L-Phenylalanine Ammonia-lyase. III. Properties of the Enzyme from Maize Seedlings*

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ABSTRACT: L-Phenylalanine ammonia-lyase (EC 4.3.1.5) has been partially purified from shoots of maize harvested 5 to 7 days after germination. Only a single species of the enzyme was detected on Sephadex G-200 chromatography and sucrose density gradient centrifugation. The size of the enzyme was similar to that of the major species isolated from the potato tuber (Havir, E. A., and Hanson, K. R. (1968a,b), *Biochemistry 7*, 1896, 1904 (this issue; papers I and II)): sedimentation constant 11.8 S, Stokes' radius 63.5 Å (significantly lower than that of the potato enzyme), and provisional mol wt 306,000. As in the case of the potato enzyme the apparent K_m and V_{max} for cinnamate formation increased with increasing substrate concentration. The observed relationship varied with the method of preparation and it seemed that the shorter the time spent in purification the lower the tangential K_m values at low and high substrate concentrations.

L-Phenylalanine ammonia-lyase catalyzes the elimination of ammonium ions from L-phenylalanine to give *trans*-cinnamate. In this paper we report certain properties of the enzyme from dark grown maize seedlings and compare these with the properties of the major species of the enzyme obtained from light-exposed slices of potato tuber (see Papers I and II: Havir and Hanson, 1968a,b).

Experimental Section

Assay. One unit (U) of enzyme is defined as the amount of enzyme catalyzing the formation of cinnamate at an initial rate of 1 μmole/min at 30° under the standard assay conditions (see Havir and Hanson, 1968a).

Growth of Plants and Preparation of Acetone Powders. The distribution and development of phenylalanine ammonia-lyase activity in maize seedlings was investigated in order to determine the best tissue to use as a source of the enzyme. Seedlings were maintained in a growth chamber under 14 hr of light (1200 ft-candles) and 10 hr of darkness; day temperature 30°; night temperature 24°. At various times after germination 30–40 seedlings were used to prepare acetone powders which were immediately extracted with borate buffer and assayed. Activity was detectable the first day after germination and increased rapidly attaining a value of 17 mU/seedling (excluding the seed) after 5 days. The specific activity of the enzyme obtained from the whole plant also increased from the first day after germination to the fourth and a plateau level of about 5.2 mU/mg of protein was then maintained for at least 4 days. The specific activity was 2.5-fold higher in the roots than the shoots, but the shoots contained 60% of the total activity. The shoots were appreciably easier to harvest and so were customarily used as a starting material for purification. No significant difference was observed in total or specific activity between preparations from dark grown plants and from plants grown under alternating light and darkness.

Acetone Powders for Purification Purposes. Seeds of *Zea mays* (var. saccharata, Bailey, Culture Early Sunglow), the gift of Agway, Inc., Syracuse, N. Y., were soaked for 24 hr in a tap-water solution of $Ca(NO_3)_2$ (0.01 M) and KH_2PO_4 (0.01 M). The seeds were then planted in vermiculite and maintained in the dark at temperatures ranging from 30 to 38°. Dark conditions were employed as a matter of convenience; facilities were not available during the winter months for growing large quantities of seedlings at elevated temperatures in the light. The shoots were harvested by cutting at the level of the vermiculite 5 to 7 days after germination, at which time the first leaf had emerged from the coleoptile and the second leaf was emerging. The fresh tissue could be stored overnight in moist toweling in a refrigerator without loss of extractable activity. The tissue was ground three times with cold (−10°) acetone (5 ml/g of tissue) in a Waring Blendor. The powder was dried at room temperature, sieved through a 20-mesh screen, weighed, and stored at −15° overnight. There was a 20–40% loss in extractable activity if the powder was stored for a week.

Enzyme Purification. All procedures (see Table I) were performed at 0–4°. The extraction with borate buffer of the acetone powder, the protamine sulfate treatment of the extract, and fractionation with $(NH_4)_2SO_4$ were carried out essentially as for the potato enzyme except that reduced glutathione did not appear to be beneficial and therefore was omitted. As much as 50% of the activity was lost upon storing the enzyme from

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TABLE I: Partial Purification of L-Phenylalanine Ammonia-lyase from Maize Shoots.[a]

Fractionation Step	Total Protein (mg)	Activity: Total (U = μmole/min)	Activity: Recov (%)	Activity: Sp Act. (mU/mg)	Purifcn/ Step (-fold)
1. Extraction	5444	28.7	100	5.27	
2. Protamine treatment and $(NH_4)_2SO_4$ fractionation (35–55%)	2036	23.6	82	11.6	2.1
3. Bio-Gel P-10 column	1542	20.3	71	13.2	1.1
4. $(NH_4)_2SO_4$ fractionation (35–55%)	761	19.3	67	25.4	1.9
5. DEAE Sephadex column and $(NH_4)_2SO_4$ fractionation (0–50%)	78.3	8.2	28	105	4.1
				Total purification	20-fold

[a] Seedlings (4000 6-day-old, 1.5 kg fresh weight) gave 100 g of acetone powder.

step 2 for 18 hr at 0°. The $(NH_4)_2SO_4$ precipitate, therefore, was dissolved in a minimal volume of 0.1 M borate (Na^+) buffer (pH 8.7) and the solution was immediately passed through a Bio-Gel P-10 column (4.8 × 13 cm) which was equilibrated and eluted with the same buffer. At this stage the ammonia-lyase was sufficiently stable for the active fractions to be combined and stored overnight at 0° with negligible loss of activity.

The $(NH_4)_2SO_4$ precipitate from step 4 was suspended in a minimal volume of 0.1 M borate (Na^+) buffer (pH 8.7) and the protein was fractionated by DEAE Sephadex A-50 chromatography. For the amount of protein indicated in Table I, three columns (2.5 × 25 cm) were employed and 250 mg of protein was added to each column. After applying the sample, each column was washed with 0.05 M phosphate (K^+) buffer (pH 6.8) until a band of protein was eluted. This band usually contained from 20 to 35% of the activity applied to the column. After the first band of protein had washed through, the adsorbed protein was removed by gradient elution using a fixed volume (150 ml) of 0.05 M phosphate (K^+) buffer (pH 6.8) in the mixing chamber and 0.5 M buffer in the reservoir; flow rate 0.5 ml/min. The protein bands which passed directly through the columns were combined, concentrated by $(NH_4)_2SO_4$ precipitation, and rechromatographed on a fourth column. The fractions containing the highest specific activities from all four columns were combined and concentrated by $(NH_4)_2SO_4$ precipitation, and the precipitate was again suspended in a minimal volume of 0.1 M borate (Na^+) buffer (pH 8.7). The enzyme obtained by this procedure is referred to below as a DEAE Sephadex preparation. The preparation could be stored for a period of weeks either at −15 or 0° without showing any loss of activity when assayed according to the standard assay procedure.

In the initial studies of the enzyme the extract was not chromatographed on a DEAE Sephadex column. Instead, after step 2, the enzyme was reprecipitated with $(NH_4)_2SO_4$ (32–43% saturation), suspended in 0.1 M borate (Na^+) buffer (pH 8.7) containing 2 mg/ml of reduced glutathione, and passed through a Bio-Gel P-100 or a Sephadex G-100 column prepared in 0.1 M pyrophosphate (Na^+) buffer (pH 8.7). Enzyme thus prepared is referred to as a Bio-Gel P-100 or a Sephadex G-100 preparation. All such preparations lost activity on storage at a rate of 2–7%/day. The Bio-Gel and Sephadex employed were purchased from Bio-Rad, Richmond, Calif., and Pharmacia, New York, N. Y., respectively.

Results

Molecular Size. The sedimentation constant for the Sephadex G-100 preparation of the enzyme was determined by sucrose density centrifugation in the same instrument and under the same conditions as employed with the phenylalanine ammonia-lyase from potato. The mean value and standard error of the mean calculated for the maize enzyme is $s^{0.725}_{20,w} = 11.76 \pm 0.07$ S ($N = 3$) with beef liver catalase as the reference protein. For the major species of the enzyme from potato $s^{0.725}_{20,w} = 11.93 \pm 0.11$ S ($N = 6$). The probability is roughly four out of ten that two such samples drawn from the same population would differ in their means by at least the observed amount (7 DF, $t = 0.92$). The technique thus failed to show any significant difference between the two enzymes.

The Stokes' radius, a, for the Sephadex G-100 preparation of the enzyme was determined as for the potato enzyme. Two runs were made using bovine glutamate dehydrogenase, bovine catalase, and yeast alcohol dehydrogenase as standards and pyrophosphate as the buffer. The pore size, r, of the column (internal volume 70.4 ml, void volume 47.2 ml) was calculated giving each observation equal weight and using 71 Å, as the Stokes' radius for glutamate dehydrogenase: $r = 256 \pm 3$ Å ($N=5$). Essentially the same value was obtained without using the glutamate dehydrogenase results in the calculation. For the ammonia-lyase $a/r = 24.8 \pm 0.1\%$ ($N = 2$) and therefore $a = 63.5 \pm$

0.9 Å. This value is appreciably lower than the value of 70.3 ± 1.8 Å found for the potato enzyme using the same buffer. The potato enzyme was eluted in the same position as glutamate dehydrogenase whereas the enzyme from maize was displaced 4 ml behind the dehydrogenase peak. In both cases the enzyme was eluted well before catalase (a = 52 Å).

The above data lead to a provisional value for the molecular weight of the maize enzyme of 306,000 (reference protein, beef liver catalase), *cf.* 340,000 calculated for the potato enzyme on the basis of a = 70 Å (pyrophosphate buffer).

Kinetic Properties. As in the case of the major species of phenylalanine ammonia-lyase from potato, the relationship between the initial velocity of cinnamate formation, v, and substrate concentration, [S], could not be described by the Michaelis–Menten equation. The observed relationship, however, varied according to the conditions of purification. In Figure 1 plots of v against v/[S] are shown for the potato enzyme (curve P) and for different samples of the enzyme from maize (curves A–D). If the Michaelis–Menten equation had been obeyed, straight-line relationships would have been observed. Confidence limits are similar to those calculated for the experiments with the potato enzyme. The fact of departure from Michaelis–Menten kinetics is therefore firmly established.

The several curves may be characterized, in part, by the tangential values of the K_m under saturating conditions of substrate (*i.e.*, at V_{satn}) and at low substrate concentrations. For the maize enzyme the ratio of these two constants was roughly the same for the different curves (threefold) and appreciably less than that for potato (6.8-fold). The tangential K_m values for the maize enzyme were all higher than the corresponding value for the potato enzyme.

The different samples of enzyme were obtained as follows: (A) Bio-Gel P-100 purified enzyme; the preparation occupied 3 days and the kinetic measurements were made on the fourth day. (C) The same preparation as A after storage for 5 further days at −10°; during this period an 11% loss in activity, as measured by the standard assay, took place. (B) DEAE Sephadex purified enzyme; the preparation took 4 days and the kinetic measurements were made on the fifth day. (D) Two preparations of DEAE Sephadex purified enzyme; the purification steps were carried out over a period of several weeks (the experimental points for both preparations are shown). All samples which had been passed through the DEAE Sephadex column, when assayed by the standard procedure, did not lose activity to a significant extent on storage at −15°. It is not known, however, whether the shape of the curve changes in such stable preparations and whether preparation B on ageing would have resembled the preparations D. The experiments suggest, however, that the shorter the time spent in purification the lower the tangential K_m values.

The inhibitory effect of D-phenylalanine on the stable enzyme preparation B was investigated, although in less detail than in the study of the potato enzyme. The plots of v against v/[S] at the various inhibitor

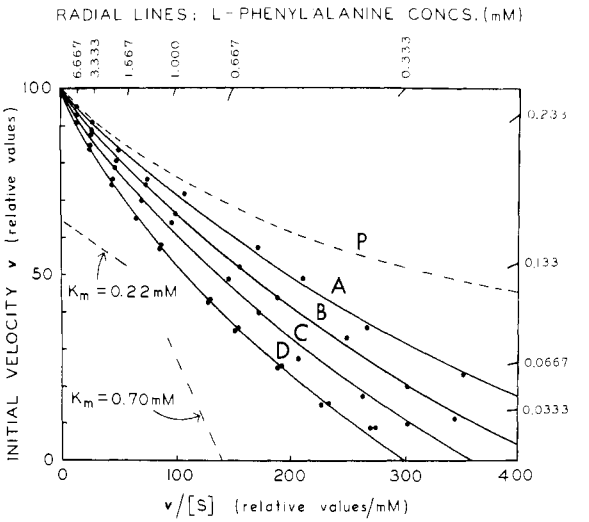

FIGURE 1: Initial velocity of cinnamate formation as a function of L-phenylalanine concentration. Temperature 30°, pH 8.7, 0.033 M borate (Na^+) buffer. The values of v were initially expressed relative to the standard assay, the saturation velocity for each curve was then estimated and the curves were redrawn relative to V_{satn} = 100. In general the points shown are the means of three observations. The samples of enzyme had specific activities of 40–106 mU/mg. Curve P shows the results for the potato enzyme (taken from Figure 5 of Havir and Hanson, 1968b); for curves A–D, see text. The tangential K_m values at V_{satn} and at low [S] differed by a factor of about 3 in cases A–D and 6.8 in case P. The values at V_{satn} were (P) 0.26, (A) 0.37, (B) 0.43, (C) 0.55, and (D) 0.70.

concentrations (0.67, 1.0, 1.67, and 2.67 mM) were approximately rectilinear and K_i for D-phenylalanine was found from the slope of the plot of K_m against [I] to be roughly 0.7 mM. However, $^{\circ}K_m$ for this plot was less than the tangential value for K_m at $\tilde{V}_{satn}$, and $\tilde{V}_{max}$ fell below $\tilde{V}_{satn}$ and then increased significantly with [I]. The kinetic properties of preparation B are thus more complex than in the case of the potato enzyme. Such complexity might be expected if preparations A–D are all mixtures of protein molecules with slightly differing kinetic properties.

Discussion

The present study establishes that the molecular weights of the enzyme from maize and that of the major species from the potato tuber are of the same order of magnitude. In view of the uncertainties in the Stokes' radii determinations (see Havir and Hanson, 1968a) and the possibility that the radii are biased by the shape of the protein, the molecular weights may well be closer than the calculations indicate. The simplest interpretation, and the one which we favor, is that in the two cases single proteins are involved, that both have the same type of quaternary structure, but that they differ slightly in the number and nature of the amino acid residues.

Two further possibilities must be considered. The Stokes' radii and therefore the molecular weights may differ by more than the calculated values and the two proteins would then be likely to differ in quaternary

structure. Quite minor changes in amino acid composition could lead to changes in the orientation of the protomer binding sets and therefore to changes in the number of protomers per molecule (Hanson, 1966a). An increase from six to eight protomers would imply an increase in molecular weight of *ca.* 100,000 whereas the calculated difference is 40,000 or less. The smaller the assumed protomer size the more plausible this explanation becomes. Lastly, in both cases mixtures of isozymes could be present with the isozymes differing slightly in molecular weight and shape so that average values are being compared. For reasons given in paper II (Havir and Hanson, 1968b) we do not think that the major species of potato enzyme is a mixture of isozymes, but the variability of the kinetic properties of the maize enzyme could be explained in terms of isozymes of differing stability. An alternative explanation for this variability is considered in the following paragraph.

The trend in kinetic properties shown in Figure 1 suggests that one form of the enzyme is initially isolated and that during purification or storage modifications of the structure occur which lead to altered kinetic behavior. Analogous changes in the kinetic properties of maize nitrate reductase were reported by Beevers *et al.* (1964). A number of agents could be responsible for the variability, *e.g.*, a proteolytic enzyme could be present which attacks some peptide bond without disrupting or completely inactivating the enzyme, also quinones derived from absorbed phenols by the action of phenolase could attack amino acid side chains. Inactivation or modification of one or more active sites could well influence the cooperative interactions between the remaining active sites. Alternatively, intramolecular rearrangements involving disulfide exchange reactions (see Pontremoli *et al.*, 1967) might occur.

A major purpose for studying the maize enzyme was to examine a tissue which might contain a phenylalanine ammonia-lyase with different properties from those of the potato enzyme. The two plants belong to separate, though relatively late, branches of the phylogenetic tree (a monocot and a dicot, respectively); also extensive lignification occurs in maize shoots, whereas in light-exposed potato tuber slices chlorogenic acid (3-*O*-caffeoylquinic acid) accumulates (Zucker, 1965; Hanson, 1966b). The finding that the major species of the enzyme in both tissues are similar may well be significant and suggests certain further avenues for investigation. If quaternary structure is not merely a packaging device, but is related to the enzyme's function, then constancy in quaternary structure throughout the phylogenetic tree would suggest constancy in function. By function we have in mind specific types of allosteric interactions, or associations with other proteins. It is clearly feasible to extend the taxanomic investigation of Young *et al.* (1966) and compare the molecular size of the phenylalanine ammonia-lyases from higher plants (Young *et al.*, 1966; Young and Neish, 1966) and fungi (Power *et al.*, 1965; Ogata *et al.*, 1966; Austin and Clark, 1966). Constancy in size would suggest constancy in quaternary structure, and such constancy would imply that a particular quaternary structure once it had appeared possessed such definite functional advantages that no further changes in quaternary structure were biologically successful.

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