant (RG-5301) from the National Institutes of Health, United States Public Health Service.

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Recently, Neish (8) has isolated an enzyme from barley, which converts L-tyrosine to *p*-coumaric acid and ammonia.

EXPERIMENTAL PROCEDURE

Sources of Plant Material—Samples of the Aravat variety of barley were obtained from a field at Davis, California. Shoots of the Mariout variety of barley, rice (*Oryza sativa* L. var. Caloro), and wheat (*Triticum vulgare* Vill. var. Ramona) were obtained as follows. Seeds that had been soaked overnight in distilled water were germinated on a perforated Nichrome support covered with cheesecloth that had been in contact with the dilute mineral solution described by Jacobson *et al.* (9). The plants were illuminated for 12 hours a day by four 40-watt fluorescent lamps placed 17 inches above the Nichrome supports, and were harvested after 7 or 8 days of growth. Sweet clover (*Melilotus alba* Desr.) was grown in soil in a greenhouse. Peas (*Pisum sativum* L. var. Alaska) were germinated in vermiculite in a greenhouse. Lupine (*Lupinus albus* L.) was germinated in vermiculite in a dark cupboard at ambient temperatures. Alfalfa (*Medicago sativa* L. var. Caliverde) was grown in a greenhouse according to the procedures outlined by Rendig and McComb (10). The nutrient solution was that given by Rendig and McComb (Table I of (10)), and the sulfur (as H₂SO₄) and nitrogen (as Ca(NO₃)₂) were present at concentrations of 8 and 28 parts per million, respectively. In all cases, acetone powders were prepared of the plant material.

Measurement of Formation of Cinnamic Acid by Phenylalanine Deaminase—The activity of phenylalanine deaminase was determined by measuring the cinnamic acid formed. In one procedure, the cinnamic acid formed was measured spectrophotometrically. The reaction mixture contained enzyme, 20 μmoles of L-phenylalanine, and 100 μmoles of borate buffer, pH 8.8, in a final volume of 2.0 ml. Occasionally, the reaction mixture contained enzyme, 50 μmoles of L-phenylalanine, and 200 μmoles of borate buffer, pH 8.8, in a final volume of 5.0 ml. The reaction mixture was incubated without shaking for 1 hour in an unstoppered test tube at 40°. The reaction was stopped by addition of 0.1 ml of 5 M HCl, and the volume was adjusted to 5.1 ml, if necessary. With enzyme preparations of low specific activity, the acidified reaction mixture was heated in a boiling water bath for 10 minutes and the coagulated protein removed by centrifugation. The removal of protein was omitted when fractions obtained by DEAE-cellulose¹ chromatography were being assayed. The acidified reaction mixture was extracted

¹ The abbreviation used is: DEAE-cellulose, diethylaminoethyl cellulose.

once with 5 ml of ether, an aliquot of the ether phase was removed, and the ether was evaporated under a stream of air. The residue that remained was dissolved in 0.05 M NaOH, and the absorbancy at 268 $m\mu$ was determined.

The second procedure was based on the conversion of radioactive L-phenylalanine to cinnamic acid. The reaction mixture contained enzyme, 25 μ moles of L-phenylalanine, 0.45 μ mole of DL-phenylalanine-3- C^{14} (specific activity, 1.3 μ c per μ mole), and 200 μ moles of borate buffer, pH 8.8, in a final volume of 5.0 ml. The reaction mixture was incubated without shaking for 1 hour in a covered test tube at 40°. The reaction was stopped by the addition of 0.5 ml of 50% trichloroacetic acid, immediately preceded by the addition of 0.5 ml of a 0.1% solution of unlabeled *trans*-cinnamic acid in 0.05 M NaOH. After 10 minutes, the reaction mixture was centrifuged to remove protein, if necessary. The acidified mixture was then extracted once with 10 ml of toluene. After centrifugation at 500 $\times g$ for several minutes at room temperature, a 5 ml aliquot of the toluene phase was transferred to a vial containing 5 ml of counting mixture in toluene and counted at -5° in a Packard Automatic Tri-Carb liquid scintillation spectrometer (Model 314-DC). A reaction mixture complete except for enzyme solution was taken through the entire assay procedure for each set of assays. The radioactivity of the blank was subtracted from the total radioactivity of each sample. Because the efficiency of counting and the specific activity of the L-phenylalanine were known, it was possible to calculate the quantity of cinnamic acid formed from the radioactivity in the toluene phase. The identification of cinnamic acid as the product of the reaction is described in "Results."

The unit of phenylalanine deaminase activity is defined as that quantity of enzyme which catalyzes the formation of 1 μ g of cinnamic acid per hour under the usual assay conditions. Usually, 10 to 100 units of enzyme (specific activity, 352 to 970 units per mg) were used in the assays. The assay of phenylalanine deaminase by the measurement of the radioactive cinnamic acid formed gives results that are identical to those obtained when the enzyme is assayed by spectrophotometric measurement of the cinnamic acid formed.

Reagents—DL-Phenylalanine-3- C^{14} (specific activity, 1.3 μ c per μ mole) was obtained from Tracerlab Inc. DL-*m*-Tyrosine, prepared according to Sealock, Speeter, and Schweet (11), was the gift of Dr. A. C. Neish. DEAE-cellulose (Cellex-D) was obtained from Bio-Rad Laboratories. Radioactive *trans*-cinnamic acid (ring and β -carbon atom) (specific activity, 0.112 μ c per μ mole) was prepared according to Brown and Neish (2) and was the generous gift of Dr. T. Kosuge.

cis-Cinnamic acid was prepared by irradiating *trans*-cinnamic acid with an ultraviolet lamp (254 $m\mu$, maximal intensity) according to Comte *et al.* (12). *cis*-Cinnamic acid (R_F , 0.75) was separated from *trans*-cinnamic acid (R_F , 0.50) on Whatman No. 3MM paper in 2% acetic acid (12). The area of the chromatogram containing the *cis* isomer was cut out, and the substance was eluted with 2% acetic acid. *cis*-Cinnamic acid was extracted into ether and was recovered as an oil after evaporation of the ether. Recovery of the *cis*-cinnamic acid after ether extraction from 2% acetic acid was possible only when peroxide-free ether was used. The concentration of the chromatographically pure *cis*-cinnamic acid was determined spectrophotometrically (13).

Analytical Methods—Protein was determined according to the spectrophotometric method of Kalckar (14). L-Phenylalanine was determined colorimetrically according to Yemm and Cocking

(15). Ammonia was determined colorimetrically after nesslerization with the reagent of Bock and Benedict (16).

Purification on Phenylalanine Deaminase

Barley Acetone Powder—Barley plants, Aravat variety, in the pre-head stage were obtained from a field at Davis, California. The acetone powder was prepared from the stems of the plant only. The yield expressed as the percentage of the fresh weight was 10.5. The purification was performed on 40 g of acetone powder, which had an activity of 6300 units per g of powder.

All procedures in the purification were performed at 0–4°. The pH of solutions being fractionated with $(NH_4)_2SO_4$ was intermittently checked and adjusted with 5 M NaOH, if necessary, so that the pH never went below 7.

Step 1. Barley stem acetone powder, 40 g, was extracted for 1 hour with 1 liter of 0.1 M borate buffer, pH 8.5. The crude preparation was strained through cheesecloth and the filtrate was centrifuged at 4100 $\times g$ to clarify it. The volume of the supernatant solution was 835 ml.

Step 2. A neutralized solution (555 ml) of saturated $(NH_4)_2SO_4$ was added to the supernatant solution of Step 1 to form a solution that was 40% saturated with respect to the salt. The precipitate was removed by centrifugation at 15,000 $\times g$ for 15 minutes and discarded. Solid $(NH_4)_2SO_4$ (165 g) was added to the supernatant solution (1300 ml) to give a solution that was 60% saturated with respect to the salt. The precipitate was removed by centrifugation at 15,000 $\times g$ for 15 minutes and dissolved in 30 ml of 0.02 M potassium phosphate buffer, pH 6.8. This solution was dialyzed overnight with stirring against 1 liter of the same buffer.

Step 3. The pH of the dialyzed solution (38 ml) of Step 2 was carefully adjusted to 6.0 with 1.0 M acetic acid. The precipitate was removed by centrifugation at 15,000 $\times g$ for 15 minutes and was discarded. The pH of the supernatant solution was immediately adjusted to 7.0 with 1 M NaOH.

Step 4. The supernatant solution (38 ml) of Step 3 was put on a DEAE-cellulose column of the following description. On the day before use, 20 g of DEAE-cellulose were washed by suspension in 0.1 M NaOH, followed by neutralization to pH 6.8 with 1.0 M H_3PO_4 . The DEAE-cellulose was collected on a Buchner funnel and washed repeatedly by filtration with 0.02 M potassium phosphate buffer, pH 6.8. The DEAE-cellulose was resuspended in more buffer and packed so that the dimensions of the column were 2.2 \times 16 cm. The column was placed in a room at 4° overnight. After addition of the enzyme, the column was washed with 5 ml of the buffer used to equilibrate the column. The enzyme was eluted from the column by using a linear gradient between equal volumes (130 ml each) of 0.02 M potassium phosphate buffer, pH 6.8, and 0.05 M potassium phosphate buffer, pH 6.8, containing 0.4 M KCl. The column was eluted at the rate of 50 ml per hour, and fractions (4 ml) were collected automatically until the enzyme had been completely eluted.

Comments on Purification—The results of the purification are summarized in Table I. The enzyme was assayed throughout Steps 1, 2, and 3 of this purification by use of radioactive phenylalanine. Earlier work with crude or partially purified extracts of sweet clover indicated that such preparations contained ether-extractable compounds that absorbed in the ultraviolet. Therefore, it was assumed that the determination of radioactive cinnamic acid formed from radioactive phenylalanine by the enzyme would be the most specific assay. However, it was clearly shown that the DEAE-cellulose fractions contained no endogenous,

ether-extractable compounds that absorbed in the ultraviolet. This information led to an assay of the enzyme based on the spectrophotometric determination of the cinnamic acid formed.

In the purification shown in Table I, the recovery of the enzyme from Step 2 is unusually low. At this stage of the purification, 50% of the activity originally present in the acetone powder was routinely recovered. However, the final purification achieved in the procedure shown in Table I was the highest obtained. Unless otherwise specified, all experiments in the present report were performed with enzyme at the DEAE-cellulose fraction step in the procedure.

RESULTS

Identification of Cinnamic Acid—*trans*-Cinnamic acid was identified as a product of the phenylalanine deaminase reaction by its absorption spectrum, chromatographic properties, and melting point. The ultraviolet absorption spectrum of the product of the phenylalanine deaminase reaction is compared with that of authentic *trans*-cinnamic acid in 0.05 M NaOH (Fig. 1). Over the range shown, the spectra are identical.

TABLE I
Summary of purification procedure

Description	Units/ml	Protein		Total units	Recovery %
		mg/ml	units/mg		
Acetone powder . . .				250,000	
Step 1	278	7.9	35	232,000	93
Step 2	1,840	15.1	122	70,000	28
Step 3	1,280	6.2	206	48,700	19
Step 4	578	0.6	970	48,700	19

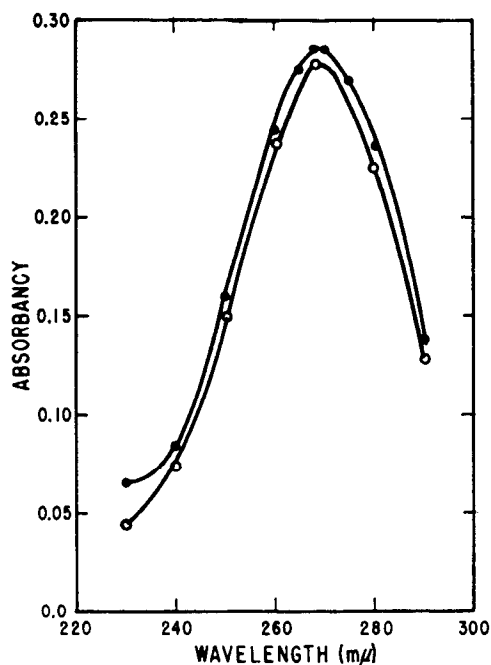


FIG. 1. Absorption spectra of *trans*-cinnamic acid (●—●) and the product of the phenylalanine deaminase reaction (○—○) in 0.05 M NaOH. Enzyme activity, 46 units, was incubated under the usual assay conditions. The ether-extractable reaction product was diluted 23-fold. The a_M of *trans*-cinnamic acid in 0.05 M NaOH at 268 $m\mu$ is 20,300.

The product of the reaction carried out under the usual assay conditions has an R_f identical to that of *trans*-cinnamic acid in three different solvents: Solvent A, *n*-propanol-concentrated NH_4OH (7:3, volume for volume); Solvent B, *n*-butanol-acetic acid-water (4:1:1.8, volume for volume); Solvent C, 2% acetic acid. When the ether-extractable radioactive product formed from phenylalanine-3- C^{14} was cochromatographed with unlabeled *trans*-cinnamic acid in Solvents A and B, the radioactive spots corresponded exactly to the ultraviolet-absorbing spots.

To obtain enough of the product of the reaction to determine its melting point, the following experiment was performed. Enzyme from Step 2 (5520 units) was incubated with 100 μ moles of L-phenylalanine at pH 8.8 at 40°. After 7 hours, the reaction mixture was heated in a boiling water bath for 10 minutes, cooled, and acidified to pH 1. The denatured protein was removed by centrifugation, and the supernatant solution was extracted once with 10 ml of ether. The ether phase was, in turn, extracted once with 5 ml of 0.1 M sodium bicarbonate. The bicarbonate phase was then acidified to pH 1 and extracted once with 10 ml of ether. The ether was allowed to evaporate, and the residue remaining was recrystallized from hot water. The melting point (corrected) of the compound was 133° which is identical with that of *trans*-cinnamic acid (17).

The possibility that the *cis* isomer of cinnamic acid is formed and is isomerized, either enzymatically or nonenzymatically, to the *trans* isomer was eliminated by the following experiment. One milligram of chromatographically pure *cis*-cinnamic acid was incubated, both with and without phenylalanine deaminase, at pH 8.8 at 40° for 2 hours. The reaction mixture, made 2% with respect to acetic acid to stop the reaction, was extracted twice with an equal volume of ether. The ether was evaporated, the residue was taken up in ethanol, and an aliquot was chromatographed in 2% acetic acid. No isomerization of *cis*-cinnamic acid was observed either in the absence or presence of enzyme in the reaction mixture. If 1% of the total *cis*-cinnamic acid had undergone isomerization, the appearance of the *trans* isomer could have been detected. Under the same conditions (both reaction and extraction), 246 μ g of *trans*-cinnamic acid were formed by the enzyme from L-phenylalanine.

Stoichiometry of Reaction—The stoichiometry of the phenylalanine deaminase reaction was determined as follows. A reaction mixture containing 122 units of enzyme, 1 μ mole of L-phenylalanine, and 100 μ moles of borate buffer, pH 8.8, in a final volume of 2.0 ml, was incubated for 4 hours at 40° in a stoppered test tube. Sufficient replicates of the reaction mixture were used to provide zero time blanks and duplicates for all determinations. The reaction was stopped by adding 0.1 ml of 5 M HCl, and the cinnamic acid formed was determined spectrophotometrically. The replicates that were to be used for the determination of L-phenylalanine were first treated with 200 mg of Permutit at neutral pH to remove the ammonia formed in the enzyme reaction. The replicate reaction mixtures which were to be used for ammonia determination were directly nesslerized. Also, the ammonia was aerated or steam-distilled out of the reaction mixture (made strongly alkaline) into a boric acid solution. Nesslerization of the boric acid solution gave the same results for ammonia formation as direct nesslerization of the reaction mixture. Typical results from such an experiment are the conversion of 0.70 μ mole of L-phenylalanine to 0.69 μ mole of cinnamic acid and 0.66 μ mole of ammonia.

Reversibility of Reaction—Preliminary attempts to demonstrate

the formation of L-phenylalanine-C¹⁴ from *trans*-cinnamic acid-C¹⁴ (ring and β -carbon atom) and ammonia were not successful. The experiments were performed at both pH 6.9 and pH 8.8.

Properties of Enzyme

1. *Effect of pH*—The optimal pH of phenylalanine deaminase was found to be 8.8 to 9.2 (Fig. 2). The enzyme is active over the pH range 8 to 10.6.

2. *Effect of Enzyme Concentration and Duration of Reaction*—In preliminary experiments, phenylalanine deaminase was found to be inhibited by cinnamic acid, one of the products of the reaction. Therefore, assay conditions were chosen so that the cinnamic acid formed never reached inhibitory concentrations. Fig. 3 shows that the rate of formation of cinnamic acid catalyzed by the enzyme is proportional to the amount of enzyme added in the range of 6 to 85 units of enzyme. Similarly, Fig. 4 shows that the formation of cinnamic acid catalyzed by 60 units of enzyme is linear for 2 hours, a period of time twice as long as the usual assay.

3. *Effect of Substrate Concentration*—The data were plotted according to Lineweaver and Burk ($1/V$ versus $1/s$) (18), and the Michaelis-Menten constant, K_m , was calculated from the intercept ($1/V$) and the slope of the line. The K_m for L-phenylalanine and phenylalanine deaminase was found to be $1.7 \pm 0.3 \times 10^{-3}$ M.

4. *Substrate Specificity*—The enzyme was specific for L-phenylalanine. Cinnamic acid was not formed when phenylalanine deaminase was incubated with D-phenylalanine. To test the ability of the enzyme to act on other amino acids, ammonia production was measured when 74 units of phenylalanine deaminase were incubated for 2 hours under the usual assay conditions with the following amino acids: L-phenylalanine, DL-aspartic acid, L-histidine, L-alanine, 3,4-dihydroxyphenyl-DL-alanine, DL-leucine, β -phenyl-DL-serine, glycine, L-cysteine, and L-tryptophan. The amino acids were present at a final concentration of 0.01 M (L isomer). Ammonia was distilled by steam, from the reaction mixture made 0.15 M with respect to $\text{Na}_2\text{B}_4\text{O}_7$, into 0.5 ml of 0.2 M boric acid, and was determined by nesslerization of the boric acid solution. Ammonia (0.94 μmole) was formed only when L-phenylalanine was the substrate. It would be well to point out here that when the enzyme has been freshly prepared, the DEAE-cellulose fractions also catalyze the deamination of L-tyrosine and DL-*m*-tyrosine to the corresponding unsaturated acids and ammonia. These conversions are probably catalyzed by the enzymes from barley described by Neish (8) which convert L-tyrosine and DL-*m*-tyrosine to the corresponding unsaturated acids and ammonia. However, the ability of the DEAE-cellulose fractions to catalyze these conversions is completely lost on storage of the fractions, although they still contain 75% of the original phenylalanine deaminase activity. For example, 97 units of enzyme (which had been stored at -10° for $3\frac{1}{2}$ months) were incubated under the usual assay conditions for 8 hours, with 0.005 M (final concentration of the L isomer) of each of the following amino acids: L-phenylalanine, L-tyrosine, and DL-*m*-tyrosine. Ammonia was determined by direct nesslerization of an aliquot of the reaction mixture. Ammonia (1.97 μmoles) was formed only when L-phenylalanine was the substrate. Thus, phenylalanine deaminase appears to be specific for L-phenylalanine.

5. *Effect of Metals*—Attempts were made to stimulate phenylalanine deaminase activity by the addition of metal ions. The

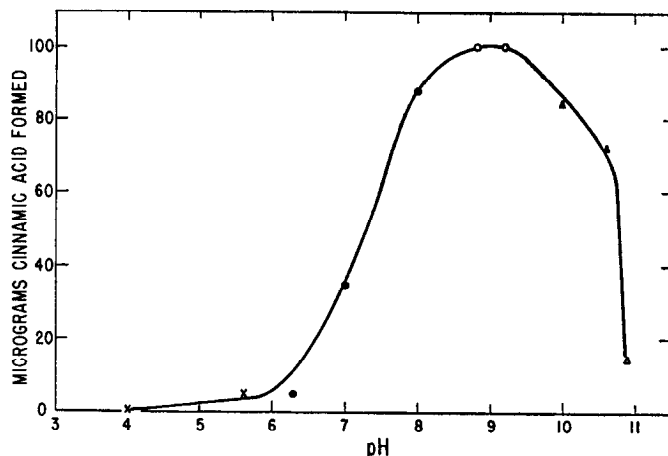


FIG. 2. The pH optimum curve of phenylalanine deaminase. For testing, 100 units of enzyme were assayed in 200 μmoles of buffer under the usual conditions. The values were measured in sodium acetate buffer (\times), potassium phosphate buffer (\bullet), sodium borate buffer (\circ), ethanolamine-HCl buffer (\blacktriangle), and methylamine-HCl (\triangle). Cinnamic acid was extracted into ether and determined spectrophotometrically.

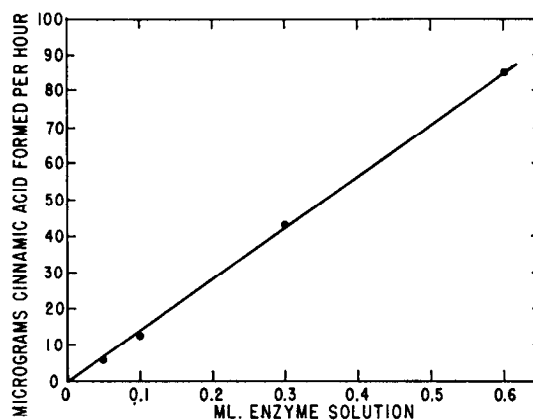


FIG. 3. The effect of the enzyme concentration on the rate of the reaction. The enzyme (142 units per ml) was assayed for 80 minutes under the usual conditions. Cinnamic acid was determined spectrophotometrically.

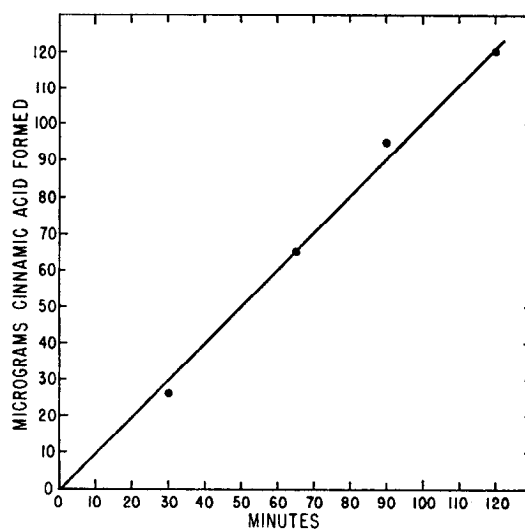


FIG. 4. The effect of time on the production of cinnamic acid. Enzyme, 60 units, was assayed under the usual conditions. Cinnamic acid was determined spectrophotometrically.

following additions, all at 10^{-3} M, were without effect: CoCl_2 , NiCl_2 , MgCl_2 , MnCl_2 , FeCl_2 , FeCl_3 , $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$, and $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2\cdot 3\text{H}_2\text{O}$. The DEAE-cellulose fractions contained KCl, and further addition of KCl did not stimulate phenylalanine

TABLE II

Inhibitory effect of certain metals on phenylalanine deaminase activity

Enzyme, 10.4 units, was incubated under the usual assay conditions. Cinnamic acid was determined spectrophotometrically

Addition	Final concentration	Inhibition
	M	%
CuSO_4	10^{-3}	18
ZnSO_4	10^{-3}	32
CaCl_2	10^{-3}	57
CdCl_2	10^{-3}	100
HgCl_2	10^{-5}	100

TABLE III

Effect of sulfhydryl compounds and sulfhydryl group reagents on phenylalanine deaminase activity

The enzyme was assayed as usual. The addition of enzyme was as follows: 10.4 units to Nos. 1 and 2, 25 units to No. 3, 17.6 units to No. 4, and 15.5 units to Nos. 5 to 10. The cinnamic acid formed was determined spectrophotometrically except for No. 2. The radioactive assay was used for No. 2.

No.	Addition	Final concentration	Effect
		M	
1	<i>p</i> -Chloromercuri-phenylsulfonic acid	1×10^{-5}	100% inhibition
2	Iodoacetamide	1×10^{-2}	67% inhibition
3	GSH	3×10^{-3}	70% stimulation
4	GSH	3×10^{-3}	40% stimulation
5	L-Cysteine	3×10^{-3}	35% inhibition
6	L-Cysteine	1×10^{-2}	76% inhibition
7	L-Cystine	1×10^{-3}	No effect
8	GSSG	4×10^{-3}	No effect
9	Mercaptoethanol	1×10^{-3}	No effect

TABLE IV

Effect of aromatic acids on phenylalanine deaminase activity

The enzyme was assayed for 2 hours under the usual conditions. The addition of enzyme was as follows: 11.3 units to Nos. 1, 3, and 4, 25 units to Nos. 2 and 7, and 37 units to Nos. 5 and 6. The radioactive assay was used for all except Nos. 4 and 5. The cinnamic acid from Nos. 4 and 5 was determined spectrophotometrically.

No.	Addition	Final concentration	Inhibition
		M	%
1	L-Tyrosine	2×10^{-4}	36
1	L-Tyrosine	6×10^{-4}	57
2	<i>trans-p</i> -Coumaric acid	2×10^{-4}	45
2	<i>trans-p</i> -Coumaric acid	6×10^{-4}	64
3	<i>trans</i> -Cinnamic acid	6×10^{-4}	25
4	3,4-Dihydroxyphenyl-DL-alanine	6×10^{-3}	22
5	β -Phenyl-DL-serine	6×10^{-3}	22
6	Phenylacetic acid	1×10^{-3}	None

deaminase activity. Table II summarizes the inhibitory effects of certain metal ions on phenylalanine deaminase activity. Although 10^{-2} M Versene (ethylenediaminetetraacetate) and 10^{-3} M α, α' -dipyridyl did not affect the reaction, 10^{-3} M KCN inhibited the reaction 85%.

6. *Effect of Sulfhydryl Compounds and Sulfhydryl Group Reagents*—As shown in Table III, phenylalanine deaminase was completely inhibited by 10^{-5} M *p*-chloromercuriphenylsulfonic acid. Iodoacetamide at a higher concentration inhibited the enzyme 67%. This evidence clearly indicates that phenylalanine deaminase is a sulfhydryl enzyme. In keeping with these observations, GSH always stimulated phenylalanine deaminase activity but the degree of stimulation varied. On the other hand, L-cysteine consistently inhibited the enzyme. The inhibition of the enzyme by L-cysteine could be an indirect one, although attempts to determine if this is the case have not been successful.

7. *Inhibition of Enzyme by Aromatic Acids*—Table IV summarizes the inhibitory effects of compounds which are structurally related to L-phenylalanine or *trans*-cinnamic acid. L-Tyrosine and both *m*- and *p*-coumaric acids markedly inhibit the enzyme reaction even when they are present in relatively low concentration. The inhibition by L-tyrosine and *p*-coumaric acid would appear to be competitive. When the concentration of L-phenylalanine was increased 3-fold, in a separate experiment, the inhibition by 6×10^{-4} M L-tyrosine and 6×10^{-4} M *p*-coumaric acid was approximately 20% less than that shown in Table IV. The reaction is inhibited to a lesser extent by β -phenyl-DL-serine and 3,4-dihydroxyphenyl-DL-alanine although they are present at a higher concentration (Table IV). *trans*-Cinnamic acid also inhibits the reaction. Because the phenylalanine deaminase reaction seems to be irreversible, however, the inhibition of cinnamic acid must be another example of the inhibition of an enzyme by a product of the reaction.

8. *Stability of Enzyme*—The stability of the enzyme depends upon the age of the plant material used for preparing the acetone powder. Acetone powders of the Aravat variety of barley in the pre-head stage are completely stable for at least 3 months at 6°. The DEAE-cellulose fractions (Step 4), which are obtained by fractionation of the enzyme from this material are also markedly stable. Storage for periods of up to 3½ months at -10° results in only 25% loss of activity. However, if the acetone powder is prepared from the Aravat variety when the heads have emerged, the powder loses most of its activity before 3 months. Acetone powders prepared from the young shoots of the Mariout variety are completely stable for at least 3 months at 6°. It has been found, however, that when the enzyme is obtained from the acetone powder of young shoots of the Mariout variety, it is unstable to fractionation.

9. *Distribution of Enzyme*—The distribution of the enzyme is shown in Table V. The enzyme was assayed by determining the cinnamic acid formed with use of the radioactive assay. In addition, the cinnamic acid formed was identified by radioautograms. The enzyme has been demonstrated in all plants tested, but occurs in various amounts. Barley, alfalfa, rice, and peas seem to be good sources of the enzyme, whereas sweet clover and lupine are less active. The amount of enzyme activity demonstrated in barley shoots and etiolated lupine seedlings was found to be reproducible from batch to batch of plants. However, the amount of enzyme activity that could be demonstrated in sweet clover varied drastically and abruptly with the age of the

TABLE V
Distribution of phenylalanine deaminase

Although the results are expressed per g of fresh weight, the assays were performed on acetone powders. The radioactive assay was used. All were green plants, except the lupine, which was etiolated. Cinnamic acid was identified by means of radioautographs as described in "Results."

Source	Variety	Part of plant used	Age of plant	Cinnamic acid formed <small>μmoles/g fresh wt/hr</small>
Sweet clover		Stems + leaves	5 weeks	0.4
Lupine		Whole plant	7 days	0.25
Rice	Caloro	Whole plant	12 days	0.6
Barley	Mariout	Shoots	7 days	1.2
Barley	Aravat	Stems	Pre-head stage	1.6
Alfalfa	Caliverde	Stems	15 days	1.5
Peas	Alaska	Whole plant	11 days	0.6

plant material. Extensive sampling of the other plants was not made.

DISCUSSION

The reaction catalyzed by phenylalanine deaminase is similar to the aspartase reaction in which one molecule of ammonia is eliminated from an α -amino acid to form an unsaturated acid. In the phenylalanine deaminase reaction, the unsaturated acid is cinnamic acid. The cinnamic acid isolated from the enzyme reaction has the *trans* configuration. The possibility that *cis*-cinnamic acid is the product of the reaction and undergoes isomerization, either enzymatically or nonenzymatically, during the course of the reaction or isolation has been eliminated. Also, in a closely analogous enzyme reaction in which L-tyrosine is converted to *p*-coumaric acid, Neish (8) has demonstrated that it is the *trans* isomer of *p*-coumaric acid which is formed.

The phenylalanine deaminase reaction is believed to occur by the direct deamination of L-phenylalanine to form cinnamic acid and ammonia. Although this reaction could occur by formation of the keto acid, followed by reduction and dehydration, it appears to occur in a single step. In addition to the stoichiometric evidence, ammonia formation is known to occur equally well under both aerobic and anaerobic conditions. Further, cinnamic acid formation is not affected by the addition of α -ketoglutarate, pyridoxal phosphate, and DPNH, when added either singly or together.

In recent years, several enzymes that catalyze aspartase-like reactions have been studied. These include aspartase (19-21), β -methylaspartase (22), histidine- α -deaminase (histidase) (23), and an enzyme that catalyzes the deamination of L-tyrosine (8). Although the reactions catalyzed by the first two enzymes are reversible (22, 24), the reactions catalyzed by the latter two enzymes are irreversible (8, 25). Although preliminary experiments suggest that the phenylalanine deaminase reaction is irreversible, this has not been conclusively demonstrated.

The stimulation of phenylalanine deaminase by GSH and the inhibition of the enzyme by certain sulfhydryl group inhibitors strongly suggest that phenylalanine deaminase requires the sulfhydryl group for activity. Aspartase (19, 20) and histidine- α -deaminase (26) have been shown to be inhibited by sulfhydryl

group reagents. The stimulation of histidine- α -deaminase by GSH has been demonstrated (26, 27).

Aspartase, β -methylaspartase, and histidine- α -deaminase require the presence of metal ions for activity (19, 22, 26-28). In addition, the inhibition of histidine- α -deaminase by Versene (26-28), and the reversal of this inhibition (27, 28) has been reported. Inasmuch as phenylalanine deaminase seems to be analogous to aspartase and aspartase-like enzymes, the possibility that phenylalanine deaminase is a metalloprotein was explored. The enzyme was found to be inhibited by cyanide, and this indicated that it could be a metalloprotein. On the other hand, phenylalanine deaminase was not stimulated by any of the metal ions added. However, the preparations were not rigorously treated for the removal of metals.

That phenylalanine deaminase is specific for L-phenylalanine is in keeping with the probable function of this enzyme in plants, namely, to provide a source of cinnamic acid for ultimate incorporation into the secondary plant products. The occurrence of phenylalanine deaminase in plants substantiates the view of Neish that L-phenylalanine is a precursor of the secondary plant products by its conversion to cinnamic acid. The enzyme occurs in both monocotyledons and dicotyledons as predicted by Neish's work (2-6). Moreover, in a joint experiment with Dr. Neish, it was shown that preparations of dicotyledons such as lupine, pea, and alfalfa lacked the enzyme that converts L-tyrosine to *p*-coumaric acid but contained phenylalanine deaminase activity. This further substantiates Neish's hypothesis that both monocotyledons and dicotyledons can convert L-phenylalanine to lignin through cinnamic acid, but only monocotyledons can convert L-tyrosine to lignin through *p*-coumaric acid.

SUMMARY

The enzyme, phenylalanine deaminase, which catalyzes the conversion of L-phenylalanine to *trans*-cinnamic acid and ammonia, has been purified 28-fold from barley (*Hordeum vulgare* L. var. Aravat) and partially characterized. Phenylalanine deaminase appears to be an aspartase-like enzyme, and some of the properties of phenylalanine deaminase are compared to those of aspartase and aspartase-like enzymes. The function of this enzyme in plants is discussed.

Acknowledgment—The authors wish to thank Dr. A. C. Neish of the National Research Council of Canada for many helpful discussions.

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J. Biol. Chem. 1961, 236:2692-2698.

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