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QUARTERLY

Alanine aminotransferase and glycine aminotransferase from maize (*Zea mays* L.) leaves°

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Alanine aminotransferase (AlaAT, EC 2.6.1.2) and glycine aminotransferase (GlyAT, EC 2.6.1.4), two different enzymes catalyzing transamination reactions with L-alanine as the amino-acid substrate, were examined in maize in which alanine participates substantially in nitrogen transport. Preparative PAGE of a partially purified preparation of aminotransferases from maize leaves gave 6 fractions differing in electrophoretic mobility. The fastest migrating fraction I represents AlaAT specific for L-alanine as amino donor and 2-oxoglutarate as amino acceptor. The remaining fractions showed three aminotransferase activities: L-alanine-2-oxoglutarate, L-alanine-glyoxylate and L-glutamate-glyoxylate. By means of molecular sieving on Zorbax SE-250 two groups of enzymes were distinguished in the PAGE fractions: of about 100 kDa and 50 kDa. Molecular mass of 104 kDa was ascribed to AlaAT in fraction I, while the molecular mass of the three enzymatic activities in 3 fractions of the low electrophoretic mobility was about 50 kDa. The response of these fractions to: aminooxyacetate, 3-chloro-L-alanine and competing amino acids promted us to suggest that five out of the six preparative PAGE fractions represented GlyAT isoforms, differing from each other by the L-glutamate-glyoxylate:L-alanine-glyoxylate:L-alanine-2-oxoglutarate activity ratio.

Alanine aminotransferase (AlaAT, EC 2.6.1.2) is an enzyme widely distributed in plants [1–6]. Two to four isoforms have been detected in green parts of higher plants [4–11]. They were mainly localized in peroxisomes [4, 8, 9] although some authors found mitochondrial and cytosolic isoforms [7–10, 12]. Often in addition to the activity with L-alanine and 2-oxoglutarate and reverse activity with L-glutamate and pyruvate, these

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Abbreviations: AlaAT, alanine aminotransferase; GlyAT, glycine aminotransferase; PHMB, *p*-hydroxymercuribenzoate; PLP, pyridoxal-5′-phospahte; PMSF, phenylmethylsulphonyl fluoride.

isoforms showed the activities with L-glutamate and glyoxylate or L-alanine and glyoxylate which are characteristic for glycine aminotransferase (GlyAT, EC 2.6.1.4) [3, 7–9]. However, the isoforms derived from mammals, fungi, or non-green tissues of higher plants were unable to use glyoxylate as an amino group acceptor [2, 7, 13] or they catalyzed these transaminations but at a very low rate [1, 14].

According to most reports the AlaAT from higher plants is a homodimer about 100 kDa [1–3, 8]. The results obtained in our laboratory allowed to conclude that GlyAT from rye seedlings showing significant L-alanine-2-oxoglutarate activity is a monomer of about 60 kDa [15].

AlaAT plays a key role in L-alanine synthesis [4, 16] as well as in degradation of this amino acid in plant cells [5]. Isoforms of the enzyme from the leaves of some C_4 pathway plants take part in the intercellular carbon shuttle associated with C_4 photosynthesis [5]. It is assumed that peroxisomal isoform of AlaAT and serine-glyoxylate aminotransferase (EC 2.6.1.45) also located in the peroxisomes participate in conversion of glyoxylate to glycine in the photorespiratory pathway [4, 17].

For several years the aminotransferases involved in photorespiration of rye, a C_3 plant, were studied in our laboratory [15, 18–20]. At present we are interested in isoenzymatic composition, physicochemical and kinetic properties and metabolic role of the enzymes involved in alanine synthesis in C_4 plants, in which photorespiration is significantly reduced [21]. Maize seemed to be an appropriate source of the enzyme, the more so that, according to Valle & Heldt [22], more than 28% of the nitrate nitrogen assimilated by this plant is transported through the phloem in the form of L-alanine.

The present studies were performed on AlaAT, GlyAT and, for comparison, a peroxisomal serine-glyoxylate aminotransferase from maize leaves.

MATERIALS AND METHODS

Materials

Plant material. Leaves of 14-day-old maize seedlings (var. Duet) were used. Seeds were sown in the garden-mould. The seedlings grew in a growth chamber at 25°C (night) and 30°C (day) in a 14 h light regime. The intensity of photosynthetically active radiation was 100 μmol (photon) $\times$ $m^{-2} \times s^{-1}$.

Reagents. 3-Chloro-L-alanine, D-cycloserine, molecular mass marker kit (12–200 kDa), and *p*-hydroxymercuribenzoate (PHMB) were purchased from Sigma (St. Louis, MO, U.S.A.). Zorbax SE-250 column was from Dionex Corp. (Salt Lake City, UT, U.S.A.). Hydroxylapatite Type II, 20 μm was purchased from Bio-Rad Laboratories (Hercules, CA, U.S.A.). Glutamate dehydrogenase, lactate dehydrogenase, phenylmethylsulphonyl fluoride (PMSF) were purchased from Boehringer Ingelheim Bioproducts (Heidelberg, F.R.G). Other reagents were from commercial sources.

Methods

Separation of the enzymatic forms showing L-alanine-2-oxoglutarate activity. All steps of purification were carried out at 4°C. Finely cut maize leaves were homogenized in a type Ultra-Turrax T25 homogenizer (4 times for 0.5 min at 20500 r.p.m.) in 50 mM K-phosphate buffer, pH 7.5, containing 20 mM 2-mercaptoethanol, 0.05 mM PLP, 1 mM EDTA and 10% sorbitol (1:5, wt/v). The homogenate was filtered through miracloth and the filtrate proteins were fractionated with ammonium sulfate. The fraction precipitated between 30–80% saturation was collected and dissolved in 10 mM K-phosphate buffer containing 20 mM 2-mercaptoethanol, 0.1 mM EDTA, 0.1 mM PMSF and 0.05 mM PLP. Then it was exhaustively dialysed against the same buffer but without PMSF,

which was added afterwards to the dialysed preparation to a concentration of 0.1 mM; then the dialysate was centrifuged at 100000 × *g* for 60 min. The supernatant obtained was concentrated using Amicon supplied with a PM 30 membrane. The concentrated supernatant was applied on the hydroxylapatite column (2.6 cm × 10 cm) equilibrated with the dialysis buffer without PLP and EDTA but supplemented with 5% sorbitol. The column was washed with the same buffer at a rate of 1.5 ml × min^{-1} and fractions of 6 ml being collected. The fractions containing proteins which were not bound to hydroxylapatite and showed L-alanine-2-oxoglutarate activity were pooled, supplemented with EDTA (up to 10 mM), PLP, (up to 0.05 mM) and concentrated (as described above) to a volume of about 1 ml. The preparation obtained was dialysed against 10 mM Tris/glycine buffer, pH 9.1, containing 10 mM EDTA, 10 mM 2-mercaptoethanol, 0.05 mM PLP and, after the addition of sorbitol (up to 20% concentration), it was subjected to preparative electrophoresis on 7.5% polyacrylamide gel column (3.7 cm × 10 cm) in the Model 491 Prep Cell of Biorad (Bio-Rad Laboratories). The gel was prepared in 50 mM Tris/glycine buffer, pH 9.1, supplemented with 10 mM 2-mercaptoethanol and 0.05 mM PLP. Electrophoresis was run at 300 V for about 20 h. The elution chamber was washed with 50 mM Tris/glycine buffer, pH 9.1, containing 5% sorbitol, 10 mM 2-mercaptoethanol, 0.05 mM PLP and 1 mM EDTA at a rate of 1 ml × min^{-1}. Fractions of 6 ml were collected. Fractions containing different peaks of L-alanine-2-oxoglutarate activity were pooled separately and concentrated (as described above).

Polyacrylamide gel electrophoresis. 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, and with 20 mM 2-mercaptoethanol and 0.05 mM PLP added.

Gels were stained for the enzymatic activity according to Hatch & Mau [23]. They were first incubated for 30 min at 4°C in 0.2 M buffer K-phosphate buffer, pH 7.5, containing lactate dehydrogenase (250 nkat × ml^{-1}). The mixture was warmed to room temperature supplemented with 33 mM L-alanine, 10 mM 2-oxoglutarate, and 0.6 mM NADH, and incubated for 20 min. Then the gels were washed with water and the appearing bands (the sites of the NADH oxidation) were photographed under the UV light using the Polaroid DS-34 with the filter 15 (deep yellow). To ascertain the aminotransferase nature of the bands some gels were incubated with 10 mM aminooxyacetate or without one of the two transamination substrates: L-alanine or 2-oxoglutarate.

Determination of aminotransferase activities. Transaminations were performed at 30°C in the incubation mixture containing in a volume of 0.65 ml: 15.4 mM L-amino acid, 5 mM 2-oxoacid, 20 mM PLP, 77 mM K-phosphate buffer, pH 7.5 or 8.0 (L-serine-glyoxylate activity) and the enzymatic protein (1–150 μg). The enzyme was preincubated with the amino-acid substrate for 10 min. The reaction was started by addition of 2-oxoacid and stopped after 15–45 min by addition of 10% trichloroacetic acid. Specific activity was expressed in nmol of the product formed or substrate used per second (nkat) at 30°C and per 1 mg of protein.

Oxoacid products were determined by spectrophotometric methods using NADH and the appropriate dehydrogenase [15]. Protein was determined according to Bradford [24] with bovine serum albumin as a standard.

Determination of molecular mass. This was determined on Zorbax SE-250 column (9.4 mm × 250 mm) attached to the HPLC system (Waters, Milford, MA, U.S.A.) and equilibrated with 50 mM K-phosphate buffer, pH 7.0, containing 0.15 M KCl. Fractions of 0.2 ml were collected at a rate of 0.5 ml/min. The column was calibrated with Sigma stan-

dards: β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (67 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa).

The results presented are mean values from at least three separate experiments, the experimental error non exceeding 10%.

RESULTS

Separation of enzymatic forms showing L-alanine-2-oxoglutarate activity

The yield after two steps of purification (ammonium sulfate fractionation and chromatography on hydroxylapatite) was 35% and the preparation obtained (8.3 nkat/mg protein), was purified about 6-fold (Table 1). It was subjected to preparative electrophoresis on 7.5% polyacrylamide gel (preparative PAGE). Analytical polyacrylamide gel electrophoresis of the six fractions obtained after preparative PAGE demonstrated the presence of 6 enzymatic forms showing L-alanine-2-oxoglutarate activity (active bands sensitive to the inhibitory action of aminooxyacetate) (Fig. 1). The lower band in fraction II was due to contamination with fraction I. The form with the lowest isoelectric point in fraction I was responsible for about 90% of the total activity after preparative PAGE amounting to 266.6 nkat. All fractions were examined for their ability to catalyze additionally transamination between L-alanine and glyoxylate or L-glutamate and glyoxylate. Neither of these activities was detected in fraction I. The remaining five fractions differed in the L-alanine-glyoxylate:L-glutamate-glyoxylate:L-alanine-2-oxoglutarate activity ratio (Table 2).

Stability of L-alanine-2-oxoglutarate activity in the preparative PAGE fractions

About 80% of alanine-2-oxoglutarate activity in fractions I–III (Table 1), was lost on storage for two weeks in the preparative PAGE elution buffer (see Materials and Methods) at 4°C, but the addition of sorbitol to fraction I to a concentration of 40% and EDTA up to 10 mM allowed to maintain the activity at the initial level for two weeks. Fractions II and III, due to low stability of low L-alanine-2-

Table 1. Separation and purification of six enzymatic forms exhibiting L-alanine-2-oxoglutarate activity

Purification step	Protein (mg)	Activity		Yield (%)	Purification factor
		Total (nkat)	Specific nkat/mg protein		
Homogenate	1674.7	2177.1	1.3	100.0	1
Ammonium sulfate 30–80% sat.	362.8	1197.4	3.3	55.0	2.5
Hydroxylapatite	91.8	762.0	8.3	35.0	6.4
Preparative electrophoresis (fractions)					
I	10.2	236.8	23.2	10.9	17.8
II	9.7	2.9	0.3	0.1	0.2
III	3.6	2.9	0.8	0.1	0.6
IV	0.6	8.5	13.3	0.4	10.2
V	0.6	11.2	20.0	0.5	15.4
VI	0.4	4.3	11.7	0.2	9.0

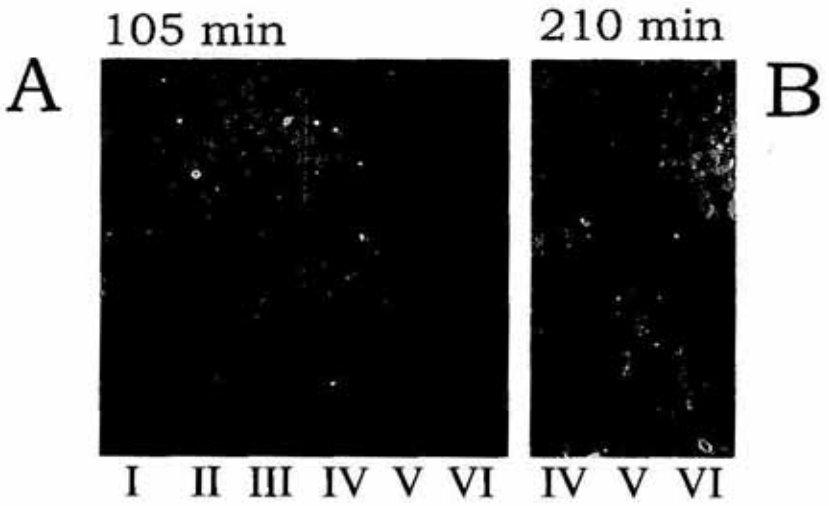

Figure 1. Polyacrylamide gel electrophoresis at pH 9.1 of preparative PAGE fractions

Electrophoresis was run for 105 min (A); or 210 min (B); A, lanes I–VI preparative PAGE fractions (0.07 nkat load); B, lanes IV–VI: preparative PAGE fractions (0.14 nkat load). Gels were stained for L-alanine-2-oxoglutarate acivity as described in Materials and Methods.

oxoglutarate activity, were not further analyzed. The alanine-2-oxoglutarate activity in fractions IV–VI was stable for over 2 weeks.

Properties of the preparative PAGE fractions

The effect of cycloserine, 3-chloro-L-alanine, *p*-hydroxymercuribenzoate (PHMB) and metal ions on the L-alanine-2-oxoglutarate activity in PAGE fractions I, IV, V and VI is presented in Tables 3 and 4. Cycloserine inhibited almost completely L-alanine-2-oxoglutarate activity in all four fractions. 3-Chloro-L-alanine was less effective and affected mainly AlaAT from fraction I. PHMB and Zn^{2+} ions inhibited AlaAT activity in all four fractions.

As can be seen from Table 2 fractions IV, V and VI showed three different aminotransferase activities. To identify the transaminating enzymes the effect of aminooxyacetate on the L-alanine-2-oxoglutarate and L-glutamate-glyoxylate activities was examined (Table 5). Aminooxyacetate strongly inhibited the two activities in fractions IV, V and VI (Table 5). The extent of inhibition of either activity in each of the three fractions at two different inhibitor concentrations was similar (Table 5). The inhibitory effects of L-alanine on L-glutamate-glyoxylate activity and of L-glutamate on L-alanine-glyoxylate activity are consistent with the supposition that the GlyAT isoforms exert AlaAT activity (Table 6).

Molecular sieving of the preparative PAGE fractions

Molecular sieving enabled to fractionate PAGE fractions into two groups differing in molecular mass: those of about 100 kDa and of about 50 kDa.

Table 2. Aminotransferase activities of six fractions obtained after preparative PAGE.

Total L-alanine-2-oxoglutarate activity after this purification step was 266.6 nkat/mg protein.

Activity ratio	Fraction					
	I	II	III	IV	V	VI
L-Alanine-glyoxylate: L-alanine-2-oxoglutarate	0	0.5	0.6	4.0	7.0	2.3
L-Glutamate-glyoxylate: L-alanine-2-oxoglutarate	0	0.8	0.8	1.8	2.1	1.3
Total L-alanine-2-oxoglutarate activity (%)	88.8	1.1	1.1	3.2	4.2	1.6

Table 3. Effect of inhibitors on L-alanine-2-oxoglutarate activity of preparative PAGE fractions.

The enzyme preparations described in Table 1 were preincubated with the inhibitor. The reaction was started by adding the mixture of two substrates. The activity is expressed as percentage of the activity determined without inhibitor taken as 100.

Fraction	Activity (%)		
	PHMB (0.1 mM)	Cycloserine (0.1 mM)	3-Chloro-L-alanine (0.1 mM)
I	28	2	22
IV	39	0	77
V	5	2	60
VI	13	0	65

Table 4. Effect of metal ions on L-alanine-2-oxoglutarate activity of fractions I, IV, V and VI obtained after preparative PAGE.

The enzyme preparations and the conditions of the assay were as described in Table 1 and legend to Table 3.

Fraction	Activity (%)			
	Metal ion concentration (mM)			
	Cd^{2+}		Zn^{2+}	
	0.1	1.0	0.1	1.0
I	0	ND	30	25
IV	51	30	60	49
V	100	15	100	75
VI	67	ND	58	ND

ND, not determined. Metal ions were applied in the form of sulfates.

Table 5. Effect of aminooxyacetate on L-alanine-2-oxoglutarate and L-glutamate-glyoxylate activities of fractions IV, V and VI obtained after preparative PAGE.

The enzyme preparations and the conditions of the assay were as described in Table 1 and legend to Table 3.

	Activity (%)	
Fraction	Concentration of aminooxyacetate (mM)	
Type of activity	0.01	0.1
IV		
L-Alanine-2-oxoglutarate	68	19
L-Glutamate-glyoxylate	73	27
V		
L-Alanine-2-oxoglutarate	53	15
L-Glutamate-glyoxylate	54	23
VI		
L-Alanine-2-oxoglutarate	66	27
L-Glutamate-glyoxylate	50	15

Table 6. Effect of L-alanine and L-glutamate on L-glutamate-glyoxylate and L-alanine-glyoxylate activities of fractions IV, V and VI obtained after preparative PAGE.

The enzyme preparations described in Table 1 were used. L-Alanine-glyoxylate and L-glutamate-glyoxylate activities are expressed as percentage of these activities without the competing amino acid, taken as 100. The concentration of the amino-acid substrates was 7.7 mM and of the competing amino acids 30.8 mM.

Fraction	Activity (%)	
	Competing amino acid	
Type of activity	Ala	Glu
IV		
L-Aalanine-glyoxylate	ND	62
L-Glutamate-glyoxylate	23	ND
V		
L-Alanine-glyoxylate	ND	43
L-Glutamate-glyoxylate	22	ND
VI		
L-Alanine-glyoxylate	ND	56
L-Glutamate-glyoxylate	11	ND

ND, not determined

As shown in Fig. 2, AlaAT in fraction I had a molecular mass of 104 kDa, and no other investigated aminotransferases were detected in this fraction (Fig. 2). Each of the remaining fractions (IV, V, VI) contained at least two aminotransferases. The first of 96 kDa showed two activities: L-serine-glyoxylate and L-alanine-glyoxylate (Fig. 2); the second of 50 kDa showed three activities: L-alanine-glyoxylate, L-glutamate-glyoxylate and L-alanine-2-oxoglutarate (Fig. 2).

DISCUSSION

Noguchi *et al.* [8, 9] thoroughly examined substrate specificity of glycine aminotransferase from spinach leaves and cucumber seedlings. They described this enzyme as identical with alanine aminotransferase. The results of these studies led some authors to the assumption that AlaAT derived from green parts of plants, besides transamination between L-alanine and 2-oxoglutarate, may also catalyze the transfer of amino group from L-alanine or L-glutamate to glyoxylate [4, 6, 10]. Consequently, AlaAT from green parts of plants was included into the group of "photorespiratory" enzymes. At the same time this explained the mainly peroxisomal localization of the enzyme [4, 7, 10]. Our results with the electrophoretic aminotransferase fractions separated from maize leaves imply that GlyAT and not AlaAT is the enzyme acting on alanine and both glyoxylate and 2-oxoglutarate as amino group acceptors. Glyoxylate did not serve as an amino group acceptor for the AlaAT form (fraction I) with the lowest isoelectric point, the form which is probably responsible for most of the L-alanine-2-oxoglutarate activity in maize leaves. Thus it seems that only this form may be, in fact, considered AlaAT (EC 2.6.1.2) and the remaining five are GlyAT isoforms (EC 2.6.1.4), two of which were not characterized in detail.

The same inhibitory effect of aminooxyacetate which proved to differentiate aminotransferases [25] and competition of alanine for glutamate-glyoxylate and *vice versa* of glutamate for alanine-glyoxylate transamination

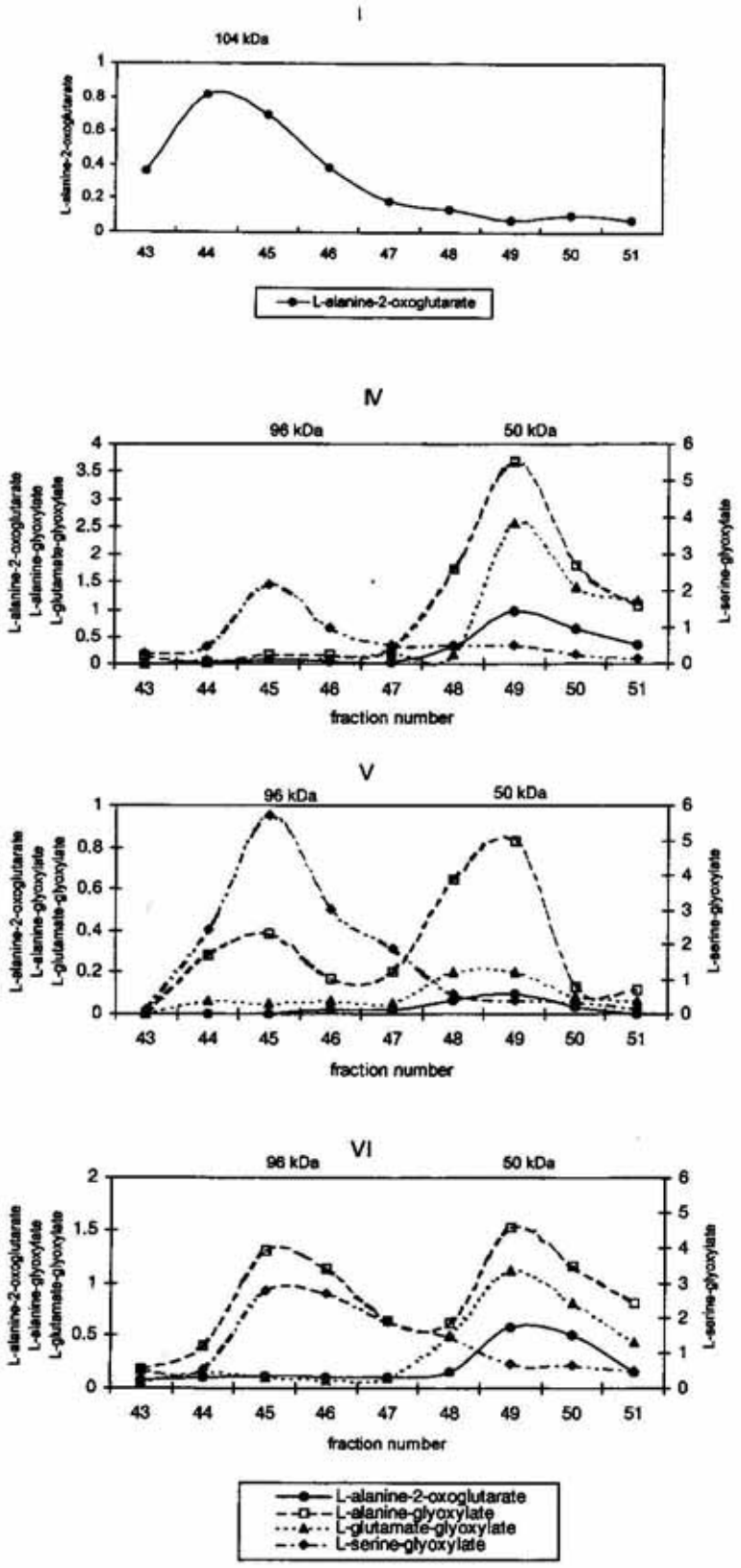

Figure 2. Molecular sieving of preparative PAGE fractions on Zorbax SE-250 column (9.4 mm × 250 mm).

The column was calibrated as described in Materials and Methods. The enzyme activities in the fractions I, IV, V, VI are expressed in nkat × ml^{-1}.

in electrophoretic fractions IV, V, VI speaks for the identity of the enzyme moiety.

All the three enzymatic forms showing three aminotransferase activities had the same molecular mass of 50 kDa. The molecular mass of AlaAT from fraction I was twice as high (104 kDa). Such a significant difference in molecular mass may imply the occurrence of two different enzymes. The molecular mass of GlyAT from rye seedlings determined earlier in our laboratory [15] was about 60 kDa which corresponds to that of the non-specific GlyAT isoforms in maize. The attempts made in our laboratory to dissociate AlaAT (fraction I) into subunits using KCl or guanidinium chloride and to change in this way the substrate specificity of the enzyme, were unsuccessful (not shown).

It can be concluded from *p*-hydroxymercuribenzoate inhibition experiments that the molecules of AlaAT and GlyAT isoforms contain reactive hydrosulfide groups essential for their enzymatic activity. SH-groups have been reported to be present in AlaAT and GlyAT derived from various sources [3, 14, 20]. Inhibition of the activities in the PAGE fractions by Cd^{2+} and Zn^{2+} ions is in agreement with the results obtained with the use of PHMB.

It should be noted that three GlyAT isoforms are accompanied in PAGE fractions IV, V and VI by three electrophoretic isoforms of serine-glyoxylate aminotransferase differing from each other in the activity ratio (L-serine-glyoxylate:L-alanine-glyoxylate). This aminotransferase was used as a reference enzyme in our studies. The common occurrence of GlyAT and serine-glyoxylate aminotransferases in peroxisomes from higher plant leaf cells and participation of both enzymes in glycine synthesis was reported by several authors [17, 26–28]. According to Heupel & Heldt [29] the structural arrangement of various enzymes within the peroxisomes allows for a controlled flow of intermediary products of photorespiration. The fact that serine-glyoxylate activity was not detected in preparative PAGE fraction I may indicate that AlaAT is not located in peroxisomes. According to Chapman & Hatch [12] most of the AlaAT activity in maize leaf cells was located in mitochondria.

Low specificity of some aminotransferases, with frequently overlapping activities and their occurrence in different isoforms makes difficult a univocal identification of individual enzymatic proteins. Polyclonal antibodies raised against particular pure homogenous enzymes may provide a clue to establish the relationships existing between the isoenzymatic forms.

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