Production and Characterization of Monoclonal Antibodies against Aspartate Aminotransferase-P₂ from Lupin Root Nodules


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ABSTRACT

Twenty-one monoclonal antibodies were raised against the aspartate aminotransferase-P₂ isoenzyme from root nodules of Lupinus angustifolius [L.] cv Uniharvest. Induction of this isoenzyme is positively correlated with the onset of N₂ fixation in effective root nodules and is associated with the assimilation of ammonia by the plant in the Rhizobium-legume symbiosis. The monoclonal antibodies produced were all of the IgG class, recognized five different epitopes on the protein, and represented greater than 90% of the available epitopes. These epitopes were not unique to lupin nodule aspartate aminotransferase-P₂ but were shown to be present on the enzyme from tobacco leaves and potato. Four of the epitopes were conformational with a fifth epitope recognized by the appropriate monoclonals in both its native and denatured forms. None of the monoclonal antibodies produced reacted with Rhizobium lupini NZP2257 extracts. Antibodies against two epitopes showed some cross-reaction with the constitutive aspartate aminotransferase-P₁ isoenzyme also found in lupin root nodules. However, affinity of these monoclonals for AAT-P₁ was three orders of magnitude lower than for AAT-P₂. Monoclonals against the other epitopes appeared to be specific for aspartate aminotransferase-P₂.

MABS have proven to be useful diagnostic reagents with many applications in the study of protein chemistry and biochemistry (24). Reports of the use of monoclonal antibodies to nodule-specific plant proteins are sparse. Brewin et al. (2) have produced MABS to a peribacteroid membrane glycoprotein and Triplett et al. (22) described MABS to xanthine dehydrogenase, a key enzyme in the synthesis of ureides. Ureides represent the major form of fixed N₂ transported from the nodule to the shoots of many legumes (10). In this paper, we report the production and characterization of MABS to another key enzyme associated with N₂ metabolism in nodule tissue, viz., AAT-P₂. AAT [EC 2.6.1.1.] catalyzes the reversible reaction:

Aspartate + 2-oxoglutarate ↔ Glutamate + oxaloacetate

Two isozymic forms of AAT have been reported in the plant cytosol fraction of soybean (15) and lupin (12) root nodules. AAT-P₁, a cytosol enzyme, is found constitutively in root tissue of legumes. AAT-P₂ is nodule specific and induced during rhizobial infection of roots concomitant with the onset of N₂ fixation and is associated with the proplastid fraction (1). Both enzymes have been separated, purified, and characterized enzymically (13). This is the first report of monoclonal antibodies prepared against plant AAT, although there is a recent report of a polyclonal antibody against an AAT isozyme from Panicum maximum (11). Polyclonal antibodies have been produced against animal enzymes (17) to study the evolution rates of vertebrate aminotransferases, but this was not extended to plants (17). MABS have been produced against porcine mitochondrial AAT (20). This paper reports the production and characterization of a range of MABS generated against AAT-P₂.

MATERIALS AND METHODS

Plant Growth and Rhizobium Inoculation

Lupin seeds (Lupinus angustifolius [L.] cv Uniharvest) were purchased from Dalgety N.Z. Ltd. Seeds were surface-sterilized, germinated on agar, and transferred to sterile pumice troughs before inoculation with Rhizobium lupini NZP2257. Plant/Rhizobium cultures were grown either in glass houses during summer (day length ≤ 12 h) or in a controlled environment growth cabinet with a day length of 12 h and a day/night temperature regime of 24°C/21°C (14).

Purification of AAT-P₂

Nodules (18–21 d old) were harvested and homogenized in two volumes of 50 mM Tris (pH 8.0) containing 0.4 m sucrose and 50 μg/mL pyridoxal phosphate. AAT-P₁ and AAT-P₂ were separated and partially purified by ammonium sulfate fractionation, gel filtration on Sepharose CL-6B, and ion exchange chromatography on DEAE Sepharose essentially as described previously (13). AAT-P₂ was further purified to homogeneity by hydrophobic interaction chromatography with a phenyl superose HR5/5 column on a FPLC system (Pharmacia Ltd). The enzyme preparation (2.4 mL, total protein = 2.4 mg) was diluted with an equal volume of 3.8 M ammonium sulfate in 50 mM potassium phosphate (pH 7.0) and loaded. The column was eluted with buffer A (1.7 m ammonium sulfate in 50 mM potassium phosphate [pH 7.0]) and buffer B (50 mM potassium phosphate [pH 7.0]). Flow rate was 0.5 mL/min; fraction size was 0.5 mL. The column was washed with buffer A for 20 min, buffer B was increased...
to 60% over the next minute, and the enzyme was eluted with a linear gradient in which buffer B increased to 100% over 14 min. AAT-P2 activity was in two fractions at 80% to 85% buffer B. Fractions containing AAT-P2 activity were combined, adjusted to 1.7 M ammonium sulfate, and subjected to a second purification on phenyl superose.

Production of Affinity-Purified Polyclonal Antibodies to AAT-P2

Polyclonal antibodies to AAT-P2 were raised in New Zealand white rabbits. Four rabbits were injected intradermally over 40 sites with a suspension of polyacrylamide containing AAT-P2 (8). Two booster injections were given at monthly intervals during which time antibodies to AAT-P2 were detectable. Rabbits were bled at weekly intervals. Immunoglobulins were purified from serum by ammonium sulfate fractionation and chromatography on DEAE Sephadex (6).

Immunoglobulins with anti-AAT activity were immunofinity purified by coupling partially purified AAT-P2 (13) to Sepharose CL-4B using carbonyl diimidazole as the coupling agent. Briefly, Sepharose CL-4B was dehydrated by solvent exchange with water/dioxane mixtures 1:2, 2:1, and pure dioxane. The gel was dried and mixed with sufficient dioxane to cover the gel, 18 g of carbonyl diimidazole was added per 240 mL of Sepharose, and the suspension was mixed on rollers for 30 min. The activated gel was washed three times with dioxane and then rehydrated through dioxane water mixtures to water. The gel was collected on a Buchner filter and quickly washed with 0.5 M Na2CO3 containing 0.5 M NaCl adjusted to pH 9.5 with NaHCO3, immediately added to an equal volume of a solution of protein in the same buffer, and mixed on rollers at 4°C for 18 h. The affinity gel was dried, suspended in 0.1 M glycine, and washed with 50 mM Tris/Cl (pH 8.0) containing 0.5 M NaCl and 0.1% NaN3. The gel was poured into a suitable column and washed with 0.2 M glycine (pH 2.0), 0.1 M Tris/Cl containing 0.5 M NaCl (pH 8.0) until the absorbance at 280 nm was below 0.01 units. Immunofinity purification was performed with antibody applied to the column in 0.1 M Tris/Cl (pH 8.0) containing 0.5 M NaCl and 0.02% Tween 20. The column was washed with the same buffer until the absorbance at 280 nm was <0.01 units, then washed with two column volumes of the same buffer without Tween 20. Immunofinity-purified anti-AAT-P2 was eluted with 0.2 M glycine (pH 2.0) into sufficient 1.7 M Tris to raise the pH to 7.5, concentrated, and dialyzed against PBS and adjusted to approximately 3 mg/mL. The antibody preparation was stored frozen at −20°C in 30-μl aliquots.

Development of Assays to Detect Monoclonal Antibodies to AAT-P2

Two assays were used to confirm the secretion of MAbsto AAT-P2: (a) an ELISA and (b) a gel electrophoretic method to show specific binding of MAbsto AAT-P2 enzyme.

ELISA

Attempts to develop a direct ELISA by coating wells with AAT-P2 failed due to the apparent inability of native AAT-P2 to adsorb to polystyrene or polyvinylchloride plates. This was shown by the inability to detect binding of polyclonal antibodies against AAT-P2 to plates that had been incubated with AAT-P2. Furthermore, native AAT-P2 was readily displaced from nitrocellulose by buffers containing detergents (TWEEN 20, Triton X-100) or physiological saline (WT Jones, unpublished results). Therefore, a sandwich ELISA was developed using polystyrene plates (Nunc) coated with polyclonal antibodies to AAT-P2 and subsequently used to capture AAT-P2. Detection of MAb was accomplished with a peroxidase-labeled rabbit anti-mouse Ig and suitable substrate.

Polystyrene microtiter wells (Immulon, Nunc) were coated with immunofinity-purified polyclonal anti-AAT-P2 (500 ng/mL) in carbonate/bicarbonate (pH 9.6) (23) and blocked with 10% rabbit serum in carbonate/bicarbonate (pH 9.6) for 3 h at 37°C, then overnight at 4°C. The plates were washed six times in PBS with 0.1% Tween 20 (wash buffer, PBST) and incubated with 50 μL of a solution of AAT-P2 (100 ng/mL; 5 ng/well) in PBST containing 10% rabbit serum (PBSTRS) for 3 h at 37°C, followed by addition of cell culture supernatants diluted one-half in 2× PBSTRS, peroxidase rabbit anti-mouse IgG (1/2000 in PBSTRS), and substrate (40 μM ortho-phenylenediamine, 0.01% H2O2 in citrate/sodium phosphate buffer [pH 6.0]) 200 μL/well for 30 min at room temperature. Color development was stopped by the addition of 50 μL/well of 4 M H2SO4, and absorbance measured at 495 nm in a Flow Multiskan MC EIA reader. Negative controls were supernatant of (a) myeloma cells, (b) splenocytes, and (c) feeder cells plated at concentrations equivalent to the concentration in the hybridoma fusion mixture.

Gel Electrophoretic Method for Detecting Specific Binding of MAbsto AAT-P2

The specificity of putative MAbsto AAT-P2 identified by ELISA was carried out as follows. Ten microliters of a crude extract of lupin nodules (1.25 g nodules in 20 mL of buffer) was mixed with 20 μL of the hybridoma supernatant and incubated for 1 h at 37°C. Controls were performed by replacing the hybridoma supernatants with culture medium or culture medium containing nonspecific mouse Ig. Following incubation, 10 μL of 50% glycerol containing 0.1% bromophenol blue was added to the samples, and these were electrophoresed on 7.5% nondenaturing polyacrylamide gels and subsequently stained for AAT activity (3). The presence of MAbsto AAT-P2 was indicated by an absence of AAT-P2 activity and/or a change in mobility of the AAT-P2/MAb complex.

Immunization Protocol

Ten female Balb/c mice, 6 to 8 weeks old, (Biotechnology Division DSIR Breeding Laboratories) were hyperimmunized with AAT-P2, purified as described above. Polyacrylamide gel was used as adjuvant (7). Initial injections with polyacrylamide suspension of AAT-P2 (~500 ng/mouse) were given intrasplenically (19), and the mice rested for 4 weeks. Three booster injections were given subcutaneously at biweekly intervals. Antibody titers were determined by the gel electrophoretic procedure. Mice with highest titers were selected for
Table I. Summary of Hybridomas Secreting MAbs to AAT-P2

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Hybridoma colonies (Typical clone)</th>
<th>Subclass (number)</th>
<th>Nature of Epitope</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(1E1)</td>
<td>IgG2b (1)</td>
<td>Conformational</td>
</tr>
<tr>
<td>2</td>
<td>4G5, 5H4, 5D5, 11D12, 2D8, 2F2, 4F6, 6B5, 6D6, (7F1), 10F12</td>
<td>IgG1 (4)</td>
<td>Sequential</td>
</tr>
<tr>
<td>3</td>
<td>4A4, 4B5, 6F10, 7E2, (10F10), 10H5, 6D5</td>
<td>IgG1 (6)</td>
<td>Conformational</td>
</tr>
<tr>
<td>4</td>
<td>(9E8)</td>
<td>IgG2a (1)</td>
<td>Conformational</td>
</tr>
<tr>
<td>5</td>
<td>(5H4)</td>
<td>IgG1 (1)</td>
<td>Mixed</td>
</tr>
</tbody>
</table>

the production of MAbs. For the second fusion, an AAT-P2/MAb complex in polyacrylamide was used as the antigen. The final injection of the antigen was given 4 d prior to fusion.

Production of Hybridomas

Murine myeloma cells (P3-NS-1-Ag4-1, Flow Laboratories Scotland) were cultured in a medium containing 8-azoguanine for 10 generations prior to fusion. Splenic lymphocytes (1 × 10⁶) were mixed with myeloma cells (4 × 10⁵) and fused with polyethylene glycol (BDH 4000) as described previously (8), and plated out into 96-well culture plates (0.1 mL, at a cell density of 3 × 10⁴/mL) in the presence of peritoneal macrophages as feeder cells (1 × 10⁴/mL, 0.1 mL/well). The culture medium was RPMI 1640 (Gibco Laboratories) supplemented with 10% NCTC109 (Difco Laboratories), 10% fetal calf serum (Gibco Laboratories), gentamycin 20 μg/mL, hypoxanthine, aminopterin, thymidine, sodium pyruvate, 2-mercaptoethanol, glutamine, and sodium bicarbonate (5). The fusion mixture and controls, consisting of individual wells containing myeloma or splenocytes at the equivalent concentration in the fused cells, were incubated at 37°C in a 5% CO₂/95% air mixture. Half the medium was replaced with fresh medium on d 5 and 7; on d 9, half the medium was replaced with medium lacking aminopterin. Cultures were tested for antibodies to AAT-P2 on d 12, when most cells were 70% confluent, by the ELISA procedure. Cultures containing cells secreting antibodies to AAT-P2 were confirmed by the gel electrophoretic procedure and, if positive, were immediately cloned by limiting dilution (average 0.5 cells/well) (5) until stable (2–3 clonings). Colonies were propagated and frozen in culture medium containing 10% DMSO by slow freezing in liquid N₂ vapor and stored in liquid N₂.

Figure 1. Gel electrophoretic method for the detection of antibodies to AAT-P2. Nodule extracts (lupin, 0.2 g nodule/mL, extraction buffer) were incubated with antibodies raised to AAT-P2 (here, monoclonal antibody 7F1) at 37°C for 1 h and 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 absence of antibody; lane 2, nodule extract incubated with antibody to AAT-P2. P₁ = AAT-P₁, P₂ = AAT-P₂.

Figure 2. Western blotting and immunoprobing with antibodies to AAT-P2. Lane 1, nodule extract probed with MAb 7F1; lane 2, nodule extract stained for protein; lane 3, mol wt markers stained for protein. Proteins and extracts were separated on a 15% SDS-polyacrylamide gel and transferred to PVDF membranes. Proteins were stained with Coomassie blue or probed with MAb and detected with phosphatase anti-mouse IgG/NBT-BCIP as described in "Materials and Methods."
Preparation of Ascites Antibodies

Hybridoma cells were propagated in vivo by injecting male Balb/c mice with $5 \times 10^5$ cells intraperitoneally. Mice had been primed by intraperitoneal injection with 0.5 mL of pristane 10 to 14 d prior to injection of cells. Ascitic fluid was collected on d 10 to 11, centrifuged 30,000$g$ for 10 min, and stored at $-20^\circ C$ or processed immediately. MAbs were partially purified by precipitation at 50% ammonium sulfate and equilibration at 4°C for 1 h, and then collected by centrifugation at 30,000$g$ for 15 min. The pellet was dissolved in 0.1 M Tris/Cl (pH 8.4) containing 0.02% NaN$_3$ and further purified by affinity chromatography on protein A Sepharose (4). The crude MAb was applied to the protein A Sepharose and washed with 0.1 M Tris/Cl (pH 8.4) until the absorbance at 280 nm was below 0.05 units. Immunoglobulins were eluted with 0.2 M glycine/HCl (pH 2.0) and collected into fraction tubes containing sufficient 1.7 M Tris to raise the pH to 7.5. Immunoglobulins were precipitated at 50% ammonium sulfate; the suspension was made to 0.1% in NaN$_3$ and stored at 4°C.

Characterization of Monoclonal Antibodies

Subclass Determination

Immunoglobulin subclasses of each clone were determined with the mouse hybridoma subsotyping kit manufactured by Bio-Rad Laboratories.

Epitope Determination

Two competitive ELISAs were developed to determine the epitopes recognized by different MAbs, in which either the MAb or AAT-P$_2$ were labeled with biotin using long arm succinyl biotin (Vector Laboratories). Each of the 21 MAbs and affinity-purified rabbit anti-AAT-P$_2$ was labeled by the protocol supplied by Vector Laboratories. Preimmune mouse sera and polyclonal mouse sera from animals used to prepare hybridomas were used as negative and positive controls, respectively.

(a) Ninety-six 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% goat
Table II. Effects of MAbs on AAT-P2 Activity

<table>
<thead>
<tr>
<th>Epitope</th>
<th>AAT-P2 Activity</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>553 (min)/553 (mL)</td>
<td>100</td>
</tr>
<tr>
<td>AAT-P2 + MAb 1E1</td>
<td>520</td>
<td>97</td>
</tr>
<tr>
<td>AAT-P2 + MAb 7F1</td>
<td>348</td>
<td>63</td>
</tr>
<tr>
<td>AAT-P2 + MAb 1OF10</td>
<td>8</td>
<td>1.4</td>
</tr>
</tbody>
</table>

serum/PBS. Biotin labeled AAT-P2 (to give \( A_{402nm} = 1.0 \) for no competition) was incubated with the same (control) MAb or different MAb over the range 10 ng to 100 \( \mu\)g for 30 min at 37°C, and 100 \( \mu\)L was added to the coated wells and incubated for 1 h at 37°C. The plates were washed with PBST and incubated with 100 \( \mu\)L of a 1/1000 dilution of peroxidase-labeled streptavidin (Amersham) in 10% goat serum/PBS for 1 h at 37°C. The plates were washed and developed with peroxidase as described above.

(b) In the second method, biotin-labeled MAbs, rather than biotin-labeled AAT-P2, were used in case biotinylation interfered with the binding of MAb to AAT-P2. Plates (96-well) were coated with affinity-purified rabbit anti-AATP2. The AAT-P2 protein (approximately 5 ng per test) was incubated with unlabeled MAb (10 ng-100 \( \mu\)g/mL) for 30 min at 37°C in PBSTRS. Biotin-labeled MAb (to give \( A_{402nm} = 1.0 \) in the absence of competing MAb) was added to the AAT-P2/unlabeled MAb and the mixture (100 \( \mu\)L) was added to the rabbit anti-AAT-P2 coated plates and incubated for 2 h at 37°C. Plates were washed, incubated with peroxidase-labeled streptavidin (1/1000), and developed with substrate, and then the optical density was measured.

With both methods (a) and (b) reduced color development indicated that the two MAbs recognized fully or in part the same epitope on the AAT-P2 molecule.

Specificity and Nature of Epitope

Specificity of MAbs for AAT-P2 versus AAT-P1, bacterial, or bacteroid AATs was indicated by the gel electrophoretic method. Further specificity was indicated by Western blot analysis of crude nodule extracts using either native or denaturing gel electrophoresis.

Samples (25 \( \mu\)L) of nodule extract (1 g/mL of extraction buffer) were electrophoresed on native 7.5% polyacrylamide slab gels using a 100 mm Tris/100 mm glycine continuous buffer (8). Denaturing gels (sodium dodecyl sulfate) were prepared according to Laemmli (9) except that a 15% polyacrylamide rather than a 10% gel was prepared. Gels were either stained with 0.25% Coomassie blue R-250 in methanol/water/acetic acid (4/4/1, v/v/v) or processed for Western blotting. Western blotting was performed with a transfer buffer (25 mm), glycine (192 mm), methanol (10% v/v) plus SDS for denaturing gels 0.003%. Proteins were transferred onto nitrocellulose by electrophoresis at 420 v/cm overnight at 4°C. Under these conditions, transfer of proteins was quantitative. Nitrocellulose was blocked with 10% rabbit serum, 1% BSA in TBS. For native gels, proteins were transferred onto nitrocellulose previously treated with polyclonal antibodies to AAT-P2, (1 mg/mL in PBS). Remaining protein binding sites were blocked with 2% BSA in TBS. The nitrocellulose was stained for AAT-P2 activity or probed with MAbs followed by phosphatase Rabbit Fab anti-mouse (Sigma; 1/1000 dilution) and detected with NBT/BCIP. Negative controls were MAbs nonspecific for AAT-P2.

Inhibition of AAT-P2 Activity by Monoclonal Antibodies

AAT enzyme activity was measured spectrophotometrically as described previously (8). Pure AAT-P2 was incubated with MAb for 1 h at 4°C after which enzyme activity was measured. Controls, AAT-P2 in the absence of MAb or with an equivalent concentration of nonspecific MAb, and MAb in the absence of AAT-P2, were run under similar conditions.

RESULTS

Production and Characterization of Anti-AAT-P2 Monoclonals

Two fusions were carried out which resulted in 21 hybridoma clones. Each of these hybridoma had been derived from separate wells of the 96-well plates that were used following the fusion and were subsequently cloned until stable. The first fusion, for which the splenocytes were obtained from mice immunized with pure AAT-P2, resulted in only one stable colony, clone 1E1. For the second fusion, mice were immunized with an AAT-P2/MAb 1E1 complex separated from excess MAb by polyacrylamide gel electrophoresis and resulted in a further 20 stable colonies secreting MAbs to AAT-P2. Immunization of the AAT-P2/MAb 1E1 complex in this second fusion precluded the possibility of any immune response to the 1E1 epitope. MAbs from each of these colonies were produced as ascitic tumors and as in vitro culture supernatants and purified by protein A affinity chromatography.

All MAbs were IgG1 class antibodies. The subclass of the antibodies were determined with the Bio-Rad isotyping kit in conjunction with the ELISA developed for screening hybridoma cultures for MAbs to AAT-P2. Results are summarized in Table I.

The specificity of MAbs for AAT-P2 was shown in two ways:

The first was by a gel electrophoretic procedure in which binding of the monoclonal to AAT results in an increased molecular mass for the complex (molecular mass AAT-P2 = 90 kDa; molecular mass AAT-P2/MAb = 330 kDa assuming two, one on each subunit, binding sites per AAT molecule) with subsequent reduction in mobility and/or total loss of enzyme activity following native-PAGE. An example for MAb 7F1 is shown in Figure 1. All MAbs removed the AAT-P2 activity band.

For those MAbs recognizing SDS-denatured AAT-P2, further specificity was shown by Western blot analysis of SDS-denatured proteins, probing nodule extracts with the monoclonal antibodies. Data are shown in Figure 2 for MAb 7F1. A single polypeptide of 46 kDa was detected. This corresponds to the subunit molecular mass of AAT-P2 (13). Monoclonals 1E1 and 1OF10 did not recognize SDS-denatured AAT-P2.
MONOCLONAL ANTIBODIES TO ASPARTATE AMINOTRANSFERASE-P2

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Figure 4. Cross-reactivities of MAbs to AATs from different organs/species. Extracts of tobacco leaves (0.5 g/mL) or lupin nodules (2 g/mL) were mixed with MAb 7F1 (epitope 2) or MAb 10F10 (epitope 3) in the absence of MAb, and incubated for 1 h at 37°C. Samples were separated on 7.5% polyacrylamide gels, and activity was detected as described in "Materials and Methods." Reaction of MAb with AAT resulted in loss or decrease in activity of the corresponding AAT bands in the control sample due to (a) increased molecular size of the AAT/MAb complex or (b) inhibition of enzyme activity. Lane 1, tobacco extract, no MAb; lane 2, tobacco extract with MAb 10F10; lane 3, potato extract with MAb 7F1; lane 4, potato extract, no MAb; lane 5, potato extract with MAb 10F10; lane 6, potato extract with MAb 7F1; lane 7, nodule extract, no MAb; lane 8, nodule extract with MAb 10F10; lane 9, nodule extract with MAb 7F1. A, MAbs used at concentration of 10 μg/mL sufficient to remove all AAT-P2-like activity; B, MAbs at 10 mg/mL to indicate reaction with AAT-P1.

whereas MAB 7F1 (as shown in Fig. 2) did. MAbs 9E8 and 5H4, while recognizing SDS-denatured AAT-P2, exhibited a much weaker response in the Western blot than did 7F1.

The epitopes of AAT-P2 recognized by the 21 monoclonal antibodies were compared by two competitive ELISAs. Each monoclonal competed with the other for AAT-P2. The 21 monoclonals recognized five different epitopes on AAT-P2. These five epitopes were recognized by monoclonal antibodies typified by 1E1 (epitope 1), 7F1 (epitope 2), 10F10 (epitope 3), 9E8 (epitope 4), and 5H4 (epitope 5). Monoclonals to epitopes 4 and 5 reacted to varying degrees with epitopes 2 and 3, indicating that epitopes 2, 3, 4, and 5 are clustered with epitopes 4 and 5 overlapping epitopes 2 and 3 (Fig. 3). Monoclonal antibodies to epitope 2 showed no cross-reaction to epitope 3 and vice versa (Fig. 3). As expected, epitope 1 showed no reactivity to monoclonals reacting with epitopes 2, 3, 4, or 5 (data not shown). Binding of a mixture of these MAbs, representing each of the five epitopes, to AAT-P2 inhibited the binding of rabbit and mouse polyclonal antibodies by greater than 90%. This indicates that most of the epitopes inducing an immune response in these animals are accounted for by the monoclonals described in this paper. Monoclonals to epitope 3 could also be shown by the gel electrophoretic procedure to react with AAT-P1, though much higher concentrations of MAb were required. MAbs 1E1 and 7F1 at this concentration did not react with AAT-P1. The affinity constant for the reaction of MAb 10F10 (epitope 3) with AAT-P2 and AAT-P1 were 6 × 10⁸ and 6 × 10⁹ mol⁻¹, respectively.

Inhibition of Aspartate Aminotransferase Activity by MAbs

Results for inhibition of AAT-P2 activity by MAbs to epitopes 1, 2, and 3 are shown in Table II. MAbs to epitope 1 indicated no inhibition, whereas MAbs to epitope 2 indicated 37% and to epitope 3 >96% inhibition of AAT-P2 activity. The MAbs to epitopes 4 and 5, which are believed to overlap epitopes 2 and 3, were similar in inhibition of AAT-P2 to epitopes 3 and 2, respectively (results not shown).

Cross-Reactivities of Monoclonals to AATs from Different Organs/Species

The gel electrophoretic method was used to examine the reaction of MAbs to epitopes 1 to 5 with aspartate aminotransferase from nodules, leaf, and tuber tissue from different species. Results for MAbs to epitopes 2 and 3 are shown (Fig. 4A). All of the MAbs reacted with at least one AAT isoenzyme, usually the faster moving species on 7.5% polyacrylamide gels, from potato tubers, tobacco leaves, and lupin nodules. Three AAT activity regions were observed in extracts of tobacco leaves (lane 1, Fig. 4A). The additional zone of intermediate mobility did not react with any of the MAbs (Fig. 4A and B). All MAbs reacted with nodule AAT-P2 and the faster moving AAT from leaf and tuber tissue. At high concentrations of MAb (1000 times that required to precipitate AAT-P2), MAbs to epitope 3 (Fig. 4B) and epitope 4 (results not shown) also precipitated nodule AAT-P2, and the corresponding lowest mobility AAT isoenzyme of leaf or tuber. No reaction was observed with AATs from Rhizobium (data not shown).

DISCUSSION

Twenty-one hybridoma cell lines secreting monoclonal antibodies to nodule AAT-P2 were produced. Of these MAbs, all were of the IgG class. Eleven were IgG1, nine were IgG2a, and one was IgG2b subclass, and recognized five different epitopes on AAT-P2, four of which are probably clustered with two of these four overlapping the remaining two distinct epitopes. The characterization of only five epitopes is not surprising in view of the ubiquitous nature of AATs. Only nonself epitopes would give rise to an immune response, and our results are consistent with there being significant homology between plant and animal AATs. The epitopes were not unique for AAT-P2 from nodules but were shown to be present on AAT from tobacco leaves and potatoes. However, no quantitative studies were carried out to measure the relative affinities of these antibodies for AATs from different species, and this study cannot make conclusions on divergence between AATs from different sources. The MAbs represent greater than 90% of the antibody activity in polyclonal serum from mice or rabbits immunized with AAT-P2.

Only antibodies recognizing epitopes 3 and 4 and, to a lesser degree, 5 (epitopes overlapping epitope 3) cross-reacted
with AAT-P₂, the enzymic form constitutively present in the roots of the legume study. The concentration of MAb to epitope 3 required to aggregate AAT-P₁ was 1000 times the concentration required to react with AAT-P₂ (the enzymic form induced in nodule tissue). Monoclonals recognizing the other epitopes of AAT-P₁ could not be shown to react with AAT-P₂ and appeared to be specific for the AAT-P₂ form of the enzyme. Monoclonals to epitope 3 were shown to inhibit AAT-P₂ enzyme activity and, to a lesser degree, AAT-P₁ enzyme activity and, therefore, either reacted with the active center of the enzyme or induced a conformational change in the active center of the AAT molecule resulting from the binding of MAbs to epitope 3. This may be related to structural differences at the active sites of the two isoforms. Indeed, differences in affinity of AAT-P₁ and AAT-P₂ for substrate have already been reported (13). AAT-P₂ has a fivefold greater affinity for oxaloacetate than AAT-P₁.

Epitopes are usually classified as being sequential, conformational, or mixed. Epitopes 1 and 3 are conformational in that AAT-P₂ denatured with SDS prior to Western blotting could no longer be detected by reaction with the MAbs against these epitopes. Epitope 2, however, was recognized by the appropriate monoclonals in both its native (Fig. 1) and denatured (Fig. 2) form. This then is a sequential epitope and, further, this epitope is accessible in both native and denatured AAT-P₂. Epitopes 4 and 5 are mixed because, while recognizing native AAT-P₂, MAbs against these two epitopes showed reduced reaction with SDS-denatured AAT-P₂. This is consistent with the overlapping nature of epitopes 4 and 5 with epitopes 2 and 3 and is supported by the competitive ELISA studies (Fig. 3).

The fact that four of the five epitopes characterized have a conformational component is in agreement with the report (16) that hybridoma cells secreting monoclonals to native proteins are best detected by a capture ELISA employing a polyclonal rather than the antigen binding to the polystyrene plate, a procedure which can cause denaturation of the antigen.

Monoclonals to epitopes 1 and 3 could be useful for developing an ELISA to measure enzymically active AAT-P₂, and monoclonals to epitopes 1 and 2 will be useful for measuring total AAT-P₂ protein and for screening λgt11 expression libraries in studies to identify the gene for AAT-P₂.

ACKNOWLEDGMENT

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LITERATURE CITED