Phytochemistry, Vol. 20, No. 12, pp. 2651–2655, 1981.
Printed in Great Britain.

0031-9422/81/122651-05 $02.00/0

SPINACH LEAF DIHYDRODIPICOLINATE SYNTHASE: PARTIAL PURIFICATION AND CHARACTERIZATION

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(*Received* 28 *February* 1981)

Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach dihydrodipicolinate synthase; lysine biosynthesis.

Abstract—The first enzyme unique to lysine biosynthesis in higher plants, dihydrodipicolinate synthase, has been partially purified from spinach leaves, using ion exchange chromatography, hydrophobic interaction chromatography and gel filtration. The spinach enzyme is moderately stable to short-term exposure to heat, in contrast to the pea leaf enzyme, but is unstable on storage even at −20°. Thiol reagents interfere with the colorimetric assay used, and so cannot be routinely used to stabilize the enzyme, which has an active sulphydryl group. The MW of the enzyme is 115000 (gel filtration). Lysine is a potent inhibitor with an $I_{(0.5)}$ of 20 μM, whilst the lysine analogue *S*-β-aminoethylcysteine has an $I_{(0.5)}$of 400 μM. The K'_m for aspartic-β-semialdehyde was determined to be 1.4 mM, but this compound demonstrated marked substrate inhibition at concentrations above 7 mM, increasing the apparent $S_{(0.5)}$for the second substrate, pyruvate.

INTRODUCTION

Higher plants synthesize lysine from aspartate via the diaminopimelate pathway [1]. It has been shown that aspartate-derived amino acids, including lysine, are synthesized by isolated chloroplasts from ^{14}C-labelled aspartate in the light [2], and the enzymes dihydrodipicolinate (DHDP)† synthase (EC 4.2.1.52) and diaminopimelate decarboxylase (EC 4.1.1.20) have been shown to be wholly located in the chloroplasts of green leaves [3, 4]. These enzymes catalyse respectively the first and last steps in the branch of the aspartate pathway that is unique to lysine synthesis.

Lysine synthesis is strictly regulated by feedback regulation in isolated chloroplasts [2] and two sites of inhibition have been demonstrated *in vitro*. Aspartate kinase (EC 2.7.2.4) from most plants is at least partially inhibited by lysine and DHDP synthase from wheat germ has been shown to be highly sensitive to inhibition by low concentrations of L-lysine [5].

In view of the potential importance of DHDP synthase as a regulatory step in lysine biosynthesis, and the close association of lysine synthesis with chloroplasts and the photochemical process, an investigation has been made of the properties of the enzyme from green tissue, specifically the leaves of spinach (*Spinacia oleracea* L.). The only previous report of the purification and characterization of a plant DHDP synthase was that from wheat germ [5].

RESULTS

Assay procedure

In the initial stages of this investigation, the assay procedure described by [5] was used, in which the reaction is stopped by the addition of trichloroacetic acid. It became apparent that a variety of compounds interfered with the colour development in this assay, without necessarily affecting the enzymic activity. This could be shown by duplicate experiments in which a compound was (a) present throughout the incubation period, and (b) added at the end of the incubation time. For example, it was found that ASA concentrations above 5 mM significantly reduced the final absorbance value when added at the end of the assay. To try and circumvent this problem the procedure of [6] was tested, where the incubation was terminated by the addition of a citrate–phosphate buffer at pH 5.0. With this procedure ASA had very much less effect on colour formation, though a slight effect was still noted, and this assay procedure was adopted for all subsequent work. Another advantage of this procedure was found, in that the absorbance was stable for a period of several hours, following a development of 2 hr. The previously used assay gave an absorbance that rapidly decreased after the peak value was obtained.

Even with this alteration, some compounds still affected colour development. As can be seen from Table 1, thiol reagents, in particular dithioerythritol, markedly inhibited colour formation. Ascorbate had a similar but lesser effect, whilst in contrast ethylene glycol increased the final absorbance value. Table 1 also shows the effect of *p*-hydroxymercuribenzoate, which at 1 mM concentration causes over 90% inhibition of enzyme activity without having any effect on the chromophore. This inhibition is indicative of an active sulphydryl group on the enzyme, which could probably be stabilized by thiol

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† Abbreviations: DHDP, dihydrodipicolinate; ASA, aspartic-β-semialdehyde; *o*-ABA, orthoaminobenzaldehyde; SAM, *S*-adenosyl methionine; HSDH, homoserine dehydrogenase (EC 1.1.1.3); AEC, *S*-2-aminoethyl-L-cysteine.

Table 1. Effect of thiol reagents and other compounds on enzyme activity and colour development

Addition	Concentration (mM)	Relative activity (%)	
		Present throughout	Added at end
None	—	100	
Dithioerythritol	0.5	26	24
β-Mercaptoethanol	1.0	27	30
Glutathione (reduced)	1.0	67	73
Ascorbic acid	5.0	82	86
Ethylene glycol	10% (v/v)	106	116
p-Hydroxymercuribenzoate	1.0	9	102

The compounds indicated were added to the standard assay mixture as described, at the final concentrations shown. Duplicate assays were run in which the compound was added at the end of the incubation time.

reagents. However, the effects of these reagents are such that enzyme preparations containing dithioerythritol or mercaptoethanol have to have these compounds removed prior to assay.

There is a significant non-enzymic absorbance in the assay, which increases with incubation time and the presence of phosphate or tricine. Thus it was necessary to routinely run a reagent blank. With this precaution, the net absorbance was linearly related to time of incubation up to 30 min and enzyme concentration, though there was significant deviation from linearity if the absorbance exceeded 1.0. The pH optimum for the spinach and pea leaf enzyme is similar to that found for the wheat germ enzyme [5].

Purification protocol

The purification protocol developed for the enzyme is described in the Experimental section and a typical purification is shown in Table 2. Chromatography on Octyl-Sepharose and Sephadex G-150 are the steps that significantly increase specific activity, but attempts to apply the redissolved $(NH_4)_2SO_4$ precipitate directly to Octyl-Sepharose were unsatisfactory. Desalting and ion-exchange chromatography were necessary for optimal results. When applying the enzyme to Octyl-Sepharose the pH of the solution and the equilibration buffer was about pH 7.2. Adjusting the pH to 8.0 almost totally abolished binding of the enzyme to the column, even though washing the column with equilibration buffer at pH 8 after the enzyme was bound did not release appreciable activity. The enzyme was bound very much more strongly to Phenyl-Sepharose, requiring the presence of 20% ethylene glycol in the elution buffer for complete release. Overall purification using Phenyl-Sepharose was no better than that achieved with Octyl-Sepharose.

The pea leaf enzyme could be partially purified using a similar procedure with the exception that the enzyme precipitated between 0 and 40% saturation with $(NH_4)_2SO_4$.

Both pea and spinach enzymes were relatively unstable in partially purified form, even on storage at −20°. The spinach enzyme lost *ca* 25% of the activity per week when stored as a 10 mg/ml solution at −20°, and more dilute solutions were very much less stable. The presence of up to 20% (v/v) ethylene glycol had little effect on stability.

The spinach enzyme was very little affected by short-term exposure to heat, as shown in Table 3. In contrast, the pea leaf enzyme was rapidly inactivated. In some batches of spinach a significant increase in activity was obtained by heating the protamine-sulphate treated extract to 60° for 1–2 min and subsequent chilling. This result was inconsistent in that for many of the spinach preparations there was no enhanced activity. This may reflect differences in the content of some heat-labile inhibitor or inactivator between batches of leaves.

Table 2. Purification of spinach leaf dihydrodipicolinate synthase

Fraction	Volume (ml)	Protein (mg/ml)	Specific activity (units/mg protein)	Recovery (%)	Purification
Crude extract	1500	4.0	9.6	—	—
Protamine sulphate	1600	3.1	12.1	104	1.3
35–55% satn. $(NH_4)_2SO_4$	140	25.6	15.7	97	1.6
Cellex D chromatography	110	14.0	20.4	54.5	2.1
Octyl-Sepharose chromatography	35	4.0	121.0	29.4	12.6
G-150	12	0.6	836.0	10.5	87.1

Table 3. The effect of heat on the spinach and pea leaf enzymes

Source	Time at 60° (min)	Relative activity (%)
Spinach leaf	0	100
	1	105
	2	109
	5	82
Pea leaf	0	100
	1	79
	5	20

Aliquots of the enzymes from the $(NH_4)_2SO_4$ precipitation step, after desalting in buffer A were placed in a water-bath at 60° for the stated times, and then rapidly transferred to an ice-bath. When cool, the samples were clarified by centrifugation and assayed in the standard system as described.

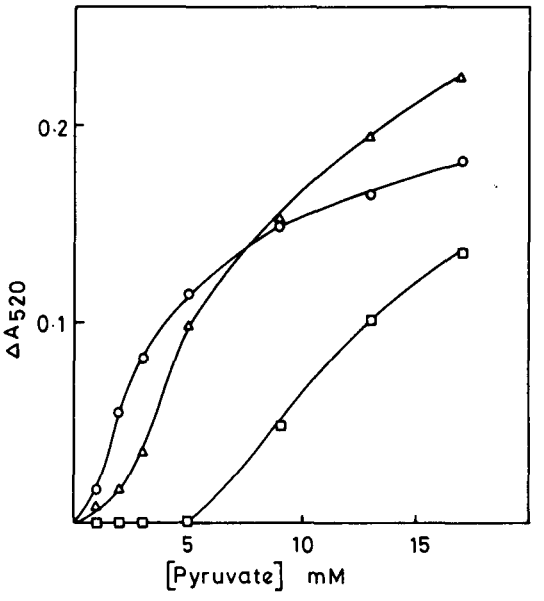

Fig. 1. Reaction rate as a function of pyruvate concentration. Activity was measured in the standard assay system, varying the pyruvate concentration, at three different concentrations of ASA. The reaction mix contained 0.14 mg protein and was incubated for 15 min at 30°. ○——○, 2 mM ASA; △——△, 5 mM ASA; □——□, 10 mM ASA. Each point represents the mean of three replicates.

The MW of the spinach enzyme was determined by gel filtration on calibrated columns of Sephadex G-150 or G-200. The mean of three determinations gave a value of 115 000 ± 5000. The enzyme activity eluted as an almost symmetrical single peak. Gel electrophoresis of the peak tubes produced two bands staining for enzyme activity. Staining for protein revealed that these bands corresponded to the two fastest-running protein bands (four or five bands were usually present), and that they made up only a small proportion of the total protein.

Kinetic properties

The relationship between pyruvate concentration and enzyme activity at three different ASA concentrations is shown in Fig. 1. As the concentration of ASA is increased, the curve displays an increasingly sigmoid shape; there is no activity with 10 mM ASA until the pyruvate concentration exceeds 8 mM. Hill plots of the data give straight lines of slope, 0.93 at 2 mM ASA and 1.81 at 5 mM ASA. Using the direct linear plot [7], estimates of the $S_{(0.5)}$ can be obtained. These are 6 mM at 2 mM ASA, and 21 mM at 5 mM ASA. In this latter case the V_{max} is approximately doubled.

The relationship between ASA concentration and enzyme activity at three different pyruvate concentrations is shown in Fig. 2. At all pyruvate concentrations the activity decreases when the ASA concentration exceeds 7 mM, and this inhibition is not due to an effect on colour formation. Using the direct linear plot, an apparent K_m for ASA of 1.4 mM is obtained at all pyruvate concentrations.

Inhibition studies

L-Lysine has a significant effect, giving almost complete inhibition at 0.1 mM, and having an $I_{(0.5)}$ of 20 uM. D-Lysine, DL-diaminopimelate, L-homoserine, L-threonine and L-alanine had no effect even at 5 mM concentrations, but the lysine analogue AEC was an effective inhibitor, with an $I_{0.5}$ of 0.4 mM. The amino acids arginine and isoleucine inhibit the reaction significantly at 5 mM. SAM has no effect on the enzyme at 0.5 mM concentration. The pyruvate homologue, 2-oxobutyrate gave no apparent activity and is only a very weak inhibitor except at high concentrations [$I_{0.5}$ 40 mM].

The kinetic and inhibitory characteristics of the pea leaf enzyme are very similar to those of the spinach leaf enzyme. The apparent K_m for ASA is 1.0 mM, and the $I_{0.5}$ for L-lysine is 20 μM. At low ASA concentrations (< 3 mM) an apparent $S_{(0.5)}$ for pyruvate of 3.5 mM was obtained.

DISCUSSION

It has previously been shown that in spinach leaves DHDP synthase is wholly localized in the chloroplast [3]. Thus the enzyme we have partially purified is the chloroplast enzyme and its characteristics can be compared to those of the *in vivo* lysine biosynthetic pathway in chloroplasts. The pH optimum is similar to that earlier reported for the wheat germ enzyme [5], and at pH 8.2 is close to the stromal pH of chloroplasts in the light [8].

The colorimetric assay is in many ways unsatisfactory, especially its sensitivity to thiol reagents. Enzymes containing an active sulphydryl group are commonly activated or stabilized by the presence of a thiol reagent, but it has been difficult to take advantage of this effect during the purification of the synthase. The requirement for a sulphydryl group for activity is strongly suggested by the complete inhibition of activity with *p*-hydroxymercuribenzoate. The low overall yield obtained may very likely be due to enzyme instability, and in this respect the spinach enzyme differs from the *Bacillus licheniformis* enzyme which is stabilized by pyruvate and NaCl but shows no requirement for thiol reagents [9]. For the spinach enzyme, pyruvate somewhat improves the extractable activity, and mercaptoethanol has a larger additional effect. Given the sensitivity of the assay's chromophore to a variety of compounds, it is important

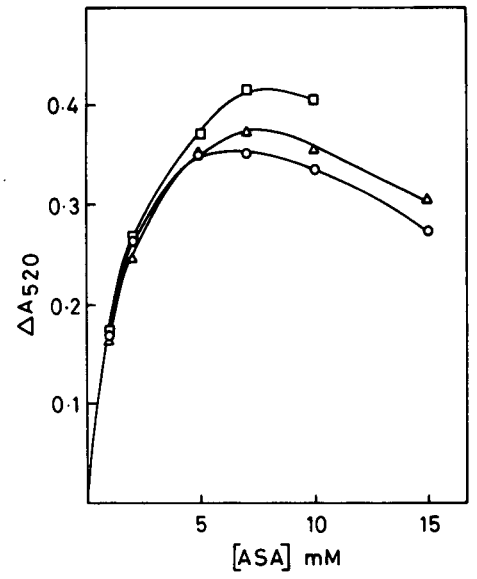

Fig. 2. Reaction rate as a function of ASA concentration. Activity was measured in the standard assay system, varying the ASA concentration at three different concentrations of pyruvate. The reaction mix contained 0.4 mg protein and was incubated for 20 min at 30°. ○——○, 50 mM pyruvate; △——△, 100 mM pyruvate; □——□, 150 mM pyruvate. Each point represents the mean of three replicates.

to test the effect of all potential modifiers of activity or stability on formation of the chromophore. Further purification and characterization of the enzyme may depend on an improved assay method without these drawbacks.

The MW of the spinach leaf enzyme is similar to that reported for *B. licheniformis* [9] and *Escherichia coli* K12 [10], 108 000–117 000 and 112 000, respectively. These represent both lysine-sensitive (*E. coli*) and insensitive enzymes [11, 6]. Bacterial dihydrodipicolinate synthases have been shown to consist of four subunits [12, 13] but it is not yet possible to investigate the sub-unit structure of the plant enzyme.

Lysine-sensitive dihydrodipicolinate synthase from *E. coli* has been shown to have four pyruvate binding sites per mol [13] and earlier results with the wheat germ enzyme were interpreted to indicate pyruvate co-operativity, with three or four pyruvate binding sites [5]. The kinetic data reported here for the spinach leaf enzyme do not support this mechanism. It seems more likely that there is substrate inhibition by ASA, a possible mechanism for this would involve an ordered binding of substrates, pyruvate binding first, and the formation of an enzyme–ASA 'dead-end' complex. Earlier work on the wheat germ enzyme also found that the apparent pyruvate co-operativity increased as the ASA concentration was raised [14], as is found for the spinach enzyme. It is, however, also possible that the ASA used in these experiments contain an inhibitor or inhibitors, even after purification by ion-exchange chromatography.

With the assay method used here, the kinetic data should be interpreted with caution. Given that incubation times of less than 10 min tend to be impractical, there is the possibility of significant breakdown of ASA during the assay, as this compound is highly unstable at neutral or alkaline pH [15]. The effects of such ASA breakdown products could be responsible for some of the observed kinetics.

The results of hydrophobic interaction chromatography are of interest because the enzyme binds very much more strongly to Phenyl-Sepharose than to Octyl-Sepharose. This is the opposite of the expected relationship [16], and suggests that a large part of the hydrophobic interaction may be due to phenyl and tyrosyl groups on the protein, which interact more strongly with Phenyl-Sepharose (see the Pharmacia booklet on Octyl- and Phenyl-Sepharoses).

The only significant inhibitors of spinach leaf DHDP synthase are L-lysine and its analogue amino-ethyl cysteine. As previously found for the wheat germ enzyme [5], DHDP synthase is very sensitive to low levels of lysine, whilst AEC is considerably less effective, as judged by the $I_{(0.5)}$. None of the other end-products of the aspartate pathway appear to affect enzyme activity, and there is no indication of DHDP synthase activation of the type found for threonine synthase [17]. Partitioning of ASA between HSDH and DHDP synthase in the chloroplast will depend on the relative concentrations of threonine and lysine in the soluble pool (and hence the activities of HSDH and DHDP synthase, respectively), and possibly also on the relative affinities of the two enzymes for ASA. The reported K'_m (ASA) for higher plants HSDHs are significantly lower than the K'_m (ASA) found for the DHDP synthases (40–250 μM) [18–20]. It is quite possible that the reported *in vitro* K'_m (ASA) for DHDP synthases are not an accurate measure of the *in vivo* affinity, so this apparent difference may not be of physiological significance.

This investigation was almost complete before the recent discovery of a synergistic effect of lysine and SAM on aspartate kinase in higher plants [21].

Lysine by itself has little effect on plant aspartate kinases until the concentration greatly exceeds the levels that affect DHDP synthase (see for example [23]). However, in the presence of SAM, aspartate kinase shows a similar sensitivity to lysine as does DHDP synthase. Hence in addition to tightly controlling its own synthesis, lysine regulates the flux through the whole aspartate pathway as the other end products accumulate.

The appearance of two bands of enzyme activity on gel electrophoretograms was unexpected, and the significance is uncertain. Crude extracts of carrot root and cell cultures have been shown to contain only one DHDP synthase as judged by gel electrophoresis [22]. It is possible that we were observing an artefact caused by the purification. There was never any indication of two peaks of activity eluting from any of the chromatographic procedures, and all attempts to separate the two activities shown on the gels were unsuccessful.

The first and last enzymes unique to lysine biosynthesis in plants, DHDP synthase and diaminopimelate decarboxylase, have now been demonstrated in a range of plant species and partially purified and characterized [5, 24]. They have also been shown to be wholly localized in the chloroplast [4, 3] and isolated chloroplasts have been shown to synthesize lysine from aspartate [2]. However, the enzymes responsible for the intermediate steps have never been demonstrated in plant tissue, and in fact the nature of the intermediates has not been determined. From past experience it would be premature to assume that the intermediates and enzymes involved

are identical to those found in bacteria which use the DAP pathway.

EXPERIMENTAL

Chemicals. ASA was prepared by the method of [15], and the L-ASA concentration assayed with a partially purified yeast homoserine dehydrogenase (EC 1.1.13) [25]. *o*-ABA was synthesized by the method of [26].

Plant material. Fresh spinach was obtained from local markets. Peas (*Pisum sativum* cv Rondo) were soaked overnight in tap H_2O and planted in shallow trays of vermiculite. After growth in the light for 10–14 days the whole shoots were harvested.

Enzyme assay. The assay used was a modification of that of [6] as previously described [3]. Crude extracts, and those containing mercaptoethanol or high salt concentrations, were dialysed or de-salted on G-25 before assay. A unit of enzyme activity is defined as an increase in E_{520nm} of 0.001/min.

Enzyme purification. 500 g of washed spinach leaves were homogenized in 2–3 vols of extraction buffer (50 mM KPi, pH 8.0, containing 10 mM Na pyruvate, 5 mM Na_2EDTA, and 10 mM 2-mercaptoethanol) in a bottom-drive blender fitted with a 'Polytron' head. The homogenate was filtered through four layers of cheese-cloth and centrifuged at 10 000 *g* for 20 min. To each 100 ml of supernatant were added 10 ml of 1% protamine sulphate, and after stirring for 15 min the ppt. was removed by centrifugation. Solid $(NH_4)_2SO_4$ was added to give 35% saturation and the ppt. removed by centrifugation. Further $(NH_4)_2SO_4$ was added to 55% saturation and the ppt. collected and redissolved in extraction buffer. After desalting the extract over Biogel P-60 equilibrated in buffer A (50 mM KPi, pH 8.0, containing 10 mM Na pyruvate) the enzyme was applied to a 30 × 2 cm column of Cellex D (Biorad) equilibrated with Buffer A. The enzyme was eluted with a linear KCl gradient from 0 to 0.3 M (300 ml total), and the active fractions pooled. Solid $(NH_4)_2SO_4$ was added to give 30% saturation and this soln was applied to a 19 × 2 cm column of Octyl-Sepharose equilibrated with Buffer A which was 30% satd with $(NH_4)_2SO_4$. The enzyme was eluted with a decreasing gradient, 30–10% saturation $(NH_4)_2SO_4$ in Buffer A (200 ml), and the active fractions pooled and concentrated by ultrafiltration. This fraction was then subjected to gel filtration using Sephadex G-150 equilibrated in Buffer A (144 × 2 cm column). The most active fractions were pooled and concentrated by ultrafiltration. All steps were carried out at 4°.

Gel electrophoresis. Samples were run in 10% gels using the standard system of [27] omitting the stacking gel. Gels were run at 3 mA/tube at 4° and proteins were stained with Coomassie Brilliant Blue R. Gels were stained for enzyme activity by incubation in a soln containing 100 mM Tris, pH 8.5, 200 mM Na pyruvate and 5 mM L-ASA, for 30 min at 30°. The gels were then transferred to 0.22 M citrate, 0.55 M NaPi, pH 5.0, containing 0.25 mg/ml *o*-ABA. After *ca* 1 hr purple-red bands appeared at the site(s) of activity and the gels were immediately scanned at 520 nm, as the colour diffuses out of the gel within a few hours.

Calibration of Sephadex columns. The following protein standards were used to calibrate the Sephadex G-150 column for MW determination: rabbit γ-globulin (156 000), chicken ovalbumin (46 000) and horse heart cytochrome *c* (12 000). Blue dextran was used to measure the void volume. For those experiments in which Sephadex G-200 was used jack bean urease (400 000) and yeast invertase (270 000) were used as additional standards.

Acknowledgement—This work was partially supported by NSF grant PCM 7810196.

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