L-Phenylalanine Ammonia-lyase (Maize)

PARTIAL PURIFICATION AND RESPONSE TO GIBBERELLIIC ACID AND CYCLOHEXIMIDE OF L-PHENYLALANINE AND L-TYROSINE AMMONIA-LYASE ACTIVITIES

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ABSTRACT

Extracts of maize leaf sheath tissue deaminate both L-phenylalanine and L-tyrosine. The activities with both substrates are enhanced by treating the plant with gibberellic acid. Both activities decrease rapidly at the same rate when tissue is incubated in a moist atmosphere, and this decrease can be slowed by treatment with cycloheximide. The ratio of the activities was constant throughout a series of purification steps which included acetone and ammonium sulfate precipitation, and passage through an agarose column. The two activities could not be separated by isoelectric focusing. These results support our earlier conclusion that both activities occur at the same catalytic site.

In this paper the purification of a stable enzyme from maize which acts on both L-phenylalanine and L-tyrosine is described. The response of both activities in vivo to GA and cycloheximide is also presented. The fact that we have not been able to separate or differentially affect the two activities by any of these means provides further evidence for our earlier hypothesis of a common catalytic site for the elimination of ammonia from L-phenylalalnine and L-tyrosine.

MATERIALS AND METHODS

Plant Material. The maize (Zea mays) tissue used in this study was grown from seeds which were produced in field plots by Herbert V. Marsh, Jr. The strain contained the d-1 dwarf mutant; however, only seed which produced normal tall plants was used. Seeds were soaked in aerated tap water for 24 hr prior to planting. Approximately 150 seeds were planted per flat in a mixture of soil, sand, and peat moss (7:3:2). The flats were placed in the greenhouse and watered daily. The plants were harvested or treated when 3 to 4 weeks old at which time they had three to four leaves. Plants were cut at the soil level and all of the tissue above the ligule of the second leaf was discarded as was the flag leaf. This left only segments of leaf sheaths which served as the experimental material.

Enzyme Assays. Conditions of assay with L-phenylalanine as substrate were as previously described by Havir and Hanson (6) and with L-tyrosine as a substrate as previously described by Havir et al. (7). A unit of activity is defined as the amount of enzyme necessary to convert 1 μ mole of substrate to product in 1 min at 30 C and pH 8.7. Protein assays were routinely carried out by the biuret method (3). Protein estimations in the fractions eluted from the agarose column employed the technique described by Warburg and Christian (16).

Enzyme Purification. Leaf sheath tissue (50 g) was ground with 150 ml of 0.1 M sodium borate buffer, pH 8.7 (hereafter referred to as borate buffer), containing 0.45 ml of 2-mercaptoethanol in a VirTis "45" homogenizer at high speed for 1 min. The homogenates were filtered through cheesecloth and spun for 10 min at 20,000g at 0 C. The supernatant fluid was decanted and 1.5 volumes of acetone (–10 C) were added to it with thorough stirring. The mixture was held at –10 C for 20 to 30 min to allow for precipitation of protein which was collected by centrifugation at 5000g for 10 min. After rinsing the pellets with 5 to 10 ml of distilled water, the precipitated pro-

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The enzyme was resuspended in 5 to 10 ml of borate buffer and centrifuged at 20,000g for 10 min. The clear supernatant solutions from several grindings were combined at this stage and dialyzed overnight against two changes of borate buffer (2 liters of buffer each change, the first containing 6 ml of 2-mercaptoethanol). The dialyzed extract was further fractionated with crystalline ammonium sulfate. The material insoluble between 32 and 70% saturation was collected by centrifugation at 5000g for 10 min, dissolved in a minimal volume of borate buffer, and placed on an agarose column (3.5 × 40 cm) which had been equilibrated with the borate buffer. Aliquots of 3-ml fractions eluted from the column with the same borate buffer were assayed for protein and enzyme activities. The active fractions were combined, crystalline ammonium sulfate was added to 70% saturation, and the protein was collected by centrifugation at 5000g for 10 min. The enzyme was dissolved in 2 to 3 ml of borate buffer.

The procedure can be modified in order to facilitate preparation of large batches of enzyme. Tissue (400 g) was ground with 1 liter of borate buffer containing 1 ml of 2-mercaptoethanol for 1 min in a large Waring Blender at low speed. Acetone was added directly to the filtrate. While the first batch was centrifuging (7000g, 10 min) a second 400 g of tissue were processed. The suspended acetone precipitates were combined, centrifuged, and the supernatant fractionated with ammonium sulfate as described above. The 32 to 70% ammonium sulfate fraction (10 to 15 ml) was placed on a large agarose column (8.5 × 50 cm; see (6) for details). Fractions containing enzymatic activity were combined and concentrated by ammonium sulfate precipitation.

The ammonia-lyase activity prepared by both methods was stable at room temperature for 24 hr and in the frozen condition for several weeks.

**Isoelectric Focusing.** The techniques of determining the isoelectric point by isoelectric focusing in ampholine buffers have been described elsewhere (4). Enzyme (150 mU)² (prepared through Step 5, Table I) was dialyzed against 1% glycine for 4 hr before adding to the isoelectric focusing apparatus (LKB Instruments, model 8101) and allowed to focus for 36 hr.

**Application of GA and Cycloheximide.** GA treatments were carried out by a drench treatment of a flat (30 × 60 cm) containing approximately 150 seedlings with 1 liter of 0.1 mM GA₃ (Eastman Chemical Co.). One drop of Triton X-100 was added as a surfactant. This application resulted in a liberal wetting of the plants as well as the soil. Control plants were treated with 1 liter of distilled water which contained 1 drop of the surfactant.

Cycloheximide was applied by adding 100 ml of a 10 mg/ml solution to a plastic box (30 × 15 × 15 cm) lined with filter paper on which approximately 100 leaf sheaths were placed. Control tissue was incubated in boxes to which 100 ml of distilled water were added.

**RESULTS**

Reid and Marsh (14) have previously shown that the specific activity of PAL increases in several plant species after GA treatment. Application of GA to intact maize seedlings resulted in an increase in the ammonia-lyase activity after a lag period of approximately 24 hr. The higher steady state level of enzyme activity persisted for several days (13). Figure 1 shows that both PAL and TAL respond to GA treatment and that the kinetics of this response were virtually identical for both activities. In this experiment there was a lag period of 24 hr followed by a 3-fold increase in specific activity in the following 48 hr.

When segments of maize tissue are harvested and incubated

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²Abbreviations: PAL: phenylalanine ammonia-lyase; TAL: tyrosine ammonia-lyase.
Each extract was assayed for both PAL and TAL activity. Reference lines indicate one standard deviation above and below the mean specific activity.

Table I. Partial Purification of L-Phenylalanine and L-Tyrosine Ammonia-lyase Activities from Zea mays

<table>
<thead>
<tr>
<th>Settling</th>
<th>PAL</th>
<th>TAL</th>
<th>PAL/TAL</th>
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<tbody>
<tr>
<td>Specific activity</td>
<td>Total activity</td>
<td>Recovery</td>
<td>Specific activity</td>
</tr>
<tr>
<td>mg/mg protein</td>
<td>mg</td>
<td>%</td>
<td>mg/mg protein</td>
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1. Acetone ppt. | 15.4 | 7140 | 100 | 1.6 | 748 | 100 | 9.6 |
2. Dialysis | 12.0 | 4768 | 67 | 1.5 | 560 | 74 | 8.0 |
3. 32-70% (NH4)2SO4 fraction | 83.8 | 4204 | 59 | 8.8 | 442 | 58 | 9.5 |
4. Agarose column | 147.0 | 3443 | 49 | 12.3 | 289 | 39 | 11.1 |
5. 0-70% (NH4)2SO4 fraction | 151.1 | 2730 | 38 | 18.3 | 316 | 48 | 8.3 |

in a moist atmosphere, PAL activity declines and the loss of activity is inhibited by cycloheximide (13). Figure 2 demonstrates that TAL activity also decreased in tissue incubated in Petri dishes in a manner similar to PAL, and that the loss of activity could also be inhibited by cycloheximide. This observation suggests the presence of an inactivating system for TAL which requires protein synthesis as previously described for PAL (2, 19, 20).

Purification of the maize leaf sheath extract was undertaken in an attempt to separate physically the two activities. Both PAL and TAL activities were assayed after each step of partial purification. Table I shows a summary of the specific activities and recovery after each step. There was a purification of about 10-fold for both activities from the acetone-precipitated extract, the first stage at which the assay was routinely made. The PAL activity in the crude extracts was about 2 mU/mg protein (Marsh, unpublished results). Thus the actual purification was about 75-fold.

Throughout these purification steps both activities were recovered proportionately and the ratio of PAL/TAL was approximately the same after each step when calculated on a specific activity basis (Table I). Unlike the earlier preparation from maize (9), this enzyme eluted from the agarose column was stable on storage at −10°C for at least several months.

Figure 3 shows the elution of both activities from an agarose column. The position of the two peaks coincided; there were no obvious minor peaks or shoulders. The ratio of PAL/TAL remained essentially constant in each fraction recovered from the column, the average PAL/TAL ratio in the aliquots recovered from this column being 9.

The active fractions eluted from the agarose column were combined, precipitated with ammonium sulfate, and the protein was redissolved in borate buffer. The solution was then placed on an isoelectric focusing column and allowed to focus for 36 hr. Initial studies were carried out with a gradient of pH 3.0 to 5.0 when placed at either the anodic or cathodic ends of the column.

A final attempt was made to distinguish between the activities by studying their inactivation. After each step of partial purification an aliquot of the extract was assayed (first two columns of numbers, Table II). Another aliquot was removed and allowed to stand at room temperature for 24 hr. A portion was assayed after 8 hr and another after 24 hr. The results are summarized in Table II. The PAL/TAL ratios were constant on a milliliter per milliliter basis throughout the purification (see also Table I). Both PAL and TAL activities were lost proportion-
ately at 25 C in the 8- and 24-hr periods. The greatest increase in stability of both activities was achieved by passage through the agarose column.

**DISCUSSION**

As was pointed out previously, resolving the question of enzyme specificity or lack of it, when working with a non-homogeneous system is a very difficult one (7). Conclusive proof that two or more enzymes are present requires either physical separation or differential inhibition of the enzymes involved. If the activities prove inseparable by physical techniques, cumulative evidence from as many types of experiments as possible must be presented in order to decide whether one or more catalytic site is responsible for both activities.

Other investigators have previously suggested that separate enzymes are responsible for catalyzing these reactions in barley (11), wheat (17), *Sporobolomyces roseus* (1), and sweet potato (10). The evidence cited in each case was primarily based on changing PAL/TAL ratios during experimental manipulation of the extracts.

It often has been pointed out that ratios possess inherent arithmetical anomalies and that therefore great care must be taken when attempting to interpret results based on such numbers. It is evident, for example, that PAL activity is virtually always greater than TAL activity. Small errors in measuring TAL activity will thus have a greater effect on the calculated ratio than small errors in measuring PAL. Further, individual workers have assayed the enzyme activities in various ways, with few of them reporting initial velocities. We would emphasize that there have been no reported cases in which an experimenter has prepared an extract which has activity for tyrosine but not for phenylalanine.

Ogata et al. (12) and Uchiyama et al. (15) have studied PAL and TAL activities from several species of *Rhodotorula* and concluded that probably only one enzyme was responsible for both activities. More recently, Hodgins (8) has described a highly purified preparation of PAL from *Rhodotorula glutinis* which had a specific activity of 1.0 U/mg protein. The *Km* was lower for tyrosine than for phenylalanine and the ratio of *V*ₘₐₓ *phe*/*V*ₘₐₓ *tyr* was 2. On acrylamide gel electrophoresis, only the major protein band was associated with enzymatic activity. For this preparation, therefore, either there was only one enzyme, or if there were two, they were so similar in properties that they were inseparable electrophoretically.

Reid and Marsh (14) have previously shown that PAL activity is enhanced by application of GA to maize seedlings. Our results now indicate that both PAL and TAL activities are enhanced by GA and that the responses of both follow identical kinetics. A similar simultaneous peaking of PAL and TAL activities during the development of *S. roseus* has been shown (1). We have also shown that treatment with cycloheximide delayed the rate of inactivation of both PAL and TAL activities in maize. Similar results have been interpreted as evidence for an inactivating system which requires protein synthesis in potato (19) and gherkin (2) tissues. Reid (13) has also shown that treatment with cycloheximide prevented the GA-mediated enhancement of both PAL and TAL activities in maize.

Unlike the earlier unstable maize enzyme (9), both PAL and TAL activities were stable in the cold for several weeks after passage through an agarose column. Both activities responded similarly in these studies and both were recovered proportionally throughout the purification. Moreover, it was not possible to separate them by the technique of isoelectric focusing. Thus the data presented here, combined with those presented earlier (7), strongly suggest that in maize the ability to eliminate ammonia from l-phenylalanine and l-tyrosine resides on the same active site.

The availability of stable enzyme from maize has made it possible to examine in detail the question of the specificity of l-phenylalanine ammonia-lyase. It is our belief that the information gained concerning the properties of the isolated enzyme is vital to a study of the functioning of the enzyme *in vivo*.

**Table II. Stability of l-Phenylalanine and l-Tyrosine Ammonia-Lyase Activities at 25 C**

<table>
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<th></th>
<th>PAL</th>
<th>TAL</th>
<th>PAL-TAL</th>
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<tr>
<td>8 hr</td>
<td></td>
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<tr>
<td>PAL</td>
<td>189.2</td>
<td>24.0</td>
<td>165.2</td>
</tr>
<tr>
<td>TAL</td>
<td>26.9</td>
<td>2.5</td>
<td>24.4</td>
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<tr>
<td>PAL-TAL</td>
<td></td>
<td></td>
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<tr>
<td>24 hr</td>
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<td>TAL</td>
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<tr>
<td>PAL-TAL</td>
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Per Cent Activity Recovered

<table>
<thead>
<tr>
<th></th>
<th>8 hr</th>
<th>24 hr</th>
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<tbody>
<tr>
<td>PAL</td>
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<tr>
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<tr>
<td>PAL-TAL</td>
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Fig. 3. Elution of PAL and TAL activities and protein from an agarose column. Enzyme (14 ml, 8.41 units) was placed on an 8.5 x 50 cm agarose column (6). Fractions (15 ml) were collected and assayed as described in "Materials and Methods."
LITERATURE CITED


