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Biochemical characterization of partially purified gaba:pyruvate transaminase from *Nicotiana tabacum*

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Abstract

Pyruvate-dependent 4-aminobutyrate transaminase (EC 2.6.1.19) activity in crude extracts or lysed mitochondrial preparations from tobacco (*Nicotiana tabacum* L. cv Samsun N.N.) leaf was separated from 2-oxoglutarate-dependent GABA-T activity by FPLC anion exchange chromatography. Pyruvate-dependent GABA-T was partially purified 1530-fold by a combination of mitochondrial isolation and FPLC anion-exchange chromatography. This enzyme preparation had an apparent K_m of 1.2 ± 0.2 mM for GABA and 0.24 ± 0.05 mM for pyruvate. Two-oxoglutarate-dependent GABA-T activity was not detected in the partially purified preparation. Our data indicate the existence of a pyruvate-specific mitochondrial GABA-T.

Keywords: *Nicotiana tabacum*; Solanaceae; Tobacco; Metabolism; Purification; GABA shunt; 4-Aminobutyrate transaminase

1. Introduction

4-Aminobutyric acid (GABA) is a ubiquitous non-protein ω-amino acid in plants (Lawrence & Grant, 1963; Selman & Cooper, 1978; Secor & Schrader, 1984; Desmaison & Tixier, 1986; Bown & Shelp, 1997). Accumulation of GABA in response to various biotic and abiotic stress conditions is quite universal (Streeter & Thompson, 1972; Wallace, Secor & Schrader, 1984; Reggiani, Aurisano, Mattana & Bertani, 1988; Crawford, Bown, Breitkreuz & Buinel, 1994). However, the role of GABA in plants remains speculative (SatyaNarayan & Nair, 1990; Bown & Shelp, 1989).

GABA is produced in the cytosol (Breitkreuz & Shelp, 1995) via the irreversible decarboxylation of L-glutamate (Chung, Bown & Shelp, 1992; Tuin & Shelp, 1994) in a reaction catalysed by the calcium/calmodulin activated enzyme, glutamate decarboxylase (GAD) (Baum, Chen, Arazi, Takatsuji & Fromm, 1993; Ling, Snedden, Shelp & Assman, 1994; Arazi, Baum, Snedden, Shelp & Fromm, 1995; Snedden, Arazi, Fromm & Shelp, 1995; Snedden, Koutsia, Baum & Fromm, 1996). GABA can be transaminated in the mitochondrion by GABA-transaminase (GABA-T) to form succinic semialdehyde (SSA) which in turn, is converted to succinate via succinic semialdehyde dehydrogenase (Breitkreuz & Shelp, 1995). These reactions constitute the GABA shunt, and provide glutamate carbon to the Krebs cycle (Tuin & Shelp, 1994; 1996).

The fate of GABA nitrogen is dependent on the transamination reaction. In vitro measurements of GABA-T activity in crude plant extracts suggest that the enzyme can use either pyruvate (Reaction (1)) or 2-oxoglutarate (Reaction (2)) as an amino acceptor, thereby producing alanine or glutamate, respectively.

$$\text{GABA} + \text{pyruvate} \rightleftharpoons \text{SSA} + \text{alanine} \quad (1)$$

Abbreviations: AOA, aminooxyacetate; BSA, bovine serum albumin; DTT, dithiothreitol; GABA, 4-aminobutyric acid; GABA-T, 4-aminobutyric acid transaminase; GAD, glutamate decarboxylase; OPA, ortho-phthalaldehyde; PLP, pyridoxal-5-phosphate; PMSF, phenylmethylsulfonyl-fluoride; PV(P)P, polyvinyl (poly)pyrrolidone; SSA, succinic semialdehyde.

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$$\text{GABA} + \text{2-oxoglutarate} \rightleftharpoons \text{SSA} + \text{glutamate} \quad (2)$$

In developing tissues such as potato tubers (SatyaNarayan & Nair, 1986), soybean cotyledons (Shelp et al., 1995) and pea seedlings (Dixon & Fowden, 1961), the ratios of pyruvate- to 2-oxoglutarate-dependent GABA-T activities range from 1.2 to 5. However, in mature leaf tissues from soybean (Wallace et al., 1984) and radish (Streeter & Thompson, 1972) the ratios of pyruvate- to 2-oxoglutarate-dependent GABA-T are 10 and 19, respectively. Changes in this ratio, as determined by in vitro measurements on tissues at different developmental stages, suggest that two proteins are responsible for the multispecificity reported. In this study we tested the hypothesis that the multispecificity of plant GABA-T for pyruvate and 2-oxoglutarate was due to more than one protein.

2. Results

2.1. Stability and solubilization of pyruvate-dependent GABA-T

The addition of Triton X-100 detergent (0.05–0.5% v/v) enhanced the total recovery of pyruvate-dependent GABA-T activity from isolated mitochondria, as well as the partitioning of GABA-T activity into the soluble fraction; at 0.1% Triton X-100 these increases were 30 and 91%, respectively (data not shown). The addition of non-ionic (octylthioglucoside and TWEEN 20) and zwitterionic (CHAPS and CHAPSO) detergents, but not NaCl and TWEEN 80, increased the recovery of pyruvate-dependent GABA-T activity in the soluble fraction by 60–100% (data not shown). Consequently, CHAPS detergent was chosen for subsequent experiments because of its mild nature (Surfact-Pak Detergent Sampler Kit Manual, Pierce). For experimental purposes, 6 mM CHAPS was chosen as a concentration that is just below the critical micelle concentration of 8 mM and which provides optimum recovery of pyruvate-dependent GABA-T activity in the soluble fraction (data not shown).

As indicated, the final purification protocol for pyruvate-dependent GABA-T includes mitochondrial isolation and anion-exchange chromatography. Where appropriate, these methods employed the use of standard protective agents such as glycerol, DTT and protease inhibitors. Numerous attempts were made to further purify the enzyme using precipitation with NH_4SO_4 or polyethylene glycol 8000, column chromatography by gel filtration, hydroxyapatite, chromatofocusing and hydrophobic interaction, and preparative isoelectric focusing. However, these were unsuccessful due to the loss of enzyme activity. The addition of substrates, BSA or ethylene glycol had no impact on enzyme stability.

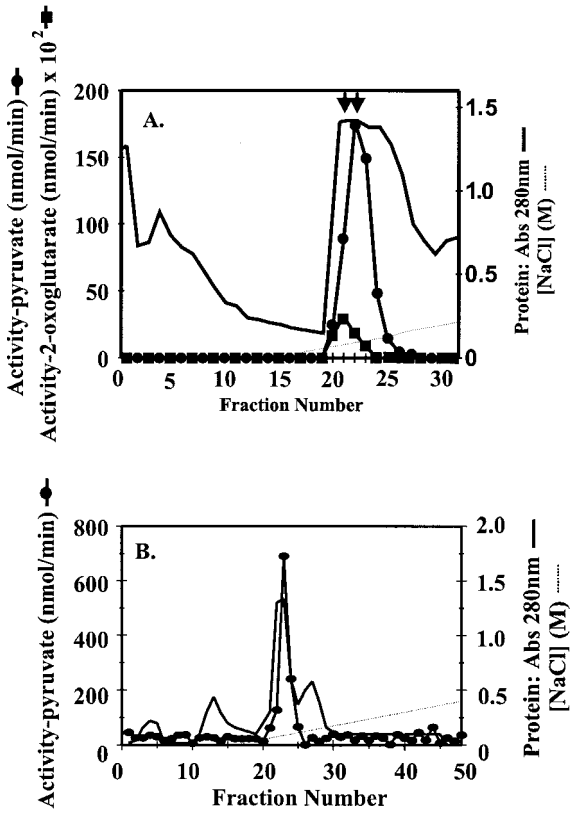

Fig. 1. Typical elution profiles for FPLC Fractogel DEAE anion exchange chromatography of tobacco leaf protein and 2-oxoglutarate and/or pyruvate-dependent GABA transaminase activities eluted with NaCl. (A) A 15-ml (100 mg protein) desalted sample of soluble mitochondrial protein derived from 500 *g* of tobacco leaf tissue was loaded onto the column and 2-ml fractions were collected and assayed for GABA-T activities via product analysis as described under Materials and Methods. The two vertical arrows represent the peak fractions for the separate activities. Note that the 2-oxoglutarate-dependent activity has been multiplied by 100 for presentation. Only activities for the first 31 fractions are shown. The experiment was repeated three times. (B) A 4-ml (16 mg protein) desalted sample representing the major activity peak from a preliminary anion exchange column was loaded onto a second Fractogel column and 2-ml fractions were collected and assayed for pyruvate-dependent GABA-T activity.

2.2. Separation of pyruvate- and 2-oxoglutarate-dependent GABA-T activities

The ratio of pyruvate- to 2-oxoglutarate-dependent GABA-T activity in crude extracts and post-mitochondrial preparations was approximately 25 and 31 times, respectively (data not shown). A single anion exchange chromatography step, performed on either crude (data not shown) or post-mitochondrial preparations, resulted in losses of about 35 and 95% of the loaded pyruvate- and 2-oxoglutarate-dependent activity, re-

Table 1
Partial purification of pyruvate-dependent GABA-T activity from 1 kg of tobacco leaf tissue (values represent the mean ± se of three experiments)

	Total activity (nkat)	Total protein (mg)	Specific activity (pkat mg^{-1})	Recovery (%)	Purification (fold)
Crude	80 ± 13	40,767 ± 1168	2 ± 0.4	100	1
Mitochondria	14 ± 19	213 ± 19	69 ± 12	18 ± 2	35 ± 3
Anion exchange	9 ± 2	16 ± 2	576 ± 104	11 ± 2	306 ± 68
Anion exchange	8 ± 1	3 ± 1	2842 ± 790	9 ± 2	1530 ± 530

spectively, giving a final ratio of 675 for pyruvate–2-oxoglutarate-dependent activity (Fig. 1A). However, this approach resulted in resolution of single pyruvate- and single 2-oxoglutarate-dependent GABA-T activities. Therefore, the inability to detect 2-oxoglutarate-dependent GABA-T activity in the highly purified preparation of pyruvate-dependent GABA-T (Fig. 1B) can be attributed to both stability and separation of the two activities (Fig. 1A).

2.3. Partial purification of tobacco pyruvate-dependent GABA-T

Although isolation of an organelle fraction enriched in mitochondria resulted in a loss of 82% of the initial activity, pyruvate-dependent GABA-T was purified 35-fold (Table 1). Further purification of GABA-T activity by two-step anion exchange chromatography gave a single peak which eluted at approximately 0.08 M NaCl (Fig. 1B). Only a further 39 and 19% of the activity was lost during the first and second anion-exchange steps, respectively (Table 1). The final pyruvate-dependent GABA-T activity from tobacco leaf tissue was purified 1530-fold with a 9% recovery of overall activity and a final specific activity of 2842 ± 790 pkat mg^{-1} protein (Table 1). This highly purified preparation did not exhibit any 2-oxoglutarate-dependent activity; consequently, it was used for biochemical characterization of pyruvate-dependent GABA-T.

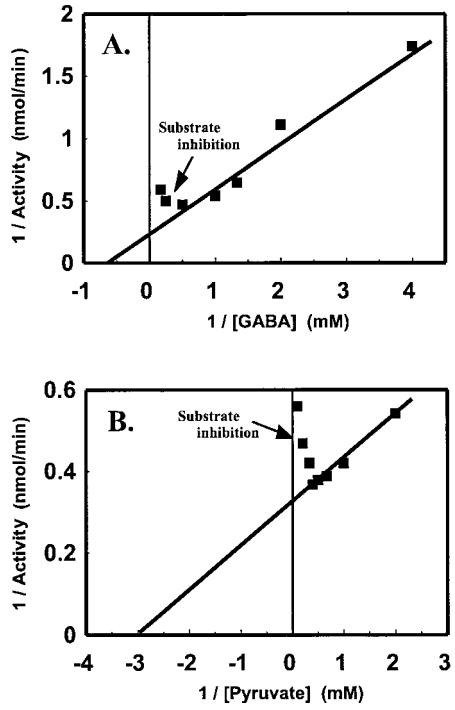

Fig. 2. Pyruvate-dependent GABA-T activity from tobacco leaf tissue as a function of (A) GABA (with 2 mM pyruvate) and (B) pyruvate (with 2 mM GABA) concentration. The assay conditions and HPLC evaluation for pyruvate-dependent GABA-T activity determination are described in Section 4.5. Values represent the mean of three replicate assays. Lines were estimated by linear regression of the rates obtained with 0.25–2 mM GABA (A) and 0.5–2.5 mM pyruvate (B).

2.4. Michaelis constant

Double reciprocal plots of pyruvate-dependent GABA-T activity versus GABA or pyruvate indicate the existence of substrate inhibition above 2 mM for GABA and above 2.5 mM for pyruvate (Fig. 2); therefore, these data points were not included in K_m estimations. The mean (±se of three independent preparations) apparent K_m values for GABA (Fig. 2A) and pyruvate (Fig. 2B) determined using non-linear re-

Table 2
Inhibition of tobacco leaf pyruvate-dependent GABA-T activity by various compounds[a]

Compound	Control %
No inhibitor	100
Beta alanine	70
Proline	98
Ornithine	60
Succinic semialdehyde	33
Glutamine	89
Succinate	93
Gabaculine	20
Aminooxyacetic acid	20

[a] The impact of all compounds was assessed at 2 mM concentration in the presence of sub-saturating pyruvate (0.3 mM) and GABA (1.0 mM). Each value is the mean of two assays.

gression techniques were 1.2 ± 0.2 and 0.24 ± 0.05 mM, respectively.

2.5. Inhibitors

The influence of various compounds, such as alternative amino donors, inhibitors and products (2 mM concentration) on pyruvate-dependent GABA-T activity in the post-anion-exchange fraction, was assessed using subsaturating GABA and pyruvate levels (Table 2). Both AOA and gabaculine (5-amino-1,3-cyclohexadienylcarboxylate) decreased pyruvate-dependent GABA-T activity by 80% (Table 2). Beta-alanine and ornithine, two other ω-amino acids, moderately inhibited (30–40%) enzyme activity, whereas the immediate product of the pyruvate-dependent GABA-T reaction, SSA, strongly inhibited (67%) its activity.

3. Discussion

Pyruvate-dependent GABA-T is apparently specific to the mitochondria of plants (Breitkreuz & Shelp, 1995), as is 2-oxoglutarate-dependent GABA-T of animals (Kim & Churchich, 1989). Consequently, the pyruvate-dependent GABA-T was partially-purified from tobacco mitochondria (Table 1). The majority of the initial loss of pyruvate-dependent GABA-T activity that occurred with mitochondrial isolation can be attributed to mitochondrial breakage and not enzyme stability, as only minimal loss occurred thereafter (Table 1). The presence of CHAPS detergent during purification improved both stability and recovery of pyruvate-dependent GABA-T suggesting that this protein is association with the mitochondrial membrane. Schein (1990) suggests that proteins isolated from membranes require replacement of the lipid environment with detergent to allow proper protein folding and maintenance of activity.

Tobacco pyruvate-dependent GABA-T had an apparent K_m for GABA of 1.2 ± 0.2 mM (Fig. 2A), a value which is of the same order of magnitude as the K_m for this substrate in GABA transaminases from other organisms (Schousboe, Wu & Roberts, 1973; Yonaha & Toyama, 1980; Der Garabedian, Lotti & Vermeersch, 1986; Jeffery, Rutherford, Weitzman & Lunt, 1988). The enzyme was unable to catalyse a reaction with 2-oxoglutarate as the amino acceptor. With pyruvate as the amino acceptor, an apparent K_m of 0.24 ± 0.05 mM was determined (Fig. 2B). In most plant transaminases, the K_m for the oxo-acid substrate is typically lower than that for the more abundant amino acid substrate (Ireland & Joy, 1985). In organisms other than higher plants, such as mouse (Schousboe et al., 1973), *Rhizobium trifolii* (Freney & Gibson, 1975) and *Saccharomyces cereviseae* (Ramon, El Guezzar, Grnason & Wiame, 1985), GABA-T is apparently specific for 2-oxoglutarate as the amino acceptor. In mammals, the K_m values for 2-oxoglutarate range from 80 to 250 μM (Schousboe et al., 1973; Jeffery et al., 1988; Scott & Jakoby, 1959; Park, Osei & Churchich, 1993). Interestingly, *Pseudomonas* sp. F-126 possesses pyruvate-dependent activity that is only 7% of the GABA-T activity with 2-oxoglutarate (Yonaha & Toyama, 1980). It can therefore be concluded that plant pyruvate-dependent GABA-T, unlike GABA transaminases from other organisms, has a higher affinity for pyruvate than for 2-oxoglutarate. Furthermore, it is subject to substrate inhibition (Fig. 2A and B), a phenomenon noted for a number of aminotransferases (Jenkins & Fonda, 1985), including GABA-T from *Pseudomonas* sp. F-126 (Yonaha & Toyama, 1980).

Our data suggest that the tobacco pyruvate-dependent GABA-T prefers GABA as an amino donor over both β-alanine and ornithine when pyruvate is the amino acceptor (Table 2). John and Fowler (1985) observed that β-alanine is a good substrate for 2-oxoglutarate-dependent GABA-T from mammals, but the molecule binds irreversibly, resulting in strong inactivation of the enzyme. β-alanine cannot substitute for GABA with bacterial or yeast GABA-transaminases when 2-oxoglutarate is the amino acceptor (Yonaha & Toyama, 1980; Yonaha, Suziki, Minei & Toyama, 1983). However, these studies did not investigate pyruvate as an amino acceptor. The fungal and bacterial ω-amino acid–pyruvate transaminases use both GABA and β-alanine as amino donors, but are inactive with 2-oxoglutarate as the amino acceptor (Yonaha et al., 1983). Since our pyruvate-dependent GABA-T was only partially purified and contained at least one other transaminase (alanine transaminase, data not shown), the present study did not attempt to determine the specificity of pyruvate-dependent GABA-T for all known amino acids. Recent research has demonstrated the existence of multiple isozymes of aspartate- and alanine-transaminases (Turano, Wilson & Mathews, 1990; Good & Muench, 1992). When the most abundant isozyme for each was purified to homogeneity and characterized, both were reported to be substrate specific. Thus, we intend to eventually demonstrate substrate specificity using purified-recombinant plant GABA-T. Strong inhibition of tobacco pyruvate-dependent GABA-T activity by AOA and gabaculine (Table 2), compounds that interfere with the binding of the coenzyme PLP (Kobayashi, Miyazawa & Endo, 1977; John, Charteris & Fowler, 1978; Yamaya & Matsumoto, 1985; Soper & Manning, 1985), indicates that tobacco GABA-T, like other GABA transaminases (Yonaha et al., 1983; Kwon, Park & Churchich,

1992; Kim, Song & Churchich, 1997), is PLP-dependent.

This study provides the first evidence from plants for separate pyruvate- and 2-oxoglutarate-dependent GABA-T activities (Fig. 1A). Other research reported the existence of two distinct isoforms of GAD (GAD1 and GAD2) in *Arabidopsis* (Turano & Fang, 1998; Zik, Arazi, Snedden & Fromm, 1998); *GAD1* is expressed in roots only, whereas *GAD2* is constitutively expressed in all tissues examined. Furthermore, GAD activity in leaves is altered by different nitrogen treatments, suggesting that GAD2 may play a unique role in nitrogen metabolism (Turano & Fang, 1998). Scott-Taggart (1997) proposed that carbon flux via 2-oxoglutarate-dependent GABA-T is enhanced by nitrogen limitation, and suggested that this pathway plays a role in maintaining the plant's carbon/nitrogen balance. With pyruvate-dependent GABA-T activity, the alanine produced may serve as an amino donor for other transamination reactions such as that involved in the glyoxylate to glycine conversion in photorespiration (Kleczkowski & Givan, 1988). The existence of multiple forms of both GAD and GABA-T suggest that there are at least two pathways for GABA metabolism in plants.

4. Experimental

4.1. Plant materials

Tobacco plants (*Nicotiana tabacum* [L.] cv. Samsun N.N.) were grown individually in 9-l pots containing Pro-mix BX. Natural light was supplemented with high-pressure sodium vapour lamps to provide a 16 h photoperiod and a photosynthetic photon flux density of 60 μmol m^{-2} s^{-1} at pot level. The average day/night temperature was 25/18°. The plants were watered bi-weekly with liquid fertilizer containing N, P and K (20:20:20) and with deionized water as required.

4.2. Solubilization of GABA-T

Preliminary experiments investigated the influence of 0.05–0.5% (v/v) Triton X-100 detergent on the partitioning of pyruvate-dependent GABA-T activity in soluble and membrane fractions of osmotically broken leaf mitochondria. The influence of several other detergents and compounds that are known to release membrane bound proteins was also tested by monitoring the recovery of pyruvate-dependent GABA-T activity in the soluble fraction of osmotically broken mitochondria. The compounds included 1 M urea, 0.5 M NaCl, 1% TWEEN 20, 1% TWEEN 80, 1% octylthioglucoside, 5 mM CHAPSO and 5 mM CHAPS. Mitochondria were isolated from 25 *g* of leaf tissue.

4.3. Subcellular fractionation of tobacco leaves

Unless indicated, all procedures were performed at 4°. One kg of tobacco leaf tissue was harvested from 8- to 10-week-old plants, deveined, sliced into small pieces (4 cm^2), and homogenized for 20–40 s (20,000 rpm) in 5 volumes of 50 mM Tris-HCl buffer (pH 8.2) containing 3 mM DTT, 1.25 mM EDTA, 2.7% (w/v) PVP, 2.5 mM $MgCl_2$, 350 mM mannitol, 30 mM ascorbate, 0.2% (w/v) BSA, 1 mM PMSF, 2.5 μg ml^{-1} leupeptin and 2.5 μg ml^{-1} pepstatin A (buffer A), using an Ultra-Turrax T25 homogenizer. For examination of total leaf GABA-T activity mannitol and BSA were omitted and 6 mM CHAPS added to the buffer (buffer B). Homogenate from either buffer system was filtered through a single layer of 80-μm Miracloth. A sample of the filtrate, referred to as the crude extract, was incubated for 20 min in 6 mM CHAPS detergent to solubilize the protein, which was then desalted using a Sephadex G-25 M PD-10 column. At each step of the purification procedure, protein concentration and pyruvate- and 2-oxoglutarate-dependent GABA-T activities were determined in the desalted sample. The crude extract prepared with buffer A was subjected to a series of five centrifugations ranging from 50 to 2500 *g*, then the resultant supernatant was centrifuged at 11,000 *g* for 20 min to pellet mitochondria and some contaminating chloroplasts which were then resuspended in 25 ml of buffer B. The solubilized, lysed mitochondrial fraction was centrifuged for 75 min at 180,000 *g* and the supernatant filtered through a 0.45-μm nylon syringe filter.

4.4. Column chromatography

The filtered mitochondrial fraction was supplemented with leupeptin and pepstatin A each to a final concentration of 2.5 μg ml^{-1}, mixed with 7.5 ml of Fractogel EMD DEAE 650 (S) pre-equilibrated in buffer B, incubated for 15 min with constant, gentle mixing and pelleted via a 3-min centrifugation at 2500 *g*. The protein-loaded Fractogel was suspended in 7.5 ml of buffer B and poured onto a Waters Protein-Pak anion exchange column (10 × 100 mm). The column was packed and washed with 50 mM Tris-HCl buffer (pH 8.2) containing 3 mM DTT, 6 mM CHAPS and 20% (v/v) glycerol (buffer C), using a flow rate of 1 ml min^{-1} (ca 600 psi) that was generated by a Waters 625 LC System. For all chromatography steps, fractions (2 ml) were collected into solution containing a final concentration of 0.2 mM pyridoxal-5-phosphate (PLP) and 2.5 μg ml^{-1} of both leupeptin and pepstatin A.

For analysis of total leaf- or mitochondrial-pyruvate- and/or 2-oxoglutarate-dependent GABA-T activities, either crude extract or mitochondrial protein was

loaded onto a Fractogel column and eluted with a 70-min linear gradient of 0–400 mM NaCl in buffer C.

For partial purification of pyruvate-dependent GABA-T activity for biochemical characterization, the proteins were eluted using a 70-min linear gradient of 0-1 M NaCl in buffer C. Fractions containing pyruvate-dependent GABA-T activity were pooled, desalted, loaded onto a second Fractogel anion exchange column equilibrated with buffer C, and the protein eluted using a 70-min linear gradient of 0–400 mM NaCl in buffer C. Fractions containing pyruvate-dependent GABA-T activity were pooled, desalted, concentrated using a Centricon-30 concentrator, and supplemented with both leupeptin and pepstatin A. After analysis, the remaining sample was used immediately or frozen in liquid nitrogen and stored at −80°.

4.5. Assays

To assay pyruvate- and 2-oxoglutarate-dependent GABA-T activity an aliquot of sample was incubated in a reaction mixture containing in a final volume of 500 μl: 50 mM Tris-HCl buffer (pH 8.2), 1.5 mM DTT, 0.75 mM EDTA, 0.1 mM PLP, 10% (v/v) glycerol, 2 mM GABA and 2 mM pyruvate or 1 mM 2-oxoglutarate to initiate the reaction. The reactions were terminated with 6 mM (final concentration) cold (4°) sulphosalicylic acid. Following centrifugation at 20,000 *g* for 5 min, the supernatant was neutralized with 1 N NaOH, filtered through a 0.45-μm filter and the amino acid products, alanine or glutamate, were separated by reverse phase HPLC on an Ultrasphere silica-based HPLC column using buffers described by Oaks, Boesel, Goodfellow and Winspear (1986), following automatic derivatization with ortho-phthalaldehyde (OPA).

Protein was precipitated from 2 μl of desalted sample by the addition of 1 ml of 12% (w/v) cold TCA; this solution was incubated overnight at 4°. The protein was pelleted by centrifugation for 30 s at 20,000 *g*, then dissolved in 0.1 M NaOH. Protein was measured using the Coomassie Plus Protein Assay Reagent with BSA as the standard.

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