Plant Physiol. (1976) 57, 101-104

# Some Physical Characteristics of the Enzymes of L-Tryptophan Biosynthesis in Higher Plants[1]

Received for publication July 15, 1975 and in revised form October 3, 1975

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## ABSTRACT

**Anthranilate synthetase, phosphoribosyltransferase, phosphoribosyl anthranilate isomerase, and indoleglycerol phosphate synthetase were examined in partially purified extracts of the monocotyledon, *Zea mays* and the dicotyledon, *Pisum sativum*. The plant extracts were chromatographed on DEAE-cellulose and Sephadex G150. The molecular weights of the enzymes were determined and found to be similar to those observed for many bacteria. None of the plant tryptophan enzyme activities was aggregated *in vitro* as is also the case with most bacteria. This is in contrast with the complex aggregation patterns observed in other eucaryotic organisms that have been examined (fungi and *Euglena gracilis*). The tryptophan enzymes from peas and corn were generally similar but some differences in stability were observed.**

The biosynthesis of L-tryptophan has recently begun to receive some attention in higher plants. The characteristics of the pathway are of particular interest in plants since L-tryptophan is a precursor of IAA. Work on the plant pathway has shown that the biochemical steps are identical to those in bacteria (4), that the enzyme activities can be obtained and some of their kinetic properties measured *in vitro* (2, 19, 20), that regulation by end product inhibition can occur *in vivo* (1), and that anthranilate synthetase mutants, relatively desensitized to feedback inhibition, can be selected by means of 5-methyl tryptophan (17, 18).

Little has been reported on the physical properties or aggregation patterns of the plant tryptophan enzymes with the exception of tryptophan synthetase. In tobacco for example, TS[2] is a bacterial-like two-component heteropolymer, in contrast to the homopolymer reported in higher fungi (5, 14). In microorganisms, a detailed analysis of the properties and varied aggregation patterns of the pathway enzymes, exemplified by studies in the *Enterobacteriaceae* (12) and the fungi (10), has established the value of such information in contributing towards the clarification of phylogenetic relationships.

As part of our continuing examination of L-tryptophan biosynthesis in photosynthetic eucaryotes, we present in this report the mol wt and aggregation patterns of the enzymes from *Pisum sativum* and *Zea mays*.

## MATERIALS AND METHODS

Golden Bantam corn seeds (*Zea mays*) were purchased from Burpee Seed Co., Riverside, California. Fresh peas (*Pisum sativum*) were purchased from a local retail grocery outlet. DEAE-cellulose (DE52) was obtained from Whatman; Sephadex G150, phosphoribosyl pyrophosphate, and ribose-5-P from Sigma Chemical Co., and L-glutamine and 2-mercaptoethanol from Calbiochem.

Chorismic acid was prepared as the free acid from culture filtrates of *Enterobacter aerogenes* 62-1 (7). Carboxyphenylamino-1-deoxyribulose-5-P was prepared by chemical synthesis as described by Smith and Yanofsky (15). Indoleglycerol phosphate was the gift of I. P. Crawford, Scripps Clinic and Research Foundation, La Jolla, Calif. Ammonium sulfate (ultrapure enzyme grade) was from Schwarz/Mann.

**Peas.** Embryos were stripped from the pods of fresh peas, washed with distilled $H_2O$, then blended with 2 volumes of ice-cold 20 mM tris-HCl (pH 7.5), supplemented with 1 mM EDTA; 10 mM 2-mercaptoethanol; 5 mM L-glutamine; and 10% (v/v) glycerol. All subsequent procedures were done at 0 to 5 C. The blended tissue was centrifuged for 60 min at 48,000*g*, and the pellet was discarded. The crude supernatant was made 0.1% (v/v) in protamine sulfate by the dropwise addition of a 1% (w/v) solution. The resulting precipitate was removed by centrifuging as before and discarded. Ammonium sulfate (472 mg/ml) was added over a 1-hr period while stirring, and the resulting precipitate was collected by centrifugation at 48,000*g* for 20 min. This precipitate served as the source of the pea tryptophan enzymes studied.

**Corn.** Corn seeds were washed with a 10% (v/v) commercial bleach solution, rinsed with 10 changes of deionized $H_2O$, and allowed to imbibe for 24 hr at room temperature. Imbibed seeds were arranged in a monolayer on moist filter paper in trays then covered with aluminum foil. The filter paper was moistened daily with deionized $H_2O$. After 3 to 4 days the approximately 5 to 8 cm radicles were harvested by manually removing the endosperm. The tissue was washed vigorously with a stream of deionized $H_2O$ to remove excess starch, then frozen with liquid nitrogen.

All extraction procedures were carried out at 0 to 5 C. The frozen corn tissue was blended to a fine powder in a chilled blender, then stirred into 1.5 volumes of 0.05 M tris-HCl (pH 7.5) supplemented with 1 mM EDTA; 10 mM 2 mercaptoethanol; 20% (v/v) glycerol; and 310 mg/ml ammonium sulfate. This mixture was stirred for 10 min, then centrifuged at 48,000*g* for 60 min and the precipitate was discarded. The supernatant solution was treated with another 210 mg/ml of ammonium sulfate and, after 30 min of stirring, the precipitate was collected by centrifuging for 10 min at 48,000*g*. This precipitate served as the source of the corn tryptophan enzymes studied.

**DEAE-cellulose Chromatography.** A column (2.2 × 30 cm) of DEAE-cellulose was equilibrated with buffer A (20 mM tris-HCl [pH 7.5], 1 mM EDTA, 10 mM 2-mercaptoethanol, 5 mM L-glutamine, and 20% [v/v] glycerol). A sample of either corn or pea ammonium sulfate precipitate was applied to the column after prior overnight dialysis against buffer A. The column was

[1] This work was supported by United States Energy Research and Development Administration P.A. 133.

[2] Abbreviations: TS: tryptophan synthetase; AS: anthranilate synthetase; PRT: phosphoribosyltransferase; PRAI: phosphoribosylanthranilate isomerase; InGPs: indoleglycerol phosphate synthetase.

eluted with a linear increasing salt gradient made of 400 ml of buffer A and 400 ml of buffer A made 0.6 M with NaCl. Approximately 10-ml fractions were collected at a flow rate of about 0.5 ml/min.

**Sephadex G150 Chromatography.** Sephadex G150 was prepared according to the manufacturers specifications. Chromatography was performed on a 2 × 100 cm column. The column was calibrated with several proteins of known mol wt by the method of Whitaker (16). Suspensions (5 ml) of corn or pea ammonium sulfate precipitate were eluted downward at a flow rate of about 10 ml/hr and approximately 5 ml fractions were collected.

**Enzyme Assays.** AS activity was measured by monitoring the fluorescence of anthranilic acid in an Aminco-Bowman spectrophotofluorometer (excitation 325 nm, emission 400 nm). The assay contained 50 μmoles of tris-HCl; 10 μmoles of $MgSO_4$; 20 μmoles of L-glutamine; 200 nmoles of chorismic acid; and enzyme in a final volume of 1 ml. The pH was 7.5 at the assay temperature of 37 C.

PRT activity was assayed by following the disappearance of anthranilate as described above. The assay contained 50 μmoles of tris-HCl; 10 μmoles of $MgSO_4$; 1 μmole of phosphoribosylpyrophosphate, 10 nmoles of anthranilic acid; and enzyme in a final volume of 1 ml. It was found that plant PRT activity was stimulated by the presence of excess PRAI. Therefore 10 μl of a derepressed bacterial extract *Enterobacter cloacae trp*-6) containing no PRT activity were routinely added to each assay. The assay was run at pH 7.5 and 37 C.

PRAI activity was measured as described previously (3).

InGPs was assayed by following the fluorescence of the reaction product indoleglycerol phosphate (excitation max, 280 nm; emission max, 350 nm). This assay is described in detail elsewhere (8).

The unit used for all the enzyme activities is defined as the disappearance of 1 nmole of substrate or the appearance of 1 nmole of product/min. Specific activity is defined as units/mg protein. Protein was estimated by the method of Lowry *et al.* (13) using desiccated BSA as the standard.

**Contamination Controls.** Freshly harvested plant tissue was washed with distilled $H_2O$ then minced in a sterile mortar with 1 ml/g sterile $H_2O$. Dilutions of this material were plated on enriched bacterial and fungal agar plates.

## RESULTS

Table I summarizes the extraction procedures described under "Materials and Methods." The specific activities and the units/g fresh tissue of the tryptophan enzymes are very similar for corn and peas with the striking exception of PRT. PRT activity was consistently 5 to 20 times higher in corn than in peas and was also 3 to 20 times more active than the other corn tryptophan enzymes. The activities for all the enzymes are too high to be the result of contamination. The contamination controls indicated that the corn extract contained 6 × $10^4$/ml bacterial and $10^4$/ml fungal contaminants. For peas the results were 2 × $10^3$/ml bacterial and 2 × $10^2$/ml fungal contaminants. Assuming that the contaminants contained levels of tryptophan enzymes comparable to that found in completely derepressed bacterial strains, and further that the extraction procedure used (blender) was efficient, there would still not be measurable levels of tryptophan enzymes in the extract.

**DEAE-cellulose Chromatography.** A sample of corn or pea dialyzed ammonium sulfate precipitate was chromatographed as described under "Materials and Methods." Figures 1 and 2 show the DEAE-profiles obtained for corn and peas, respectively. With the exception of PRAI, the corn and pea tryptophan enzymes give nearly identical DEAE-chromatographs. None of the pea tryptophan enzyme activities co-elute under these conditions. Although corn PRT and PRAI co-elute (with the void of the gradient) under these conditions, when chromatographed with buffers of lower ionic strength (not shown) the two activities are also separable. The latter conditions were not

Table I. *Summary of Extraction Procedures*

| Enzyme | Units/ml | Units/g fresh tissue | Specific activity |
|---|---|---|---|
| Corn ammonium sulfate precipitate | | | |
| AS | 14 | 2.8 | 0.50 |
| PRT | 330 | 66 | 13 |
| PRAI | 71 | 14 | 2.7 |
| InGPs | 130 | 26 | 4.9 |
| Pea crude extract | | | |
| AS | 3.6 | 5.9 | .38 |
| PRT | 7.1 | 12 | .76 |
| PRAI | 12 | 19 | 1.3 |
| InGPs | 16 | 26 | 1.7 |
| Pea ammonium sulfate precipitate | | | |
| AS | 12 | | 0.5 |
| PRT | 27 | | 1.2 |
| PRAI | 43 | | 1.9 |
| InGPs | 36 | | 1.6 |

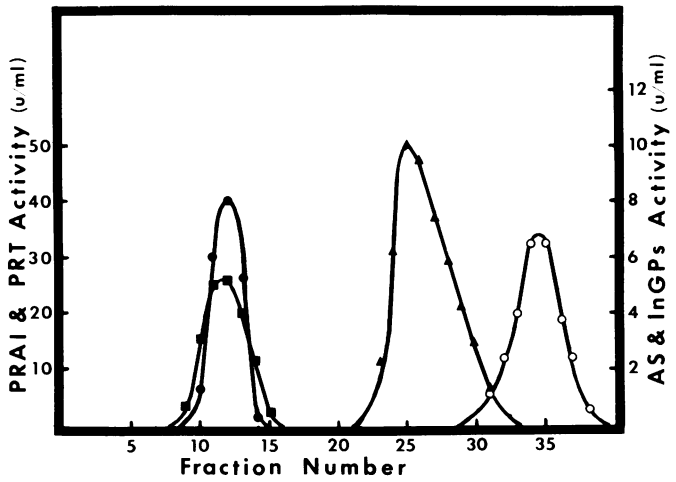


FIG. 1. DEAE-profile of corn tryptophan enzymes. ○: AS; ●: PRT; ▲: InGPs; ■: PRAI. Recovery of applied activity was: AS, 30%; PRT, 50%; InGPs, 75%; PRAI, 55%.

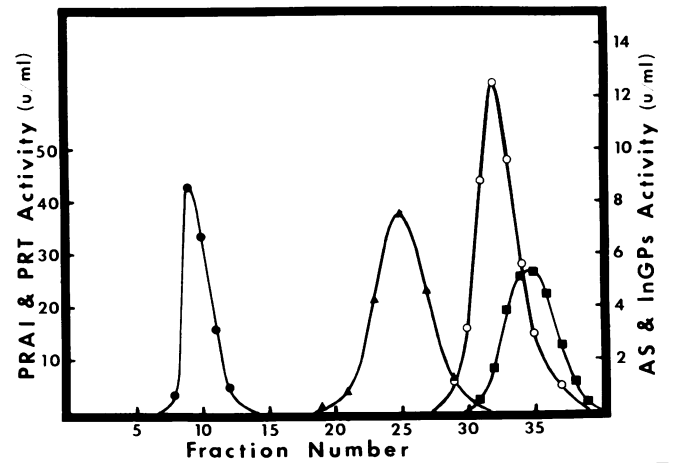


FIG. 2. DEAE-profile of pea tryptophan enzymes. ○: AS; ●: PRT; ▲: InGPs; ■: PRAI. Recovery of applied activity was: AS, 90%; PRT, 75%; InGPs, 100%; PRAI, ≥100%.

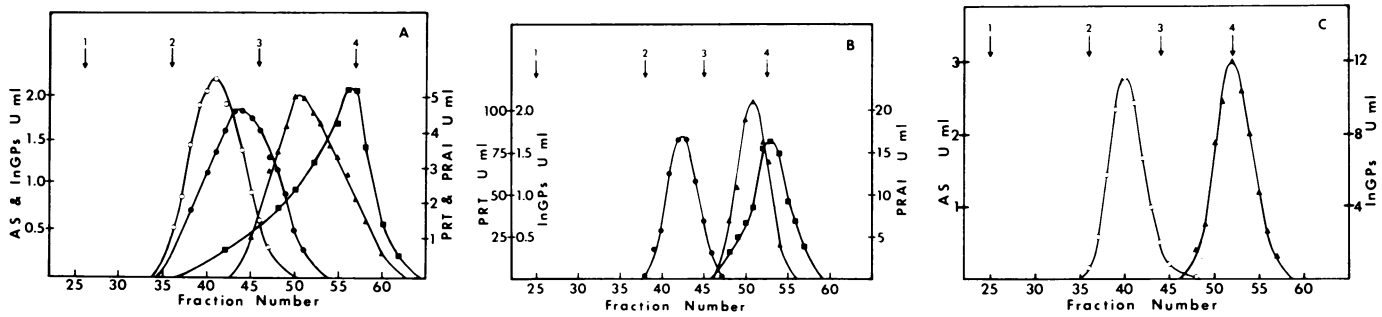


FIG. 3. Sephadex G150 chromatography. ○: AS; ●: PRT; ▲: InGPs; ■: PRAI. Calibration markers and their molecular weights were: 1: blue dextran (void); 2: lactate dehydrogenase (136,000); 3: malate dehydrogenase (66,000); 4: α-protein, tryptophan synthetase (29,000). A: Pea extract eluted with buffer A. Approximate recovery of activity was: AS, 55%; PRT, 75%; InGPs, 60%; PRAI, 50%. B: Corn extract eluted with buffer A also 1 M in KCl. Recovery of activity was: PRT, 60%; InGPs, 15%; PRAI, ≥ 100%; AS, <5%. C: Corn extract eluted with buffer A. Recovery of activity was: AS, 100%; InGPs, 70%; PRT and PRAI, <10%.

Table II. *Summary of Molecular Weights Determined by Gel Filtration on Sephadex G150*

| Enzyme | Average Molecular Weight | | | |
|---|---|---|---|---|
| | Peas | Corn | *Pseudomonas putida*[2] | *Bacillus subtilis*[2] |
| | *daltons* × $10^{-3}$ | | | |
| AS | 95.3 ± 5(3)[3] | 95.5 ± 1(2) | 75 | 67 |
| PRT | 83.5 ± 3(4) | 82.6 ± 5(3) | 64 | 57 |
| PRAI | 28.0 ± 5(3) | 27.2 ± 1(2) | 39 | 27 |
| InGPs | 52.0 ± 2.8(2) | 32.0 ± 2.6(3) | 32 | 30.5 |

[1] Data from ref. 6.

[2] Data from ref. 9.

[3] Numbers in parentheses show number of determinations used for these calculations.

routinely employed as they led to relatively large losses of PRT activity.

**G150 Sephadex Chromatography.** Ammonium sulfate precipitates containing all the activities were chromatographed on Sephadex G150. Figure 3A shows the results for *P. sativum*. It was not possible to measure all four enzymic activities from maize on a single G150 column since AS activity was lost when extracts were chromatographed in 1 M KCl buffers and PRT was not recovered from low ionic strength columns. Thus corn extracts were chromatographed in both high and low ionic strength buffers and the results are given in Figure 3, B and C. None of the plant tryptophan enzymes co-eluted in these experiments.

Table II summarizes the mol wt extrapolated from these experiments. In some experiments corn InGPs, activity was found in a peak corresponding to a mol wt of 52,000 as well as the peak eluting at about 32,000 daltons. We have been unsuccessful in attempts to observe reproducibly corn InGPs activity that elutes at this position (52,000) on Sephadex G150.

## DISCUSSION

Although only one monocotyledonous and one dicotyledonous plant were included in this study, some interesting results are apparent. No *in vitro* aggregation of any of the tryptophan enzymes was observed. This is in striking contrast to the results observed with the other eucaryotic organisms examined (fungi and *Euglena*).

A systematic study of the fungi revealed that, with the exception of the *oomycetes*, all other taxonomic groups had AS aggregated with one or more other tryptophan enzymes (10). In the *oomycetes* the PRAI and InGPs activities are found together (10). In *Euglena gracilis* a stable complex containing all the tryptophan enzymes except AS has been observed (11). Our observation with plants rules out the possibility that stable aggregation within the tryptophan pathway is a general feature of eucaryotic cells. As for procaryotic cells, it is of some interest to note that the majority of the bacteria are similar to plants with respect to the tryptophan pathway enzymes.

The bacterial tryptophan enzymes have mol wt similar to those found in plants (Table II). The exception is the *Enterobacteraceae* which, because of the inclusion of *Escherichia coli*, may have received a disproportionate amount of attention.

In the studies reported here corn InGPs was generally observed at a peak corresponding to a mol wt of about 32,000 daltons. Occasionally, a 52,000 dalton species was observed. Corn InGPs may exist in a second form, perhaps a dimer of the smaller mol wt species; we have been unable to define the conditions required for its stabilization. The possibility exists that some or all of the plant tryptophan enzymes may be associated *in vivo*, but that our *in vitro* conditions disrupt these associations. Nonetheless, similar *in vitro* conditions have revealed the existence of aggregates in bacteria, fungi and *Euglena*.

While the pathway enzymes of both plants are, in common, unaggregated *in vitro* they differ in other respects. As shown in Table II differences in mol wt are apparent and, causing us some experimental difficulty, was the sharp difference in stability of the PRTs. It was not until we had discovered the requirement for high salt as indispensable for the stability of the corn PRT that we were able to measure its properties. Our preliminary examination of the green alga, *Chlamydomonas reinhardti* indicated that PRT, PRAI and InGPs are separate activities with mol wt very similar to those for plants. Information on AS and TS in this organism is not yet available.

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