



Figure 1: Time course of rat brain glutamate decarboxylase activity by manometric and fluorimetric methods. One ml aliquots of purified rat brain GAD with protein concentration of 0.12 mg were incubated in the presence of 100 μ l glutamate solution (10 mM) at 37°C and the rate of glutamate decarboxylation or changes of fluorescence intensities were measured by manometric (••) and fluorimetric (•••) methods respectively for a period of up to 60 min. Each point represents mean \pm SD of 6 determinations.

Fractions (total protein)	Manometric method μ l CO ₂ /min/ mg protein	Fluorimetric method Δ F /min/mg protein	Purity fold
Brain Supernatant	0.05 (0.01)	0.23 (0.01)	-
DEAD-cellulose eluate	0.25 (0.11)	1.36 (0.09)	5.1
Hydroxyapatate eluate	2.24 (0.47)	10.51 (0.78)	45.5
Gel filtration eluate	7.06 (1.92)	32.50 (2.04)	140.5

Table 1: Specific activity of rat brain glutamate decarboxylase in different steps of the purification. The rate of CO₂ production and the changes of fluorescence intensities in each step of purification were measured by manometric and fluorimetric methods respectively. 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.

Samples	Manometric assay μ l CO ₂ /min/ mg protein	Fluorimetric assay Δ F /min/mg protein
Purified GAD + Control serum	6.50(1.46)	31.48(1.06)
Purified GAD +IDDM serum	2.89*(1.08)	11.05*(0.59)

Table 2: Inhibition of rat brain glutamate decarboxylase activity by antiserum of IDDM patients. The activity of the purified GAD was measured by manometric and fluorimetric techniques in the presence of serum samples from IDDM patients (n=21) or non diabetic subjects (n=21). Values are mean with SD in round brackets. Statistically significant differences between the patients and control groups were determined by Student's t-test. * $p < 0.005$

protein concentrations were performed by the method of Lowry et al. [16]. The protein concentrations in supernatant and eluted fractions collected from three chromatographic steps were 12.88 ± 2.16 , 2.14 ± 0.35 , 0.57 ± 0.80 and 0.12 ± 0.03 mg/ml respectively. The enzyme eluted in the last step of purification (Sephadex G-200 gel filtration) was used to evaluate antiserum in IDDM patients.

Enzyme assays

Two different methods were employed to measure the activity of GAD in the rat brain preparation; a Warburg manometric method [15] and a fluorimetric method [10]. In manometric method 1 ml aliquots of the brain supernatant or eluted partially purified enzyme 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 addition of 100 μ l glutamate solution (10 mM) and CO₂ production was measured for 15 min. The results are expressed as μ l CO₂ released/ min/mg protein. In the fluorimetric method, 1 ml aliquots of the enzyme preparations with the same protein and pH levels were transferred in the fluorimetric cuvet (LSE spectrofluorimeter, Perkin-Elmer, Norwalk, CT) and pre-incubated in for 5 min at 37°C. The reaction was then started by addition of 100 μ l glutamate solution (10 mM) and increasing of fluorescence intensities (Δ F) was monitored

at the excitation wavelength of 495 nm and emission wavelength of 540 nm, for 15 min against a blank containing all components except glutamate. Using the purified enzyme preparations (Sephadex G-200 gel filtration step), the rate of glutamate decarboxylation or changes of fluorescence intensities were linear up to more than 40 min (Figure 1) with protein concentration of 0.12 mg/ml. of The results are expressed as Δ F /min/mg protein.

Because the protein concentrations of the enzyme preparations decreased during purification steps, in order to examine the possible interaction between GAD and serum samples taken from IDDM patients, the experiments were carried out as above using purified enzyme preparations (approximately 0.12 mg protein/ml) and the rate of glutamate decarboxylation or changes of fluorescence intensities were measured after addition of 100 μ l of serum sample taken from either IDDM patient or non diabetic subjects

Results

The specific activity of GAD in rat brain supernatant and the fractions eluted from each chromatographic step as measured by both manometric and fluorimetric assays is summarized in Table 1. A positive correlation was observed between the rate of CO₂ formation and the changes of fluorescence intensities in different steps of GAD purification ($r=98$). Total protein content of the rat brain supernatant (5 forebrains) that applied to the three chromatographic purification steps (see Methods) was 640 mg with the enzyme specific activity of 0.05 μ l CO₂/min/mg protein. The enzyme protein recovered from the last step chromatographic step was 0.67 mg which its enzyme specific activity increased approximately 140 folds of that in the supernatant (Table 1). This is in good agreement with the results previously reported [13]. As can be seen in Table 1, the increase in the purification folds of the enzyme as measured by manometric and fluorimetric techniques were quite similar. Pooled enzyme eluted from the gel filtration step was used as the purified GAD to assess the interaction between the enzyme and antiserum from IDDM patients. The results are shown in Table 2. Based on the enzymatic methods, the patient's serum samples caused approximately a 56% inhibition of CO₂ production in manometric method and approximately a 65% reduction in the enhancement of the fluorescence intensities of the enzyme /substrate complex in the fluorimetric method. Whereas, serum samples from healthy control individuals had little effect on the GAD activity.

Discussion

The results reported in this paper demonstrated that the release of CO₂ and the changes of the fluorescence intensities of the assay mixture increased markedly as purification proceeded (Table 1). The rate of CO₂ production by rat brain supernatant and the eluted fractions from three chromatographic steps, positively correlated with the increasing rate of fluorescence intensities [$r=99\%$] of the enzyme preparations. The increasing of the fluorescence intensities of the purified GAD upon the addition of glutamate reveals the formation of GAD/glutamate complex [12] can be taken as a measure for GAD activity. As can be seen in Table 1 the ratio of the standard deviation to its corresponding value obtained from the manometric technique was markedly greater as compared to that of the fluorimetric method. These results demonstrate that the fluorimetric method have advantage of higher sensitivity and reliability as compared with conventional manometric method which involved CO₂ determination [15].

However, the subsequent addition of serum samples from the IDDM patients inhibited the enzyme activity significantly (Table 2).

Reduction in the enzyme activity by IDDM serum samples interpreted as being consistent with the enzyme/ anti-enzyme antibody interaction [1]. The inhibition of the enzyme might be a consequence of the probable conformational changes arising from binding of anti-GAD antibodies on the enzyme active site and the subsequent inhibition in formation of the enzyme/substrate complex. In the fluorimetric method the signal produced by the enzyme/substrate is quenched by a specific anti-enzyme antibody, and then a measurement can be made after addition of antibody directly [i.e.17].

The high degree of fluorescence sensitivity and enzyme specificity indicate that enzyme inhibition assay is simpler, more accurate and sensitive enough for screening of a large number of serum samples for the presence of anti-GAD antibodies for identification of people who are at risk for IDDM.

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