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A novel Enzyme Inhibition Assay for Screening of Type 1 Diabetes Mellitus

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Abstract

Glutamate decarboxylase (GAD) antibody has been found in patients with recent-onset insulin dependent diabetes mellitus (IDDM). Several analytical methods are described for detection and quantitation of anti-GAD antibodies whereas the inhibition of the enzyme activity by anti-enzyme antibody is given little attention. In this study a quenching fluorimetric method based on changes of fluorescence intensities upon addition antiserum into the enzyme/substrate medium is described. The activity of the purified rat brain GAD was measured by both manometeric and fluorimeteric methods in the presence of serum samples from IDDM patients (n=21) or non diabetic subjects (n=21). Significant differences between the patients and control groups were observed using both methods. However, high degree of fluorescence sensitivity of fluorimetric technique allows for less error, less time and satisfactory useful and economical for screening of IDDM in the large populations.

Introduction

Type 1 diabetes, known as insulin dependent diabetes mellitus (IDDM) is well documented as an autoimmune disease in which pancreatic islet cells are progressively destroyed [1-3]. A membrane-bound protein expressed by the islet cells is shown to act as a major autoantigen in IDDM. This protein is reported to be the enzyme glutamate decarboxylase [GAD] [4-6]. Pancreatic islet cells are the major site of extraneural GAD expression [4-7]. The enzyme catalyzes the formation of γ-aminobutyric acid [GABA] from glutamate, which can be derived from extracellular glutamine [7]. Two isoforms of the enzyme [GAD65 and GAD67] are shown to be expressed both in rat brain and pancreatic beta cells, and GAD65 is predominantly expressed in the islet cells [8]. Since discovery of GAD as an antigen in IDDM, and detection of anti-GAD antibodies prior to the clinical onset of the disease, several analytical methods have been described for detection and quantitation of anti-GAD antibodies for identification of people who are at risk for IDDM [4-11]. These include radioimmunoprecipitation assay, radioimmunoassay, time-resolved fluorometric assay and ELISA in which a complex of either radioisotope- or enzyme-labeled recombinant GAD65 is required to be used as labeled-ligand. Although these methods are reported to be successful, a simpler, more rapid, sensitive and inexpensive method is needed for screening of large number of serum samples for the presence of anti-GAD antibodies for the risk assessment of IDDM. Fluorogenic enzyme /substrate methods for the determination of enzyme activities have been known for a relatively long time [12]. The method involves determination of changes of fluorescence intensities upon addition antiserum into the enzyme/substrate medium. Because the expression of GAD65 in human pancreatic beta cell and rat brain is reported to be similar [13], in the present study direct inhibition of rat brain GAD activity by antiserum in IDDM patients was investigated.

Method and Materials

Materials

DEAE-cellulose, Sephadex –G200, hydroyapatite, phenyl methyl sulfonylflouride (PMST), dithio-trathiol (DTT), aprotonin, pyridoxal 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. Serum samples from 21 diagnosed patients with type 1 insulin depended diabetes were examined. The office guide to diagnosis and classification of IDDM, outlined by the American Diabetes Association [14] was used for diagnosis of the patients. Serum samples from apparently healthy subjects (age and sex were matched), with no familial background of diabetes were assayed as control.

Rat Brain GAD Preparation

Male Wistar rats weighing (100-150) gram were purchased from Pasteur Institute (Tehran, Iran) and maintained in animal house until the desired weight (200-220) gram was attained. All rats were fed with food and water under standard condition. Purification of rat brain GAD was essentially carried out as described by Nathan et.al. [13]. Animals were killed by decapitation and brains were carefully removed and placed on a Petri dish over crashed ice and chopped into the consistency of mince, which was rapidly transferred into 50 ml phosphate buffer [pH 7] containing 25 mM potassium phosphate,0.2mM PLP, 1mM EDTA, 0.1 mM PMST, 5 mM DTT, and 1% aprotonin, and homogenized in crashed ice. The homogenate was centrifuged at 54000 g for 60 min at 4 C (LS-50 Beckman) 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 (pH 7) from 0.03 to 0.30 M containing 5 mM DTT. The protein fractions were detected by spectrophotometer at 280 nm and the activity of the enzyme was assayed. The active GAD fractions were combined and chromatographed on a 0.5 cm x 30 cm hydroxapatite resin column, which was eluted as above with the same gradient of phosphate buffer. The active fractions were then collected and pooled for Sephadex G-200 gel filtration chromatography. The determination of the enzyme.

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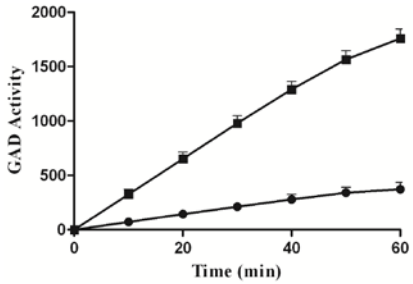

Figure 1: Time course of rat brain *glutamate decarboxylase* activity by manometric and fluorimetric methods. One ml aliquots of purified rat brain GAD with protein concentration of 0.12 mg were incubated in the presence of 100 µl glutamate solution (10 mM) at 37°C and the rate of glutamate decarboxylation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

Fractions (total protein)	Manometric method µl CO_2/min/ mg protein	Fluorimetric method ΔF /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 CO_2 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 µl CO_2/min/ mg protein	Fluorimetric assay ΔF /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 ± o.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 asays

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 100µl glutamate solution (10 mM) and CO_2 production was measured for 15 min. The results are expressed as µl CO_2 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 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 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 ΔF /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 100µl 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 CO_2 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 0.05µl $_{CO2}$/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 CO_2 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 CO_2 and the changes of the fluorescence intensities of the assay mixture increased markedly as purification proceeded (Table 1). The rate of CO_2 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 fluorimeteric method have advantage of higher sensitivity and reliability as compared with conventional manometric method which involved CO_2 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.

References

1. Backkeskov S, Neiles JH, Marner B, Bile T, Ludvigsson J, et al. (1982) Autoantibodies in newly diagnosed diabetic children, Immunoprecipitate human pancreatic Islet cell. Nature 298: 167-169.
2. Maclren NK, Schatx DS, Drash A, Graves G (1988) Initial pathogenenic events in IDDM. Diabetes 38: 534-638.
3. Todd JA (1990) Genetic control of autoimmunity in type 1 diabetes. Immonol Today 11: 122-129.
4. Clare-Salzier MJ, Tobin AJ, Kaufman DL (1992) Glutamate decarboxylase as an autoantigen in IDDM. Diabetes Care 15: 132-133.
5. Christie MR. Peipeleers DG. Lemmark A. Backkeskove S (1990) Cellular and subcellular localization of an M64000 protein autoantigens in insulin dependent diabetes. J Biol Chem 256: 376-381.
6. Fernandez-Pascual S, Mukala-Nsengu-Tshibangu A, Martin Del Rio R, Tamarit-Rodriguez J (2004) Conversion into GABA (γ-aminobutyric acid) may reduce the capacity of L-glutamine as an insulin secretagogue. Biochem J 379: 721–729.
7. Erdo SL, Wolff JR (1999) Gama aminobutric acid outside the mammalian brain. J Neurochem 54: 363-372.
8. 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.
9. Roll U, Christie MR, Standl E, Ziegler AG (1994) Associations of anti-GAD antibodies with islet cell antibodies and insulin autoantibodies in first-degree relatives of type I diabetic patients. Diabetes 43: 154-160.
10. Seidel D, Ziegler AG (1996) Prediction of type 1 diabetes. Horm Res 45 Suppl 1: 36-9.
11. Ankelo M, Westerlund-Karlsson A, Ilonen J, Knip M, Savola K, et al.(2003) Time-resolved fluorometric assay for detection of Autoantibodies to Glutamic acid Decarboxylase (GAD65) Clinical Chemistry 49 : 908-915.
12. Stryer L (1995) Enzymes: Basic concepts and kinetics: Biochemistry. (4th edn), W.H. Freedman and Company, New York.
13. 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.
14. American Diabetes Association (1995) Office guide to diagnosis and classification of diabetes mellitus and other categories of glucose intolerance. Diabetes Care (suppl. 18) 1: 4-7
15. Newman D (1965) Manometric device, in experimental methods of experimental biology, Macmillan Company.
16. Lowry OH, Rosenrough NN, Farr AL, Randall RJ (1951) Protein measurement with the folin-phenol reagent. J Biol Chem 193: 265-276.
17. Nargessi RD, Landon J, Smith DS (1979) Use of antibodies against the label in non-separation non-isotopic immunoassay: indirect quenching fluoroimmunoassay of proteins. J Immonol Methods 26:307-313.