Partial Purification and Characterization of Aspartate Aminotransferases from Seedling Oat Leaves

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SUMMARY

As relatively little information is available on the properties of aspartate aminotransferase from photosynthetic tissue, isolation and characterization of the two major electrophoretically distinct forms of this enzyme from seedling oat leaf homogenates were undertaken. These two forms are designated I for the more anionic form and II for the less anionic form. Form I, 80 to 90% of the total activity, has been purified to a specific activity of 120 μmol/min/mg of protein (1100-fold) and is estimated to be 90 to 95% homogeneous, as judged by analytical polyacrylamide gel electrophoresis. Form II, 10 to 20% of the total activity, has been purified to a specific activity of approximately 6 μmol/min/mg of protein (300-fold).

Both forms exhibit optimal activity at pH 7.5. Michaelis constants do not differ greatly between forms I and II and are similar to those reported for the pig heart cytosolic enzyme as well as aspartate aminotransferase from other plant sources. A molecular weight of 130,000 for the purified aspartate aminotransferase I was estimated by sedimentation equilibrium centrifugation; molecular weights of the two forms are similar as estimated by sucrose density gradient centrifugation. No activation by pyridoxal phosphate has been observed during purification.

Leading to the hypothesis that this enzyme plus malate dehydrogenase may be serving to shuttle cytoplasmic reducing equivalents into the peroxisome to complete the photosynthetic process. The importance of photorespiration, a nonmitochondrial photosynthesis-related process of CO₂ evolution which lowers net carbon fixation in most higher plant species, alone calls for further investigation of the role of aspartate aminotransferase in photosynthetic tissue.

Reports on the properties of aminotransferases from plant tissue have been limited, the most thorough studies being the purification of aspartate aminotransferase from cauliflower florets (a tissue lacking photosynthetic activity) to a final specific activity of approximately 34 μunits/mg of protein (4, 5). Kinetic constants are available for the cauliflower floret enzyme (5), cytosolic enzyme from soybean root nodules (6), and the bundle sheath and mesophyll isoenzymes from Atriplex spongiosa, a plant exhibiting C₄ metabolism (7). Only in the study of A. spongiosa have separation, localization, and kinetic properties of the enzyme from photosynthetic tissue been investigated; however, aspartate aminotransferase from Atriplex cannot be considered typical of all plant species because of its involvement in the auxiliary CO₂ fixation process via phosphoenolpyruvate carboxylase and oxalacetate formation. Thus, available information on properties of plant aspartate aminotransferase does not clarify the cell fractionation studies which indicate spatial separation of electrophoretically distinct forms of this enzyme within photosynthetic cells.
assay of the enzyme by the absorbance of oxalacetate in the 250 to 280 nm region (24). It was observed that the enzyme was not stable to heat, so the determination was carried out at 5.55 and 4.65°C, respectively. The enzyme preparation was dialyzed 12 hours against stabilizer buffer before centrifugation. A density of 1.020 g/ml for the solvent was determined by weighing aliquots of the dialysis solution. The partial specific volume of aspartate aminotransferase I was assumed to be 0.73.

Molecular weight estimations of partially purified preparations by the sucrose density gradient technique of Martin and Ames (21) were carried out using yeast alcohol dehydrogenase (molecular weight 150,000) as a marker.

Treatment of Kinetic Data—Kinetic data were fitted by computer analysis (22) to the equation for a bireactant system exhibiting a ping-pong mechanism:

$$v = \frac{VAB}{K_{AB} + KA + AB}$$

where $v = $ velocity, $V = $ maximum velocity, $A = $ concentration of the variable substrate, $B = $ concentration of the changing fixed substrate, $K_m = $ Michaelis constant for substrate A, and $K_i = $ Michaelis constant for substrate B (Ref. 25, pp. 46-50).

RESULTS

Preliminary Studies

In preliminary differential centrifugation studies isotonic (24) oat leaf homogenates yielded 4% of the total aspartate aminotransferase activity in the chloroplast-enriched fraction (pellet from 1,000 x g centrifugation for 1 min, washed), 2% in the mitochondria-enriched fraction (pellet from 2,000 to 15,000 x g for 10 min, washed), and 88% in the soluble fraction (15,000 x g, supernatant). Acrylamide gel electrophoretic patterns showed two major bands (I and II) in both the soluble fraction and the total homogenate. Only I occurred in the chloroplast-enriched fraction, whereas both I and II plus two intermediate bands occurred in the mitochondria-enriched fraction.

Purification of Enzyme Forms

The crude glycerol supernatant was immediately saturated with solid (NH$_4$)$_2$SO$_4$ (52 g/ml) to separate rapidly the soluble protein from other plant materials, specifically phenolics or tannins. After centrifugation the precipitated protein was redisolved in stabilizer buffer which consisted of 0.1 M succinate and 0.1 M K$_2$HPO$_4$, adjusted to pH 7.5 with KOH; when frozen in this buffer, activity is stable for several months. After desalting by passing through a Sephadex G-15 column, the protein fraction was immediately applied to a DEAE-cellulose column (Fig. 1) and eluted with a linear gradient of increasing ionic strength. Fractions were concentrated by pressure ultracentrifugation and stored frozen in stabilizer buffer. Data are summarized in Steps 1 to 5 of Table I.

Fraction I containing form I of the enzyme (Fig. 1) was fractionated by (NH$_4$)$_2$SO$_4$, approximately 50% of the activity being recovered in the 50 to 55% (NH$_4$)$_2$SO$_4$ fraction. Sephadex G-200 gel filtration or preparative density gradient ultracentrifugation accomplished a similar degree of purification but was less reproducible and more time-consuming. Activity not appearing in the 50 to 55% fraction was distributed among the other fractions; attempts to recover more enzyme at a higher specific activity by repeated fractionation were not successful.

The (NH$_4$)$_2$SO$_4$ fraction was well suited for final purification of form I by preparative polyacrylamide gel electrophoresis. Analytical polyacrylamide gels of the prep gel fraction containing the enzyme are shown in Fig. 2. Data for purification are summarized in Table I, Steps 5B, 7, and 8. This purified fraction contained malate dehydrogenase with a specific activity of 40 units/mg of protein as compared with a specific activity of 120 units/mg of protein for the aminotransferase I.

Detection of Enzyme Activity on Analytical Polyacrylamide Gels—The aspartate aminotransferase activity stain for polyacrylamide gels (18) was modified for use with oat enzyme. Solution B was revised to contain: polyvinylpyrrolidone (PVP 40), 1.5 g; 0.1 M phosphate, pH 7.5, 0.7 ml, with the omission of pyridoxal 5'-phosphate, NADH, and 30", in 0.1 M Tris-Cl containing 5 mM oxalacetate and 0.13 mM NADH. Yeast alcohol dehydrogenase was assayed at 340 nm, pH 9.6, and 30", with 0.1 M sodium glycinate containing 0.1 M ethanol and 1 mM NaADP.

Homogenization of Oat Tissue in Glycerol Medium—For enzyme purification, chopped oat tissue was homogenized for 15 to 30 s in a Waring Blender in glycerol medium containing 0.10 X K$_2$HPO$_4$-0.002 M EDTA-0.002 M isoascorbic acid (added immediately prior to use), pH adjusted to 7.6, plus glycerol to give a final concentration of 20%. The crude homogenate was filtered through 8 layers of cheesecloth and centrifuged at 16,000 x g for 15 min to yield the crude glycerol supernatant.

Protein Determination—Protein measurements of fractions not subjected to Sephadex G-15 chromatography were carried out using a modification of the technique of Lowry et al. (13), in which the 0.1 n NaOH and 2% Na$_2$CO$_3$ were added separately in that order. After removal of plant pigments and phenolic material by Sephadex G-15 chromatography, protein concentration was estimated by ultraviolet absorbance measurements (14).

Analytical Polyacrylamide Gel Electrophoresis—Analytical polyacrylamide disc gel electrophoresis was performed using either the pH 8.9-7.0% gel standard analytical system (omitting stacking gel) (Ref. 15, p. 44) or an analytical system derived from the standard pH 8.9 preparative system (Ref. 15, p. 149), utilizing an 8% polyacrylamide gel, 0.03% methylenebisacrylamide, and a 10-fold dilution of electrode buffer. Gels were fixed 30 min or longer in 20% ethanol and 30% acetic acid. Electrophoresis was performed using the Canalco Prep Disc apparatus (Canalco, Itockville, Md.). The pH 8.9 Tris-glycinate gel (2.1 X 8 cm) was modified for use with oat enzyme. Solution B was revised to contain: polyvinylpyrrolidone (PVP 40), 1.5 g; 0.1 M phosphate. Gels were stained for malate dehydrogenase activity with anionic and II for the less anionic forms in accordance with recommendations of the Commission on Biochemical Nomenclature (17).

Detection of Enzyme Activity on Analytical Polyacrylamide Gels—The aspartate aminotransferase activity stain for polyacrylamide gels (18) was modified for use with oat enzyme. Solution B was revised to contain: polyvinylpyrrolidone (PVP 40), 1.5 g; 0.1 M Tris-Cl, pH 7.5, 11.3 ml; 0.2 mg aspartate, pH 7.5, 3.4 ml; and 0.2 M a-ketoglutarate, pH 7.5, 0.7 ml, with the omission of pyridoxal phosphate. Gels were stained for malate dehydrogenase activity as described by Shaw and Prasad (19) with the omission of NaCN.

Preparative Polyacrylamide Gel Electrophoresis—Preparative gel electrophoresis was performed using the Canalco Prep Disc apparatus (Canalco, Rockville, Md.). The pH 8.9 Tris-glycinate preparative system (15) was used with an 8% acrylamide separator gel (2.1 X 8 cm). A sample containing no more than 30 mg of protein was dialyzed against upper reservoir buffer and made 5% in sucrose before layering it between the upper reservoir buffer and the stacking gel. The system was subjected to electrophoresis at less than 2 mA until the bromophenol blue had migrated 3 to 4 cm, at which time the current was increased to 20 mA. The column was eluted at a flow rate of 1.8 ml/min and fractions containing 25% or greater of the maximum activity were pooled. Protein was concentrated on a DEAE-cellulose column (0.9 X 2 cm) equilibrated with 0.05 M Tris-Cl, pH 7.5, and was eluted with 0.1 M succinate-0.1 M K$_2$HPO$_4$, pH 7.5.

Molecular Weight Estimation—Sedimentation equilibrium ultrafiltration of the purified enzyme preparation was carried out on the Spinco model E analytical ultracentrifuge using the Yphantis meniscus depletion method of analysis (20). Two separate determinations were carried out at 5.55 and 4.65°C, respectively. The enzyme preparation was dialyzed 12 hours against stabilizer buffer before centrifugation. A density of 1.020 g/ml for the solvent was determined by weighing aliquots of the dialysis solution. The partial specific volume of aspartate aminotransferase I was assumed to be 0.73.

Molecular weight estimations of partially purified preparations by the sucrose density gradient technique of Martin and Ames (21) were carried out using yeast alcohol dehydrogenase (molecular weight 150,000) as a marker.
Further purification of form II (DEAE-cellulose Fraction A, Fig. 1) was achieved (Fig. 3) utilizing the gradient focusing technique (9). Data for purification of form II are given in Table I. Because of the low quantity of enzyme present, estimated to account for only 10 to 20% of the total activity (see Table I, footnote a), further purification has not been achieved at this time.

Characterization Studies

pH Optima—Both enzyme forms exhibit optimum activity at pH 7.5 in the presence of either Tris or phosphate buffer; however, both forms are approximately 20% more active in the presence of Tris than of phosphate.

Table I

<table>
<thead>
<tr>
<th>Step and fraction</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Recovery</th>
<th>Purity</th>
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<td><strong>Forms I and II</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1. Crude homogenate</td>
<td>20.0</td>
<td>2500</td>
<td>0.13</td>
<td>100</td>
<td>1</td>
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<tr>
<td>2. Crude supernatant</td>
<td>18.0</td>
<td>2000</td>
<td>0.11</td>
<td>80</td>
<td>1</td>
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<td>3. (NH₄)₂SO₄ concentrate</td>
<td>5.6</td>
<td>1400</td>
<td>0.25</td>
<td>57</td>
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<td>4. Sephadex G-15</td>
<td>4.9</td>
<td>1500</td>
<td>0.31</td>
<td>60</td>
<td>1</td>
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<tr>
<td>5. DEAE-cellulose, Fractions A + B</td>
<td>1.8</td>
<td>1100</td>
<td>44</td>
<td></td>
<td></td>
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<tr>
<td><strong>Form II</strong></td>
<td></td>
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<tr>
<td>5A. DEAE-cellulose, Fraction A</td>
<td>0.29</td>
<td>180</td>
<td>0.61</td>
<td>7</td>
<td>30⁰</td>
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<td>6. DEAE-Sephadex</td>
<td></td>
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<tr>
<td><strong>Form I</strong></td>
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<tr>
<td>5B. DEAE-cellulose, Fraction B</td>
<td>1.5</td>
<td>910</td>
<td>0.61</td>
<td>37</td>
<td>5⁰</td>
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<td>7. 50 to 55% (NH₄)₂SO₄</td>
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<td>420</td>
<td>8.2</td>
<td>17</td>
<td>70⁰</td>
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<tr>
<td>8. Preparative gel electrophoresis</td>
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<td>180</td>
<td>100</td>
<td>7</td>
<td>1100⁰</td>
</tr>
</tbody>
</table>

* Fold purity is calculated assuming a specific activity of 0.02 μmol/min/mg of protein for form II in the crude homogenate based on the proportions of 16% form II in the DEAE-cellulose Fractions A and B.

** Fold purity is calculated assuming a specific activity of 0.11 μmol/min/mg of protein for form I in the crude homogenate based on the proportions of 84% form I in the DEAE-cellulose Fractions A and B.

These values are representative of several combined preparations.

Kinetic Studies—Reciprocal plots of kinetic data are shown in Figs. 4 to 6. For form II, levels of glutamate and oxalacetate were not found which would yield linear data when plotted in reciprocal form; thus, the Michaelis constants reported for this form with these substrates are estimates derived from data such as those shown in Fig. 7. No reciprocal plot for form II with oxalacetate is presented as the data were scattered and irreproducible. Due to the low Km of the enzyme for oxalacetate and low extinction coefficient for oxalacetate (1.0 mm⁻¹ cm⁻¹ in 0.1 M Tris-Cl, pH 7.5), the sensitivity of the direct assay was marginal even when 5-cm path length cuvettes and full scale measurements of 0.1 absorbance were used. At lower oxalacetate concentrations, the total absorbance of oxalacetate represented less than 0.1 A;
thus, initial rates (10% or less of substrate utilization) could not be measured reliably.

It should be noted that in all cases a reaction was observed only in the presence of all reactants after correction for oxalacetate utilization. Rates of nonenzymatic decarboxylation of oxalacetate were determined independently for each reaction condition. In the direction of oxalacetate formation, rates from the direct measurement of oxalacetate were very difficult to interpret because of significant deviation from linearity (the rates decreasing with increasing time of reaction). The initial rates obtained from the coupled assay where oxalacetate is continually being removed from the reaction mixture show no such deviations; thus, these data were used to determine $K_m$ values. A summary of these results is presented in Table II.

**Molecular Weight Determination**—A molecular weight of $(198 \pm 9) \times 10^3$ was calculated from two separate sedimentation equilibrium ultracentrifugation experiments (Fig. 8), omitting the data set for the fringe closest to the rotor center. The molecular weights of forms I and II, estimated by sucrose density gradient centrifugation (Fig. 9), are identical, being $1.0 \times 10^5$ based on a molecular weight of $1.5 \times 10^3$ for yeast alcohol dehydrogenase.

**Pyridoxal Phosphate Involvement**—No activation of aspartate aminotransferase I has been observed after addition of pyridoxal phosphate to the crude supernatant, the DEAE-cellulose chromatography, or the preparative gel electrophoresis fractions.

**DISCUSSION**

To provide a basis for the study of the role of aspartate aminotransferase in metabolism by photosynthetic tissue, the proper-

![Fig. 3. DEAE-Sephadex A-25 ascending flow focusing chromatography of Fraction A from DEAE-cellulose (form II).](image)

![Fig. 4 (left). Initial velocity reciprocal plot for aspartate aminotransferase I with a-ketoglutarate (mM) as varied substrate, coupled assay.](image)

![Fig. 5 (center). Initial velocity reciprocal plot for aspartate aminotransferase I with glutamate (mM) as varied substrate, direct assay.](image)

![Fig. 6 (right). Initial velocity reciprocal plot for aspartate aminotransferase II with a-ketoglutarate (mM) as varied substrate, coupled assay.](image)

![Fig. 7. Initial velocity reciprocal plot for aspartate aminotransferase II with glutamate (mM) as varied substrate, direct assay.](image)
Purification of the two forms involves essentially two processes, separation of plant-soluble protein from low molecular weight and polyphenolic material before significant browning can occur followed by separation of the enzyme from other proteins. Glycerol in the medium aids in retarding the browning reactions for a length of time sufficient to remove the protein by ammonium sulfate precipitation.

The final preparation of form I is estimated from gel electrophoretic patterns to be 90 to 95% homogeneous (Fig. 2); note the less anionic, Coomassie blue-positive material in the gel containing 50 µg of protein. That a low level of contaminating protein exists is supported by the nonlinearity of the sedimentation data (Fig. 8) and the presence of malate dehydrogenase activity. Although the specific activity of malate dehydrogenase seems high, 30% that of aspartate aminotransferase, the turnover number of malate dehydrogenase is probably an order of magnitude higher than for the aminotransferase, thus accounting for the low levels of contaminating protein. Form I, at a specific activity of 120 µmol/min/mg of protein, represents the most highly purified plant aspartate aminotransferase reported to date; the cauliflower enzyme has been purified to a specific activity of only 34 µmol/min/mg of protein (4).

Form II, only 10 to 20% of the total aspartate aminotransferase activity, has not yet been purified to complete homogeneity; however, a complete separation of form II from form I since both forms show identical mobilities by sucrose density gradient ultracentrifugation. The primary distinguishing feature of these enzyme forms is their different electrophoretic mobilities, whereas pH optima, Michaelis constants, and molecular weights are similar for both forms. The molecular weight of 130,000 for form I is similar to that reported for aspartate aminotransferase from mammalian systems (25). Form II is not an aggregate or subunit multiple of form I since both forms show identical mobilities by sucrose density gradient ultracentrifugation. Thus, differences in mobility during electrophoresis are not due to molecular weight differences but rather must be due to electrophoretic properties as determined by other structural features, e.g. amino acid composition.

Kinetic constants (Table II) are similar to those reported for the mammalian cytoplasmic enzyme (25) and to those reported for the mammalian cytoplasmic enzyme (25) and to those reported...
for aminotransferases from other plant tissues (5–7). Forms I and II do not exhibit an inversion in magnitude of the $K_m$ values for aspartate and α-ketoglutarate as do the mammalian cytoplasmic and mitochondrial isoenzymes (25). The otherwise similar properties of the plant and mammalian enzymes suggest that the $K_m$ differences seen for the mammalian isozymes may not be a major consideration in the function of the malate-aspartate shuttle which could be an energy driven process (26).

Although ammonium sulfate precipitation results in substantial resolution of pyridoxal phosphate from both the pig heart cytosolic isozyme (25) and the cauliflower enzyme (4), no comparable activity loss is observed for oat leaf aspartate aminotransferase. In addition, no reactivation of the oat leaf enzyme forms by pyridoxal phosphate has been observed.

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Partial purification and characterization of aspartate aminotransferases from seedling oat leaves.
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