Aminotransferases of *Agrobacterium tumefaciens*

TRANSAMINATION BETWEEN TRYPTOPHAN AND PHENYLPYRUVATE

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Tryptophan is recognized to be the precursor of the plant hormone indolylacetic acid. The role of tryptophan in the biosynthesis of the auxin and also the probable intermediates involved have been discussed by Fawcett (1961). Indolylacetic acid has also been implicated in tumour formation in plants and this aspect has been reviewed by Braun (1962). The hyperauxinic condition of tomato crown-gall tissues has been demonstrated by Link & Eggers (1941) and Dye, Clarke & Wain (1962). It was therefore decided to study the metabolism of tryptophan by a virulent strain of the crown-gall organism, *Agrobacterium tumefaciens*.

The conversion of tryptophan into the plant hormone may involve either the initial transamination or deamination of the amino acid followed by decarboxylation (Thimann, 1935; Wildman, Ferri & Bonner, 1947), or initial decarboxylation followed by deamination (Clarke & Mann, 1957; Crady & Wolf, 1959; Dannenburg & Liverman, 1957). The available evidence (Stowe, 1955, 1959; Dannenburg & Liverman, 1957; Liverman & Dannenburg, 1957) seems to support the view that the first step involves the formation of the oxo acid which in turn forms indolylacetic acid via indolyl-acetaldehyde.

Kaper & Veldstra (1958) have reported the accumulation of indolylpyruvic acid in culture filtrates of *A. tumefaciens* grown in media containing tryptophan, which further suggests that the conversion of tryptophan into indolylacetic acid involves the formation of indolylpyruvic acid as an intermediate, probably by transamination.

Preliminary studies in our Laboratory showed that crude dialysed extracts of *A. tumefaciens* contained transaminase activity with various amino acids as amino donors and a number of oxo acids as acceptors. Of the oxo acids tested as acceptors with tryptophan as amino donor, phenylpyruvate was the most effective, the next in order being dimethylpyruvate, *p*-hydroxyphenylpyruvate and α -oxoglutarate. Pyruvate, glyoxylate and oxaloacetate were completely inactive as amino acceptors with tryptophan. The reverse reaction with indolylpyruvic acid as amino acceptor and various amino acids as donors was also highest when phenylalanine was used as the amino donor. However, the

tryptophan- α -oxoglutarate-transaminase activity could be separated from the other activities by the method of purification reported below.

MATERIALS AND METHODS

Organism. *Agrobacterium tumefaciens* strain B₆ was used throughout this work. The virulence of the organism was shown by the development of galls after the inoculation of a suspension of the bacterium on tomato (*Lycopersicon esculentum*) and sunflower (*Helianthus annuus*) plants.

Bacteria were grown for 48 hr. on potato-sucrose-agar slants [200 g. of potatoes was autoclaved with 1 l. of water, and sucrose (1%) and agar (British Drug Houses Ltd.) (1.5%) were added to the extract and autoclaved for 30 min. at 121°], and the 48 hr. cultures were transferred to 500 ml. Erlenmeyer flasks each containing 100 ml. of media with nitrate as the sole nitrogen source (Salle, 1954) and incubated under static conditions for 48 hr. at 30°. Bacteria were collected by centrifugation at 5000g for 15 min. and washed with 0.1 M-phosphate buffer, pH 7.0. Extracts were prepared by suspending the cells in the same buffer, at a cell density of 100 mg. wet wt. of cells/ml., and exposing them for 20 min. to sonic vibration in a Raytheon 10 kcyc./sec. oscillator. After the removal of cell debris by centrifugation in the cold (0–4°) at 13 000g for 15 min. the supernatant was used for further purification.

Substrates and cofactors. L-Tryptophan, α -oxoglutarate and pyridoxal phosphate were obtained from Hofmann-La Roche Co. Ltd. Phenylpyruvate was prepared by the method of Herbst & Shemin (1943); the purity of the phenylpyruvate was checked spectrophotometrically according to the method of Lin, Pitt, Civen & Knox (1958).

Assay of tryptophan-phenylpyruvate-transaminase activity. The enzyme assay was performed in a reaction mixture (2.0 ml.) containing 1 μ mole of L-tryptophan, 2 μ moles of phenylpyruvate, 0.05 μ mole of pyridoxal phosphate, 25 μ moles of veronal-HCl buffer, pH 9.6, 0.5 ml. of enzyme preparation and water. Incubation was carried out at 45° for 60 min. The reaction was stopped by the addition of 0.5 ml. of 15% (w/v) trichloroacetic acid. Then 4 ml. of peroxide-free ether was added to each tube. The tubes were shaken and the layers separated by centrifugation. Samples from the aqueous layer were taken for the colorimetric estimation of tryptophan according to the method of Horn & Jones (1945). In the controls the enzyme was added after the addition of trichloroacetic acid.

Estimation of phenylpyruvate. Phenylpyruvate was estimated spectrophotometrically by the method of Lin *et al.* (1958). The deproteinized reaction mixture was suitably diluted and treated with hog-kidney tautomerase

Table 1. *Progress of purification of the aminotransferase*

Experimental details are given in the text.

Step	Protein ($\mu\text{g./ml.}$)	Specific activity ($\mu\text{g. of}$ tryptophan disappeared/hr./ mg. of protein)	Purification	Total activity	Recovery (%)
Crude extract	13.5	20	(1.0)	2700	(100)
First negative adsorption on calcium phosphate gel	2.26	133	6.6	3000	111
pH 5.0 supernatant	2.0	150	7.5	3000	111
Calcium phosphate-gel eluate	1.0	300	15.0	3000	111
Second negative adsorption on calcium phosphate gel	0.801	370	18.5	3000	111
Alumina C_γ -gel eluate	0.171	1350	67.5	2300	85

prepared by the method of Knox & Pitt (1957). Borate was added to give a final concentration of 0.57 M, the pH adjusted to 6.3 and the extinction measured at 310 m μ .

Estimation of phenylalanine. The amino acid was identified and estimated by a circular-paper-chromatographic technique (Giri & Rao, 1952) with Whatman no. 1 paper and *n*-butanol-acetic acid-water (4:1:5, by vol.) as the solvent system.

Estimation of protein. The protein content of enzyme preparations was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin as standard.

RESULTS

Purification procedure for the aminotransferase.

All the steps were carried out in the cold (0–5°).

Step 1. It was observed that treatment of the crude extract with tricalcium phosphate gel at pH values above 6.5 resulted in the retention of some of the inactive proteins by the gel but little adsorption of the aminotransferase. The total activity of the supernatant, compared with that of the crude extract, increased, presumably owing to the removal of an inhibitor.

The crude extract was treated with half its volume of tricalcium phosphate gel (14 mg./ml.) (Keilin & Hartree, 1938). After being stirred for 15 min., the suspension was centrifuged and the precipitate discarded. This negative adsorption of the extract resulted in a 6.6-fold purification.

Step 2. The pH of the supernatant from step 1 was adjusted to 5 by the dropwise addition of 6*N*-acetic acid. The precipitated inactive protein was removed by centrifugation at 13 000*g* for 15 min.

Step 3. The supernatant from step 2 was treated with twice its volume of tricalcium phosphate gel. The suspension was stirred for 20 min. and centrifuged. The supernatant was discarded. The enzyme was eluted by stirring the gel with 0.1 M-phosphate buffer, pH 8.0, for 15 min. and centrifuging.

Step 4. The eluate was subjected to a second negative adsorption by adjusting the pH of the

enzyme to 7.0 with acetic acid and treating with an equal volume of tricalcium phosphate gel for 15 min. The supernatant obtained after centrifugation of the suspension was used for further purification.

Step 5. The supernatant from step 4 was adjusted to pH 5.0 with 6*N*-acetic acid and treated with alumina C_γ gel (0.2 ml. of gel/ml. of enzyme) (Willstätter, Kraut & Erbacher, 1925). The mixture was centrifuged after 15 min. and the supernatant discarded. The enzyme was eluted from the gel by treatment with 0.1 M-phosphate buffer, pH 8.0, for 15 min., followed by centrifugation.

A summary of the steps involved, the degree of purification achieved and the percentage recovery obtained are given in Table 1.

Though the crude extract was stable for more than 3 months at –30°, the partially purified preparation lost activity either on freezing or on storing at 0–4° for more than 72 hr.

Specificity. As mentioned above, the crude extract was active with tryptophan as donor and α -oxoglutarate, phenylpyruvate, dimethylpyruvate and *p*-hydroxyphenylpyruvate as amino acceptors. The tryptophan- α -oxoglutarate aminotransferase could be completely eliminated during the second step of purification, which involved lowering of the pH of the extract to 5.0, when the tryptophan- α -oxoglutarate transaminase was precipitated.

The activities with respect to the other three oxo acids followed the same pattern during purification.

The ability of phenylpyruvate to act as an acceptor of the amino group from tryptophan, histidine, methionine, leucine, valine, isoleucine and tyrosine was studied in more detail. Table 2 shows that, although the activities with the various amino acids follow the same trend during purification and during heat denaturation, the ratios for heat-treated enzyme to heat-denatured enzyme vary from 2.7 to 8. Similarly, the ratios for purified enzyme to crude enzyme vary from 1.8 to 3.3. This suggests the involvement of more than one enzyme in the transamination reactions.

Table 2. *Phenylalanine formation from phenylpyruvate and different amino donors with crude, partially purified, heat-treated and heat-denatured enzyme preparations*

The reaction mixture (2.0 ml.) contained 2 μ moles of phenylpyruvate, 2 μ moles of the amino donor, 0.1 μ mole of pyridoxal phosphate (the cofactor was omitted in the heat-treated samples), 25 μ moles of veronal-HCl buffer, pH 9.6, and 0.5 ml. of enzyme. Incubation was for 60 min. at 45°. The heat-treated enzyme was prepared from partially purified enzyme (0.5 ml.) by heating at 60° for 5 min. in the presence of pyridoxal phosphate and then chilling. The heat-denatured enzyme was prepared from partially purified enzyme (0.5 ml.) by heating at 60° for 5 min. in the absence of substrates or cofactor.

Amino donor	Phenylalanine formed (μ mole)		B/A ratio	Phenylalanine formed (μ mole)		
	With crude enzyme (A)	With purified enzyme (B)		With heat-treated enzyme (C)	With heat-denatured enzyme (D)	C/D ratio
L-Tryptophan	0.054	0.098	1.8	0.102	0.036	2.9
DL-Leucine	0.048	0.097	2.0	0.106	0.034	3.1
DL-Valine	0.044	0.102	2.3	0.102	0.033	3.1
DL-Isoleucine	0.030	0.098	3.3	0.106	0.033	3.2
DL-Methionine	0.022	0.056	2.5	0.060	0.015	4.0
L-Tyrosine	0.018	0.054	3.0	0.046	0.017	2.7
DL-Histidine	0.018	0.036	2.0	0.032	0.004	8.0

Table 3. *Specificity of the partially purified aminotransferase*

The reaction mixtures were the same as those in Table 2 except that 0.5 ml. of alumina C_γ-gel eluate was used.

Amino donor	Phenylalanine formed (μ mole)
L-Tryptophan	0.50
DL-Histidine	0.09
DL-Methionine	0.20
DL-Leucine	0.51
DL-Valine	0.41
DL-Isoleucine	0.51
L-Tyrosine	0.45
L-Glutamic acid	0.02
DL-Aspartic acid	0.00
DL-Alanine	0.00
DL-Threonine	0.00
L-Arginine	0.00
L-Glutamine	0.00
DL-Serine	0.00

Glutamic acid, aspartic acid, alanine, threonine, arginine, glutamine and serine were practically inactive (Table 3).

Stoichiometry. A correlation was observed in the disappearance of tryptophan and phenylpyruvate (Table 4). Indolylpyruvic acid, being very unstable under the experimental conditions, continued to decompose with time and hence was not estimated.

pH-activity curve. The activity of the enzyme at various pH values was studied by using 0.1M-phosphate buffer (pH 5.8–8.0), 0.05M-tris-hydrochloric acid buffer (pH 8.0–9.1), 0.05M-veronal-hydrochloric acid buffer (pH 8.0–9.6) and 0.05M-glycine-sodium hydroxide buffer (pH 8.6–10.6). A steady increase in activity was observed up to pH 9.6 and there was a fall in activity at higher pH values. However, the activity was significant even at pH 10.6 (Fig. 1).

Effect of temperature. The activity of the enzyme increased with temperature from 25° to 50°. At higher temperatures the activity levelled off (Fig. 2).

Heat stability of the enzyme. The enzyme was found to be markedly heat-stable in the presence of substrates or pyridoxal phosphate. Heating the enzyme in the absence of substrates or pyridoxal phosphate for even 5 min. resulted in a fall in activity, and complete inactivation was observed at 70° (Table 5).

Though all the three components of the reaction mixture, namely tryptophan, phenylpyruvate and pyridoxal phosphate, afforded protection against heat inactivation, pyridoxal phosphate was the most effective, phenylpyruvate and tryptophan being less effective in that order.

Table 4. *Stoichiometry of the tryptophan-phenylpyruvate-aminotransferase reaction*

The reaction mixture (2.0 ml.) contained 1.5 μ moles of tryptophan, 2 μ moles of phenylpyruvate, 0.05 μ mole of pyridoxal phosphate, 25 μ moles of veronal-HCl buffer, pH 9.6, and 0.5 ml. of enzyme preparation. Incubation was for the time-intervals mentioned at 45°.

Time (min.)	Tryptophan disappeared (μ mole)	Phenylpyruvate disappeared (μ mole)
5	0.172	0.169
10	0.330	0.325
15	0.526	0.529
20	0.663	0.650
30	0.669	0.675

Since incubation of the enzyme with pyridoxal phosphate at 45° for 30 min. did not result in any decrease in activity, routine enzyme assays were performed at this temperature.

Effect of enzyme concentration. The activity of the enzyme was proportional to enzyme concentration when 25–100 μ g. of protein was used.

DISCUSSION

Accumulation of indolylpyruvic acid in culture filtrates of *A. tumefaciens* grown on media containing tryptophan has been demonstrated by Kaper &

Veldstra (1958). Their conclusion was based on the comparison of the chromatographic pattern of the decomposition products of indolylpyruvic acid with those of the bacterial metabolites. But the enzymic conversion of tryptophan into indolylpyruvic acid has not been demonstrated. Though indolylpyruvic acid formation could be estimated in the transaminase system involving tryptophan and α -oxo-glutarate by using a colorimetric method (N. K. Sukanya & C. S. Vaidyanathan, unpublished work), the method was not suitable in the present system owing to interference of the colour development

Fig. 1. pH-activity curve for the tryptophan-phenylpyruvate-transaminase reaction. The reaction mixture (2.0 ml.) contained 1 μ mole of L-tryptophan, 2 μ moles of phenylpyruvate, 0.05 μ mole of pyridoxal phosphate, glycine-NaOH buffer (0.05M) at the pH values indicated and 0.5 ml. of the partially purified enzyme. Incubation was at 45° for 60 min.

Fig. 2. Effect of temperature on the rate of the tryptophan-phenylpyruvate-transaminase reaction. The reaction mixture (2.0 ml.) contained 1 μ mole of L-tryptophan, 2 μ moles of phenylpyruvate, 0.05 μ mole of pyridoxal phosphate, 25 μ moles of veronal-HCl buffer, pH 9.6, and 0.5 ml. of enzyme. Incubation was for 60 min. at the indicated temperatures.

Table 5. Heat inactivation in the presence and in the absence of substrates and cofactor

The reaction mixture (2 ml.) contained 1 μ mole of L-tryptophan, 2 μ moles of phenylpyruvate, 0.05 μ mole of pyridoxal phosphate, 25 μ moles of veronal-HCl buffer, pH 9.6, and 0.5 ml. of enzyme. It was heated at the indicated temperatures with or without the cofactor or substrate for 5 min. and chilled immediately. Incubation was continued for 60 min. at 45° after the addition of the other constituents.

Substance present during heat treatment ...	Tryptophan disappeared (μ mole)			
	None	Pyridoxal phosphate	Tryptophan	Phenylpyruvate
Temp. 30°	0.22	0.24	0.22	0.22
35	0.22	0.24	0.22	0.24
40	0.22	0.28	0.22	0.24
45	0.22	0.28	0.19	0.24
50	0.14	0.28	0.19	0.24
60	0.12	0.32	0.19	0.24
70	0.00	0.31	0.17	0.24

by phenylpyruvic acid. However, since the stoichiometry was convincing, the rate of tryptophan disappearance was followed in the estimation of enzyme activity.

The pathway for the metabolism of tryptophan to indolylacetic acid by *A. tumefaciens* appears to be via transamination, since the tryptamine pathway could not be demonstrated and more than one enzyme catalysing transamination reactions with tryptophan and various oxo acids was present in crude extracts. Though the oxo acid which is the amino acceptor for the reaction is generally derived from intermediates in carbohydrate metabolism, in the present investigation phenylpyruvate was found to be a better acceptor of the amino group; α -oxoglutarate was less active and pyruvate and oxaloacetate were completely inactive.

A partially purified transaminase preparation has been observed not to be capable of utilizing α -oxoglutarate as amino acceptor. Though higher activity with phenylpyruvate was observed with tryptophan as amino donor, an absolute specificity for the two compounds was lacking, since the enzyme was equally active with isoleucine, valine or leucine as amino donor. In this respect the enzyme resembles the transaminase isolated from *Neurospora* by Seecof & Wagner (1959) and by Fincham & Boulter (1956) from the same source. But the latter workers studied glutamate formation with various amino donors.

The optimum pH for the activity of the enzyme was 9.6. Though a decrease in activity was observed at higher pH values, the activity was significant even at pH 10.6. Lack of a sharp fall in activity at high pH values can be compared with that of the tyrosine- α -oxoglutarate transaminase reported by Sentheshanmuganathan (1960).

In its heat stability the enzyme resembles glutamate-oxaloacetate transaminase (Jenkins, Yphantis &Sizer, 1959) and tyrosine- α -oxoglutarate transaminase (Sentheshanmuganathan, 1960; Kenney, 1962). This property has been advantageously employed by these authors for the removal of inactive proteins from their enzyme preparations.

Protection against thermal denaturation afforded by substrates is similar to that observed with glutamate-oxaloacetate transaminase from pig heart (Jenkins *et al.* 1959) and tyrosine- α -oxoglutarate transaminase isolated from rat liver (Kenney, 1962).

The activity of the enzyme at elevated temperatures and the prevention of denaturation even at such a high temperature as 70° in the presence of pyridoxal phosphate, phenylpyruvate or tryptophan could be explained as being due to protection afforded by cofactors or substrates against thermal denaturation (Burton, 1951).

SUMMARY

1. Evidence is presented to show that the first step involved in the conversion of tryptophan into indolylacetic acid by *Agrobacterium tumefaciens* involves transamination.

2. A 68-fold purification of an aminotransferase, exhibiting a broad specificity with comparable transaminase activities with tryptophan, valine, leucine and isoleucine as amino donors and phenylpyruvate as acceptor, was achieved.

3. The optimum pH for the enzyme activity is 9.6. The activity at higher pH values, though less than optimum, is significant.

4. Substrates and pyridoxal phosphate stabilize the enzyme against thermal denaturation.

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