ENZYMES IN AMINO ACID METABOLISM. VI.*

PURIFICATION AND SOME PROPERTIES OF L-ASPARTATE: α-OXOGLUTARATE AMINOTRANSFERASE FROM *Nicotiana tabacum* L.

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In cell-free extracts from leaves of *Nicotiana tabacum* L. cv. "Havana II" plants a 282-fold concentration of L-aspartate: α-oxoglutarate aminotransferase was obtained. The procedure included ammonium sulphate precipitation, gel filtration on Sephadex G-100 columns and fractionation on DEAE Sephadex A-50. The pH-optimum of the gel filtrated enzyme is pH 6·7 and K_m = = 4·3 mM. The enzyme stability and inhibition by hydroxylamine and semicarbazide was studied. The enzyme catalyses also the transfer of amino groups of L-lysine and L-ornithine to α-oxoglutarate and pyruvate.

L-Aspartate: α-oxoglutarate aminotransferase (E.C.2.6.1.1) occurs in many plants[1,2] and plays an important role in the metabolism both of amino acids and α-oxoacids. The importance of this enzyme in tobacco plants especially is given by the participation of L-aspartic acid in the biosynthesis of nicotinic acid[3] and of nicotine and other tobacco alkaloids[4,5].

The presence of α-aspartate: α-oxoglutarate aminotransferase in crude leaf-extracts of tobacco plants has already been reported[6]. In this paper results are given on isolation, purification and a more detailed characterization of this aminotransferase.

EXPERIMENTAL

Chemicals. L-Aspartic acid, L-ornithine hydrochloride and L-lysine hydrochloride as well as pyridoxal 5-phosphate were purchased from Fluka A.G. α-Oxoglutaric acid and sodium glyoxalate were purchased from C. Roth, Karlsruhe. NADH and malate dehydrogenase — E.C. 1.1.1.37 — were obtained from C. F. Boehringer and Soehne, Mannheim. All other chemicals were analytical reagents from E. Merck, Darmstadt.

Material. Tobacco plants, *Nicotiana tabacum* L. cv. "Havana II C" (Tabakforschungsinstitut

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Forchheim, GFR) were cultivated in the Botanical Gardens, University Karlsruhe, where they were grown under normal soil and climate conditions. For preparative work the top-leaves of the 8—10 weeks old plants were used in the stage of rosetting.

Determination of proteins. Proteins in all purification steps were determined according to Warburg and Christian[7] spectrophotometrically at 280 and 260 nm, respectively.

Enzyme Assay

L-*Aspartate*: α-*oxoglutarate transaminase activity.* The incubation mixture contained in a final volume of 3·0 ml 255 μmol of potassium phosphate buffer, pH 7·6, 120 μg pyridoxal 5-phosphate, 0·7 μmol of NADH, 20 μmol of sodium α-oxoglutarate, 0·2 ml of malate dehydrogenase diluted with glycerol–water (50% v/v) to 0·5 mg protein/ml and variable amounts of enzyme, 0·2—0·5 ml. After preincubation at 25°C for 5 minutes the reaction was started by adding 636 μmol of sodium L-aspartate. The oxalacetate formed was measured according to Bergmeyer and Bernt[8] by using a Zeiss Spectrophotometer PMQ II. The unit of enzyme activity is the amount of enzyme that causes a change in absorbance of 0·001/minute at 340 nm. L-Aspartate transaminated in the reaction (in μmol) was found using an extinction coefficient of ε_{340} 6·22 $cm^2/\mu mol$ for NADH (ref.[9]). In acceptor specificity experiments various ketoacids as sodium salts instead of α-oxoglutarate were used.

L-*Ornithine*: α-*oxoglutarate aminotransferase activity.* The incubation mixture consisted of 200 μmol of L-ornithine or L-lysine, 80 μmol of α-oxoglutarate, 120 μg of pyridoxal 5-phosphate, 25 μmol of *o*-aminobenzaldehyde, 300 μmol of Tris-HCl buffer, pH 7·7 and the enzyme in a final volume of 3·0 ml. After incubation (120 minutes at 37°C) the reaction was stopped by addition of one ml of 20% trichloroacetic acid and the precipitate removed by centrifugation. The optical density of the supernatant was measured at 435 nm for L-ornithine and at 460 nm for L-lysine.

The unit of enzyme activity is the amount of enzyme that causes in one ml of the incubation mixture in one minute a change in absorbance of 0·01 at given wavelengths[10]. Specific activity is expressed as enzyme units (U) per mg of protein.

The amount of μmol of L-ornithine and L-lysine resp. transaminated in the incubation mixture was obtained from the molar extinction coefficients of the *o*-aminobenzaldehyde adduct of Δ^1-pyrroline-2-carboxylic acid — $1{\cdot}9 \cdot 10^3$ cm^2/mol at 435 nm — and of Δ^1-piperideine-2-carboxylic acid — $2{\cdot}5 \cdot 10^3$ cm^2/mol at 460 nm resp.[10].

Column chromatography. Sephadex G-100 obtained from Pharmacia (Uppsala) was allowed to swell for 72 h in 0·01M potassium phosphate buffer, pH 7·6, introduced to a column and a bed of 2·5 × 40 cm was prepared.

DEAE Sephadex A-50 (Pharmacia) was allowed to swell for 12 h in water and prepared in the usual way; equilibration followed with 0·01M potassium phosphate buffer at pH 7·6. The gel thus prepared was then used to prepare 1·5 × 27 cm beds in suitable columns.

Some Properties of the Enzyme

Inhibition. As inhibitors of L-asparate: α-oxoglutarate aminotransferase hydroxylamine — in final concentrations from $5 \cdot 10^{-3}$M to $3{\cdot}3 \cdot 10^{-5}$M — and semicarbazide — in final concentrations of $1 \cdot 10^{-3}$ and $5 \cdot 10^{-3}$M — were used.

Temperature stability. The effect of heat on the activity of the appropriate enzyme preparation at various temperatures was studied. The enzyme preparations were heated in tubes in a water bath at temperatures indicated (from 40°—65°C) for 15 minutes. After removal from the

bath the tubes were immediately chilled to 0°C and kept at this temperature until examined; control samples were kept at 0°C.

pH-*Optimum.* The pH-optimum of L-aspartate: α-oxoglutarate aminotransferase was determined by varying the pH of the standard incubation mixture using 0·1M potassium phosphate buffer from pH 5·4 to pH 8·0.

RESULTS AND DISCUSSION

Enzyme Purification

Step I. Extraction of tobacco plant leaves. 65–70 g of leaves without the main nervature were after repeated washings with cold distilled water homogenized with 200–250 ml of 0·1M potassium phosphate buffer, pH 7·6 at 0°C in a chilled waring

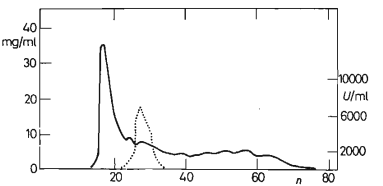

FIG. 1

Elution Pattern from a Sephadex G-100 Column

Proteins (full line), L-aspartate: α-oxoglutarate aminotransferase activity (dotted line). Other conditions are given in the text.

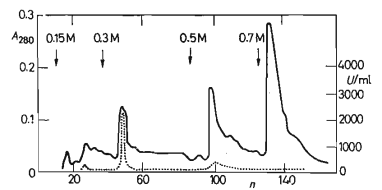

FIG. 2

Elution Pattern from a DEAE Sephadex A-50 Column

Proteins (full line) as estimated by the absorbance at 280 nm, L-aspartate: α-oxoglutarate aminotransferase activity (U/ml, dotted line). Arrows indicate addition of KCl to the eluting buffer. For further conditions see text.

blendor for 2 minutes. The crude preparation was then strained through a cheesecloth and centrifuged (20 000 *g*/20 min) in a Christ Universal III KS centrifuge at 0°C.

Step II. Ammonium sulphate fractionation. The extract from step I was placed in an ice-bath and solid ammonium sulphate was added (pH was controlled – pH 7·0–7·6 – and when necessary diluted ammonia was added) and fractionated in three steps: from 0–31%, 31–62% and from 62–100% saturation. After standing

TABLE I

Ammonium Sulphate Fractionation of L-Aspartate: α-Oxoglutarate Aminotransferase

Fractionation	Volume, ml	Protein mg/ml	Activity U/ml	Specific activity U/mg protein	Purification
Crude extract	268	36	3 500	97·2	1
0– 31%	8	51	5 600	111·5	1·2
31– 62%	10·7	167	39 680	237	2·4
62–100%	7·2	68	5 200	76	0·8

TABLE II

Enzyme Purification

Fraction	Volume, ml	Protein mg/ml	Activity U/ml	Specific activity U/mg protein	Purification
Crude extract (step I)	268	36	3 500	97·2	1
$(NH_4)_2SO_4$ 31–62% (step II)	10·7	167	39 680	237	2·4
Sephadex G-100 (step III)	6	4·8	9 074	1 674	17·3
DEAE Sephadex A-50 (step IV)	3	0·09	2 560	27 300	282

TABLE III

Effect of Inhibitors

Final concentration M	Inhibition, %
hydroxylamine	
$3{\cdot}3 \cdot 10^{-5}$	41
$1 \cdot 10^{-4}$	71
$3{\cdot}3 \cdot 10^{-4}$	83
$1 \cdot 10^{-3}$	91
$5 \cdot 10^{-3}$	100
semicarbazide	
$1 \cdot 10^{-3}$	48
$5 \cdot 10^{-3}$	80

TABLE IV

Substrate Specificity

Amino acid	Acceptor	Aminotransferase activity, %
L-Aspartate	α-oxoglutarate	100
	glyoxalate	13
	pyruvate	0
L-Ornithine	α-oxoglutarate	39
	pyruvate	11
L-Lysine	α-oxoglutarate	2
	pyruvate	10

for 20 minutes in the cold the samples at each saturation were centrifuged as given above. The supernatants after the first and second saturation step resp. were further fractionated and the precipitates from all 3 steps dissolved in 0·1M potassium phosphate buffer, pH 7·6 and dialysed against 5 l of 0·001M potassium phosphate buffer,

pH 7·6 overnight at 2°C. The fraction of 31 – 62% saturation with the highest activity (Table I) was used for further purification.

Step III. Gel filtration. 5 – 6 ml of the above given fraction was applied to a Sephadex G-100 column equilibrated with 0·1M potassium phosphate buffer, pH 7·6 and eluted with the same buffer. The effluents were collected automatically in 3 ml portions at a rate of 10 ml/h and the absorbance at 280 nm recorded by means of Uvicord II (LKB Produkter, Stockholm). The elution pattern is given in Fig. 1.

Step IV. Fractionation on DEAE Sephadex A-50. About 29 mg of protein as estimated from two Sephadex G-100 columns were applied on a DEAE Sephadex A-50, column, equilibrated with 0·01M potassium phosphate buffer, pH 7·6. After washing the column with the same buffer the enzyme was eluted stepwise with the same buffer supplemented with potassium chloride in final concentrations from 0·15 to 0·7M. The elution pattern is given in Fig. 2.

The results of the purification of L-aspartate: L-oxoglutarate aminotransferase are summarized in Table II.

Properties of the Purified Enzyme

Studying the properties of the purified enzyme – preparations after gel filtration (purification step III) were used – a high temperature lability (Fig. 3) and a high sensibility against carbonyl reagents was observed (Table III); a pH optimum of pH 6·6 – 6·7 was determined (Fig. 4). The K_m for L-aspartate was determined according to the method of Lineweaver and Burk[11] and was found to be 4·3 mM.

Using purified enzyme preparations from extracts of germinating *Lupinus angustifolius* plants a pH optimum for the same enzyme of pH 5·5 – 6·0 was reported[12].

As to substrate specificity the purified L-aspartate: α-oxoglutarate aminotransferase catalyses also the transamination of L-ornithine and L-lysine, the activity towards the last two amino acids being much lower.

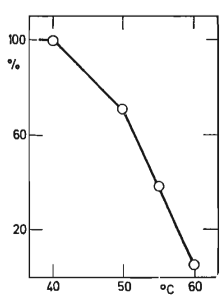

FIG. 3
Effect of Temperature on Enzyme Stability

In experiments with L-aspartate α-oxoglutarate was more effective as acceptor of the amino group than glyoxalate; on the other hand pyruvate was not capable to substitute α-oxoglutarate in this reaction. When L-lysine as substrate was used pyruvate was more effective than α-oxoglutarate; on the other hand L-ornithine was transaminated more effectively in the presence of α-oxoglutarate than of pyruvate (Table IV).

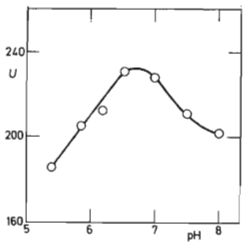

FIG. 4
The pH-Optimum Curve of L-Aspartate: α-Oxoglutarate Aminotransferase
U Enzyme activity in units.

The enzyme activities studied may play an important role in metabolic processes leading to biosynthesis of the pyridine moiety (L-aspartate) of nicotine and anabasine[3] and the pyrrolidine (L-ornithine) or piperidine (L-lysine) moiety of these alkaloids[13,14], resp.

Interesting are the results of this paper as compared with results of a previous paper[15] in which isolation of the same enzyme from *Nicotiana tabacum* L. tissue culture was reported. L-Aspartate: α-oxoglutarate aminotransferase from this material behaves in a similar way during purification and the same pH optimum and temperature-lability were shown. Differences were found in specific activities of crude extracts (about twice as high in tobacco tissue culture extracts than in leaves); in tissue culture experiments only a 29-fold concentration of the enzyme could be achieved compared with the 282-fold purification described in this paper.

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