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Aspartate Aminotransferase in Alfalfa Root Nodules[1]

I. Purification and Partial Characterization

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ABSTRACT

Aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1 [AAT]), a key enzyme in the assimilation of C and N compounds, was purified from the cytosol of alfalfa (*Medicago sativa* L.) root nodules. Isoforms that increased during nodule development, AAT-2a, AAT-2b, and AAT-2c, were purified greater than 447-fold to apparent homogeneity, and high titer polyclonal antibodies were produced. The native molecular weight of the AAT-2 isoforms was approximately 80 kilodatons with a subunit molecular weight of 40 kilodatons, indicating that the holoenzymes are dimers. The AAT-2 isoforms comprised approximately 0.4% of the total soluble nodule protein. The AAT specific activity was measured in leaf, stem, root, and nodule organs, and zymograms of each were compared. Enzyme activity was 4- to 37-fold greater in effective (nitrogen fixing) nodules than in leaves, stems, and roots. Effective nodule AAT-specific activity was 3- to 8-fold greater than that of plant-controlled ineffective nodules. No differences in K_m were observed between AAT-1 and AAT-2. Antibodies raised against AAT-2 were more selective against AAT-2 than AAT-1. Evidence obtained from zymograms suggests that the expression of alfalfa nodule AAT is controlled at two different gene loci, AAT-1 and AAT-2, resulting in different dimeric isoforms.

Effective (N_2-fixing) alfalfa (*Medicago sativa*) root nodules export recently fixed N_2 primarily in the form of asparagine and aspartate (12, 27). By contrast, plant-controlled ineffective nodules, that fix little if any N_2, produce little if any aspartate and asparagine (12, 29). Effective alfalfa nodules rapidly convert exogenously applied [^{14}C]aspartate and $^{14}CO_2$ into ^{14}Casparagine and [^{14}C]TCA cycle acids, while ineffective nodules do not (12, 27). Using aminooxyacetate, an inhibitor of AAT[3] (EC 2.6.1.1.), Snapp and Vance (27) showed that the flow of C between the organic acid and amino acid pools was regulated by AAT activity. They suggested that multiple forms of AAT may regulate endogenous pools of aspartate in effective root nodules.

In plants, AAT has been characterized as a dimeric enzyme that catalyzes the reversible reaction: aspartate + α-ketoglutarate $\leftrightharpoons$ glutamate + oxaloacetate (OAA) (32). Multiple forms of AAT have been reported in soybean (*Glycine max* L. Merr.) (22, 23), lupine (*Lupinus angustifolius* L.) (19, 20), cowpea (*Vigna unguiculata* L.) (26), and alfalfa (8; CP Vance, unpublished data). In soybean root nodules, Ryan and Fottrell (23) reported AAT specific activity associated with the plant cytosolic, mitochondrial, and bacteroid fractions, with 87% of the total AAT-specific activity confined to bacteroids. By contrast, Boland *et al.* (2) reported that soybean nodules contained two isozymes of AAT, one plant cytosolic and the other located in proplastids. The cytosolic form accounted for 70% of the total specific activity. Lupine nodule AAT occurred as three forms, plant cytosolic (AAT-P_1), plastid (mitochondrial) (AAT-P_2), and bacteroid, with, again, the plant cytosolic form predominating (19–21). Reynolds and Farnden (20) reported that lupine nodule AAT-P_2 increased in activity during nodule development, concurrent with increases in activity of other nodule enzymes of ammonia assimilation. Cowpea nodule AAT-specific activity was localized to both infected and uninfected cells and was primarily of host origin (26). Plant cytosolic AAT comprised 91 to 94% of that in alfalfa nodules with the remaining portion in the bacteroid fraction (8). Thus, most AAT activity in legume nodules appears to be of host origin.

Although the role of AAT in the assimilation of fixed N_2 into aspartate and asparagine appears clear, this enzyme probably plays other important roles in root nodule function. Ryan *et al.* (22) noted that the multiple forms of nodule AAT might constitute a portion of a shuttle system to maintain a supply of OAA to bacteria. Similarly, Akkermans *et al.* (1), Snapp and Vance (27), and Kahn *et al.* (9) suggested that nodule AAT could function as part of a malate-aspartate shuttle and regulate C and N flow between microbial symbiont and host plant cytoplasm. Similarly, Wightman and Forest (32) indicated that aminotransferase activity is an important link between C and N metabolism in plant cells.

In legume nodules, AAT has been purified and partially characterized in soybean (22) and lupine (19). A preliminary report showed production of antibodies to lupine nodule AAT

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[3] Abbreviations: AAT, aspartate aminotransferase; DAZ, days after inoculation; DAP, days after planting/inoculation; FPLC, fast protein liquid chromatography; GS, glutamine synthatase; PEPC, phosphoenolpyruvate carboxylase.

(21). However, the specificity of these antibodies was not addressed. The objectives of this study were to: (a) purify the isoforms of alfalfa nodule AAT associated with the effective N_2-fixing condition, (b) characterize AAT isoform patterns, (c) determine if AAT activity is reduced or absent in plant-controlled ineffective alfalfa nodules, (d) determine some physical and kinetic characteristics of the nodule isoforms, and (e) produce antibodies to AAT.

MATERIALS AND METHODS

Plant Material

Alfalfa (*Medicago sativa* L.) plants were grown in a sand bench under glasshouse conditions as previously described (29). Micro- and macronutrients (except for N) and lime ($CaCO_3$) were incorporated into the sand. The sand was inoculated before seedling emergence with the effective *Rhizobium meliloti* strain 102F51. Mature nodules were harvested from plants at approximately 35 to 45 DAP. Nodules used were either fresh or frozen at −70°C.

Plants for the study of nodule development were inoculated at the first trifoliolate leaf stage. Roots or nodules were harvested at 0, 4, 7, 11, 17, and 31 DAI. On 0 and 4 DAI, nodules were not visible, so the entire root was taken.

Enzyme Extraction and Purification

Tissue of interest was extracted (200 mg fresh weight · mL buffer^{-1}) with 20 mM K^+-phosphate buffer (pH 7.5), 5 mM DTT, 10 μM antipain, and 1 mM PMSF using sand and a mortar and pestle. All extraction and purification steps were performed at 4°C. The resulting homogenate was centrifuged at 26,000*g* for 20 min. For purification of root nodule AAT-2, 40 mL of K^+-phosphate buffer-equilibrated hydroxyapatite was added to the supernatant, and the slurry was incubated for 1 h with occasional stirring. Aspartate aminotransferase did not bind to hydroxyapatite and was present in the supernatant following centrifugation at 10,000*g*. Aspartate aminotransferase activity was precipitated with a saturated solution of $(NH_4)_2SO_4$ (50–70% saturated fraction), and the pellet was resuspended in 5 mL 20 mM K^+-phosphate buffer (pH 7.5) containing 3 mM DTT and applied to a gel filtration column (Sephacryl S-200 by Pharmacia; 2.6 × 93 cm). Fractions containing 3 AAT were applied to a Affi-Gel Blue column (1.5 × 6 cm) equilibrated in 20 mM K^+-phosphate buffer (pH 8.0). The AAT did not bind to the column and eluted in the void volume. Following the Affi-Gel Blue step, AAT was applied to an FPLC anion exchange column (5 × 50 mm, Mono-Q HR 5/5 by Pharmacia, Uppsala, Sweden) equilibrated in 20 mM Tris-HCl (pH 8.0), and AAT isoforms eluted with a linear salt gradient from 0 to 200 mM NaCl in 20 mM Tris-HCl (pH 8.0).

Enzyme Assay

The routine assay to measure AAT (EC 2.6.1.1) activity involved a NADH-dependent malate dehydrogenase (MDH)-linked reaction as described by Ryan *et al.* (22). This assay was chosen for its greater sensitivity and was in the direction of aspartate utilization. Oxaloacetate produced was converted to malate by MDH. The oxidation of NADH was monitored spectrophotometrically at 340 nm. One unit of activity is defined as that forming 1 μmol of product min^{-1}. This assay was also used to determine the K_m values for aspartate and α-ketoglutarate (α-KG). An alternative, but less sensitive assay was used to determine the K_m values for glutamate and OAA. This assay involved measuring the direct production or utilization of OAA in the reaction mix by monitoring the change in absorbance at 280 nm as described by Cammarata and Cohen (3). The specific activity of alfalfa nodule AAT-1 or AAT-2 did not change following incubation for several hours with pyridoxal phosphate. Therefore, this coenzyme was not included in any of the above assays. The kinetics of each major isoform, AAT-1 and AAT-2, were determined using a mixture of each subform, AAT-1b and 1c and AAT-2a, 2b, and 2c, respectively. The relationship between velocity and substrate concentration showed hyperbolic Michaelis-Menten kinetics. Values of K_m were determined from Lineweaver-Burk linear plots. Soluble protein was measured as described by Lowry *et al.* (10).

Gel Electrophoresis

Nondenaturing PAGE was performed with the standard Tris-glycine system of Ornstein (17). Soluble plant extracts were used immediately after extraction. The AAT activity on nondenaturing PAGE gels was identified by incubating the gel in 100 mM Tris-HCl (pH 7.5), 40 mM aspartate, 5 mM α-KG, and Fast Violet B salt (1 mg mL^{-1} buffer) as previously described (7, 25). Electrophoresis of proteins by SDS-PAGE was performed as described by Maizel (11). Protein samples were diluted 1:1 (v/v) prior to electrophoresis with Maizel (11) buffer containing 5% β-mercaptoethanol and 4% SDS. The SDS protein samples were boiled for 2 min. Proteins were separated in a 10% SDS-polyacrylamide gel. Gels were run at a constant current of 18 mA for 3 h with constant cooling. Markers for mol wt determination consisted of β-galactosidase (116 kD), phosphorylase b (94 kD), bovine serum albumin (68 kD), and creatine phosphokinase (40 kD). Proteins were visualized by silver staining (13).

Antiserum Production

Antiserum against alfalfa nodule AAT-2 isoforms was produced and prepared as described previously (14). Rabbits (NZW) were initially immunized by a subcutaneous injection of a total of 100 μg nondenatured purified AAT-2 proteins in Freund's complete adjuvant. Five booster injections of 300 μg purified SDS treated AAT-2 protein in incomplete adjuvant were given at bimonthly intervals.

Immunotitration of AAT Activity

A constant quantity of AAT activity from different sources was incubated for 1.5 h at 4°C with varying quantities (25–200 μL) of AAT antiserum as described by Miller *et al.* (14). The AAT remaining in the supernatant following centrifugation was assayed spectrophotometrically at 340 nm. Preim-

mune serum was incubated with AAT activity from each source as a control.

RESULTS

AAT Specific Activity

Alfalfa nodules had higher AAT specific activity than either leaves, stems, or roots, whether expressed on a protein or fresh weight basis (Table I). Enzyme activity was expressed on both a protein and fresh weight basis because of unequal protein concentrations measured for each tissue. Over 90% of the total soluble AAT activity extracted from alfalfa nodules was associated with the plant fraction.

The AAT activity of alfalfa plant-controlled ineffective alfalfa nodules, both early-senescing (in_1) and tumor-like (in_2 and in_3) genotypes, was reduced 65 to 80% on a protein and fresh weight basis, respectively, as compared to that of effective nodules (Table I). Ineffective nodules also contained lower total protein concentrations on a fresh weight basis than effective nodules. These reductions in AAT activity and total protein concentrations coincided with previously reported reductions in leghemoglobin and N_2-fixation rates among the ineffective nodule genotypes (5, 29).

AAT Zymograms

Native-PAGE of equal amounts of AAT activity from crude extracts from leaves, stems, roots, and nodules (30 DAI) showed two major regions of AAT activity, AAT-1 and AAT-2 (Fig. 1). Soluble protein preparations from nodules showed intense staining for both AAT-1 and AAT-2, whereas leaves stained more intensely for AAT-2 and less so for AAT-1. Stem and root tissue showed greater staining for AAT-1 than for AAT-2.

The AAT-2 region could be subdivided into three AAT isoforms. The distribution and intensity of AAT-2 isoforms varied between tissues. Nodules showed intense staining for all three forms of the faster-migrating AAT-2 region. Leaf, stem, and root tissue also showed staining for all three forms of AAT-2, but the intensity of staining was strikingly reduced as compared to nodules.

Table I. *Alfalfa Aspartate Aminotransferase Activity in Effective (cv. Saranac) Leaf, Stem, Root, and Nodule Tissue, and in Plant-Controlled Ineffective Early-Senescing (in_1sa and in_1ag) and Tumor-Like (in_2 and in_3) Nodules*

Source	Total Protein	AAT Activity	
	$mg \cdot g^{-1}$ fresh wt	$\mu mol \cdot min^{-1} \cdot g^{-1}$ fresh wt	$\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein
Leaf	35.5 ± 2.1[a]	2.5 ± 0.1	0.12 ± 0.0
Stem	1.2 ± 0.7	0.5 ± 0.0	0.40 ± 0.0
Root	2.2 ± 0.1	0.3 ± 0.0	0.13 ± 0.0
Nodule			
Effective	10.3 ± 0.5	11.0 ± 0.6	1.07 ± 0.1
Ineffective in_1sa	4.1 ± 0.2	1.3 ± 0.1	0.34 ± 0.0
Ineffective in_1ag	5.0 ± 0.3	2.2 ± 0.1	0.38 ± 0.0
Ineffective in_2	5.9 ± 0.4	2.0 ± 0.1	0.35 ± 0.0
Ineffective in_3	3.1 ± 0.2	1.5 ± 0.1	0.50 ± 0.0

[a] Each value represents the mean of three replicates ± one SE.

Reduced AAT activity in plant-conditioned ineffective in_1, in_2, and in_3 nodules appeared to be due to a reduction in AAT-2 isoforms (Fig. 1) Ineffective nodules contained both AAT-1 and AAT-2. However, the staining intensity of the AAT-2 region from ineffective nodules was less than that of effective nodules. By comparison, staining intensity of the AAT-1 region of effective and ineffective nodules appeared comparable.

Alfalfa nodule bacteroids and free-living *Rhizobium meliloti* AAT activity measured 20 and 40 nmol min^{-1} mg^{-1} protein, respectively. *R. meliloti* bacteroids and free-living bacteria showed a single AAT activity band on a native-PAGE gel (data not shown). These bands migrated slightly faster than AAT-2c.

AAT Activity during Nodule Development

A zymogram of AAT activity of infected roots and developing nodules showed that AAT-1 predominated in young roots from 0 to 4 DAI and in nodules at 7 and 11 DAI (Fig. 2A). In young roots, AAT-2 often was not detectable. However, in mature roots (30 DAI) AAT-2 isoforms were present but low in activity (Fig. 1). By 11 DAI, all three AAT-2 isoforms were detected in nodules. The staining intensity of the AAT-2 isoforms increased by 17 DAI and then remained relatively constant to 31 DAI. Similarly, the nodule AAT-1 isoforms also appeared to increase in staining intensity by 7 DAI.

The AAT specific activity in infected roots (0–4 DAI) was low prior to the appearance of visible white nodules at 7 DAI (Fig. 2B). The AAT activity increased in nodules fivefold between 7 and 17 DAI. The greatest rate of change in AAT activity occurred between 7 and 11 DAI. From 17 to 31 DAI, nodule AAT activity continued to increase gradually. Increase in nodule AAT activity between 7 to 17 DAI coincided with the development of effective nodules and the synthesis of nitrogenase and enzymes associated with the GS/GOGAT pathway (5).

Purification

Since AAT-2 appeared to be closely related to nodule organogenesis and N metabolism, the isoforms of AAT-2 were further purified to homogeneity and partially characterized (Table II). Soluble plant AAT activity was extracted from 35 g of mature effective nodules harvested approximately 35 to 45 DAP. Although AAT did not bind to hydroxyapatite, this step removed 66% of the protein and resulted in a 2-fold purification. Fractionation with $(NH_4)_2SO_4$ gave a further 3-fold purification. Gel filtration on Sephacryl S-200 resulted in a total 15-fold purification. Since MDH, an abundant nodule enzyme, copurified with AAT-2, an Affi-Gel Blue step was used to remove MDH. This step effectively removed MDH and resulted in a further 1.8-fold purification. Anion exchange chromatography with FPLC allowed exceptional separation of the AAT-1 and AAT-2 isoforms (Fig. 3A). To achieve greater separation and purification of the AAT-2 isoforms, pooled peak fractions of the AAT-2 isoforms were again bound on the FPLC anion exchange column and eluted with a more shallow salt gradient (Fig. 3B). Pooled AAT-1

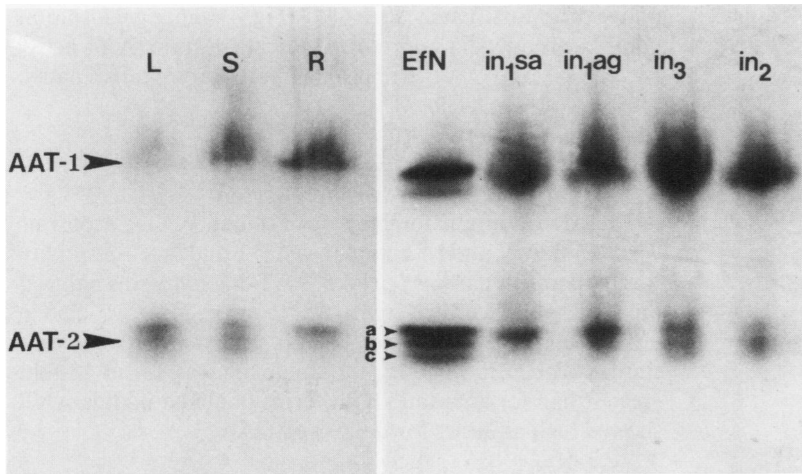

Figure 1. Zymogram of AAT activity from cell free extracts from effective 'Saranac' leaf (L), stem (S), root (R), nodule (EfN), and nodules of plant-controlled, ineffective, early-senescing (*in*$_1$sa and *in*$_1$ag) and tumor-like (*in*$_3$ and *in*$_2$) genotypes. The two major regions of AAT activity were designated as AAT-1 and AAT-2 and banding within the AAT-2 region was further subdivided into bands a, b, and c. All lanes contained 0.02 units of AAT activity.

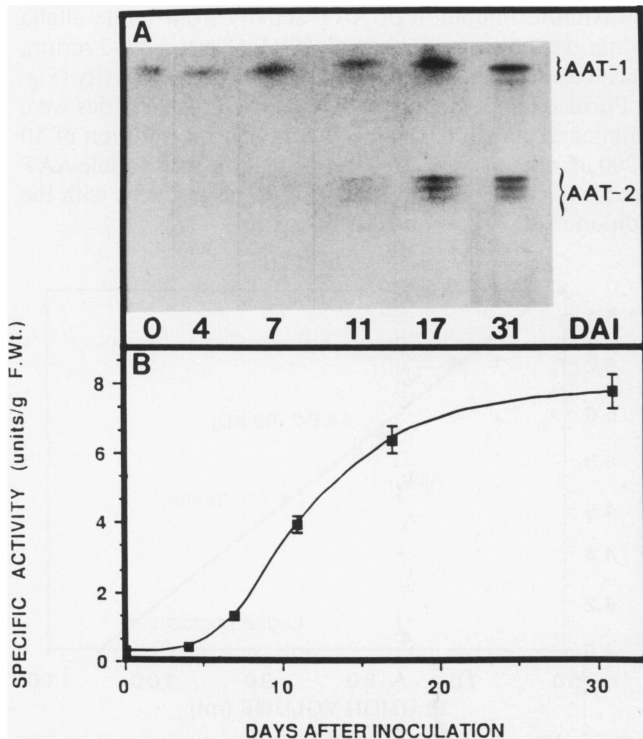

Figure 2. AAT zymogram (A) and activity (B) during effective alfalfa nodule development. At 0 and 4 DAI, nodules were not visible; by 7 DAI tiny white nodules were collected; and on 11, 17, and 31 DAI, pink, actively N_2-fixing nodules were harvested. Each lane of the zymogram contained 0.03 unit of AAT activity. One unit of activity is defined as that forming 1 μmol of product min^{-1}. Each point is the mean of three replicates ± 1 SE.

Table II. *Purification of Aspartate Aminotransferase-2 (AAT-2) Isoforms from Alfalfa (Medicago sativa L. c.v. Saranac) Root Nodules. Values are Representative of Four Separate Purifications.*

Purification Step	Total Activity[a]	Total Protein	Specific Activity	Purification	Yield
	units	mg	units mg^{-1} protein		%
Crude Extract[b]	150	390	0.38	1.1	100
Hydroxylapatite	105	131	0.80	2.1	70
50–70% $(NH_4)_2SO_4$	92	37.7	2.44	6.4	61
Gel Filtration	88	15.4	5.71	15.0	59
Affi-Gel Blue	51	5.1	10	26.3	34
Anion Exchange (AAT-2a,b,c)	47	0.23	169	447	31
AAT-2a	17	0.13	130		
AAT-2b	12	0.05	240		
AAT-2c	7	0.05	140		

[a] One unit of activity is defined as that forming 1 μmol of product min^{-1}. [b] Alfalfa nodule crude extract was prepared using 35 g fresh weight of nodules.

fractions from the first FPLC elution were pooled and saved for later analysis. Following anion exchange chromatography, peaks of AAT-2 isoform activity were pooled and electrophoresed under nondenaturing PAGE conditions to assess the extent of isoform purity in terms of isoform separation and protein content. Results showed excellent isoform separation with little or no contamination from neighboring AAT-2 isoforms. Final purification of AAT-2a, 2b, and 2c was greater than 447-fold, with a yield of 31%. The AAT-2 isoforms were estimated to comprise approximately 0.4% of the total plant soluble protein from N_2-fixing nodules harvested 35 to 45 DAP.

Extraction buffers in our study contained antipain and PMSF, proteolytic enzyme inhibitors, thus reducing the possibility that the isoforms resulted from proteolytic cleavage of the holoenzyme. To ensure that proteolysis was not a factor in generating the isoforms, we extracted nodules in both the absence of proteolytic enzyme inhibitors and the presence of the proteolytic enzyme Proteinase K. Even after 12 h of treatment we found no difference in resolution of AAT-2a, 2b, and 2c (data not shown).

Mol Wt Determination

By SDS-PAGE, the subunit mol wt was determined for the purified forms of AAT-2a, 2b, and 2c. All three forms had a subunit mol wt of 40 kD (Fig. 3C). Silver staining of the purified AAT-2 forms confirmed the purity of the proteins.

Native AAT-1 and AAT-2 mol wt was estimated by gel

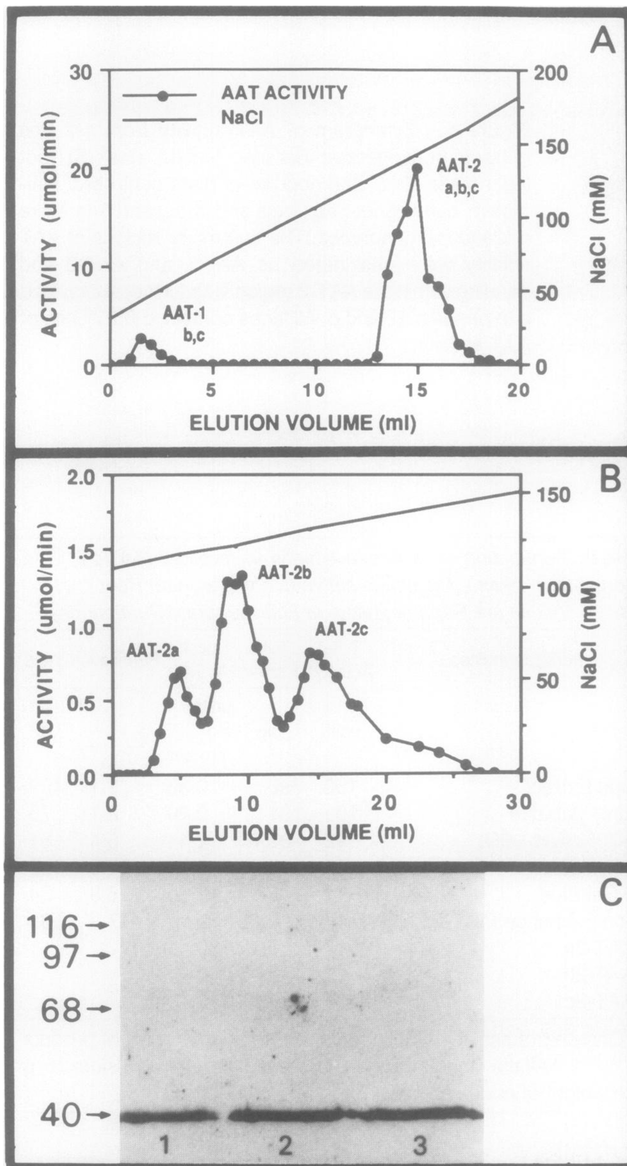

Figure 3. Chromatographic separation of AAT-1 and -2 and evidence for AAT-2 isoform purification to homogenity. A, The FPLC elution profile of effective alfalfa root nodule AAT-1 and AAT-2 activities following anion exchange chromatography. One unit of activity is defined as that forming 1 μmol of product min^{-1}. B, the elution profile of purified AAT-2a, 2b, and 2c from a more shallow gradient on FPLC anion exchange chromatography. C, A silver-stained 10% SDS-PAGE gel containing 7.5 μg of purified AAT-2a (lane 1), 2b (lane 2), and 2c (lane 3) protein. Mol wt of β-galactosidase (116 kD) phosphorylase b (94 kD), BSA (68 kD), and creatine phosphokinase (40 kD) are indicated by arrows. The figure is representative of four separations.

filtration (exclusion) chromatography. The elution of known mol wt standards showed a linear relationship ($R^2 = 0.99$) when plotted against their log mol wt. Gel filtration chromatography of an alfalfa nodule extract yielded a single peak of AAT activity. This extract was previously subjected to hydroxyapatite and ammonium sulfate purification steps and contained all of the nodule plant isoforms AAT-1 and AAT-2. This eluted activity peak corresponded to an estimated native of approximately 80 ± 4 kD (Fig. 4). Since gel filtration chromatography did not resolve separate peaks of AAT activity, AAT-1 and AAT-2 appear to have very similar native mol wt.

pH Optimum and Substrate Kinetics

The pH optimum for both AAT-1 and AAT-2 isoforms was 8.0. Between pH 5.5 to 8.0, AAT-1 and AAT-2 activity was reduced similarly. At pH 8.5, AAT-1 activity was reduced only 2%, while AAT-2 activity was reduced 28%.

The substrate kinetics for AAT-1 and AAT-2 were very similar (Table III). The K_m for glutamate was 13- to 15-fold greater than for aspartate. Both forms of alfalfa nodule AAT showed high affinities for α-KG and OAA.

Immunotitration of AAT Activity

Maximum inhibition of AAT activity from crude alfalfa nodule extract was achieved with 50 μL of anti-AAT-2 serum, which resulted in a 83% reduction of total AAT activity (Fig. 5). Purified alfalfa nodule AAT-1 and AAT-2 activities were inhibited 58 and 100%, respectively, with the addition of 50 to 200 μL of antiserum. Soybean and lupine root nodule AAT activities were reduced by 42 and 51%, respectively, with the addition of 100 μL of anti-AAT-2 serum.

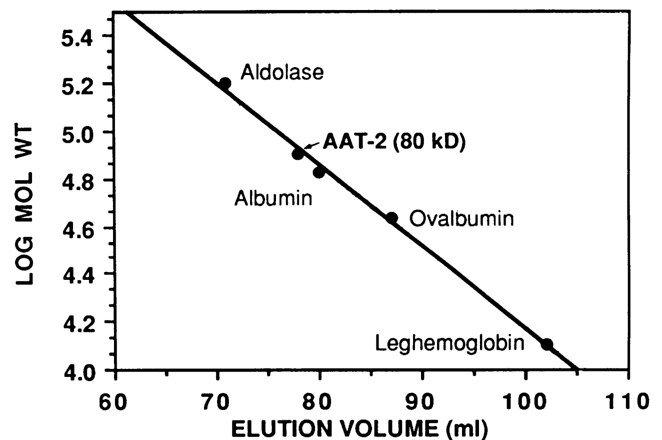

Figure 4. Mol wt determination of a partially purified root nodule AAT-2. The protein preparation contained all isoforms of both AAT-1 and -2. Native AAT and protein mol wt standards, aldolase (158 kD), BSA (68 kD), ovalbumin (43 kD), and leghemoglobin (13 kD) were eluted from a 2.6 × 9.3 cm Sephacryl S-200 column in 3 mL fractions with 20 mM K^+-phosphate buffer (pH 7.5). The graph is representative of three determinations.

Table III. *Kinetic Values for Alfalfa Nodule Plant AAT-1 and AAT-2*

Isoform	Km			
	Asp	α-KG	Glu	OAA
	mM			
AAT-1[a]	1.2	0.55	18.5	0.031
AAT-2[b]	1.5	0.39	19.4	0.027

[a] Contained a mixture of AAT-1b and 1c. [b] Contained a mixture of AAT-2a, -2b, and -2c.

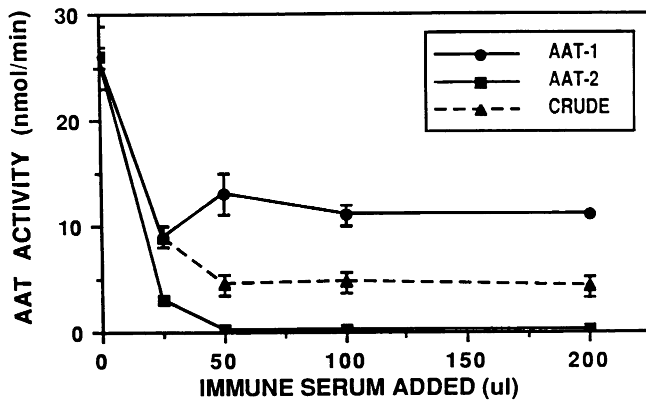

Figure 5. Immunotitration of AAT activity from alfalfa root nodule crude extract and purified AAT-1 and AAT-2 using antiserum prepared against alfalfa nodule AAT-2 isoforms. A constant quantity of activity from each source was incubated with anti-AAT serum (25–200 μL). Goat anti-rabbit IgG (90 μg) was added to each reaction to precipitate the immune complexes and the activity of AAT remaining in the supernatant following centrifugation was assayed pectrophotometrically as described in "Materials and Methods." Preimmune serum was incubated with proteins from each source as a control. Each value is the mean of three replicates ± 1 SE.

DISCUSSION

Although the essentiality of AAT to the assimilation of NH_4^+ and AAT activity had been documented in many species of legume nodules (2, 8, 18–23, 26), the enzyme has been purified and partially characterized from only lupine nodules (19). Our studies of alfalfa nodule AAT confirm lupine data showing that two forms of the enzyme (AAT-1 and -2) occur in effective nodules and that increased AAT activity during effective nodule development is associated with expression and enhanced activity of the AAT-2 form. The alfalfa nodule enzyme, similar to lupine (19), has a much higher affinity for aspartate, α-KG, and OAA than for glutamate. We have further extended the knowledge and understanding of root nodule AAT by (a) showing that alfalfa nodule AAT-2 is comprised of three isoforms, (b) purifying all three AAT-2 isoforms to homogeneity and showing that they are dimers with identical subunit mol wt of 40 kD, (c) showing that plant-controlled ineffective nodules have reduced AAT activity and this reduction is associated with an apparent decrease or absence of specific isoforms of AAT-2, and (d) producing high titer polyclonal antibodies to AAT-2.

Since the primary function of root nodules is associated with N metabolism (9), it is not surprising that effective nodules have the highest AAT specific activity of any alfalfa organ examined. The high AAT activity in nodules and the appearance of AAT-2 isoforms during effective nodule development (20, and this report) lead us to believe that nodule-specific (nodulin) forms of AAT accounted for our observations. However, zymogram analysis showed that both forms of AAT occurred in all tissues and in ineffective nodules. This suggests that alfalfa nodule AAT, in contrast to GS in *Phaseolus* nodules (4) and uricase in *Glycine* nodules (31), is nodule enhanced rather than completely nodule specific. Nodule-enhanced expression of enzyme forms found in other plant organs has also been demonstrated for GS in *Pisum* (4) and PEPC in alfalfa (14).

There did appear to be tissue specific differential expression within the three isoforms comprising AAT-2. The finding that all three forms of AAT-2 are expressed in effective nodules, while ineffective nodules and other organs either lacked or had much reduced amounts of one or more of the isoforms, implies both organogenic and chemical regulation of root nodule AAT activity. Such differential expression may reflect subcellular distribution of AAT and/or the presence of NH_4^+. Cytosolic, mitochondrial, and plastid forms of AAT have been documented (2, 18, 20, 24, 32). Increased AAT activity during effective lupine nodule development was associated with a plastid form of the enzyme (19–21). Application of N fertilizer inhibited expression of the lupine nodule plastid AAT. Differential expression of GS in *Pisum* has been shown to be a function of plastid and cytosolic forms of the enzyme (4).

Maximum expression of AAT activity in nodules appears to require some product of the effective symbiosis. This product may be NH_4^+. Expression of nodule-specific uricase requires continued production of NH_4^+ by nodules (31). Ineffective nodules usually have reduced activity for enzymes of C and N assimilation and reduced expression of many nodulins (4, 29, 31). By contrast, in nodules of some species, mRNAs and polypeptides for GS are induced in ineffective nodules (4) suggesting that the nodule morphogenesis, irrespective of effectiveness, can enhance expression of selected proteins. Resolution of whether AAT is comprised of any nodule specific polypeptides will require immunoblot analysis of AAT purified from various alfalfa organs, organelles, and from effective and ineffective nodules.

Purification and characterization of alfalfa nodule AAT-2 showed that the enzyme has a native mol wt of 80 kD (Fig. 4) and a subunit of mol wt of 40 kD. These data indicate that the enzyme is a homodimer. The native mol wt of alfalfa nodule AAT-2 is similar to that reported for wheat (*Triticum aestivum*) germ AAT (75 kD) but somewhat less than that reported for bushbean (*Phaseolus vulgaris* L.) roots (125 kD) and soybean nodules (100 kD) (6, 22, 30). Reynolds *et al.* (19) reported that lupine nodule AAT-P_1 and -P_2 appeared to be dimers comprised of polypeptides with subunit of 47 and 45 kD, respectively. We could not resolve a mixture of alfalfa nodule AAT-1 and -2 into two activity peaks by exclusion chromatography, but zymograms containing sequentially collected exclusion chromatography fractions showed a greater portion of AAT-1 eluting just prior to AAT-2 (SM Griffith, CP Vance, unpublished data). This suggests a slightly higher native mol wt for AAT-1 and is consistent with the small difference in mol wt seen for lupine nodule AATs. Mammalian soluble and mitochondrial AATs also appear to be dimers with native mol wt ranging from 90 to 100 kD and subunit mol wt of 40 to 46 kD (15, 16).

Native forms of alfalfa nodule AAT-1 and AAT-2 differ in electrical charge, based on their mobility during nondenaturing PAGE (Fig. 1) and anion exchange chromatography (Fig. 3). The AAT-2 isoforms are considerably more anionic than the AAT-1 forms. This may indicate major differences in amino acid composition between AAT-1 and -2. Antigenic

differences are also evident between AAT-1 and -2 (Fig. 5), further evidence of amino acid differences at epitope sites. Similar ionic and antigenic differences have been shown with lupine nodule (19–21) and mammalian AATs (15, 16). These differences suggest that AAT-l and -2 are encoded by separate, distinct genes (7). This suggestion is supported by evidence showing that mammalian cytoplasmic and mitochondrial AATs are encoded by separate genes (16) In addition, a putative AAT-P_2 cDNA has been isolated from lupine nodules (21). Independent segregation of soluble, mitochondrial, and glyoxysomal isozymes of AAT in maize (*Zea mays* L.) has shown that each of these forms of the enzyme is under the control of separate genetic loci (24). Our data are consistent with the interpretation that AAT-l and -2 are encoded by two distinct loci with different alleles at each locus encoding slightly different forms of the enzyme and resulting in separate dimeric forms. Verification of this hypothesis requires evaluation of allozyme patterns in progeny of cross- and self-pollinations within at least two genotypes.

Alfalfa nodule AAT-1 and -2 have similar K_m values for all substrates tested (Table III). Similar to lupine nodule AAT-P_1 and -P_2 (19), the alfalfa enzymes had a much higher affinity for α-KG and OAA than for aspartate and glutamate, and the affinity of the enzymes for aspartate was 10- to 15-fold greater than that for glutamate. Soybean nodule-soluble AAT shows similar properties (22). Concentrations of aspartate and glutamate within alfalfa nodules approach 3.5 and 8.5 mM, respectively (12, 28; CP Vance unpublished data). At such concentrations AAT would be saturated for aspartate and nearly 50% saturated for glutamate, suggesting that the enzyme favors glutamate production. Support for this interpretation is drawn from studies of Snapp and Vance (27) showing that alfalfa nodules rapidly convert exogenously applied [^{14}C] aspartate to [^{14}C]TCA cycle acids and $^{14}CO_2$. Furthermore, inhibition of AAT activity by aminooxyacetate blocked conversion of aspartate to TCA cycle acids and stimulated asparagine production. Inconsistent with the AAT reaction favoring glutamate production is the fact that alfalfa nodules synthesize high concentrations, 43.7 μmol·g nodule fresh weight^{-1} (28), of asparagine, indicating that AAT must also favor aspartate production. This inconsistency can be explained by subcellular compartmentalization of the various forms of the enzyme and concentration of reactants at the enzyme site. Cytosolic, plastid, mitochondrial, and glyoxysomal forms of AAT have been documented (2, 21, 24, 32), and these various forms may be exposed to different concentrations of substrates. Reynolds *et al.* (19) suggested that the differences in K_ms of lupine nodule AAT-P_1 and -P_2 reflected their cytosolic and plastid locations, respectively.

We have successfully produced polyclonal antibodies against purified alfalfa nodule AAT-2 protein. Although a preliminary report indicated production of antibodies to lupine nodule AAT-P_2 (21), ours is the first to show immunotitration of root nodule *in vitro* AAT activity by a highly specific antibody preparation. Inhibition of 40 to 50% of the *in vitro* AAT activity from lupine and soybean nodules show that our antibodies recognize a form of AAT in these species and suggests genetic conservation of a portion of the coding region of the AAT genes. While our AAT-2 antibodies completely removed AAT-2 *in vitro* activity, they only partially recognized AAT-l. This explains the incomplete immunotitration of *in vitro* AAT activity from crude enzyme extracts from alfalfa nodules. Morino *et al.* (15) demonstrated a similar phenomenon with mammalian AATs. They showed that antibodies to soluble AAT fully recognized soluble AATs from several mammalian sources but mitochondrial AATs were much less readily recognized. Likewise, antibodies to mitochondrial AAT recognized other mitochondrial AATs but less so soluble AATs. Our antibodies should be useful in studying the genetic regulation of AAT expression in alfalfa nodules.

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