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Research Article

A fluorescence enhancement assay for measurement of glutamate decarboxylase activity

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Abstract

Glutamic Acid Decarboxylase (GAD) is an enzyme that converts glutamate to γ-aminobutyric acid (GABA) both in the brain and pancreatic β-cells. Several analytical methods are described for quantitative assay of GAD, where little attention has been given to the enzyme regulation in tissues, in part, due to the complexity of the methods. In this study, a novel fluorimetric method based on changes of fluorescence intensities upon the addition of glutamate substrate into the assay mixture is described. Rat brain GAD was purified and the enzyme activity was determined fluorimetrically in different stages of the enzyme purification. Results showed that during purification steps, changes in fluorescence emission intensities (ΔF/min/mg protein) increased in the paralleled to purification folds of the enzyme. In support of these findings, the levels of CO2 production were measured by Warburg manometric method. The close correlation between the new fluorimetric method and the conventional manometric assay method was demonstrated. Because the proposed fluorimetric method is simple, accurate, and sensitive enough for measuring GAD activity in different stages of the enzyme purification, it would be recommended for the clinical and pharmaceutical investigations.

Introduction

Glutamate (Glu) is the main excitatory and γ-aminobutyric acid (GABA) the main inhibitory neurotransmitter in the mammalian nervous system. Excessive levels of extracellular Glu in the nervous system are excitotoxic and lead to several neurodegenerative processes [1-3]. Conversely, GABA is known to have several physiopathological functions such as epilepsy, anti-anxiety, and anti-diabetic effect in humans [4-7]. The Glu and GABA have a complex homeostatic relationship that brings balance to the level of brain activity. In GABAergic neurons, Glu is converted into GABA by glutamate decarboxylase (GAD, EC 4.1.1.15), the rate-limiting enzyme in the synthesis of GABA. The enzyme is present in several non-neuronal tissues including the pancreatic β-cells, [8-11]. The enzyme exists as two isoforms, named GAD67 and GAD65 [12,13]. GAD67 is essentially active, while GAD65 can be activated in response to an additional demand for extra GABA in neurotransmission [14,15]. GAD65 exists mainly in an inactive form that can be activated by its coenzyme, pyridoxal 5'-phosphate (PLP) [16]. It has been demonstrated that there are many similarities in identity of rat brain GAD 65 with the human GAD 67 [17]. Since the discovery of GAD as an antigen in Type 1 Diabetes (T1D) several analytical methods have been described for the detection of its anti-GAD antibodies [18]. However, regulation of GAD activity in pancreatic β-cells plays a key role in governing the cell function for production and secretion of insulin [10,19]. There are still many unanswered questions that need to be investigated in the regulation of GAD activities in the therapy of both T1D and type 2 diabetes (T2D). Considerable studies support the evidence regarding the localization of GAD and physiological function of GABA, given the original suggestion that certain human pathological disorders is associated with the alteration in GAD activity both in central and peripheral disorders. Despite its importance, the precise mechanism underlying the regulation of GAD activity and the chemical mechanisms for PLP-mediated reactions with an emphasis on the chemical steps processed by enzyme in the specific cells

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are not fully elucidated. From a technical point of view, it gives the impression that most of the enzyme assay protocols are complex, time-consuming, and expensive. Various methods, such as; conventional Warburg monomeric technique where glutamate is converted to GABA and Carbon dioxide (CO_2) under the specified conditions, radiochemical methods by liberating $^{14}CO_2$ from [14C]glutamate [20] sequential enzymatic reactions for conversion of GABA to succinate [21] or HPLC measurement of GABA production [22,23]. These methods need complex chemical substances or radiolabeled materials that are often expensive and/or health hazardous. For clinical diagnostic purposes and pharmaceutical industries a simpler, more sensitive and reliable enzyme assay is needed to clearly delineate the enzymatic activity and metabolic roles of the enzyme in tissue samples.

The interaction between the phosphate group of PLP and the active site of GAD, maintains PLP molecule in the fluorogenic site that upon addition of glutamate conformational changes can be detected fluorimetrically [24]. Because of the rate of fluorescence emission of GAD is likely limited upon addition its substrate the present study extends the initial works on the use of the fluorimetric properties to determine GAD activity in the small amounts of biological samples.

Materials and methods

Materials

DEAE-cellulose, Sephadex –G200, hydroyapatite, phenyl methyl sulfonylflouride (PMST), Dithio-trathiol (DTT), aprotonin, pyridoxal 5'-phosphate (PLP), and bovine serum albumin were obtained from Aldrich Chemical Company, Dorsel, U.K. All other reagents used were unless stated otherwise of analar grade (or the highest available) and made up in double-distilled water.

Purification of glutamate decarboxylase

Purification of rat brain GAD was essentially carried out as described by Nathan, et.al. [25], briefly, in each experiment, five male Wistar rats (200-2S0g) were killed by decapitation and between 8:00 and 9:00 AM. forebrains were removed and chopped into the consistency of mince and rapidly transferred into 50mL buffer (pH7) containing 25 mM potassium phosphate, 0.2 mM pyridoxal -5 phosphate, ImM EDTA, 0.1 mM phenyl methyl sulfonylfluoride, 5mM dithiotrathiol, and 1 % aprotonin and homogenized in crushed ice. The homogenate was centrifuged at 54000g for 60 min at 4 C and the supernatant was poured into a column of EDTA-cellulose (1.5 x 40 cm) and eluted with a linear gradient of phosphate buffer (pH7) from 0.03 to 0.30 M containing 5 mM DTT. The protein fractions were detected by the spectrophotometer at 280. The positive fraction was chromatographed on a 0.5 cm x 30 cm hydroxyapatite resin column and was eluted as above with the same gradient buffer. The positive fractions were pooled for Sephadex G-200 gel filtration chromatography. The protein concentrations in the supernatant and eluted fractions were performed by the method of Lowry, et al. [26] with bovine plasma albumin as a standard.

Manometric measurement of GAD activity

In order to compare the results of the fluorimetric method with a non- fluorimetric one, conventional mercury manometric technique (Warburg) was chosen. The most commonly measured end product has been CO_2, and this gas has been measured by techniques which include Warburg manometry [27]. Briefly, 1 ml aliquot of the brain supernatant or eluted partially purified enzyme preparations with protein concentration as described above were adjusted to pH 7 and transferred in the manometric cell and incubated at 37°C for 5 min. The reaction was then started by the addition of 100µl glutamate solution (10 mM) and CO_2 production was measured for 15 min. The results are expressed as µlCO_2 released/min/mg protein.

Fluorimetric measurement of GAD activity

In the fluorimetric method, 1 ml aliquot of the brain supernatant or eluted partially purified enzyme preparations with protein concentrations as shown in Table 1 were adjusted to pH 7 and transferred in the fluorimetric cuvet (LSE specrrophotofluorimeter, Perkin-Elmer, Norwalk, CT) and pre-incubated in for 5 min at 37°C. The reaction was then started by addition of 100µl glutamate solution (10 mM) and increasing of fluorescence intensities (ΔF) was monitored at the excitation wavelength of 495 nm and emission the wavelength of 540 nm, for 15 min against a blank containing all components except for glutamate. The results are expressed as ΔF/min/mg protein.

Table 1: Rat brain GAD acyivity in different steps of the purification.

Fractions	mg protein/ ml	Manometric method µlCO_2/min/ mg protein	Fluorimetric method ΔF/min/mg protein	Purity Fold
Supernatant	12.88 ± 2.16	0.05 ± 0.01	0.23 ± 0.01	0.00
Cellulose eluate	2.14 ± 0.35	0.25 ± 0.11	1.36 ± 0.09	5.1
H.apatate eluate	0.57 ± 0.80	2.24 ± 0.47	10.51 ± 0.78	45.5
Filtration eluate	0.12 ± 0.03	7.06 ± 1.92	32.50 ± 2.04	140.5

Results

The specific activity of GAD in rat brain supernatant and the fractions eluted from each chromatographic step as measured by two different methods a Warburg manometric method and a fluorimetric method. Data are summarized in Table 1. Both fluorescence emission and CO_2 release of the experimental mixture containing tissue preparations were markedly increased after the addition of glutamate. Whereas, the total protein content of the supernatant that applied to the three chromatographic purification steps was 640 mg which recovered from the last chromatographic step was 0.67 mg. which its specific enzyme activity increased approximately 140 folds of that in the supernatant. This is in good agreement with the results previously reported [25]. As shown in Table 1 both fluorescence emission and CO_2 release of the assay mixture

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containing tissue preparations were markedly increased after the addition of glutamate. During efficient purification steps, specific activity as expressed by fluorescence emission or CO2 release/mg of protein significantly increased, indicating that the GAD protein is getting more abundant (Table 1). The increase in the purification folds of the enzyme as measured by manometric and fluorimetric techniques were quite similar.

The rate of CO2 production and the changes of fluorescence intensities in each step of purification were measured by manometric and fluorimetric methods. The results are mean of 6 separate experiments with SD in round brackets. In each experiment 5 forebrains were processed as described in the Method section.

Discussion

The results reported in this paper demonstrated that the release of CO2 and the changes of the fluorescence intensities (ΔF) of the assay mixture increased markedly as purification proceeded (Table 1). The increasing of the fluorescence intensities of the purified GAD upon the addition of glutamate indicates the formation of GAD/glutamate complex [24]. Changes in fluorescence intensities (ΔF)/min/mg protein by the supernatant and the eluted fractions from three chromatographic steps of purifications positively correlated with the levels of µlCO2 released/min/mg protein CO2 of the enzyme preparations(r=99%). However, the results in Table 1 showed that the levels of Standard Deviation (SD) related to the specific CO2 production of manometric method to its corresponding values of specific ΔF obtained from the fluorimetric technique was markedly higher. This suggests a higher sensitivity for the fluorimetric method compared to the manometric method. There are several methods for the determination of GAD activity using different procedures and some of these studies are reporting the advantageous properties of fluorimetric methods over the other ones [28-31]. The results of this study providing further support for the previous report that the fluorimetric method has advantages of being easy and rapid, with higher quantitative sensitivity and reliability as compared with the conventional manometric method which involved CO2 determination.. In conclusion, the fluorometric assay of GAD seems to be the most accessible, simple with low-cost and may be used in clinical and pharmaceutical investigations.

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