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L-Phenylalanine Ammonia-Lyase Activity During Germination of Phaseolus vulgaris

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Abstract. L-Phenylalanine ammonia-lyase (PAL) activity develops in excised bean axes after approximately 5 hours of incubation and reaches a maximum level after 14 hours of incubation. Light does not affect the development of activity, but puromycin, cycloheximide, actinomycin D, and 5-fluorouracil inhibit.

During this period of incubation both D- and L-*p*-fluorophenylalanine stimulate fresh weight increase and both inhibit the development of PAL activity. Neither L- nor D-phenylalanine stimulates fresh weight increase while the former inhibits development of PAL activity and the latter has no effect. Neither D isomer is deaminated while L-*p*-fluorophenylalanine is deaminated at about one-half the rate of L-phenylalanine. It is suggested that fluorophenylalanine does not stimulate the fresh weight increase by its effects on the phenolics pathway.

Trans-cinnamic acid was found to inhibit both the development of PAL activity and the *in vitro* deamination of L-phenylalanine. Various hydroxycinnamic acids, although inhibiting the development of PAL activity, had little or no effect on the *in vitro* deamination of L-phenylalanine.

No tyrosine ammonia-lyase activity was found in the axes and L-tyrosine had no effect on the *in vitro* deamination of L-phenylalanine.

The pattern of PAL development in intact seedlings differs markedly from that which occurs in the excised axes, although light also has no effect on the course of activity.

In a recent paper, I reported that DL-*p*-fluorophenylalanine stimulated the fresh weight increase of excised bean axes by 20 to 40 % (16). The suggestion was made that the stimulation by fluorophenylalanine might be due to effects on the metabolism of various phenylalanine-derived compounds, such as the flavonoids and coumarins. Since L-phenylalanine ammonia-lyase (E.C. 4.3.1.5), (PAL) catalyzes the conversion of L-phenylalanine to trans-cinnamic acid, the postulated initial reaction on the pathway from L-phenylalanine to the phenolics (7), it was of interest to determine if the axes contained PAL activity during the period under observation, and if so, what the effects of fluorophenylalanine on this activity would be. Examination of the axes has shown that PAL activity does develop during incubation and a recent communication discussed the effects of abscisin II, cycloheximide, and actinomycin D on the development and retention of activity (17).

This report concerns the course of PAL activity in excised bean axes and in seedlings germinated in the intact seed. Described are the effects of D-*p*-fluorophenylalanine, and various compounds which may be involved in the phenolic metabolic pathway, on both the development of PAL activity and the *in vitro* conversion of L-phenylalanine to trans-cinnamic acid.

Materials and Methods

The embryonic axes were excised from the dry seed of *Phaseolus vulgaris* L. (var. White Marrowfat). The incubation procedures and determination of fresh weight were as previously described (15). Since no effect of light on the course of axis PAL activity was found, incubation was under ordinary laboratory light conditions, except where otherwise specified in the Results section. Intact seeds were germinated in vermiculite either in complete darkness or under normal laboratory light conditions at 26 ± 1°. The extraction of PAL from the axes has been described (17).

PAL activity was determined either spectrophotometrically (20) or with the radioactive assay of Koukol and Conn (7) as modified by Young and Neish (18). In both procedures the reaction mixture consisted of 150 μmoles borate buffer, pH 8.8, and 0.5 ml extract in a total volume of 3.0 ml. In the spectrophotometric assay, 15 μmoles of L-phenylalanine were added, while in the radioactive assay 10 μmoles were added. In the radioactive assay, 0.25 μc (0.02 μmoles) of L-phenylalanine-1-^{14}C were also added. Incubation was for 2 hours at 37°.

In the spectrophotometric assay, activity was determined by the increase in absorbance at 290 mμ. The unit of activity is defined as that amount of

enzyme which produces an increase in absorbance at 290 mμ of 0.01 per hour with a 1-cm light path.

In the radioactive assay, the reaction was stopped by the addition of 0.5 ml 0.1 % cinnamic acid in 0.05 M KOH, followed by 0.5 ml HCl. The precipitated protein was removed by centrifugation and the supernatant extracted with 10 ml ether. The ether layer was reextracted with 5 ml 0.1 N HCl, decanted, and then shaken with anhydrous sodium sulfate. The ether layer was then decanted into a scintillation vial, and the ether evaporated. Ten ml of toluene containing 2,5-diphenyloxazole and 1,4-*bis*-2-(5-phenyloxazolyl)-benzene were added to the vial and the vial counted in a Packard Tricarb, Model 3375, liquid scintillation spectrometer, at approximately 80 % efficiency. The activity was corrected for quenching and the counts from a boiled enzyme control subtracted.

Tyrosine ammonia-lyase activity was determined according to Neish (10). The general procedure used was the same as that described for the radioactive assay, except that 15 μmoles of L-tyrosine were used as substrate, and the evaporated ether extract was taken up in 3 ml 0.05 N NaOH and the absorbance measured at 333 mμ.

DL-*p*-Fluorophenylalanine was resolved by carboxypeptidase treatment of *N*-chloroacetyl-DL-*p*-fluorophenylalanine (4). Fluorocinnamic acid was synthesized from fluorobenzaldehyde (8). Protein was estimated according to Lowry *et al.* (9).

Results

Figure 1 shows the course of PAL activity in the excised bean axes. Initially, there is little or no detectable activity, but at about 5 hours after the beginning of incubation measurable activity appears and continues to increase until about the fourteenth or fifteenth hour. Continued incubation leads to a sharp reduction in activity so that by 24 hours the activity is only about 10 to 35 % of the maximum. The pattern in which the development of PAL activity is followed by a decline has been noted in other tissues, and the implication of the decline in the bean axes has been discussed (17). Tyrosine ammonia-lyase activity could not be detected in the axes at the time at which PAL activity reached its maximum level.

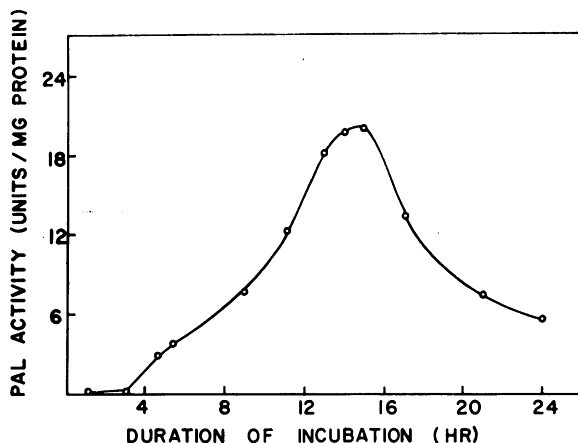

FIG. 1. Course of PAL activity in excised axes incubated under laboratory light conditions at 26°.

Inhibitors of Protein and/or RNA Synthesis. Several compounds known to be inhibitors of protein and/or RNA synthesis in the axes (15, unpublished results) were tested for their effect on development of PAL activity (table I). All of these compounds inhibit the development of PAL activity, while all but 5-fluorouracil inhibit fresh weight increase. These results suggest that the development of PAL activity in the axes may be dependent on *de novo* protein synthesis. The inhibition of the development of PAL activity by 5-fluorouracil, a compound which inhibits ribosomal RNA synthesis but not protein synthesis in the axes (unpublished results), may be due to its incorporation into m-RNA with consequent misreading of a critical amino acid during translation (1). 5-Fluorouracil has been reported to be without effect on the development of activity of several plant enzymes and to have a selective inhibitory effect on the development of enzyme activities in *Bacillus subtilis* (5,6). Although the results obtained with the antibiotics suggest that the development of PAL activity requires protein synthesis, a variety of amino acid analogues had no inhibitory effect when used at 10^{-3} M. They included L-ethionine, L-azetidine-2-carboxylic acid, L-canavanine sulfate and hydroxy-L-proline.

Table I. *Effects of Inhibitors of RNA and/or Protein Synthesis on Development of PAL Activity and Increase in Fresh Weight*

100 mg tissue dry weight incubated 14 hours at 26° in 2 ml solution containing 0.01 M K PO_4, pH 6.0, 100 units penicillin G and additions as indicated. Laboratory light conditions.

Treatment	Fr wt increase	PAL specific activity
	% of control[1]	*% of control*[2]
Actinomycin D, 25 μg/ml	25	5
Puromycin, 5×10^{-4} M	0	5
Cycloheximide, 3×10^{-5} M	0	0
5-Fluorouracil, 2×10^{-3} M	100	10

1 Control fresh weight increase = 160 mg.
2 Control PAL specific activity = 23 units/mg protein.

In Vivo *and* in Vitro *Effects of Phenylalanine and* p-*Fluorophenylalanine.* The results in table II indicate that both D and L isomers of *p*-fluorophenylalanine can stimulate growth although the D isomer is more effective. Neither D- nor L-phenylalanine stimulates growth. Of the 4 compounds, only D-phenylalanine does not inhibit the development of

Table II. *Effect of* D- *and* L-*p-Fluorophenylalanine on Fresh Weight Increase and PAL Activity of Excised Axes*

100 mg tissue dry weight incubated 14 hours at 26° in 2 ml solution containing 0.01 M K PO_4, pH 6.0, 100 units penicillin G, and additions as indicated. Laboratory light conditions were employed.

Compound	Conc	Fr wt increase	PAL specific activity
		% of control	*% of control*
L-Phenylalanine	5×10^{-4} M	100	71
	10^{-3} M	90	52
D-Phenylalanine	10^{-3} M	100	100
D-*p*-Fluorophenylalanine	10^{-5} M	108	...
	2.5×10^{-4} M	120	87
	10^{-3} M	122	50
	5×10^{-3} M	100	10
L-*p*-Fluorophenylalanine	10^{-5} M	100	...
	2.5×10^{-4} M	115	37
	5×10^{-4} M	111	20
	10^{-3} M	100	10
	5×10^{-3} M	80	0

PAL activity. Neither of the D isomers is deaminated while L-*p*-fluorophylalanine is deaminated at approximately one-half the rate of L-phenylalanine (table III). Both D isomers inhibit the *in vitro* deamination of L-phenylalanine suggesting that they can bind to the enzyme although not be deaminated (table III).

Table III. *Relative Rates of Deamination and Effects on* L-*Phenylalanine Deamination of* D-*Phenylalanine and of* D- *and* L-*p-Fluorophenylalanine*

Enzyme from axes incubated 11 hours as described in table II. All compounds used at 5×10^{-3} M. Details of spectrophometric assay are given in Methods Section.

Compound	Rate of deamination	Inhibition of L-phenylalanine deamination
	units/mg protein	%
L-Phenylalanine	11.7	...
D-Phenylalanine	0.1	30
D-*p*-Fluorophenylalanine	0.0	53
L-*p*-Fluorophenylalanine	7.4	...

In Vivo *and* in Vitro *Effects of* L-*Tyrosine and Cinnamic Acids.* L-Tyrosine, trans-cinnamic acid, *p*-fluorocinnamic acid, the deamination product of *p*-fluorophenylalanine, and several hydroxycinnamic acids important in the biosynthesis of phenolic compounds, were tested for their effects on the development of PAL activity and fresh weight increase. Table IV shows that all of these compounds except L-tyrosine inhibit both the development of PAL activity and fresh weight increase to some extent. Trans-cinnamic acid inhibits both PAL activity and fresh weight increase strongly and to about the same extent. *p*-Fluorocinnamic acid inhibits fresh weight increase more strongly than the development of PAL activity, while the hydroxycinnamic acid derivatives inhibit the development of PAL activity more strongly than the fresh weight increase. L-tyrosine, unlike L-phenylalanine, stimulated the development of PAL activity although it had no significant effect on fresh weight increase.

L-Tyrosine, trans-cinnamic acid, and *p*-coumaric acid have been reported to inhibit the *in vitro* deamination of L-phenylalanine (7, 18). The effects of these compounds, and also ferulic and caffeic acids, were determined on the *in vitro* deamination of L-phenylalanine by extracts from the bean axes (table V). Cinnamic acid and caffeic acid inhibited deamination, ferulic acid and L-tyrosine had no significant effect, while *p*-coumaric acid appeared to have a slight stimulatory effect. The inhibition of deamination by caffeic acid, although not by either *p*-coumaric or ferulic acids, suggests that caffeic acid may be converted to a quinone with subsequent reaction with essential sulfhydryl groups on PAL (7, 11). The reduction of the caffeic acid inhibition by barbituric acid (table V) lends support to this hypothesis, since barbituric acid has been reported to react with quinones (11).

Light Effects. Since most reports about PAL have indicated that light is necessary for maximum development of activity (2, 3, 12, 20), the effect of light on both the development and retention of activity in the axes was determined. Table VI

Table IV. *Effects of* L-*Tyrosine, Cinnamic Acid, and Substituted Cinnamic Acids on Fresh Weight Increase and PAL Activity of Excised Axes*

Incubation conditions are described in table II.

Compound	Conc	Fr wt increase	PAL specific activity
		% of control	*% of control*
Trans-cinnamic acid	10^{-4} M	67	65
	10^{-3} M	0	0
Fluorocinnamic acid	[illegible]	88	100
	3×10^{-4} M	40	85
p-Coumaric acid	5×10^{-4} M	80	38
Caffeic acid	5×10^{-4} M	88	40
Ferulic acid	5×10^{-4} M	90	62
L-Tyrosine	10^{-3} M	100	145

Table V. *Effects of* L-*Tyrosine, Trans-cinnamic Acid and Cinnamic Acid Derivatives on Deamination of* L-*Phenylalanine*

Incubation mixture contains 3.3×10^{-3} M L-phenylalanine-^{12}C + 0.25 μc L-phenylalanine-1-^{14}C and added compounds as noted. Other details of radioactive assay are given in Methods section. Enzyme from axes incubated 14 hours as described in table II.

Addition	Effect on deamination of L-phenylalanine	
	dpm	*% of control*
None	2396	...
4.3×10^{-3} M L-Tyrosine	2876	116
1.4×10^{-3} M Trans-cinnamic acid	1675	73
3.3×10^{-3} M *p*-Coumaric acid	2696	103
1.4×10^{-3} M Caffeic acid	860	36
1.4×10^{-3} M Caffeic acid + 6×10^{-3} M Barbituric acid	2304	95
1.4×10^{-3} M Ferulic acid	2420	101

compares the PAL activity in the axes after 14 and 24 hours of incubation in complete darkness, and under either approximately 5 or 1000 ft-c of continuous fluorescent and incandescent light. The results indicate that light is not required for development of PAL activity nor does it prevent the loss of activity after longer incubation periods. The development of PAL activity in axes incubated under 1000 ft-c was actually slightly inhibited. Engelsma has reported that removal of the cotyledons from gherkin seedlings reduced the light requirement for development of PAL activity (3). In order to determine if the lack of light sensitivity was due to excission of the axes, intact seeds were germinated in the dark or under normal laboratory light conditions for up to a period of 4 days (fig 2). There is almost no detectable activity in the seedlings until about 20 hours after the beginning of imbibition. At 48 hours, the PAL specific activity is about the same in the hypocotyls as in the roots. However, the activity in the roots continues to rise over the next 48 hours, while that in the hypocotyls decreases to an almost undetectable level. As was found for the axes, there is no effect of light on the apparent PAL activity in either the roots or hypocotyls during this period. Although the results have been expressed on a specific activity basis, the conclusions are essentially the same if the results are presented on a seedling basis.

Table VI. *Effect of Light on Development and Retention of PAL Activity in Excised Axes*

100 mg tissue dry weight incubated at 26° in 2 ml solution containing 0.01 M K PO_4, pH 6.0, and 100 units of penicillin G. Incandescent and fluorescent light were continuous throughout the incubation.

	PAL specific activity 14 hrs	24 hrs
	units/mg protein	
Expt. 1		
Dark	22.5	8.7
5 ft-c	20.5	4.4
Expt. 2		
Dark	24.7	7.6
1000 ft-c	17.2	5.6

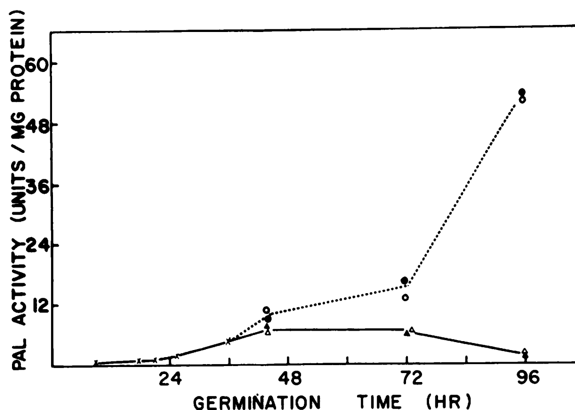

FIG. 2. Course of PAL activity in intact seed germinated in dark or under laboratory light. Roots + hypocotyls, light (x). Hypocotyls, dark (▲) or light (△). Roots, dark (●) or light (○).

Discussion

The results show that the excised axes develop PAL activity during the period of time in which both D- and L-*p*-fluorophenylalanine are effective in promoting growth. However, the results suggest that the growth promotive effect is independent of phenolic metabolism if the deamination of L-phenylalanine by PAL is the sole method of entry into the pathway. Development of PAL activity is inhibited by 5-fluorouracil and stimulated by L-tyrosine although neither affects growth, suggesting that PAL activity is not important for growth during the period of incubation. The fact that D-*p*-fluorophenylalanine is not deaminated suggests that the growth stimulation is not due to the production of fluorinated cinnamic acid derivatives unless racemization occurs. The striking difference between the effects of D- and L-phenylalanine on the development of PAL activity indicates that racemization occurs to a limited extent if at all.

The inhibition of development of PAL activity by L-phenylalanine has been reported by Zucker for potato slices (20). The inhibition was attributed to the formation of trans-cinnamic acid, which as he reported and is confirmed here is an inhibitor of the development of enzyme activity. The lack of inhibition by D-phenylalanine, which is not deaminated, is consistent with this hypothesis. The effects of cinnamic acid on the development of activity in the bean axes are complicated, however, by the strong and parallel inhibitory effects on growth, suggesting the possibility that cinnamic acid may have a broader effect on metabolism than inhibition of PAL synthesis. Trans-cinnamic acid has been reported to have an inhibitory effect on the induction of nitrate reductase in corn seedlings but the rate of overall protein synthesis was inhibited to at least the same extent (13). Trans-cinnamic acid has also been reported to have anti-auxin properties (14).

The lack of an inhibitory effect by either L-tyrosine or *p*-coumaric acid on the *in vitro* deamination of L-phenylalanine differs from the results reported by Koukol and Conn for barley (7) and by Young and Neish for wheat (18). Both groups reported that these compounds were effective inhibitors of L-phenylalanine deamination and the latter workers also reported that L-phenylalanine had little or no effect on the deamination of L-tyrosine. The bean axes also differ from either wheat or barley in that they do not contain any measurable tyrosine ammonia-lyase activity. The lack of such activity in the stems and leaves of *Phaseolus vulgaris* has also been recently reported by Young *et al.* (19). These differences between the enzymes from bean, barley, and wheat suggest that not only have the latter 2 acquired the ability to deaminate L-tyrosine, but that deamination of L-phenylalanine may be regulated by their level of L-tyrosine.

As noted before, the apparent lack of a light effect on the development of PAL activity in either excised embryonic axes or intact seedlings differs from the results reported by several investigators. At present, it is not known what factors may contribute to this difference.

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