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Regulation of L-Phenylalanine Ammonia-Lyase from *Rhizoctonia solani*

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Maximal levels of L-phenylalanine ammonia-lyase activity were observed when the mycelial felts of *Rhizoctonia solani* were grown for 4.5 days on Byrde synthetic medium containing 3.5% glucose and 0.3% L-phenylalanine. Differential centrifugation studies have indicated that the enzyme is localized in the soluble fraction. The time course of induction of L-phenylalanine ammonia-lyase activity by L-phenylalanine showed a lag period of 1 to 1.5 h and reached a maximum around 4 to 6 h after the addition of the inducer to the medium. L-Phenylalanine, L-tyrosine, and L-tryptophan were nearly equally efficient inducers of the enzyme. D-Phenylalanine was as efficient as the L-isomer, whereas D-tyrosine was a poor inducer. Light, gibberellic acid, indole 3-acetic acid, and kinetin had no effect on the induction of L-phenylalanine ammonia-lyase activity. Cycloheximide did not inhibit the uptake of amino acids by the mycelia but completely blocked the incorporation of radioactive amino acids into soluble proteins and the development of L-phenylalanine ammonia-lyase activity. Actinomycin D inhibited both the incorporation of ^{32}P into ribonucleic acid and the enzyme activity. Conclusive evidence for de novo synthesis of L-phenylalanine ammonia-lyase was obtained by the incorporation of radioactive amino acids into the enzyme. Electrophoretic analysis of the purified preparation showed a single protein band that coincided with radioactivity and L-phenylalanine ammonia-lyase activity. Glucose and intermediates of the tricarboxylic acid cycle, like citric acid, α-ketoglutaric acid, and succinic acid, and the metabolites of L-phenylalanine, like *o*-coumaric acid, *o*-hydroxyphenylacetic acid, and protocatechuic acid, significantly repressed L-phenylalanine ammonia-lyase activity. The observed repression was not relieved by cyclic adenosine 5'-triphosphate.

Nonoxidative deamination of L-phenylalanine to *trans*-cinnamate is catalyzed by L-phenylalanine ammonia-lyase found in most higher plants and some microorganisms. The enzyme is implicated in a number of functions including lignification, biosynthesis of flavonoids, chlorogenic acid, isochlorogenic acid, and biochemical changes in diseased conditions and injury (10). The microbial enzyme apparently has a degradative role, although in some instances it is involved in the synthesis of cinnamic acid derivatives (4, 6, 29).

Extensive studies have been carried out on the regulation of L-phenylalanine ammonia-lyase in higher plants. Light, plant growth regulators, wounding, and a number of other factors lead to profound increase in the enzyme levels (10). Although the light-mediated changes in L-phenylalanine ammonia-lyase activity have been attributed to de novo synthesis of the enzyme (14, 39), conclusive evidence is available for the presence of a pool of inactive L-phenylalanine ammonia-lyase in some higher plant systems (3, 7). There is no published report on the regulation of L-phenylalanine ammonia-lyase from microbial sources. In a previous paper (16), we have shown that the mycelial felts of *Rhizoctonia solani* grown on Byrde synthetic medium did not possess any detectable levels of L-phenylalanine ammonia-lyase and L-tyrosine ammonia-lyase activities, and the culture filtrates were free from phenolic metabolites except for traces of *p*-hydroxybenzoic acid which could be derived from chorismate by the biosynthetic pathway for aromatic compounds. Supplementation of the medium with L-phenylalanine resulted in the appearance of both L-phenylalanine ammonia-lyase and L-tyrosine ammonia-lyase activities in the mycelia, as well as the synthesis of an array of phenolic intermediates. The present communication describes some of the factors that regulate the levels of this enzyme in the plant pathogen *R. solani*.

MATERIALS AND METHODS

Chemicals. *Chlorella* ^{14}C-labeled protein hydrolysate (specific activity, 39.3 mCi/mmol) and carrier-free ^{32}P were purchased from Bhabha Atomic Research Centre, Trombay, India. D-Phenylalanine, D-tyrosine, L-phenylalanine, L-tyrosine, L-tryptophan, *p*-coumaric acid, *o*-coumaric acid, caffeic acid, dihydrocaffeic acid, and phenylpyruvic acid were from Sigma Chemical Co., St. Louis, Mo. Agar and Freund adjuvant were obtained from Difco Laboratories, Detroit, Mich. Actinomycin D was a product of Merck Sharp Dohme and Co., West Point, Pa. Cycloheximide was obtained from Upjohn & Co., Kalamazoo, Mich. All other chemicals used were of analytical reagent grade.

Organism. *R. solani* was obtained from the Department of Plant Pathology, Indian Agricultural Research Institute, New Delhi, India.

Media and culture conditions. The stock cultures were maintained on potato-dextrose-agar slants containing boiled potato extract (100 g), 200 ml; glucose, 6 g; and agar, 5 g. *R. solani* was grown on Byrde medium (8), 1 liter of which contained glucose, 50 g; KNO_3, 5 g; KH_2PO_4, 2.5 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; Na_2SO_4, 1.0 g; $FeCl_3 \cdot 6H_2O$, 20 mg; $ZnSO_4 \cdot 7H_2O$, 10 mg; $MnSO_4 \cdot H_2O$, 3 mg; $Na_2MoO_4 \cdot 2H_2O$, 1.5 mg; and $CuSO_4 \cdot 5H_2O$, 1.0 mg. The medium was adjusted to pH 5.5 with 1 N NaOH and filtered and sterilized by autoclaving at 120 C for 15 min. The flasks were inoculated with small bits of the mycelium and incubated for 6 days under static conditions at room temperature (23 ± 4 C).

Enzyme assays. L-Phenylalanine ammonia-lyase and L-tyrosine ammonia-lyase activities were determined spectrophotometrically according to the method of Kalghatgi and Subba Rao (17). One unit represents the formation of 1 μmol of cinnamate or *p*-coumarate per min under the standard assay conditions. Absorbancies were recorded on a Unicam Sp-500 spectrophotometer.

Crude extract preparation. The mycelial felts of *R. solani* were harvested by decanting the culture filtrates and then washing the mycelia several times with sterile glass-distilled water. The crude extracts were prepared by crushing the mycelia with equal amount of glass powder (70 to 80 mesh, wt/wt) along with 25 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 8.8, in a precooled mortar (0 C). The homogenate was passed through a cheesecloth and centrifuged for 10 min at 10,000 × *g*. The clear supernatant fluid was used to determine the enzyme activities.

Purification of phenylalanine ammonia-lyase. The enzyme was purified from the extracts of acetone-dried mycelium according to the previously described procedure (17); the enzyme was extracted from protamine sulfate complexes of crude extract, precipitated with ammonium sulfate, fractionated with acetone, and then chromatographed on diethylaminoethyl (DEAE)-cellulose.

Induction experiments. *R. solani* was grown on Byrde medium containing 3.5% glucose. The mycelium was washed several times with sterile-distilled water and cut into small bits (0.5 cm by 0.5 cm). Ten such bits were transferred to test tubes each containing 3.0 mg of L-phenylalanine in 1.0 ml of 50 mM potassium phosphate, pH 7.0, and then incubated on a wrist action shaker at 25 C. The enzyme activities were tested in the crude extract preparations. The protein content of the crude extracts was around 2.0 mg per 10 bits of the mycelium. These conditions were precisely used in all the experiments unless stated otherwise.

Labeling procedures. Uptake of ^{14}C-labeled amino acids. The conditions were similar to those described for induction. Each tube contained 1.0 μCi of ^{14}C-labeled *Chlorella* protein hydrolysate. The experiment was terminated by decanting the solution and washing the mycelial bits with ice-cold 50 mM potassium phosphate buffer, pH 7.0. The mycelial bits were crushed with 1.0 ml of 5% trichloroacetic acid to precipitate the proteins. After allowing to stand for 8 to 10 h at 0 to 4 C, the homogenate was centrifuged, and the clear supernatant was extracted several times with diethyl ether to remove trichloroacetic acid. Aliquots (0.1 ml) of the aqueous layer were transferred directly to vials containing 6 ml of Yardley (38) scintillation fluid and the radioactivity as determined in a Beckman LS-100 liquid scintillation counter at an efficiency of 50%.

Incorporation of ^{14}C-labeled amino acids into soluble proteins. The residue obtained after the addition of trichloroacetic acid was washed successively with (i) ice-cold 5% trichloroacetic acid (3 × 10 ml), (ii) 5% trichloroacetic acid at 100 C for 2 min (2 ml), (iii) distilled ethanol (3 × 10 ml), (iv) chloroform-diethyl ether-ethanol (1:2:1) (vol/vol) (3 × 10 ml), and (v) diethyl ether (2 × 10 ml). The pellet was dried and suspended in 1.0 ml of 0.1 N NaOH and 0.1-ml aliquots of the clear supernatant were used to determine radioactivity in 6 ml of Yardley scintillation fluid.

Incorporation of ^{32}P into total ribonucleic acid. The conditions were similar to those described for the uptake of ^{14}C-labeled amino acids except that 50 mM Tris-hydrochloride buffer, pH 7.0, was used instead of potassium phosphate buffer. The residue obtained after the addition of trichloroacetic acid was washed with ethanol and chloroform-ethanol-diethyl ether mixture and finally was dried with diethyl ether. The pellet was suspended in 2.0 ml of 0.3 N KOH and the ribonucleic acid (RNA) was hydrolyzed for 16 h at 37 C. Equivalent amounts of perchloric acid (10%, vol/vol) were added to neutralize the alkali and left at 0 to 4 C for 3 h. The samples were centrifuged and 0.1-ml aliquots of the supernatant were used to measure radioactivity in 6 ml of Yardley liquid scintillation fluid. The efficiency of radioactivity counting was around 48%.

Preparation of radioactive L-phenylalanine ammonia-lyase. *R. solani* was grown for 4 days on Byrde medium containing 3.5% glucose. The mycelium (2.0 g) was cut into small bits and distributed into conical flasks (100 ml) containing 20 ml of 50 mM potassium phosphate buffer, pH 7.0, and L-phenylalanine (3.0 mg/ml). The flasks were incubated on a wrist action shaker. At the end of 1 h, 7.5 μCi of ^{14}C-labeled *Chlorella* protein hydrolysate was added and the incubation was continued for 7 h. The

mycelial bits were filtered through a Buchner funnel and then washed with distilled water. The mycelial bits were mixed with fresh mycelium (2.0 g) grown on L-phenylalanine, blended with chilled acetone (−20 C) in a Waring blender, and dried after repeated washing. The acetone-dried powders were used for the purification of L-phenylalanine ammonia-lyase according to the procedure described earlier (17). The fractions at each stage of purification were analyzed for protein, L-phenylalanine ammonia-lyase activity, and radioactivity.

Polyacrylamide gel electrophoresis. The purified enzyme preparation (35 μg) was subjected to polyacrylamide disc gel electrophoresis (7.5%) at pH 8.3 (0.6 by 6 cm) according to the method of Davis (13).

Protein determination. Protein was determined by the method of Lowry et al. (21) with bovine serum albumin as standard. Appropriate corrections for the interference of Tris in the estimation of protein were made by using proper controls.

RESULTS

Optimal conditions of growth. Age. *R. solani* was grown in 250-ml Erlenmeyer flasks containing Byrde medium (30 ml) supplemented with 0.1% L-phenylalanine and harvested during different stages of growth. Fresh weight of the mycelium increased until day 6 of growth and then remained constant. L-Phenylalanine ammonia-lyase and L-tyrosine ammonia-lyase activities increased up to day 4 after inoculation and then declined. Specific activities of these enzymes showed similar patterns with the optima around day 3 (Fig. 1).

Concentration of glucose. The organism was grown for 4.5 days on L-phenylalanine (0.1%)-supplemented Byrde medium containing different amounts of glucose. The growth increased up to 3.5% glucose and further increase in the concentration of the sugar was found to be inhibitory. Higher specific activities of L-phenylalanine ammonia-lyase and L-tyrosine ammonia-lyase observed at lower concentrations of glucose decreased up to 2.5% of glucose and then increased to give a peak at 3.5% glucose. The enzyme activities were inhibited at higher concentration of the sugar (Fig. 2). Optimal yields of the enzyme were obtained with 3.5% glucose in the medium.

Concentration of L-phenylalanine. *R. solani* was grown for 4.5 days on Byrde medium containing 3.5% glucose and different amounts of L-phenylalanine. Slight decrease in the fresh weight was observed beyond 0.3% L-phenylalanine. L-Phenylalanine ammonia-lyase and L-tyrosine ammonia-lyase activities increased with the increase in the concentration of L-phenylalanine (Fig. 3). The optimal concentration of L-phenylalanine chosen was 0.3%.

Temperature. The organism was grown for 4.5 days on Byrde medium containing 3.5% glucose and 0.3% L-phenylalanine. The flasks were incubated at different temperatures. The fresh weight of the mycelium as well as L-phenylalanine ammonia-lyase and L-tyrosine

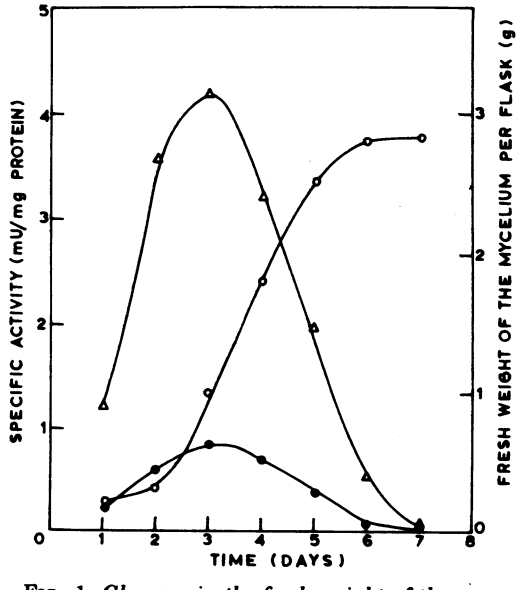

FIG. 1. *Changes in the fresh weight of the mycelium (○), L-phenylalanine ammonia-lyase (△), and L-tyrosine ammonia-lyase (●) activities in the crude extracts during growth of R. solani.*

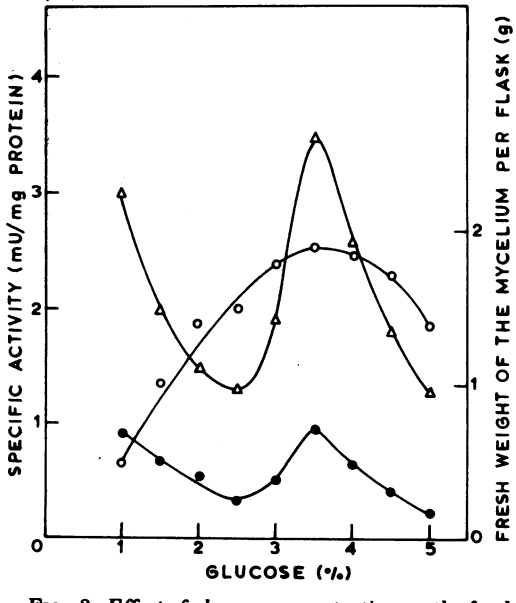

FIG. 2. *Effect of glucose concentration on the fresh weight of the mycelium (○), L-phenylalanine ammonia-lyase (△), and L-tyrosine ammonia-lyase (●) activities in the crude extracts during growth of R. solani.*

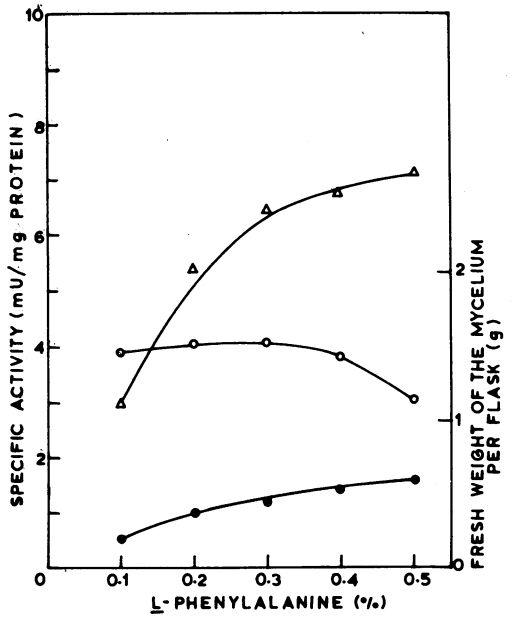

FIG. 3. *Effect of* L-*phenylalanine concentration on the fresh weight of the mycelium* (○), L-*phenylalanine ammonia-lyase* (△), *and* L-*tyrosine ammonia-lyase* (●) *activities in the crude extracts during growth of R. solani.*

ammonia-lyase activities were maximal when the organism was grown at 25 C. *R. solani* grown at room temperature (23 ± 4 C) showed more or less the same amounts of the enzyme activity (Fig. 4).

Light. The growth conditions were the same as described for optimal temperature. One set of flasks was kept under fluorescent light and the second was covered with aluminum foil and left in a dark chamber. The organism in the third set was grown under normal light and dark conditions. L-Phenylalanine ammonia-lyase, L-tyrosine ammonia-lyase, and other growth parameters were nearly the same for *R. solani* grown under fluorescent light, complete darkness, and neutral photoperiod conditions.

Intracellular localization of L-phenylalanine ammonia-lyase. The homogenates of mycelial felts of *R. solani* grown under optimal conditions were made in buffered sucrose medium (12) (sucrose, 0.4 M; tricine, 0.165 M, adjusted to pH 7.5; KCl, 10 mM; $MgCl_2$, 10 mM; ethylenediaminetetraacetic acid [EDTA], 1 mM; dithiothreitol, 10 mM; and bovine serum albumin, 0.2%) and subjected to differential centrifugation. The pellet obtained at various stages was suspended in 10 ml of the buffered sucrose medium and centrifuged. The washed pellet was homogenized in a Potter-Elvehjem homogenizer with 10 ml of the buffered sucrose medium, and the enzyme activity was determined in the homogenate. The results given in Table 1 indicate that L-phenylalanine ammonia-lyase is a soluble enzyme and is not associated with any organelle of the cell. Succinate dehydrogenase which was taken as a marker enzyme was found to be mainly localized in the 8,700 × *g* sediment where more than 90% of the activity in the crude homogenate could be recovered.

Time course of induction of L-phenylalanine ammonia-lyase and L-tyrosine ammonia-lyase activities. The time course of induction of L-phenylalanine ammonia-lyase and L-tyrosine ammonia-lyase activities is given in Fig. 5. Both these activities reached maximum around 6 h of incubation with L-phenylalanine. These enzyme activities, however, were absent when L-phenylalanine was omitted from the medium. The enzyme activities were one-tenth of the original values if the induction experiments were carried out under static conditions.

Effect of various factors on the induction of L-phenylalanine ammonia-lyase activity. (i) Amino acids. Among the various amino acids and the standard analogues tested (Table 2), only the aromatic amino acids, namely, phenylalanine, tyrosine, and tryptophan, were capable

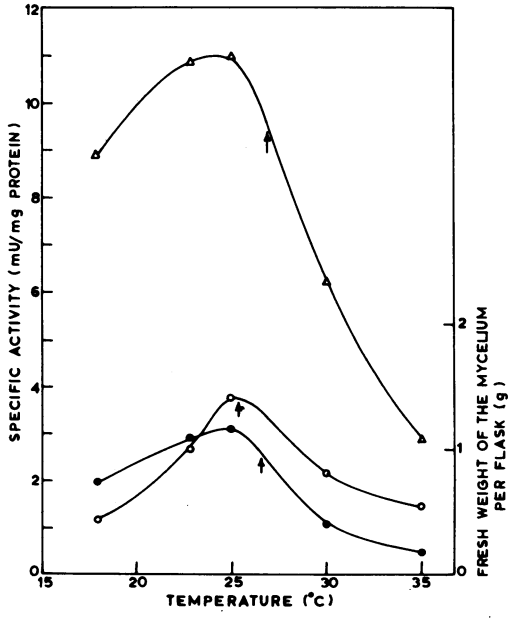

FIG. 4. *Effect of temperature on the fresh weight of the mycelium* (○), L-*phenylalanine ammonia-lyase* (△), *and* L-*tyrosine ammonia-lyase* (●) *activities in the crude extracts during growth of R. solani. The arrow marks indicate the values obtained with the organism grown at room temperature.*

TABLE 1. *Intracellular localization of L-phenylalanine ammonia-lyase in R. solani*[a]

Sample	Vol (ml)	Total protein (mg)	Total L-phenyl-alanine ammonia-lyase activity (mU)
Fresh mycelium (20 g):			
Homogenate	72	568	2,600
1. Centrifugation (750 × *g* for 10 min):			
Residue	10	21	8
Supernatant	70	518	2,333
2. Centrifugation (8,700 × *g* for 10 min):			
Residue	10	13	4
Supernatant	65	460	2,200
3. Centrifugation (37,000 × *g* for 10 min):			
Residue	10	4	3
Supernatant	61	423	2,135
4. Centrifugation (100,000 × *g* for 60 min):			
Residue	10	11	4
Supernatant	55	380	1,978

[a] The crude homogenates were prepared by grinding the mycelial felts of the organism grown on L-phenylalanine-supplemented medium with glass powder and buffered medium and passing through a cheesecloth. The residue obtained after centrifugation was washed with 10 ml of the buffered medium, recentrifuged, and homogenized with 10 ml of the above medium. Details are given in the text.

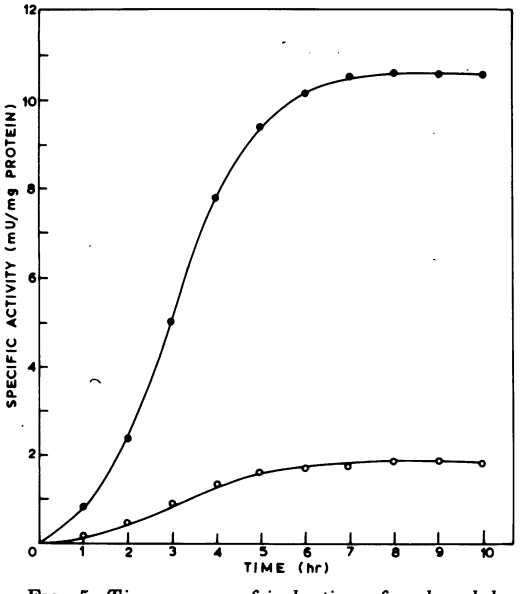

FIG. 5. *Time course of induction of L-phenylalanine ammonia-lyase (●) and L-tyrosine ammonia-lyase (○) activities in R. solani. Concentration of the inducer used was 3.0 mg of L-phenylalanine per ml.*

of inducing L-phenylalanine ammonia-lyase activity. Both D- and L-isomers of L-phenylalanine were equally effective in inducing L-phenylalanine ammonia-lyase activity. D-Tyrosine was a poor inducer, whereas a number of other structural analogues like L-histidine, L-alanine, L-serine, DL-β-phenylserine, and indole failed to induce L-phenylalanine ammonia-lyase activity.

(ii) Plant growth regulators. Growth promoters like indole-3-acetic acid (1 μg/ml and 10 μg/ml), gibberellic acid (1 μg/ml and 10 μg/ml), and kinetin (0.5 μg/ml and 2 μg/ml) were unable to induce the enzyme activity.

(iii) Metabolites of L-phenylalanine by *R. solani*. A number of aromatic compounds, mostly the degradative metabolites of L-phenylalanine by *R. solani* (16), were tested for their effect on the development of L-phenylalanine ammonia-lyase activity. Cinnamic acid, *o*-coumaric acid, *p*-coumaric acid, caffeic acid, dihydrocaffeic acid, benzoic acid, *p*-hydroxybenzoic acid, phenylacetic acid, and *m*-hydroxyphenylacetic acid were unable to induce L-phenylalanine ammonia-lyase activity.

Evidence for de novo synthesis of L-phenylalanine ammonia-lyase. (i) Effect of cycloheximide. The induction of the enzyme activity was completely abolished when the mycelial bits of *R. solani* were incubated in the presence of L-phenylalanine (3.0 mg/ml) and cycloheximide (3 μg/ml). Addition of the inhibitor at various stages of induction prevented further development of L-phenylalanine ammonia-lyase activity (Fig. 6).

The uptake of ^{14}C-labeled amino acids by the mycelial bits and incorporation of the radioactive label into soluble protein were studied in the presence or absence of cycloheximide. The results shown in Fig. 7 suggest that the uptake of the amino acids was not affected by cycloheximide, whereas the incorporation of the amino acids into soluble proteins was totally inhibited by the inhibitor.

(ii) Effect of actinomycin D. Experiments with actinomycin D are often unsatisfactory with bacterial, fungal, and higher plant sys-

tems due to permeability factors. It is reported that a pretreatment with EDTA (10 mM) at 40 C would give better results with some systems (19, 20, 36, 37). The effect of actinomycin D on the development of L-phenylalanine ammonia-lyase activity in *R. solani* was studied either by incubating the mycelium directly with actinomycin D or after a pretreatment with EDTA. The results are summarized in Table 3. Significant inhibition of L-phenylalanine ammonia-lyase activity could be observed when the mycelia were treated with actinomycin D. The inhibition was, however, more pronounced in EDTA-treated mycelia. A progressive decrease in the enzyme activity was observed with an increase in the concentration of actinomycin D. Nearly 70% inhibition of the enzyme activity could be observed at 100 μg of the inhibitor per ml.

TABLE 2. *Effect of structural analogues of L-phenylalanine on the induction of L-phenylalanine ammonia-lyase activity in R. solani*[a]

Compound	Phenylalanine ammonia-lyase activity (%)
L-Phenylalanine	100
D-Phenylalanine	95
L-Tyrosine	100
D-Tyrosine	10
L-Tryptophan	80

[a] The mycelial bits were incubated with various compounds (5 mM) for 6 h. Details are given in Materials and Methods.

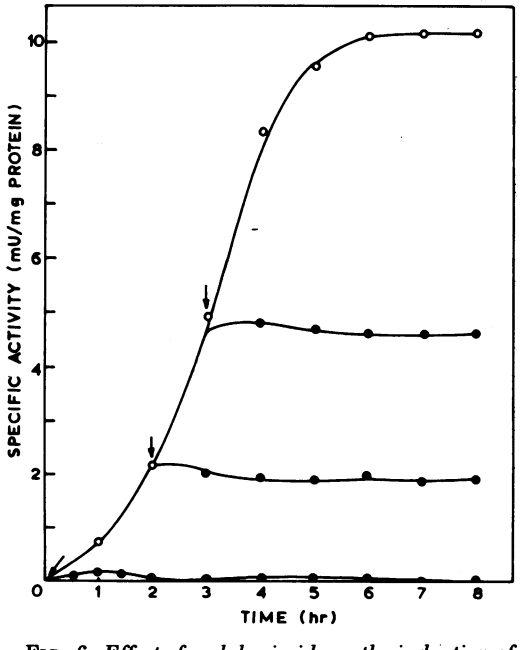

FIG. 6. *Effect of cycloheximide on the induction of L-phenylalanine ammonia-lyase in R. solani. The arrow marks indicate the time of addition of cycloheximide. Symbols: (○) L-phenylalanine (3.0 mg/ml); (●) L-phenylalanine (3.0 mg/ml) + cycloheximide (3.0 μg/ml).*

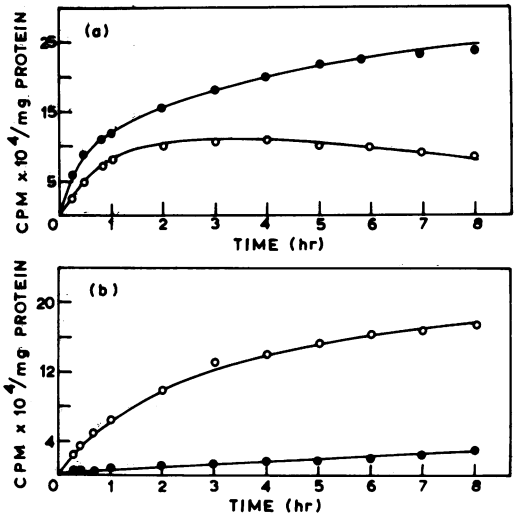

FIG. 7. *Uptake of ^{14}C-labeled amino acids (a) and incorporation of the radioactive label into the soluble proteins (b) of R. solani. Control samples (○) contained L-phenylalanine and test samples (●), in addition, contained cycloheximide (3.0 μg/ml).*

TABLE 3. *Effect of actinomycin D on the induction of L-phenylalanine ammonia-lyase in R. solani*[a]

Conditions	L-Phenylalanine ammonia-lyase activity (%)
Set A: No pretreatment with EDTA but the mycelial bits were replaced directly with 50 mM potassium phosphate buffer, pH 7.0, containing L-phenylalanine (3.0 mg/ml) and:	
No actinomycin D	100
16 μg of actinomycin D	84
40 μg of actinomycin D	55
Set B: Pretreated with EDTA (1 mM) at 40 C for 3 min, washed and replaced with 50 mM potassium phosphate buffer, pH 7.0, containing L-phenylalanine (3.0 mg/ml) and:	
No actinomycin D	100
16 μg of actinomycin D	70
40 μg of actinomycin D	40

[a] The mycelial bits were incubated for 6 h with actinomycin D under various conditions indicated below. Details are given in text.

The effect of actinomycin D on the incorporation of ^{32}P into RNA was studied by incubating the mycelial bits with ^{32}P and L-phenylalanine. The results shown in Fig. 8 indicate that there was considerable inhibition in the incorporation of ^{32}P into total RNA.

(iii) Incorporation of ^{14}C-labeled amino acids into L-phenylalanine ammonia-lyase. Phenylalanine ammonia-lyase was purified from the acetone-dried powders of the mycelial bits incubated with L-phenylalanine, the inducer, as well as ^{14}C-labeled amino acids. Details of the purification are given in Table 4.

The elution profile of L-phenylalanine ammonia lyase from the DEAE-cellulose column is given in Fig. 9. From this it is apparent that both L-phenylalanine ammonia-lyase activity and radioactivity were eluted together. The active fractions (no. 19 to 23) were pooled, desalted by passing through a column (40 cm by 2 cm) of Sephadex G-50 equilibrated with glass-distilled water, and freeze dried. The final preparation was subjected to polyacrylamide gel electrophoresis at pH 8.3. One set of the gels was stained for protein and other was cut into 2-mm thick slices and analyzed for L-phenylalanine ammonia-lyase activity. Both L-phenylalanine ammonia-lyase activity and radioactivity coincided with the protein band on the gel (Fig. 10).

Repression of L-phenylalanine ammonia-lyase. Glucose and intermediates of tricarboxylic acid cycle were ineffective as inducers of L-

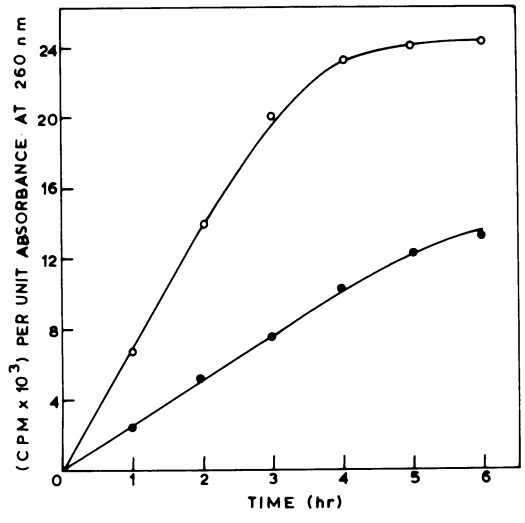

FIG. 8. *Effect of actinomycin D on the incorporation of ^{32}P into total RNA of R. solani. Control samples (○) contained L-phenylalanine (3.0 mg/ml) and test samples (●), in addition, contained actinomycin D (100 μg/ml). The incubations were carried out in 50 mM Tris-hydrochloride buffer, pH 7.0.*

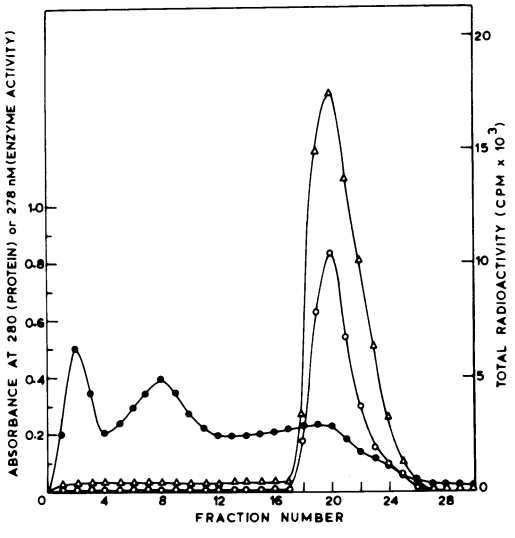

FIG. 9. *DEAE-cellulose chromatography of radioactive L-phenylalanine ammonia-lyase from R. solani. Experimental details are same as those reported earlier (17). Each fraction was analyzed for protein (●), L-phenylalanine ammonia-lyase activity (○), and radioactivity (△). L-Phenylalanine ammonia-lyase activity was expressed as E_{278} by incubating 0.1 ml of each fraction for 15 min under standard assay conditions.*

TABLE 4. *Summary of purification of radioactive L-phenylalanine ammonia-lyase from R. solani*[a]

Step	Vol (ml)	Total protein (mg)	Total activity (mU)	Sp act (mU/mg)	Total radioactivity (counts/min)	Specific radioactivity (counts/min per mg)
1. Crude extract	90	1,188	4,320	3.64	4,680,000	3,937
2. Protamine sulfate reextraction	100	120	4,000	33.34	500,000	4,167
3. Ammonium sulfate fractionation (0–55%)	31	81	3,888	48.00	330,000	4,074
4. Acetone fractionation (40–60%)	10	40	3,332	83.30	175,000	4,375
5. DEAE-cellulose chromatography	25	4.1	2,466	601.50	64,000	15,610

[a] The details of purification of the enzyme are essentially the same as those described earlier (17). Details are given in Materials and Methods.

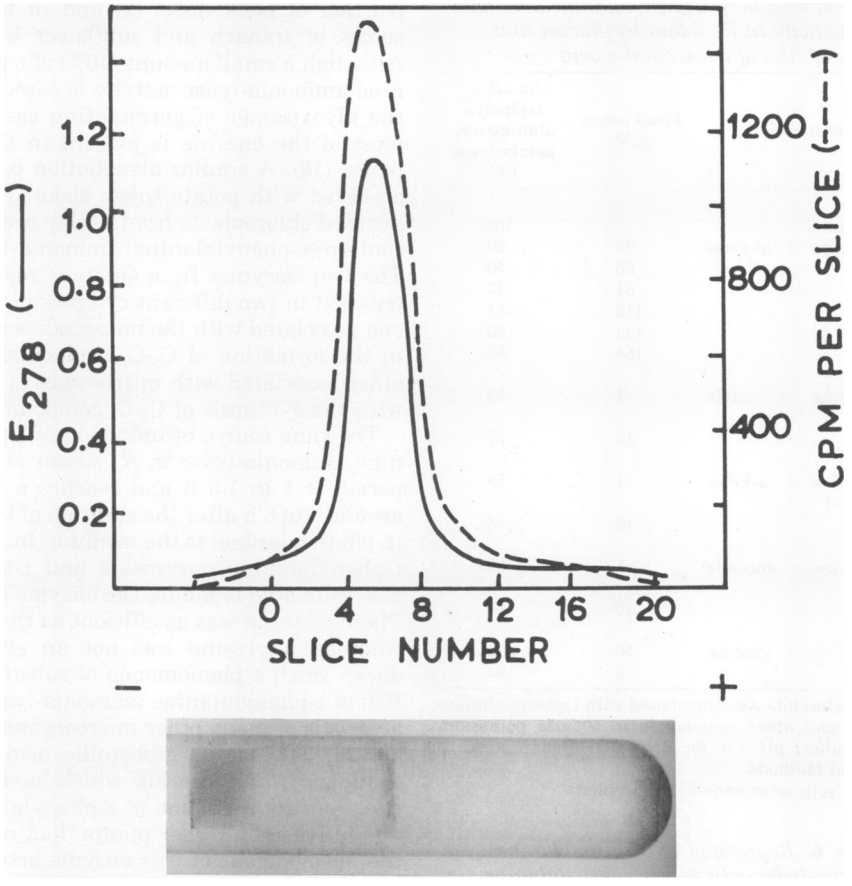

FIG. 10. *Polyacrylamide gel electrophoresis of radioactive* L-*phenylalanine ammonia-lyase from R. solani. Aliquots (0.1 ml) of buffer extracts of the gel slices were used to determine the radioactivity (- - -) and* L-*phenylalanine ammonia-lyase activity (——) (E_{278}). The period of incubation was 60 min.*

phenylalanine ammonia-lyase. The presence of these compounds in the incubation medium, however, repressed the phenylalanine-dependent synthesis of L-phenylalanine ammonia-lyase in *R. solani*. The results are summarized in Table 5. Repression by glucose increased up to 84 mM, whereas higher concentrations were less effective. Citric acid, α-ketoglutaric acid, and succinic acid caused significant inhibition in the development of L-phenylalanine ammonia-lyase activity. Addition of cyclic adenosine 5'-triphosphate (cAMP) (5 mM) to a repressed system had no effect on L-phenylalanine ammonia-lyase levels in *R. solani*.

Repression of L-phenylalanine ammonia-lyase by the metabolites of L-phenylalanine in *R. solani* was tested and the results are given in Table 6. The enzyme levels decreased considerably by the presence of *o*-coumaric acid, *o*-hydroxyphenylacetic acid, or protocatechuic acid in the medium.

DISCUSSION

Maximal levels of L-phenylalanine ammonia-lyase and L-tyrosine ammonia-lyase and L-tyrosine ammonia-lyase activities were obtained when *R. solani* was grown on Byrde synthetic medium containing 3.5% glucose and 0.3% L-phenylalanine. Highest levels of these enzyme activities were observed with the organism grown at 25 C and nearly equal yield was obtained at room temperature (23 ± 4 C). Although the ratio of L-phenylalanine ammonia-lyase and L-tyrosine ammonia-lyase activities varied between 3.0 and 6.0 during the various conditions of growth of *R. solani*, the two activities went hand in hand. These results, in addition to those reported earlier (16, 17), prove that L-phenylalanine ammonia-lyase for *R. solani* has a common catalytic site for both L-phenylalanine and L-tyrosine. In this respect, it resembles the enzyme isolated from *Sporobolomyces*

TABLE 5. *Repression of L-phenylalanine ammonia-lyase activity in R. solani by glucose and intermediates of tricarboxylic acid cycle*[a]

Addition	Final concn (mM)	Sp act of L-phenylalanine ammonia-lyase (%)
L-Phenylalanine		100
L-Phenylalanine + glucose	28	80
	56	50
	84	43
	112	51
	140	60
	168	55
L-Phenylalanine + citric acid	1	83
	10	57
L-Phenylalanine + α-ketoglutaric acid	1	89
	10	58
L-Phenylalanine + succinic acid	1	92
	10	61
L-Phenylalanine + glucose + cAMP[b]	56 5	53

[a] The mycelial bits were incubated with L-phenylalanine (3.0 mg/ml) and other compounds in 50 mM potassium phosphate buffer, pH 7.0, for 6 h. Details are given in Materials and Methods.

[b] cAMP, Cyclic adenosine 5'-triphosphate.

TABLE 6. *Repression of L-phenylalanine ammonia-lyase activity by L-phenylalanine metabolites of R. solani*[a]

Addition	Sp act of L-phenylalanine ammonia-lyase (%)
L-Phenylalanine	100
L-Phenylalanine + cinnamic acid	74
L-Phenylalanine + *p*-coumaric acid	83
L-Phenylalanine + *o*-coumaric acid	62
L-Phenylalaine + *p*-hydroxybenzoic acid	95
L-Phenylalanine + protocatechuic acid	51
L-Phenylalanine + phenylacetic acid	98
L-Phenylalanine + *m*-hydroxyphenylacetic acid	95
L-Phenylalanine + *o*-hydroxyphenylacetic acid	50

[a] The mycelial bits were incubated with L-phenylalanine (3.0 mg/ml) and various metabolites (1 mM) of L-phenylalanine for 6 h. Details are given in Materials and Methods.

pararoseus (28), maize shoots (15), and wheat seedlings (25).

Differential centrifugation studies with the crude extracts of *R. solani* have indicated that L-phenylalanine ammonia-lyase is localized in the soluble fraction. The enzyme has been shown to be associated with the microsomal fraction of buckwheat (2) and in the peroxisomes of spinach and sunflower leaves (31). Although a small amount (10%) of L-phenylalanine ammonia-lyase activity is associated with the glyoxisomes of germinating castor beans, most of the enzyme is present in the soluble phase (18). A similar distribution pattern was observed with potato tuber disks (11). Highly purified chloroplasts from barley are known to contain L-phenylalanine ammonia-lyase (33). The two isozymes from *Quercus* roots are distributed in two different cytoplasmic fractions; one associated with the microbodies is involved in the formation of C_6-C_1 compounds and the other associated with microsomes is concerned with the synthesis of C_6-C_1 compound (1).

The time course of induction of L-phenylalanine ammonia-lyase in *R. solani* shows a lag period of 1 to 1.5 h and reaches a maximum around 4 to 6 h after the addition of the inducer (L-phenylalanine) to the medium. In addition to L-phenylalanine, L-tyrosine and L-tryptophan also were able to induce the enzyme activity. D-Phenylalanine was as efficient as the L-isomer, whereas D-tyrosine was not an effective inducer. Such a phenomenon of substrate induction of L-phenylalanine ammonia-lyase is characteristic of many other microorganisms (9, 23, 26, 28, 34). Light, gibberellic acid, indole-3-acetic acid, and kinetin, which have profound effect on the induction of L-phenylalanine ammonia-lyase in higher plants, had no effect on the development of this enzyme activity in *R. solani*. *Polyporous hispidus* happens to be the only microorganism in which L-phenylalanine ammonia-lyase activity is increased on exposure to light (35). Similarly, the degradative metabolites of L-phenylalanine by *R. solani* are not effective as inducers of L-phenylalanine ammonia-lyase.

The development of L-phenylalanine ammonia-lyase activity is completely blocked by cycloheximide, an inhibitor of protein synthesis. Cycloheximide, in addition to the inhibition of protein synthesis, is known to have a number of side effects (5, 22, 24, 30, 32). For example, it inhibits the uptake of small-molecular-weight compounds like glucose, nucleoside bases, amino acids, and ions (22, 27, 30, 32). It is quite possible that the inability to observe L-phenylalanine ammonia-lyase activity in the mycelia of *R. solani* could be as well due to lack of entry of the inducer (L-phenylalanine) into the cell. This, however, was ruled out by the uptake studies which suggested that the intracellular levels of amino acids increased considerably more in cycloheximide-treated samples than in the controls. Further, cycloheximide inhibited the incorporation of amino acids into the solu-

ble proteins by more than 90%. Induction of L-phenylalanine ammonia-lyase in *R. solani* is also inhibited by actinomycin D. Generally, many fungal and higher plant systems are not permeable to actinomycin D and treatment with EDTA facilitates the entry of the drug into the cell (19, 20, 36, 37).

Significant inhibition of L-phenylalanine ammonia-lyase activity as well as incorporation of ^{32}P into total RNA was observed with the mycelia of *R. solani* treated directly with actinomycin D, and this inhibition increased when the mycelia were pretreated with EDTA. These results indicate that the induction of L-phenylalanine ammonia-lyase activity in *R. solani* requires de novo RNA and protein synthesis. Conclusive evidence for de novo synthesis of L-phenylalanine ammonia-lyase in *R. solani* was obtained by the incorporation of radioactive amino acids into the enzyme. However, there are a few points in the radioactive incorporation studies that require some explanation. From the data presented in Table 4 it is apparent that the proteins in the crude extracts were extensively labeled and much of the label was lost during the purification of L-phenylalanine ammonia-lyase. The total radioactivity at step 4 was 175,000 counts/min, which on further purification by DEAE-cellulose chromatography yielded 75,000 counts/min in the enzyme fraction. DEAE-cellulose chromatography, as published earlier (17), is indeed an effective purification step. In the radioactive experiment, although much of the contaminating radioactivity came out unadsorbed after application to the DEAE-cellulose column before gradient elution, it is likely that some of the radioactive impurities remained on the column and were not eluted at the salt concentration used. Although many proteins, apart from L-phenylalanine ammonia-lyase, were labeled in the present studies, it is not necessary that all proteins in the mycelium, already grown on glucose before incorporation with ^{14}C-labeled amino acids, should contain radioactivity. Also the 280-nm absorbing material shown in the elution profile of L-phenylalanine ammonia-lyase (Fig. 9) is indeed due to protein, and it is likely that these could be proteins which have been synthesized before ^{14}C labeling.

It is normally expected that L-phenylalanine ammonia-lyase, which is induced during the incubation with L-phenylalanine and ^{14}C-labeled amino acids, will be more radioactive than all other proteins. However, as shown in Table 4, the specific radioactivity of the proteins present was more or less the same during purification from step 1 through step 4. In the final step, however, there was about fourfold increase in the specific radioactivity. The failure to observe considerable increase in specific radioactivity during enrichment of L-phenylalanine ammonia-lyase in the early stages of purification may be partly explained if it is realized that L-phenylalanine in *R. solani* is metabolized by two divergent pathways (16) that require the de novo synthesis of several enzymes. Furthermore the mycelium subjected to radioactive incorporation was mixed with nonradioactive mycelium grown in the presence of L-phenylalanine and then processed for the isolation of radioactive L-phenylalanine ammonia-lyase. Electrophoretic analysis of the purified preparation showed a single protein that coincided with L-phenylalanine ammonia-lyase and radioactivity. Recently, Hahlbrock and Scbroder (14) have provided similar evidence to establish the de novo synthesis of L-phenylalanine ammonia-lyase in parsley cell cultures grown under light.

Glucose and intermediates of tricarboxylic acid cycle like citric acid, succinic acid, and α-ketoglutaric acid significantly repressed L-phenylalanine ammonia-lyase activity in *R. solani*, as also did the end products of phenylalanine degradation like *o*-coumaric acid, *o*-hydroxyphenylactic acid, and protocatechuic acid. Repression by these compounds could not be relieved by cAMP. This could be either due to lack of sensitivity of the organism to cAMP or an experimental artifact where cyclic adenosine 5'-triphosphate is unable to enter the cell.

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