THE ENZYMOLOGY OF LYSINE BIOSYNTHESIS IN HIGHER PLANTS

The occurrence, characterization and some regulatory properties of dihydrodipicolinate synthase

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1. Introduction

Lysine, an essential amino acid for human nutrition, is frequently the limiting amino acid in plant protein in terms of nutritional quality. It is the limiting amino acid in all the important cereal grains [1]. The biosynthesis of this amino acid in higher plants and its regulation are of considerable practical importance.

In vivo studies of the incorporation of radioactive precursors into lysine indicate that higher plants utilize the diaminopimelate pathway [2,3]. For definitive proof of the existence of this route, the individual enzymes for each step must be demonstrated. Diaminopimelate decarboxylase (EC 4.1.1.20) which catalyses the final step in the sequence, has been found in a large number of higher plant tissues [4–7]. It is the only enzyme in the pathway which has been extensively characterized [4–7]. In addition the presence of dihydrodipicolinate synthase (EC 4.2.1.52) in maize seedlings has been reported [8]. This enzyme catalyses the first step in the pathway, the condensation of ASA and pyruvate to dihydrodipicolinate.

The occurrence of dihydrodipicolinate synthase in a number of higher plants is now demonstrated and some of the properties of the enzyme obtained from wheat germ are reported.

Abbreviations: ASA, aspartate-β-semialdehyde; Tricine, *N*-tris[hydroxymethyl]methylglycine; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DTE, dithioerythritol

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2. Materials and methods

2.1. *Preparation of ASA*

ASA was synthesized by the method of Black and Wright [9]. The purity of the final product was determined by paper chromatography and a ninhydrin spray. The amount of L-ASA produced was assayed by homoserine dehydrogenase (EC 1.1.1.3) using a partially purified yeast preparation [10].

2.2. *Dihydrodipicolinate assay*

The *o*-aminobenzaldehyde method developed by Yugari and Gilvarg [11] was used with slight modifications. The reaction was terminated by the addition of 10% trichloroacetic acid, the denatured protein removed by centrifugation, and the color due to the adduct between dihydrodipicolinate and the *o*-aminobenzaldehyde allowed to develop for 60 min at room temperature. The A_{550} was measured in a spectrophotometer. One unit of enzyme activity is defined as an A_{550} increase of 0.001 unit/min.

2.3. *Plant materials and preparation and assay*

Maize seeds, variety Pioneer 3780, and red kidney bean seeds were germinated in vermiculite and harvested after 6–8 days. The maize shoot and coleoptile were removed and ground in 0.2 M Hepes buffer, pH 7.4, containing 5 mM DTE. The slurry was passed through cheese-cloth and centrifuged at 23 000 × *g* for 30 min. The supernatant solution was decanted and made to 75% saturation with solid

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$(NH_4)_2SO_4$. The precipitate was collected by centrifugation and dissolved in 0.02 M Hepes, pH 7.4, containing 20% ethylene glycol and 5 mM DTE. If necessary the protein solution was clarified by centrifugation before use as the enzyme source. Wheat germ was extracted and prepared in the same manner.

All other plant materials, purchased in a local market, were blended in the extracting buffer above and then treated by the above protocol. The standard reaction mixture used contained the following in final vol. 3 ml: 67 mM Tris buffer, pH 8.5; 10 mM Na pyruvate; 4 mM DL-ASA; 1.5 mg *o*-aminobenzaldehyde; enzyme preparation containing 2–3 mg protein. The usual incubation time was 2 h and the reaction stopped by the addition of 1 ml 10% trichloroacetic acid. The *o*-aminobenzaldehyde was made up in 95% ethanol as a 1.5% solution just prior to use. The DL-ASA after synthesis was kept as a 0.8 M solution in 1 N HCl frozen at −20°C. It is stable for many months under these conditions. It was not necessary to neutralize the ASA before addition since the amount added to the reaction mixture is so small that it does not affect the pH drastically. The final pH of the assay mixture was between 8.0 and 8.3 which coincides with the optimum pH for the reaction.

2.4. *Partial purification of the wheat germ enzyme*

Wheat germ, 20 g, was ground in a mortar and pestle with 100 ml 0.2 M Hepes buffer, pH 7.4, containing 5 mM DTE. After passage through cheesecloth the filtrate was centrifuged at 23 000 × *g* for 30 min and the supernatant decanted through glasswool. For each 100 ml supernatant, 15 ml 1% protamine sulfate solution was added, and the resulting precipitate removed by centrifugation. The supernatant solution was then treated with a saturated $(NH_4)_2SO_4$ solution so as to obtain the protein precipitating between 35% and 50% saturation. This precipitate was collected by centrifugation and dissolved in 0.01 M K phosphate buffer, pH 7.5. In experiments involving this fraction the standard reaction mixture consisted of the following in final vol. 1 ml: 100 mM Tris-HCl buffer, pH 8.5; 40 mM Na pyruvate; 4 mM DL-ASA; 0.5 mg *o*-aminobenzaldehyde; enzyme preparation containing up to 3 mg protein. Incubation time was usually for 30 min and the reaction stopped by the addition of 0.5 ml 10% trichloroacetic acid.

2.5. *Protein determination*

The amount of protein in the enzyme fractions was measured spectrophotometrically [12].

2.6. *Chemicals*

All chemicals were obtained from commercial sources and were the highest grade available.

3. Results

3.1. *Limited survey of enzyme distribution*

A number of plant materials were prepared and assayed for the enzyme activity as described above. Representatives of 6 families including both dicotyledonous and monocotyledonous species were tested. The results are summarized in table 1. Activity was present in every species tested. Because of the convenience of preparation and availability of large amounts of uniform material, wheat germ was selected for more detailed examination.

3.2. *Properties of the wheat germ enzyme*

3.2.1. Rate as a function of time

Previous workers have reported that there is a definite lag in the rate of the reaction against time before linearity is reached when using the *o*-aminobenzaldehyde assay [8,11]. When an aliquot of the 0–75% saturated $(NH_4)_2SO_4$ protein fraction was passed through a short Sephadex G-25 gel filtration column, and the protein eluate assayed for activity,

Table 1
Survey of distribution of synthase activity

Plant material	Family	Enzyme activity (spec. act.)
Wheat germ	Graminae	3.22
Corn seedling	Graminae	2.95
Bean shoot	Leguminosae	1.15
Cabbage leaf	Cruciferae	0.96
Spinach leaf	Chenopodiaceae	0.73
Potato tuber	Solanaceae	3.75
Squash fruit	Cucurbitaceae	3.04

The assay method and reaction mixture were as described in Materials and methods. The assay time was 2 h and the 0–75% saturated $(NH_4)_2SO_4$ protein fraction was the enzyme source

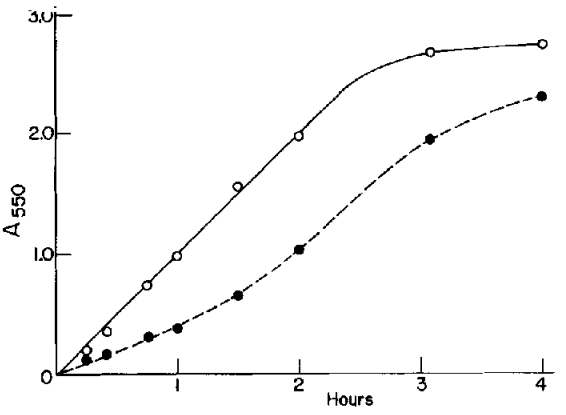

Fig.1. The progress of the synthase reaction with time. (o-o-o) 0–75% $(NH_4)_2SO_4$ fraction (1.7 mg protein/ml reaction mixture). (•-•-•) Eluate from the gel filtration column (1.6 mg protein/ml reaction mixture). A 1 ml sample withdrawn at an appropriate time from a 9 ml batch of the standard reaction mixture described in Materials and methods was used to determine each point.

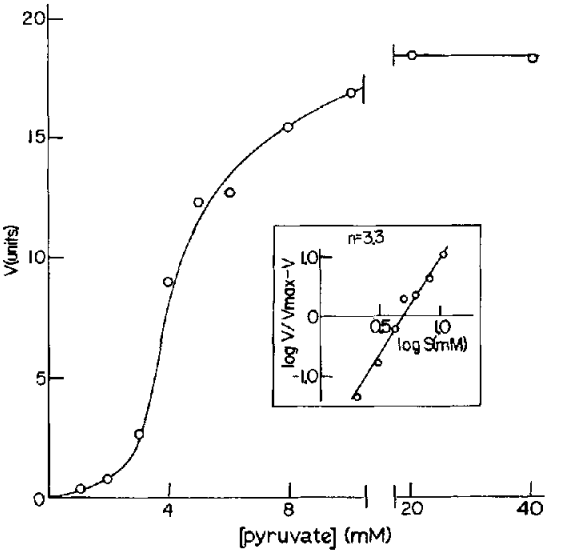

Fig.2. Reaction rate as a function of pyruvate concentration. The reaction conditions were those described in Materials and methods except that the reaction time was 30 min. The standard reaction mixture (1 ml final vol.) was used, except that the amounts of pyruvate added were varied, as noted. Of the 35–50% $(NH_4)_2SO_4$ fraction, 0.8 mg was used for each determination.

such a lag was observed. A linear rate was found, however, if the 0–75% fraction was used directly as the enzyme source. This rate continued until the ASA was completely utilized (fig.1).

3.2.2. pH optimum

Using the 0–75% $(NH_4)_2SO_4$ fraction as enzyme source a rather sharp optimum in activity was found between pH 7.9 and pH 8.2 with Tris–HCl as the buffer. The buffer used had a considerable effect. At pH 7.5 the activity found with Tris–HCl was 3 times that found with a phosphate buffer.

3.2.3. Effect of substrate concentration

Only L-ASA acted as a substrate. The D-form was totally inactive. The K_m for L-ASA was determined using either DL- or L-ASA as substrate. At higher concentrations both DL-ASA and L-ASA were inhibitory. The K_m in each case was calculated by use of a computer program for linear regression analysis of a straight line form of the Michaelis-Menten equation. A K_m for L-ASA of 1.0 mM was obtained using L-ASA as substrate, and a K_m of 2.1 mM with DL-ASA as substrate. The activity as a function of the pyruvate concentration is shown in fig.2. There is a very sigmoid shape to the curve indicating a high degree of cooperativity between pyruvate and the enzyme. The Hill plot gives a slope of 3.3 suggesting at least 3 and possibly 4 binding sites for pyruvate. The $[S]_{0.5}$ value is 4.2 mM.

3.2.4. Inhibition by lysine and other amino acids

Low concentrations of L-lysine strongly inhibited the reaction (table 2). This appeared to be a very specific property since, of the amino acids tested, only L-lysine had such a marked effect. L-Arginine also gave a significant inhibition at the high concentration tested (table 2). D-Lysine, DL-diaminopimelic acid and L-ornithine had comparatively little effect. The inhibition by L-lysine was examined in greater detail. From fig.3 one can obtain an $[I]_{0.5}$ of 11 μM, which may be equivalent to the K_i [13], although the present data are not sufficient to make this judgement. D-ASA also acts as an inhibitor at high concentrations. At the level present in the reaction, i.e., equimolar with L-ASA, there is only a small inhibitory effect.

Table 2
Effect of various amino acids on the synthase reaction

Amino acid added	Concn. (mM)	% Inhibition
None	–	0
L-Lysine	1.0	97
L-Lysine	0.1	97
D-Lysine	1.0	14
D,L-Diaminopimelate	1.0	0
L-Ornithine	1.0	20
D,L-Threonine	1.0	3
L-Methionine	1.0	3
L-Homoserine	1.0	13
L-Arginine	1.0	69
L-Aspartate	1.0	0

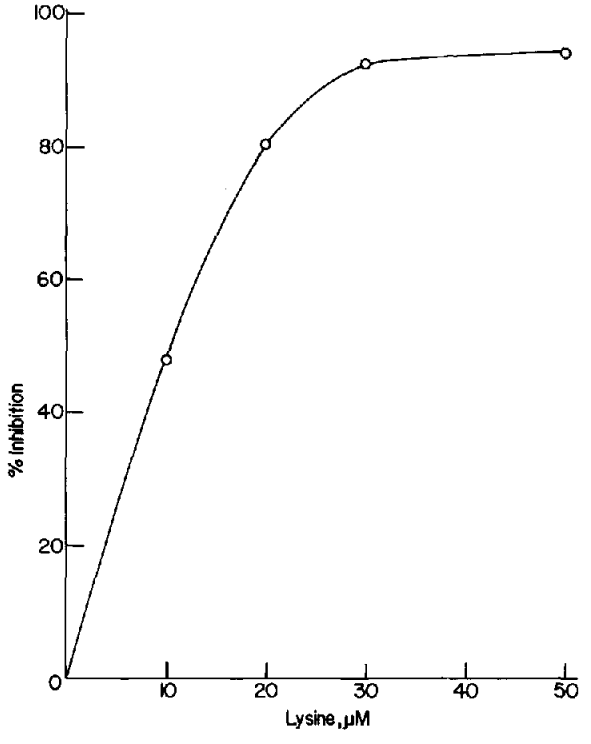

Fig.3. Inhibition by L-lysine. Of the 35–50% $(NH_4)_2SO_4$ fraction, 0.9 mg was used in each determination. The assay conditions are described in Materials and methods except for the addition of the designated amounts of L-lysine and an incubation time of 60 min.

Table 3
Calculation of the provisional molar extinction coefficient of the reaction product

L-ASA (mM)	A_{550}	E ($M^{-1} \times cm^{-1}$)
0.27	0.724	2680
0.53	1.403	2650
0.80	1.942	2430
1.07	2.605	2430
	Avge	2550

The assay mixture was as described in the text except for variations in the amount of L-ASA. The final volume was 1 ml. Incubation time was 3 h. The 35–50% saturated $(NH_4)_2SO_4$ fraction was the enzyme source

3.3. *Provisional molar extinction coefficient of adduct*

The calculation of a provisional molar extinction coefficient for the *o*-aminobenzaldehyde-dihydrodipicolinate adduct appears possible due to the linear rate with time and the complete utilization of L-ASA with time. Table 3 shows the final absorbance obtained at 4 different levels of L-ASA. A molar extinction coefficient (ϵ_{550}) of 2550 can be calculated from this data. This offers the possibility of expressing future results in terms of actual amounts of product rather than in arbitrary units.

4. Discussion

It is now definitely proven that dihydrodipicolinate synthase is of common occurrence in higher plants. The pH optimum is quite similar to that found for the enzyme in *E. coli* [11] and *Bacillus licheniformis* [14]. There was no activity with maize seedling preparations at pH 6.5 in phosphate buffer, contrary to a previous report [8].

The wheat germ enzyme shows a strong positive cooperativity with pyruvate. This is an unusual property. Although pyruvate cooperativity was originally proposed for the enzyme from *B. licheniformis* [14] and *B. subtilis* [15] (though not from other bacteria) later findings have raised doubts about this conclusion [16,17]. The wheat germ synthase is also very sensitive to lysine. Although the maize enzyme was also reported to be inhibited by lysine

[8] at pH 6.5, a much higher concentration was needed for complete inhibition. The combination of pyruvate cooperativity and lysine inhibition appears to be unique to this enzyme. The two bacterial enzymes for which pyruvate cooperativity has been proposed are not sensitive to lysine [15,17], and none of those inhibited by lysine exhibit pyruvate site cooperativity. The inhibition of the reaction by L-arginine, acting as a lysine analogue, could be of physiological significance.

This combination of properties indicates that the synthase is an important site for regulatory control of lysine biosynthesis in wheat. The pyruvate concentration must first be above a certain level for the enzyme to function. Once this minimal value is reached only a relatively small increase in concentration is necessary for maximal activity. The strong inhibition by small amounts of lysine explains why free lysine is so rarely found in plant extracts. As soon as lysine begins to accumulate it will shut down this branch of the pathway from aspartate without interfering with the synthesis of the other amino acids arising from this common precursor.

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