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Microbial L-Phenylalanine Ammonia-Lyase

PURIFICATION, SUBUNIT STRUCTURE AND KINETIC PROPERTIES OF THE ENZYME FROM *RHIZOCTONIA SOLANI*

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1. Phenylalanine ammonia-lyase (EC 4.3.1.5) was purified to homogeneity from the acetone-dried powders of the mycelial felts of the plant pathogenic fungus *Rhizoctonia solani*. 2. A useful modification in protamine sulphate treatment to get substantial purification of the enzyme in a single step is described. 3. The purified enzyme shows bisubstrate activity towards L-phenylalanine and L-tyrosine. 4. It is sensitive to carbonyl reagents and the inhibition is not reversed by gel filtration. 5. The molecular weight of the enzyme as determined by Sephadex G-200 chromatography and sucrose-density-gradient centrifugation is around 330000. 6. The enzyme is made up of two pairs of unidentical subunits, with a molecular weight of 70000 (α) and 90000 (β) respectively. 7. Studies on initial velocity versus substrate concentration have shown significant deviations from Michaelis–Menten kinetics. 8. The double-reciprocal plots are biphasic (concave downwards) and Hofstee plots show a curvilinear pattern. 9. The apparent K_m value increases from 0.18mM to as high as 5.0mM with the increase in the concentration of the substrate and during this process the $V_{max.}$ increases by 2–2.5-fold. 10. The value of Hill coefficient is 0.5. 11. Steady-state rates of phenylalanine ammonia-lyase reaction in the presence of inhibitors like D-phenylalanine, cinnamic, *p*-coumaric, caffeic, dihydrocaffeic and phenylpyruvic acid have shown that only one molecule of each type of inhibitor binds to a molecule of the enzyme. These observations suggest the involvement of negative homotropic interactions in phenylalanine ammonia-lyase. 12. The enzyme could not be desensitized by treatment with $HgCl_2$, *p*-chloromercuribenzoic acid or by repeated freezing and thawing.

L-Phenylalanine ammonia-lyase (EC 4.3.1.5) which catalyses the formation of *trans*-cinnamate from L-phenylalanine by non-oxidative deamination is present in most higher plants and in some fungi. It has been extensively purified from potato tubers (Havir & Hanson, 1968*a*), maize shoots (Marsh *et al.*, 1968), wheat seedlings (Nari *et al.*, 1972) and certain strains of yeast (Hodgins, 1971; Parkhurst & Hodgins, 1971). The enzyme preparations from different sources exhibit considerable variations in kinetic behaviour. The purified preparations from potato tubers (Havir & Hanson, 1968*b*), maize shoots (Marsh *et al.*, 1968) and wheat seedlings (Ricard *et al.*, 1972; Nari *et al.*, 1974) show significant deviations from Michaelis–Menten kinetics, whereas those obtained from sweet potato roots (Minamikawa & Uritani, 1965), *Ustilago hordei* (Subba Rao *et al.*, 1967), *Rhodotorula glutinis* (Hodgins, 1971), *Sporobolomyces pararoseus* (Parkhurst & Hodgins, 1972) and *Streptomyces verticillatus* (Emes & Vining, 1970) obey the classical Michaelis–Menten kinetics. These observations led Hanson & Havir (1972) to suggest that the non-linear kinetics are either peculiar to phenylalanine ammonia-lyase from higher plants only or the observations made with the enzyme preparations from microbial sources were confined to limited ranges of initial-velocity studies. Further, phenylalanine ammonia-lyase from potato, maize (Havir & Hanson, 1973) and *Rhodotorula glutinis* (K. R. Hanson, personal communication) is made up of four identical subunits whereas the wheat enzyme (Nari *et al.*, 1972) with a molecular weight of 330000, is composed of two pairs of unidentical subunits each having a molecular weight of 75000 and 85000 respectively. In view of these discrepancies, we have undertaken experiments to examine, in a homogeneous preparation, the kinetic behaviour and subunit structure of L-phenylalanine ammonia-lyase from the plant pathogen *Rhizoctonia solani*. Kalghatgi *et al.* (1974) reported that *Rhizoctonia solani* degrades phenylalanine by the phenylpyruvate and cinnamate pathways and that phenylalanine ammonia-lyase is concerned with the metabolism of phenylalanine. The present paper is an extension of this work which is ultimately aimed at understanding the regulation of L-phenylalanine ammonia-lyase in this plant pathogen.

Experimental

Chemicals

L-Phenylalanine was a product of Calbiochem Inc., Los Angeles, Calif., U.S.A. DL-[1-^{14}C]Phenylalanine

(sp. radioactivity 34.7mCi/mmol) was obtained from Bhabha Atomic Research Centre, Trombay, India. D-Phenylalanine, D-tyrosine, urease (type VI), alcohol dehydrogenase (yeast), catalase (C-100), peroxidase (type VI), γ-globulin, ovalbumin, bovine serum albumin and lysozyme were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. L-Tyrosine was supplied by Reanal Biochemicals, Medimpex, Budapest, Hungary. DEAE-cellulose was from H. Reeve Angel and Co., London E.C.4, U.K. All other chemicals were of analytical-reagent grade.

Methods

Organism and culture conditions. Rhizoctonia solani was grown on Byrde's medium (Byrde *et al.*, 1956) supplemented with (0.3%, w/v) L-phenylalanine. Other growth conditions were the same as those described by Kalghatgi *et al.* (1974).

Enzyme assay. Phenylalanine ammonia-lyase activity was measured in two ways. (i) In a reaction mixture (2.0ml) containing 2.5mM-L-phenylalanine or -L-tyrosine, 25mM-Tris–HCl buffer, pH8.8 and enzyme at 30°C. The reaction was terminated after 30min by the addition of 1.0ml of 0.5M-HCl. The product of the reaction, cinnamate or *p*-coumarate respectively, was measured at 278 or 308nm. The molar extinction coefficients for cinnamic acid and *p*-coumaric acid were 20100 and 15000 litre·mol^{-1}·cm^{-1} respectively. (ii) Alternatively the enzyme activity was assayed in a reaction mixture (1.0ml) containing 0.05μCi of DL-phenylalanine, 2.5mM-L-phenylalanine, 25mM-Tris–HCl buffer, pH8.8 and enzyme. The reaction was terminated after 30min of incubation at 30°C by the addition of 1.0ml of 0.5M-HCl and the reaction mixture was extracted with 5ml of toluene. Portions (3ml) were transferred to vials containing 3ml of diphenyloxazole solution (1%, w/v) in toluene and the radioactivity was measured in Beckman LS-100 liquid-scintillation counter.

Purification of enzyme. The washed mycelial felts of the organism were blended with chilled acetone and dried after repeated washing. The powder (10g) was crushed in a precooled mortar along with glass powder and 25mM-Tris–HCl buffer, pH8.8 (150ml), passed through a cheese cloth and centrifuged at 10000*g* for 10min. The enzyme from the supernatant was precipitated by aq. protamine sulphate solution (13ml of 2%, w/v) and re-extracted by suspending the residue for 1h in 200mM-potassium phosphate buffer, pH8.0 (200ml). Solid $(NH_4)_2SO_4$ (65.2g) was added to bring the supernatant to 55% saturation and centrifuged after 30min. The precipitate was dissolved in 25mM-Tris–HCl buffer, pH8.8 (30ml), and the enzyme from the clear supernatant was subjected to acetone precipitation at −15°C. The precipitate obtained with 40–60% (v/v) acetone was dissolved in 25mM-Tris–HCl buffer, pH8.8 (10ml), and centrifuged. The clear supernatant (10ml) was loaded on a column (15cm×1cm) of DEAE-cellulose pre-equilibrated with the same buffer. The enzyme was eluted with a linear gradient of 75ml of 25mM-Tris–HCl buffer, pH8.8, and 75ml of the same buffer containing 300mM-NaCl. The elution profile of DEAE-cellulose column chromatography is given in Fig. 1. The active fractions (21–26) from the DEAE-cellulose column were pooled, desalted against glass-distilled water on a column (40cm×2cm) of Sephadex G-50 and freeze-dried.

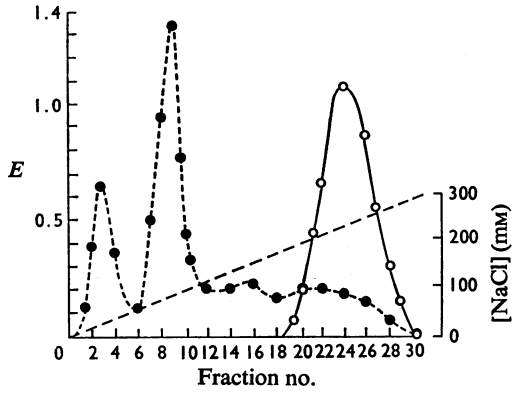

Fig. 1. *DEAE-cellulose chromatography of* L-*phenylalanine ammonia-lyase from Rhizoctonia solani*

Enzyme (10ml; 5.921 units; 62.4mg of protein) after the acetone precipitation step was applied to a column (15cm×1cm) of DEAE-cellulose equilibrated with 25mM-Tris–HCl buffer, pH8.8. The enzyme was eluted with a linear gradient of 0–300mM-NaCl in the above buffer (75ml of each). Fractions (5ml) were collected and protein (E_{280}; ●) and L-phenylalanine ammonia-lyase activity (○) were measured for each fraction. L-Phenylalanine ammonia-lyase activity was expressed as ΔE_{278} by incubating 0.1ml of each fraction for 30min under standard assay conditions. ——, NaCl concn.

Electrophoresis. Polyacrylamide-disc-gel electrophoresis was carried out by the method of Davis (1964) in 7% (w/v) gels. Coomassie Brilliant Blue R was used for staining.

Determination of molecular weight. The molecular weight of the enzyme was determined by the procedure of Andrews (1965) using a column (135cm×1.4cm) of Sephadex G-200 standardized with the following protein markers. Urease, mol.wt. 483000 (Sehgal *et al.*, 1965); catalase, mol.wt. 244000 (Samejima & Shibata, 1961); yeast alcohol dehydrogenase, mol.wt. 151000 (Kagi & Valee, 1960) and peroxidase, mol.wt. 40200 (Maehly, 1955). The column was equilibrated with 25mM-Tris–HCl buffer, pH8.8. Fractions (1ml) were collected and each marker was followed by E_{620} measurement and

urease was assayed by nesslerization. Standard procedures were used to determine the activities of alcohol dehydrogenase (Valee & Hoch, 1955), catalase (Beers & Sizer, 1952) and peroxidase (Maehly & Chance, 1954).

Sucrose-density-gradient centrifugation. A linear gradient of 30ml (5–20%, w/v, sucrose) was prepared in 20mM-potassium phosphate buffer, pH7.0. Samples were applied on the top of the gradient and centrifuged in Beckman L-2 model preparative ultracentrifuge for 15h at 60000*g* and 4°C. The tube was punctured at the bottom of the gradient and 1ml fractions were collected. Catalase was the standard marker used in this experiment. The mathematical assumptions of Martin & Ames (1961) were used to calculate the molecular weight.

Molecular weight of the subunits. The molecular weight of subunits were determined by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis by the method of Weber *et al.* (1972) with 7.5% (w/v) gels. The standard marker polypeptides for this purpose were γ-globulin (unreduced), mol.wt. 150000 (Weber *et al.*, 1972); bovine serum albumin, monomer mol.wt. 68000 (Tanford *et al.*, 1967); catalase, monomer mol.wt. 58000 (Sund *et al.*, 1967); ovalbumin, mol.wt. 43000 (Castellino & Barker, 1968) and lysozyme, mol.wt. 14000 (Weber & Osborn, 1969). Each protein was dissociated into subunits by treatment with sodium dodecyl sulphate (1%, w/v) containing 2-mercaptoethanol (1%, v/v) at 100°C for 2min. Bromophenol Blue was the marker dye and the gels were stained with Coomassie Brilliant Blue R.

Determination of protein. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results

Purity of the enzyme

The final preparation moved as a single band on polyacrylamide-disc-gel electrophoresis at pH8.3.

General properties

The summary of purification of phenylalanine ammonia-lyase is given in Table 1. Phenylalanine ammonia-lyase and tyrosine ammonia-lyase activities could not be separated during the purification procedure. The final preparation had the same optimum pH (8.7–8.9) and temperature (44–46°C) for both phenylalanine ammonia-lyase and tyrosine ammonia-lyase activities. The activation energy as determined by Arrhenius plots (Sizer, 1943) for L-phenylalanine and L-tyrosine were 25.5J (6.1Kcal) and 39.3J (9.4Kcal) per mol respectively. Phenylalanine ammonia-lyase retained full activity on heating at 60°C for 1h. It was sensitive to metals like Zn^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Co^{2+} and was not affected by treatment with metal-chelating agents (8-hydroxyquinoline, ethylenediaminotetra-acetic acid, *o*-phenanthroline, αα-dipyridyl, diethyldithiocarbamate, azide and salicylaldoxime). Thiol reagents like *p*-chloromercuribenzoic acid and thiol compounds, like L-cysteine, GSH, 2-mercaptoethanol and dithiothreitol, caused only slight inhibition.

Substrate specificity

In addition to L-phenylalanine, the enzyme deaminated L-tyrosine and a few other substrate analogues (Table 2). D-Phenylalanine, *p*-chlorophenylalanine, D-tyrosine, *o*-tyrosine, L-histidine, L-tryptophan, β-phenylalanine and β-phenylserine did not serve as substrates as tested by the u.v. spectra of the products in ethanol solutions.

Effect of carbonyl reagents

As in the case of phenylalanine ammonia-lyase from other sources (Hanson & Havir, 1972), the *Rhizoctonia* enzyme was also sensitive to carbonyl reagents. The effective concentration required to give almost 100% inactivation of the enzyme differed

Table 1. *Summary of purification of phenylalanine ammonia lyase from Rhizoctonia solani*

Details are given in the Experimental section. One unit is the amount of enzyme that will catalyse the formation of 1 μmol of cinnamate or *p*-coumarate/min.

Step	Vol. (ml)	Total protein (mg)	Total activity (munits)	Specific activity (munits/mg)	Recovery (%)	Purification (fold)	Phenylalanine ammonia-lyase/ tyrosine ammonia-lyase ratio
Crude extract	146	3022	9096	3.0	100	1.0	6.3
Protamine sulphate precipitation and re-extraction	200	180	7134	39.6	78.4	13.2	5.9
0–55% $(NH_4)_2SO_4$ fractionation	30	120	6170	51.4	68.3	17.1	6.4
40–60% Acetone precipitation	10	62.4	5921	94.9	65.1	31.6	6.0
DEAE-cellulose active fractions	30	2.4	1632	680.0	17.9	227.0	6.5

from one reagent to another (Table 3). The reversibility of inhibition by carbonyl reagents was tested by passing the enzyme pretreated with such reagents to cause 50% inactivation or less, through a column (20cm×1cm) of Sephadex G-25 equilibrated with 25mM-Tris–HCl buffer, pH8.8, and the eluates were assayed for enzyme activity. The inhibition caused by each of the carbonyl reagents tested was found to be irreversible.

Table 2. *Substrate specificity of L-phenylalanine ammonia-lyase from Rhizoctonia solani*

The enzyme was incubated with various substrates (2.5mM) in 25mM-Tris–HCl buffer, pH8.8, for 30min under the standard assay conditions. Cinnamic acid and *p*-coumaric acid were determined by the standard assay method described in the Experimental section. *m*-Coumaric acid and caffeic acid were determined in ethanol solutions at E_{278} (ε 19000 litre·mol^{-1}·cm^{-1}) and E_{328} (ε 13000 litre·mol^{-1}·cm^{-1}) respectively. *p*-Fluorocinnamic acid and *o*-fluorocinnamic acid were estimated by method A of Emes & Vining (1970). The ammonia-lyase activity is expressed as a percentage of the activity observed with L-phenylalanine (=100). The values are means±S.E.M. ($n = 3$).

Substrate	Activity (%)
L-Tyrosine	15±1
m-Tyrosine	10±1
3,4-Dihydroxyphenylalanine	29±2
p-Fluorophenylalanine	31±3
o-Fluorophenylalanine	14±1

Effect of aromatic compounds

A large number of aromatic compounds, mostly the catabolites of L-phenylalanine by *Rhizoctonia solani* were tested for their effect on phenylalanine ammonia-lyase activity and the results are summarized in Table 4.

Molecular weight

The molecular weight of the enzyme from gel-filtration studies was around 330000. The values obtained in three determinations with two different preparations were 315000, 332000 and 338000 and the calculated value was 328000 with a S.E.M. of ±6890. This was in good agreement with that obtained by density-gradient centrifugation (340000).

Subunit structure

Electrophoresis of the enzyme under denaturing conditions yielded two distinct bands (α and β). The values obtained with four preparations of the enzyme for the molecular weight of subunits were $\alpha = 69000$, 71300, 68100 and 69800 and $\beta = 91000$, 88000,

Table 3. *Effect of carbonyl reagents on L-phenylalanine ammonia-lyase and L-tyrosine ammonia-lyase activities from Rhizoctonia solani*

The enzyme was incubated with each of the reagents for 10min at pH8.8 and 30°C. The residual activity was determined after a 20-fold dilution with 25mM-Tris–HCl buffer, pH8.8. The enzyme activities were expressed as percentage of respective controls processed in the absence of carbonyl reagents.

Compound	Final concn. (M)	Enzyme activity (%) Phenylalanine ammonia lyase	Tyrosine ammonia lyase
$NaBH_4$	5×10^{-6}	60	64
	1×10^{-5}	40	40
	2×10^{-5}	12	15
	1×10^{-4}	0	0
NaCN	1×10^{-4}	86	85
	5×10^{-4}	30	30
	2×10^{-3}	0	0
$NaHSO_3$	1×10^{-4}	90	95
	5×10^{-4}	41	38
	2×10^{-3}	0	0
Semicarbazide–HCl	1×10^{-4}	90	95
	4×10^{-3}	86	86
	6.7×10^{-3}	80	75
Hydroxylamine–HCl	1×10^{-4}	90	94
	4×10^{-3}	80	79
	1×10^{-2}	56	57
Hydrazine–H_2SO_4	1×10^{-4}	92	96
	4×10^{-3}	85	82
	1×10^{-2}	77	77

Table 4. *Effect of aromatic compounds on phenylalanine ammonia-lyase activity from Rhizoctonia solani*

The enzyme was preincubated for 10min with each compound (0.1mM) and the reaction was started by the addition of substrate (0.4mM). Cinnamate was determined by the radioactivity method described in the Experimental section. The enzyme activity was expressed as percentage of control. The values are means±S.E.M. ($n = 3$).

Compound	Activity (%)
Cinnamic acid	76±3
p-Coumaric acid	86±1
Phenylpyruvic acid	83±3
Caffeic acid	81±4
Dihydrocaffeic acid	64±2
D-Phenylalanine	79±2
β-Phenylalanine	98±1
p-Chlorophenylalanine	99±1
o-Coumaric acid	93±2
Phenylpropionic acid	94±2
Phenylacetic acid	96±2
m-, *o*- and *p*-Hydroxyphenylacetic acid	98±2
β-Phenyl-lactic acid	98±1
Benzoic acid	99±1
p-Hydroxybenzoic acid	98±2
Salicyclic acid	99±1
Protocatechuic acid	96±3
Coumarin	98±2
4-Hydroxycoumarin	99±1

86700 and 90300. The mean values and S.E.M. for the subunits were $\alpha = 69550 \pm 560$ and $\beta = 89000 \pm 990$ respectively. Since the molecular weight of the native phenylalanine ammonia-lyase is about 330000, the observed values equate with a subunit pattern of $\alpha_2\beta_2$ for the enzyme.

Initial-velocity studies

Studies on initial velocity versus substrate concentration showed significant deviations from Michaelis–Menten kinetics. Substrate-saturation curves indicated that the saturation velocity typical of a Michaelis–Menten enzyme was not achieved and the double-reciprocal plots (Lineweaver & Burk, 1934) were biphasic (concave downwards). The same data when plotted as v versus $v/[S]$ (Hofstee, 1959) showed a curvilinear pattern (Fig. 2). The apparent K_m increased from 0.18mM to as high as 5mM depending on the substrate concentration and during this process the $V_{max.}$ increased 2–2.5-fold. The value of the Hill coefficient was 0.5, as evident from the Hill-plot (Loftfield & Eigner, 1969; Atkinson, 1966) constructed from the above kinetic data (Fig. 3). The R_s value was approximately 1000. A similar kinetic pattern was observed with L-tyrosine.

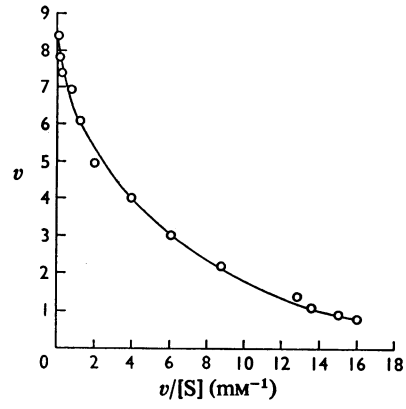

Fig. 2. *Effect of the concentration of* L-*phenylalanine on the initial velocity of* L-*phenylalanine ammonia-lyase from Rhizoctonia solani*

L-Phenylalanine ammonia-lyase activity was measured by incubating the purified enzyme with different concentrations of L-phenylalanine for 30min under standard assay conditions. Initial velocities were expressed as nmol of cinnamate formed/min and the results are presented in a Hofstee plot (Hofstee, 1959).

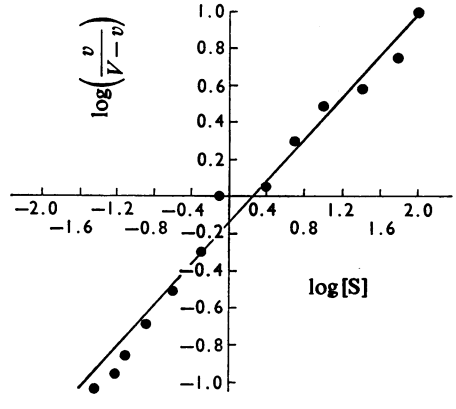

Fig. 3. *Hill plot for phenylalanine ammonia-lyase from Rhizoctonia solani*

The conditions were the same as in Fig. 2. The value of the Hill coefficient was 0.5.

Inhibition studies

The kinetic behaviour of phenylalanine ammonia-lyase was studied in the presence of D-phenylalanine, cinnamic acid, *p*-coumaric acid, caffeic acid, dihydrocaffeic acid and phenylpyruvic acid. The K_i values for these inhibitors are given in Table 5. Substrate-saturation velocity studies were carried out in the

Table 5. *K_i values for inhibitors of phenylalanine ammonia-lyase from Rhizoctonia solani*

The K_i values were determined by a Dixon plot (Dixon, 1953) at three different concentrations of the substrate. The inhibitors were added 10min before starting the reaction. Phenylalanine ammonia-lyase activity was determined by the radioactivity method given in the Experimental section.

Inhibitor	K_i value (mM)
D-Phenylalanine	0.75
Cinnamic acid	0.21
p-Coumaric acid	0.36
Caffeic acid	1.78
Dihydrocaffeic acid	0.29
Phenylpyruvic acid	3.00

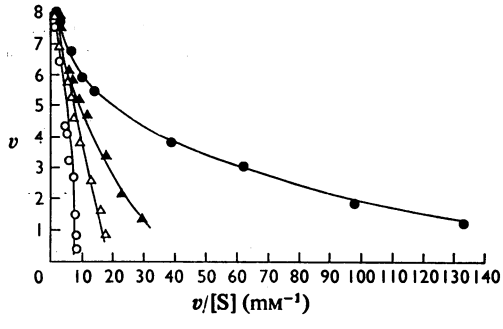

Fig. 4. *Inhibitory effect of* D-*phenylalanine on* L-*phenylalanine ammonia-lyase from Rhizoctonia solani*

The results were expressed in Hofstee (1959) plots. The purified enzyme was preincubated with D-phenylalanine for 10min. Phenylalanine ammonia-lyase activity was measured by incubating the enzyme with a fixed concentration of the inhibitor and at different concentrations of L-phenylalanine for 45min. Initial rates of the reaction were expressed as nmol of cinnamate formed/min. D-Phenylalanine concentrations used were: ●, none; ▲, 2mM; △, 4mM; ○, 10mM.

presence of these inhibitors. The double-reciprocal plots which were concave downwards at lower concentrations of the inhibitors progressively changed to straight lines and concave upwards as the concentration of the inhibitor was increased. This effect was more apparent in Hofstee (1959) plots. The effect of D-phenylalanine is shown in Fig. 4. If v_s and v_i represent the steady-state rates in the absence and presence of the inhibitor, it is clear from Fig. 5 that the plots of $[(v_s/v_i)-1]$ versus [I] were linear over a wide range of D-phenylalanine concentrations. Similar effects were observed with other inhibitors.

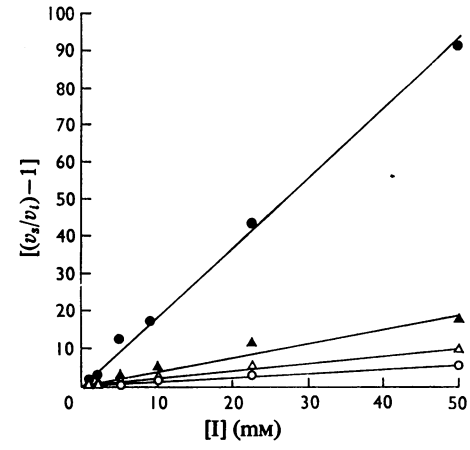

Fig. 5. *Effect of* D-*phenylalanine on the steady-state rate of* L-*phenylalanine ammonia-lyase from Rhizoctonia solani*

The purified enzyme was preincubated with D-phenylalanine for 10min. The reactions were carried out by incubating the enzyme at a fixed concentration of L-phenylalanine and different concentrations of D-phenylalanine for 45min. The steady-state rates were expressed as nmol of cinnamate formed/min. Substrate concentrations used were: ●, 0.25mM; ▲, 1.25mM; △, 2.5mM; ○, 5mM. Details are given in text. v_s and v_i, rates in the absence and presence of the inhibitor respectively.

Desensitization studies

Attempts were made to desensitize phenylalanine ammonia-lyase by heating at 60°C for 1h, treating with *p*-chloromercuribenzoic acid, $HgCl_2$ or by repeated freezing and thawing. In each case the kinetic plots were similar to those of the untreated enzyme.

Discussion

There are two important spectrophotometric methods for the assay of phenylalanine ammonia-lyase activity. In Zucker's (1965) method the increase in E_{290} due to the formation of cinnamate is followed at pH8.8. The molar extinction coefficient reported under these conditions is about 10000 litre·mol^{-1}·cm^{-1}. The other method used by Koukol & Conn (1961) involves the extraction of cinnamic acid from the reaction mixture by diethyl ether under acidic conditions. The residue obtained after evaporation of the ether extract is dissolved in 0.05M-NaOH and spectrophotometric determinations are made at 268nm using a molar extinction coefficient of 20300 litre·mol^{-1}·cm^{-1}. It is obvious that Zucker's (1965) method is half as sensitive as that of Koukol & Conn (1961) but the wavelength, 290nm, chosen by Zucker (1965) does not represent $\lambda_{max.}$ for

cinnamate. It may also be noted that the method of Koukol & Conn (1961) becomes laborious and is subject to errors in taking reproducible samples of the ether extract. These difficulties are overcome by using the direct assay method of recording the absorbance of cinnamic acid or *p*-coumaric acid under acidic conditions. A similar method was used by O'Neal & Keller (1970) who estimated cinnamic acid after stopping the reaction with trichloroacetic acid.

A useful modification in protamine sulphate treatment has been tried successfully for the purification of phenylalanine ammonia-lyase from *Rhizoctonia solani*. To our knowledge, it is the first time that an enzyme has been precipitated quantitatively by protamine sulphate and re-extracted with good recovery. This leads to a 13-fold purification of the enzyme in a single step.

The purified enzyme catalyses the deamination of both L-phenylalanine and L-tyrosine and the two substrates seem to have a common catalytic site. In this respect the *Rhizoctonia* enzyme resembles phenylalanine ammonia-lyase isolated from *Sporobolomyces pararoseus* (Parkhurst & Hodgins, 1971). A similar pH profile with an optimum around 8.8 is found for the deamination of both L-phenylalanine and L-tyrosine with the purified enzyme. This observation, however, is in contrast with the finding of Havir *et al.* (1971) that the maize enzyme, although having a common catalytic site for L-phenylalanine and L-tyrosine exhibits different pH optima (8.7 and 7.7 respectively).

In spite of several reports on the carbonyl reagents as inhibitors of phenylalanine ammonia-lyase, the mechanism by which they bring about the inhibition is largely obscure. $NaBH_4$ causes irreversible inactivation of the potato enzyme whereas the inhibition caused by NaCN, semicarbazide and phenylhydrazine could be reversed by removing these reagents (Havir & Hanson, 1968*b*). Both CN^- and HSO_3^- bring about irreversible inactivation of phenylalanine ammonia-lyase from *Rhodotorula glutinis* (Hodgins, 1971).

The *Rhizoctonia* enzyme is made up of two pairs of unidentical subunits ($\alpha = 70000$ and $\beta = 90000$) and resembles the wheat enzyme (Nari *et al.*, 1972) in its subunit structure. Initial-velocity studies with the *Rhizoctonia* enzyme showed significant deviations from Michaelis–Menten kinetics. Biphasic double-reciprocal plots (concave downwards), a Hill coefficient of less than 1 and a R_s value of greater than 81 are strong indications of negative co-operativity (Convay & Koshland, 1968; Levitzki & Koshland, 1969). The substrate-saturation studies revealed the presence of negative homotropic interactions and the phenomenon of 'activation at high substrate concentrations' in the *Rhizoctonia* enzyme. Although similar observations have been made with phenylalanine ammonia-lyase preparations from potato (Havir & Hanson, 1968*b*) and maize (Marsh *et al.*, 1968), systematic analysis of the subunit interactions in wheat enzyme was done only recently (Nari *et al.*, 1974). The present studies showed that similar subunit interactions are prevalant in the *Rhizoctonia* enzyme, the first of its kind to be reported from a microbial source. The *Rhizoctonia* enzyme is inhibited by a number of aromatic compounds and the studies with these inhibitors showed that only one molecule of the inhibitor is able to bind to a molecule of the enzyme without forming any hybrid complexes with substrate and inhibitor molecules. We were, however, unable to find an inhibitor like benzoic acid (Nari *et al.*, 1974), which can bind two molecules per molecule of the enzyme. The results obtained with the *Rhizoctonia* enzyme could be explained neither by the model of Monod *et al.* (1965) nor that of Koshland *et al.* (1966), but by a partially concerted mechanism of subunit interactions described for the wheat phenylalanine ammonia-lyase (Nari *et al.*, 1974).

Desensitization, another important property of the polymeric enzyme is yet to be studied in the case of phenylalanine ammonia-lyase. Partial desensitization of phenylalanine ammonia-lyase activity against inhibition by flavonoids could be observed in crude extracts of peas treated with *p*-chloromercuribenzoate, $HgCl_2$ or by repeated freezing and thawing (Attridge *et al.*, 1971). Our attempts to desensitize the enzyme against subunit interactions were, however, unsuccessful.

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