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Rapid Purification and Thermostability of the Cytoplasmic Aspartate Aminotransferase from Carrot Suspension Cultures[1]

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ABSTRACT

Several isoenzymic forms of aspartate aminotransferase (AAT) have been identified in protein extracts from carrot (*Daucus carota*) cell suspension cultures. The cellular location of the major form (form I) of AAT in carrot suspension cultures was determined by heat inactivation, subcellular fractionation, and amino acid sequence analysis. In mammalian systems, there are two forms of AAT, a heat-stable cytoplasmic form and a heat-labile form in the mitochondria. The thermostability of three isoenzymes of carrot AAT was examined, and the results showed that form I was more thermostable than forms II or III. Organelles were separated in sucrose gradients by isopynic centrifugation. Activity for form I was identified in the soluble fractions and not in fractions containing peroxisomes, proplastids, or mitochondria. Form I was purified to homogeneity and endoproteolytically cleaved, and the peptide fragments were separated by reverse phase chromatography. Analysis of the sequence data from two of the polypeptides showed that the amino acid identity of form I is more conserved to the animal cytoplasmic AAT than to animal mitochondrial AAT sequences. These data strongly suggest that form I of AAT from carrot is the cytoplasmic isoenzyme. Additionally, a rapid purification scheme for form I of AAT from carrot is presented using selective heat denaturation and anion-exchange chromatography.

AAT[3] (EC 2.6.1.1) catalyzes the reversible transfer of an amino group from aspartate to α-ketoglutarate to form oxaloacetate and glutamate. In plants, AAT is involved in numerous metabolic functions, which include hydrogen shuttles, carbon shuttles, and nitrogen distribution (8, 16). AAT has been studied in various plants, and there are numerous isoenzymic forms (8, 16). These different forms are located in the cytoplasm, mitochondria, chloroplast, and peroxisomes. Genetic analyses of varieties of maize (24) and wheat (4) have shown that some of these isoenzymic forms are under independent genetic control. Further evidence for the presence of independent AAT genes in plants includes the preparation of immunologically distinct antibodies to two different forms of AAT isolated from alfalfa nodules (6, 9). Likewise, in *Panicum maximum,* AAT isoenzymes may be encoded by distinct genes (19).

In animals, there are two isoenzymic forms of AAT; one has been localized in the cytoplasm and the other in the mitochondrion. The two isoenzymes are encoded by different genes that have 53% nucleotide identity and 47% amino acid identity (20).

Three AAT isoenzymes from carrot suspension culture cells have been identified and separated by this laboratory (27). The relative mol wts on native PAGE are 111,000, 105,000, and 94,000 for forms I, II, and III, respectively. Form I, the predominant isoenzyme, was physically and biochemically characterized. Form I was purified to homogeneity using ammonium sulfate precipitation, gel filtration, several anion-exchange columns, and finally, immunoaffinity chromatography with rabbit anti-pig AAT antibodies. It is composed of two 43,000 D subunits and is similar to other plant AATs in that it has a broad pH optimum and similar K_m values and the mode of action is a ping-pong-bi-bi mechanism. Additionally, we presented data suggesting that form I may be the cytoplasmic isoenzyme.

A goal of this laboratory is to determine the molecular mechanism(s) that regulate and orchestrate the expression of AAT isoenzymes. Subcellular localization of the different isoenzymes is necessary to (a) understand the specific metabolic roles of the isoenzymes in the cell; (b) assign the gene(s) to their product(s); and (c) understand differential regulation at the level of transcription and translation. In this paper, we present a method for the rapid purification of AAT form I from carrot cell suspension culture. Additionally, we present data for the cellular location of form I based on results from heat stability tests, subcellular fractionation, and amino acid sequence analysis. The data strongly suggest that AAT form I from carrot is a cytoplasmic isoenzyme.

MATERIALS AND METHODS

Cell Cultures

Cell suspension cultures of carrot (*Daucus carota* L. cv Danvers) were transferred and maintained as previously described (27).

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[3] Abbreviation: AAT, aspartate aminotransferase.

Protein Purification

Forms I, II, and III were separated by anion-exchange HPLC as previously described (27). Form I was purified to homogeneity using heat denaturation and anion-exchange chromatography as described below. Cell cultures were harvested 5 d after inoculation by filtration using 3 MM Whatman[4] 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 diethyldithiocarbamic acid/L) per g fresh weight. Cells were lysed by two passes of 20 min through a cell disruption bomb (1000 psi). Cellular debris was removed by centrifugation at 18,000*g* for 15 min at 4°C. Proteins precipitated with 60% saturated ammonium sulfate at 4°C were collected by centrifugation at 18,000*g* at 4°C for 20 min. Protein pellets were resuspended and dialyzed in extraction buffer minus diethyldithiocarbamic acid at 4°C overnight. The volume was adjusted to one-half of the original gram fresh weight of the cells (w/v). The sample was heated to 55°C for 30 min, with occasional mixing. The sample was chilled on ice and two volumes of cold buffer were added. Denatured proteins were pelleted by centrifugation at 19,000*g* for 20 min at 4°C. The supernatant was mixed with one-third volume DEAE-Sephacel (Pharmacia) to allow protein binding. The DEAE-Sephacel beads and supernant were poured into a Büchner funnel and AAT form I was eluted from the beads using 100 mM KCl, pH 7.5. The fraction was diluted with three volumes of 10 mM Tris, pH 7.5, and applied to a Waters DEAE-5PW HPLC (2.15 × 15 cm) column. The column was washed with 10 mM Tris, pH 7.5, for 30 min. AAT was eluted with the same buffer using a linear NaCl gradient, 5 min from 0 to 37.5 mM NaCl, a 5 min hold at 37.5 mM NaCl, then a 40 min gradient to 100 mM NaCl at a flow rate of 1 mL/min. Fractions were assayed for AAT activity by measuring the formation of NAD in a coupled enzyme assay with malate dehydrogenase, and analyzed by native and SDS-PAGE as previously described (27).

Thermostability of AAT Isoenzymes

Separate samples containing either form I, II, or III (900–1000 units/mL) were incubated at 55°C for 0, 5, 10, 20, and 30 min. One unit of AAT activity equals 1 μmol of product generated per min at 25°C. Samples, each isoenzymic form at each time point, were performed in triplicate. After the incubation period, denatured protein was removed from the samples by centrifugation at 14,000*g* for 10 min at 4°C. The average of the total AAT activity was determined for each time point and divided by the average AAT activity for the zero time point (control). The total activity was expressed as a percentage of the control for each time point and plotted *versus* the incubation time at 55°C. Similar experiments were conducted at 60 and 65°C.

Samples containing all three forms of AAT were analyzed by anion-exchange HPLC before and after incubation at 55°C for 30 min. Fractions containing AAT activity were analyzed on 10 to 15% PAGE and stained for AAT activity as previously described (27) to determine the presence of the different forms.

Subcellular Localization

Five-day-old carrot cells from suspension cultures were collected by centrifugation at 200*g* for 2 min. Cells were resuspended (4 mL packed cells in 50 mL enzyme solution) in 0.5% Onozuka RS 0.05%, pectolyase Y23, 300 mM sorbitol, 300 mM mannitol, 0.6 mM calcium chloride, and 0.7 mM sodium phosphate, pH 5.7. The cells and enzyme solution were incubated on a shaker at 28°C for 3.5 h. Cell wall debris was removed by filtration through a 150 μm screen. The protoplasts were washed two times in lysis butter (300 mM sorbitol, 100 mM Tris [pH 7.8], 1 mM EDTA, 0.1% BSA, and 0.1% β-mercaptoethanol). Protoplasts were ruptured and the organelles were liberated by gently passing the sample through an 18 g needle. Organelles were separated in a sucrose gradient by centrifugation at 113,000*g* for 4 h. Sucrose gradients were poured 24 h in advance as follows: 2 mL each of 60, 49, 43 (8 mL), 37, 36, 34, and 25% (6 mL) sucrose. Ten to 12 mL of the organelle filtrate wash was layered on the sucrose gradient. After centrifugation, 300 to 500 μL fractions were collected. The sucrose concentration of each fraction was determined by refractive index. Each fraction was assayed for AAT, and the marker enzymes for glyoxyosome, mitochondria, and plastids: catalase (2), Cyt *c* oxidase (26), and triose phosphate isomerase (12), respectively.

Peptide Cleavage and Amino Acid Sequence Analysis

Pure AAT, form I, was cleaved with Lys-C. The resulting polypeptide fragments were separated by reverse phase chromatography and purified fragments were sequenced by a commercial vendor (Dr. William Lane, Microchemistry Department, Harvard University, Cambridge, MA). Amino acid sequences from two of the polypeptides were compared to amino acid sequences in the National Biomedical Research

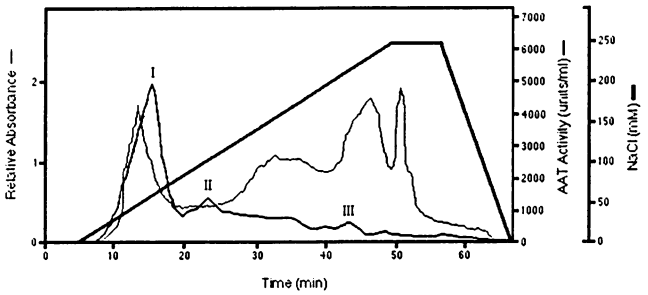

Figure 1. Separation of carrot AAT isoenzymes by anion-exchange chromatography. Three isoenzymes of AAT were separated with a linear gradient from 0 to 250 mM NaCl over 45 min. Relative absorbance of proteins at 280 nm is represented by a narrow line; AAT activity expressed in units/mL is represented by a medium line; and NaCl concentration is represented by a thick line. Peaks of AAT activity associated with the different isoenzymes are labeled I, II, and III.

[4] 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 other products or vendors that may be suitable.

Foundation database and to amino acid sequences derived from DNA sequences in GenBank using the Putsell Sequence Analysis Software (IBI).

RESULTS

AAT isoenzymes were separated using anion-exchange chromatography (Fig. 1). Forms I, II, and III were eluted separately at 79, 110, and 160 mM NaCl, respectively. The thermostability of each form was determined by incubating enzyme extracts at 55°C for 0, 5, 10, 20, and 30 min (Fig. 2). Form I retained 100% AAT activity after incubation at 55°C for 30 min, whereas forms II and III retained 69 and 33% activity, respectively. Similar experiments were conducted at 60 and 65°C (not shown). At both 60 and 65°C, the enzymatic stability of the three forms was similar to that observed at 55°C, *i.e.* form I retained AAT activity greater than form II, which retained activity greater than form III.

As a separate experiment to verify the thermostability of form I, samples containing all three forms were analyzed by HPLC anion-exchange chromatography before and after heating at 55°C. The elution profiles of the unheated controls showed three peaks of AAT activity at 79, 110, and 160 mM NaCl. The elution profiles of the samples treated by heating at 55°C for 30 min showed a higher ratio of form I to forms II and III as compared with nonheated controls. The major peak of AAT activity eluted at 79 mM NaCl (Fig. 3). Fractions from the peak of AAT activity were analyzed by native PAGE; only one protein band was observed at 111,000 D when the gel was stained with silver (Fig. 4A, lane 1). The same protein band also stained for AAT activity (Fig. 4A, lane 2). On an SDS-PAGE gel, one band was observed at 43,000 D (Fig. 4B, lane 2). These relative mol wts correspond to those that were previously reported for form I (27). These data also show that the heat stability of form I is greater than that of forms II and III.

To determine the subcellular location of form I, carrot protoplasts were gently lysed and the organelles were separated by centrifugation on a sucrose gradient. Fractions were collected, and sucrose concentrations and AAT activities were determined for each fraction (Fig. 5). The activities of catalase, triose phosphate isomerase, and Cyt *c* oxidase were used as marker enzymes for the peroxisomes, plastids, and mitochondria, respectively. Peaks of AAT activity were observed in fractions 8 through 12, 16 through 24, and 30 through 38. Maximum catalase activity was associated with fractions 8 through 12, Cyt *c* oxidase activity was associated with fractions 19 through 24, and triose phosphate isomerase activity was associated with fractions 18 through 24. Fractions 30

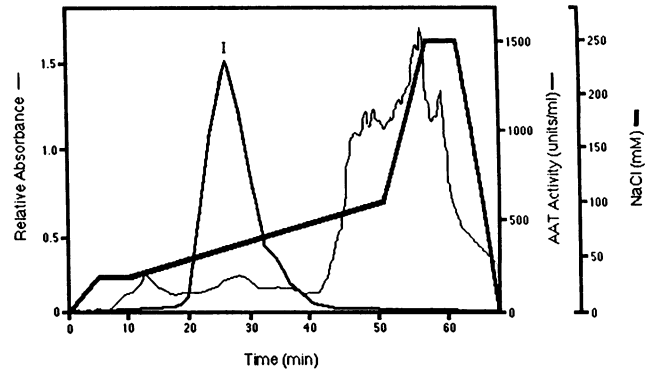

Figure 3. Anion-exchange chromatography of a carrot cell suspension extract after incubation at 55°C for 30 min. Relative absorbance, AAT activity, and NaCl concentration are represented as described in Figure 1.

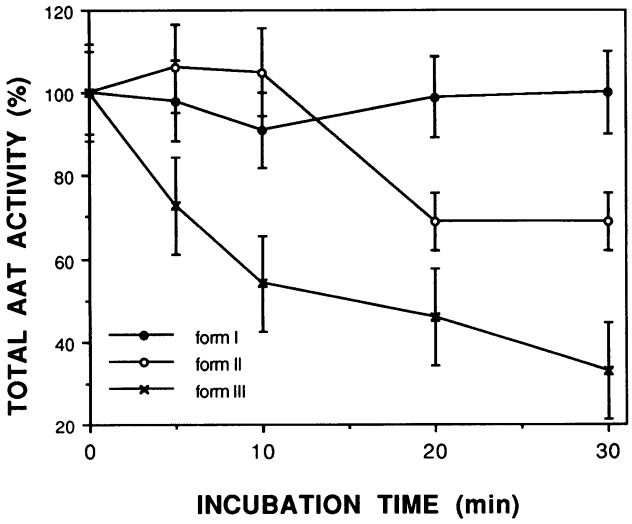

Figure 2. Thermostability of carrot AAT isoenzymes. Separate samples containing either form I, II, or III (900–1000 units/mL) were incubated at 55°C for 0, 5, 10, 20, and 30 min. Each time point represents the average of three trials; at each, the average of the total activity for each isoenzyme was determined and expressed as a percentage of the control (100%). Total AAT activity was plotted *versus* the incubation time at 55°C. Form I retains 100% activity after incubation for 30 min, whereas forms II and III retain 69 and 33% activity, respectively. These data are representative of three separate experiments. Vertical bars represent ± SE.

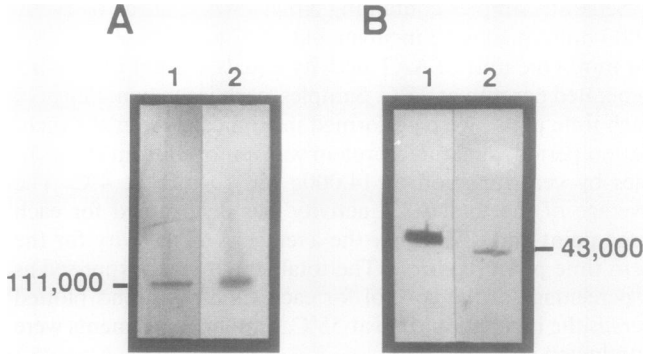

Figure 4. Native (A) and SDS (B) PAGE of purified AAT form I from carrot cell suspension cultures. Proteins were separated on 10 to 15% gradient gels. A, 50 ng of protein was loaded per lane, samples were stained for protein (lane 1) and AAT activity (lane 2). B, 50 ng of protein was loaded per lane, samples were stained for protein. Egg albumin (45,000) was used as a mol wt marker (lane 1) with pure carrot AAT form I (lane 2).

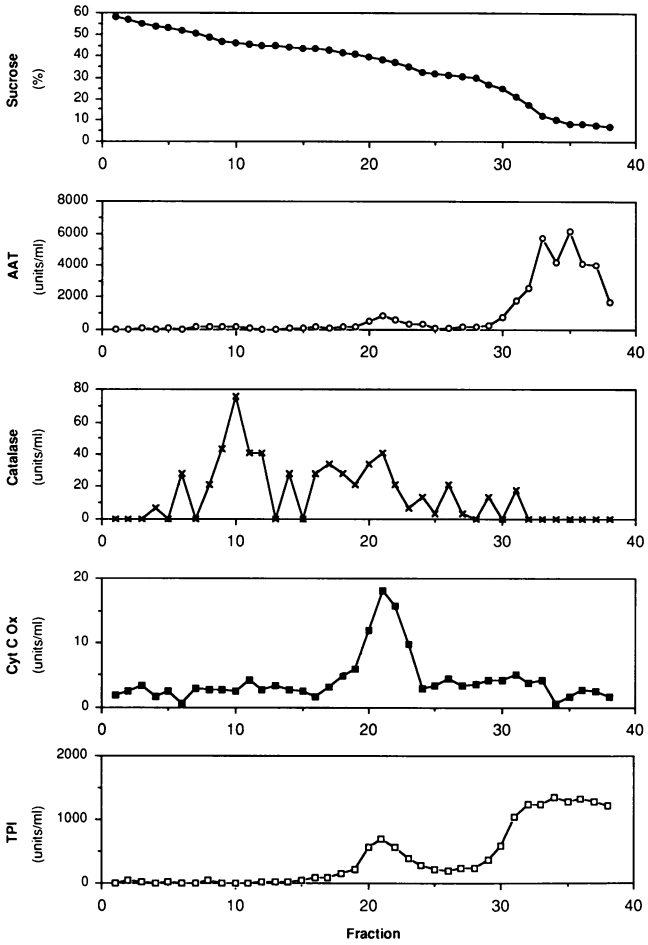

Figure 5. Subcellular localization of form I of carrot AAT was determined by sucrose gradient centrifugation. Sucrose concentration and total AAT activity were determined for each fraction. Peaks of AAT activity from fractions 8 through 12 were associated with peaks of catalase activity. AAT activity in fractions 16 through 24 is associated with triose phosphate isomerase (16–24) and Cyt *c* oxidase (19–24) activity. Form I was associated with the soluble fraction (30–38), and it was not observed in the fractions containing peroxisomes (7–10), plastids (16–24), or mitochondria (19–24).

through 38 contain the soluble enzymes and enzymes released from lysed organelles. Fractions containing peaks of AAT activity were pooled and concentrated. The proteins were separated by electrophoresis and the gels were stained for AAT activity (Fig. 6). Form I was not associated with the peroxisomal (7–10), plastid (16–24), or mitochondrial (19–24) fractions. Form I was found only in soluble fractions (30–38). These data suggest that form I is the cytoplasmic isoenzyme of AAT in carrot. Furthermore, the soluble fraction contains approximately 85% of the total AAT activity applied to the gradient, whereas 2 and 9% of the AAT activity is associated with the peroxisomal and plastid/mitochondrial fractions, respectively. These data are similar to those observed in peas (28), in which 90% of the AAT activity is soluble. Forms II and III were identified in both the poorly resolved plastid and mitochondrial fractions.

Because form I could not be associated with an organelle,

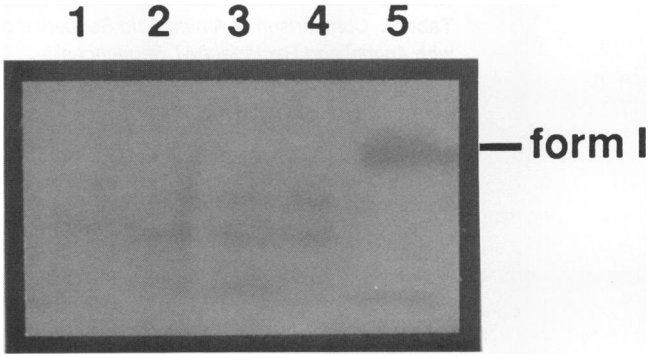

Figure 6. Native PAGE of fractions from a sucrose gradient containing AAT activity. Approximately 20 units of AAT activity was loaded per lane. Proteins from fractions 10 (lane 1), 17 (lane 2), 23 (lane 3), 26 (lane 4), and 31 (lane 5) from a sucrose gradient were separated on 10 to 15% gradient gels and stained for AAT activity. Form I is present only in fraction 31, a soluble fraction, and not in the organelle fractions.

we obtained amino acid sequence data from portions of the polypeptide to compare with known cytoplasmic and mitochondrial AAT amino acid sequences. AAT form I was purified to homogeneity using ammonium sulfate precipitation, heat denaturation, and anion-exchange chromatography. The purity and integrity of the enzyme was verified by native and SDS-PAGE: only one band of 111,000 D was visualized by silver stain of a native PAGE gel, whereas one band at 43,000 D was visualized on the SDS-PAGE gel (Fig. 4A, B). The pure AAT was cleaved with Lys-C, and the resulting polypeptide fragments were separated by reverse phase chromatography (Fig. 7). The amino acid sequence of two peptides, peaks A and B, were determined. The peptides in peaks A and B consisted of 21 and 11 amino acid residues, respectively. The

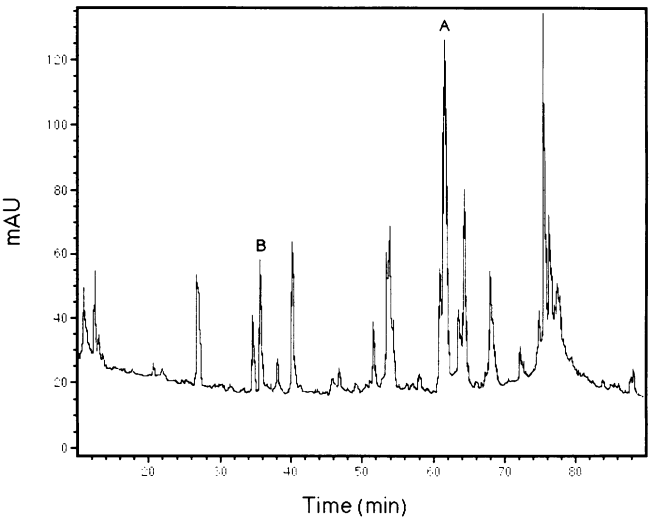

Figure 7. Chromatograph of polypeptide fragments of AAT form I separated by reverse phase chromatography after cleavage with Lys-C. Peaks A and B are indicated. The amino acid sequence of the polypeptide fragments associated with peaks A and B were determined.

Table I. *Comparison of Amino Acid Sequence of Polypeptide Fragment in Peak A of Carrot AAT Form I with Animal and Bacterial AAT Sequences*

Dashes represent gaps inserted to maximize homology. Amino acid residues that are identical to the carrot AAT residues are underlined.

Source	Sequence	% Identity	Reference
Carrot form I (peak A)	L I F G A D S P A I Q E N R V S T V Q C L		
Cytoplasmic AAT			
Chicken	I A L G D D S P A I A Q K R V G S V Q G L	52	25
Rat	L V L G D N S P A L R E N R V G S V Q S L	57	11
Horse	L A L G D D S P A L Q E K R V G G V Q S L	62	5
Mouse	L V L G D N S P A I R E N R V G G V Q S L	62	20
Pig	L A L G D D S P A L Q E K R V G G V Q S L	62	21
Mitochondrial AAT			
Chicken	L A L G E N S E A F K S G R Y V T V Q G I	38	10, 14
Rat	L A L G E N S E V L K S G R F V T V Q T I	33	13
Horse	L A L G E N S E A L K S G R Y V T V Q S I	38	5
Mouse	L A L G E N N E V L K S G R F V T V Q T I	29	20
Pig	L A L G E N N E V L K S G R Y V T V Q T I	29	15, 17
Turkey	L A L G E N S E A F K S G R Y V T V Q G I	38	1
Human	L A L G E N S E V L K S G R F V T V Q T I	33	18, 25
Bacterial AAT			
E. coli	L L F G K G S A L I N D K R A R T A Q T P	33	7
S. solfataricus	Y L N T R Y G T D V - - - K K E E V I V T P	4	3

amino acid sequences from the carrot AAT form I peptides were compared with published amino acid sequences (Tables I and II). The amino acid sequence from peptide A (Table I) has homology to a region corresponding to amino acid residues 85 to 106 in the pig cytoplasmic AAT sequence (21). The amino acid identity with animal cytoplasmic AAT sequences was 57 to 62%, while the identity with mitochondrial AAT sequences was only 39 to 48%. The amino acid sequence from peptide B (Table II) has sequence homology to a region corresponding to amino acid residues 344 to 355 in the pig cytoplasmic AAT sequence. The fragment had an amino acid identity of 55 to 64% with animal cytoplasmic AAT sequences and 36 to 45% identity with the animal mitochondrial sequences. These data further suggest that form I is a cytoplasmic isoenzyme.

The amino acid sequences of peptide A and B were also compared to known AAT sequences from microorganisms to show divergence and variability of these regions. The amino acid sequence of peptide A had 33 and 4% identity with *Escherichia coli* AAT (7) and *Sulfolobus solfataricus* AAT (3) amino acid sequences, respectively. The identity of the amino acid sequence of peptide B was 45% with *E. coli* AAT and 9% with *S. solfataricus* AAT.

DISCUSSION

One isoenzymic form of AAT, form I, from carrot suspension cell cultures has been purified to homogeneity using selective heat denaturation and anion-exchange chromatography. This isoenzyme was cleaved with Lys-C. The resulting polypeptides were separate by reverse phase chromatography and two fragments were subjected to amino acid sequence

Table II. *Comparison of Amino Acid Sequence of Polypeptide Fragment in Peak B of Carrot AAT Form I with Animal and Bacterial AAT Sequences*

Source	Sequence	% Identity	Reference
Carrot form I (peak B)	G T P G D W S H I V K		
Cytoplasmic AAT			
Chicken	G T P G T W N H I T D	64	25
Rat	K T P G T W S H I T E	64	11
Horse	K T P G T W N H I T E	55	5
Mouse	K T P G T W S H I T E	64	20
Pig	K T P G T W N H I T D	55	21
Mitochondrial AAT			
Chicken	G S S H N W Q H I T D	36	10, 14
Rat	G S S H N W Q H I T D	36	13
Horse	G S S H S W Q H I A D	36	5
Mouse	G S S H N W Q H I T D	36	20
Pig	G S S H N W Q H I V D	45	15, 17
Turkey	G S S H N W Q H I T D	36	1
Human	G S T H N W Q H I T D	36	18, 23
Bacterial AAT			
E. coli	G A N R D F S F I I K	45	7
S. solfataricus	K P N G A F Y M F D N V	9	3

analysis. A comparison of the carrot amino acid sequences with animal and bacterial AAT sequences shows that the carrot sequences have a higher amino acid sequence identity with the animal cytoplasmic AAT sequences than with the animal mitochondrial or bacterial AAT sequences.

Previously, we (27) reported the identification and separation of three AAT isoenzymes from carrot suspension culture cells by HPLC. The relative mol wts on native PAGE were 111,000, 105,000, and 94,000 for forms I, II, and III, respectively. Form I, the predominant isoenzyme, was purified to homogeneity using ammonium sulfate precipition, gel filtration, several anion-exchange columns, and finally, immunoaffinity chromatography with rabbit anti-pig AAT antibodies. The procedure was effective for purification of form I; however, it was time consuming and gave low overall yields. In the procedure described here, selective heat denaturation and anion-exchange chromatography were used to purify AAT form I to homogeneity; it is less complex, faster, and gives higher yields than the procedure described previously.

Previously, we (27) suggested that AAT form I from carrot suspension cultures may be a cytoplasmic isoenzyme, based on several lines of indirect evidence. Form I was 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 observations of the soluble form of AAT in pea, which accounted for 90% of the total extractable AAT activity (28). Additionally, we presented preliminary data showing that form I was heat stable. In mammals, the cytoplasmic form of AAT is heat stable, whereas the mitochondrial form is heat labile (22). In the present study, the thermostability of several forms of carrot AAT was tested either separately or together at 55°C. The results show that form I is more stable than forms II or III after heating at 55°C for 30 min. Greater thermostability of form I was observed at both 60 and 65°C for up to 10 min (data not shown). These data suggest that form I may be the cytoplasmic form of AAT in carrot. In mammalian systems, 3.7 mM α-ketoglutarate was added to the samples during the heat treatment to stabilize the cytoplasmic form of AAT (22). The carrot AAT samples did not contain α-ketoglutarate during incubation at the elevated temperatures. Form I does not require α-ketoglutarate as a stabilizer. In the presence of 3.7 mM α-ketoglutarate, all forms of AAT (I, II, and III) retain total activity at 55 and 65°C (data not shown).

Subcellular localization of carrot AAT isoenzymes shows that form I was identified in the soluble fraction and not with a specific organelle. The specific subcellular localization of forms II and III have not been determined; they both appear in fractions containing plastids and mitochondria.

Due to the cross-reactivity of antibodies prepared to pig heart AAT with form I of carrot, we (27) previously suggested that there may be a high degree of conservation between the plant and animal AAT amino acid sequences. The amino acid sequence data presented here verify the existence of a high degree of similarity between animal and plant AAT amino sequences as compared with bacterial AAT sequences. Furthermore, the data suggest that the homologous AAT isoenzymes between the animal and plant kingdoms, *i.e.* cytoplasmic or mitochondrial isoenzymes between organisms, have a higher degree of similarity than nonhomologous AAT isoenzymes.

In summary, our approach to identify definitively the cellular location of form I AAT from carrot included heat stability tests, subcellular fractionation, and amino acid sequence analysis. In mammals, cytoplasmic isoenzyme AAT is heat stable (22). Our results show that, after heating, form I AAT from carrot is more stable than forms II or III. Upon subcellular fractionation, activity for form I was identified in the soluble fraction and not in fractions containing perioxisomes, proplastids, or mitochondria. We presented a rapid method for the purification of form I in carrot that included selective heat denaturation and anion-exchange chromatography. Pure form I was endoprotolytically cleaved, the peptide fragments were separated by reverse phase chromatography, and two polypeptides were sequenced. Analysis of the sequence data from two of the polypeptides showed that the amino acid identity is more conserved to animal cytoplasmic AAT than to animal mitochondrial AAT sequences or bacterial AAT sequences. These data strongly suggest that form I of AAT from carrot is a cytoplasmic isoenzyme.

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