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TRYPTOPHAN SYNTHASE IN MAIZE (Zea mays L.) : I. in vivo & in vitro DEMONSTRATION OF ENZYME ACTIVITY

P.R.L. Gupta, V.S. Rathore*, S.V. Saradhi and G.K. Garg
College of Basic Sciences and Humanities, G.B. Pant University of Agriculture & Technology, Pantnagar 263 145, India.

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(3-^{14}C)-serine was fed through cut ends of 10-day-old shoots of normal (Ganga-2) and opaque (opaque-2) maize (Zea mays L.) and allowed to be metabolized in light for 24 hours. Subsequent methanolic extraction, radiochromatographic separation and radioautographic analysis showed that 10.4 and 10.3 percent of the absorbed ^{14}C-serine activity was incorporated into L-tryptophan by the shoot tissues of the normal and opaque maize respectively.

By coupling extraction of the enzyme with ammonium sulfate precipitation (75% saturation) it was possible to show a serine-dependent indole utilization by the enzyme preparations from coleoptiles (4-day-old), kernels (18 and 28 days after anthesis) and first nodal callus tissues (one-month-old) of the two corn varieties. Furthermore, the indole disappearance from reaction mixture occurred with a concomitant transfer of (3-^{14}C)-serine appearance into tryptophan.

The enzyme tryptophan synthase (TS) which catalyzes the terminal step in tryptophan biosynthesis has been investigated in great detail in microorganisms (1). In higher plants the enzyme has been purified and separated into subunit components only in case of pea and cultured tissues of tobacco (2,3).

Normal maize seeds are known for long to be deficient in tryptophan (4-6). The first four enzymes of tryptophan pathway namely anthranilate synthetase, PR transferase, PRA isomerase, and InGP synthetase, have been demonstrated and partially purified from maize seedlings (7). However, there has so far been no convincing in vitro demonstration of TS activity in maize tissue extracts. Our earlier studies in this direction led to the detection of an indole oxidizing system which may probably interfere with the assay of TS (8). We have attempted to circumvent this problem by in vivo determination of TS activity and by

* To whom requests for reprints should be sent.

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utilizing maize tissues having low indole oxidase activity to extract and assay the enzyme activity and the results of these studies are reported herein.

MATERIALS & METHODS

Source of seeds : Maize (Zea mays L.) seeds were obtained from Department of Plant Breeding of the University.

Source of chemicals : (3-^{14}C)L-serine (49.6 mCi/m mole) was purchased from ICN Pharmaceuticals, U.S.A. All other chemicals used were of analytical grade.

Plant culture : Seeds were surface sterilized with 0.1% $HgCl_2$. After washing thoroughly with distilled water, the seeds were germinated in petridishes lined with moist filter paper in dark. When the whole plants were desired, the 3-4-days-old seedlings were transferred to plastic trays filled with washed quartz sand and supplied with Hoagland's nutrient solution on alternate days. The plants were raised in a growth chamber at 28-30°C and at a photoperiod of 14 hours.

Callus culture : Callus cultures of the first nodal tissues of 2 maize varieties were developed on modified Murashige & Skoog medium supplemented with 2,4-D (2,4-dichlorophenoxyacetic acid) (9). 30-days-old callus tissues were used for enzyme extraction.

In vivo feeding of labelled serine (3-^{14}C-serine) : 10-days-old maize (Zea mays L. variety Ganga-2 and opaque-2) seedlings were harvested and shoots were cut off under water. The shoots were then placed in small glass bottles containing one ml of feeding solution composed of indole (10^{-3}M), L-serine (10^{-3}M), Pyridoxal-5'-phosphate (PALP) (10 µg/ml), chloramphenicol (10 µg/ml) and L(3-^{14}C)-serine (0.9 µCi). The shoots were allowed to absorb feeding solution in light with a gentle current of air to expedite absorption of solution through transpiration stream. When the entire solution was taken up, the shoots were transferred to a beaker of water and allowed to metabolize in light for another 24 hours at 28±2°C.

Extraction and identification of ^{14}C-metabolites : At the expiry of absorption period, the shoots were extracted in 80% methanol repeatedly and the methanol was evaporated in vacue at 40°C. The residue was made to a small volume. The methanol extract was subjected to one dimensional ascending paper chromatography in n-butanol, acetic acid, water (90:10:29 v/v). Standard L-serine and L-tryptophan were chromatographed and were localized by spraying with ninhydrin and Ehrlich's reagent, respectively. The Rf regions of the methanol extract corresponding with Rf regions of the standard serine and tryptophan were cut, eluted with 80% methanol and radioassayed with liquid scintillation counter using Bray's liquid scintillation fluid (10).

In vitro demonstration of TS activity:

Enzyme extraction : Enzyme was extracted from 3-days-old coleoptiles and developing grains (kernels) (6, 18 and 28 days after anthesis) according to Hanking et al. (7) (ratio 1:2 w/v). The supernatant was brought to 75% ammonium sulfate saturation. The precipitate was collected and dissolved in phosphate buffer (pH 7.8) and used for enzyme assays.

The callus tissues were extracted according to Widholm (11). The supernatant was used for enzyme assay without further purification.

Assay procedure : TS activity was assayed in 10 ml screw-capped glass tubes. The reaction mixture contained indole 0.4 µ moles, L-serine 40 µ moles, pyridoxal-5'-phosphate (PALP) 20 µg, enzyme and phosphate buffer (pH 7.8) in a final volume of one ml. The reaction was initiated by the addition of enzyme. With each assay, two blanks were run, one with boiled enzyme and

another without enzyme. The tubes were incubated at 37°C for 30 minutes in a water bath after which 0.2 ml of 5% NaOH was added to stop the reaction. The unused indole was extracted in toluene, and estimated according to Yanofsky (12).

In vitro incorporation of ^{14}C-serine into tryptophan : This experiment was done with the enzyme from callus tissues of the 2 maize varieties. The acetone powder from callus tissues was extracted in the extraction medium of Hankins et al. (7). After bringing the supernatant to 75% saturation with ammonium sulfate, the precipitate was collected and dissolved in phosphate buffer (pH 7.8) and used as the enzyme preparation. The reaction mixture contained 0.2 μ moles indole, 4 μ moles serine, 20 μg PALP, 2 μCi 3-^{14}C-serine, phosphate buffer and enzyme preparation in a final volume of 0.13 ml. The unused indole was extracted in toluene and the tubes were kept overnight at 0°C. Toluene was then carefully decanted off. The frozen aqueous phase was melted at room temperature and was gassed briefly with N_2 to remove toluene. An appropriate volume of the aqueous phase was used for paper chromatography alongwith reference spots of serine and tryptophan. The chromatograms were developed and radioassayed as described earlier.

Protein determination : Protein in the crude enzymes and partially purified enzymes was determined according to Lowry et al. (13).

RESULTS

In vivo demonstration of TS activity : The distribution of radioactivity on the chromatograms developed from methanol extracts of shoots fed with ^{14}C-serine are depicted in Figure 1. Two major peaks of radioactivity were found which corresponded with the positions of L-serine (Rf = 0.1-0.2) and L-tryptophan (Rf = 0.4-0.5) as confirmed by co-chromatography with authentic compounds. The Rf region corresponding to that of tryptophan (0.4-0.5) contained 10.4 and 10.3% of the total radioactivity in case of variety Ganga-2 and opaque-2, respectively (Figure 1). In a corresponding experiment with pea seedlings, the tryptophan region contained 22% of the radioactivity.

In vitro demonstration of TS activity:

Coleoptiles : Although in vivo incorporation of ^{14}C-serine into tryptophan was easily shown, our attempts to detect TS activity in crude or partially purified leaf homogenates of 2-3 weeks-old maize plants were not successful. Therefore, attempts were made to detect the activity in young, developing tissues. The extraction of enzyme was coupled with ammonium sulphate precipitation to protect the enzyme from inactivation during isolation. In this manner it was possible to show a serine-dependent indole disappearance with extracts of 3-day-old maize coleoptiles (Table 1). The activity was more or less same in the two maize varieties.

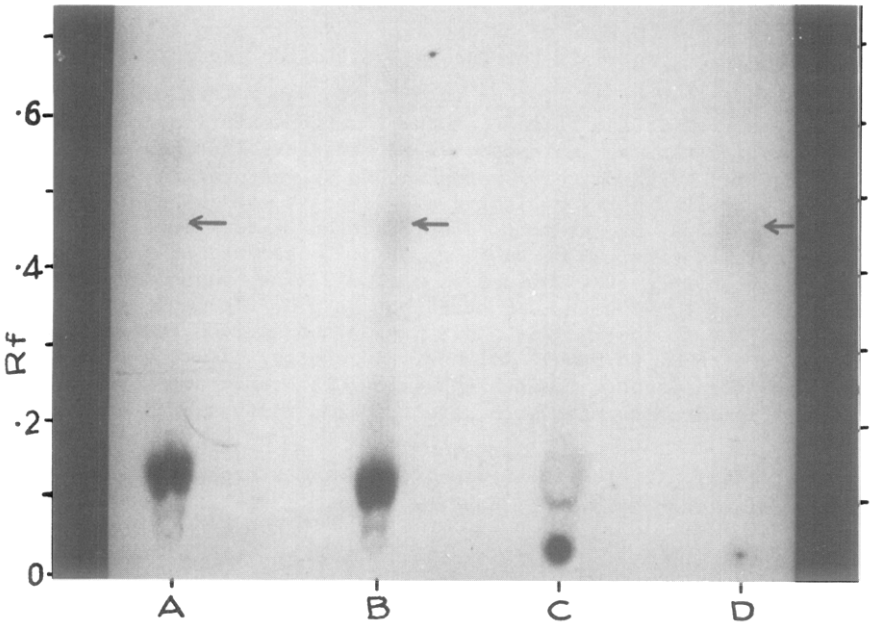

Figure 1. Autoradiographic detection of ^{14}C-serine incorporation into L-tryptophan by intact shoots of two maize varieties.

A : Ganga-2 B : Opaque-2 C : 2-^{14}C-serine reference

D : 5-^{3}H-tryptophan reference

Arrows indicate the position of L-tryptophan on the chromatograms. For further details see Materials and Methods.

Table 1 : Tryptophan synthase activity in maize (Zea mays L.) coleoptiles and developing kernels

Maize variety	Coleoptiles		Kernels			
	4-days-old		18-days-old		28-days-old	
	+serine	-serine	+serine	-serine	+serine	-serine
	Indole disappearance (nanomoles, mg protein^{-1}, hour^{-1})					
Ganga - 2	36	N.D	114	38	65	50
Opaque - 2	32	N.D	110	20	64	39.5

3-days-old coleoptiles or 6 to 28 days old developing kernels were extracted according to Hankins et al. (7). The 75% ammonium sulfate precipitate was dissolved in buffer (pH 7.8) and assayed for TS activity. The reaction mixture contained indole (0.4 u moles), L-serine (40 u moles), pyridoxal-5'-phosphate (20 ug) and phosphate buffer in a final volume of 1.0 ml at pH 7.8. In one set L-serine was omitted. In case of 6-days-old kernels no enzyme activity could be detected.

Table 2 : Tryptophan synthase activity in maize (Zea mays L.) callus tissues

Maize variety	Indole disappearance, (nanomoles, gm. fr.wt.$^{-1}$hr^{-1})	
	+serine	-serine
Ganga - 2	100.0	none
Opaque - 2	112.5	none

Fresh callus tissues (one-month-old) were extracted according to Widholm (11). The cell free supernatant was used for enzyme activity. The details for TS assay were same as given under Table 1.

Developing grains : 6, 18 and 28 days-old developing maize grains (kernels) were extracted by the same method as used for coleoptiles and TS activity in the presence and absence of serine was assayed. No detectable activity was observed in 6-day-old kernels. 18-days-old kernels, however, showed a marked increase in TS activity in both the maize varieties. The enzyme, activity as well as its serine dependency, however, declined when the kernels reached the age of 28 days. At this age of kernels, indole disappearance was much higher, when serine was included in assay mixture, but significant amount of indole also disappeared when no serine was added to assay mixture. The latter activity represents the indole oxidase activity which is showing increase with age of kernel.

Callus tissue : Extracts of 30-days-old callus tissues developed from maize shoots were assayed for TS in the presence and absence of L-serine. The data are given in Table 2. The activity was serine dependent in both varieties. There was no marked difference in the enzyme activity in the two varieties.

In vitro incorporation of (3-^{14}C)-serine into tryptophan : With a view to further confirm that indole disappearance under assay conditions is due to its conversion into L-tryptophan, incorporation of ^{14}C-serine into tryptophan was followed in callus tissue homogenates at pH 7.8. Results of the distribution of radioactivity on the chromatogram of the aqueous phase of assay mixture are given in Figure 2. It may be see that two major peaks of radioactivity are

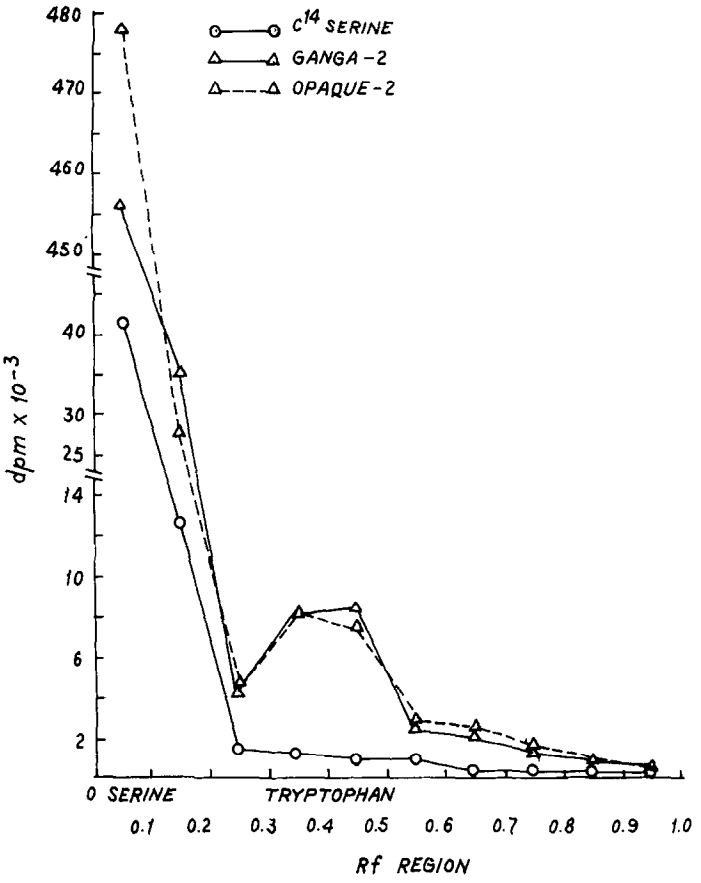

Figure 2. Incorporation of 2-^{14}C-serine into L-tryptophan by maize callus tissue extract. For further details see Materials and Methods.

observed; one corresponding to serine (Rf = 0.1-0.2) and the other corresponding to L-tryptophan (Rf = 0.4-0.5). Furthermore, the incorporation of (3-^{14}C)-serine into L-tryptophan occurred maximally at the expected pH 7.8 and at this incorporation declined to a ngligible level at pH 5.0.

DISCUSSION

The present study has demonstrated that when (3-^{14}C)-serine is allowed to be metabolized by intact maize plants, a significant amount of radioactivity is incorporated into L-tryptophan (Figure 1) thus providing evidence that serine serves as a precursor for tryptophan synthesis by TS system in maize like other plant and microorganisms. This observation coupled with the demonstration of 4 enzymes of TS pathways (7) leave little doubt that pathway of

tryptophan biosynthesis in maize is same as in other microorganisms and higher plants (1-3).

The TS system (L-serine hydrolyase (adding indole), EC 4.2.1.20) has been used in E. coli and Neurospora for the biochemical studies and to reveal the gene enzyme relationship (14,15). Such studies, although very desirable, have, however, not been possible in higher plant systems. The major difficulty in undertaking such studies has been lack of a suitable procedure for purifying TS from higher plants. Major difficulties in purifying TS include i) very low activity of the enzyme in plant tissues and ii) the instability of TS during extraction and purification. In addition in the case of maize (Zea mays L.) plant, the presence of an indole oxidase system (7) which brings about a rapid indole oxidation, further makes the task of extraction and purification of TS system more complicated. Earlier attempts, therefore, to demonstrate serine-dependent indole disappearance in maize leaf tissue extracts have not been successful (8). The problem arising out of the interference by indole oxidase was circumvented in this study by using young, developing tissues such as coleoptiles, young kernels and developing calli. Using these tissues, it was possible to demonstrate in vitro TS activity (Table 1 & 2). Both in coleoptile and callus tissues, the indole disappearance was strictly serine dependent and no indole disappearance occurred in the absence of serine indicating that it was not due to its oxidation by oxidase system. However, in case of developing kernels some indole oxidase activity was observed at pH 7.8. These results, therefore, demonstrate that young coleoptiles or callus tissues offer suitable material for further purification of TS system, because in these tissues, interference due to oxidase system is minimal. These young tissues of maize may, therefore, be good starting materials for purification of TS system from maize and for detailed study on this enzyme.

Convincing proof that indole disappearance in the in vitro assay system represented its utilization by TS is also provided by the demonstration that radioactivity of ^{14}C-serine added to assay medium was transferred to tryptophan (Figure 2) and this transfer was maximal at pH 7.8 and declined to

negligible level at pH 5.C. However, it must be mentioned that the efficiency of ^{14}C incorporation in tryptophan was rather low (ca. 2%) which may indicate the low activity of the reaction 2 (i.e. Indole + L-serine ⟶ L-tryptophan) in TS of maize. An interesting observation made in the present study is that there was no marked difference in the TS activity of the normal (low tryptophan) and opaque-2 (high tryptophan) varieties either in coleoptile or in callus tissues. In case of kernels also there appears to be no large difference in the TS activity (serine-dependent-indole disappearance). However, there was a marked serine-independent indole disappearance in kernels and this was particularly high in normal maize varieties (Table 1). The roles played by TS and indole oxidase in determining tryptophan levels in maize seeds are under further investigation in our laboratory.

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