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Biochemical characterization of aspartate aminotransferase allozymes from common wheat

Research Article

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Abstract: Six allozymes of aspartate aminotransferase (AAT, EC 2.6.1.1): three plastidial (AAT-2 zone) and three cytosolic (AAT-3 zone) were isolated from common wheat (*Triticum aestivum*) seedlings and highly purified by a five-step purification procedure. The identity of the studied proteins was confirmed by mass spectrometry. The molecular weight of AAT allozymes determined by gel filtration was 72.4±3.6 kDa. The molecular weights of plastidial and cytosolic allozymes estimated by SDS-PAGE were 45.3 and 43.7 kDa, respectively. The apparent Michaelis constant (K_m) values determined for four substrates appeared to be very similar for each allozyme. The values of the turnover number (k_{cat}) and the k_{cat}/K_m ratio calculated for allozymes with *L*-aspartate as a leading substrate were in the range of 88.5–103.8 s^{-1}/10,412–10,795 s^{-1} M^{-1} for AAT-2 zone and 4.6–7.0 s^{-1}/527–700 s^{-1} M^{-1} for AAT-3 zone. These results clearly demonstrated much higher catalytic efficiency of AAT-2 allozymes. Therefore, partial sequences of cDNA encoding AATs from different zones were obtained using the RT-PCR technique. Comparison of the AAT-2 and AAT-3 amino acid sequences from active site regions revealed five non-conservative substitutions, which impact on the observed differences in the isozymes catalytic efficiency is discussed.

Keywords: *Active site • Allozyme • Aspartate aminotransferase • Triticum aestivum*

1. Introduction

Aspartate aminotransferase (AAT, EC 2.6.1.1) catalyses the fully reversible transamination reaction between *L*-aspartate and 2-oxoglutarate with formation of oxaloacetate and *L*-glutamate, by operating through a ping-pong Bi-Bi mechanism. The enzyme plays a pivotal role in the regulation of carbon and nitrogen flux in all organisms. In *eukaryotes,* AAT along with malate dehydrogenase comprise the malate-aspartate shuttle [1]. In C_4 plants (PEP carboxykinase and NAD-malic enzyme types) two forms of AAT participate in the transport of oxaloacetate (the CO_2 fixation product) from mesophyll into the bundle sheath cells [2]. Taking into consideration the broad range of AAT functions, it is not surprising that several AAT isozymes localized in at least four cellular compartments (cytosol, mitochondria, plastids and microbodies) have been detected in different plant species [3].

Plant AAT is active only as an association of two subunits, and similarly to the animal enzyme, contains two active sites. Each active site is formed from components of both subunits and includes a pyridoxal 5`-phosphate (PLP) as a cofactor [4]. AAT has been purified from several plant species (among them wheat), however obtained preparations were not pure enough to be used in X-ray crystal structure determination. These types of experiments, using AAT from *Escherichia coli* [5], chick [6] and pig [7], with previous biochemical, kinetic and spectroscopic studies [8], together with a more recently used site-directed mutagenesis [9,10] have resulted in the identification of 10 key amino acid residues, which directly interact with PLP in the active site of AAT: SerGlyThr107–109, Trp140, Asn194, Asp222, Tyr225, Ser255, Arg266 and protruding from the second subunit *Tyr70 form non-covalent bonds with PLP. Additionally, Lys258 covalently binds (*via* Schiff base linkage) to the carbonyl carbon of this cofactor in the absence of the substrate at the active site [11]. These 11 amino acid residues take identical positions in the sequences of AAT from different origins, and form the active site together with the side chains

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of amino acids located within their closest proximity, as well as with *Arg292 and Arg386 interacting with substrate carboxylate groups [11-13]. Few studies, using mainly site-directed mutagenesis, have shown that the character of the residues in AAT polypeptide chain adjacent to those binding cofactor and substrate also influence the enzyme catalytic efficiency [14-16]. It seems that substitutions within these residues might be crucial for the differences in the kinetic parameters values (k_{cat} or k_{cat}/K_m) determined for recombinant AAT of several origins: bacterial [17], animal [18], plant [19] and native enzymes from three different bacteria species [16,20,21], or from sheep's liver [22].

Zymographic analysis of AATs in crude extracts from hexaploid wheat (*Triticum aestivum*, 2n=6x=42, AABBDD) seedlings performed in our laboratory revealed three enzymatic zones (isozymes) [23]. Each AAT zone is encoded by genes located in one locus out of three loci, which are fully independent from each other. One locus contains three allelic genes of a homeological genomes origin: A, B and D [24]. High similarity of the sequences of two out of three allelic genes from a single locus provides evidence that one zone is formed from only two types of subunits (α and β). These subunits are associated into pairs in three different combinations (αα, αβ and ββ) forming three allozymes [24]. In our previous study, we examined on zymograms the relation between the activity levels of three AAT zones, and within them between allozymes [23]. The aim of the present study was to elucidate whether the differences in the amino acid sequence (causing significantly different electrophoretic mobility) of AATs from various zones alter their catalytic properties, and whether the changes in the amino acid sequence are within the AATs active site. It was also important to clarify whether the different activity levels of allozymes from one zone are caused by their different catalytic efficiency, or by a different level of AAT α and β subunits biosynthesis as it was assumed in the previous study [23].

For these purposes a five-step purification procedure was developed, which allowed separation, partial purification and reliable biochemical characteristics of six AAT allozymes from wheat seedlings: three of them from plastidial zone (AAT-2) and three from a cytosolic one (AAT-3). The mitochondrial zone (AAT-1) was not included in the studies due to a very low initial activity of this isozyme, which constituted less than 1% of total AAT activity in crude extracts (data not shown). For the comparative analysis of amino acid sequences, the partial sequences (about 90%) of cDNA encoding AAT isozymes were obtained.

2. Experimental Procedures

2.1 Plant material

Seeds of the common wheat (*Triticum aestivum* cv. Jasna, 2n=6x=42, AABBDD, Kobierzyce Seed Centre, Poland) were surface sterilized with 0.5% hypochlorite solution, washed with distilled water and placed in plastic containers with soil. Seedlings were grown in a growth chamber (12 h photoperiod; 22/18°C; 400 μE).

2.2 Aspartate aminotransferase allozymes purification

All steps except HPLC were carried out at 4°C. Green sections of the 14 days old seedlings (500 g) were homogenized in 1.5 L of the 100 mmol L^{-1} Tris-HCl buffer pH 7.5. The proteins in the homogenate were fractionated with ammonium sulfate (40–80% saturation). Collected protein precipitate was dissolved in approximately 80 mL of 10 mmol L^{-1} Tris-HCl buffer pH 7.5 (this buffer was used as a column buffer during all further chromatography steps) and after centrifugation (20,000x*g*; 30 min) it was applied to a Sephadex G-150 column (2.6 x 95 cm). The flow rate was 27 mL h^{-1}, and fractions of 9 mL were collected. The fractions exhibiting the highest enzyme activity were pooled and directly loaded onto a DEAE-cellulose column (2.6 x 10 cm) attached to a BioLogic system (Bio-Rad Laboratories; Richmond, CA, USA). The bound allozymes were eluted with a 240 mL linear gradient of KCl from 0 to 250 mmol L^{-1} at a flow rate of 1.5 mL min^{-1}, and the fractions of 4.5 mL were collected. The fractions containing plastidial (AAT-2) or cytosolic (AAT-3) allozymes were separately pooled and concentrated using Amicon 8010 (Millipore Corporation; Bedford, MA, USA) supplied with PM 30 membrane. Then, AAT-2 preparation was additionally applied to a *L*-ornithine-Sepharose 4B (*L*-ornithine linked to Epoxy-activated Sepharose 4B *via* its δ-amino group) column (0.8 x 8 cm) and eluted with a 240 mL linear gradient of KCl from 20 to 120 mmol L^{-1} at a flow rate of 1.5 mL min^{-1}, and the fractions of 4.5 mL were collected. Partially purified AAT-2 and AAT-3 preparations were separately applied to a Protein-Pak Q 8HR column (1 x 10 cm) attached to a HPLC system (Waters Corporation; Milford, MA, USA). The bound allozymes were eluted using a 240 mL linear gradient of KCl from 20 to 100 mmol L^{-1} (AAT-3) and from 120 to 200 mmol L^{-1} (AAT-2) at a flow rate of 1.5 mL min^{-1}, and fractions of 4.5 mL were collected. The fractions containing separated allozymes were dialyzed against 10 mmol L^{-1} Tris-HCl buffer pH 7.5, concentrated using Amicon (as described above) and enriched with sorbitol to a final concentration of 10% (w/v). The final preparations of the AAT allozymes were stored at -80°C.

2.3 Determination of aminotransferase activities and protein concentration

L-aspartate:2-oxoglutarate transamination activity was assayed according to Bergmayer and Bernt [25]. The reaction mixture consisted of 100 mmol L^{-1} Tris-HCl buffer pH 7.5, 200 mmol L^{-1} *L*-aspartate, 10 mmol L^{-1} 2-oxoglutarate, 0.12 mmol L^{-1} NADH and 0.4 U mL^{-1} malate dehydrogenase in a total volume of 1.2 mmol L^{-1}. *L*-glutamate:oxaloacetate transamination activity was determined according to Wilkie and Warren [19]. Here, the reaction mixture contained 100 mmol L^{-1} Tris-HCl buffer pH 7.5, 15 mmol L^{-1} *L*-glutamate, 1 mmol L^{-1} oxaloacetate, 3 mmol L^{-1} NH4Cl, 0.12 mmol L^{-1} NADH and 3 U ml^{-1} glutamate dehydrogenase. AAT activity was calculated from a decrease of NADH absorbance at 340 nm. Specific activity was expressed as 1 µmol of the oxoacid product formed per minute and per mg protein at 25°C. Protein was determined according to Bradford [26] with bovine serum albumin as a standard.

2.4 Native PAGE and AAT activity staining

Native PAGE was performed according to Laemmli [27] using 7.5% resolving and 4% stacking gel. Electrophoresis (30 mA per plate) was run in 50 mmol L^{-1} Tris-glycine buffer pH 9.1 for 180 min. The AAT activity bands were visualized according to Stejskal [28] by incubating gels in reaction mixture containing 100 mmol L^{-1} Tris-HCl buffer pH 7.5, 8 mmol L^{-1} *L*-cysteine sulfinic acid, 5 mmol L^{-1} 2-oxoglutarate, 0.1 mmol L^{-1} PLP, 0.5 mmol L^{-1} MTT and 0.16 mmol L^{-1} PMS.

2.5 Kinetic studies

Apparent Michaelis constant (K_m) values for *L*-aspartate, 2-oxoglutarate, *L*-glutamate and oxaloacetate were determined using the Lineweaver-Burk plot. In the forward reaction (*L*-aspartate:2-oxoglutarate) *L*-aspartate concentration varied from 2 to 20 mmol L^{-1} (at 10 mmol L^{-1} 2-oxoglutarate concentration) and 2-oxoglutarate concentration varied from 0.1 to 1.0 mmol L^{-1} (200 mmol L^{-1} *L*-aspartate). In the reverse reaction (*L*-glutamate:oxaloacetate) *L*-glutamate concentration varied from 1 to 20 mmol L^{-1} (1 mmol L^{-1} oxaloacetate) and oxaloacetate concentration varied from 0.01 to 0.10 mmol L^{-1} (20 mmol L^{-1} *L*-glutamate). Maximum velocity (V_{max}) values were calculated using the Michaelis-Menten equation. The turnover number (k_{cat}) and the k_{cat}/K_m ratio values were determined for the reaction with *L*-aspartate as a leading substrate.

2.6 Molecular weight determination

The molecular weight of AAT under native conditions was estimated on a Sephadex G-150 column (2.6 x 85 cm) equilibrated with 10 mmol L^{-1} Tris-HCl buffer pH 7.5 using the enzyme preparation obtained after ammonium sulfate fractionation. The flow rate was 9 mL h^{-1}, and fractions of 3 mL were collected. The column was calibrated with blue dextran (2 MDa), alcohol dehydrogenase from *Methylophilus methylotrophus* (150 kDa), bovine albumin dimer (134 kDa) and monomer (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa). The molecular weight of AAT under denaturing conditions was determined by SDS-PAGE according to Laemmli [27] using a 15% resolving gel. Protein bands were silver stained according to Blum *et al*. [29]. The gels were calibrated with Bio-Rad low molecular mass standards (14.4–94.7 kDa). The gels with stained protein bands were scanned and analyzed by a Gel Analyser computer software package implemented to ImageJ.

2.7 Identification of AATs

The silver stained protein bands corresponding to the AAT allozymes were excised from the SDS gel, digested using semi-trypsin and analyzed by liquid chromatography coupled to electrospray ionization Fourier transform ion cyclotron resonance mass spectrometer in the Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland. Obtained results were used to search the NCBI protein sequence database using the Mascot program.

2.8 Obtaining a partial sequence of the genes encoding AATs

Total RNA was isolated with TRIzol reagent according to the manufacturer's protocol (Invitrogen; Carlsbad, CA, USA) and was subsequently reverse transcribed with oligo$(dT)_{18}$ primers (RevertAid™ First Strand cDNA Synthesis Kit; Fermentas; Vilnius, Lithuania). The synthesized cDNA was used as a template in the PCR reactions. The PCR amplifications were performed using a PCR Master Mix (Fermentas) under the following conditions: 94°C for 2 min; 35 x (94°C for 1 min; 50°C for 0.5 min; 72°C for 1 min) and 72°C for 7 min. Primer sets were derived from wheat Expressed Sequence Tags (EST) database: 5`-GACGGTCGCGTACAACAAGG-3` (fwd) and 5`-GCATGTATGGCATCTGCAAG-3` (rev) for cytosolic AAT gene (EST database accession number: TC232219); 5`-GAAGCTCAACCTTGGTGTTG-3` (fwd) and 5`-TCGATGATGGCATCGGCAAG-3` (rev) for plastidial AAT gene (EST database accession number: TC251581). The resulting PCR products were cloned into vector (pGEM-T Easy Vector System II, Promega Corporation; Madison, WI, USA) and sequenced in the Laboratory of DNA Sequencing and Oligonucleotide Synthesis, Institute of Biochemistry and Biophysics, Polish Academy of Sciences.

3. Results

Six AAT allozymes were separated and highly purified in a five-step purification procedure consisting of ammonium sulfate fractionation, gel filtration and combination of low and high pressure anion exchange chromatography (Table 1). Ammonium sulfate fractionation between 40 and 80% saturation followed by gel filtration resulted in the removal of considerable amounts of contaminant proteins from a crude extract without substantial loss of AATs activity. The molecular weight of AATs estimated by gel filtration was 72.4±3.6 kDa (both AAT isozymes eluted from the column as a single peak of activity). The next stage of purification, anion exchange chromatography on DEAE-cellulose, allowed obtaining two separate AAT activity peaks corresponding to plastidial (AAT-2) and cytosolic (AAT-3) isozymes. AAT-2 preparation obtained after this step was additionally subjected to chromatography on resin composed of *L*-ornithine linked to epoxy-activated Sepharose 4B *via* its δ-amino group (*L*-ornithine-Sepharose 4B), synthesized in our laboratory. Finally, six allozymes from both AAT zones were independently separated on Protein-Pak Q 8HR column attached to a HPLC system (Figure 1). Applied purification procedure yielded at least 2.3 U of each allozyme from AAT-2 zone (purification factors ranged from 135.9 to 159.4-fold) and at least 0.4 U of each allozyme from AAT-3 zone (purification factors ranged from 106.7 to 146.0-fold).

The final AAT preparations were subsequently subjected to SDS-PAGE. Protein bands corresponding to subunits forming AAT allozymes from the same zone migrated the same distance in the gel (Figure 2). The molecular weights of these subunits were 45.3 kDa for plastidial and 43.7 kDa for cytosolic allozymes. To confirm their identity, silver stained protein bands corresponding to allozymes consisting of two identical subunits (AAT-2a, -2c, AAT-3a and -3c) were excised

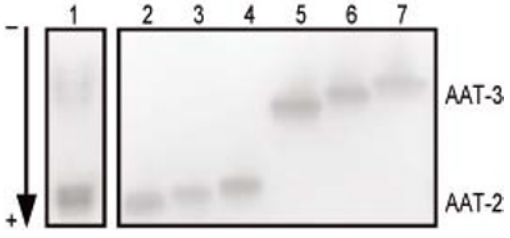

Figure 1. AAT activity zymograms of crude extract and final HPLC preparations of plastidial (AAT-2) and cytosolic (AAT-3) allozymes from wheat seedlings. Lanes: 1, crude extract; 2, AAT-2a; 3, AAT-2b; 4, AAT-2c; 5, AAT-3a; 6, AAT-3b; 7, AAT-3c. Approximately 50 mU of AAT activity was loaded in each lane.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1} protein)	Yield (%)	Purification (-fold)
Crude extract	1,529.90	2,258.75	0.68	100.0	1.0^{0}
Ammonium sulfate	701.50	710.00	0.99	45.9	1.5^{0}
Sephadex G-150	320.00	64.35	4.97	20.9	7.3^{0}
DEAE-cellulose					
AAT-2	150.26	10.93	13.75	11.3^{1}	23.3^{2}
AAT-3	5.10	2.40	2.13	2.6^{1}	23.7^{2}
L-ornithine-Sepharose					
AAT-2	110.45	5.40	20.45	8.3^{1}	34.7^{2}
Protein-Pak Q 8HR					
AAT-2a	2.26	0.13	17.39	0.8^{1}	144.9^{2}
AAT-2b	7.88	0.20	39.40	1.2^{1}	135.9^{2}
AAT-2c	2.87	0.10	28.70	0.7^{1}	159.4^{2}
AAT-3a	0.48	0.15	3.20	0.8^{1}	106.7^{2}
AAT-3b	0.72	0.15	4.80	0.7^{1}	120.0^{2}
AAT-3c	0.38	0.13	2.92	0.9^{1}	146.0^{2}

Table 1. Summary of AAT allozymes purification from wheat seedlings. [1] It was assumed that in a crude extract total AAT activity of each zone and of the individual allozymes was: 1,331.01 U (AAT-2 zone), 198.89 U (AAT-3), 268.86 U (AAT-2a allozyme), 658.85 U (AAT-2b), 403.30 U (AAT-2c), 60.26 U (AAT-3a), 98.45 U (AAT-3b), and 40.18 U (AAT-3c). [2] It was assumed that in a crude extract AAT specific activity of each zone and of the individual allozymes was: 0.59 U mg^{-1} protein (AAT-2 zone), 0.09 U mg^{-1} protein (AAT-3), 0.12 U mg^{-1} protein (AAT-2a allozyme), 0.29 U mg^{-1} protein (AAT-2b), 0.18 U mg^{-1} protein (AAT-2c), 0.03 U mg^{-1} protein (AAT-3a), 0.04 U mg^{-1} protein (AAT-3b), and 0.02 U mg^{-1} protein (AAT-3c). These assumptions are based on the relation between allozymes activity levels within zones observed on zymograms [23].

from the gel and analyzed using mass spectrometry. As a result 29 peptides (representing 41% of the total protein sequence) for AAT-2 allozymes and nine peptides (representing 32% of the total protein sequence) for AAT-3 allozymes were identified (Figure 3). Densitometric analysis of the gels revealed that the protein bands corresponding to AAT-2a, -2b, -2c, AAT-3a, -3b and -3c constituted respectively 15.9%, 31.4%, 26.5%, 53.6%, 55.7% and 50.6% of the total protein content in the final enzyme preparations.

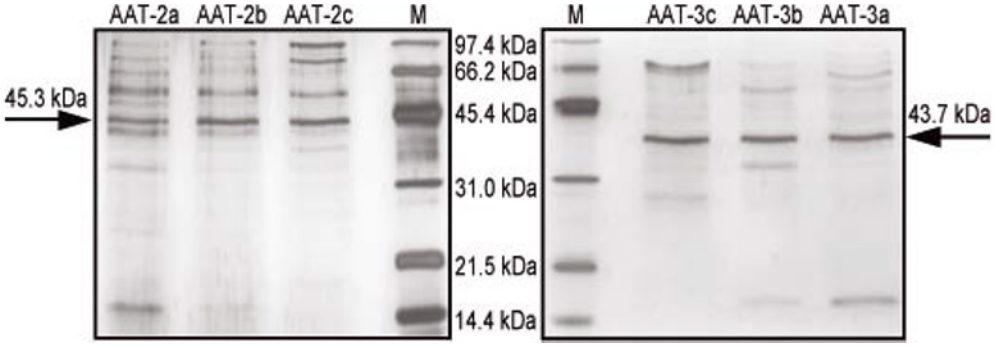

Figure 2. SDS-PAGE of purified AAT allozymes preparations from wheat seedlings. Approximately 2 μg of protein of each allozyme preparation was loaded in each lane; M, molecular mass standards.

A

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                10                  20                  30                  40                  50
MALAVDASRFEGVPMAPPDPILGVSEAFKADTSDLKLNLGVGAYRTEELQ

                60                  70                  80                  90                  100
PAVLNVVKKAEKLMLEKGENKEYLPIEGFAAFNKATADLLLGADNPVIKQ

                110                 120                 130                 140                 150
GRVATLQSLSGTGSLRLAAAFIQRYFPDAKVLISSPTWGNHKNIFNDARV

                160                 170                 180                 190                 200
PWSEYRYYDPKTVGLDFEGMIADIQAAPEGSFVLLHGCAHNPTGIDPTPE

                210                 220                 230                 240                 250
QWEKLADVIEEKKHMPFFDVAYQGFASGSLDEDASSVRLFVKRGLEVFVA

                260                 270                 280                 290                 300
QSYSKNLGLYAERIGAINVICSAPEVADRVKSQLKRLARPMYSNPPIHGA

                310                 320                 330                 340                 350
KIVANVVGDPTMFGEWKEEMEQMAGRIKNVRQKLYDSLTAKDQSGKDWSF

                360                 370                 380                 390                 400
ILSQIGMFSFTGLNRPQSDNMTDKWHIYMTKDGRISLAGLNLAKCEYLAD

                410
AIIDSFHNVN
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B

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                10                  20                  30                  40                  50
MASPSVFAGIAQGPEDPILGVTVAYNKDPSPVKVNLGVGAYRTEEGKPLV

                60                  70                  80                  90                  100
LNVVRRAEQMLIQNESRVKEYLPITGLADFNKLSAKLIFGADSPAIQENR

                110                 120                 130                 140                 150
VATVQCLSGTGSLRVGGEFLARHYHERTIYIPQPTWGNHPKVFTLAGLTA

                160                 170                 180                 190                 200
RSYRYYDPATRGLDFQGLLEDLSSAPSGAIVLLHACAHNPTGVDPTLEQW

                210                 220                 230                 240                 250
EQIRQLMRSKALLPFFDSAYQGFASGSLDKDAQSVRMFVADGGELLMAQS

                260                 270                 280                 290                 300
YAKNMGLYGERVGALSIVCGSADIAVKVESQLKLVIRPMYSNPPLHGASI

                310                 320                 330                 340                 350
VATILKDSAMFDEWTLELKAMADRIISMREQLFNALKIRETPGDWSHIIK

                360                 370                 380                 390                 400
QIGMFTFTGLNSDQVAFMRQEYHIYMTSDGRISMAGLSSRTVPHLADAIH

                407
AAVTKLK
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Figure 3. Plastidial (A) and cytosolic (B) AAT sequences coverage by peptides identified using mass spectrometry. The deduced amino acid sequences of plastidial (accession number: TC251581) and cytosolic (accession number: TC232219) AAT were derived from EST database for Chinese Spring wheat. Analysis of highly purified AAT-2a and AAT-2c allozymes (plastidial zone) yielded 29 peptides that matched with the TC251581 sequence and represented 41% of this sequence (the identified sequence regions are boxed). Analysis of highly purified AAT-3a and AAT-3c allozymes (cytosolic zone) yielded nine peptides that matched with the TC232219 sequence and represented 32% of this sequence (the identified sequence regions are boxed).

Highly purified allozymes preparations were used for the determination of the K_m values for the substrates of the forward and reverse transamination reactions. The estimated K_m values for these substrates appeared to be very similar for each of the six allozymes and were an average of 8.9±1.1 mmol L^{-1} for *L*-aspartate, 0.17±0.05 mmol L^{-1} for 2-oxoglutarate, 12.6±3.8 mmol L^{-1} for *L*-glutamate and 0.048±0.009 mmol L^{-1} for oxaloacetate (Table 2). The k_{cat} and k_{cat}/K_m values were also calculated for all studied AAT allozymes. In order to obtain the molar concentration of particular allozyme, the molecular weights of the allozymes estimated by SDS-PAGE (45.3 kDa for AAT-2 and 43.7 kDa for AAT-3) were taken into consideration, as well as the results of a densitometric analysis of the gel after SDS-PAGE. Values of k_{cat} and k_{cat}/K_m for allozymes from the same zone differed slightly, however, the values of these parameters for allozymes from the AAT-2 zone were almost 17-fold higher than those determined for AAT-3. It should also be noted that for all the studied allozymes the maximum velocities in the direction of *L*-aspartate utilization were similar to those in the reverse direction.

In order to determine the differences in the amino acid sequence of AAT-2 and AAT-3 active site regions, which might resulted in the observed discrepancy in allozymes catalytic efficiency, 1,107 bp and 1,140 bp fragments of the genes encoding plastidial and cytosolic AATs were amplified and sequenced. These sequences, which represented 91.4% (AAT-2) and 93.4% (AAT-3) of the total gene sequences, have been deposited in the GenBank under the accession numbers: EU346759 and EU885207, respectively. The similarity between deduced amino acid sequences of AAT-2 and AAT-3 reached approximately 60%. However, when only the sequence of the polypeptide chain fragments composing the active site and these in its neighborhood were taken into consideration, the similarity was much higher and reached about 80% (this data is discussed in Figure 4).

It is also worth mentioning that the obtained EU346759 and EU885207 sequences exhibited high similarity (about 98%) to the equivalent genes sequences (accession numbers: TC251581 and TC232219) from Chinese Spring wheat, which were available in the EST database.

4. Discussion

In this paper we present data concerning purification and some biochemical properties of six allozymes isolated from wheat leaves. Previously reported procedures for AAT purification from wheat (*Triticum aestivum*, 2n=6x=42, AABBDD) were not satisfactory. Verjee and Evered [30] used in their studies ammonium sulfate fractionation, treatments with hydroxyapatite and alumina Cγ gels, as well as chromatography and rechromatography on DEAE-cellulose. This procedure resulted in a 60-fold purified AAT preparation, which contained only one enzyme form. Orlacchio *et al.* [31], by applying ammonium sulfate fractionation, chromatographic separation on DEAE-cellulose and Sephadex G-200 columns, obtained 160-fold purified AAT preparation with two active enzyme forms. Most probably those were: plastidial (AAT-2) and cytosolic (AAT-3) zones, which exhibit the highest activity in wheat [23]. Despite the use of several different methods of proteins fractionation (acidification-alkalization, protamine sulfate and ammonium sulfate precipitation) in combination with ion exchange and hydroxyapatite chromatography, Kochkina [32] obtained only one AAT form characterized by a low level of purification (42-fold). The AAT purification procedure developed in our laboratory allowed to get six highly purified AATs: three allozymes from AAT-2 zone (purified approximately 150-fold) and three allozymes from AAT-3 zone (purified approximately 120-fold). To the best of our knowledge

Allozyme	K_m (mmol L^{-1})				$V_{max(Asp+2\text{-}OXO)}/V_{max(Glu+OAA)}$ (-fold)	$V_{max(Asp+2\text{-}OXO)}$ (U mg^{-1} protein)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
	Asp	2-OXO	Glu	OAA				
AAT-2a	8.3	0.17	12.0	0.052	0.8	18.85	89.6	10,795
AAT-2b	9.9	0.13	8.2	0.039	1.3	43.30	103.8	10,485
AAT-2c	8.5	0.13	16.0	0.057	0.9	31.10	88.5	10,412
AAT-3a	9.3	0.13	8.3	0.037	1.0	3.50	4.9	527
AAT-3b	10.0	0.25	17.0	0.057	1.3	5.28	7.0	700
AAT-3c	7.1	0.18	14.0	0.043	1.3	3.13	4.6	648

Table 2. Steady-state kinetic parameters of plastidial (AAT-2) and cytosolic (AAT-3) AAT allozymes from wheat seedlings. The results are average values from at least three separate experiments. Asp, *L*-aspartate; 2-OXO, 2-oxoglutarate; Glu, *L*-glutamate; OAA, oxaloacetate.

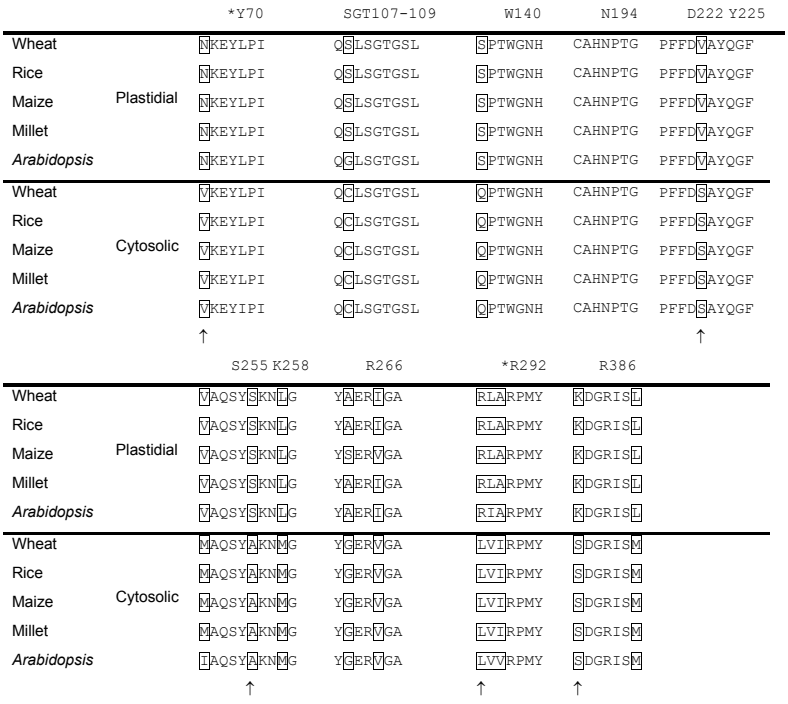

Figure 4. Comparison of the active site region of plastidial and cytosolic AAT sequences from different plant sources. Nine peptide fragments (71 amino acids residues in total) each containing at least one amino acid residue identified as the one taking part in binding PLP (*Tyr70, SerGlyThr107–109, Trp140, Asn194, Asp222, Tyr225, Ser255, Lys258, and Arg266) or dicarboxylic substrate (*Arg292 and Arg386), and six adjacent residues (three toward the N- and three toward the C-terminus) from wheat (EU885207/EU346759), rice (gi29468084/gi115440075), maize (gi195623894/gi194703362), millet (gi633095/gi20599) and *Arabidopsis* (gi15236129/gi15239772) were compared. Identified inconsistencies are boxed and these non-conservative were marked additionally by arrows. The amino acid numbering was as for mitochondrial AAT from chicken [11]. The amino acids designated with an asterisk are protruding from the second subunit.

this is the first report demonstrating separation of six wheat AAT allozymes from plastidial and cytosolic zones, and their individual biochemical characterization.

The molecular weights of allozymes subunits from AAT-2 and AAT-3 zones determined by SDS-PAGE were 45.3 kDa and 43.7 kDa, respectively. The molecular weight of AATs estimated by molecular sieving on a Sephadex G-150 column under non-denaturing conditions was 72.4±3.6 kDa. This value is very similar to that obtained by Verjee and Evered [30] on a Sephadex G-100 column for wheat AAT – 75 kDa. Taking into consideration that AAT can be catalytically active only as an association of two subunits [11], these results indicate that the shape of active form of wheat AAT molecule is far from globular.

Table 3 presents the comparison of the kinetic parameters (K_m, $V_{max(Asp+2\text{-}OXO)}/V_{max(Glu+OAA)}$, k_{cat} and k_{cat}/K_m) values determined in this study for plastidial and cytosolic AATs, with the values determined for the corresponding isozymes isolated from other cereals: millet, rice and oat. K_m values for 2-oxoglutarate, glutamate and oxaloacetate, for both AAT-2 and AAT-3 zones, are comparable with values obtained for relevant isozymes from other cereals. Only the K_m values for *L*-aspartate were about 3-fold higher for wheat AATs than for isozymes from millet, rice and oat. The studied wheat allozymes exhibited a ratio of maximal reaction rates for forward and backward reaction (V_{max} of the reaction directed towards the formation of *L*-glutamate to V_{max} of reverse reaction) from 0.8 to 1.3. Analogical values for AATs from millet ranged from 0.5 to 0.7 [34]. This indicates that AAT allozymes from wheat, contrary to its equivalents from millet, display similar catalytic efficiency in both directions when fully saturated. Furthermore, the comparison of the k_{cat} and the k_{cat}/K_m values for the forward and backwards reactions for all six studied allozymes leads to the same conclusions. The value of the k_{cat}, contrary to the V_{max} value, is independent of enzyme concentration, and the k_{cat}/K_m value indicates on the enzyme ability to act in live cells (under conditions of incomplete saturation with substrate). Up to date k_{cat} value has not been determined for AAT of plant origin. The only available k_{cat} values were determined for the native AATs isolated from sheep's liver – k_{cat}=322 s^{-1} [22], *Thermus thermophilus* – k_{cat}=120 s^{-1} [20] and *Escherichia coli* – k_{cat}=259 s^{-1} [16]. The k_{cat} values for the studied plastidial allozymes (88.5 to 103.8 s^{-1}) are only slightly lower than those depicted in scientific literature.

Source of the enzyme	K_m (mmol L^{-1})				$V_{max(Asp+2\text{-}OXO)}/V_{max(Glu+OAA)}$ (-fold)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	Reference
	Asp	2-OXO	Glu	OAA				
	Plastidial isozyme							
Wheat	7.1-10.0	0.13-0.25	8.3-17.0	0.037-0.057	0.8-1.3	88.5-103.8	10,412-10,795	Table 2
Millet	1.7-7.5	0.20-0.23	12.2-17.4	0.200-0.320	–	–	–	[33]
Millet	2.2-2.6	0.07-0.09	18.0-32.0	0.023-0.033	0.51-0.55	–	–	[34]
Rice	3.7	0.11	13.2	0.048	–	–	–	[35]
Oat	4.1	0.22	32.9	0.057	–	–	–	[36]
	Cytosolic isozyme							
Wheat	8.3-9.9	0.13-0.17	8.2-16.0	0.039-0.057	1.0-1.3	4.6-7.0	527-700	Table 2
Millet	2.3	0.11	13.0	0.049	–	–	–	[37]
Millet	2.6-3.2	0.14-0.20	13.0-17.0	0.065-0.080	0.55-0.65	–	–	[34]
Rice	2.4	0.11	5.0	0.043	–	–	–	[35]
Millet	3.3-5.0	0.12-0.17	–	–	–	–	–	[38]
Millet	3.3-5.0	0.40-0.50	–	–	–	–	–	[38]

Table 3. Kinetic parameters of plastidial and cytosolic AAT isozymes from wheat and three other cereals. Asp, *L*-aspartate; 2-OXO, 2-oxoglutarate; Glu, *L*-glutamate; OAA, oxaloacetate.

However, the k_{cat} values determined for cytosolic allozymes (4.6 to 7.0 s^{-1}) differ significantly. Moreover, the average value of k_{cat}/K_m for ATTs from AAT-2 zone is 17-fold higher than analogical value estimated for AATs from AAT-3 zone. Such prominent differences in catalytic efficiency (k_{cat} and k_{cat}/K_m values) between allozymes from different zones might have resulted from different amino acid sequences of the regions, which build the active site of the studied proteins. In order to verify this hypothesis a comparison of nine fragments (enclosing in total 71 amino acids residues) of plastidial and cytosolic AAT sequences from five different plant species, which contain at least one amino acid involved in PLP or substrate binding and six adjacent amino acids (three toward the N- and three toward the C-terminus) was carried out (Figure 4). Literature data [14-16] indicates that these six amino acid residues neighboring to those directly taking part in transamination reaction may also influence the course of the catalytic reaction. The comparison of the chosen nine sequence fragments revealed only three and four cases of inconsistency for plastidial and cytosolic isozyme, respectively. This outcome showed that the amino acid residues building the active site of the particular AAT isozyme represent a great level of conservation. The comparison of the discussed sequence fragments between plastidial and cytosolic isozymes from wheat revealed 14 cases of inconsistency per 71 amino acid residues (about 80% of similarity). By analyzing these differences an observation was made that most of them (nine substitutions per 14) are conservative – one amino acid is replaced by another with similar physicochemical properties (*e.g.* Ala264 and *Leu290 in AAT-2 are substituted respectively by Gly and *Val in AAT-3), which allow rather to exclude their effect on the kinetic properties of AAT isozymes. Among the other five substitutions, which are non-conservative (in Figure 4 pointed with arrows), two of them are within the polypeptide chain fragments engaged in PLP binding (*Asn-67-*Val and Val-223-Ser). Three remaining changes (Ser-257-Ala, *Arg-289-*Leu and Lys-383-Ser) took place in the regions directly associated with the transamination reaction, closely to highly conservative Lys258 residue which takes part in external aldimine formation with substrate, and two arginins (*Arg292 and Arg386) of which guanidine groups form hydrogen bonds with carboxylate groups of substrate (*Arg292 with a distal group and Arg386 with proximal group) and play a crucial role in ATT substrate binding [11]. Substitution of one of these two arginines, or both of them simultaneously, with amino acids having different physicochemical properties resulted in a decrease of k_{cat}/K_m values for at least three or six orders of magnitude, respectively [9,10,39-41]. The occurrence of Ser257, *Arg289 and Lys383 in AAT-2 instead of Ala, *Leu and Ser in AAT-3 should increase the positive electrostatic charge (*Arg289 and Lys383) and the potential to form the hydrogen bonds (Ser257, *Arg289) in the area of arginine (*Arg292 and Arg386) side chains of plastidial allozymes comparing with those from the cytosol. Unexpectedly, the presence of these amino

acid residues had no effect on the binding strength of the dicarboxylic substrates by AAT-2 because the K_m values for allozymes from both AAT zones were almost identical. The effect of Ser257, *Arg289 and Lys383 on higher catalytic efficiency of AAT-2 comparing to AAT-3 must therefore be related to the contribution of these three amino acid residues in better substrate positioning during the catalysis. The change in the k_{cat} value reflects the change in the slowest step of the reaction [42]. Experiments with α-deuteroamino acids have shown that during the transamination reaction the slowest step is the tautomerization, which is catalyzed by the acid/base group [11]. Thus, the 17-fold higher catalytic efficiency of plastidial isozyme comparing to cytosolic one can be interpreted as the effect of the participation in the catalysis, as acid/base catalyst, of ε-amino group of the Lys383 instead of the most commonly proposed Lys258 residue. The proximity of Lys383 and Arg386 (binds proximal carboxylate group of substrate) in the AAT-2 polypeptide chain indicates the possibility that ε-amino group of Lys383 is in the more advantageous position for the contribution in the proton transfer between Cα of the substrate and C4 of the PLP, comparing to the analogical group of the Lys258 that forms the internal aldimine with cofactor.

Presented considerations regarding the catalytic role of some amino acid residues are based on the comparison of the plastidial and cytosolic AAT sequences and their kinetic parameters values. This study indicates that further research should involve site-directed mutagenesis methods. This approach provides supporting evidence that amino acid residues like His143 [14], Cys191 [15], or *Lys68 and Glu265 [16] are very important for the course of AAT catalysis, despite the fact that they do not bind PLP or substrate molecules directly. Similarly to the Lys383, *Arg289 and Ser257 present in AAT-2 allozymes from wheat, these amino acid residues are located in the proximity of the amino acid residues that are responsible for the catalysis. In conclusion, we would like to point out that different subunit composition of allozymes (αα, αβ or ββ) from a single electrophoretic zone have no impact on their affinity for the substrates and the catalytic efficiency. These data confirm our previous hypothesis regarding different α and β subunits biosynthesis level, which results in the different activities of allozymes from one zone observed on zymograms [23]. On the other hand, differences in the active site sequences of the allozymes (especially three observed non-conservative substitutions: Ser-257-Ala, *Arg-289-*Leu and Lys-383-Ser) from plastidial and cytosolic zones are the most probable cause of their different catalytic efficiency.

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