Purification and Characterization of Aspartate Aminotransferase Isoenzymes from Carrot Suspension Cultures

Frank J. Turano, Barbara J. Wilson, and Benjamin F. Matthews*

U. S. Department of Agriculture, Agricultural Research Service, Plant Molecular Biology Laboratory, Beltsville, Maryland 20705

ABSTRACT

Three aspartate aminotransferase isoenzymes were identified from extracts of carrot (Daucus carota L.) cell suspension cultures. These isoenzymes were separated by DEAE chromatography and were analyzed on native gradient polyacrylamide gels. The relative molecular weights of the isoenzymes were 111,000 ± 5000, 105,000 ± 5000, and 94,000 ± 4000 daltons; they were designated forms I, II, and III, respectively. Form I, the predominant form, has been purified to apparent homogeneity (>300-fold) using immunoaffinity chromatography with rabbit anti-pig AAT antibodies. Form I has a subunit size of 43,000 M r, as determined on sodium dodecyl sulfate polyacrylamide gel electrophoresis. Isoelectric focusing (IEF)-PAGE has resolved three bands at pH of approximately 5.2. Form I may be composed of subunits of similar molecular weight and different charges, and the three bands with AAT activity on the IEF-PAGE gel are a combination of hetero- and homodimers. Form I has a broad pH optimum of 7.5 to 10.0. K m values of 23.6, 2.8, 0.05, and 0.22 millimolar were obtained for glutamate, aspartate, oxaloacetate, and α-ketoglutarate, respectively. The mode of action is a ping-pong-bi-bi mechanism.

Aspartate aminotransferase (EC 2.6.1.1) catalyses the reversible transfer of an amino group from aspartate to α-ketoglutarate to form oxaloacetate and glutamate:

\[ \text{aspartate} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{oxaloacetate} + \text{glutamate}. \]

AAT \(^1\) plays a key role in hydrogen shuttles, carbon shuttles, and nitrogen distribution in plants (7, 11). In hydrogen shuttles, AAT is involved in the exchange of reducing equivalents across organelar membranes to the cytoplasm via the malate-aspartate shuttle (9). In “C 4” plants a carbon shuttle (8) is utilized to transport fixed carbon from mesophyll cells to bundle sheath cells. Carbon dioxide is fixed into OAA in the mesophyll cells. In some C 4 plants the OAA is transaminated by AAT to form aspartate, which is transported from the mesophyll cells to the bundle sheath cells and then deaminated by AAT to form OAA. Asparagine, which is frequently used to distribute nitrogen in plants, is synthesized from aspartate. Aspartate is also necessary for the biosynthesis of nucleotides and the essential amino acids lysine, methionine, threonine, and isoleucine in higher plants.

AAT has been studied in various plants (7, 11) and as many as five different isoenzymic forms of AAT have been reported. Additionally, the activity of the different forms varies among different tissues. The enzyme has been extensively characterized only in cauliflower (3, 6), pea (19), soybean (16), oats (14), and lupine (15); however, none of these plants are particularly amenable to cell culture techniques nor can they be readily regenerated from protoplasts and cells into plants. Selection of cells resistant to analogs of substrates and products of AAT to obtain mutant cell lines containing AAT with modified activity would be of less value because they could not be regenerated into plants. Numerous mutants have been selected using cell culture techniques (18) and have been used to gain valuable insights into cellular metabolism. Cell suspension cultures also have been valuable for altering precursor and product levels to understand their roles in regulation of enzyme activity.

To use cell culture techniques to examine the regulation of AAT, carrot has been chosen as our plant source because it is readily manipulated in cell suspension cultures, it has been used in other related enzyme studies, and it can be readily regenerated into plants from protoplasts and single cells. In this paper, we describe the separation of three forms of AAT from carrot suspension cultures and the purification and characterization of the major isoenzymic form of the enzyme. We discuss the possible physiological functions and compare the properties with the AAT forms from other plants.

MATERIALS AND METHODS

Cell Cultures

Daucus carota L. cv Danvers cells were grown in 200 mL of Murashige-Skoog medium (12) containing 2,4-D and BAP in 500 mL flasks illuminated and shaken at 27°C. Cells were transferred weekly into fresh medium.

Crude Protein Extractions

Cell cultures were harvested 5 d after inoculation by filtration using 3MM Whatman paper. Cells were resuspended in two volumes of extraction buffer (50 mM Tris-HCl adjusted to pH 7.5 with KOH, 5 mM threonine, 10 mM β-mercaptoethanol, 20% glycerol, and 1.728 g DDCA/L) per gram fresh

---

\(^1\) Abbreviations: AAT, aspartate aminotransferase; BAP, 6-benzylaminopurine; DDCA, diethyldithiocarbamic acid; IEF, isoelectric focusing; PLP, pyridoxal 5-phosphate.
weight. Cells were lysed by 2 passes of 20 min through a cell disruption bomb (1000 psi). Cellular debris was removed by centrifugation at 18,000g for 15 min at 4°C. Proteins precipitated with 60% saturated ammonium sulfate were collected by centrifugation at 18,000g at 4°C for 20 min. Protein pellets were resuspended and dialyzed in extraction buffer minus DDCA at 4°C overnight. Aliquots of the dialyzed enzyme were applied to a Biogel A-0.5 m column (25 mm × 50 cm) equilibrated in 50 mM Tris-HCl (pH 7.5), 20% glycerol with a flow rate of 6 mL/h. Fractions were assayed for AAT activity. Peaks of AAT activity were combined and PLP was added to a final concentration of 15 μg/mL, and the pooled sample was applied to a Waters DEAE-5PW (21.5 mm × 15 cm) HPLC column equilibrated with 10 mM Tris (pH 7.5). AAT was eluted with a linear gradient from 0 to 250 mM NaCl over 45 min. Peaks of AAT activity were further resolved and purified on an Altex DEAE-5PW (7.5 mm × 7.5 cm) HPLC column by nonlinear gradients of 0 to 250 mM NaCl over 45 min.

Enriched Mitochondrial Fraction

The pellet from the 18,000g centrifugation after cell rupture was resuspended in 0.05% Triton X-100 in 50 mM Tris-HCl adjusted to pH 7.5 with KOH, 10 mM β-mercaptoethanol, 20% glycerol. The suspension was centrifuged at 18,000g for 30 min and the supernatant was applied to the Biogel column. Further purification steps were as above.

Gel Electrophoresis and Staining Procedures

PAGE was performed on the Pharmacia Phastsystem2 using 10 to 15% gradient gels for native and SDS conditions. Isoelectric focusing was performed on 5% polyacrylamide gels with ampholytes ranging from pH 4 to 6.5. Proteins were visualized by silver staining on the Phastsystem. Protein bands containing AAT activity on acrylamide gels were visualized by incubation with aspartate, α-ketoglutarate, PLP, and fast blue dye (5). Identical gels incubated in the absence of aspartate or α-ketoglutarate were used as controls for AAT-specific staining.

Protein and Enzymatic Activity Assays

Protein concentrations were determined using a BioRad or Pierce protein assay kit. The forward reaction and reverse reaction were used to determine AAT activity, substrate specificity, and enzyme kinetics (10). In the forward reaction, AAT activity was coupled with 10 units of malate dehydrogenase with 10 mM aspartate and 10 mM α-ketoglutarate as the substrates for AAT. In the reverse reaction, AAT activity was coupled with 5 units of glutamate dehydrogenase with 10 mM glutamate and 10 mM oxaloacetate as substrates for AAT and 3.1 mM NH4Cl as a substrate for glutamate dehydrogenase. In both cases the assays contained 0.06 mM PLP, 0.14 mM NADH, and 60 mM KPO4 (pH 7.5), and the decrease in absorbance at 340 nm was recorded over 1 min. One unit of AAT activity equals 1 μmol of product generated per minute at 25°C.

Antibody Production

Pig heart AAT was purchased from Boehringer Mannheim Biochemicals and analyzed by PAGE. Identical native gels were stained separately for protein (silver stain) and AAT activity. The pig heart AAT preparation was found to be >99% pure. Pig heart AAT (500 μg) was injected with Freund’s complete adjuvant intramuscularly into female New Zealand rabbits. Injections were repeated every 14 d; after 51 d the rabbit serum was collected. Antiserum and preimmune serum were tested by Western blot analysis.

Western Blot Analysis and Immunoaffinity Chromatography

Proteins were separated by native PAGE on 10 to 15% gradient gels. Proteins were transferred to nitrocellulose filters by electrotransfer in 25 mM Tris-20 mM glycine (pH 8.3), at 25 to 35 V for 1 h at 4°C. AAT activity was visualized as described above. Nonspecific protein binding sites were blocked by incubating the filter in TBS (20 mM Tris [pH 7.5] and 150 mM NaCl) containing 1.0% powdered milk and 0.5% BSA (block solution) for 1 h at room temperature or overnight at 4°C. Nitrocellulose filters were incubated in primary antibody (1:500, rabbit anti-AAT) and 10% block solution for 1 h at room temperature or 16 h at 4°C. Filters were washed four times with TBS. The filters were incubated in a secondary antibody (alkaline phosphatase conjugated goat anti-rabbit) and 10% block solution for 1 h at room temperature. Filters were washed in TBS as previously described. Bands were visualized by incubating the filters in 100 mM Tris (pH 9.5), 100 mM NaCl, and 5 mM MgCl2, 0.035% nitroblue tetrazolium, and 0.0175% 5-bromo-4-chloro-3-indolyl phosphate for 10 min at room temperature. The phosphatase reaction was stopped by incubating the filters in 20 mM Tris (pH 7.5), and 1 mM EDTA for 5 to 10 min.

The immunochromatography matrix was made using cyanogen bromide-activated cross-linked agarose beads (Sigma) which were washed and swollen in 1 mM HCl overnight at 4°C while rotating gently on an end-over-end mixer. AAT antiserum was dialyzed overnight in coupling buffer (0.1 M NaHCO3, 0.5 M NaCl [pH 8.8]). The beads were resuspended in two bed volumes of coupling buffer, and AAT antiserum was immediately added to the beads. The antiserum was incubated with the beads for 2 h at room temperature or overnight at 4°C on an end-over-end mixer. Excess active groups were blocked by incubating the beads in 0.1 M Tris, 0.2 M glycine (pH 8.0), for 2 h at room temperature or overnight at 4°C. Excess uncoupled proteins were removed with alternating low (0.1 M acetate [pH 4.0], 0.5 M NaCl) and high (coupling buffer) pH buffers for a total of five washes each. AAT was dialyzed overnight at 4°C in loading buffer (10 mM Tris, 2 mM DTT, 20 μg/mL PLP, 20% glycerol [pH 7.5]), prior to loading on the column. AAT was bound to an immunoaffinity column. Excess proteins were removed by washing the column with 5 to 10 bed volumes of loading

---

2 Mention of trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of the products or vendors that may be suitable.
buffer. AAT was eluted with 3 to 5 volumes of elution buffer (50 mM potassium citrate [pH 4.5], 50 mM KCl, 2 mM DTT, 20 μg/mL PLP, 20% glycerol). Columns were regenerated by washing the column with 15 to 25 volumes of acetate buffer and 5 to 10 volumes of TBS. Immunoaffinity columns were stored in TBS, 0.02% azide at 4°C.

### Enzyme Kinetics and Substrate Specificity

The $K_m$ values for oxaloacetate, aspartate, α-ketoglutarate, and glutamate were determined at various concentrations from 0.01 and 80 mM. For each $K_m$ determination, one substrate concentration was varied while the other substrate concentration was maintained near saturation. The change in absorbance at 340 nm was recorded. Reaction velocities were plotted on double reciprocal plots and $K_m$ values were determined by linear regression.

The pH optimum of carrot AAT form I was determined in assay buffers that ranged from pH 5.5 to 10.5. The buffer contained 50 mM citrate, 50 mM potassium phosphate, and 50 mM glycine, and the pH was adjusted with NaOH. Controls for pH effects on malate dehydrogenase were also performed at each pH to determine the effects of pH on the enzyme, since the AAT assay is coupled with malate dehydrogenase.

Substrate specificity was tested with various keto and amino acids. The keto acids used in the experiments were oxaloacetate, α-ketoglutarate, pyruvate, and glyoxylate. Aspartate, glutamate, glutamine, alanine, glycine, leucine, asparagine, methionine, tryptophan, and phenylalanine were the amino acids used in the experiments. Combinations of one keto acid and one amino acid were individually tested for aminotransferase activity. The experimental conditions, i.e. concentrations and components of each reaction, were conducted as described by Reynolds et al. (15).

### Results

#### Purification of AAT

Extracts of carrot cell suspension cultures were separated into three major peaks of AAT activity by anion-exchange chromatography. These peaks of activity were designated forms I, II, and III according to their order of elution from the anion-exchange column, with increasing salt concentration. Form I was the major form of AAT activity, representing approximately 80% of the total activity, with forms II and III representing 15 and 5%, respectively. The order of elution from the anion-exchange column coincided with the order of migration of the forms on an 10 to 15% native polyacrylamide gradient gel, with form I migrating the least and form III migrating the most (Fig. 1). These data indicate that form I is the least negatively charged and the largest form, whereas form III is the most negatively charged and smallest form.

To further purify AAT, an immunoaffinity column was constructed using rabbit anti-pig AAT antibodies. The antibodies cross-reacted with carrot AAT (Fig. 2). The specificity of the rabbit anti-pig AAT was high, and there was little cross-reactivity of the antibody with other carrot proteins. Peak fractions representing form I from the anion-exchange column were bound to the immunoaffinity column and after
Figure 3. Native PAGE of purified AAT form I from carrot suspension cultures. Proteins were applied to a 10 to 15% polyacrylamide gel and were separated by PAGE. Fifty ng of each of the following proteins were added as mol wt standards (lane 1): thyroglobulin (669,000), ferritin (440,000), catalase (232,000), lactate dehydrogenase (140,000), and bovine serum albumin (67,000). Approximately 20 units of purified form I was loaded in each lane. The samples were silver stained for protein (lane 2) and for AAT activity (lane 3). The relative mol wt of form I is indicated.

Figure 4. SDS-PAGE of purified AAT form I from carrot suspension cultures. Protein samples were treated with SDS and β-mercaptoethanol and applied to a 10 to 15% polyacrylamide gel. Protein samples were separated by SDS-PAGE. Approximately 50 ng of each protein was added per lane. The low mol wt markers (lane 1) are as follows: bovine albumin (66,000), trysinogen (24,000), β-galactoglobulin (18,400), and lysozyme (14,000). Lane 2 contains purified carrot AAT form I. The high molecular markers (lane 3) are as follows: mysin (205,000), β-galactosidase (116,000), bovine albumin (66,000), egg albumin (45,000), and carbonic anhydrase (29,000). The relative mol wt for AAT form I is indicated.

When this highly purified fraction of AAT was subjected to IEF-PAGE, form I was resolved into three individual bands between pH of 5.1 to 5.3 (Fig. 5). Each protein band possessed AAT activity as indicated by AAT-specific staining (data not shown). These data support the conclusion that AAT has been purified to homogeneity, but also suggest that AAT form I of carrot is composed of nonidentical subunits.

During purification and separation of the different forms of carrot AAT isoenzymes, the ratios of the different forms varied among isolations. In all cases, form I was the major form (between 95–80%) observed in the extracts from carrot cell suspension cultures. Also, we observed that the pressure disruption method did not rupture mitochondria, which were pelleted in the first centrifugation step in the protocol. This protocol therefore selected against isolating a mitochondrial isoenzyme of AAT. When a fraction enriched for mitochondria was prepared by low speed centrifugation (see Materials and Methods), form I was still the predominant form (about 60%) but forms II and III constituted a higher percentage of the total activity than in the cell extracts. Since the mitochondrial fraction was prepared from material which also con-

<table>
<thead>
<tr>
<th>Table 1. Purification of AAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step</td>
</tr>
<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>Crude</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
</tr>
<tr>
<td>Biogel</td>
</tr>
<tr>
<td>DEAE</td>
</tr>
<tr>
<td>Forms I &amp; II</td>
</tr>
<tr>
<td>Form III</td>
</tr>
<tr>
<td>DEAE</td>
</tr>
<tr>
<td>Form I</td>
</tr>
<tr>
<td>Form II</td>
</tr>
<tr>
<td>Form III</td>
</tr>
<tr>
<td>Immunoaffinity</td>
</tr>
</tbody>
</table>
PURIFICATION OF CARROT ASPARTATE AMINOTRANSFERASE

591
values were determined for highly purified (>240-fold) form I. The effect of pH from pH 5.5 to 10 was determined in buffers containing 50 mM citrate, 50 mM potassium phosphate, 50 mM glycine. Total activity was determined at each pH tested (Fig. 6). Form I had a broad range of activity from pH 7.5 to 10.0. AAT activity at pH 9.0 was approximately 3.5 times higher than at pH 6.5.

Aminotransferases require PLP for enzymatic activity. Carrot AAT activity was completely inhibited by the addition of aminooxyacetate (final concentration 2 mM), indicating the enzyme may require PLP. Substrate specificity experiments were performed in both the forward and reverse reactions with various keto and amino acids, to establish form I as aspartate aminotransferase and not a nonspecific aminotransferase. The keto acids used in the experiments were oxaloacetate, α-ketoglutarate, pyruvate, and glyoxylate. Aspartate, glutamate, glutamine, alanine, glycine, leucine, asparagine, methionine, tryptophan, and phenylalanine were the amino acids used in the experiments. Combinations of one keto acid and one amino acid were individually tested for aminotransferase activity. Form I only used aspartate as an amino acid substrate in the presence of α-ketoglutarate. Likewise, only α-ketoglutarate was used as a keto acid substrate in the presence of aspartate. In the opposite direction, in the presence of oxaloacetate only glutamate was used as an amino acid substrate and in the presence of glutamate only oxaloacetate was used as a keto acid substrate. Form I did not utilize the amino acids glutamine, glycine, alanine, leucine, asparagine, methionine, tryptophan, and phenylalanine nor the keto acids pyruvate and glyoxylic acid as substrates. These data indicated that form I is specific for the substrates aspartate, α-ketoglutarate, oxaloacetate, and glutamate; thus, form I is an aspartate aminotransferase.

Further kinetic characterization was performed to compare the enzyme mechanism and enzyme localization of form I to other known AAT isoenzymes. Km values for the substrates oxaloacetate, aspartate, α-ketoglutarate, and glutamate were individually determined (Fig. 7, A-D). The Km values for form I are 2.8, 0.22, 0.05, and 23.6 mM for aspartate, α-ketoglutarate, oxaloacetate, and glutamate, respectively. The Km values for carrot AAT form I were compared to those of other plant AAT isoenzymes (Table II).

The kinetic parameters K_m and V_max can be used to determine the reaction mechanism. Reciprocal plots of the initial velocity versus variations in the concentration of α-ketoglutarate at fixed aspartate concentrations were utilized to determine the reaction mechanism (Fig. 8). An increase in apparent V_max values with an increase in aspartate concentrations results in parallel lines on the double reciprocal plot which are characteristic of a ping-pong-bi-bi mechanism (1).

Results of product inhibition studies were consistent with a ping-pong-bi-bi mechanism. Reciprocal plots of the initial velocity versus aspartate concentration at fixed glutamate concentrations showed that glutamate was a competitive inhibitor with respect to the amino acid aspartate (Fig. 9A). In the experiment, aspartate concentrations were varied from 0.5 to 6.0 mM at fixed concentrations of glutamate (0, 5, 10, and 20 mM) with a constant α-ketoglutarate concentration of 0.2 mM. Additionally, glutamate was a noncompetitive inhibitor with respect to the organic acid α-ketoglutarate (Fig. 9B).

Figure 5. IEF-PAGE of purified AAT form I from carrot suspension cultures. Purified AAT form I was applied to a 5% polyacrylamide gel with pH 4 to 6.5 ampholytes. Proteins were separated by PAGE and were visualized by silver stain. Three bands, each with AAT activity, were resolved at a pI of approximately 5.2.

Figure 6. The effect of pH on the activity of carrot AAT form I. The buffer contained 50 mM citrate, 50 mM potassium phosphate, 50 mM glycine, and the pH was adjusted with NaOH. Assays were conducted in the forward direction with 10 mM aspartate, 10 mM α-ketoglutarate, 1.4 mM NADH, 0.006 mM PLP, and 25 units of malate dehydrogenase.

The presence of form I, a putative cytoplasmic form, is to be expected. Additionally, when proteins were extracted from carrot tissue by other means, all three forms were present. A higher percentage of forms II and III was observed in extracts from carrot seedlings (data not shown).

Analysis of AAT Form I Kinetic Parameters

To further characterize the isoenzyme and to compare the carrot isoenzyme to other plant AAT forms various kinetic parameters, i.e. pH optimum, substrate specificity, and K_m were determined nonbroken cells, the presence of form I, a putative cytoplasmic form, is to be expected. Additionally, when proteins were extracted from carrot tissue by other means, all three forms were present. A higher percentage of forms II and III was observed in extracts from carrot seedlings (data not shown).
Figure 7. Determination of $K_m$ values for carrot AAT form I from cell suspension cultures. One substrate concentration was varied while the other substrate concentration was maintained at saturation. Results were plotted on Lineweaver-Burke double reciprocal plots and $K_m$ values were determined by linear regression. A. The $K_m$ value of glutamate was determined to be 23.6 mM. Oxaloacetate OAA was maintained at 10 mM and glutamate was varied from 5 to 80 mM. B. The $K_m$ value of aspartate was determined to be 2.8 mM. $\alpha$-Ketoglutarate was maintained at 10 mM and aspartate was varied from 1 to 5 mM. C. The $K_m$ value of OAA was determined to be 0.05 mM. Glutamate was maintained at 80 mM and OAA was varied from 0.005 to 0.1 mM. D. The $K_m$ value of $\alpha$-ketoglutarate was determined to be 0.22 mM. Aspartate was maintained at 10 mM and $\alpha$-ketoglutarate was varied from 0.05 to 0.5 mM.

Table II. Values of $K_m$ for AAT from Different Sources

<table>
<thead>
<tr>
<th>Source</th>
<th>Aspartate</th>
<th>$\alpha$-Ketoglutarate</th>
<th>Oxaloacetate</th>
<th>Glutamate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carrot, Form I</td>
<td>2.8</td>
<td>0.22</td>
<td>0.05</td>
<td>23.6</td>
<td></td>
</tr>
<tr>
<td>Cauliflower</td>
<td>7.5</td>
<td>0.76</td>
<td>0.105</td>
<td>38.0</td>
<td>(2)</td>
</tr>
<tr>
<td>Pea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soluble</td>
<td>0.87</td>
<td>0.67</td>
<td>0.048</td>
<td>4.4</td>
<td>(19)</td>
</tr>
<tr>
<td>Particulate</td>
<td>1.60</td>
<td>0.73</td>
<td>0.45</td>
<td>6.4</td>
<td>(19)</td>
</tr>
<tr>
<td>Lupine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAT-P1</td>
<td>2.2</td>
<td>0.26</td>
<td>0.10</td>
<td>22.0</td>
<td>(15)</td>
</tr>
<tr>
<td>AAT-P2</td>
<td>2.6</td>
<td>0.20</td>
<td>0.02</td>
<td>12.0</td>
<td>(15)</td>
</tr>
<tr>
<td>Oats</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Form I</td>
<td>4.14</td>
<td>0.22</td>
<td>0.057</td>
<td>32.9</td>
<td>(14)</td>
</tr>
<tr>
<td>Form II</td>
<td>2.3</td>
<td>0.30</td>
<td>0.030</td>
<td>13.7</td>
<td>(14)</td>
</tr>
<tr>
<td>Soybean cytosol</td>
<td>17.0</td>
<td>1.8</td>
<td>1.1</td>
<td>65.0</td>
<td>(16)</td>
</tr>
</tbody>
</table>

In the experiment, $\alpha$-ketoglutarate concentrations were varied from 0.1 to 0.8 mM at fixed concentrations of glutamate (0, 5, 10, and 20 mM) with a constant aspartate concentration of 2.0 mM. The data were replotted (not shown) according to the method of Dixon (4). A $K_i$ for aspartate of 6.0 mM and for $\alpha$-ketoglutarate of 15.3 mM was obtained. These data, kinetic parameters, and inhibition studies are consistent with a ping-pong-bi-bi mechanism for carrot AAT form I.
DISCUSSION

Three AAT isoenzymes were separated and purified from carrot suspension culture cells. The major form was purified to homogeneity using ammonium sulfate precipitation, gel-filtration, anion-exchange, and immunoaffinity chromatography techniques with a final 333-fold purification and 21% recovery.

We have shown that three forms of AAT were present in extracts of carrot cell suspension cultures. The mol wt determined on PAGE for forms I, II, and III are 111,000, 105,000, and 94,400 M_r, respectively. The mol wt of the carrot AAT isoenzymes are similar to those that were observed in other plants and animals (7, 11). Two AAT isoenzymes from lupine (15) have very similar mol wt to forms II and III from carrot. However, it is premature to say that the isoenzymes with similar mol wt between the two species are localized in the same cellular location, have the same function and/or have the same kinetic parameters.

Form I has similar physical characteristics to other AAT isoenzymes. It has an apparent mol wt of 111,000 D and is composed of 43,000 D subunits. In animals and in nearly all plants (7, 11) AAT is composed of two identical subunits. However, in Bermudagrass (Cynodon dactylon) the cytoplasmic forms of AAT are composed of both heterodimers and homodimers (17). The data from the IEF-PAGE suggest that form I is composed of nonidentical subunits. Furthermore, it can be postulated that carrot form I is composed of subunits of similar mol wt and different charges, and that three bands with AAT activity on the IEF-PAGE gel are a combination of hetero- and homodimers. It is unknown at this point whether the two subunits are a result of differential processing of a single gene product or are they encoded by different genes.

Form I AAT from carrot suspension cultures has similar kinetic parameters as other plant AAT isoenzymes. The pH optimum for carrot AAT form I activity is from pH 7.5 to 10. Similarly, broad pH optima have been observed in lupine (15), particulate AAT in pea (19), soybean (16), and cauliflower (3). The pK at 6.5 and 10.0 could correspond to the ionization of PLP or a lysine ε-amino group on the enzyme, respectively. However, it is not possible to attribute the decrease in AAT activity at either pH to the ionization of a specific substrate or residue on the protein. Form I is specific for the substrates aspartate, glutamate, oxaloacetate, and α-ketoglutarate and utilizes PLP.

The K_m values determined for form I are consistent with values reported for other plants (3, 14-16, 19). Like other plant AAT isoenzymes, the K_m values for the keto acids are lower than the K_m values for the amino acids. These data suggest that all the aspartate aminotransferases identified to date may perform similar functions. Previously, different K_m values for the various isoenzymes have been attributed to different cellular function. Likewise, the different K_m values could be related to compartmentalization in the cell and the need for the reaction to proceed more in one direction than the other. The K_m values for the keto acids are 10 to 450 times lower than the K_m values for the amino acids, indicating that the
enzyme is involved in nitrogen assimilation. Furthermore, the isoenzyme may be involved in the formation of glutamate, since the $K_m$ value for glutamate is eightfold higher than the $K_m$ for aspartate. Similar $K_m$ values were observed for AAT-P$_1$ in lupine (15) and form I AAT in oats (14). In lupine, AAT has been postulated to be involved in nitrogen assimilation. However, it is difficult to elaborate on the specific role of form I in carrot until the $K_m$ values for other nitrogen assimilation enzymes (glutamine synthetase, glutamate synthase, and asparagine synthetase) and accurate estimates of the concentrations of the substrates in carrot are obtained.

Ping pong mechanisms are common among substitution reactions. The proposed mechanism of action for many plant AAT isoenzymes is the ping-pong-bi-bi mechanism (14–16, 19). Based on the presence of parallel lines on double-reciprocal plots from kinetic data for form I, the carrot isoenzyme follows the ping-pong-bi-bi mechanism reported for other AAT isoenzymes. Product inhibition studies showed that glutamate was a competitive inhibitor with respect to the amino acid aspartate and a noncompetitive inhibitor with respect to the keto acid $\alpha$-ketoglutarate. These data are also consistent with a ping-pong-bi-bi mechanism. $K_i$ for aspartate of 6.0 mm and for $\alpha$-ketoglutarate of 15.3 mm was obtained from glutamate inhibition studies. These data are similar to the $K_i$ values of 12 mm for aspartate and 10 mm for $\alpha$-ketoglutarate obtained for AAT-P$_1$ in lupine (15).

Several observations suggest that carrot AAT form I may be a cytoplasmic protein. Form I is the predominant form in all cell extracts (95–80%), but the percentage of form I in organelle enriched fractions decreased (60%). These data correspond with the soluble form of AAT in pea, which accounted for 90% of the total extractable AAT activity. Furthermore, in mammals the cytoplasmic form of AAT is heat stable (13). Carrot extracts that contained all three forms of AAT were heated at 55°C for 30 min. The sample was analyzed by anion-exchange chromatography and native PAGE; form I was the only active form present after the heat treatment (data not shown). While these data suggest that form I may be a cytoplasmic isoenzyme, subcellular fractionation of the carrot AAT forms has yet to be attempted.

Immunological data indicate that regions of the AAT amino acid sequence and structure are conserved between plant isoenzymes and pig AAT, since rabbit antibodies to pig AAT cross-react with carrot AAT and soybean AAT. Cross-reactivity between the antibodies to pig AAT is stronger with carrot than with soybean (our unpublished results). The antibodies made to native pig AAT did not cross-react with denatured protein extracts from carrot or soybean. A comparison of the amino acid sequences of *Escherichia coli* and several animal cytoplasmic and mitochondrial AAT isoenzymes shows that regions of AAT are highly conserved (2). Our data suggest that some degree of conservation of the amino acid sequence may extend into the plant kingdom.

LITERATURE CITED

1. Cleland WW (1963) The kinetics of enzyme-catalyzed reactions with two or more substrates or products. Biochim Biophys Acta 67: 104–137