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 approximately 34 μmol/min/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 (2), 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 oxaloacetate 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.

EXPERIMENTAL PROCEDURES

Materials—Oat seeds, Avena sativa var. Lodi, were generously provided by H. L. Shands, Department of Agronomy, University of Wisconsin, Madison, Wis. Leaves from 10- to 14-day-old oat seedlings grown in vermiculite medium provided material for these studies. Reagent grade chemicals were obtained from commercial sources.

Sephadex G-15 was regenerated by extensive washing with distilled water and then equilibrated with the 0.05 M Tris-Cl-0.02% NaN3, pH 7.5, elution buffer. DEAE-cellulose was washed before use with 1 N NaOH and 1 N HCl (8), as was DEAE-Sephadex A-25 anion exchanger (9).

Enzyme Assays—Unless otherwise specified, aspartate aminotransferase was assayed at pH 7.5 and 30°C in 0.1 M Tris-Cl containing 2 mM α-ketoglutarate and 25 mM aspartate, 6.5 units/ml of pig heart malate dehydrogenase (approximately 5 μg of protein/ml), and 0.13 mM NADH. Reactions were initiated by addition of enzyme and followed by the decrease in absorbance of NADH at 340 nm.

After consideration of the available information on the direct
assay of the enzyme by the absorbance of oxalacetate in the 250 to 290 m/Jm range (12), the following procedure was used for our kinetic studies. In the direction of oxalacetate formation, the increase in absorbance of a reaction mixture containing 0.1 M Tris-Cl (pH 7.5), enzyme, and the specified concentrations of aspartate and α-ketoglutarate was followed at 260 nm and 32°. In the direction of oxalacetate utilization 5-cm path length cuvettes were used; the decrease in absorbance at 260 nm was measured for a 10-ml reaction mixture containing 0.1 M Tris-Cl (pH 7.5), enzyme, and the specified concentrations of oxalacetate and glutamate, at 32°. Oxalacetatic acid was dissolved in water immediately before use and maintained at 0-5°. Rates of oxalacetate utilization were corrected for nonspecific decarboxylation of oxalacetate; the latter was determined to be a function of both oxalacetate and glutamate concentrations.

Malate Dehydrogenase activity was assayed at 340 nm, pH 7.5, and 30°, in 0.1 M Tris containing 5 mM oxalacetate and 0.13 mM NADH. Yeast alcohol dehydrogenase was assayed at 340 nm, pH 9.0, and 30°, in 0.1 M sodium glycinate containing 0.1 M ethanol and 1 mM NAD+. 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% Na2CO3 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).

Preparative Polyacrylamide Gel Electrophoresis—Analytical polyacrylamide 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 50 min or longer in 20% trichloroacetic acid-7% acetic acid, then protein-stained with Coomassie blue (16). The two major bands of aspartate aminotransferase activity found in oat leaf homogenates after polyacrylamide gel electrophoresis are designated I for the more anionic and II for the less anionic forms in accordance with recent recommendations of the Commission on Biochemical Nomenclature (17).

Detection of Enzyme Activity on Analytical Polyacrylamide Gels—The aspartate aminotransferase activity stain for polyacrylamide 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% acrylamide gel, 0.03% methylenebisacrylamide, and a 10-fold dilution of electrode buffer. Gels were fixed 50 min or longer in 20% trichloroacetic acid-7% acetic acid, then protein-stained with Coomassie blue (16). The two major bands of aspartate aminotransferase activity found in oat leaf homogenates after polyacrylamide gel electrophoresis are designated I for the more anionic and II for the less anionic forms in accordance with recent recommendations of the Commission on Biochemical Nomenclature (17).

Preparative Polyacrylamide Gel Electrophoresis—Preparative gel electrophoresis was performed using the Canaco Prep Disc apparatus (Canaco, Rockville, Md.). The pH 8.9 Tris-glycine preparative system (15) was used with an 8% acrylamide separator gel (2.1 cm, 15 cm).

Protein concentration was no more than 10 mg of protein per 5 ml of aspartate, pH 7.5, and 0.2 M α-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 Canaco Prep Disc apparatus (Canaco, Rockville, Md.). The pH 8.9 Tris-glycine preparative system (15) was used with an 8% acrylamide separator gel (2.1 cm, 15 cm). A sample containing no more than 10 mg of protein was dialyzed against upper reservoir buffer and made 6% in sucrose before layering it between the upper reservoir buffer and the stacking gel. The system was subjected to electrophoresis at 3 to 4 cm, 0.1 M K2HPO4, pH 7.5.

Preliminary Studies

In preliminary differential centrifugation studies isotonic (24) oat leaf homogenates yielded 4% of the total aspartate aminotransferase activity in the chloroplast-enchriched fraction (pellet from 1,000 × g centrifugation for 1 min, washed), 2% in the mitochondria-enriched fraction (pellet from 2,000 to 15,000 × g for 10 min, washed), and 88% in the soluble fraction (15,000 × g for 10 min, supernatant). Acrylamide gel electrophoretic patterns of the 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 (NH4)2SO4 (52 g/ml) to separate rapidly the soluble protein from other plant materials, specifically polyphenols or tannins. After centrifugation the precipitated protein was redissolved in stabilizer buffer, which consisted of 0.1 M succinate and 0.1 M K2HPO4 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 ultrafiltration and stored frozen in stabilizer buffer. Data are summarized in Steps 1 to 5 of Table I.

Fraction II containing form I of the enzyme (Fig. 1) was fractionated by (NH4)2SO4, approximately 50% of the activity being recovered in the 50 to 55% (NH4)2SO4 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 (NH4)2SO4 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 were 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 for the aminotransferase I.
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.

**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 $K_m$ of the enzyme for oxalacetate and low extinction coefficient for oxalacetate (1.0 mm$^{-1}$ cm$^{-1}$), 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;

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**Table I**

**Purification of oat leaf aspartate aminotransferase**

| Step and fraction | Total protein | Total activity | Specific activity | Recovery | Purity |
|-------------------|---------------|----------------|-------------------|----------|--------|
|                   | g             | $\mu$mol/min   | $\mu$mol/min/mg of protein | %        | -fold  |

**Forms I and II**

| Step and fraction | Total protein | Total activity | Specific activity | Recovery | Purity |
|-------------------|---------------|----------------|-------------------|----------|--------|
| 1. Crude homogenate | 20.0          | 2500           | 0.13              | 100      |        |
| 2. Crude supernatant | 18.0          | 2000           | 0.11              | 80       |        |
| 3. $(NH_4)_2SO_4$ concentrate | 5.6          | 1400           | 0.25              | 60       |        |
| 4. Sephadex G-15 | 4.9           | 1500           | 0.31              | 60       |        |
| 5. DEAE-cellulose, Fractions A + B | 1.8       | 1100           |                    | 44       |        |

**Form II**

| Step and fraction | Total protein | Total activity | Specific activity | Recovery | Purity |
|-------------------|---------------|----------------|-------------------|----------|--------|
| 5A. DEAE-cellulose, Fraction A | 0.29       | 180            | 0.61              | 7        | 30$^a$ |
| 6. DEAE-Sephadex | 1.5           | 910            | 6 ± 2$^a$         | 5-7$^b$  | 300$^a$|

**Form I**

| Step and fraction | Total protein | Total activity | Specific activity | Recovery | Purity |
|-------------------|---------------|----------------|-------------------|----------|--------|
| 5B. DEAE-cellulose, Fraction B | 1.5             | 910            | 0.61              | 37       | 5$^c$ |
| 7. 50 to 55% $(NH_4)_2SO_4$ | 0.051         | 420            | 8.2               | 17       | 70$^c$|
| 8. Preparative gel electrophoresis | 0.0014       | 180            |                    | 7        | 1100$^c$|

* Fold purity is calculated assuming a specific activity of 0.02 $\mu$mol/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 $\mu$mol/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.
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 $1.3 \times 10^4$ 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-

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**Fig. 3.** DEAE-Sephadex A-25 ascending flow focusing chromatography of Fraction A from DEAE-cellulose (form II). A 50-ml gradient of 0 to 0.2 M KCl in 0.02 M Tris-Cl, pH 7.5, was applied to a column (2.5 x 70 cm) of DEAE Sephadex A-25 equilibrated with 0.02 M Tris-Cl, pH 7.5. A 4.2-ml sample of aspartate aminotransferase II, specific activity 0.22 $\mu$mol/min/mg of protein and 240 mg of protein total, was applied and eluted with 0.2 M KCl in 0.02 M Tris-Cl at room temperature and a flow rate of 1 ml/min. ○—○, activity; ●—●, absorbance at 280 nm; □—□, relative conductivity. Fractions between the arrows were combined to give a DEAE-Sephadex fraction of a specific activity of 7.0 $\mu$mol/min/mg of protein (72% recovery of activity).

**Fig. 4 (left).** Initial velocity reciprocal plot for aspartate aminotransferase I with a-ketoglutarate (mM) as varied substrate, coupled assay. Velocity units are $\Delta A_{245}$ min/1-ml reaction mixture. Enzyme units are $\Delta A_{245}$ min/1-ml reaction mixture (specific activity, 120 $\mu$mol/min/mg of protein). Reaction rates were determined at 32° in 5-cm path length cuvettes at these aspartate concentrations: 1, 1.23 mM; 2, 0.727 mM; 3, 0.416 mM; and 4, 0.500 mM. Lines drawn through the data points were obtained from a computer fit of the data to the ping-pong mechanism (22).

**Fig. 5 (center).** Initial velocity reciprocal plot for aspartate aminotransferase I with glutamate (mM) as varied substrate, direct assay. Velocity units are $\Delta A_{245}$ min/1-ml reaction mixture. Enzyme units are $\Delta A_{245}$ min/1-ml reaction mixture (specific activity, 120 $\mu$mol/min/mg of protein). Reaction rates were determined at 32° in 5-cm path length cuvettes at these oxalacetate concentrations: 1, 0.045 mM; 2, 0.0209 mM; 3, 0.0130 mM; 4, 0.0101 mM; and 5, 0.008 mM. Lines drawn through the data points were obtained from a computer fit of the data to the ping-pong mechanism (22).

**Fig. 6 (right).** Initial velocity reciprocal plot for aspartate aminotransferase II with a-ketoglutarate (mM) as varied substrate, coupled assay. Velocity units are $\Delta A_{245}$ min/1-ml reaction mixture. Enzyme units are $\Delta A_{245}$ min/1-ml reaction mixture (specific activity, 5.0 $\mu$mol/min/mg of protein). Reaction rates were determined at 32° in 1-cm path length cuvettes at these aspartate concentrations: 1, 4.00 mM; 2, 1.23 mM; 3, 0.727 mM; 4, 0.500 mM; and 5, 0.400 mM. Lines drawn through the data points were obtained from a computer fit of the data to the ping-pong mechanism (22).
tions for a length of time sufficient to remove the protein by protein. Glycerol in the medium aids in retarding the browning reaction 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 protein. Glycerol in the medium aids in retarding the browning reactions for a length of time sufficient to remove the protein by ammonium sulfate precipitation.

Table II

Kinetic constants for aspartate aminotransferases I and II

| Constant                  | Form I          | Form II         |
|---------------------------|-----------------|-----------------|
|                           | μmol/min/mg protein | μmol/min/mg protein |
| K_m (aspartate)           | 4.14 ± 0.24     | 2.3 ± 0.09      |
| K_m (α-ketoglutarate)     | 0.22 ± 0.01     | 0.30 ± 0.01     |
| K_m (glutamate)           | 32.9 ± 3.0      | 13.7 ± 3.2^a    |
| K_m (oxalacetate)         | 0.057 ± 0.005   | 0.030 ± 0.008^a |
| V_i                       | 119 ± 6         | —^b             |
| V_a                       | 246 ± 20        | —^b             |

^a These values are estimations of K_m at a single concentration of the fixed substrate.

^b These values are irrelevant as the enzyme preparations are not homogeneous.

Fig. 8. Sedimentation equilibrium ultracentrifugation of purified aspartate aminotransferase I in 0.10 M potassium succinate-0.01 M potassium phosphate, pH 7.5. Data were obtained from two measurements of a photograph of the interference pattern taken after 24 hours at 5.6^a and 12,590 rpm. The distance from the rotor center, r, is expressed in centimeters; f is the fringe number.

Fig. 9. Molecular weight estimation by sucrose density gradient ultracentrifugation. Of the three gradients centrifuged simultaneously, Gradient 1 contained 37 μg of yeast alcohol dehydrogenase, Gradient 2 contained 41 μg of aspartate aminotransferase I (specific activity, 2.8 μmol/min/mg of protein) plus 37 μg of yeast alcohol dehydrogenase, and Gradient 3 contained 38 μg of aspartate aminotransferase II (specific activity, 5.2 μmol/min/mg of protein) plus 37 μg of yeast alcohol dehydrogenase.

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 and a 300-fold purification has been achieved.

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 (23). Form II is not an aggregate or subunit multiple of I since both forms show identical mobilities by sucrose density gradient ultracentrifugation. Thus, differences in mobility during polyacrylamide gel 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 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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