Purification and Characterization of Dihydrodipicolinate Synthase from Pea

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ABSTRACT

Dihydrodipicolinate synthase (EC 4.2.1.52), the first enzyme unique to lysine biosynthesis in bacteria and higher plants, has been purified to homogeneity from etiolated pea (*Pisum sativum*) seedlings using a combination of conventional and affinity chromatographic steps. This is the first report on a homogeneous preparation of native dihydrodipicolinate synthase from a plant source. The pea dihydrodipicolinate synthase has an apparent molecular weight of 127,000 and is composed of three identical subunits of 43,000 as determined by gel filtration and cross-linking experiments. The trimeric quaternary structure resembles the trimeric structure of other aldolases, such as 2-keto-3-deoxy-6-phosphogluconic acid aldolase, which catalyze similar aldol condensations. The amino acid compositions of dihydrodipicolinate synthase from pea and *Escherichia coli* are similar, the most significant difference concerns the methionine content: dihydrodipicolinate synthase from pea contains 22 moles of methionine residue per mole of native protein, contrary to the *E. coli* enzyme, which does not contain this amino acid at all. Dihydrodipicolinate synthase from pea is highly specific for the substrates pyruvate and L-aspartate-β-semialdehyde; it follows Michaelis-Menten kinetics for both substrates. The pyruvate and L-aspartate-β-semialdehyde have Michaelis constant values of 1.70 and 0.40 millimolar, respectively. L-Lysine, S-(2-aminoethyl)-L-cysteine, and L-α-(2-aminoethoxyvinyl)glycine are strong allosteric inhibitors of the enzyme with 50% inhibitory values of 20, 160, and 155 millimolar, respectively. The inhibition by L-lysine and L-α-(2-aminoethoxyvinyl)glycine is noncompetitive towards L-aspartate-β-semialdehyde, whereas S-(2-aminoethyl)-L-cysteine inhibits dihydrodipicolinate synthase competitively with respect to L-aspartate-β-semialdehyde. Furthermore, the addition of (2R,3S,6S)-2,6-diamino-3-hydroxy-heptandioic acid (1.2 millimolar) and (2S,6R/S)-2,6-diamino-6-phosphono-hexanic acid (1.2 millimolar) activates dihydrodipicolinate synthase from pea by a factor of 1.4 and 1.2, respectively. This is the first reported activation process found for dihydrodipicolinate synthase.

The biosynthetic pathway of lysine, methionine, and threonine shares a common part between L-aspartate and ASA[1] in the beginning of the pathways (1). The lysine-specific part

of the pathway between ASA and lysine has not been completely elucidated yet. It is usually assumed that lysine is synthesized by the same diaminopimelic acid pathway that exists for bacteria (1), although only a few of the six enzymes involved in lysine biosynthesis in *Escherichia coli* have been found to be present in plants.

One of these is DHDPS, the first enzyme unique to lysine biosynthesis in bacteria and plants. DHDPS is feedback-regulated by lysine and thus plays a regulatory role in lysine biosynthesis (12). The enzyme catalyzes the condensation of pyruvate and ASA to dihydrodipicolinic acid. Attempts (17) to identify the reaction product indicated that an equilibrium is established between 2,5-dihydrodipicolinic acid, 2,3-dihydrodipicolinic acid, and 4-hydroxy-2,3,4,5-tetrahydrodipicolinic acid or even a noncyclic form of the compound.

Up to now, DHDPS has been isolated from various bacterial strains (2, 8, 9, 17, 18, 19, 21, 25) and been purified to homogeneity from *E. coli* (17). The *E. coli* enzyme has a mol wt of 134,000 and is composed of four identical subunits of 35,000. Mechanistic studies performed on *E. coli* DHDPS (17) indicated that the reaction is catalyzed via an ordered ping-pong mechanism: in a first step, pyruvate binds to a lysine residue in the active site forming a Schiff base and releasing one molecule of water. In a second step, the binding of ASA is followed by condensation and release of two products, dihydrodipicolinic acid and water.

DHDPS activity has also been found in a variety of plant tissues (1, 4–7, 12, 14, 16, 22, 23) and located at the level of chloroplasts (6, 22, 23). Plant DHDPS have been partially purified from wheat (12), maize (5), and spinach (23); denatured homogeneous subunits have been obtained from *Nicotiana sylvestris* (6).

DHDPS from wheat, maize, and *N. sylvestris* are oligomeric enzymes with mol wts of 123,000, 130,000, and 164,000, respectively; all are composed of four identical subunits. The spinach enzyme has a mol wt of 115,000, but its oligomeric structure is still unclear.

Kinetic studies with DHDPS from wheat (12), spinach (23), and *N. sylvestris* (6) indicate that pyruvate binds allosterically to the enzyme, while ASA binds in a nonallosteric way. There are three, or possibly four, binding sites for pyruvate on the enzyme.

DHDPS from higher plants is feedback inhibited by micromolar concentrations of L-lysine (5, 6, 16, 23). The enzyme from wheat is inhibited by L-lysine and AEC, a lysine analog. This inhibition is competitive with respect to ASA and noncompetitive with respect to pyruvate.

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[1] Abbreviations: ASA, L-aspartate-β-semialdehyde; DHDPS, dihydrodipicolinate synthase (EC 4.2.1.52); o-ABA, o-aminobenzaldehyde; AEC, S-(2-aminoethyl)-L-cysteine; AVG, L-α-(2-aminoethoxyvinyl)glycine; IEF, isoelectric focusing; $I_{0.5}$, 50% inhibitory concentration.

The purpose of this study was to establish a purification procedure for homogeneous native DHDPS to carry out a detailed characterization and investigation of the structural and functional properties of this key enzyme in lysine biosynthesis.

MATERIALS AND METHODS

Plant Material

Pea (*Pisum sativum* cv Medullare Alef) was grown in the dark on vermiculite at 37°C. After 10 d, the plants were harvested and immediately frozen at −80°C.

Chemicals

ASA was synthetized using the method of Bold (Ciba Geigy Ltd., Basel, CH). EDTA, PMSF, Tris-HCl, KH$_2$PO$_4$, Na-pyruvate, benzamidin, glutathione, DTE, and pepstatin A were from Fluka Biochemica (Buchs, Switzerland). *o*-ABA was from Sigma Chemical Co. (St. Louis, MO). Pyruvate analogs 3-bromo-pyruvate, 3-bromo-pyruvate ethyl ester, and β-hydroxy-pyruvate were obtained from Sigma. Lactic acid, 2-keto-isovaleric acid, phosphoenolpyruvate, 2-oxo-glutaric acid, and 2-oxo-butyric acid were from Fluka Biochemica (Buchs, Switzerland). Chelidamic and chelidonic acids were from Sigma. Lysine and diaminopimelic analog δ-hydroxyly-sine, *S*-2-aminoethylcysteine, *N*-ε-formyl-L-lysine, L-cysteine, L-asparagine, DL-α-aminopimelic acid, L-aspartic acid, D-lysine, L-lysine, L-glutamic acid, α-amino-*n*-butyric acid, L-glutamine, and L-arginine were from Sigma. *S*-Carboxy-ethyl-L-cysteine, L-2,4-diaminobutyric acid, *S*-aminoethyl-L-cysteine, and L-α-(2 aminoethoxyvinyl)glycine were purchased from Fluka. The synthesis of all the other lysine and diaminopimelic acid analogs was described by Bold (will be published elsewhere). Fractogel TSK butyl 650 (M), Sephacryl S 200 sf, Mono Q HR 5/5, Superose 12 HR 10/30, the fast protein liquid chromatography, and the phastgel electrophoresis systems were from Pharmacia/LKB (Uppsala, Sweden). Eupergit C was from Roehm.

Enzyme Assay

A modification of the ABA method developed by Yugari and Gilvarg (25) was used to assay DHDPS. The reaction mixture consisted of 100 mM Tris-HCl (pH 8), 20 mM Na-pyruvate, 1.6 mM ASA, and 0.35 mg *o*-ABA (dissolved in 10 μl ethanol and added just before incubation). The reaction was stopped by the addition of 100 μl TCA 15% (w/w)/HCl 3 *N* (1:1). The color was allowed to develop for 40 min at room temperature. One unit of enzyme activity is arbitrarily defined as the amount of enzyme that produces a change in A_{520} of 0.001/min at 35°C. Reaction mixtures lacking ASA were used as controls.

The DHDPS activity was located on zymograms using native PAGE soaked in double strength reaction mixture after electrophoresis and incubated until a faint colored band appeared. The gels were then transferred into 0.22 M citrate/0.55 M Na$_2$HPO$_4$, pH 5.0, to enhance the color of the band.

Preparation of Aminoethylcysteine Eupergit

The affinity chromatography matrix was prepared by adding 3 g of dried Eupergit C to 3 g of AEC dissolved in 10 mL of water. The reaction mixture was allowed to sit 7 d at room temperature without shaking. The gel was then washed with 1 liter of 0.1 M K-phosphate buffer, pH 7.5. The coupling reaction was followed by using the qualitative trinitrobenzene sulfonic acid test described by Inmann (10).

Crude Extract Preparation

All procedures were carried out at 4°C; 1.2 kg of pea plants were frozen in liquid nitrogen and powdered, 3 liters of buffer A (69 mM K-phosphate buffer [pH 7.5], 3 mM DTE, 2 mM EDTA, 10 mM Na-pyruvate, 0.1 μM pepstatin, 1 mM PMSF, 1 mM benzamidine, 10 mM glutathione) were added and the suspension mixed for 5 min in a blender (Turmix). This was followed by a filtration through four layers of cheesecloth (Miracloth).

Fractogel TSK Butyl Batch Extraction

The crude extract was brought to 30% saturation by the addition of solid (NH$_4$)$_2$SO$_4$ and stirred for 1 h; 300 mL of Fractogel TSK Butyl 650 (M) equilibrated with buffer B (20 mM K-phosphate buffer [pH 7.5], 2 mM DTE, 1 mM EDTA, 5 mM Na-pyruvate) containing (NH$_4$)$_2$SO$_4$ (30% saturation), were added to the crude extract. After 1 h of stirring, the mixture was filtered on a buchner funnel. The gel was washed with 2 liters of buffer B containing (NH$_4$)$_2$SO$_4$ (30% saturation) and 2 liters of buffer B 20% saturated in (NH$_4$)$_2$SO$_4$. The enzyme was eluted with 1 liter of buffer B 10% saturated in (NH$_4$)$_2$SO$_4$.

Fractogel TSK Butyl Chromatography

The eluate was concentrated by fractional (NH$_4$)$_2$SO$_4$ precipitation. The pellet resulting from the 10 to 75% saturation (NH$_4$)$_2$SO$_4$ precipitation was resuspended in a minimal volume of buffer B and loaded onto a Fractogel TSK Butyl 650 (M) (50 × 130-mm) column equilibrated with buffer B 30% saturated with (NH$_4$)$_2$SO$_4$. The enzyme activity was eluted with a gradient from 30 to 0% saturation (NH$_4$)$_2$SO$_4$.

Sephacryl S 200 of Chromatography

Fractions containing DHDPS activity were pooled and concentrated by (NH$_4$)$_2$SO$_4$ precipitation [80% saturation (NH$_4$)$_2$SO$_4$]. The pellet was dissolved in buffer B and loaded onto a Sephacryl S 200 sf (5 × 100 cm) column equilibrated with buffer B.

AEC-Eupergit Chromatography

Fractions containing DHDPS activity were pooled and concentrated in a Centripep 30 (Amicon). The pH of the concentrated solution was adjusted to 6.0 with 1 M K-phosphate buffer and loaded onto an AEC-Eupergit (13 × 100 mm) column equilibrated with buffer C (20 mM K-phosphate buffer [pH 6.0], 2 mM DTE, 5 mM Na-pyruvate). The column was washed with 2 volumes of buffer C, followed by 1 volume

of buffer C containing 100 mM KCl, and 1 volume of buffer C. The enzyme activity was eluted with buffer D (100 mM Tris-HCl [pH 8.5], 2 mM DTE, 5 mM Na-pyruvate).

Mono Q HR 5/5 Chromatography

Fractions containing DHDPS activity after affinity chromatography step were pooled and loaded directly onto a Mono Q HR5/5 column equilibrated with buffer B. The enzyme was eluted by a 0 to 500 mM KCl gradient, DHDPS eluting with 150 mM KCl. Protein concentrations were determined with a Bio-Rad protein assay kit using BSA as a standard.

PAGE

Native PAGE and SDS-PAGE were carried out on a Pharmacia Phastgel electrophoresis system. Samples were applied to 8 to 25% (w/v) acrylamide gradient Phastgels, and the appropriate buffer strips were used according to the supplier's instructions. Prior to application on SDS-PAGE gels, the samples were boiled in a denaturing buffer (10 mM Tris-HCl, 1 mM EDTA, 1% [w/w] SDS, 5% [w/w] β-mercaptoethanol [pH 8.0]) for 10 min. Phastgels were stained with Coomassie brilliant blue or silver stained, according to the supplier's instructions. Mol wts were determined by using high mol wt native and SDS calibration kits from Pharmacia/LKB.

Cross-Linking Reaction

The cross-linking method used was described by Davies and Stark (3). Immediately before use, dimethyl suberimidate hydrochloride was dissolved in 0.2 M triethanolamine HCl, pH 8.5 (3 mg/mL). Dimethyl suberimidate and enzyme solutions were mixed to give 1 mg/mL of dimethyl suberimidate and 0.2 mg/mL protein in a volume of 300 μL. The pH of the solution was 8.5. The reaction mixture was incubated at room temperature for 3 h. To follow the kinetics of cross-linking, a portion (10 μL) of the reaction mixture was removed every 30 min, denatured, and analyzed by SDS-PAGE and silver staining.

Inhibition Tests

Based on their structural homology to lysine, ASA, pyruvate, dihydrodipicolinic acid, or diaminopimelic acid, compounds were selected and tested for their effect on DHDPS activity. The enzyme was preincubated for 10 min with the potential inhibitor (dissolved in water), Tris-HCl buffer, and

Na-pyruvate in concentrations used for the activity test. After preincubation, ASA and o-ABA were added and the activity test carried out as described above. For each substance tested, controls without ASA and without enzyme were included. For tests using pyruvate analogs, the enzyme solution was gel filtrated on PD-10 before use to eliminate pyruvate present in the solution. The preincubation solution contained Tris-HCl, enzyme solution, and potential inhibitor. To start the activity test, o-ABA, ASA, and pyruvate were added. All inhibitors were tested at a concentration of 1.2 mM. The concentrations of ASA and pyruvate were 1.6 and 20 mM, respectively.

Amino Acid Analysis

The amino acid analysis of the purified enzyme was carried out according to the method of Knecht and Chang (11). The enzyme solution was first gel filtrated on PD 10 with 0.1 M $(NH_4)HCO_3$ and lyophilized to dryness. After gas-phase hydrolysis of the protein, the amino acids were derivatized using (dimethyl amino)-azobenzenesulfonyl chloride and measured by liquid chromatography using a Lichrosphere 100 CH-18/2 column.

RESULTS

Enzyme Purification

Our purification procedure is presented in Table I. Probably due to the presence of free lysine, the crude extract always shows a lower total activity than the preparation obtained after the Fractogel TSK Butyl batch step. Therefore, this latter step was taken as starting point for further purification. The pea DHDPS obtained by the described procedure was homogeneous as demonstrated by the single protein band observed on IEF and SDS gels (Fig. 1). The band on IEF gels retains DHDPS activity in a zymogram. The low purification factor obtained was a consequence of the great loss of enzyme activity after the run on AEC-Eupergit C. This can be explained by the fact that, to get optimal binding, the enzyme solution had to be brought to pH 6 prior to loading onto the affinity column. Because these conditions are not optimal for enzyme stability, losses in enzyme activity were observed.

The amino acid composition of the purified enzyme is shown in Table II. The composition of the pea enzyme shows some similarities to that of the *Escherichia coli* enzyme. The amino acid compositions of dihydrodipicolinate synthase from pea and *E. coli* are similar, the most significant differ-

Table I. *Purification of DHDPS*

Purification Step	Volume	Activity	Protein	Specific Activity	Purification Factor	Yield
	ml	units	mg	units/mg		%
Crude extract	3,950	52,831	8,374	6.3		
Butyl-Fractogel batch	770	123,200	1,807	68.1	1	100
Butyl-Fractogel column	82	114,287	264	433	6.3	92
Sephacryl S 300 sf	31	85,443	58.7	1,455	21.3	69
AEC-Eupergit C HR 5/5	6.5	16,722	11.3	1,480	21.7	14
MonoQ HR 5/5	5	9,856	0.86	11,458	168	8

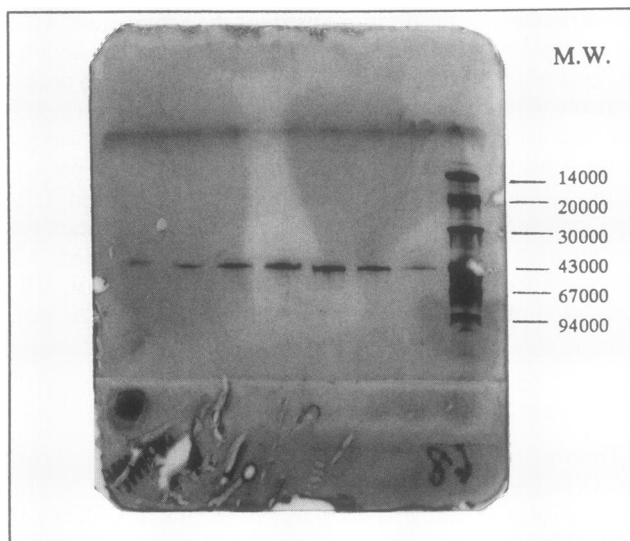**Figure 1.** SDS-PAGE of purified DHDPS.

Figure 2. Mol wt of DHDPS determined on Superose 12.

ence concerns the methionine content: dihydrodipicolinate synthase from pea contains 22 mol of methionine residue per mol of native protein, contrary to the *E. coli* enzyme, which does not contain this amino acid at all.

Pea DHDPS is relatively unstable at all stages of purification, even during storage at −80°C; 40% of the activity was lost after 1 d at +4°C and 15% after 4 d at −20°C. In addition, this instability is further increased for highly diluted solutions. We found that addition of DTE (up to 5 mM), glycerol 30% (v/v), 100 mM KCl, 10% saturation $(NH_4)_2SO_4$, 5% (w/w) sucrose, or bivalent ions such as Mn^{2+}, Mg^{2+}, or Ca^{2+} to the

buffers did not prevent activity loss. A similar stability problem was previously reported for DHDPS from spinach (23). Bacterial DHDPS seems to be more stable than DHDPS from plant sources.

Mol Wt

A mol wt of 127,000 ± 6,000 for the native enzyme was estimated by gel filtration on Superose 12 (Fig. 2). This result was confirmed by native PAGE (data not shown). The band containing DHDPS activity corresponded to the same mol wt of 127,000. This mol wt was similar to that reported for DHDPS from other plant sources: 130,000 for maize (5), 123,000 for wheat germ (12), and 115,000 for spinach leaf (23). Interestingly, at all stages of purification, two activities were observed in the zymogram, one of them at the expected mol wt of 127,000, and the other at a mol wt between 40,000 and 50,000, which corresponded to the subunit mol wt. A similar result was reported for DHDPS from maize (5) and
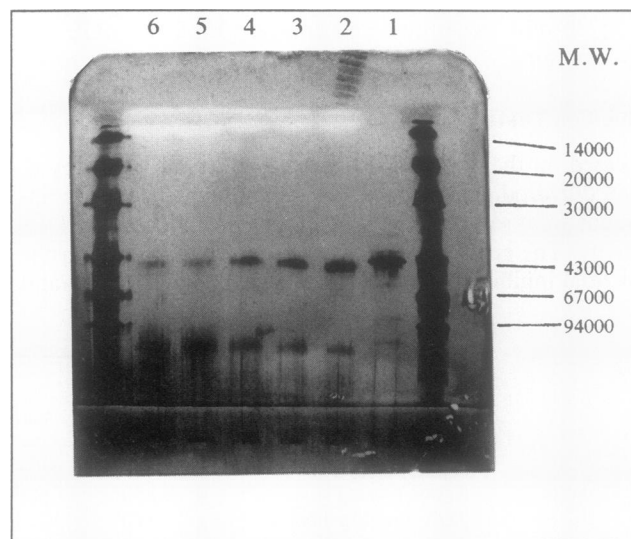
Table II. *Amino Acid Composition of DHDPS from E. coli and Pea*

Amino Acids	DHDPS from *E. coli*[a]	DHDPS from Pea
	mol amino acid/mol native protein	mol amino acid/mol native protein
L-Lys	53	55
L-His	14	16
L-Arg	72	52
L-Asp + L-asn	107	119
L-Thr	66	69
L-Ser	46	83
L-Glu + L-gln	105	104
L-Pro	54	55
L-Gly	96	153
L-Ala	100	123
L-Half-Cys	14	0
L-Val	93	60
L-Met	0	22
L-Ile	61	41
L-Leu	105	88
L-Tyr	18	25
L-Phe	56	35
L-Trp	ND[b]	ND

[a] Composition obtained by Schedlarski and Gilvarg (17).   [b] ND, not determined.

Figure 3. Cross-linking experiment with the purified DHDPS on SDS-PAGE; cross-linking reaction times are: 1, 0 min; 2, 30 min; 3, 60 min; 4, 90 min; 5, 120 min; 6, 150 min.
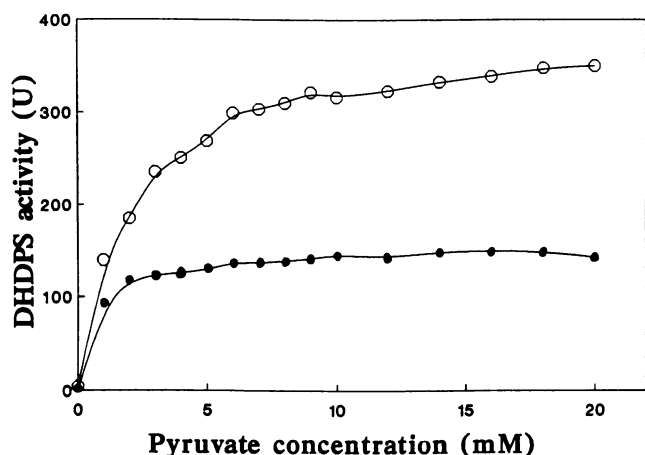
Figure 4. DHDPS activity as a function of pyruvate concentrations at two constant ASA concentrations: 0.4 mM (●), 1.6 mM (O).
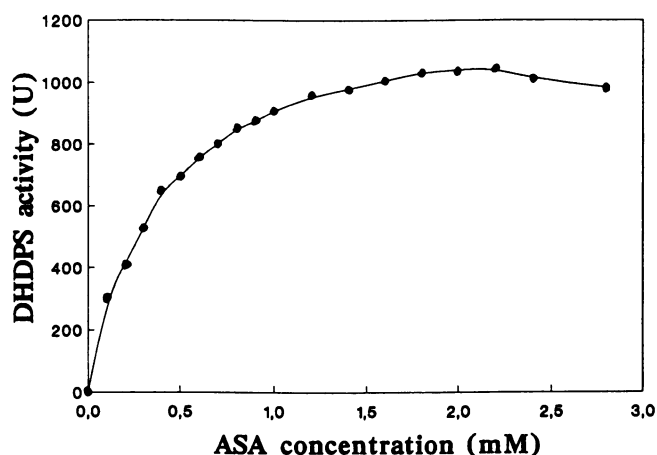

Figure 6. DHDPS activity as a function of ASA concentrations at a fixed pyruvate concentration of 20 mM.

spinach (23). In both cases, the additional active band corresponded to the fastest running bands in the gel.

A single protein band with an apparent mol wt of 41,000 ± 2,000 was obtained on SDS-PAGE (Fig. 1). The kinetics of the cross-linking with dimethylsuberimidate followed by SDS-PAGE showed a decreasing band corresponding to a mol wt of 43,000 and an increasing band at 127,000 ± 3,000 (Fig. 3). These results suggested a trimeric structure.

Optimum pH, Isoelectric Point, Optimal Temperature

The optimal pH for DHDPS activity is pH 8.0. The isoelectric point is 4.5 (determined on IEF-PAGE gel), and the optimal reaction temperature is 35°C.

Kinetic Studies

The enzyme activity as a function of Na-pyruvate concentration at two different ASA concentrations is shown in Figure

4. At fixed ASA concentrations of 0.4 and 1.6 mM, the enzyme follows Michaelis-Menten kinetics in response to increasing Na-pyruvate concentrations. It has been reported for maize (5), wheat germ (12), and spinach leaf (23) DHDPS, that the saturation curve changes from a Michaelis-Menten type to a sigmoid shape as the fixed ASA concentration is increased. A double-reciprocal plot of our data showed parallel linear curves (Fig. 5). The apparent K_m for Na-pyruvate of 1.7 mM is lower than the K_m values for wheat germ and spinach leaf DHDPS, which were reported to be 11.7 and 6 mM, respectively.

DHDPS activity with regard to ASA concentration at fixed Na-pyruvate concentrations showed a Michaelis-Menten type saturation curve (Fig. 6). From double-reciprocal plots, we obtained a K_m value for ASA of 0.40 mM (Fig. 7). This result was consistent with that observed for wheat germ DHDPS (12).
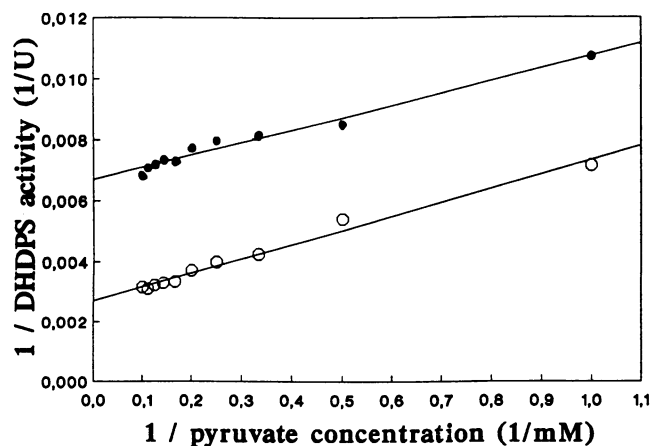

Figure 5. Double-reciprocal plot of initial velocities of DHDP formation against pyruvate concentration at fixed ASA concentrations: 0.4 mM (●), 1.6 mM (O).
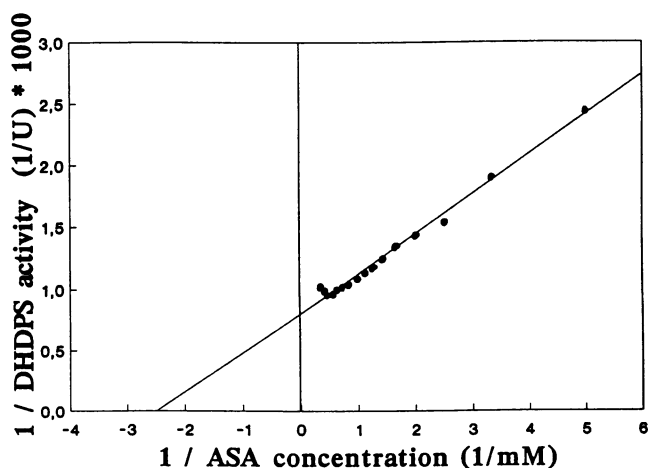

Figure 7. Double-reciprocal plot of initial velocities of DHDP formation against ASA concentration at a fixed concentration of pyruvate of 20 mM.

Inhibitor Structure	Name	Concentr. tested (mM)	% relative activity
H₃C–CO–COOH	2-oxobutyric acid	1.2	100
HOOC–CO–COOH	2-oxoglutaric acid	1.2	100
H₂O₃PO–C(CH₂)–COOH	phosphoenol-pyruvate	1.2	100
H₃C–CO–COOC₂H₅	ethyl-pyruvate	1.2	100
H₃C–CH(OH)–COOH	L-lactic acid	1.2	100
H₃C–CH(CH₃)–CO–COOH	2-keto-isovaleric acid	1.2	100
H₃C–CO–COOH	2-keto-caproic acid	1.2	100
H₃C–CO–CO–NH₂	2-oxo-propionic acid amide	1.2	100
H₃C–CO–COOCH₃	methyl-pyruvate	1.2	97
HO–CH₂–CO–COOH	3-hydroxy-pyruvate	1.2	91
Br–CH₂–CO–COOC₂H₅	3-bromo-pyruvate-ethylester	1.2	16
Br–CH₂–CO–COOH	3-bromo-pyruvate	1.2	0

Figure 8. Inhibition of DHDPS by compounds related to pyruvate.

Inhibitor Structure	Name	Concentr. tested (mM)	% relative activity
H₂N–(CH₂)₃–CH(NH₂)–COOH	D-lysine	1.2	100
HOOC–CH₂–CH₂–S–CH₂–CH(NH₂)–COOH	S-carboxyethyl-L-cysteine	1.2	100
H₂O₃P–CH=CH–CH(NH₂)–COOH	(R/S)-2-amino-5-phosphono-3-pentenoic acid	1.2	100
N-{[3-hydroxy-2-methyl-5-((phosphonoxy)methyl)-4-pyridinyl]methyl}-D/L-alanine structure	N-{[3-hydroxy-2-methyl-5-((phosphonoxy)methyl)-4-pyridinyl]methyl}-D/L-alanine	1.2	80
OHC–NH–(CH₂)₃–CH(NH₂)–COOH	N-ε-formyl-L-lysine	1.2	75
Nⁿ-{[3-hydroxy-2-methyl-5-((phosphonoxy)methyl)-4-pyridinyl]methyl}-L-lysine structure	Nⁿ-{[3-hydroxy-2-methyl-5-((phosphonoxy)methyl)-4-pyridinyl]methyl}-L-lysine	1.2	61
HN=C(NH₂)–NH–(CH₂)₃–CH(NH₂)–COOH	L-arginine	1.2	38
H₂N–CH₂–CH₂–S–CH₂–CH(NH₂)–COOH	S-aminoethyl-cysteine	1.2	9
H₂N–CH₂–CH₂–O–CH=CH–CH(NH₂)–COOH	L-α-(2-amino-ethoxy vinyl)-glycine	1.2	0

Figure 9. Inhibition of DHDPS by lysine-related compounds and other amino acids.

Inhibition Studies

A variety of compounds structurally related to pyruvate were tested as putative inhibitors (Fig. 8). Complete inhibition was found with 1.2 mM 3-bromo-pyruvate and 84% inhibition was obtained with 1.2 mM 3-bromo-pyruvate ethylester. These results were in agreement with those obtained with DHDPS from wheat germ (13). The inhibition is probably due to alkylation of the enzyme since it was irreversible, and pyruvate ethylester did not inhibit DHDPS activity. β-Hydroxypyruvate is a weak inhibitor of DHDPS inasmuch as only 9% of inhibition was obtained at 1.2 mM concentration. DHDPS of spinach was weakly inhibited by oxobutyrate ($I_{0.5}$, 40 mM) (23). We obtained no inhibition of the pea enzyme with 1.2 mM oxobutyrate. None of the other pyruvate analogs tested inhibited DHDPS either. The two dihydrodipicolinic acid-related compounds, chelidamic and chelidonic acids, did not affect DHDPS at 1.2 mM. Figure 9 summarizes the inhibition studies obtained with various lysine analogs and other amino acids. Complete inhibition was observed with AVG and L-lysine at 1.2 mM concentration. This effect of AVG on DHDPS has not been reported before. AEC inhibited DHDPS by 91%, δ-hydroxylysine by 74%, L-arginine by 62%, and N-ε-formyl-L-lysine by 25%, each at the concentration of 1.2 mM.

Inhibition of DHDPS by L-lysine, AEC, and AVG were studied in more detail. Inhibition of DHDPS by increasing L-
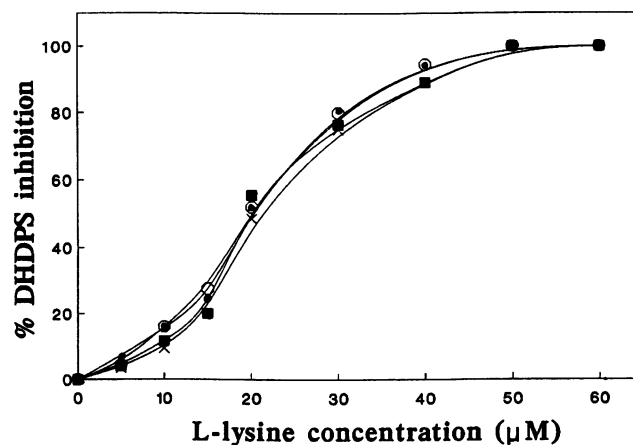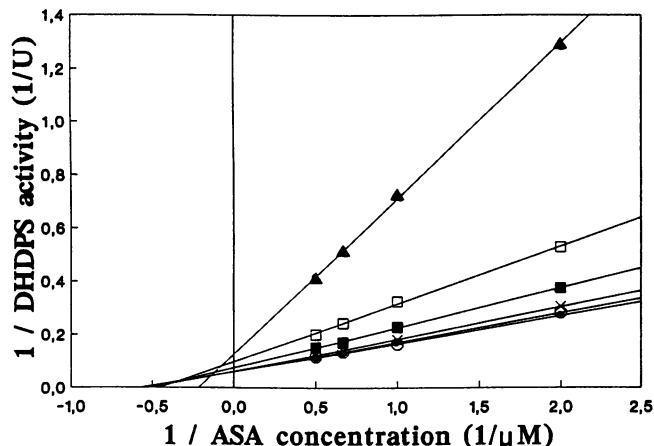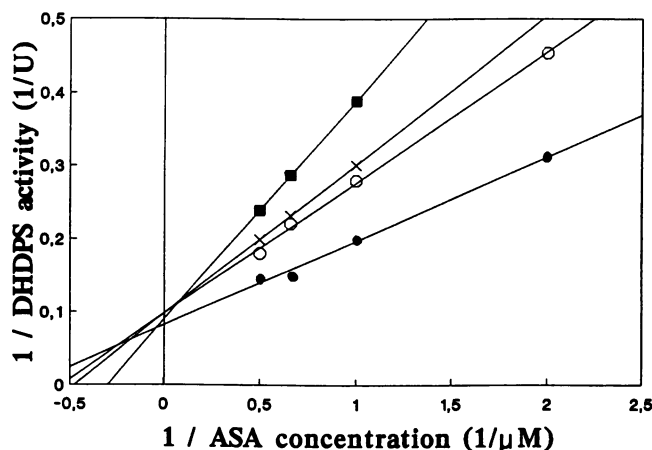

Figure 10. Effect of L-lysine on DHDPS activity at different fixed ASA concentrations of 0.5 mM (●), 1.0 mM (○), 1.5 mM (×), 2 mM (■).

Figure 11. Double-reciprocal plot of the inhibition of DHDPS at different fixed L-lysine concentrations and varying ASA concentrations. L-Lysine concentrations used are 0 μM (●), 5 μM (O), 10 μM (×), 15 μM (■), 20 μM (□), 30 μM (▲).

Figure 13. Double-reciprocal plot of inhibition of DHDPS at different fixed AEC concentrations and varying ASA concentrations. AEC concentrations used are 0.0 μM (●), 20 μM (O), 40 μM (×), and 60 μM (■).

lysine concentration showed a sigmoid shape (Fig. 10). From a Hill plot, a slope of 2.95 and a $I_{0.5}$ value of 20 μM were determined. These values were similar to those reported for spinach leaf (23) and wheat germ (12) DHDPS. The double-reciprocal plot indicated that L-lysine is a noncompetitive inhibitor with respect to ASA (Fig. 11). This result implies that L-lysine and ASA do not share the same binding site on the enzyme, and is in contrast to the results described for DHDPS from maize (5) and wheat (12), where L-lysine inhibits DHDPS competitively with respect to ASA.

An identical study carried out with AEC also indicated an allosteric inhibition (Fig. 12). A slope of 2.8 and an $I_{0.5}$ of 160 μM were determined from a Hill plot. However, using a double-reciprocal plot, unlike L-lysine, it seemed that AEC was a competitive inhibitor toward ASA (Fig. 13).

Allosteric inhibition of DHDPS was also observed with increasing AVG concentrations (Fig. 14). The Lineweaver-Burk plot indicated a noncompetitive inhibition with regard

to ASA (Fig. 15). The slope and $I_{0.5}$ values derived from a Hill plot were 2.45 and 155 μM, respectively. Pea DHDPS activity was not affected by the diaminopimelic acid analogs tested (Fig. 16). Figure 17 lists compounds that activated DHDPS. (2R,3S,6S)-2,6-Diamino-3-hydroxy-heptandioic acid and (2S,6R/S)-2,6-diamino-6-phosphono-hexanic acid activated DHDPS 40, 13, and 10%, respectively, at a concentration of 1.2 mM. This is the first time that an activator of DHDPS has been reported.

DISCUSSION

The key step in the purification of DHDPS from pea is a newly developed affinity chromatography based on the immobilized competitive DHDPS inhibitor AEC. Specific elution of DHDPS is achieved by an increasing pH gradient. Under the conditions used, other proteins remain adsorbed on the column. To avoid time-consuming procedures needed
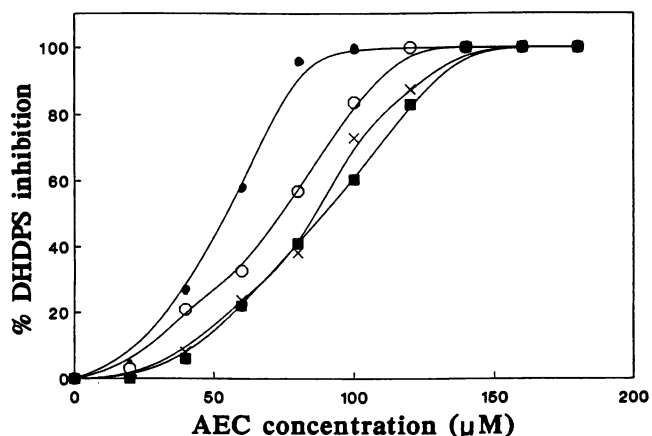
Figure 12. Effect of AEC on DHDPS activity at different fixed ASA concentrations of 0.5 mM (●), 1.0 mM (O), 1.5 mM (×), and 2.0 mM (■).
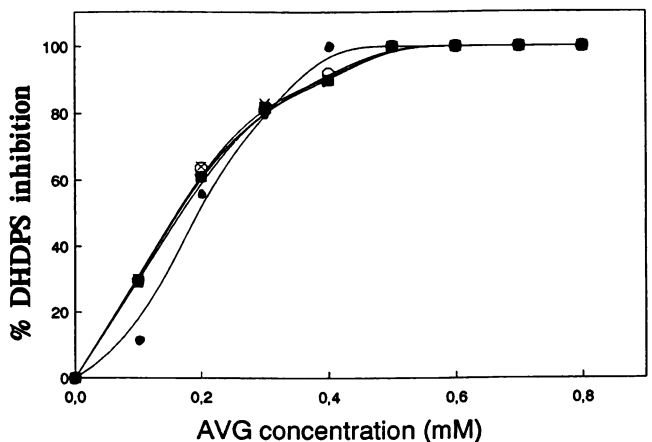
Figure 14. Effect of AVG on DHDPS activity at different fixed ASA concentrations of 0.5 mM (●), 1.0 mM (O), 1.5 mM (×), and 2.0 mM (■).
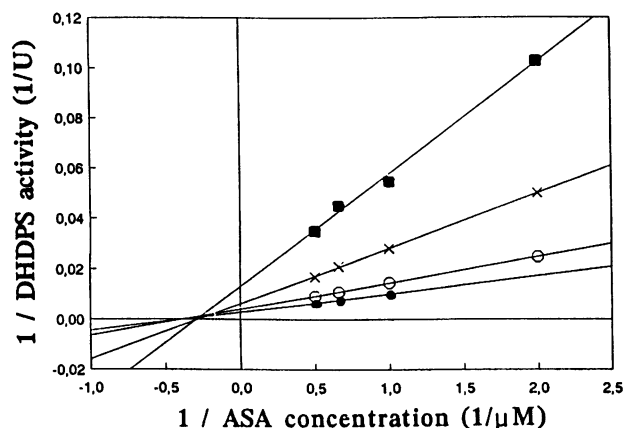
Figure 15. Double-reciprocal plot of the inhibition of DHDPS at different fixed concentrations of AVG and varying concentrations of ASA. AVG concentrations used are 0.0 mM (●), 0.1 mM (○), 0.2 mM (×), and 0.3 mM (■).

Figure 16. Diaminopimelic acid and related compounds tested for DHDPS inhibition.

Figure 17. Activators of DHDPS.

to remove the inhibitor from the relatively unstable enzyme, free AEC was not used in the elution of DHDPS.

Interestingly, at all stages of purification, DHDPS shows two bands of activity in zymograms. The mol wts of these two bands correspond to the native enzyme (127,000) and to the subunit (43,000). As suggested by similar results obtained with DHDPS from maize (5) and spinach (23), we assume that this observation is an artefact due to the conditions applied specifically during Native-PAGE that seem to favor dissociation of the enzyme. Indeed on IEF gel followed by Coomassie as activity staining agent only one single band is found. A trimeric enzyme is unexpected but not all together surprising, inasmuch as DHDPS is known to be closely related to the enzymatic reaction mechanism of aldolases. Aldolases of class 1, which are found in plants, have mol wts between 108,000 and 140,000. They bind pyruvate via a Schiff base, and are not inhibited by complexing agents such as EDTA. It has been shown that this class of aldolases includes a number of trimeric enzymes (20, 24). Moreover, three-dimensional structures of aldolases are known, and some, like 2-keto-3-deoxy-6-phosphonogluconate aldolase (15), are definitively trimeric enzymes.

We found that 3-bromo-pyruvate irreversibly inactivates DHDPS from pea, as was the case for DHDPS from wheat (12). We obtained 100% inhibition with 3-bromo-pyruvate and 84% inhibition with 3-bromo-pyruvate ethylester at a concentration of 1.2 mM. These compounds probably alkylate DHDPS on the active site. In contradiction to results obtained for DHDPS from spinach, we saw no inhibition of pea DHDPS by 1.2 mM 2-oxobutyrate. None of the other pyruvate analogs tested inhibited DHDPS either. None of the diaminopimelic acid analogs tested affected DHDPS activity. So far, no activator of DHDPS has been reported. We obtained an increase of activity of 40% with (2R,3S,6S)-2,6-diamino-3-hydroxy-heptane-dioic acid and 20% with (2S,6R/S)-2,6-diamino-6-phosphono-hexanoic acid. These two compounds were tested at only one concentration, which was perhaps not the one that causes maximum activation. Because diaminopimelic acid or its 3-chloro derivative showed no activation or inhibition of DHDPS, it seems that the 3-hydroxyl function is important for activation of DHDPS from pea.

We obtained an allosteric inhibition of DHDPS with L-lysine, AEC, AVG ($I_{0.5}$, 20 μM; 160 μM; 155 μM, respectively).

L-Lysine and AEVG are noncompetitive inhibitors of the enzyme with regard to ASA, whereas AEC is competitive with ASA for the same binding site. These inhibitors give a Hill value between 2.45 and 2.95, indicating three binding sites per enzyme molecule. This is consistent with the presence of a trimeric enzyme structure.

ACKNOWLEDGMENTS

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