Production and Characterization of Monoclonal Antibodies against Aspartate Aminotransferase-P1 from Lupin Root Nodules


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Six hybridoma clones were obtained that secreted monoclonal antibodies against the aspartate aminotransferase-P1 (AAT-P1) isoenzyme from root nodules of *Lupinus angustifolius* [L.] cv Uniharvest. This enzyme is found constitutively in the plant cytosol fraction. The monoclonal antibodies produced were all of the immunoglobulin G1 class, recognized two distinct epitopes on the protein, and represented the major paratopes found in the immunoglobulin fraction of sera taken from mice and rabbits immunized with the pure AAT-P1 protein. One of these epitopes was unique to lupin nodule AAT-P1. The other epitope was shown to be present on enzyme from lupin bean, white clover and tobacco leaves, lupin roots and nodules, and potato tubers. Both epitopes were recognized by the appropriate monoclonal antibodies in both their native and denatured forms. None of the monoclonal antibodies produced reacted with *Rhizobium lupini* NZP2257, *Escherichia coli* extracts, or with the inducible aspartate aminotransferase-P2 (AAT-P2) isoform also found in root nodules. A sandwich enzyme-linked immunosorbent assay utilizing two monoclonal antibodies recognizing the two distinct epitopes was developed and was capable of quantitating AAT-P1, in plant extracts. The limit of detection of AAT-P1 was less than 15 pg/mL and AAT-P1 protein could be quantified in the range 80 to 1000 pg/mL. Using this assay, AAT-P1 protein was shown to remain relatively constant during nodule development. Use of an AAT-P2-specific monoclonal antibody that inhibits the enzyme activity of this isoform enabled the direct determination of AAT-P1 enzyme activity in nodule extracts. Using these assays, specific activities of the individual isoforms were calculated; that of the AAT-P2 isoform was shown to be 7.5-fold higher than that of the AAT-P1 isoform.

MABs have proven to be useful diagnostic reagents with many applications in the study of protein chemistry and biochemistry (Vora, 1985). Reports of the use of MABs to detect nodule-specific plant proteins are sparse. MABs have been produced to a peribacteroid membrane glycoprotein (Brewin et al., 1985) and to xanthine dehydrogenase (Triplet et al., 1986), a key enzyme in the synthesis of ureides in the nodules of the tropical legumes. Recently, we reported the production of MABs to AAT-P2; another key enzyme associated with N2 metabolism in nodule tissue (Jones et al., 1990). We have used these antibodies to quantitate the induction of the AAT-P2 isoform during nodule development (Jones et al., 1992) and to isolate a nearly full-length cDNA encoding the AAT-P2 protein (Reynolds et al., 1992). Polyclonal antibodies have been raised against AATs from alfalfa root nodules (Farnham et al., 1990b).

AATs (EC 2.6.1.1) catalyze the reversible reaction:

\[
\text{aspartate} + \text{2-oxoglutarate} \rightleftharpoons \text{glutamate} + \text{oxaloacetate}
\]

Two isozymic forms of AAT have been reported in the plant cytosol fraction of soybean (Ryan et al., 1972), lupin (*Lupinus angustifolius*) (Reynolds and Farnden, 1979), and alfalfa (Griffith and Vance, 1989) root nodules. AAT-P1, a cytosolic enzyme, is found constitutively in root tissue of lupin. AAT-P2 is nodule specific, is induced during rhizobial infection of roots concomitant with the onset of N2 fixation (Jones et al., 1992), and is associated with the proplastid fraction (Boland et al., 1982). Both enzymes have been separated, purified, and characterized enzymically (Reynolds et al., 1981). This paper reports the production and characterization of a range of MABs generated against the constitutive isoform, viz. AAT-P1.

**MATERIALS AND METHODS**

**Preparation of Nodule Extracts and Purification of AAT-P1**

Plants were inoculated with *Rhizobium* and maintained as previously described (Jones et al., 1990). Nodules (18–21 d old) were harvested and homogenized in 2 volumes of 50 mm Tris (pH 8.0) containing 0.4 mm Suc and 50 μg/mL pyridoxal phosphate. This homogenate was filtered through muslin and then centrifuged at 50,000g to yield a nodule extract substantially free of bacteroids and other organelles. Extracts from other plants and plant tissues were prepared similarly. Protein was determined according to the dye-binding method of Bradford (1976).

AAT-P1 and AAT-P2 were partially purified from nodule extracts by ammonium sulfate fractionation and gel filtration on Sepharose CL-6B (Pharmacia). The two isoforms were then separated by ion-exchange chromatography on DEAE Sepharose (Pharmacia) (Reynolds et al., 1981). AAT-P1 was further purified to homogeneity by hydrophobic interaction chromatography with a Phenyl-Superose HR5/5 column on

Abbreviations: AAT, aspartate aminotransferase; MAB, monoclonal antibody; PBST, PBS + 0.1% (v/v) Tween 20; %CV, percent- age coefficient of variation of the mean.
a fast-performance liquid chromatography system (Phar-
macia) as described previously (Jones et al., 1990).

Production of Affinity-Purified Polyclonal
Antibodies to AAT-P1

Polyclonal antibodies to AAT-P1 were raised in New Zea-
land White rabbits. Four rabbits were injected intradermally
over 40 sites with a suspension of polyacrylamide containing
AAT-P1 (Jones et al., 1982). Two booster injections were
given at monthly intervals, during which time antibodies to
AAT-P1 were detectable. Rabbits were bled at weekly inter-
vals. Immunoglobulins were purified from serum by ammo-
nium sulfate fractionation and chromatography on DEAE
Sephadex (Pharmacia) (Harboe and Ingold, 1973).

Immunoglobulins with anti-AAT activity were immunoaf-
finity purified by coupling partially purified AAT-P1 (Rey-
nolds et al., 1981) to Sepharose CL-4B (Pharmacia) using
carbonyl diimidazole as the coupling agent as described
previously (Jones et al., 1990).

Assays to Detect MAbs to AAT-P1

The two assays, (a) an ELISA and (b) an electrophoretic
procedure, to confirm the secretion of MAbs to AAT-P1 (Jones
et al., 1990), were modified to enable their use for detecting
MAbs to AAT-P1.

(a) ELISA. Polystyrene microtiter plates were coated with
affinity-purified, polyclonal anti-AAT-P1 and incubated with
AAT-P1, then with hybridoma culture fluid, and finally with
peroxidase-labeled anti-mouse IgG antibodies as previously
described (Jones et al., 1990).

(b) Gel electrophoretic method (Jones et al., 1990). Briefly,
plant extracts were incubated with hybridoma culture fluid,
separated on native PAGE, and stained for AAT activity
(Decker and Rau, 1963). The presence of MAbs to AAT-P1
was indicated by a retardation of AAT-P1 due to the forma-
tion of AAT-P1/MAb aggregates.

Immunization Protocol

Ten female Balb/c mice, 6 to 8 weeks old, were hyperim-
munized with pure AAT-P1. Polyacrylamide gel was used as
adjuvant (Jones et al., 1982). Initial injections with a poly-
acrylamide suspension of AAT-P1 (approximately 250 ng/
mouse) were given intrasplenically (Spitz et al., 1984), and
the mice rested for 4 weeks. Three booster injections were
given at monthly intervals subcutaneously. Antibody titer
was determined by the gel electrophoretic procedure. Mice
with the highest titers were selected for the production of
MAbs. The final injection of the antigen was given 4 d prior
to fusion.

Production of Hybridomas

Hybridoma cells secreting MAbs to AAT-P1 were prepared
as described previously (Jones et al., 1990) using murine
myeloma cells (P3-NS-1-Ag4-1, Flow Laboratories, Glasgo,
Scotland) as the fusion partner. Briefly, azoguanine-resistant
myeloma cells were fused with splenocytes prepared from
mice immunized with AAT-P1 using PEG (BDH 4000). The
fused cells (3 × 10⁶ cells/well), together with negative controls
consisting of individual wells containing either myeloma or
splenocytes at the equivalent concentrations in the fusion
mixture, were cultured as described previously (Jones et al.,
1990). Cultures were tested, using the ELISA, for antibodies
to AAT-P1 on d 12, when most wells were 70% confluent.
Cultures containing cells secreting antibodies to AAT-P1 were
confirmed by the gel electrophoretic procedure and, if posi-
tive, were immediately cloned by limiting dilution (average
0.5 cells/well) (Goding, 1983) until stable (two to three clon-
ings). Colonies were propagated and frozen in culture media
containing 10% (v/v) DMSO by slow freezing in liquid N₂
vapor and storage in liquid N₂.

Preparation and Labeling of MAbs

MAbs were prepared from ascitic tumors as described
previously (Jones et al., 1990) or by culture of immobilized
hybridoma cells in an in vitro hollow-fiber culture system (G.
Ryan, unpublished results). MAbs were purified using am-
nonium sulfate fractionation followed by affinity chroma-
tography on protein A Sepharose (Repligen) as previously
reported (Ey et al., 1978). Polyclonal antibodies and AAT-P1
were labeled with biotin using long-arm succinyl biotin (Vec-
tor Laboratories) and the protocol supplied by the manufac-
turer. MAbs were labeled with peroxidase (Sigma Chemicals,
type VII) using succinimidyl pyridyl dithiopropionate as de-
scribed previously (Jones et al., 1992).

Characterization of MAbs

Subclass Determination

The immunoglobulin subclass of MAbs secreted by each
clon was determined using the mouse hybridoma isotyping
kit manufactured by Bio-Rad Laboratories.

Epitope Determination

A competitive ELISA (Jones et al., 1990), in which AAT-P1
was labeled with biotin, was used to identify MAbs that
recognized different epitopes on the AAT-P1 protein. Preim-
mune and polyclonal mouse sera from animals used to pre-
pare hybridomas were used as negative and positive controls,
respectively. Briefly, 96-well plates were coated with each of
the unlabeled MAbs or with polyclonal antibody at optimal
plating concentration (37°C, 3 h) and blocked with 10% (v/v)
goat serum/PBS. Biotin-labeled AAT-P1 (to give an A492
of 1.0 for no competition) was incubated with the same (control)
MAb or a different MAb over the range 10 ng to 100 µg for
30 min at 37°C, and 100 µL was added to the coated wells
and incubated for 1 h at 37°C. The plates were washed with
PBS and incubated with 100 µL of a 1/1000 dilution of
peroxidase-labeled streptavidin (Amersham) in 10% (v/v)
goat serum/PBS for 1 h at 37°C. The plates were washed
and peroxidase developed as described above. Reduced color
development indicated that the two MAbs recognized fully
or in part the same epitope on the AAT-P1 molecule.
Specificity and Nature of Epitope

Specificity of MAbs for AAT-P\textsubscript{1} versus AAT-P\textsubscript{2} and bacterial and bacteroid AATs was indicated by the gel electrophoretic method (Jones et al., 1990). Further specificity was indicated by western blot analysis (Towbin et al., 1979) of crude nodule extracts using either native or denaturing gel electrophoresis. Native and denaturing PAGE and western transfer conditions were as described previously (Jones et al., 1990), except that polyvinylidene difluoride rather than nitrocellulose membrane was used.

Inhibition of Purified AAT-P\textsubscript{1} Activity by MAbs

AAT enzyme activity was measured spectrophotometrically (Bergmeyer and Bernt, 1963). Pure AAT-P\textsubscript{1} was incubated with MAb (either 7D9C6 or 2B5E5) for 1 h at 4°C, after which enzyme activity was measured. Incubation was at 4°C to preserve activity of the purified enzyme, which is unstable in dilute solution. Controls, AAT-P\textsubscript{1} in the absence of MAb or with the equivalent concentration of nonspecific MAb and MAb in the absence of AAT-P\textsubscript{1}, were run under similar conditions.

Measurement of AAT-P\textsubscript{1} Enzyme Activity in Nodule Extracts

Nodule extracts (20 µL) were preincubated with MAb 7E2 (10 µg) for 30 min at 37°C, after which enzyme activity was measured spectrophotometrically (Bergmeyer and Bernt, 1963). MAb 7E2 has previously been shown to be specific for the AAT-P\textsubscript{1} isoform and to inhibit enzyme activity totally (Jones et al., 1990). Total AAT activity was determined in the absence of MAb 7E2, and the enzyme activity of the AAT-P\textsubscript{1} isoform was calculated by difference.

Development of a Sandwich ELISA for AAT-P\textsubscript{1}

Microtiter wells were coated with MAbs in PBS (100 µL/well). Optimal plating concentration was determined for each of the six MAbs using a previously described method (Munoz et al., 1986). BSA, 2% (w/v) in PBS, was used to block remaining protein-binding sites on the microwell and in PBST as the dilution buffer for antigen and detecting antibody.

Optimal conditions for ELISA were determined by varying the incubation time, temperature, and reagent concentrations. Between addition of reagents, the plates were washed six times with PBST. Substrate (200 µL/well) was added for 30 min. The peroxidase reaction was stopped by addition of 4 M sulfuric acid (50 µL/well), and the \(A_{492}\) was read using a Titertek MC microplate reader (Flow Laboratories). Precision of the assay (Tijssen, 1990) was calculated as the %CV \(A_{492}\) for each AAT-P\textsubscript{1} concentration. Intra-assay precision was measured on assays using one batch of MAb-coated plates, by one operator in a single experiment. Inter-assay precision refers to the %CV calculated from assays carried out by different operators at different times using different batches of microwell plates.

RESULTS

Production and Characterization of Anti-AAT-P\textsubscript{1}, MAbs

Six stable hybridoma clones, secreting MAbs recognizing epitopes on AAT-P\textsubscript{1} purified from lupin (Lupinus angustifolius) root nodule tissue and derived from separate wells following fusion, were isolated. MAbs from each of these colonies were produced as ascitic tumors and as in vitro culture supernates and purified by protein A affinity chromatography. All MAbs were IgG\textsubscript{1} antibodies. The specificity of MAbs for AAT-P\textsubscript{1} was shown using the gel electrophoretic procedure (Fig. 1) for all six MAbs. In this assay, the MAbs retarded the mobility of the AAT-P\textsubscript{1} isoform without affecting the AAT-P\textsubscript{2} isoform, indicating a specific reaction between AAT-P\textsubscript{1} and each of the six MAbs. Binding of MAbs to purified AAT-P\textsubscript{1} did not inhibit enzyme activity (data not shown). This can also be seen in Figure 1, in which it can be seen that incubation of nodule extracts with MAb-containing culture fluids increased the size of the AAT-P\textsubscript{1} activity band due to the formation of a soluble MAb-enzyme complex. Because the MAb-enzyme complex was visible with the AAT activity stain, it is believed that the complex retained enzymic activity.

Further specificity was shown by western blot analysis of SDS-denatured proteins. Data are shown in Figure 2 for MAbs 7D9C6 and 2B5E5. A single polypeptide of 46 kD was detected. This corresponded to the subunit molecular mass of AAT-P\textsubscript{1} (Reynolds et al., 1981). All six MAbs recognized SDS-denatured AAT-P\textsubscript{1} (data not shown).

The epitopes of AAT-P\textsubscript{1}, recognized by the six MAbs were compared using the competitive ELISA format. Each MAb was competed with each of the others for AAT-P\textsubscript{1}. Results are shown in Figure 3. The six MAbs recognized two different epitopes on AAT-P\textsubscript{1}. One epitope was recognized by the MAb secreted by clone 7D9C6 (epitope 1), whereas the second epitope was recognized by the remaining five MAbs and typified by clone 2B5E5 (epitope 2). Binding of a mixture of these MAbs, representing each of the two epitopes, to biotin-labeled AAT-P\textsubscript{1}, inhibited the reaction of affinity-purified rabbit and mouse polyclonal antibodies (plated as in the competitive ELISA) by 85%.

Figure 1. Gel electrophoretic method for the detection of MAbs to AAT-P\textsubscript{1}. Lupin nodule extracts (15 µg of protein) were incubated with hybridoma culture fluid containing MAbs to AAT-P\textsubscript{1} at 37°C for 1 h and were then separated by electrophoresis on 7.5% polyacrylamide gels. Isoenzymes were detected by activity stain as described in "Materials and Methods." Lane 1, Nodule extract incubated in the presence of a nonspecific MAb(\textsuperscript{IgG\textsubscript{1}}); lanes 2 to 7, nodule extract incubated with AAT-P\textsubscript{1}-specific MAbs 2B5E5, 288E6, 3G7H17, 6D1E5, 7D9C6, and 10E1A12, respectively; lane 8, nodule AAT-P\textsubscript{1} lane 9, native AAT-P\textsubscript{1}.
Cross-Reactivities of MAbs to AATs from Different Organs and Species

The gel electrophoretic method was used to examine the reaction of MAbs to the two epitopes of AAT from tissues of different species (Fig. 4, A and B). MAbs recognizing both epitopes reacted with AAT-Pi isoenzyme from lupin leaves, roots, and nodule tissue (Fig. 4A). In addition, MAB 7D9C6, recognizing epitope 1, reacted with AAT-Pi isozyme, the slower-moving isoenzyme from extracts of potato tubers, tobacco leaves and roots, and bean or white clover leaves (results for lupin, clover, and tobacco leaves shown in Fig. 4B), although the reaction with the enzyme from potato tuber and tobacco leaf and root extracts only partially changed the mobility of the free enzyme. In contrast, MAbs recognizing epitope 2 (2B5E5, etc.) reacted only with AAT-Pi from lupin tissue (results for lupin, clover, and tobacco leaves shown in Fig. 4B). No reaction was observed between any of the MAbs and extracts from Rhizobium or Escherichia coli (data not shown).

Development of a Two-Site ELISA for AAT-Pi

The principle of the two-site ELISA for AAT-Pi protein is that AAT-Pi binds to a specific ‘capture’ MAb coupled to a microwell plate. A second MAb, labeled with peroxidase and recognizing a different epitope to that used to capture the AAT-Pi, is allowed to react with the ‘captured’ AAT-Pi. Addition of peroxidase substrate allows the peroxidase-labeled MAb, and hence bound AAT-Pi, to be quantitated. MAB 2B5E5 was selected as the capture MAb and peroxidase-labeled 7D9C6 was selected as the detecting antibody. The optimal concentration of the capture MAb (2B5E5) was 5 μg/mL in PBS (100 μL/well), with an incubation time of 3 h at 37°C. After any remaining protein-binding sites were blocked, the plates were either stored at 4°C or used in the assay.

Solutions (100 μL) of AAT-Pi in dilution buffer or plant extracts were added to the coated microwells and incubated either at 37°C for 3 h or overnight at 4°C. Wells were washed six times with PBST, then peroxidase-labeled MAb 7D9C6 (100 μL/well, 150 ng/mL) was added and the wells were incubated for 1 h at 37°C. After the wells were washed, AAT-Pi protein was quantified by measurement of bound peroxidase activity. Figure 5 shows a typical standard curve for the assay of lupin AAT-Pi.

With this assay, AAT-Pi protein could be measured with an intra-assay precision of ±4% and an inter-assay precision of ±9% in the range 80 to 1000 pg AAT-Pi/mL. The mean of 10 data points was used for each concentration. Assays performed in the absence of AAT-Pi (n = 10) gave an absorbance mean of 0.020 ± 0.003/cm. The significance level

Figure 3. Epitope analysis using solid-phase biotin-streptavidin ELISA. Wells of microtiter plates were coated with MAB and incubated with biotinylated AAT-Pi, and increasing concentrations of MAbs. The bound biotinylated AAT-Pi was detected with biotin-streptavidin-peroxidase conjugate. Binding was expressed as

\[ 1 - \frac{B}{B_0} \]

where \( B_0 \) = absorbance in the absence of MAB in soluble phase and \( B \) = absorbance in the presence of competing MAB. A, Wells coated with 2B5E5. Competing MAbs were 2B5E5 (●), 2B8E6 (Δ), 3G7H7 (○), 6D1E5 (◇), 7D9C6 (○), and 10E1A12 (*). B, Wells coated with 7D9C6. Competing MAbs were 2B5E5 (●), 2B8E6 (Δ), 3G7H7 (○), 6D1E5 (◇), 7D9C6 (○), and 10E1A12 (*).
Monoclonal Antibodies to Aspartate Aminotransferase-P₁

Figure 4. Cross-reactivities of MAbs to AATs from different organs and species. Extracts of lupin nodules (15 μg of protein), lupin roots (20 μg of protein), and white clover leaves (10 μg of protein) were mixed with 10 μg of MAb 7D9C6 (epitope 1) or 10 μg of MAb 2B5E5 (epitope 2) and incubated for 1 h at 37°C. Samples were resolved on 7.5% polyacrylamide gels and activity was detected as described in “Materials and Methods.” Reaction of MAb with AAT resulted in reduction of mobility of the AAT/P, activity band due to increased molecular size of the AAT/MAb complex. A, Extracts of lupin leaf (lanes 1–3), root (lanes 4–6), and nodule (lanes 7–9) were incubated with no MAbs (lanes 1, 4, and 7), MAb 2B5E5 (lanes 2, 5, and 8), and MAb 7D9C6 (lanes 3, 6, and 9). B, Extracts of lupin leaf (lanes 1–3), white clover leaf (lanes 4–6), and tobacco leaf (lanes 7–9) were incubated with no MAbs (lanes 1, 4, and 7), MAb 2B5E5 (lanes 2, 5, and 8), and MAb 7D9C6 (lanes 3, 6, and 9).

Figure 5. Two-site ELISA for AAT-P₁. Plates were coated with MAb 2B5E5 (5 μg/mL), blocked with BSA, and then incubated with purified AAT-P₁ (15–1000 pg/mL). The detecting MAb was peroxidase-labeled MAb 7D9C6 (150 ng/mL).

Figure 6. AAT-P₁ enzyme activity and protein during lupin nodule development. A, Lupin nodule extracts were assayed spectrophotometrically in the presence of MAb 7E2 to determine AAT-P₁ activity (○). AAT-P₂ activity (■) was calculated as the difference between AAT activity measured in the absence and in the presence of MAb 7E2. B, Lupin nodule extracts were assayed for AAT-P₁ protein using the two-site ELISA. Plates were coated with MAb 2B5E5 (5 μg/mL), blocked with BSA, and then incubated with nodule extract. The detecting MAb was peroxidase-labeled MAb 7D9C6 (150 ng/mL).

Rhizobial and E. coli extracts (10¹⁰ bacteria/mL) gave no apparent response (data not shown).

Direct Measurement of AAT-P₁, Protein and Enzyme Activity during Nodule Development

We have previously described a number of MAbs that recognize different epitopes on the AAT-P₁ isofrom (Jones et al., 1990). One of these MAbs, 7E2, has been shown to abolish AAT-P₂ activity totally. Hence, preincubation of crude extracts with this 7E2 antibody has allowed direct assay of AAT-P₁ activity in a crude nodule extract. These results are shown in Figure 6A. Between d 10 and 15, the specific activity of AAT-P₁ increased by 50%, whereas the AAT-P₂ activity increased by more than 7-fold. Direct measurement of AAT-P₁ protein over this time course revealed little change (Fig. 6B). The specific activity of the AAT-P₁ isoform was calculated from the d-19 enzyme activity and enzyme protein data and gave a value of 2.1 μmol min⁻¹ mg⁻¹ AAT-P₁ protein. In contrast, the d-19 specific activity of AAT-P₂ was 7.5-fold lower at 0.28 μmol min⁻¹ mg⁻¹ AAT-P₂ protein. This
value was calculated from the AAT-P₂ enzyme activity (Fig. 6A) and AAT-P₁ protein determined by a specific ELISA (Jones et al., 1992), which on d 19 of the time course was 1.9 μg/mg total protein.

**DISCUSSION**

**Characteristics of AAT-P₁ MAbs**

Six hybridoma cell lines secreting MAbs to nodule AAT-P₁ were produced. All of these MAbs were of the IgG₁ subclass and recognized two different epitopes on AAT-P₁. AATs are ubiquitous in nature and show a high degree of homology. Because only nonself epitopes would give rise to an immune response, it is not surprising that only a small number of unique epitopes could be characterized, and our results are consistent with there being significant homology between plant and animal AATs. The MAbs represent greater than 85% of the antibody activity in polyclonal sera from mice and rabbits immunized with AAT-P₁.

One of the epitopes, recognized by MAb 7D9C6, was not unique for AAT-P₁ from lupin nodules, but was shown to be present on AAT from leaves and roots from tobacco, leaves of beans and white clover, and potato tubers. However, results from the gel method described in this paper indicated that the concentration of MAB 7D9C6 required to react with AAT-P₁ from tobacco or potatoes was greater than that required to react with AAT-P₁ from any of the legumes tested in this report. These results indicate that the relative affinity of MAB 7D9C6 for AATs from nonlegumes is considerably lower than for AATs from the legumes examined. The remaining larger group of MAbs, typified by MAB 2B5E6, appeared to identify only AAT-P₁ from lupin leaves, roots, or nodule tissue. No reaction was found using either the gel method or the quantitative ELISA with extracts of tissue from plants other than lupin. Thus, it would appear that within the limited group of species studied in this report, MAbs such as 2B5E6 recognize a unique epitope found only on lupin AAT-P₁. This is in contrast to the distribution of epitopes found by MAbs raised against the other isoform, viz. AAT-P₂ (Jones et al., 1990), in which unique epitopes were not found among different species.

Both epitopes were recognized by the appropriate MAbs in both their native (Fig. 1) and SDS-denatured (Fig. 2) form, and are therefore sequential or mixed epitopes (Jones et al., 1990). However, detectability of AAT-P₁ on western blots was variable and sensitivity was lower than expected. This could suggest that a varying degree of epitope renaturation is occurring during transfer to the membrane and that these antibodies are in fact not recognizing sequential epitopes. They have proven to be useful reagents for the screening of cDNA expression libraries from which to clone the mRNA for AAT-P₁ (C. Winefield, unpublished results).

**Quantitation of AAT-P₁ and AAT-P₂ Protein and Enzyme Activity in Nodule Extracts**

The epitopes identified by the MAbs to AAT-P₁ were unique to the AAT-P₁ isoform and did not recognize AAT-P₂. Furthermore, these MAbs did not recognize any AAT present in *Rhizobium* or in *E. coli* (data not shown). These facts have proven useful in the development of a two-site sandwich ELISA for specifically quantitating AAT-P₁ in lupin tissue extracts. The assay is extremely sensitive (limit of detection of AAT-P₁ less than 15 pg/mL) and is specific for lupin AAT-P₁. The ELISA assay circumvents the need to separate the isoforms found in nodule tissue and has enabled the first direct measurements of the AAT-P₁ isoform to be performed on nodule extracts. AAT-P₁ protein has previously been measured by rocket immunoelectrophoresis (Farnham et al., 1990a) and by the use of a MAb (Jones et al., 1992).

The use of a specific MAb to inhibit the enzyme activity of the AAT-P₁ isoform has enabled direct measurement of AAT-P₁ activity to be made in a crude extract. Such measurements have previously been made following immunoprecipitation of AAT-2 using an alfalfa polyclonal antiserum (Farnham et al., 1990a). We have previously estimated the relative amounts of AAT-P₁ and AAT-P₂ enzyme activity in lupin nodules by relying on the heat lability of the AAT-P₂ isoform (Jones et al., 1992); these data showed the activity of the two isoforms to be approximately equal 17 d after infection on a gram nodule tissue basis. In this work, we have shown the specific activity of AAT-P₁, on a total soluble protein basis to be higher than that of AAT-P₂, after the activity of this isoform is maximal (d 15). This could simply reflect the inherent inaccuracy of reliance on heat lability to totally inhibit the activity of the AAT-P₁ isoform in the earlier study. The data presented in this work show that AAT-P₁ and AAT-P₂ contribute 60 and 40%, respectively, of the total AAT activity detected in the plant fraction of lupin nodules after the AAT-P₂ activity is fully induced. This compares with the immunoprecipitation study of Farnham et al. (1990a), in which AAT-P₂ activity was shown to range from 25% of the total in soybean to 71% of the total in alfalfa.

The availability of MAbs for the AAT-P₁ (this work) and the AAT-P₂ (Jones et al., 1990) isoforms present in lupin nodules, together with the enzyme-inhibition properties of the 7E2 AAT-P₂-specific MAb, has enabled accurate determination of the specific activity of the individual isoforms in a simple nodule extract. Interestingly, the specific activity of AAT-P₁ is 7.5-fold higher than that of AAT-P₂. This is consistent with the large increase in AAT-P₂ protein that has been observed previously (Jones et al., 1992) and explains how, in this study, AAT-P₂ protein is 9-fold higher than AAT-P₁ protein at d 19 while making only a 40% contribution to the total AAT enzyme activity.

These assays will have applications in studies undertaken to investigate the developmental regulation of the expression of these isoenzymes as well as the chemical and environmental factors leading to the induction of the enzymes. The information gained from such studies will further our understanding of the role of the individual AAT isoenzymes in the assimilation of symbiotically fixed nitrogen and in the possible role of AAT in regulating the flow of carbon between organic and amino acids.

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LITERATURE CITED


