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THE RAPID DETERMINATION OF γ-AMINOBUTYRIC ACID

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Key Word Index—*Glycine max*; Leguminosae; soybean; γ-aminobutyric acid (GABA) determination; stress.

Abstract—A rapid procedure for the extraction and assay of γ-aminobutyric acid (GABA) is described. The extraction procedure prevents rapid GABA accumulation during the sampling of tissue for analysis. It also removes over 95% of pigments absorbing at 340 nm which otherwise reduce the sensitivity of the spectrophotometric coupled enzyme assay. The absorbance increase at 340 nm was linear for GABA levels ranging from 10 to 100 nmol per cuvette. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

γ-Aminobutyric acid (GABA) is a ubiquitous non-protein amino acid produced through the α-decarboxylation of L-Glu in a reaction catalysed by glutamate decarboxylase (GAD, EC 4.1.1.15). Rapid and large accumulations of GABA in response to diverse environmental factors have been documented [1, 2]. For example, GABA levels increased 10–25-fold within 1–4 min of reduced temperature, darkness or mechanical manipulation of soybean leaves [3, 4].

Plant GAD is a cytosolic enzyme [5] which, unlike GAD from other sources, has a Ca^{2+}/calmodulin binding domain [6, 7]. GABA accumulation results from GAD activation mediated by increases in the cytosolic level of Ca^{2+} [8] or H^+ [9, 10]. Mechanical manipulation results in rapid increases in cytosolic Ca^{2+} levels [11]. Consequently, rapid GABA accumulation in response to manipulation during tissue sampling will lead to an overestimation of GABA levels. For example, GABA synthesis in slices of pea cotyledon was many times faster than in intact cotyledons [12]. An initial report of a seven-fold increase in GABA level in phloem sap in response to water stress was later attributed to the mechanical procedures required for sampling [13]. In addition, GAD activity survives grinding in liquid N_2 and GABA accumulates in tissue homogenates which are allowed to reach room temperature [3, 4].

A commercially available mixture of GABA transaminase (EC 2.6.1.19) and succinic semialdehyde dehydrogenase (EC 1.2.1.16) allows for a spectrophotometric assay of GABA (GABASE, Sigma):

1. GABA + α-ketoglutarate → glutamate + succinic semialdehyde
2. Succinic semialdehyde + $NADP^+$ → succinate + NADPH

GABA values are related to the increase in absorbance due to NADPH production. Older tissues, however, contain water-soluble phenolic pigments which absorb strongly at 340 nm, and aqueous extracts raise the absorbance of the assay system to values where absorbance increases cannot be determined accurately. These interfering compounds are not removed by organic solvents, and removal by ion-exchange chromatography [4] is time-consuming and incomplete. A method of GABA determination is reported which eliminates both GABA accumulation subsequent to tissue sampling and the interfering pigments.

RESULTS AND DISCUSSION

Soybean leaves were detached from the plant and placed in liquid N_2 within 2 sec. Frozen leaves were then ground using a mortar and pestle containing liquid N_2 until a fine powder was obtained. About 0.1 g of the frozen homogenate was transferred into a preweighed 1.5 ml Eppendorf tube containing 400 μl methanol at 25°. The tube was weighed again to obtain the precise weight of the homogenate transferred. After 10 min, the sample was vacuum dried, and 1 ml of 70 mM lanthanum chloride was added. The sample was then shaken for 15 min, centrifuged at 13000 *g* for 5 min, and 0.8 ml of the supernatant fluid removed to a second Eppendorf tube. To this was added 160 μl of 1 M KOH, followed by shaking for 5 min, and centrifugation as before. The resulting supernatant

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fluid was used in the spectrophotometric GABA determination (see Experimental section); and the pellet consisting of lanthanum hydroxide and yellow pigment was discarded. The GABA value and the weight of the homogenate were used to estimate the GABA level (as nanomole GABA per gram fresh weight).

Frozen homogenate was transferred to methanol to inactivate GAD. The efficiency of inactivation was tested by comparing transfer to methanol as described with direct transfers to 1 ml of 30 mM HCl at 60°, or to 1 ml of 70 mM lanthanum chloride at 60°. After 10 min, these samples were centrifuged and treated as described above. HCl has been used to inactivate animal GAD [14]. These three treatments resulted in GABA levels of less than 7133 ± 52 S.D. and 287 ± 11 S.D. nmol GABA/g fresh weight, respectively. To demonstrate GABA synthesis subsequent to grinding in liquid N_2, homogenates were stored at 0 or 25° in duplicate experiments. Whereas the initial values were less than 7 nmol GABA/g fresh weight, values after 5 min at 0° and 25° were 32 and 34, and 2000 and 2200 nmol GABA/g fresh weight, respectively. These data demonstrate that determination of *in vivo* GABA levels requires both rapid freezing in liquid N_2 after tissue sampling and rapid inactivation of GAD with methanol after tissue homogenization.

Water-soluble phenolic pigments can be removed from solution by precipitation with lead acetate [15]. To avoid lead or acetate inactivation of the enzymes employed in the subsequent GABA assay (data not shown), pigment removal by lanthanum chloride was investigated. Pigments absorbing between 260 and 500 nm were effectively removed and the absorbance at 340 nm declined over 95% as the concentration of lanthanum chloride employed was increased. Maximum removal was attained when concentrations reached 70 mM (Fig. 1).

In the procedure described, lanthanum is removed by precipitation with KOH, and the supernatant fluid is used for GABA determination. When standard GABA was added to this fluid, the GABA assay gave an absorbance increase at 340 nm which was no different from control samples. Lanthanum chloride was not inhibitory at concentrations below 20 mM. Standard GABA was also added to an Eppendorf tube containing methanol and tissue homogenate, and subjected to the procedure described. The absorbance increase at 340 nm indicated a GABA recovery of 100.2 ± 2.5%. The calibration graph indicates a linear relationship between the absorbance increase and GABA values varying from 10 to 100 nmol in the 1 ml assay system (see Experimental section).

Considerable current interest in GABA has resulted from papers demonstrating that plant GAD is Ca^{2+}/calmodulin activated [6–8] and that rapid GABA accumulation may function as a pH-stat mechanism [9, 10], a defence against phytophagous insects [4] and/or the regulation of plant development [16, 17]. The method described is rapid and inexpensive, because it does not require the chromatographic separation and derivatization of amino acids. It avoids overestimating GABA by eliminating synthesis subsequent to tissue sampling and eliminates pigments that interfere with the spectrophotometric assay.

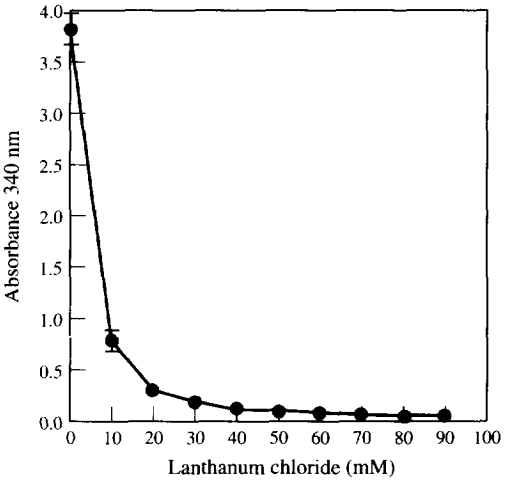

Fig. 1. Lanthanum chloride removal of water-soluble pigments. Frozen tissue homogenate (0.1 g) was added to 1 ml of lanthanum chloride at the concentration indicated. Subsequent procedures are described in the text. For absorbance measurements, 550 μl of the final supernatant fluid was diluted to 1 000 μl with 200 μl 0.5 M pyrophosphate buffer (pH 8.6) and 250 μl distilled water. The values indicated are the means of three experiments.

EXPERIMENTAL

The 1 ml assay system contained 550 μl of a sample containing 10–100 nmol GABA, 150 μl 4 mM $NADP^+$, 200 μl 0.5 MK^+ pyrophosphate buffer (pH 8.6), 50 μl of 2 units GABASE per ml and 50 μl of 20 mM α-ketoglutarate. The initial *A* was read at 340 nm before adding α-ketoglutarate, and the final *A* was read after 60 min. The difference in *A* values was used to construct a calibration graph. The commercial GABASE enzyme preparation was dissolved in 0.1 M K-P_i buffer (pH 7.2) containing 12.5% glycerol and 5 mM 2-mercaptoethanol. The resulting soln was divided into 0.5 ml aliquots each containing 1 unit of activity. These were frozen until used.

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