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THE CHARACTERIZATION OF PHENYLALANINE AMMONIA-LYASE FROM SEVERAL PLANT SPECIES

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Key Word Index—*Setaria glauca*; phenylalanine ammonia-lyase; herbicides.

Abstract—Inhibition of the enzyme phenylalanine ammonia-lyase is considered as a target for the design of herbicides. A reliable and simple assay for the enzyme has been used and the kinetics of the enzyme from several sources compared. Purification of the enzyme from the grass green foxtail (*Setaria glauca*) did not change its kinetic behavior. The distribution of phenylalanine ammonia-lyase and tyrosine ammonia-lyase activity in various plant species was determined.

INTRODUCTION

IN DESIGNING herbicides, at least three criteria must be considered. The design must result in an agent that is (a) effective against the target plant or plant group, (b) selective or less effective against important crop species, and (c) relatively non-toxic to insects, birds, and mammals. It seemed probable that the development of a specific and effective inhibitor of phenylalanine ammonia-lyase (PAL, E.C. 4.3.1.5), which catalyzes one step in the biosynthesis of lignin, may produce a herbicide having these characteristics.[1,2] Lignin is an important component of higher plant vascular tissue, which imparts structural rigidity to the plant [3,4] and inhibition of its biosynthesis should seriously impair plant growth. Selectivity between species might be expected since it has been reported that grasses might use an alternate reaction, catalyzed by tyrosine ammonia-lyase (TAL), in the formation of lignin.[5–7] A specific PAL inhibitor might thus not adversely affect lignin synthesis and plant growth in those plants which utilize TAL. PAL has not been detected to date either in bacterial or animal tissues, so a specific PAL inhibitor might be relatively non-toxic to these organisms.[7]

This paper deals with methods for activity determination, taxonomic distribution, and kinetics of PAL and TAL. A subsequent paper will report on the effect of some herbicides and plant growth regulators on these enzymes.[8]

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RESULTS

Prior to studying the distribution and kinetics of PAL and TAL, the validity of the assay system was established. The initial reaction rate was found to be constant over a wide range of substrate concentrations. The pH optimum for the reaction was 8·8. Addition of polyvinylpyrollidone to the reaction mixture was without effect on rate, which indicates that inhibition of the enzyme by phenolics present in the enzyme preparation was insignificant.

TABLE 1. RELATIVE RATIOS OF PAL/TAL IN SEVERAL PLANT SPECIES

Plant species	Cinnamic acid (μmol)	*p*-Coumaric acid (μmol)	Units/ml* prep. PAL	Units/ml* prep. TAL	Protein (mg/ml)	Sp. act. PAL	Sp. act. TAL	Ratio PAL/TAL
Soybean (*Glycine max* L.)	7·7	2·7	38·5	13·5	2·9	13·3	4·7	2·8
Barnyard grass (*Echinochloa crusgalli* Beauv.)	7·3	2·4	36·5	12·0	1·7	21·5	7·1	3·0
Cotton (*Gossypium hirsutum* L.)	7·7	1·8	38·5	9·0	3·0	12·9	3·0	4·3
Purple nutsedge (*Cyperus rotundus* L.)	13·1	2·8	65·5	14·0	7·8	8·4	1·8	4·7
Johnsongrass (*Sorgham halepeuse* Pers.)	66·2	12·9	66·2	12·9	2·2	30·5	5·9	5·2
Corn (*Zea mays* L.)	23·1	3·9	115·2	19·6	3·0	56·6	9·6	5·9
Cocklebur (*Xanthium pensylvanicum* Wallr.)	8·4	1·4	42·0	7·0	2·8	15·0	2·5	6·0
Wheat (*Triticum vulgare* Vill.)	12·3	1·8	61·5	9·0	2·6	23·4	3·4	6·9
Yellow nutsedge (*Cyperus esculentus* L.)	19·6	2·7	49·0	6·7	5·3	9·2	1·3	7·1
Crabgrass, hairy (*Digitaria sanguinalis* Scop.)	33·5	4·2	33·5	4·2	2·3	14·5	1·8	8·0
Yellow foxtail (*Setaria glauca* Beauv.)	15·6	1·5	78·0	7·5	1·6	48·1	4·6	10·5
Green foxtail (*Setaria viridis* Beauv.)	18·4	1·7	91·8	8·5	2·6	34·9	3·2	10·9
Pigweed (*Amaranthus retroflexus* L.)	11·9	0·8	59·3	4·1	2·9	20·4	1·4	14·6
Bracken fern (*Pteridium* spp.)	75·2	1·3	187·5	3·3	7·4	25·5	0·5	55·0
Morning glory (*Ipomoea purpurea* Roth)	91·6	—	91·6	—	7·0	13·1	—	—

* One enzyme unit catalyzed the formation of 1 μmol product in 2 hr at 37°.

The relative ratios of PAL and TAL in a number of plant species are shown in Table 1. Young plants, 3–5 weeks of age, were used in these experiments. In every case the PAL specific activity of PAL was greater than that of TAL. Two economically important crop species, cotton and soybeans, were among those plants having relatively low PAL/TAL ratios. Pigweed, yellow foxtail and green foxtail were among the plants having higher PAL/TAL ratios. Bracken and other ferns examined had extremely high PAL/TAL ratios and morning glory contained no detectable TAL. All of these results were obtained using saturating concentrations of phenylalanine.

Hill plots[9,10] of PAL from corn, yellow foxtail, and wheat reveal that the Michaelis constant (K_m) of the enzymes from these sources ranges from $1{\cdot}1$–$1{\cdot}6 \times 10^{-4}$ M. The slope

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[10] HILL, A. J. (1913) *Biochem. J.* **7**, 471.

of the curve (n) is approximately 1 in each case, indicating that the enzymes display normal Michaelis–Menton kinetics. The K_m values for PAL reported in the literature range from $3{\cdot}8 \times 10^{-5}$ M[15] to $1{\cdot}7 \times 10^{-3}$ M.[1] A Hill plot of TAL isolated from corn gave a K_m for tyrosine of $2{\cdot}7 \times 10^{-5}$ M, about 1/4 of the corresponding value for PAL ($1{\cdot}1 \times 10^{-4}$). The slope of the line, n, is 1·25.

The elution pattern of green foxtail PAL, TAL, and protein from a DEAE-cellulose column is shown in Fig. 1. The major PAL activity peak coincides with the TAL peak activity and both peaks are associated with the major protein peak. There are two minor PAL activity peaks in addition to the major one. Purification did not affect the kinetic behavior of the enzyme.

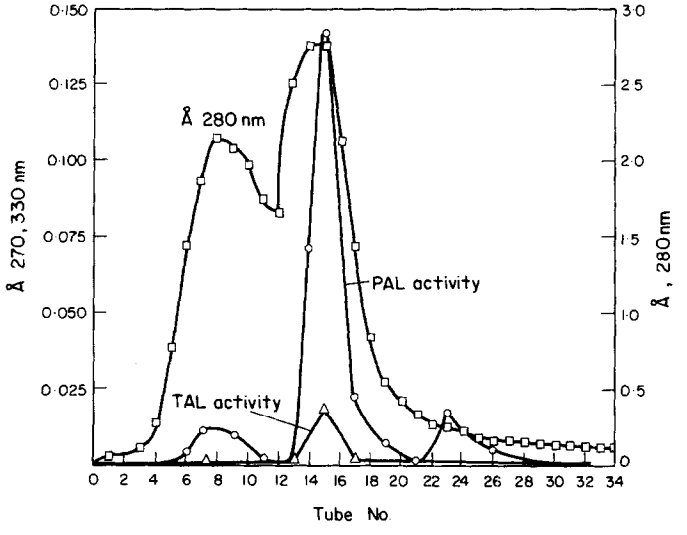

FIG. 1. ELUTION PATTERN OF PAL, TAL, AND PROTEIN FROM GREEN FOXTAIL USING A DEAE-CELLULOSE COLUMN.

35·0 ml of enzyme prepared according to the method of Koukol and Conn[1] were applied to a 2·2 × 16 cm DEAE-cellulose column that had been equilibrated with 0·02 M potassium phosphate buffer (pH 6·8). The enzyme was eluted from the column using a linear gradient of equal volumes 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. Flow rate 60 ml per hr and fractions 9 ml.

DISCUSSION

Phenylalanine ammonia lyase has been isolated from a number of sources since it was first purified by Koukol and Conn.[1] Tyrosine ammonia lyase was first isolated at approximately the same time by Neish,[5] but has not been as extensively studied.

The general PAL/TAL distribution pattern found in this study is very similar to that reported by others,[6,7] with the highest TAL activity found in grasses.[11] Soybean and cotton have relatively low PAL/TAL ratios and for this reason might be able to survive complete PAL inhibition, but species such as the foxtails and pigweed have a very high PAL/TAL ratio and would, therefore, be more susceptible to PAL inhibition. This differential selectivity is based on the premise that the deamination of phenylalanine and the deamination of tyrosine are catalyzed by different enzymes.

[11] HIGUCHI, T., HO, Y. and KAWAMURA, I. (1967) *Phytochemistry* **6**, 875.

There is conflicting evidence regarding the independent existence of PAL and TAL. Young and Neish[12] could not find a preparation active with tyrosine which was not also active with phenylalanine. They found, however, that the ratio of PAL/TAL varied from 4 to 20. Boudet and coworkers[13] found two PAL isoenzymes in *Quercus pedunculata*, while Hahlbrock and coworkers[14] were unable to find any isoenzymes of PAL in *Glycine max*. Havir *et al.*[15] reported that maize contained a single protein having common catalytic sites for tyrosine and phenylalanine, while the potato PAL appeared to be inactive against tyrosine. The results in this paper do not allow a clear differentiation between the two possibilities (Table 1, Fig. 1).

The relative distribution of PAL and TAL in crop and weed species suggests that a herbicide acting through PAL inhibition may display a desirable spectrum of selectivity. The methods developed in this study will be used to assess the effects of various herbicides and plant growth regulators on PAL and TAL activity.

EXPERIMENTAL

Materials. L-tyrosine and L-phenylalanine were obtained from Sigma Chemical Company, *p*-coumaric acid from K & K Laboratories, and *trans*-cinnamic acid from Aldrich Chemical Company. Phenol reagent (Folin-Ciocalteau) was obtained from Fisher Scientific Company. Bio-Rad Laboratories was the source for the DEAE-cellulose anion exchange resin of 0·90 meq/g capacity. Enzyme grade ammonium sulfate was purchased from Nutritional Biochemicals Company.

Enzyme preparation. Fresh plant material was homogenized at a ratio of 1 g fresh wt/4 ml 0·1 M sodium borate buffer (pH 8·8, 5 mM glutathione) in a Virtis homogenizer. The homogenate was filtered through cheesecloth and centrifuged for 30 min at 29 000 *g*. The supernatant was decanted and used as the enzyme preparation. Protein determinations were according to Lowry *et al.*[16]

Wheat PAL preparation. 15 g. of wheat were minced with scissors and homogenized in a Virtis homogenizer containing 50 ml of borate buffer. The homogenizing flask was maintained at 3–6°. After homogenization the preparation was filtered through cheesecloth and centrifuged for 30 min at 14 000 *g* in a refrigerated centrifuge. $(NH_4)_2SO_4$ was slowly added with stirring to the supernatant until 30% saturation (21·2 g/100 ml). The preparation was centrifuged and the supernatant was brought to 70% saturation by adding additional $(NH_4)_2SO_4$ (28·2 g/100 ml) and recentrifuged. The pellet was resuspended in 10 ml of borate buffer.

Corn acetone powder. Young corn plants (100 g; 3–4 weeks of age) were harvested and homogenized in cold acetone (600 vol −20°). The homogenate was filtered through No. 1 filter paper and the corn powder was dried at 25°, passed through a No. 18 mesh screen, and stored in a sealed container at −20°.

PAL Assay. The assay mixture contained 2·5 ml of 0·1 M Na borate buffer (pH 8·8, 5 mM glutathione), phenylalanine (final conc $1·5 \times 10^{-3}$ M) or tyrosine (normally saturated), and enzyme preparation in a total volume of 3·0 ml. This mixture was incubated for 2 hr at 37° and 0·5 ml conc HCl added. The mixture was extracted with 10 ml Et_2O. The Et_2O was dried and the residue dissolved in 3 ml of 0·05 N NaOH. The amount of *trans*-cinnamic acid or *p*-coumaric acid produced was then determined by measuring the absorbance at 270 or 330 nM, respectively. Tubes containing enzyme preparation without substrate were used as blanks. The identity of the cinnamic acid and *p*-coumaric acid produced was confirmed by TLC.[17]

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