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L-alanine:2-oxoglutarate aminotransferase isoenzymes from *Arabidopsis thaliana* leaves

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Abstract

The occurrence of four L-alanine:2-oxoglutarate aminotransferase (AOAT) isoenzymes (AOAT-like proteins): alanine aminotransferase 1 and 2 (AlaAT1 and AlaAT2, EC 2.6.1.2) and L-glutamate:glyoxylate aminotransferase 1 and 2 (GGAT1 and GGAT2, EC 2.6.1.4) was demonstrated in *Arabidopsis thaliana* leaves. These enzymes differed in their substrate specificity, susceptibility to pyridoxal phosphate inhibitors and behaviour during molecular sieving on Zorbax SE-250 column. A difference was observed in the electrostatic charge values at pH 9.1 between GGAT1 and GGAT2 as well as between AlaAT1 and AlaAT2, despite high levels of amino acid sequence identity (93 % and 85 %, respectively). The unprecedented evidence for the monomeric structure of both AlaAT1 and AlaAT2 is presented. The molecular mass of each enzyme estimated by molecular sieving on Sephadex G-150 and Zorbax SE-250 columns and SDS/PAGE was approximately 60 kDa. The kinetic parameters: K_m (Ala) = 1.53 mM, K_m (2-oxoglutarate) = 0.18 mM, k_{cat} = 124.6 s^{-1}, k_{cat}/K_m = 8.1 x 10^4 $M^{-1} \cdot s^{-1}$ of AlaAT1 were comparable to those determined for other AlaATs isolated from different sources. The two studied GGATs also consisted of a single subunit with molecular mass of 47.3-70 kDa. The estimated K_m values for L-glutamate (1.2 mM) and glyoxylate (0.42 mM) in the transamination catalyzed by putative GGAT1 contributed to indentification of the enzyme. Based on these results we concluded that each of four AOAT genes in *Arabidopsis thaliana* leaves expresses different AOAT isoenzyme, functioning in a native state as a monomer.

List of abbreviations: AlaAT, alanine aminotransferase; AOAT, L-alanine:2-oxoglutarate aminotransferase; GGAT, L-glutamate:glyoxylate aminotransferase; PLP, pyridoxal phosphate

Introduction

There are four genes that encode L-alanine:2-oxoglutarate aminotransferase (AOAT, EC 2.6.1.2; EC 2.6.1.4) isoenzymes in *Arabidopsis thaliana* genome. These enzymatic proteins, called AOAT(1-4)-like proteins by Igarashi *et al.* (2003), catalyze the transamination reaction between L-alanine and 2-oxoglutarate. Two of them, AOAT1 and AOAT2, can also catalyze the transfer of -amino group between L-glutamate or L-alanine and glyoxylate (Paszkowski and Niedzielska 1989, Orzechowski *et al.* 1999, Liepman and Olsen 2003). AOAT1 and AOAT2 have a targeting signal (PTS1) at the C-terminus that directs them into peroxisomal matrix and exhibit amino acid sequences corresponding to L-glutamate:glyoxylate aminotransferases (GGAT1 and GGAT2, EC

2.6.1.4; NCBI protein accession nos AAK25905 and AAL34156, respectively) (Igarashi *et al.* 2003). The other two isoenzymes, AOAT3 and AOAT4, have been identified as alanine aminotransferases (AlaAT2 and AlaAT1, EC 2.6.1.2; NCBI protein accession nos AAF82781 and AAF82782, respectively) (Igarashi *et al.* 2003). The distinction of two groups among AOAT-like proteins of plant origin may be based not only on the substrate specificity. GGAT1 and GGAT2 exhibit 93 %, and AlaAT1 and AlaAT2 85 % of amino acid identity, whereas the sequence identity between the two groups of aminotransferases from *A. thaliana* ranges about 44 %. They also differ in their subunit composition and molecular mass. GGAT aminotransferases have a monomeric structure and a molecular mass of about 50 kDa (Paszkowski and Niedzielska 1989, Orzechowski *et al.* 1999a), while AlaAT aminotransferases are dimers with a molecular mass of about 100 kDa (Noguchi and Hayashi 1981, Lain-Guelbezu 1991, Son *et al*. 1991,Good and Muench 1992, Orzechowski *et al.* 1999a, Orzechowski *et al.* 1999b). Moreover, GGAT proteins are localized to peroxisomes (Orzechowski *et al.* 1999c, Igarashi *et al.* 2003, Liepman and Olsen 2003), whereas AlaAT is usually found in the cytoplasm (Noguchi and Hayashi 1981, Penther 1991) or mitochondria (Chapman and Hatch 1981, Orzechowski *et al.* 1999c). The cellular localization pattern is associated with roles of these enzymes in different metabolic processes. GGAT aminotransferases along with L-serine:glyoxylate aminotransferase (E.C. 2.6.1.45) take part in photorespiratory pathway (Leegood *et al.*1995, Liepman and Olsen 2001, 2004, Truszkiewicz and Paszkowski 2004). Taler *et al.* (2004) have recently reported that two *Cucumis melo* peroxisomal photorespiratory glyoxylate aminotransferases confer resistance against an oomycete pathogen. In turn, AlaATs may participate in the general amino acid transformation reactions (Sechley *et al.* 1992) or have specialized functions such as transferring three-carbon units between mesophyll and bundle sheath cells in C_4 plants (Son *et al.*1991, Son and Sugiyama 1992); storage of N and C, that could be lost due to alcoholic fermentation (Good and Muench 1992, Muench *et al.*1998) or acting as the key enzymes during biosynthesis of amino acids utilized to produce the reserve proteins in seeds (Kikuchi *et al.* 1999). Transcripts of all four AOAT-like proteins genes have been identified in green leaves and flowers of *Arabidopsis thaliana* (Igarashi *et al.* 2003). Only one of two L-glutamate:glyoxylate aminotransferases (most probably GGAT1) was isolated from this plant leaves, purified to near homogeneity and studied concerning some of its physicochemical and kinetic properties (Liepman and Olsen 2003). To date, there has been no information at all on the three remaining homologs from *Arabidopsis thaliana.* The present study was undertaken to find out whether all four AOAT enzymes are expressed in *Arabidopsis* leaves. Here we describe a purification method and a preliminary characterization of these aminotransferases.

Materials and methods

Materials

Leaves of *Arabidopsis thaliana* L. ecotype *Landsberg erecta* originating from 31-day-old seed culture carried out on a solid medium were used for study. 2MS medium (Murashige and Skoog 1962) was applied. Seeds of *Arabidopsis thaliana* were sterilized in 96 % ethanol and 50 % sodium hypochlorite, and after washing out, they were suspended in 0.1 % agarose water solution. Such prepared seeding material was placed on Petri dishes (= 11 cm) half-filled with medium (0.5 ml of seeds per dish) and remained in growing chamber (12-hour photoperiod, 26/20 °C, 200 E). Plants were harvested at the stage of inflorescence shoot setting.

Purification of L-alanine:2-oxoglutarate aminotransferase isoenzymes from Arabidopsis thaliana leaves

All steps except HPLC were carried out at 4 °C. Finely cut *Arabidopsis thaliana* leaves (90 g) were homogenized in a type Ultra-Turrax T25 homogenizer from IKA-Labortechnik Staufen, Germany (4 times for 30 s at 20500 r.p.m.) in 450 ml of 50 mM K-phosphate buffer pH 7.5 containing 10 mM 2-mercaptoethanol, 50 M PLP, 100 M PMSF, 1 mM EDTA and 20 % sorbitol. The proteins in the homogenate were fractionated with ammonium

sulfate. The fraction precipitated between 25 - 65 % saturation was collected and dissolved in homogenate buffer. Then it was dialyzed against the same buffer. The dialysate was applied to Sephadex G-150 column (2.6 x 81cm) equilibrated with 50 mM K-phosphate buffer pH 7.5 containing 10 mM 2-mercaptoethanol, 50 M pyridoxal phosphate (PLP) and 5 % sorbitol. The column was washed with the same buffer at a rate 6 ml/h and fractions of 3.8 ml being collected. The fractions showing highest L-alanine:2-oxoglutarate activity were pooled and concentrated using Amicon 8010 supplied with PM 10 membrane. The concentrated enzyme preparation was dialysed against 5 mM K-phosphate buffer pH 7.5 containing 10 mM 2-mercaptoethanol, 50 M PLP and 5 % sorbitol and then applied to two combined, 1 ml EconoPac hydroxyapatite CHT II columns from Bio-Rad (Hercules, CA, USA) equilibrated with dialysis buffer and attached to the Biologic LP (Bio-Rad) chromatography system. The columns were washed with equilibrating buffer. The fractions of 2 ml at a rate 0.6 ml/min were collected. The enzymatically active proteins did not bind to the hydroxylapatite and the fractions containing them were pooled and concentrated (as described above). The obtained enzyme preparation was applied to Protein-Pak Q 8HR anion exchange column (1 x 10cm) attached to HPLC system from Waters (Milford, MA, USA) equilibrated with 50 mM buffer Tris/glycine buffer pH 9.1 containing 10 mM 2-mercaptoethanol, 50 M PLP and 5 % sorbitol. The enzymes were eluted from the column in 240 ml linear gradient of 0 - 0.5 M KCl in column buffer at flow rate 1.5 ml/min and 4 ml fractions being collected. To each fraction 60 l of 20 mM PLP was added and additionally to the fractions 40 - 47 from the second activity peak, 400 l of 100 mM EDTA which showed stabilizing effect on L-alanine:2-oxoglutarate activity within this peak. The final preparations obtained after HPLC were stored at -80 °C.

Determination of aminotransferase activities

L-alanine:2-oxoglutarate aminotransferase activity was determined at 25 °C in a continuous assay using NADH and lactate dehydrogenase (Horder and Rej 1983).

L-glutamate:glyoxylate activity was determined at 30 °C in a discontinuous assay according to Rowsell *et al.* (1972) with modifications (Paszkowski and Niedzielska 1989) using NADH and glutamate dehydrogenase.

Specific activity was expressed in mol of the oxoacid product formed per minute at 25 °C (L-alanine:2-oxoglutarate activity) (U) or at 30 °C (L-glutamate:glyoxylate activity) (U) and per 1 mg of protein. Protein was determined according to Bradford (1976) with bovine serum albumin as a standard.

Determination of molecular mass

This was determined on Sephadex G-150 (2.6 x 78 cm) column equilibrated with 50 mM K-phosphate buffer pH 7.5 containing 5 % sorbitol and 0.04 % sodium azide with the use of enzyme preparation obtained after third step of purification (Sephadex G-150). Fractions of 3.2 ml were collected at a rate 6.4 ml/hr. The column was calibrated with blue dextran 2000 (2 000 kDa), alcohol dehydrogenase from *Methylophilus methylotrophus* (150 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen (25 kDa).

Zorbax SE-250 (Dionex Co., Sunnyvale, CA, USA) column (9.4 x 250 mm) attached to the HPLC system (Waters, Milford, MA, USA) equilibrated with 20 mM K-phosphate buffer pH 7.0 containing 0.3 M KCl was also used. The final enzyme preparations I and II obtained after ion exchange HPLC have been applied and fractions of 0.18 ml collected at a rate 0.5 ml/min. The column was calibrated with blue dextran 2000 (2 000 kDa), alanine aminotransferase (100 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) , chymotrypsinogen (25 kDa) and ribonuclease (13.7 kDa).

SDS/PAGE

The gels were prepared and run according to Laemmli (1970). Protein bands were silver stained according to the method of Blum *et al.* (1987). The gels were calibrated with Bio-Rad low molecular mass standards (14.4 kDa-94.7 kDa).

Native PAGE and detection of enzymatic activity in gels

Polyacrylamide gel (7.5 %) was prepared in 50 mM Tris/glycine buffer, pH 9.1 containing 10 % glycerol. Electrophoresis was run in the same buffer but without glycerol. Gels were stained for the L-alanine:2-oxoglutarate aminotransferase activity according to Hatch and Mau (1972) with modifications described previously (Truszkiewicz and Paszkowski 2004) . They were first incubated for 30 min at 4 °C in 0.15 M K-phosphate buffer pH 7.5 containing lactate dehydrogenase (10 U/ml). The mixture was warmed to room temperature, supplemented with 35 mM L-alanine, 10 mM 2-oxoglutarate and 0.6 mM NADH and incubated for 30 min. Then the gels were washed with water and appearing bands (the sites of NADH oxidation) were photographed under UV light. To ascertain the aminotransferase nature of the band some gels were incubated with 10 mM aminooxyacetate or without one of two transamination substrates.

Identification of proteins in the final enzyme preparations

The proteins from enzyme preparations I and II obtained after ion exchange HPLC were digested by using trypsin and analyzed by liquid chromatography coupled to Q-TOF (Quadrupole Time-Of--Flight) mass spectrometer in the Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland. The results were used to search NCBI *Arabidopsis thaliana* protein sequence database using the MASCOT program.

Table 1. Purification of AOAT isoenzymes from *Arabidopsis thaliana* leaves.

Purification step	Total activity	Total protein	Specific activity	Yield	Purification
	(U)	(mg)	(U/mg protein)	(%)	(-fold)
Crude extract					
Ala:2-oxoglutarate[a]	134.4	118.23	1.1	100.0	1.0
Glu:glyoxylate[b]	150.9		1.3	100.0	1.0
Ammonium sulfate (25–65% sat. fraction)					
Ala:2-oxoglutarate	115.0	81.02	1.4	85.6	1.3
Glu:glyoxylate	114.1		1.4	75.6	1.1
Sephadex G-150					
Ala:2-oxoglutarate	73.4	14.99	4.9	54.6	4.3
Glu:glyoxylate	76.8		5.1	51.0	3.9
Hydroxyapatite					
Ala:2-oxoglutarate	67.9	5.60	12.1	50.5	10.7
Glu:glyoxylate	65.9		11.8	43.0	9.1
Protein-Pak Q 8HR					
Enzyme preparation I					
Ala:2-oxoglutarate	5.1	0.18	28.3	3.8	25.7
Glu:glyoxylate	2.6		14.4	1.7	11.1
Enzyme preparation II					
Ala:2-oxoglutarate	9.4	0.11	85.5	7.0	77.7
Glu:glyoxylate	0.3		2.7	0.2	2.1

[a] L-Alanine:2-oxoglutarate aminotransferase activity
[b] L-Glutamate:glyoxylate aminotransferase activity

Results

The identification of four L-alanine:2-oxoglutarate aminotransferase (AOAT)-like mRNA transcripts in *Arabidopsis thaliana* leaves by Igarashi *et al.* (2003) prompted us to investigate the putative occurrence of four AOAT isoenzymes in *A. thaliana* leaves by various purification and separation methods. The fractionation of the homogenate using ammonium sulfate during the purification procedure (Table 1) was inefficient to remove various plant pigments, which resulted in green color of the preparation and hindered further purification.

An attempt to adsorb the pigments on the insoluble polyvinylpolypyrrolidone added to the extraction buffer proved only partially successful (data not shown). During the molecular sieving on Sephadex G-150 gel these impurities were eluted from the column at the same volume as L-alanine:2-oxoglutarate and L-glutamate:glyoxylate aminotransferase activities (not shown). Thus, this chromatography also failed to separate the isoenzymes from the pigments. Moreover, the achieved enzyme preparation still contained large amounts of unrelated proteins of molecular masses from approximately 20 kDa up to over 100 kDa as shown by SDS – polyacrylamide gel electrophoretic analyses (SDS/PAGE) depicting the progress of the purification process (Figure 3). A zymogram for detection of L-alanine:2-oxoglutarate activity, following native PAGE, indicated the presence of only two enzymatically active proteins in the preparation collected from the Sephadex G-150 column (Figure 1). Possibly, more than one AOAT isoenzyme was hidden in each activity band.

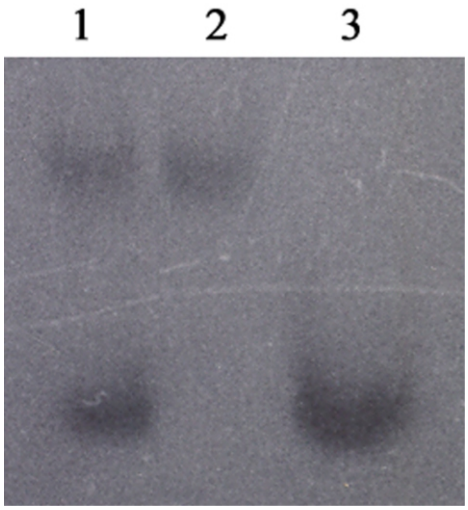

Fig. 1. Native PAGE at pH 9.1 of AOAT isoenzyme preparations. Lanes: 1, enzyme preparation obtained after Sephadex G-150 column (2 g of protein load); 2, final enzyme preparation I (0.3 g of protein load); 3, final enzyme preparation II (0.1 g of protein load). Gel was stained for L-alanine: 2-oxoglutarate activity. 8 mU of this activity was loaded per lane.

Subsequently, adsorption chromatography on the two combined 1 ml columns with hydroxyapatite CHT II was applied. High efficiency of this method (Table 1) enabled the elimination of large amounts of protein impurities (Figure 3) and the plant pigments. However, it did not reveal any individual AOAT-like proteins. The last step of the purification, ion-exchange chromatography on Protein Pak Q 8HR column attached to HPLC system, finally brought the expected results, as predicted by the native PAGE of the preparation after Sephadex G-150 column (Figure 1). Two peaks: I and II, further referred to as enzyme preparation I and enzyme preparation II respectively, were achieved, each exhibiting L-alanine:2-oxoglutarate and L-glutamate:glyoxylate aminotransferase activities (Figure 2).

The activities determined in the second HPLC peak had different sensitivities towards cyclic freezing and thawing. A significant decrease of the L-glutamate:glyoxylate activity accompanied by the stabile L-alanine:2-oxoglutarate activity (data not shown) was observed after a single thawing of

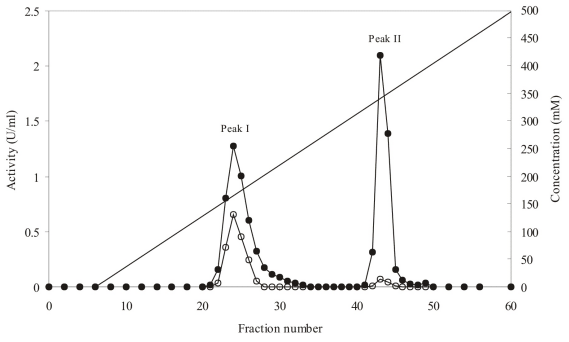

Fig. 2. Anion exchange HPLC on Protein-Pak Q 8HR column. Enzyme preparation obtained after hydroxyapatite column was applied. Aminotransferase activities: L-glutamate: glyoxylate (m); L-alanine:2-oxoglutarate (l); KCl (–).

Table 2. Effect of inhibitors reacting with carbonyl group of pyridoxal phosphate on aminotransferase activities of final enzyme preparations I and II. Enzyme preparations were preincubated with amino acid substrate and inhibitor or without (control) at 25 °C (L-alanine:2-oxoglutarate activity) or at 30 °C (L-glutamate:glyoxylate activity) for 10 min.

Enzyme preparation	Inhibitor	Inhibitor concentration (mM)	Inhibition of L-alanine:2-oxoglutarate activity (%)	Inhibition of L-glutamate:glyoxylate activity (%)
I	Aminooxyacetate	0,001	9	43
		0,01	47	89
	-Chloro-L-alanine	0,1	0	3
		1,0	0	62
II	Aminooxyacetate	0,001	4	16
		0,01	21	66
		0,1	94	100
	-Chloro-L-alanine	0,1	3	36
		1,0	43	84

the final enzyme preparation II originating from this peak. Two subsequent freeze-thaw cycles led to the complete exclusion of L-glutamate:glyoxylate activity, whereas L-alanine:2-oxoglutarate activity remained at about 95 % of the initial level (data not shown). The final purification factor for L-glutamate:glyoxylate activity in the enzyme preparation I, from the first HPLC peak was greater than 11-fold and for L-alanine:2-oxoglutarate activity in the enzyme preparation II, from the second peak, ranged over 77-fold (Table 1). A zymogram for detection of L-alanine:2-oxoglutarate activity developed after native PAGE of these two final preparations is presented on Figure 1. The homogeneity of the preparations was checked by SDS/PAGE (Figure 3).

As determined by silver staining, the separation of proteins from the enzyme preparation I under denaturing conditions yielded three main bands with the apparent molecular masses of 56 kDa, 49 kDa, 47.3 kDa, as well as at least three additional faint bands, whereas enzyme preparation II produced a clear, single band of 59.5 kDa and three faint, diffused bands, which were estimated by densitometric de-

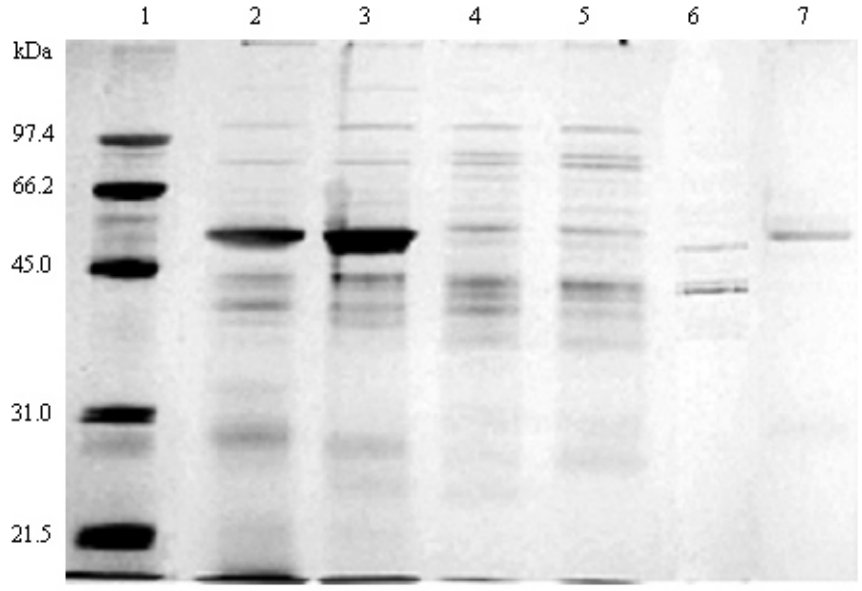

Fig. 3. Progress of AOAT isoenzymes purification as determined by SDS/PAGE. Lanes: 1, molecular mass markers; 2, crude extract; 3, ammonium sulfate fraction; 4, enzyme preparation after Sephadex G-150 column; 5, enzyme preparation after chromatography on hydroxyapatite column; 6, final enzyme preparation I; 7, final enzyme preparation II. 3 g of protein was loaded in each lane.

Table 3. LC/MS/MS analysis of final enzyme preparations I and II. 6 proteins identified by Mascot program in each preparation with highest score are shown.

Sample	Score	Queries matched	GI	Protein	Mass (Da)
Enzyme preparation I	573	15	47600741	cobalamin-indepependent methionine synthase	84604
	556	13	6006863	putative methionine synthase	84873
	422	10	9755746	formate dehydrogenase (FDH)	42668
	410	**11**	**13430566**	**putative alanine aminotransferase**	53776
	396	9	693690	aspartate aminotransferase	44497
	311	**8**	**17104535**	**putative alanine aminotransferase**	53980
Enzyme preparation II	**523**	**15**	**9082270**	**alanine aminotransferase**	60396
	505	13	6690395	phosphoglucose isomerase precursor	67506
	429	9	1256534	GF14 chi chain	29997
	336	8	487791	GF14omega isoform	29224
	319	8	166717	GF14 psi chain	28703
	295	9	21554058	14-3-3 protein GF14mu (grf9)	29616

termination (not shown) to constitute for 35 % of a total amount of protein (3 g) loaded (Figure 3). In a subsequent stage of the studies, a series of inhibition experiments (Table 2) were focused on determining whether a single isoenzyme or several enzymatic proteins were responsible for the aminotransferase activities (L-alanine:2-oxoglutarate and L-glutamate:glyoxylate) detected in each of the two analyzed preparations from ion exchange HPLC (Figure 2).

We used aminooxyacetate and -chloro-L-alanine, compounds that specifically react with the carbonyl group of pyridoxal phosphate (PLP), an aminotransferase cofactor (Morino and Tanase 1985), to inactivate the enzymes. The experimental results presented in Table 2 indicate that both activities in the first and the second peak were inhibited with apparently different efficiency by individual inhibitors.

The enzyme preparations I and II achieved after ion exchange HPLC were subjected to the trypsin digestion procedure. The resulting peptide mixture

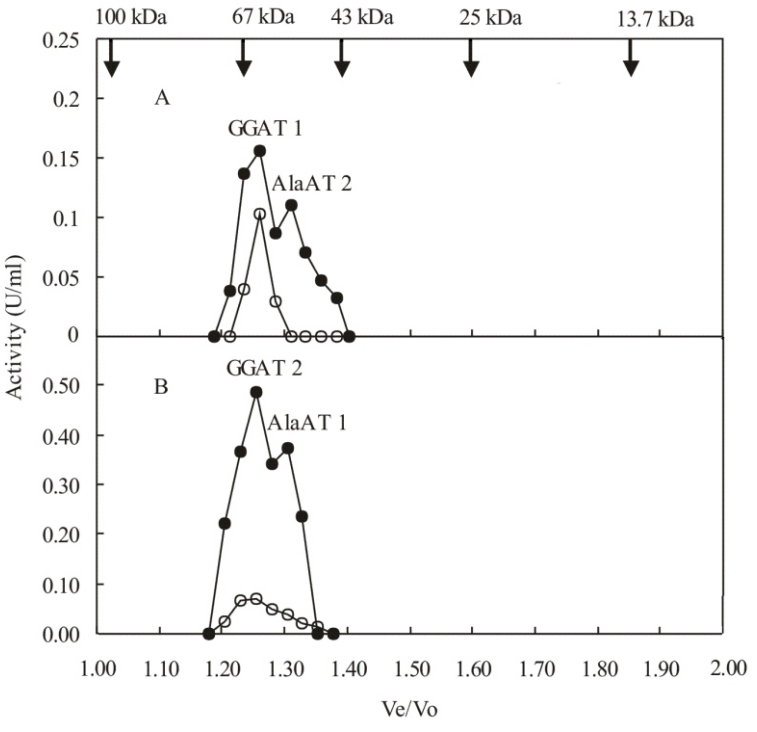

Fig. 4. Separation of AOAT isoenzymes by molecular sieving on Zorbax SE-250 column. Final enzyme preparations I (A) and II (B) were applied. Aminotransferase activities: L-glutamate:glyoxylate (m); L-alanine:2-oxoglutarate (l).

Table 4. Kinetic parameters of AlaAT1 and GGAT1 Apparent K_m values were estimated under conditions of a pseudo-first-order reaction. Lineweaver-Burk plots were plotted using triplicate data points and least-squares approach.

AOAT isoenzyme	Substrates	K_m (mM)	V_{max} (U/mg protein)	k_{cat} (1/s)	k_{cat}/K_m (1/M s)
GGAT1 (Enzyme preparation I)	L-Glutamate	1.20	15.5	–	–
	Glyoxylate	0.42			
AlaAT1 (Enzyme preparation II)	L-Alanine	1.53	81.5	124.6	81449.5
	2-Oxoglutarate	0.18			

was analyzed using LC/MS/MS. The comparison of the obtained results with the NCBI *Arabidopsis thaliana* protein sequence data base indicates that GGAT1 (AOAT1) was most possibly present in the enzyme preparation I, as well as AlaAT1 (AOAT 4) in the preparation II (Table 3). Three peptides specifically indentified GGAT1 presence in the sample of the enzyme preparation I (data not shown). Only one dodecapeptide residue could identify GGAT2 presence unambiguously, however, it was not possible to distinguish it from its analog in GGAT1 amino acid sequence in this analysis. Thus, only the presence of GGAT1 was confirmed.

The results of the Sephadex G-150 column chromatography step (both aminotransferase activities: L-alanine:2-oxoglutarate and L-glutamate:glyoxylate eluted at the same volume (not shown) suggested that all AOAT-like enzymes from *Arabidopsis thaliana* leaves retained the common molecular mass of about 70 kDa (data not shown). The molecular masses of the aminotransferases eluted from Q-type anion exchange HPLC column by a KCl gradient in two peaks, as assayed for L-alanine:2-oxoglutarate and L-glutamate:glyoxylate activities (Figure 2), were estimated by molecular sieving on Zorbax SE-250 column (Figure 4). During molecular filtration of the enzyme preparation I (from the first HPLC peak), as well as the enzyme preparation II (from the second HPLC peak) on this column, two separate activity peaks were obtained for each of the ion exchange HPLC peaks (Figure 4). When the enzyme preparation I was applied to the Zorbax column, the first peak exhibited both aminotransferase activities (L-alanine:2-oxoglutarate and L-glutamate:glyoxylate) and corresponded to a molecular mass of about 61.8 kDa (Figure 4A). The second peak exhibited only L-alanine:2-oxoglutarate activity with a molecular mass of about 54.7 kDa (Figure 4A). A similar situation developed during the analysis of the enzyme preparation II (Figure 4B). An initial peak contained both aminotransferase activities eluted at a position consistent with a molecular mass of about 62.9 kDa. The second peak at a molecular mass of about 55.3 kDa exhibited L-alanine:2-oxoglutarate activity only (Figure 4B).

As previously concluded from the results presented in Table 3, LC/MS/MS analysis confirmed the presence of AlaAT1 (AOAT4) within the enzyme preparation II (second HPLC peak). After the AlaAT1 homolog (probably GGAT2) in this preparation was inactivated by three cycles of freezing/thawing, the turnover number (k_{cat}) for AlaAT1 was calculated (Table 4). To obtain the molar concentration of this enzyme in the enzyme preparation II its molecular mass estimated by SDS/PAGE to be 59.5 kDa (Figure 3), as well as the results of the densitometric analysis of the gel after SDS/PAGE, showing about 35 % of unrelated protein contaminations, were taken into account. The V_{max} value was calculated using the Michaelis-Menten equation. The values of apparent constants K_m (further called K_m) were determined for both AlaAT substrates: L-alanine and 2-oxoglutarate (Table 4). The experiment was performed using 15 mM 2-oxoglutarate with four L-alanine concentrations (from 10 mM up to 500 mM) and 500 mM L-Ala with six 2-oxoglutarate concentrations (from 0.05 mM up to 15 mM). It should be added that at the Ala concentration of 2.5-5 mM, the slight inhibition by 15 mM 2-oxoglutarate was observed. The calculated k_{cat}/K_m value for AlaAT1 in the reaction of pyruvate formation was $8.1 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ (Table 4). Furthermore, in the enzyme

preparation I, which most possibly contained GGAT1 (Table 3 and Figure 4), K_m values for L-glutamate and glyoxylate were determined (Table 4). Four concentrations of L-Glu, from 0.5 mM up to 10 mM, with 5 mM glyoxylate and four concentrations of glyoxylate, from 0.5 mM up to 5 mM, with 15.4 mM L-Glu were used.

Discussion

Here we present the methods for identification and separation of the products of all four genes encoding L-alanine:2-oxoglutarate aminotransferase (AOAT) isoenzymes (AOAT-like proteins) in *Arabidopsis thaliana* leaves. The results of the experiments applying inhibitors that specifically react with pyridoxal phosphate indicated that at least two AOAT isoenzymes differing in substrate specificity occurred in each of the two activity peaks obtained from ion exchange HPLC. Molecular filtration on Zorbax SE-250 column yielded one L-glutamate:glyoxylate aminotransferase (GGAT; L-alanine:2-oxoglutarate and L-glutamate:glyoxylate activities) and one alanine aminotransferase (AlaAT; L-alanine:2-oxoglutarate activity) in each of the ion exchange HPLC peaks.

The results of the enzyme preparation I analysis using LC/MS/MS indicated the presence of GGAT1 in the first HPLC peak. Liepmann and Olsen (2003) purified L-glutamate:glyoxylate activity from *Arabidopsis thaliana* leaves by employing cation and anion exchange chromatographic techniques but observed only a single peak of this activity. Using MALDI-TOF they identified an enzyme within the peak fraction to be most probably GGAT1. The comparison of our studies showing the distribution of L-glutamate:glyoxylate activity between the fractions from the first (92 %) and the second (8 %) peak obtained after ion exchange HPLC, with the results achieved by these two authors strongly confirms the fact of GGAT1 (AOAT1) localization within the first peak (where the activity was obviously higher) and puts forward the putative GGAT2 (AOAT2) presence in the second one. Although mRNA levels do not often correlate with the protein levels due to the differences in the mRNA and protein turnover, it should be noted that Igarashi *et al.* (2003) established the relative quantity of each AOAT mRNA measured by real time RT PCR in green leaves of *Arabidopsis thaliana*. AOAT2 (GGAT2) mRNA represented not more then 2 % of AOAT1 (GGAT1) mRNA detected in this experiment. The spectrometric analysis, which identified AlaAT1 in the enzyme preparation II, clearly indicated that the second peak obtained by us after anion exchange HPLC contained AlaAT1.

To date, AlaAT of plant origin, *i.e*. enzyme that catalyzes the transfer of -amino groups between L-alanine and 2-oxoglutarate and is unable to utilize glyoxylate as an acceptor of this group (Liepman and Olsen 2004), was characterized as a dimer (Rech and Crouzet 1974, Lain-Guelbenzu *et al.* 1991, Good and Muench 1992, Orzechowski *et al.*1999a, Orzechowski *et al*. 1999b). Generally, the members of the aspartate aminotransferase family of pyridoxal phosphate dependent enzymes, including AlaAT (Grishin *et al.* 1995), are recognized to be catalytically active as dimers (Schneider *et al.* 2000). However, there are some reports about the plant aromatic aminotransferases that also belong to this family and consist of a single active subunit of 45-59 kDa (Koshiba *et al.* 1993, Simpson *et al.* 1997). Our report presents an unprecedented evidence that plant AlaAT, *i.e*. *A. thaliana* AlaAT1 eluted in the second HPLC peak, as well as putative AlaAT2 from the first HPLC peak, can exist in a native state as a monomer with a molecular mass of about 60 kDa. This assumption is based on the data acquired by molecular filtration under non-denaturing conditions on Sephadex G-150 gel column and Zorbax SE-250 column, in the comparison to the results obtained under denaturing conditions (SDS/PAGE). Two studied GGATs also exhibited the monomeric structure, which agrees with our previous findings about this aminotransferase from the rye (Paszkowski and Niedzielska 1989) and maize (Orzechowski *et al*. 1999a) leaves.

GGAT1 (481 amino acids), as well as hypothetical GGAT2 (481 amino acids) were eluted earlier from Zorbax SE-250 than putative AlaAT2 (540 amino acids) and AlaAT1 (543 amino acids), respectively. This observation may indicate the processing of the investigated isoenzymes. We think the effect of the higher axial ratio characterizing GGAT molecules should be also considered. A deviation of a protein

molecule standard behavior during gel filtration seems to increase with a growing axial ratio (Andrews 1965).

Interestingly, we observed differences in the electrostatic charge values at pH 9.1 between GGAT1 and GGAT2, as well as between AlaAT1 and AlaAT2, despite of a high level of amino acid sequence identity within each pair of isoenzymes (93 % and 85 %, respectively). It seems to comply with a well known rule that electrostatic charge of protein mainly depends on the amino acid residues present on the molecule surface (Sheehan and Fitz-Gerald 1996). However, some unspecific interactions with contaminating proteins can not be excluded.

When searching for additional nucleotide sequences encoding AOAT-like proteins, Liepman and Olsen (2003) identified three sequences in *Arabidobsis thaliana* homologous to the gene encoding aminotransferase L-alanine:glyoxylate 2 in rat (Lee *et al*. 1995). The cDNA corresponding to one of the sequences was cloned and expressed in *Escherichia coli* but it did not result in any gain of L-alanine:2-oxoglutarate aminotransferase activity (Liepman and Olsen 2003). In the present study, despite using five different separation methods taking into account various properties of proteins (solubility, size of a molecule, affinity for hydroxyapatite, electrostatic charge and electrophoretic mobility), we did not manage to identify more than four L-alanine:2-oxoglutarate aminotransferase isoenzymes (AOAT-like proteins). Earlier (Orzechowski *et al*. 1999a), we found six apparently distinct isoenzymes in maize seedlings based on differences in their electrophoretic mobility at pH 9.1. All appeared to be AOAT-like proteins, one of the isoenzymes exhibited high L-alanine:2-oxoglutarate activity but was unable to catalyze the transamination reaction between L-glutamate or L-alanine and glyoxylate.

In most of the studies concerning the subcellular localization of AOAT-like proteins in higher plants the peroxisomal isoenzymes were found to dominate in green tissues; additional isoenzymes were attributed to the cytosol and/or mitochondria (Biekmann and Feierabend 1982, Penther 1991, Other *et al.* 1992). However, Orzechowski *et al.* (1999c) proposed that most of the L-alanine:2-oxoglutarate activity in the maize leaf extract was brought about by mitochondrial AlaAT, while GGAT was responsible for the same activity (although substantially lower) in the peroxisomes. According to Liepman and Olsen (2003) L-glutamate:glyoxylate activity of the *Arabidopsis thaliana* leaf extract was localized exclusively to peroxisomes. The same authors (Liepman and Olsen 2004) reported later about a computer prediction study, which suggested that one of *Arabidopsis thaliana* AlaATs is a mitochondrial and the other – a cytosolic form. Among three AlaAT forms with different cellular localization found in leaves of *Panicum milaceum* the cytosolic isoenzyme was a predominant one (Son *et al.*1991). When AOAT1 (GGAT1) and AOAT4 (AlaAT1) cDNAs, each cloned into the green fluorescent protein (GFP) expression vector, were expressed in BY2 cells, the fluorescence of GFP-GGAT1 was detected in peroxisomes and that of GFP-AlaAT1 in cytoplasm (Igarashi *et al*. 2003). Thus, the previous literature and our results indicate that the great part of the L-alanine:2-oxoglutarate aminotransferase activity in *Arabidopsis thaliana* leaves comes from AlaAT1 localized in the cytoplasm. The mitochondrial AlaAT2 and peroxisomal GGAT1 and GGAT2 are responsible for the rest of this activity. This assumption is quite convincing referring to the fact of GGAT1/GGAT2 occurrence in plant peroxisomes, including *Arabidopsis thaliana* leaves, which was reported by several authors (Biekmann and Feierabend 1982, Ohnishi *et al.*1983, Liepman and Olsen 2003).

The kinetic parameters of the isoenzyme identified by us as AlaAT1 were comparable to those determined for other AlaATs from different sources (Son *et al.* 1991, Orzechowski *et al*. 1999b, Ward *et al*. 2000). Similarly to what was described for most of aminotransferases (Givan 1980), we observed that the K_m of AlaAT1 for the amino acceptor was lower than that of amino donor. The inhibition of the AlaAT catalyzed reaction by 2-oxoglutarate, which occurred during our kinetic experiments, is also a well known phenomenon (Horder and Rej 1983). Assuming a similar overall AlaAT1 efficiency (k_{cat}/K_m) in the reaction of pyruvate production

and that of 2-oxoglutarate formation, as it was reported for alanine aminotransferase from *Pyrococcus furiosus* (Ward *et al.*2000), one can suggest that the cytosolic isoenzyme from *Arabidopsis thaliana* plays pivotal roles in the biosynthesis of alanine and in its degradation, proposed earlier for other plant alanine aminotransferases (Son *et al.*1991, Sechley *et al.*1992, Kikuchi *et al*.1999). The range of the calculated k_{cat}/K_m value ($8.1 \cdot 10^4$ $M^{-1} \cdot s^{-1}$) suggests that the formation of pyruvate is proceeded *via* the Michaelis-Menten equilibrium mechanism (Price and Stevens 1999). The putative GGAT1, partially purified in the present study, had the K_m values for L-glutamate and glyoxylate very similar to those estimated by others for GGAT1 purified from *Arabidopsis thaliana* leaves, as well as for two nearly identical recombinants, GGAT1 and GGAT2 (Liepman and Olsen 2003). It adds to our confidence in these results. Noteworthy, the kinetic parameters we determined for two GGAT substrates matched well the concentration ranges for these compounds observed in the plant cells (Winter *et al.* 1994).

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