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...
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 monometric technique where glutamate is converted to GABA and Carbon dioxide (CO2) under the specified conditions, radiochemical methods by liberating 14CO2 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, hydroxapatite, phenyl methyl sulfonflyfluoride (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 (200–250g) 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 sulfonflyfluoride, 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 hydroxapatite 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 CO2, 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 concentrations as shown in Table 1 were adjusted to pH 7 and transferred in the fluorimetric cuvet (LSE spectrophotofluorimeter, Perkin–Elmer, Norwalk, CT) and pre-incubated in for 5 min at 37°C. The reaction was then started by the addition of 100μl glutamate solution (10 mM) and CO2 production was measured for 15 min. The results are expressed as μlCO2 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 spectrophotofluorimeter, Perkin–Elmer, Norwalk, CT) and pre-incubated in for 5 min at 37°C. The reaction was then started by the 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.

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 CO2 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 speci

| Fractions       | mg protein/ml | Manometric method µCO2/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        |
| Hapatate 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      |

Citation: Messripour M, Mesripour A (2020) A fluorescence enhancement assay for measurement of glutamate decarboxylase activity. Open J Anal Bioanal Chem 4(1): 007-010. DOI: https://dx.doi.org/10.17352/ojabc.000018
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 μCO2 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.

**References**

1. Mazaud D, Kottler BD, Goncalves Pimentel CS, Proelss S, et al. (2019) Transcriptional regulation of the Glutamate/GABA/Glutamine cycle in adult glia controls motor activity and seizures in Drosophila. J Neurosci 39: 5269-5283. Link: https://bit.ly/3g3Gvaw2

2. Otto N, Marejza J, Schoofs A, Kraneburg H, Bittner J, et al. (2018) The sulfite oxidase shopper controls neuronal activity by regulating glutamate homeostasis in Drosophila ensheathing glia. Nat Commun 9: 3514. Link: https://bit.ly/3GsaJX1

3. Rowley M, Madsen KK, Schousboe A, Steve White H (2012) Glutamate and GABA synthesis, release, transport and metabolism as targets for seizure control. Neurochem Int 61: 546-558. Link: https://bit.ly/3IinHc1O

4. Boison DR, Steinhauser C (2018) Epilepsy and astrocyte energy metabolism. Glia 66: 1235-1243. Link: https://bit.ly/2YC0xKak

5. Chao H, Chen H, Samaco R, Xue M, Chahrou M, et al. (2010) Dysfunction in GABA signalling mediates autism-like stereotypies and Rett syndrome phenotypes. Nature 468: 263-269. Link: https://go.nature.com/2Vl48HK

6. Wong CG, Bottiglieri T, Sneed OC (2003) GABA, γ-aminobutyric acid, and neurological disease. Ann Neurol 54: S12-S14. Link: https://bit.ly/21AAZRq

7. Chen L, Zhao H, Zhang C, Lu Y, Zhu X, et al. (2016) γ-Aminobutyric acid-rich yogurt fermented by Streptococcus salivarius subsp. thermophilus fmb5 appears to have anti-diabetic effect on streptozotocin-induced diabetic. Func Foods 20: 267-275. Link: https://bit.ly/2A75kC3

8. Cho JH, Lee KM, Lee YI, Nam HG, Jeon WB (2019) Glutamate decarboxylase contains different enzymes in diverse pancreatic islets. Islets 11: 33-43. Link: https://bit.ly/2NCia8B

9. Zhu Y, Qian L, Liu Q, Jing Z, Ying Z, et al. (2020) Glutamic Acid Decarboxylase Autoantibody Detection by Electrochemiluminescence Assay Identifies Latent Autoimmune Diabetes in Adults with Poor Islet Function. Diabetes Metab J 44: 260-266. Link: https://bit.ly/3Vhn50b

10. Braun M, Ramacharya R, Bengtsson M, Clark A, Walker JN, et al. (2010) Gamma-aminobutyric acid (GABA) is an autocrine excitatory transmitter in human pancreatic β-cells. Diabetes 59: 1694-1701. Link: https://bit.ly/3eP7igu

11. Yi Z, Waseem Ghani M, Ghan I, Jiang W, Waseem Birmann M, et al. (2020) Gimmicks of gamma-aminobutyric acid (GABA) in pancreatic β-cell regeneration through transdifferentiation of pancreatic α- to β-cells. Cell Biol Int 44: 926-935. Link: https://bit.ly/3i3bXnA

12. Bu DF, Erlander MG, Hitz BC, Tillakaratne NJ, Kaufman DL, et al. (1992) Two human glutamate decarboxylase 65Kda and 67 Kda are each encoded by a single gene. Proc Natl Acad Sci USA 89: 2115-2119. Link: https://bit.ly/2YF7LbB

13. Christie MR, Peipeleers DG, Lemmark A, Backkeskove S (1990) Cellular and subcellular localization of an M64000 protein autotagins in insulin dependent diabetes. J Biol Chem 265: 376-381. Link: https://bit.ly/2B6lW7Z

14. Chattopadhyaya B, Di Cristo G, Wu CZ, Knott G, Kuhlman S, et al. (2007) GAD67-mediated GABA synthesis and signaling regulate inhibitory synaptic innervation in the visual cortex. Neuron 54: 889-903. Link: https://bit.ly/384UgxD

15. Lazarus MS, Krishnan K, Huang ZJ (2015) GAD67 deficiency in parvalbumin interneurons produces deficits in inhibitory transmission and network disinhibition in mouse prefrontal cortex. Cereb Cortex 25: 1290-1296. Link: https://bit.ly/3g3u0kO

16. Messripour M, Mesripour A (2011) Effects of vitamin B6 on age associated changes of rat brain glutamate decarboxylase activity. Afr J Pharm pharmacol 5: 454-456. Link: https://bit.ly/2YC8keZ

17. Kim J, Richter W, Aanstoot HJ, Shi Y, Fu Q, et al. (1993) Differential expression of GAD65 and GAD67 in human, rat, and mouse pancreatic islets. Diabetes 42: 1799-1808. Link: https://bit.ly/3dCteKr

18. Towns R, Pietropaolo M (2011) GAD65 autoantibodies and its role as a biomarker of Type 1 diabetes and Latent Autoimmune Diabetes in Adults (LADA). Drugs Future 36: 847. Link: https://bit.ly/3l0a5jD

19. Abarkan M, Gaitan J, Lebret F, Perrier R, Jaffredo M, et al. (2019) The glutamate receptor GluK2 contributes to the regulation of glucose homeostasis and its deterioration during aging. Mol Metab 30: 152-160. Link: https://bit.ly/2Vp3CIF

20. Quinn MR, Chan MM (1979) Effect of vitamin B−6 deficiency on glutamic acid decarboxylase activity in rat olfactory bulb and brain. J Nutr 109: 1694-1702. Link: https://bit.ly/3g3l2EJ

21. De Blase D, Tramonti A, John, RA, Bossa F (1996) Isolation, overexpression, and biochemical characterization of the two isoforms of glutamic acid de
22. Holdiness MR (1982) Determination Of Glutamic Acid Decarboxylase Activity In Subregions Of Rat Brain By High Pressure Liquid Chromatography. J Liq Chro 5: 479-487. Link: https://bit.ly/311omLZ

23. Martin DL, Rimvall K (1993) Regulation of gamma-aminobutyric acid synthesis in the brain. J Neurochem 60: 395-407. Link: https://bit.ly/2YAB0K0

24. Stryer L (1998) Integration of metabolism. In: Stryer L (ed.), Biochemistry, New York: W.H. Freedman and Company, 764-784.

25. Nathan B, Bao J, Hsu CC, Yarom M, Deupree DL, et al. (1994) An integral membrane protein form of L-glutamate decarboxylase: Purification and its relationship to insulin-dependent diabetes mellitus. Brain Res 642: 297-302. Link: https://bit.ly/2YCK2WT

26. Lowry OH, Rosenrough NN, Farr AL, Randall RJ (1951) Protein measurement with the folin-phenol reagent. J Biol Chem 193: 265-276. Link: https://bit.ly/2Zd5X1g

27. Newman D (1965) A manometer devices. In: Newman D, (ed.), Experimental Methods of Experimental Biology. New York, Macmillan Company 394-404.

28. Royer CA (1995) Fluorescence spectroscopy. Methods Mol Biol 40: 65-89. Link: https://bit.ly/3gBWFZ

29. Berezin MY, Achilefu S (2010) Fluorescence lifetime measurements and biological imaging. Chem Rev 110: 2641-2684. Link: https://bit.ly/3i695EL

30. Valeur B, Berberan-Santos MRN (2011) A Brief History of Fluorescence and Phosphorescence before the Emergence of Quantum Theory. J Chem Educ 88: 731-738. Link: https://bit.ly/3g2lIP0

31. Franco PG, Adamo AM, Mathieu P, Pérez MJ, Setton-Avruij PC, et al. (2017) Update on the fluorometric measurement of enzymatic activities for Lysosomal Storage Disorder detection: The example of MPS VI. J Rare Dis Treat 2: 56-61. Link: https://bit.ly/2NyyM2L