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BIOCHEMICAL AND MOLECULAR ANALYSIS OF SUGARCANE GLUTAMATE DECARBOXYLASE

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Abstract: Glutamate decarboxylase [GAD (EC 4.1.1.15)], a γ-aminobutyric acid (GABA) metabolizing enzyme is characterized in sugarcane (*Saccharum officinarum* L.) Var. Co 86032. The sugarcane GAD was purified by 7.8 folds and SDS-PAGE analysis revealed presence of apparent 65 and 52 kD GAD isomers. Biochemical characterization of Sugarcane GAD revealed the *Km* values of 1.6 mM for L-glutamate, 2 µM for PLP, 3.5 µM for Ca^{+2} and 6.3 nM for CaM at a sharp optimum pH of 6.0. Ca^{+2}/CaM induced sugarcane GAD by 360%, however Ca^{+2} alone was ineffective. In absence of Ca^{+2}, CaM induced the activity by 150% at pH 6.0, but no such induction was found at neutral pH. Metal ion and inhibitor studies revealed that the sugarcane GAD gets induced by Co^{+2}, 1-10 phenanthroline and requires -SH groups. Isolation of GAD gene through cDNA yielded 1481 bp stretch of sequence occupying distant position in the phylogram of plant GADs. Further analysis confirmed the presence of a plant specific C-terminal extension of 30-amino acid lacking authentic CaM binding domain. The results indicate the presence of at least two forms of GAD in sugarcane.

Keywords: cDNA, γ-aminobutyric acid, Glutamate decarboxylase, metal ions and inhibitors, *Saccharum officinarum*, purification.

INTRODUCTION

γ-aminobutyric acid (GABA), a four carbon, non-protein amino acid, is a significant component of free amino acid pool. It is conserved in prokaryotes to eukaryotes and acts as a major neurotransmission inhibitor in vertebrates [1]. Glutamate decarboxylase (GAD, EC 4.1.1.15) catalyzes the conversion of L-Glutamate to GABA and provides an alternative route for linking glutamate to TCA cycle. In vitro GAD is a pyridoxal-5' phosphate (PLP) dependent enzyme, specific for L-glutamate and exhibits a sharp pH optimum of 5.8 [2,3,4]. GAD is characterized in various plant species [2,3,5, 6, 7, 8, 9, 10, 11] and found to get elevated in its activity by the *in vitro* addition of Ca^{+2}/CaM [5,9,12,13]. Despite a better understanding of the cellular factors stimulating GAD activity, the physiological roles of GAD or GABA have not been clearly established in plants.

The C-terminal CaM binding domain, absent in bacterial and animal GAD genes, have been found in dicotyledonous[7,10,12,14,15] and monocotyledonous plant species[8,16]. Further studies indicated that GAD expression is developmentally controlled at both the transcriptional and translational levels[17] and CaM binding domain is critical for the regulation of the enzyme activity via Ca^{+2} signaling. But, a novel GAD lacking such CaM binding domain has been reported in rice[18] In order to assess the properties and molecular mechanism of GAD regulation we carried the biochemical analysis of sugarcane GAD and isolated gene encoding a distinct GAD lacking CaM binding domain.

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MATERIALS AND METHODS

General Experimental Procedures: The reagent grade chemicals were purchased from Sigma-Aldrich, Sisco Research Laboratory, and Hi-Media, Mumbai.

Extraction of GAD: All the procedures were carried at 4°C, unless stated. 500 g of leaf tissue from greenhouse grown, 40 days old sugarcane Var. Co 86032 was ground in equivalent tissue weight of extraction buffer consisting 50 mM sodium phosphate buffer (pH 5.8), 200 µM PLP and 1 mM PMSF. Insoluble PVP (50 g) of was added separately while grinding. The extract was filtered through four layers of cheesecloth and centrifuged at 12,000 g for 15 min. The supernatant was used as the crude enzyme source.

GAD assay: The activity of GAD was assayed according to Brandon et al., [19] at pH 6.0. Total protein was estimated according to Bradford [20].

Partial purification of sugarcane GAD: The crude enzyme was saturated to 80% with ammonium sulphate and centrifuged at 10,000 g for 10 min. The pellet was dissolved in minimal volume of extraction buffer and dialyzed for 8h against dialysis buffer containing 5mM sodium phosphate buffer (pH 6.0), 200µM PLP, 200 µM DTT and 1 mM PMSF. The dialyzed enzyme was loaded on to a DEAE-cellulose column pre-equilibrated with extraction buffer. The column was washed with 3 column volumes of the same buffer and the bound enzyme was eluted with linear gradient of 50-500mM extraction buffer. The 2ml fractions containing GAD were pooled and loaded onto Sephadex G-75 column pre-equilibrated with extraction

buffer. The fractions were assayed for GAD, separated on native and SDS-polyacrylamide gel[21] and used for further characterization.

Table.I: Fold purification of sugarcane GAD at various purification steps

Purification step	Protein concentrations (mg/ml)	Specific activity U/mg protein	Yield (%)	Fold purity (%)
Crude	1.2	1.8	100	1
Dialysis	2.8	2.78	81.8	154
DEAE-Cellulose	0.44	8.96	9.0	490
Sephadex-G75	0.185	14.05	5.4	780

Characterization of Sugarcane GAD: The pH for the sugarcane GAD was optimized by assaying at different pH using 50mM acetate buffer (pH 3.0-5.8), 50mM sodium phosphate buffer (pH 6.0-7.8) and 50mM tris buffer (pH 8.0-9.0).

The *Km* of sugarcane GAD was determined against different concentrations of L-glutamate, PLP, CaCl2 and CaM. Apo-GAD obtained by exhaustive dialysis of GAD against dialysis buffer without PLP was used for the determination of *Km* for PLP.

The sugarcane GAD was further analyzed for the effect of metal ions and inhibitors on activity (Table-II). The pH of each metal ion and inhibitor solutions (25 mM) were brought to 6.0 while assaying.

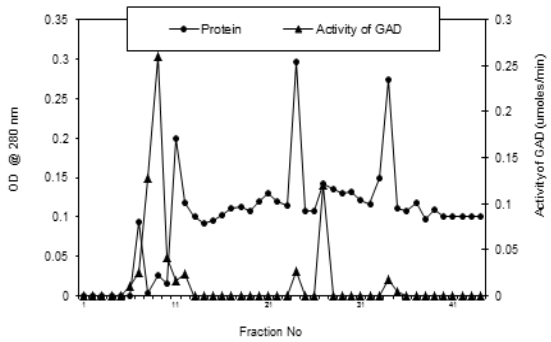

Fig.1: The dialyzed GAD was loaded on to DEAE-Cellulose column pre-equilibrated with 50mM sodium phosphate buffer and the fractions were eluted by establishing a buffer gradient of 50-500mM.

Table.II: Effect of various metal ions and inhibitors on sugarcane GAD (each test is the carried in triplicates)

Metal ion/inhibitor	% activity found
Control	100
DTT	100
α-Ketoglutarate	100
Pyruvate	90
Cysteine-HCL	95
Proline	85
PMSF	100
$MnSO_4$	100
$MgSO_4$	90
$CuSO_4$	15
$ZnSO_4$	90
$CoCl_2$	120
$FeCl_3$	5
$AgNO_3$	105
1-10phenathroline	120
p-hydroxymercuribenzoate	0
Dithionitrobenzoate	0
$HgCl_2$	0

Isolation and characterization of GAD gene: Total RNA was isolated from 40 days old, green house grown leaf tissue from sugarcane var. Co 86032 with an mRNA purification kit (Bangalore Genei Ltd, Bangalore). One µg purified mRNA was used for the synthesis of single-stranded cDNA (MBI Fermentos kit) using oligodt primers at 37°C for 60 min. Subsequently double stranded cDNA was PCR amplified with 5´-ATG AAG CTT GTG CTC TCG CAC GCG AGC-3´ and 3´-TAC CGG CCT AGC CTA CCC T T T CGA AGA CT-5´ primer pair. The PCR product was eluted from 1.2% low-melting agarose gel and sequenced at Bioserve Biotechnologies (India) Pvt. Ltd, Hyderabad.

The sequence obtained was identified by BLAST[22] search at NCBI. The homologous sequence hits were further analyzed with query sequence by multiple alignment with ClustalW 1.7.

RESULTS AND DISCUSSION

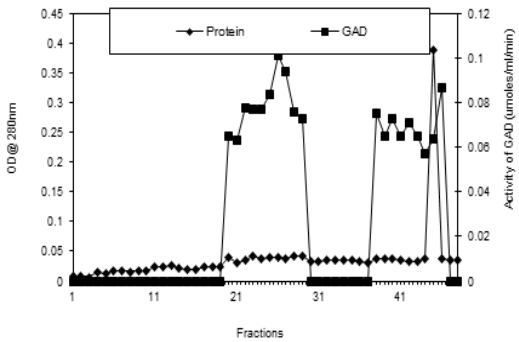

Fig.2: The active fractions collected from DEAE-Cellulose column were loaded onto Sephadex G-75 column pre-saturated with 50mM sodium phosphate buffer. The fractions were collected and assayed for GAD activity.

Sugarcane GAD was partially purified and the elution patterns are shown in Fig.1 and 2. The experiments yielded 7.8 fold purified GAD. SDS-PAGE (Fig.3) shows two GAD isomers of apparent 65 and 52 kD, which can be compared to the reports of plant GAD with multiple identical sub units of 58 kD from squash [23] or 45.5 kD GAD from potato [24] and Cowpea [19]. The 52 kD of sugarcane GAD may be a proteolytic product of 65 kD protein as GAD is a sensitive enzyme to partial proteolysis [13]. Previously reported GAD from many other plants has diversified molecular weights. Barley

had 256 and 120 kD forms of GAD [25], potato GAD is a homodimer of 91 and 43 k Mw on native and SDS-PAGE, respectively [26]. GAD isolated from squash had different Mw on elution with buffer of different pH [24]. It was 340 k Mw in pH 5.8 eluent and 120 k Mw in pH 7.2 eluent, but SDS-PAGE analysis showed a single band of 58 k Mw. The existence of multiple forms of GAD suggests the operation of at least two pathways for GABA metabolism in plants [27]. The presence of two forms of GAD in sugarcane strongly supports this possibility.

pH dependence of sugarcane GAD: The optimum pH for sugarcane GAD was studied using different buffers (Fig.4). The enzyme was optimally active in 50 mM sodium phosphate buffer of pH 6.0, though the activity was between pH 5.2-6.2. The cowpea GAD is active between 5.5-6.0 [19], the potato [23] and wheat GAD is active at sharp pH 5.8 [28].

***Km* of sugarcane GAD:** A double reciprocal plot of sugarcane GAD against different concentrations of L-glutamate showed *Km* of 1.6 mM (Fig. 5) which is comparable to cowpea GAD [19]. Sugarcane apo-GAD has 2 µM *Km* for PLP (Fig.6) similar to the potato apo-GAD [23].

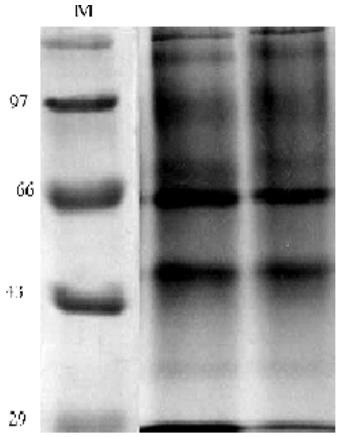

Fig.3: The purified sugarcane GAD was separated on 10% non-denaturing polyacrylamide gel.

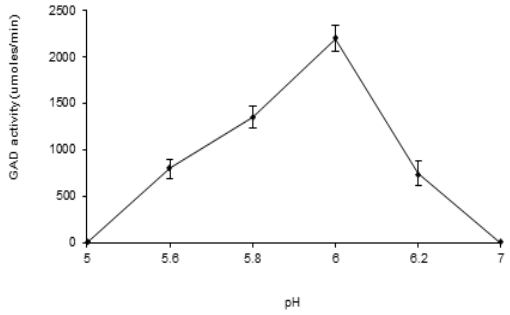

Fig.4: The pH dependence of sugarcane GAD was analyzed using different buffer compositions. The optimum activity was found in 50mM sodium phosphate buffer at pH 6.0 (each value is a mean of triplicate readings)

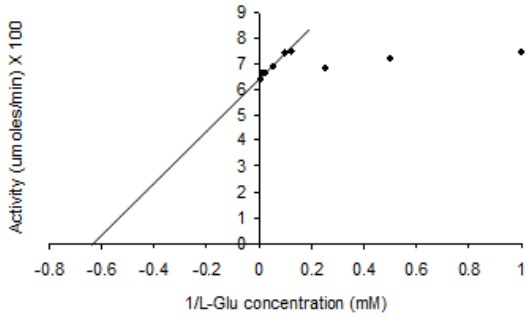

Fig.5: The *Km* of sugarcane GAD for L-glutamate was analyzed against various concentrations of L-glutamate in presence of 500 µM $CaCl_2$ and 200 µM CaM

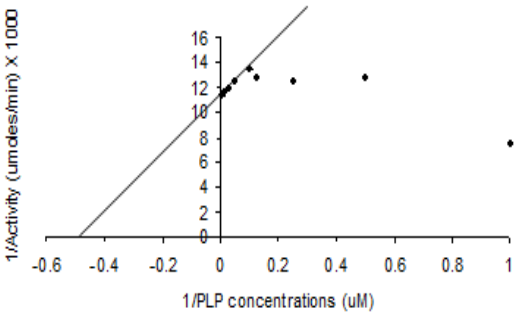

Fig.6: The *Km* of sugarcane GAD for PLP. The purified GAD was exhaustively dialyzed against the 5 mM sodium phosphate buffer (pH 6.0) containing 200 µM DTT and 1 mM PMSF to remove bound PLP and assyed for the *Km* against different concentrations of PLP

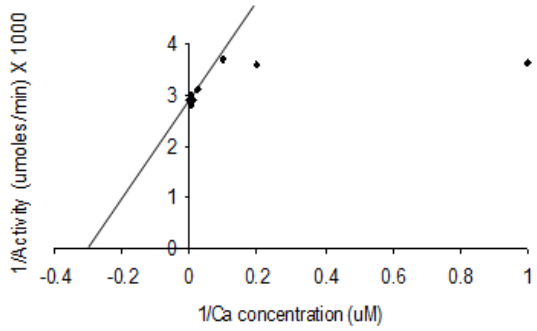

Fig.7: The *Km* of sugarcane GAD for Ca^{+2} was analyzed against different concentrations of $CaCl_2$ in presence of 200 µM CaM and 20 µM PLP.

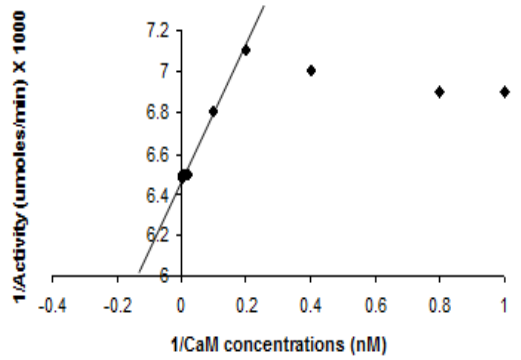

Fig.8: *Km* value of sugarcane GAD for CaM was analyzed against different concentrations of bovine brain CaM in presence of 500 µM $CaCl_2$ and 20 µM PLP

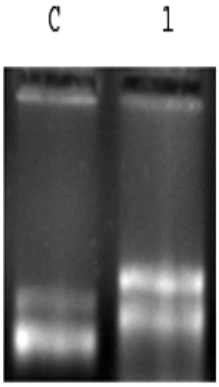

Fig. 9: Total RNA isolated (C-control; 1- Co 86032)

The effect of different concentrations of CaCl2 on GAD activity was also analyzed (Fig.7). The sugarcane GAD displayed a *Km* value of 3.5 µM for Ca^{+2} in presence of 200 nM CaM. The purified soybean GAD was stimulated at saturating concentration of 50-100 µM Ca^{+2} and showed a *Km* value between 7-11 µM [13] but purified GAD from Cowpea did not show any stimulation by Ca^{+2} [19]. It supports the views of Snedden [13] that unprotected GAD from proteolysis is insensitive to Ca^{+2}/CaM.

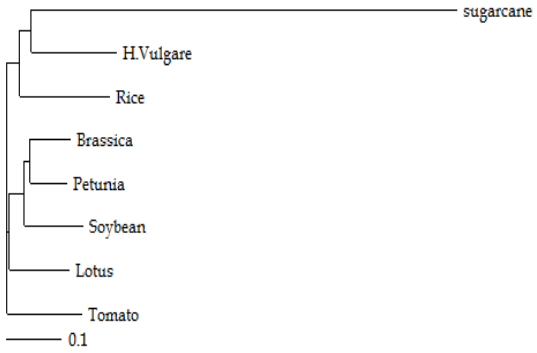

Fig.11: Phylogram of deduced sugarcane GAD with other plant GADs

The sugarcane GAD activity improved with the increasing concentrations of CaM between 10-200 nM, in presence of 500 µM CaCl2. Sugarcane GAD showed a *Km* value of 6.2 nM (Fig.8), which has more specificity for CaM than Soybean GAD [13]. In presence of Ca^{+2}/CaM the activity of crude sugarcane GAD increased by 360%. Interestingly, various environmental stimuli such as hypoxia, water, temperature and mechanical stress trigger an increase in the intracellular Ca^{+2} therefore the possibility of a correlation between GABA production and Ca^{+2} signaling has been suggested [29]. Sugarcane is known for varied responses to stress conditions with imbalanced metabolic activities [30] and the present results indicate the need for the characterization of the specific relation between stress and GABA shunt. In presence of CaM alone, partially purified sugarcane GAD induced by 150%. According to the suggestion of Snedden et al [13] that purified GAD loses its sensitivity towards Ca^{+2}, partially purified sugarcane GAD did not show any elevation in presence of Ca^{+2} alone.

Effect of metal ions and inhibitors: The effect of various metal ions and inhibitors on sugarcane GAD is shown in Table II. Like potato GAD [23], sugarcane GAD was inhibited by typical SH- directed compounds like p-hydroxy mercuribenzoate, dithinitrobenzoate, iodoacetate, Hg^{+2}, Cu^{+2} and Fe^{+3} suggesting the requirement of SH- group for the enzyme activity which facilitate the binding of the inhibitor to enzyme-PLP complex. Pyruvate- one of the immediate amino acceptors of GABA catabolism moderately inhibited the activity but another amino acceptor 2-ketoglutarate was ineffective. Among other compounds analyzed Cysteine hydrochloride, proline, Mg^{+2}, Zn^{+2} inhibited the activity moderately where as PMSF and Mn^{+2} did not alter the activity, but highest induction was observed in presence of Co^{+2} and 1-10 phenathroline.

Characterization of GAD gene:

Sequence analysis of PCR amplified product (Fig. 9) yielded a 1481 bp open reading frame which encodes a 495 amino acid peptide with a molecular mass of ~56 kD which can be referred to the smaller subunit (Fig.3) characterized in the present study. The size of the deduced protein is close to those of *Brassica juncea* (493 amino acids; Accession No. AAS79671), rice (500 amino acid) [18], tomato (502 amino acids) [15] and petunia (500 a.a.) [14] and the sequence surrounding a putative translation initiation codon in these ORFs matched a consensus sequence found in monocot genes [18], suggesting that the cDNA encode a full length GAD sequence. Figure 11 shows an alignment of eight plant GADs. Sugarcane GAD is similar to *OsGAD2* rice GAD clone [18] and occupied the most distant position (Fig.12). We could detect only one GAD gene in sugarcane but Arabidopsis and rice have 5 and 2 genes, respectively [11, 18]. Like other plant GADs [7, 10, 11, 12, 14, 18] the deduced protein from sugarcane carry a C-terminal extension of about 30 amino acids with significant variations and strong conservation of the putative active site domain with a conserved S-X-X-K motif, which is common to PLP dependent enzymes [18]. The C-terminal extension is specific for plant GADs and absent in microbial and animal counterparts. Although there is a little homology among these peptides, previous work indicated that they consist of a potentially forming a basic amphiphilic α-helix, which is characteristic of CaM-binding proteins examined so far [14]. The extensive variation in this motif of present GAD with other GADs (Fig. 13) suggests that we have characterized a gene encoding GAD lacking authentic

CaM binding domain. In rice one such GAD form has been reported and our results indicate that one of GAD form in grasses does not need CaM for its activity. The novel character of sugarcane GAD is reminiscent of the suggestion that some plant GAD activities are Ca^{+2}/CaM independent [9, 12]. It should be noted that plants have more varied CaM isoforms than bacteria and animals and hence we agree with the possibility of interaction between endogenous CaM and GAD inducing the activity. Our biochemical studies have clearly demonstrated the stimulation of sugarcane GAD by Ca^{+2}/CaM indicating the presence of genes encoding Ca^{+2}/CaM dependent GAD in sugarcane. Further functional analyses of the structurally diverged sugarcane GAD isoforms will contribute to a detailed understanding of the fundamental roles of plant GADs.

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