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PURIFICATION AND CHARACTERIZATION OF ASPARTATE AMINOTRANSFERASES FROM SOYBEAN ROOT NODULES AND *RHIZOBIUM JAPONICUM*

E. RYAN, F. BODLEY and P. F. FOTTRELL

Department of Biochemistry, University College, Galway, Republic of Ireland

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Abstract—At least four electrophoretically distinct forms of aspartate aminotransferase were detected in root nodules of soybeans (*Glycine max* L.). Two forms originated from the cytosol of the host plant, a third from the mitochondria of the host and a fourth from the bacterial component (*Rhizobium japonicum*). The properties of aspartate aminotransferase purified from nodule cytosol and from *R. japonicum* were compared. Both aminotransferases had many similar features including mol. wt., pH optimum and mode of action. However, the cytosol enzyme was much more resistant to a wide variety of inhibitors. Possible roles of the transaminases in ammonia assimilation in nodules is discussed.

INTRODUCTION

SOYBEAN (*Glycine max*) root nodules are the sites of nitrogen fixation and subsequent assimilation of the first stable product of fixation, ammonia.[1] Although it is known that glutamate and aspartate are important intermediates in the assimilation of fixed nitrogen in soybean nodules,[1] relatively little information is available about the enzymes which catalyse the formation of these amino acids in root nodules. The present paper deals with the purification and characterization of two aspartate aminotransferases (L-aspartate: 2-oxoglutarate aminotransferase, E.C. 2.6.1.1) from soybean nodules; one from the nodule cytosol of the host legume and the other from its symbiont *Rhizobium japonicum* (strain 392) which was grown in culture outside the nodules.

RESULTS

Electrophoresis

Four areas of aspartate aminotransferase (AAT) activity were detected after starch gel electrophoresis of crude nodule extracts (Fig. 1). It was found by differential centrifugation that band α was from the bacterial fraction, bands γ and δ from nodule cytosol and band β from nodule mitochondria. The α band had the same electrophoretic mobility as AAT from *R. japonicum* grown outside the nodule; AAT from both of these latter sources had several other properties in common.[2]

General Properties of AAT from Cytosol and Rhizobium

Purified AAT from *R. japonicum* and from the cytosol fraction of soybean nodules was used in these studies. AAT from both sources was free from malate dehydrogenase, glutamate dehydrogenase and alanine aminotransferase activities.[3] During purification, a striking

[1] F. J. BERGERSEN, *Ann. Rev. Plant Physiol.* **22**, 121 (1971).
[2] E. RYAN and P. F. FOTTRELL, in preparation.

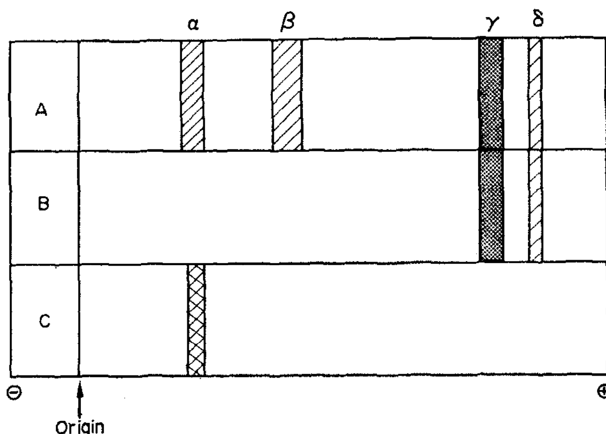

FIG. 1. ASPARTATE AMINOTRANSFERASES FROM SOYBEAN ROOT NODULES SEPARATED BY STARCH GEL ELECTROPHORESIS.
A, crude nodule extract; B, nodule cytosol fraction; C, rhizobial fraction from soybean nodules or extract from *Rhizobium japonicum* (strain 392). The relative intensities of the aminotransferase bands is indicated by shading.

difference was noticed in the stability of AAT from the two sources. Cytosol AAT was highly stable whereas rhizobial AAT lost activity relatively quickly. It was found however that rhizobial AAT was stabilized by the addition of aspartate and pyridoxal-5′-phosphate (PLP). This property was used in a heat purification step (Table 3).

Effect of pH on Enzyme Activity

The cytosol enzyme had maximum activity at pH 7·5 whereas the optimum pH of the rhizobial enzyme was pH 8·0 (Fig. 2).

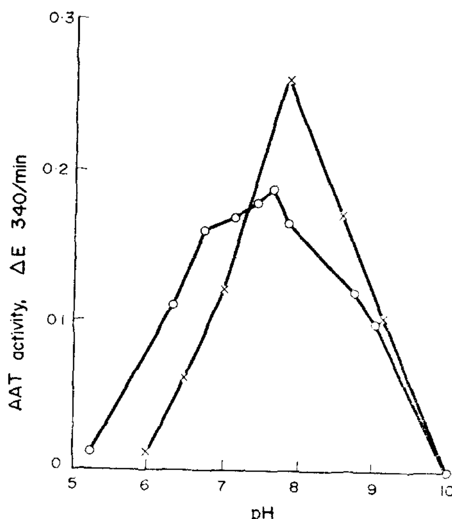

FIG. 2. EFFECT OF pH ON ASPARTATE AMINOTRANSFERASE ACTIVITY.
Cytosol aminotransferase (O); bacterial aminotransferase (×).

[3] H. U. BERGMEYER, *Methods of Enzymatic Analysis*, Academic Press, New York (1965).

Molecular Weights

The molecular weights were determined[4] with a column of Sephadex G.200 (42 × 2·5 cm) calibrated with the following proteins, α-chymotrypsinogen A (MW 25,000), pepsin (MW 36,000), ovalbumin (MW 44,000–46,000), guinea-pig haemoglobin (MW 60,000), bovine serum albumin (MW 65,000–70,000), pig heart aspartate aminotransferase (MW 100,000), L-amino acid oxidase (MW 130,000). The molecular weights of AAT from both sources were similar to values previously reported[5] for AAT from pig heart, i.e. about 100,000 (±10%).

Inhibitors of Cytosol Aminotransferase

Cytosol AAT appeared to be much more resistant to inhibition than rhizobial AAT. The stability of the former was examined further by studying the effect of various inhibitors including pyridoxal antagonists on the enzyme. In these experiments the enzyme was incubated beforehand at 30°, with the reagent except in the case of divalent metals, the effects of which were measured directly in the assay mixture. Only the sulphydryl reagents sodium mersalyl and *para*chloromercuribenzoate (PCMB) at relatively high concentrations inhibited the enzyme within 1 hr. (Table 1). Inhibition by pyridoxal antagonists such as L-cycloserine occurred only after incubation with the enzyme for 24 hr. AAT was protected from these antagonists and the inhibition reversed by 10^{-4} M PLP. In common with AAT from other sources,[6,7] inhibition of the cytosol enzyme by PCMB was partially reversed by

TABLE 1. EFFECT OF VARIOUS COMPOUNDS ON THE ACTIVITY OF CYTOSOL ASPARTATE AMINOTRANSFERASE

Compound	Mode of action	Conc. (M)	Exposure (min)	Inhibition (%)
$HgCl_2$ N-Ethylmaleimide	Mercaptide forming agent	10^{-2}	30	0
Iodoacetamide	Alkylating agent	10^{-2}	30	0
NH_2OH CN^-	Carbonyl binding agent	5×10^{-3}	30	30
Isonicotinic acid hydrazine	Pyridoxyl antagonist	5×10^{-3}	30	< 5
L-Cycloserine	Pyridoxyl antagonist	10^{-3}	30	< 5
Isonicotinic acid hydrazine	Pyridoxyl antagonist	10^{-3}	24 (hr)	22
L-Cycloserine	Pyridoxyl antagonist	10^{-3}	24 (hr)	83
EDTA		10^{-3}	30	0
αα-Dipyridyl		10^{-3}	30	0
1,10-Phenanthroline		10^{-3}	30	0
8-Hydroxyquinoline		10^{-3}	30	0
Pyrophosphate		10^{-3}	30	0
Citrate		10^{-2}	30	< 5
Na Mersalyl		5×10^{-3}	60	40
PCMB		10^{-3}	10	50

[4] P. ANDREWS, *Biochem. J.* **96**, 595 (1965).
[5] Z. H. M. VERJEE and D. F. EVERED, *Biochim. Biophys. Acta* **185**, 103 (1969).
[6] C. TURANO, A. GIARTOSIO, F. RIVA and P. VECCHINI, in *Chemical and Biological Aspects of Pyridoxal Catalysis* (edited by E. E. SNELL, P. FASELLA, A. BRAUNSTEIN and A. ROSSI-FANELLI), p. 149, Pergamon Press, Oxford (1963).
[7] O. POLYANOVSKI and YU. M. TORCHINSKY, in *Chemical and Biological Aspects of Pyridoxal Catalysis* (edited by E. E. SNELL, P. FASELLA, A. BRAUNSTEIN and A. ROSSI-FANELLI), p. 157, Pergamon Press, Oxford (1963).

L-cysteine (6×10^{-2} M). However, in contrast with other enzymes containing PLP, no inhibition of enzyme activity occurred after incubation of nodule cytosol AAT with L-cysteine.[8] Although activation of AAT from other sources by divalent metals has been reported[9,10] no stimulation of cytosol AAT was detected with $MnCl_2$, $MgCl_2$ or $CaCl_2$. The lack of inhibition by chelating agents also argues against the involvement of metal ions in the catalytic action. Likewise, rhizobial AAT was not affected by chelating agents such as EDTA. A number of other enzymes containing PLP was not influenced by divalent metal ions.[11]

In marked contrast to the cytosol AAT the rhizobial enzyme was completely inhibited by $1{\cdot}5 \times 10^{-4}$ M PCMB or 3×10^{-4} M isonicotinic acid hydrazide and the inhibition was only partially reversed by PLP.

Michaelis Constants

Apparent Michaelis constants for the substrates were determined for AAT from both sources and plotted according to the method of Lineweaver and Burk. In agreement with data for AAT from other sources[10,12] both cytosol and rhizobial AAT had lower K_m values for keto acids than for amino acids (Table 2). In this regard, the relatively high K_m of both enzymes for glutamate should be noted. The kinetics of the reaction involving 2-oxoglutarate and aspartate were examined with the cytosol enzyme as described by Alberty.[13] Parallel lines were obtained when initial reaction velocities were plotted by the Lineweaver–Burk method against different concentrations of aspartate at two fixed concentrations of 2-oxoglutarate (Fig. 3). This pattern is indicative of successive binding of the two latter substrates and indicates a binary or 'ping-pong' mechanism of action. Similar results were obtained for rhizobial AAT.

TABLE 2. MICHAELIS CONSTANTS FOR ASPARTATE AMINOTRANSFERASES FROM *Rhizobium japonicum* AND CYTOSOL FRACTION OF SOYBEAN NODULES

Substrate	K_m (moles/l. $\times 10^4$) Cytosol AAT	Bacterial AAT
L-Glutamate	65	130
L-Aspartate	17	19
2-Oxoglutarate	1·8	5·3
Oxaloacetate	1·1	1·0

[8] N. KATUNUMA, M. OKADA, T. KATSUNUMA, A. FUJINO and T. MATSUZAWA, in *Enzymes and Model Systems* (edited by E. E. SNELL, A. E. BRAUNSTEIN, E. S. SEVERIN and YU. M. TORCHINSKY), p. 258, Interscience, New York (1968).
[9] S. R. NADKARNI and K. SOHONIE, *Indian J. Chem.* **1**, 220 (1963).
[10] K. F. WONG and E. A. COSSINS, *Phytochem.* **8**, 1327 (1969).
[11] B. M. GUIRARD and E. E. SNELL, in *Comprehensive Biochemistry* (edited by M. FLORKIN and E. H. STOTZ), Vol. XV, p. 138, Elsevier, Amsterdam (1964).
[12] Y. MORINO and H. WADA, in *Chemical and Biological Aspects of Pyridoxal Catalysis* (edited by E. E. SNELL, P. FASELLA, A. BRAUNSTEIN and A. ROSSI-FANELLI), p. 157, Pergamon Press, Oxford (1963).
[13] R. A. ALBERTY, *Adv. Enzymol.* **17**, 1 (1956).

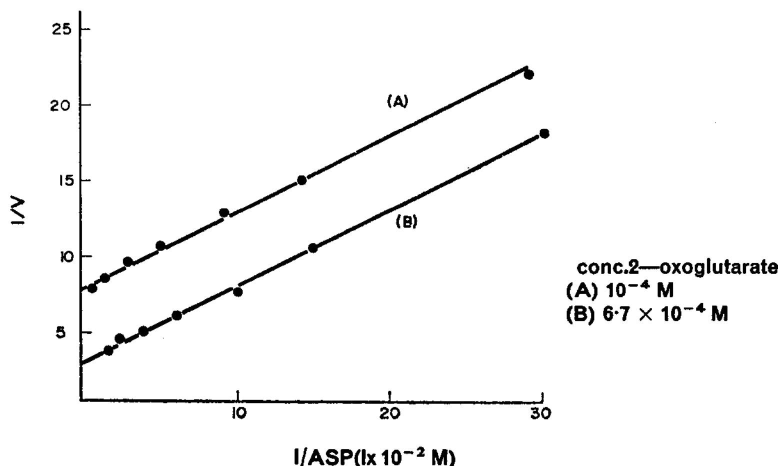

FIG. 3. INITIAL REACTION VELOCITIES OF THE CYTOSOL ASPARTATE AMINOTRANSFERASE ENZYME V ASPARTATE CONCENTRATION AT TWO FIXED CONCS. OF 2-OXOGLUTARATE, LINEWEAVER–BURK PLOT.

DISCUSSION

At least four distinct forms of AAT were found in soybean nodules; three forms originating from the host legume (mitochondrial and cytosol forms) and the fourth located within the *Rhizobium* bacteria. The cytosol form of AAT had many properties in common with AAT from *R. japonicum* and both of the latter enzymes also resembled AAT from other sources.[5,6,10] These properties include mol. wts., higher affinities for keto acids than for amino acids and 'ping-pong' mechanism of action. The most striking difference between the cytosol and rhizobial AAT was the high degree of stability displayed by the former towards various inhibitors (Table 2).

The role of the three aspartate aminotransferases in ammonia assimilation in soybean nodules remains to be clarified. The primary assimilation reaction in soybean and serradella nodules is the incorporation of ammonia into glutamate[1] and nodule rhizobia contain an active glutamate dehydrogenase for this purpose.[14] The next assimilatory reaction is probably a transamination between glutamate and oxaloacetate[1] and the present studies suggest that this step also occurs in the bacterial fraction of nodules which contains a highly active AAT. In previous studies we showed that both glutamate dehydrogenase and AAT activity was induced in *R. japonicum* by NH_4Cl and 2-oxoglutarate.[15]

AAT in the host cytosol might constitute part of a 'shuttle' system where aspartate and 2-oxoglutarate react to maintain a supply of oxaloacetate to the bacteria. Alternatively, the bacterial and host-cytosol forms of AAT might be involved in ammonia assimilation in separate sites within the nodules. There is evidence for two pools of ammonia in root nodules; one pool is in rapid equilibrium with newly-fixed nitrogen, probably in the bacteria, whereas the other, which is not, is probably in the host cytoplasm.[1]

EXPERIMENTAL

Plants. Soybeans (*Glycine max* var Kent; seed obtained from the United States Department of Agriculture, Beltsville, Md.) were grown in a medium free of nitrogen and inoculated as described previously[16] with *R. japonicum*, strain 392 (supplied by C. L. Masterson, Agricultural Institute, Wexford, Republic of

[14] P. F. FOTTRELL and P. MONTGOMERY, *Abstr. 5th Meet. Fed. Europ. Biochem. Soc., Prague*, p. 57 (1968).
[15] P. F. FOTTRELL and P. MOONEY, *J. Gen. Microbiol.* **59**, 211 (1969).
[16] P. F. FOTTRELL, *Nature, Lond.* **210**, 198 (1966).

Ireland). Bacterial and cytosol fractions were prepared from nodules as described by Bergersen[17] and mitochondria were isolated by the method of Muecke and Wiskich.[18] Cytochrome oxidase[19] was used as a marker for the mitochondrial fraction and β-hydroxybutyrate dehydrogenase[20] as a marker for the rhizobial fraction.[21]

Bacteria. Rhizobium japonicum (strain 392) was grown in a defined medium with NH_4Cl + 2-oxoglutarate.[15] Bacterial extracts were prepared by shaking dense suspensions of cells with Ballotini beads in a Braun (Melsungen, Germany) disintegrator for 40 sec. The bacterial suspension was cooled with solid CO_2 during disruption of the cells.

Enzyme assays. Aspartate aminotransferase was estimated[3] in an assay system containing aspartate and 2-oxoglutarate with malate dehydrogenase and NADH included to measure oxaloacetate formation. Net synthesis of glutamate during this reaction was demonstrated with ^{14}C-2-oxoglutarate.[15] Protein was estimated by a modified Biuret method.[22]

Electrophoresis. Extracts were electrophoresed for 16 hr at 4° in 14% starch gels (25 × 9 × 0·5 cm) prepared with 0·005 M Tris-malate buffer pH 8·4. The bridge buffer was 0·10 M Tris-malate pH 8·4 and the conditions for electrophoresis were 100 V and 20 m.a.

After electrophoresis, gels were sliced longitudinally and each of the cut surfaces overlaid with 30 ml 1% agar prepared in 0·1 M phosphate buffer pH 7·5, in which the following were dissolved: aspartate (130 mg), 2-oxoglutarate (30 mg), NAD (60 mg), phenazine methosulphate (1·0 mg), nitrobluetetrazolium (15 mg), glutamate dehydrogenase, type I, (3 units) and pyridoxal-5′-phosphate (PLP) (2 mg). About 1 mg/ml PLP was also added to crude extracts before electrophoresis. Areas of AAT activity appeared on gels after incubation at 35° for 2 hr.

Purification of AAT from R. japonicum

Heat and $(NH_4)_2SO_4$ *steps.* All steps were carried out at 4°. Aspartate and PLP were added to the crude extract at final concentrations of 0·5 M and 0·3 mM respectively after which the extract was heated to 55° for 15 min. The heated extract was then centrifuged at 34,000 *g* for 5 min and the precipitate discarded. Saturated AnalaR $(NH_4)_2SO_4$ (adjusted to pH 7 with 0·1 M NH_4OH) was added slowly with stirring to the supernatant to give a final concentration of 80%. The precipitate was collected by centrifugation at 34,000 *g* for 5 min after which it was extracted twice with 60% $(NH_4)_2SO_4$ and finally with a small volume (usually equal to volume of original crude extract) of 40% $(NH_4)_2SO_4$ solution.

Chromatography. Sephadex G-200 was left to swell for 4 days at 18°. The enzyme preparation (2 ml) was applied to a Sephadex G-200 column (42 × 2·5 cm) and the column eluted at 30 ml/hr with 0·1 M NaCl. Fractions containing AAT activity were combined, concentrated by ultrafiltration (Dialfo, UM-10 membrane) and dialysed for 16 hr against 5 mM potassium phosphate buffer pH 6·5 containing 10^{-5} M PLP.

A microgranular DEAE-cellulose (Whatman, type DE-52) was used to prepare a 25 × 1·5 cm column which was then equilibrated with 5 mM potassium phosphate buffer pH 6·5. The enzyme solution (about

TABLE 3. PURIFICATION OF ASPARTATE AMINOTRANSFERASE FROM *Rhizobium japonicum*

Fraction	Total units*	Total protein (mg)	Sp. act. (units/ mg. prot.)	Yield (%)	Purification
Crude Extract	132·0	175·2	0·75	100	1
Heat + {asp. + PLP}	156·6	105·0	1·49	118	2·0
60–40% $(NH_4)_2SO_4$	104·3	59·5	1·75	79	2·3
Sephadex G-200	101·2	17·5	5·72	77	7·7
DEAE-cellulose	96·5	1·39	69·40	73	92·0

* Aminotransferase units were expressed as micromoles oxaloacetate formed/min/ml enzyme solution.

[17] F. J. BERGERSEN, *J. Gen. Microbiol.* **22,** 671 (1960).
[18] P. S. MUECKE and J. T. WISKICH, *Nature, Lond.* **221,** 674 (1969).
[19] L. SMITH, in *Methods of Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. II, p. 732 (1955).
[20] H. U. BERGMEYER, K. GAWEHN, H. KLOTZSCH, H. A. KREBS and D. H. WILLIAMSON, *Biochem. J.* **102,** 423 (1967).
[21] P. F. FOTTRELL and A. O'HORA, *J. Gen. Microbiol.* **57,** 287 (1969).
[22] A. G. GORNAL, C. J. BARDAWILL and M. M. DAVID, *J. Biol. Chem.* **177,** 751 (1949).

10 ml) was applied to the column and eluted stepwise with 100 ml of 0·1 M, 0·2 M and 0·3 M NaCl in potassium phosphate buffer. AAT activity was eluted from the column with 0·3 M NaCl. A purification of about 90-fold was obtained (Table 3).

Purification of AAT from Soybean Nodules

Heat and $(NH_4)_2SO_4$ *steps.* Soybean nodules were gently crushed in 0·02 M Tris-HCl buffer pH 7·4 and a supernatant fraction free from rhizobia and mitochondria[18] was heated to 60° for 8 min and rapidly cooled. All subsequent steps were carried out at 4°. The heated enzyme preparation was centrifuged at 34,000 *g* for 10 min and solid $(NH_4)_2SO_4$ was added slowly to the resulting supernatant. AAT activity was precipitated between 30–60% $(NH_4)_2SO_4$ saturation. The latter precipitate was collected by centrifugation, resuspended in 0·01 M potassium phosphate buffer pH 8·0 containing 5 mM EDTA, 0·4 mM glutathione (reduced) and 10 mM PLP and dialysed for 16 hr against two changes of this buffer.

Chromatography. After dialysis the enzyme preparation was applied to a column of DEAE–cellulose (Whatman, type DE-52) (20 × 2·0 cm) previously equilibrated with the aforementioned potassium phosphate buffer pH 8·0. The column was eluted with a linear phosphate gradient (10–150 mM phosphate) in pH 8·0 phosphate buffer and AAT activity was eluted at 100 mM phosphate. Tubes containing AAT were pooled and the enzyme was dialysed with 0·01 M phosphate buffer pH 7·0 for 16 hr. The dialysed enzyme preparation was then applied to a column of DEAE–Sephadex (A-50) (20 × 2·0 cm) previously equilibrated with 0·01 M phosphate buffer pH 7·0 and the column was eluted with a linear phosphate gradient (10–300 mM). AAT activity was eluted at 200 mM phosphate. A purification of about 120-fold was obtained (Table 4).

TABLE 4. PURIFICATION OF CYTOSOL ASPARTATE AMINOTRANSFERASE FROM SOYBEAN NODULES

Fraction	Total units*	Total protein (mg)	Sp. act. (units/ mg prot.)	Yield (%)	Purification
Crude extract	202·6	42,000	0·005	100	1
30–60% $(NH_4)_2SO_4$	186·2	6624	0·028	90	5·8
DEAE–cellulose	130·2	773	0·168	64	35
DEAE–sephadex	91·1	158	0·576	45	120

* Units were the same as in Table 3.

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Key Word Index—*Glycine max*; Leguminosae; *Rhizobium japonicum*; nitrogen fixation; aspartate aminotransferases.