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Tryptophan-phenylpyruvate aminotransferase of *Agrobacterium tumefaciens*: Purification and general properties of the enzyme

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Abstract

Evidence has been presented to show that the first step in the conversion of tryptophan to indole acetic acid by *Agrobacterium tumefaciens* involves transamination. The properties of a 58-fold purified tryptophan-phenylpyruvate amino transferase preparation with respect to specificity, optimum pH, temperature and protection against thermal denaturation by substrates and cofactors have been studied.

Key words: Aminotransferase, transamination and plant tumours.

1. Introduction

The production of plant tumours by the genus *Agrobacterium* is a well known phenomenon. Tumorigenesis in plants by *Agrobacterium tumefaciens* has received the attention of several research groups because of the great virulence and wide host range of the organism. In recent years several excellent reviews have appeared which deal in detail with the biochemical and molecular aspects of tumour induction by *Agrobacterium tumefaciens*[1-3].

It has been shown by several authors that hormone-like substances are synthesized by *Agrobacterium tumefaciens* and these in turn may be responsible for tumour production in plants. The role of IAA in tumour formation has been reviewed by Braun[4]. The hyper-auxic condition of tomato crown-gall tissues has been demonstrated by Link and Eggers[5] and Dye *et al*[6]. Lippincotts[7] have clearly established that bacteria of *Agrobacterium* genus actually produce the hormones *in* plant cells.

That IAA is clearly involved in tumour production by *Agrobacterium tumefaciens* is shown by the fact that some strains of this bacterium fail to induce tumour on tomato stems without added auxin[8-9]. Klein and Link[9] have suggested that bacterial auxin is required either to promote transformed cells into continued multiplication or as cocarcinogen in the transformation process.

Production of other hormones such as cytokinin and cytokinin active substances[10–11] and cytokinesin[12] have also been shown.

The role of tryptophan in the biogenesis of auxin and also the probable intermediate involved have been discussed by Fawcett[13]. The conversion of tryptophan to the plant hormone may involve either the initial deamination of the amino acid followed by decarboxylation[14–15] or initial decarboxylation followed by deamination[16–18]. The available evidence[19–22] seems to support the view that the first step involves the formation of the keto acid which in turn forms indole acetic acid *via* indole acetaldehyde.

Kaper and Veldstra[23] have reported the accumulation of indole pyruvic acid in the culture filtrates of *A. tumefaciens* grown in media containing tryptophan, which further suggests that the conversion of tryptophan to indole acetic acid involves the formation of indole pyruvic acid as an intermediate, probably by transamination.

Preliminary studies in our laboratory showed that crude extracts of *A. tumefaciens* contained transaminase activity with various aminoacids as amino donors and a number of keto acids as amino group acceptors. It was observed that transaminase activity with tryptophan and either α-oxo-glutarate or phenylpyruvate was significant. Further, it was found that the two activities could be readily separated.

The present communication deals with the partial purification of the tryptophan-phenylpyruvate transaminase.

2. Materials and methods

2.1. *Organism*

Agrobacterium tumefaciens, strain B_6, was used throughout this work. The virulence of the organism was shown by the development of galls subsequent to inoculation of a suspension of the bacterium on the tomato (*Lycopersicum esculentum*) and sunflower (*Helianthus annuus*) plants.

48-hour cultures of bacteria grown on potato-sucrose-agar slants were transferred to liquid media containing nitrate as the sole nitrogen source[24] and incubated for 48 hours at 30° C. The bacterial cells were collected by centrifugation at 5,000 g for 15 min. Cells were washed with 0·1 M phosphate buffer pH 7·0. Extracts were prepared by suspending the cells in the same buffer and exposing them for 20 min to sonic vibration in a Raytheon 10 kc oscillator. After removal of the cell debris by centrifugation in the cold (0–4° C) at 13,000 g for 15 min the supernatant was used for further purification.

2.2. *Assay of the tryptophan-phenylpyruvate transaminase*

The enzyme assay was performed in 2 ml reaction mixture containing 1 μmole of L-tryptophan, 2 μmoles of phenylpyruvate, 0·05 μmoles of pyridoxal phosphate, 25 μmoles of veronal : HCl buffer pH 9·6, 0·5 ml of enzyme preparation and water up to 2·0 ml. Incubation was carried out at 45° C for 60 min. The reaction was stopped by the addi-

tion of 0·5 ml of 15% trichloroacetic acid. 4 ml of peroxide-free ether was added to each tube. The tubes were shaken and the layers separated by centrifugation. Aliquots from the aqueous layer were taken for the colorimetric estimation of tryptophan according to the method of Horn and Jones[25]. In the case of controls, the enzyme was added after the addition of trichloroacetic acid.

2.3. *Estimation of phenylpyruvate*

Phenylpyruvate was estimated spectrophotometrically by the method of Lin *et al*[26]. The deproteinized reaction mixture was suitably diluted and treated with hog kidney tautomerase prepared by the method of Knox and Pitt[27]. Borate was added at a final concentration of 0–57 M, pH adjusted to 6·3 and the absorption measured at 299 mμ.

2.4. *Estimation of phenylalanine*

The amino acid was identified and estimated by circular paper chromatographic technique[28] using Whatman No. 1 paper and butanol : acetic acid : water (4 : 1 : 5) as the solvent system.

2.5. *Estimation of protein*

The protein content of the enzyme preparations was determined by the method of Lowry *et al*[29], using crystalline bovine serum albumin as standard.

2.6. *Substrates and cofactor*

L-tryptophan, α-oxoglutarate and pyridoxal phosphate were obtained from Hoffman-La-Roche Co. Ltd. Phenylpyruvate was prepared by the method of Herbst and Shemin[30]. The purity of the phenylpyruvate was checked spectrophotometrically according to the method of Lin *et al*[26].

3. Results

3.1. *Purification procedure for tryptophan-phenylpyruvate transaminase*

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

Step 1 : It was observed that treatment of the crude extract with tricalcium phosphate gel at pH above 6·5 resulted in retention of some of the inactive proteins by the gel but little adsorption of the transaminase. The total activity of the supernatant, compared to the crude, increased, presumably due to the removal of an inhibitor.

The crude extract (pH 7·0) was treated with half its volume of tricalcium phosphate gel (15 mg/ml)[31]. After stirring 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 of step 1 was adjusted to 5 by the dropwise addition of 6N acetic acid. The precipitated inactive protein was removed by centrifugation at 13,000 g for 15 min.

Step 3 : The supernatant from the previous step 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 dilute 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 6N acetic acid and treated with alumina C_γ gel[32] (0·2 ml gel/ml enzyme). The mixture was centrifuged after 15 min and the supernatant discarded.

The enzyme was eluted 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 is given in Table I.

Though the crude extract was stable for over three months at $-20°$ C, the partially purified preparation was found to lose activity either on freezing or storing at 0–4° C for more than 72 hr.

Table I

Progress of purification of tryptophan-phenylpyruvate transaminase from *A. tumefaciens*.

Step	Sp. activity μg. tryp. dissapp./mg protein	Fold purification	Total activity	% recovery
Crude	20	—	2700	—
Calcium phosphate negative adsorption	133	6·6	3000	111
pH 5·0 supernatant	150	7·5	3000	111
Calcium phosphate eluate	300	15·0	3000	111
Second negative adsorption on calcium phosphate gel	370	18·5	3000	111
Alumina C_γ eluate	1350	67·5	2300	90

3.2. *Specificity*

The partially purified preparation was completely inactive with tryptophan, histidine, methionine, leucine, valine or isoleucine as the amino donor and α-oxo-glutarate as acceptor. However, phenylpyruvate, dimethyl-pyruvate and hydroxyphenylpyruvate were very similar in their ability to act as amino group acceptors, with the above-mentioned amino acids as amino group donors.

Of the various amino acids tested with phenylpyruvate, tryptophan, leucine, valine, isoleucine and tyrosine showed higher activity while methionine and histidine were less active. DL-aspartic acid, DL-alanine, DL-threonine, L-arginine, L-glutamine and DL-serine were not effective as amino donors (Table II).

3.3. *Stoichiometry*

Under the conditions of incubation up to 90 min, within reasonable limits, there was a correlation in the disappearance of tryptophan and phenylpyruvate. Because of its very unstable nature and progressive decomposition with time indolepyruvate was not estimated.

3.4. *pH-activity curve*

The activity of the enzyme at various pH values was studied by using phosphate (5·8 to 8·0), tris : HCl (8·0 to 9·1), veronal : HCl (8·0 to 9·6) and glycine : NaOH (8·6 to 10·6). A steady increase in activity was observed up to pH 9·6 and there was a drop in activity at higher pH values. The activity was quite significant even at pH 10·6 (Fig. 1).

Table II

Phenylalanine formation with various amino donors

Reaction mixtures : 2 μmoles of phenylpyruvate, 2 μmoles of the amino donor, 0·1 μmoles of pyridoxal phosphate, 25 μmoles of veronal: HCl buffer pH 9·6 and enzyme ; incubation was at 45° C for 60 min.

Amino donor	μmoles phenylalanine formed	Amino donor	μmoles phenylalanine formed
L–Tryptophan	0·50	L–Glutamic acid	0·02
DL–Histidine	0·09	DL–Aspartic acid	0·00
DL–Methionine	0·20	DL–Alanine	0·00
DL–Leucine	0·51	DL–Threonine	0·00
DL–Valine	0·41	L–Arginine	0·00
DL–Isoleucine	0·51	L–Glutamine	0·00
L–Tyrosine	0·45	DL–Serine	0·00

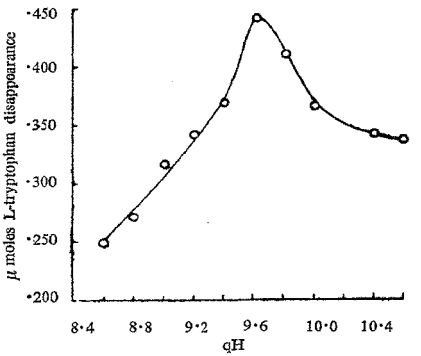

FIG. 1. pH activity curve.

Reaction mixtures were the same as mentioned under Materials and Methods except that glycine: NaOH buffer was used at the pHs indicated (*i.e.*, pH 8·6 to 9·6).

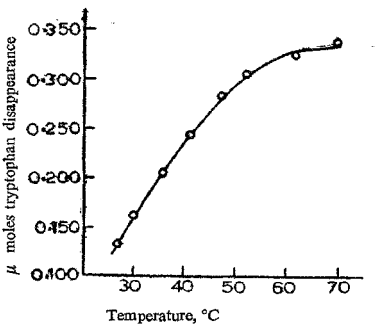

FIG. 2. Effect of temperature.

Reaction mixtures were same as in Materials and Methods, incubated at different temperatures for 60 min.

3. 5. *Effect of temperature*

The activity of the enzyme was found to increase with temperature from 25 to 50° C. At higher temperatures the activity levelled off (Fig. 2).

Table III

Heat inactivation in the presence and in the absence of substrates and cofactors.

Reaction mixtures : Same as mentioned under Materials and Methods (Section 2). The enzyme was heated at the indicated temperatures with or without the substrates or cofactor for 5 min and chilled immediately. Incubation was continued for 60 min at 45° C after the addition of other constituents.

Temperature °C.	μmoles tryptophan disappeared when the enzyme was heated with			
	No addition	Pyridoxal phosphate	Trypto-phan	Phenyl-pyruvate
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

3.6. *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 cofactor for even 5 min at 50° C resulted in a drop in activity, and complete inactivation was observed at 70° C (Table III).

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

Incubation of the enzyme with pyridoxal phosphate at 45° C for 30 min, under the onditions involved, did not result in any reduction in activity. Incubation was therefore routinely performed at this temperature.

3.7. *Effect of enzyme concentration*

The activity of the enzyme increased with increase in protein concentration from 10 to ¢100 μg (Fig. 3).

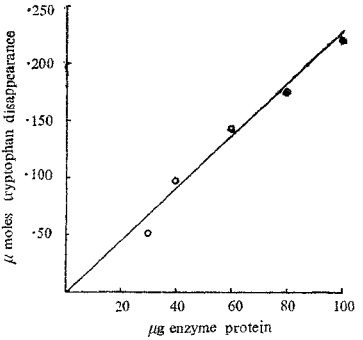

FIG. 3. Relationship of enzyme concentration to activity.
Reaction mixtures were the same as mentioned in the text except for the variation in enzyme concentrations.

4. Discussion

Accumulation of indolepyruvic acid in culture filtrates of *A. tumefaciens* grown on tryptophan containing media has been demonstrated by Kaper and Veldstra[23]. Their conclusion was based on the comparison of the chromatographic pattern of the decomposition products of indolepyruvic acid with those of the bacterial metabolites. More recently intermediates in this biosynthetic sequence have been studied by paper chromatography[33]. But the enzymic conversion of tryptophan to indolepyruvic acid has not been demonstrated. However, recent studies on the formation of IAA by *A. tumefaciens* suggest that more than one pathway may operate. Rodriguez *et al*[24] have reported a sequence

Tryptophan → Indole-3-pyruvate → Indole-3-glycolic acid.
↓
IAA

It is of interest to note that the sequence of steps in the formation of IAA is different in *A. tumefaciens* from its fixed L. form[35]. The authors[35] have put forward a novel scheme for the synthesis of IAA in the L. forms of *A. tumefaciens*, the sequence being

Tryptophan → Indole-3-acetonitrile → Indole-3-acetic acid.

In addition, indole 3 acetonitrile has been identified as intermediate in the biosynthetic sequence tryptophan to IAA in several other tumour producing bacteria also[36].

The formation of indolepyruvic acid has been demonstrated in the tryptophan α-oxoglutarate system (unpublished results). The method was not found suitable in the present system due to the interference of colour development by phenylpyruvate. However, since the stoichiometry was quite convincing, the rate of tryptophan disappearance was routinely followed in the estimation of enzyme activity.

Results of the present investigation support the view that the first step in the metabolism of tryptophan to indole acetic acid by *A. tumefaciens* involves transamination and the formation of indolepyruvic acid. Indole acetic acid is formed from indolepyruvic acid presumably by oxidative decarboxylation. Extracts of *A. tumefaciens* seem to contain more than one transaminase which converts tryptophan to indolepyruvic acid. Though an absolute specificity for tryptophan as amino donor and phenylpyruvate as acceptor was lacking for the enzyme purified by the present method, a complete separation from a tryptophan-α-oxoglutarate amino transferase could be achieved.

The optimum pH for the activity of the enzyme was found to be 9·6. Though decrease in activity was observed at higher pH values, the activity was quite significant even at pH 10·6. Lack of sharp drop in activity at high pH values could be compared to that of the tyrosine-α-oxoglutarate enzyme reported by Sentheshanmuganathan[37].

In its heat stability the enzyme resembles the glutamic-aspartic transaminase reported by Jenkins *et al*[38] and the tyrosine-α-oxoglutarate transaminase[37,39]. This property has been advantageously employed by these authors for the removal of inactive proteins from their enzyme preparations.

Protection against heat denaturation afforded by substrates is similar to that observed in the case of glutamic-aspartic transaminase of pig heart[38] and the tyrosine-α-oxoglutarate transaminase isolated from rat liver[39].

The activity of the enzyme at elevated temperatures and prevention of denaturation even at such a high temperature as 70° C in the presence of pyridoxal phosphate, phenylpyruvate or tryptophan could be explained as due to the protection afforded by the substrates or cofactors against thermal denaturation[40].

5. Summary

1. Evidence has been presented to show that the first step involved in the conversion of tryptophan to indole acetic acid by *A. tumefaciens* involves transamination.

2. A 58-fold purification of the enzyme has been achieved.

3. The optimum pH for the enzyme activity has been shown to be 9·6. The activity at higher pHs, though less than at optimum, was quite significant.

4. Substrates and pyridoxal phosphate were found to stabilize the enzyme against heat denaturation.

6. Acknowledgements

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References

1. Lippincott, J. A. and Lippincott, B. B. — *Ann. Rev. Microbiol.*, 1975, **29**, 377.
2. Kado, C. I. — *Ann. Rev. Phytopath.*, 1976, **14**, 265.
3. Schell, J. and Van Montagu, M. — *Brookhaven. Symp. Biol.*, 1977.
4. Braun, A. C. — *Ann. Rev. Plant Physiol.*, 1962, **13**, 533.
5. Link, G. K. K. and Eggers, V. — *Bot. Gaz.*, 1941, **103**, 87.
6. Dye, M. H., Clarke, G. and Wain, R. L. — *Proc. Roy. Soc. (B)*, 1962, **155**, 478.
7. Lippincott, J. A. and Lippincott, B. B. — *Phytopath.*, 1968, **58**, 1058.
8. Braun, A. C. and Laskaris, T. — *Proc. Natl. Acad. Sci. (USA)*, 1942, **28**, 468.
9. Klein, R. M. and Link, G. K. K. — *Proc. Natl. Acad. Sci. (USA)*, 1952, **38**, 1066.
10. Miller, C. O. — *Proc. Natl. Acad. Sci. (USA)*, 1974, **71**, 334.
11. Kaiss Chapman, R. W. and Morris, R. O. — *Biochem. Biophys. Res. Comm.*, 1977, **76**, 463.
12. Wood, H. N., Renne Kamp, M. E., Bowen, D. B., Field, F. H. and Braun, A. C. — *Proc. Natl. Acad. Sci. (USA)*, 1974, **71**, 4140.
13. Fawcett, C. H. — *Ann. Rev. Plant. Physiol.*, 1961, **12**, 345.
14. Thimann, K. V. — *J. Biol. Chem.*, 1935, **109**, 279.
15. Wildmann, S. G., Ferri, M. G. and Bonner, J. — *Arch. Biochem. Biophys.*, 1947, **13**, 131.
16. Clark, A. J. and Mann, P. J. G. — *Biochem. J.*, 1957, **65**, 763.
17. Cardy, E. E. and Wolfe, F. J. — *Physiol. Plantarum*, 1959, **12**, 526.
18. Dannenburg, W. N. and Liverman, J. L. — *Plant. Physiol.*, 1957, **33**, 263.

19. STOWE, B. B. — *Biochem. J.*, 1955, **61**, ix.

20. STOWE, B. B. — *Progress in the chemistry of organic natural products*, Ed. Zechmeister, Springer Verlag, Vienna, 1959, **17**, 248.

21. DANNENBURG, W. N. AND LIVERMAN, J. L. — *Plant. Physiol.*, 1957, **33**, 263.

22. LIVERMAN, J. L. AND DANNENBURG, W. N. — *Plant. Physiol.*, 1957, **32**, Suppl. xviii.

23. KAPER, J. M. AND VELDSTRA — *Biochim. Biophys. Acta*, 1958, **30**, 401.

24. SALLE, A. J. — *Fundamental principles of bacteriology*, McGraw-Hill Book Co., Inc., 1954, 135.

25. HORN, M. J. AND JONES, D. B. — *J. Biol. Chem.*, 1945, **157**, 153.

26. LINN, E. C. C., PITT, B. M., CIVEN, M. AND KNOX, E. — *J. Biol. Chem.*, 1958, **233**, 668.

27. KNOX, W. E. AND PITT, B. M. — *J. Biol. Chem.*, 1957, **225**, 675.

28. GIRI, K. V. AND RAO, N. A. N. — *J. Indian Inst. Sci.*, 1952, **34**, 95.

29. LOWRY, O. H., ROSEBOROUGH, M. J., FARR, A. L. AND RANDALL, R. J. — *J. Biol. Chem.*, 1951, **193**, 265.

30. HERBST, R. M. AND SHEMIN, D. — *Organic synthesis coll.* Vol. 2, John Wiley and Sons, 1943, 519.

31. KEILIN, D. AND HARTREE, E. F. — *Proc. Roy. Soc.* (*B*), 1937, **124**, 397.

32. WILLSTATTER, R., KRANT, H. AND ERBASHER, O. — *Ber.*, 1925, **58**, 2448.

33. OHARA, H., AND KOYAMA, M. — *Doshisha Joshi Daigahu Gakujutsee Kenkyu Nempo*, 1970, **21**, 38-53.

34. BELTRA, R., MARCILLA, P., RODRIGUEZ DE LECEA, J. AND DELA ROSA, C. — *Microbiol. Exp.*, 1970, **23**, 1.

35. RODRIGUEZ DE LECEA, J. DELA ROSA, C. AND BELTRA, R. — *Phyton (Buenos Aires)*, 1972, **29**, 119.

36. GALACH'YAN, R. M. — *Vop. Mikrobiol.*, 1969, **4**, 129.

37. SENTHESHANMUGANATHAN, S. *Biochem. J.*, 1960, **77**, 619.

38. JENKINS, W. T., YPHANTIS, D. A. AND SIZER, I. W. *J. Biol. Chem.*, 1959, **234**, 51.

39. KENNEY, F. T. *J. Biol. Chem.*, 1962, **237**, 1605.

40. BURTON, K. *Biochem. J.*, 1951, **48**, 458.