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## *Full Length Research Article*

# GABA: PYRUVATE-DEPENDENT TRANSAMINASE DOMINATES GABA: 2-OXOGLUTARATE DEPENDENT TRANSAMINASE IN SUGARCANE AND THEIR MOLECULAR CHARACTERIZATION

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**ABSTRACT**

GABA occupies a significant component of cytosolic free amino acid pool. GABA-Transaminase (GABA-T, EC 2.6.1.19), the second enzyme of GABA shunt, converts GABA to Succinic semialdehyde (SSA). GABA pyruvate- (GPT) and 2-oxoglutarate- (GOT) dependent gamma-aminobutyrate transaminase (GABA-T) activities were noted in crude leaf extract of sugarcane var. Co 86032. The enzyme was partially purified by DEAE-Cellulose and Sephadex G-100 columns. Non-denaturing PAGE analysis of ~90 folds purified GABA-T showed 65, 60 and 55 kD proteins, which were further confirmed by activity staining. GPT and GOT forms were active at pH 6.2 and 8.2 respectively. The Km values of both forms were determined. Sugarcane GPT was found to prefer beta-alanine and ornithine to GABA but GOT was specific to 2-oxoglutarate. GOT was induced in presence of $Cu^{+2}$ and GPT in presence of $Fe^{+3}$ while proline and succinic semialdehyde inhibited both forms. Sugarcane GABA-T is a pyridoxal 5'-phosphate (PLP) dependent enzyme. A 1458 bp GABA-T gene was isolated and characterized by comparing with the gene sequences from other sources. A 463 amino acid long deduced protein possessed mitochondrial sorting signal but PLP binding site was missing.



## INTRODUCTION

GABA is a four carbon, non-protein ω-amino acid that is ubiquitous in plants, animals, bacteria and fungi (Lawrence and Grant, 1963; Selman and Cooper, 1978; Secor and Schrader, l984; Desmaison and Tixier, 1986; Bown and Shelp, 1989, 1997; Satyanarayan and Nair, 1990). It was discovered in plants more than half a century ago (Steward *et al*., 1949), but interest in GABA shifted to animals when it was revealed that GABA occurs at high levels in the brain, playing a major inhibitory neurotransmitter. Accumulation of GABA in response to various biological and mechanical stress conditions is universal (Streeter and Thompson, 1972; Wallace *et al*., 1984; Reggiani *et al*., 1988; Crawford *et al*., 1994), but its role in plants remains uncertain (Satyanarayan and Nair, 1990; Bown and Shelp, 1997). GABA catabolism begins with its transamination to succinic semialdehyde in a reaction catalyzed by GABA-T. The plant enzyme is able to use both pyruvate and 2-oxoglutarate as amino acceptors, but the affinity varies between tissues (Satyanarayan and Nair, 1990). With pyruvate, the product is alanine, a compound that can be employed as an amino donor for synthesis of glycine and serine (Kleczkowski and Givan, 1988). Alternatively, with 2-oxoglutarate, the product is glutamate; this recycling of nitrogen may allow the plant to eliminate excess carbon (Bown and Shelp, 1997). The inability in early reports to detect GABA-T activity in many plants may be attributed to incorrect (e.g., using 2-oxoglutarate, rather than pyruvate as an amino accepter) and insensitive assay conditions (Satyanarayan and Nair, 1990). With little information available regarding plant GABA-T, any discussion on the regulation of this enzyme is at best speculative. In plants, GABA transamination occurs via different types of GABA-T which probably have specialized functions. The alkaline pH optimum, mitochondrial localization and substrate specificity may play a role in the regulation of GABA-T activity. Feedback inhibition by SSA may also regulate GABA-T, but accumulation of this toxic compound has not been observed (Satyanarayan and Nair, 1990). The present paper reports biochemically characterization the purifed, GABA: pyruvate-transaminase (GPT) and GABA:2-oxoglutarate-transminase (GOT), isolation and characterization of the gene encoding the enzyme

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## MATERIALS AND METHODS

The reagent grade chemicals were purchased from Sigma-Aldrich, Sisco Research Laboratory and Hi-Media, Mumbai, Bangalore Genei, Bengaluru.

### Plant Material

The 40 days old green house grown sugarcane variety Co 86032 served as a source of GAD was used for studying GABA-T. During the present research, all the procedures were carried at $4^0$C, unless stated.

### Extraction of GABA-T

The 500 g of leaf tissue pooled from different sugarcane plants was ground in equivalent tissue weight of extraction buffer consisting 50 mM Tris buffer (pH 8.2), 0.1 µM PLP. The extract was filtered through four layers of cheesecloth and centrifuged at 12,000 g for 20 min. The supernatant served as the crude enzyme source. Total protein was estimated according to Bradford (1976).

### GABA-T Assay

Sugarcane GABA-T isoforms (GPT and GOT) were assayed according to Yonaha *et al*., (1983). GPT was assayed by incubating the enzyme solution at $37^0$C for 30 min in 1 ml reaction mixture containing 50 mM potassium phosphate (pH 6.2), 50 mM GABA, 0.1 µM PLP and 2 mM pyruvate. GOT was assayed in 50 mM Tris buffer at pH 8.2, by replacing pyruvate with 2 mM 2-oxoglutarate from the reaction mixture. The reactions were terminated by adding 100 µl of 25% Trichloro acetic acid (TCA) followed by centrifugation at 6,000 g for 10 min. To 0.5 ml of the supernatant solution 0.1 ml of 0.8 N KOH, 1.0 ml of 1 M glycine-KCl-KOH buffer (pH 8.0) and 1 ml of 25 mM *o*-aminobenzaldehyde in 10% ethanol were added and incubated at $37^0$C for 30 min to develop a yellow colour which was measured at 440 nm. The activities of transaminases were calculated as µ moles of SSA liberated/min; by referring to standard curve, the protein was estimated.

### Partial Purification of Sugarcane GABA-T

The 500 ml crude enzyme extract was saturated to 80% (w/v) with ammonium sulphate. The precipitated protein was separated by centrifuging at 10,000 g for 10 min. The protein pellet was dissolved in minimal volume of extraction buffer and dialyzed against dialysis buffer containing 5 mM Tris buffer (pH 8.2), 0.1 µM PLP for 8 h with three changes in the buffer.

### Ion-exchange Chromatography

The prepared DEAE-cellulose slurry was packed in a column avoiding the formation of air bubble. The column was allowed to settle and washed with 4 bed volume of 50 mM extraction buffer (pH 8.0). The dialyzed enzyme (3 ml) was loaded on to a column pre-equilibrated with extraction buffer. The column was washed with 3 bed volumes of the same buffer and the bound enzyme was eluted with linear gradient of 50-500 mM extraction buffer. The 50 fractions of 2 ml were collected. The fractions were analyzed for the eluted protein by reading the absorbance at 280 nm. All the fractions were screened for GPT and GOT activities and active fractions were pooled. The protein concentration of the pooled fractions was determined as said before.

### Gel filtration Chromatography

The Sephadex G-100 column material was activated by heating the matrix at $50^0$C for 30 min with 50 mM Tris buffer followed by overnight incubation at $4^0$C. The floating fines were removed and loaded into a column of 1.5x60 ml. The column was washed with 3 bed volumes of extraction buffer. The active fractions collected from ion-exchange column were pooled and saturated with 80% ammonium sulphate. The precipitate was separated by centrifuging at 12,000 g for 20 min at $4^0$C and dissolving in 3 ml of extraction buffer. This enzyme was loaded onto Sephadex G-100 column followed by elution with 3 bed volumes of extraction buffer with simultaneous fraction collection. 50 fractions of 2 ml each were collected and analyzed for the eluted protein by measuring the absorbance at 280 nm followed by screening for the active fractions by finding the activities of GPT and GOT. The specific activity of the enzyme eluted at every step of purification was measured and the total fold purification was calculated. The eluted sugarcane GABA-T was further analyzed for determination of its molecular mass by polyacrylamide gel electrophoresis.

### Polyacrylamide gel Analysis of Sugarcane GABA-T

The GABA-T fractions collected from gel filtration column were analyzed on non-denaturing and SDS-polyacrylamide gel (10%) according to Laemmli (1970). The 50 µl of column eluent was mixed with 50 µl of sample buffer and loaded on to the gel. The gel was run at 100V and stained with Coomassie Brilliant Blue R-250.

### Activity Staining

The activity staining of GOT and GPT was performed according to Van Cauwenberghe *et al.*, (2002) in 10% non-denaturing polyacryalimide gel. A filter paper soaked in 100 mM Tris-HCl (pH 8.2) containing 0.15 mM PLP, 200 mM GABA, 12 mM 2-oxoglutarate, 1mM $NADP^+$ and 1.5 units of SSADH was overlaid onto the gel and incubated at $37^0$C for 20 min. GPT activity staining was performed in 50 mM potassium phosphate buffer (pH 6.2), replacing 2-oxoglutarate with 12 mM pyruvate from GOT staining mixture. The NADPH produced in both the reactions was viewed as a fluorescent band under short wavelength ultra violet light (Vilber Lourmate).

### Biochemical Characterization of Sugarcane GABA-T
### Determination of Optimum pH

The pH for the sugarcane GABA-T isoforms was optimized by assaying at different pH using different buffer systems. 50 mM acetate buffer was used to determine the GABA-T activity between pH 3.0-5.8, 50 mM sodium phosphate buffer was used for pH 6.0-7.8, 50 mM Tris buffer was used for pH 8.0-9.0 and 50 mM Glycine-NaOH buffer for pH 9.0-10.0.

### Kinetic Studies

The *Km* values of sugarcane GABA-T isoforms were determined against different concentrations of GABA, amino acceptors pyruvate and 2-oxoglutarate.

The *Km* of sugarcane GPT against substrate GABA was determined by assaying the enzyme against 0.1 to 100 mM GABA. The reaction mixture contained everything as mentioned in the initial method stated for GABA-T assay. The *Km* value was calculated by plotting the 1/GPT activity against 1/[GABA]. The affinity of GPT for pyruvate was analyzed by assaying the enzyme against 0.1 µM to 1 mM pyruvate. The activities obtained were applied into a LB plot and the *Km* value was calculated. The *Km* of GOT for substrate was calculated by assaying the enzyme against varying concentrations of GABA (0.1-100 mM). The reciprocal values of activities obtained were plotted against 1/[GABA] and the affinity of enzyme for the substrate was calculated. The effect of different concentrations of 2-oxoglutarate on the activity of GOT was found by assaying GOT against 0.1 µM to 1 mM 2-oxoglutarate. The values obtained were subjected to LB plot analysis and the *Km* value was calculated.

**Effect of Inhibitors, other Amino Acceptors and Metal ions on GABA-T**

The effect of various inhibitors and metal ions on the sugarcane GABA-T isoforms was analyzed by pre-incubating the enzyme in presence of following compounds of 2 mM reagents at $4^0$C for 20 min. The inhibitors like 1-10phenathroline, p-hydroxymercuribenzoate, PMSF Aminooxyacetate Phenylhydrazine, EDTA and metal ions like $MnSO_4$, $MgSO_4$, $CuSO_4$, $ZnSO_4$, $CoCl_2$, $FeCl_3$, $AgNO_3$', $HgCl_2$ were studied for their effect. The specificity of the GABA-T isoforms for different amino acceptors was analyzed by measuring the activity by replacing pyruvate and 2-oxoglutarate with other amino acceptors like beta alanine, ornithine, glutamine and cysteine. The effect of GABA shunt products on GABA-T was measured by finding the activities in presence of succinic semialdehyde and succinate. The readings were recorded in triplicates and statistically analyzed.

**Isolation and Molecular Characterization of GABA-T 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 $37^0$C for 60 min. Subsequently double stranded cDNA was PCR amplified with 5'-ATGGTCGTTATCAACAGTCTCCG-3' and 5´-TCACTTCTTGTGCTGAGCCTTG-3´ primer pair.

**Amplification of Sugarcane GABA-T Gene**

A PCR reaction mixture of 10 µl contained 1 µl 10 X Taq buffer, 1 µl cDNA template, 0.4 µl forward primer, 0.4 µl reverse primer (10 pM) 0.4 µl dNTP mix, 0.3 µl Taq DNA polymerase (1 u) and 6.5 µl deionized water. The contents of the mixture were mixed thoroughly and added with a layer of 20 µl mineral oil. The PCR involved initial denatruation at $94^0$C for 1 min, and 35 cycles of denatruation at $94^0$C for 30 sec annealing at $62^0$C for 1 min, extension at $72^0$C for 90 sec with a final extension at $72^0$C for 10 min. The amplified products were analyzed on 1.2% (w/v) agarose gel. Agarose gel preparation, electrophoresis, staining was done as said before.

**Sequencing of the Amplified Product**

The amplified product was eluted from the agarose gel and was sent for commercial sequencing at Bioserve Biotechnologies (India) Pvt. Ltd, Hyderabad.

**Sequence Analysis**

The sequence result was further analyzed by submitting for the BLASTn search (Altschul *et al.*, 1997) at NCBI server and the homologous hits were studied. The homologous sequences, across the species were retrieved and analyzed by multiple alignment using Clustal W (1.8) at EBI server. The dendrogram generated was visualized using Tree View 4.0. The sequence was submitted to Gen Bank. The CDs encoding the protein were deduced and compared with translated sequences at NCBI by submitting to tBLASTn. The homologous hits were retrieved and analyzed by multiple alignment using ClustalW and simultaneously a dendrogram was generated. The tree was visualized by TreeView4.0 and studied for the evolutionary relatedness with other similar sequence across the species. The characters of the deduced GABA-T enzyme sequence were analyzed with the help of P Sort.

## RESULTS

**GABA-T Assay**

The sugarcane var. Co 86032 was used as the plant material for the studies. The crude extract contained 5.8 mg/ml protein with 70.7 and 56.6 U/mg of GOT and GPT activities, respectively.

**Partial Purification of Sugarcane GABA-T**

The partial purification steps improved the specific activities of GOT and GPT by about 31 and 32 U/mg respectively. The fold purity of the enzyme was improved by 0.55 fold but lead to 36.7% loss in yield (Table 1).

**Ion-exchange Purification**

The DEAE-Cellulose fractions (Fig.1) were screened for the eluted protein. The eluted protein increased constantly from fraction 3-17. The highest protein was eluted in the fraction 24. The fractions were then screened for the GOT and GPT activities.

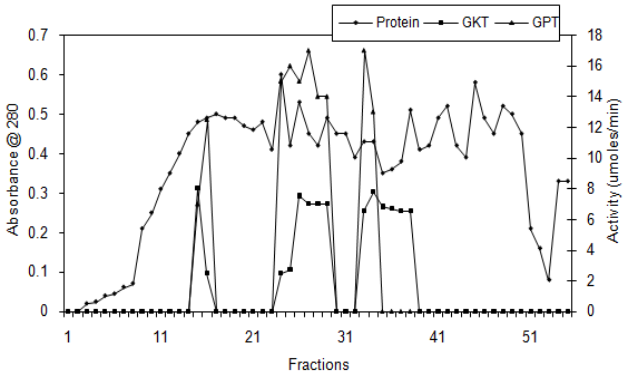


**Fig.1: Elution pattern of GOT and GPT from DEAE-Cellulose column**

**Table 1: Fold purification of sugarcane GABA-T at various purification steps**

| Purification step | Protein concentrations (mg/ml) | Specific activity U/mg protein | | Yield (%) | | Fold purity (%) | |
|---|---|---|---|---|---|---|---|
| | | GOT | GPT | GOT | GPT | GOT | GPT |
| Crude | 5.8 | 70.7 | 56.6 | 100 | 100 | 1 | 1 |
| Dialysis | 3.7 | 101.35 | 88.1 | 63.7 | 63.7 | 143 | 155 |
| DEAE-Cellulose | 0.74 | 202.7 | 305.9 | 12.75 | 11.5 | 286 | 540 |
| Sephadex-G75 | 0.27 | 629.6 | 500 | 4.65 | 2.41 | 890 | 883 |

The GOT was eluted in three series of fractions between 15-16, 24-29 and 33-38. The highest GOT activity (8 µ moles/min) was noted in the fraction number 34. The GPT was eluted in three series of fractions- 15-16, 24-29 and infractions 33-34. The highest GPT (17 µ moles/min) activity was found in fractions 27 and 33. The ion-exchange chromatography step improved the GOT activity by 101 U/mg and GPT activity by 218 U/mg. The fold purity of GOT and GPT was increased by 1.4 and 3.9 folds for GOT and GPT, respectively. But 50.9% and 52.2% loss in the yield was noted for GOT and GPT, respectively.

### Gel filtration Chromatography

The 100 ml eluent was collected in 50 fractions of 2 ml each and initially screened for protein (Fig.2). The protein was continuously eluted from the fraction 8-50, with highest concentration in the 31st fraction. Later the fractions were analyzed for GOT and GPT activities. GOT activities were noted in the fractions 28-33. The highest 15 µ moles/min activity was noted in fraction number 31. GPT activities were detected in fractions 28-38 with highest 9 µ moles/min activity in 30th fraction. The gel filtration purification improved the specific activities of GOT and GPT by 427 U/mg and 194 U/mg respectively. The fold purification of GOT and GPT was increased by 6.1 and 3.4 folds respectively with a loss of 8.1% and 9.1% yield loss. The overall purification experiments yielded 8.9 and 8.8 fold purified GOT and GPT with a loss of 95.4% and 97.6% loss in the yield. The specific activities were increased by 629.6 and 500 U/mg for GOT and GPT respectively.

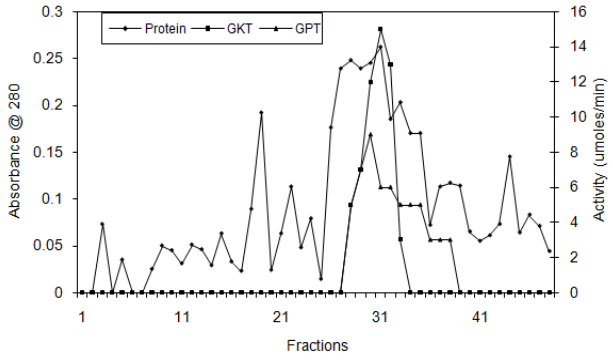


**Fig.2: Elution pattern of GOT and GPT from Sephadex G-100 column**

### Polyacrylamide gel Analysis of GABA-T

The partially purified sugarcane GABA-T was analyzed for the determination of its molecular mass by non-denaturing PAGE. The active protein fraction eluted from Sephadex column showed the presence of three prominent peptides of 65, 60 and 55 kD (Fig.3). These peptides were confirmed as GABA-T by activity staining. The activity stained gel showed the presence of one more GABA-T of ~50 kD which could not be detected by CBB staining as the concentration of this subunit was less. The activity staining of the purified GABA-T was performed separately for GOT and GPT, but the banding pattern did not show any difference. The CBB staining displayed the presence of two more peptides with more than 70 kD molecular weight, which were not GABA-T. The SDS-PAGE analysis did not reveal any difference with the banding pattern.

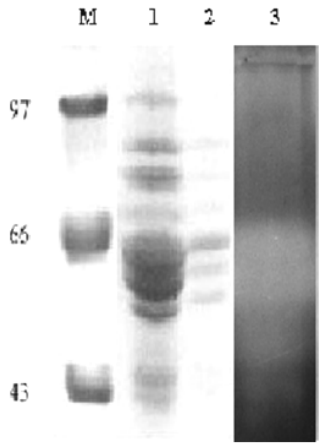


**Fig.3: The photograph showing the partially purified GABA-T and activity staining. (M- Marker; 1-Elute from DEAE-cellulose column; 2- Elute from Sephadex-G100 column; 3-activity staining of partially purified fractions)**

### Biochemical Characterization of GABA-T

### Characterization of GOT

### Analysis of GOT pH Dependence

The optimum pH for the GOT activity was analyzed by assaying in the enzyme at pH 3.0 to 10.0 in different buffer systems. The GOT displayed activity at pH 6.0 to 8.8 (Fig.4).

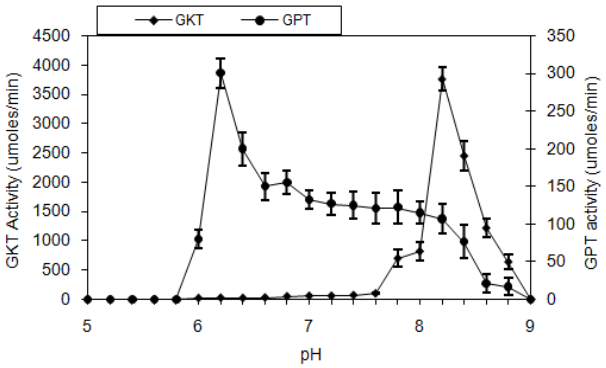


**Fig.4: Characterization of GOT and GPT for optimum pH**

It was sensitive to pH less than 6.0 as it did not show any activity at pH 5.8, suddenly a sharp rise in the activity was

observed at pH 6.0 and 6.2, from where a decline in the activity was seen. It maintained nearly constant activity between pH 6.6 to 8.2 but no activity was found beyond pH 9.0.

### Kinetic Studies of GOT

The GOT obtained from Sephedex G-100 column was used for determination of kinetic constants. The kinetics of purified GOT was studied against various concentration of substrate GABA. A plot of initial velocity V versus substrate concentration [S] showed bell shaped curve indicating that the enzyme was inhibited by excess GABA. The double reciprocal plot (Fig.5) constructed for the calculation of Michaelis-Menten constant (Km) of partially purified GOT for GABA was found to be 1.6 mM. The Michaelis-Menten constant of sugarcane GOT for the amino acceptor 2-oxoglutarate was studied against various concentrations of 2-oxoglutarate. Initial analysis displayed a bell shaped curve indicating that GOT gets inhibited by high concentrations of 2-oxoglutarate. The reciprocal values of initial velocity and [2-oxoglutarate] were plotted on to a double reciprocal plot and a *Km* of 1.4 mM was determined (Fig. 6).

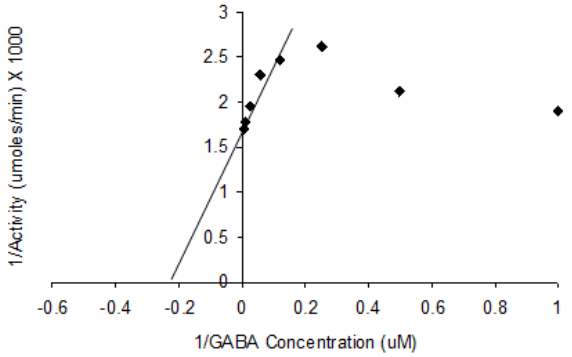


**Fig.5: *Km* graph of GOT against various concentrations of GABA (Each value is the mean of triplicate readings)**

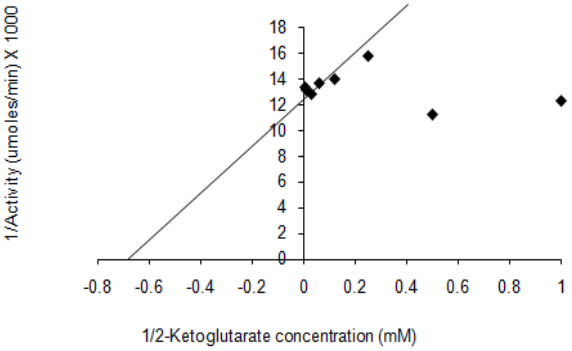


**Fig.6: *Km* graph for GOT against different concentrations of 2-ketoglutarate (Each value is the mean of triplicate readings)**

### Effect of Inhibitors, other Amino Acceptors and Metal ions on GOT

Sugarcane GOT was significantly inhibited in presence of inhibitors like aminooxyacetate, phenylhydrazine, cystein hydrochloride, PMSF, 1-10phenathroline and EDTA. Sugarcane GOT preferred 2-oxoglutarate as the amino acceptor as inhibition of the activity was found in presence of amino acceptors like beta alanine, ornithine and glutamine. Among the metal ions analyzed, $Cu^{+2}$ and $Fe^{+3}$ induced the GOT but all other metal ions inhibited the activity. The other compounds like SSA and DTT also inhibited the GOT activity significantly. Proline was the most potent inhibitor and $Cu^{+2}$ was the significant inducer of the enzyme (Table-2).

**Table 2: Effect of various metal ions and inhibitors on sugarcane GABA-T (each value is a mean of triplicates)**

| Metal ion/inhibitor | % activity found | |
|---|---|---|
| | GOT | GPT |
| Control | 100 | 100 |
| Beta-alanine | -87 | +100 |
| Proline | -89 | -50 |
| Ornithine | -74 | +33 |
| Succinic semialdehyde | -53 | -40 |
| Glutamine | -68 | -59 |
| Succinate | +19 | -75 |
| Aminooxyacetate | -79 | -66 |
| Phenylhydrazine | -11 | +450 |
| DTT | -21 | -100 |
| Cystein hydrochloride | -53 | -100 |
| PMSF | -58 | -100 |
| $MnSO_4$ | -53 | -34 |
| $MgSO_4$ | -53 | -34 |
| $ZnSO_4$ | -32 | +166 |
| $CuSO_4$ | +74 | -100 |
| $CoCl_2$ | -26 | -34 |
| $FeCl_3$ | +21 | +850 |
| 1-10phenathroline | -5 | +166 |
| EDTA | -21 | 100 |

### Characterization of GPT

### Analysis of pH dependence of GPT

The GPT was analyzed at pH 3.0 to 10.0 to find the optimum pH, by assaying the enzyme in different buffer systems. The GPT displayed activity in the pH range 7.8-8.8 (Fig. 4). The highest activity was observed at pH 8.2. It was found to be more sensitive to pH as no activity was found in the pH less than 7.8 and more than 8.8. A sharp raise and decline in the activity was observed from pH 8.0 to 8.2 and from pH 8.2 to 8.6, respectively.

### Kinetic Studies of GPT

The kinetics of purified GPT was studied to find the affinity for the substrate by assaying against various concentration of substrate GABA. Like GOT, GPT also displayed a bell shaped curve when a plot of initial velocity V versus substrate concentration [S] was drawn indicating that the enzyme was inhibited by excess GABA.

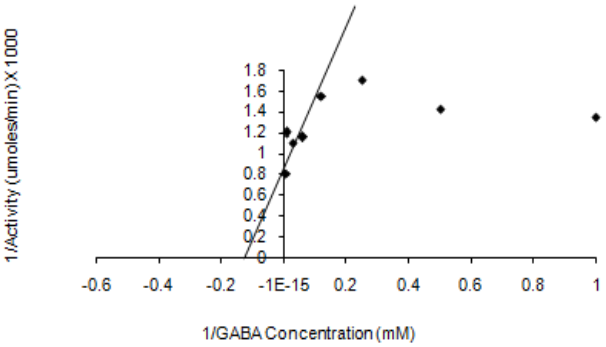


**Fig.7: *Km* graph for GPT against different concentrations of GABA (Each value is the mean of triplicate readings)**

The double reciprocal plot constructed for the calculation of Michaelis-Menten constant (Km) of partially purified GPT for GABA was found to be 9 mM (Fig.7). The effect of different concentrations of amino acceptor pyruvate on GPT was analyzed. Initial analysis displayed a bell shaped curve indicating that GPT gets inhibited by high concentrations of pyruvate. Michaelis-Menten constant of sugarcane GPT for the amino acceptor pyruvate was calculated by plotting the reciprocal values of initial velocity and [pyruvate] on to a double reciprocal plot and a *Km* of 5.2 mM was determined (Fig. 8).

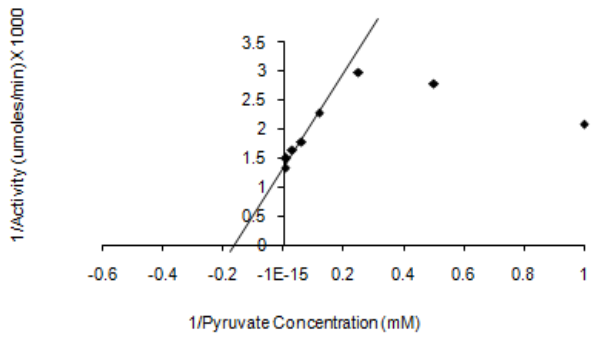


**Fig.8: *Km* graph for GPT against different concentrations of pyruvate (Each value is the mean of triplicate readings)**

### Effect of Inhibitors, other Amino Acceptors and Metal ions on GPT

Sugarcane GPT was significantly inhibited in presence of inhibitors like aminooxyacetate, cystein hydrochloride and PMSF but, 1-10phenathroline and phenyl hydrazine induced the activity where as EDTA was ineffective (Table-2). Sugarcane GPT preferred beta alanine as the amino acceptor as 100% induction in the GPT activity was found. In presence of another amino acceptor Ornithine the induction was observed but it was not as significant as in beta alanine. But, glutamine inhibited the GPT activity. Among the metal ions analyzed, $Zn^{+2}$ and $Fe^{+3}$ induced the GPT where as all other metal ions proved as inhibitors of GPT activity. The other compounds like SSA, succinate, proline and DTT inhibited the GPT activity significantly. Phenylhydrazine induced the activity but highest induction was observed in presence of $Fe^{+3}$. The DTT, Cystein hydrocholoride, PMSF and $Cu^{+2}$ were most potent inhibitors of sugarcane GPT.

### Isolation and Molecular Characterization of Sugarcane GABA-T Gene

The sugarcane GABA-T gene was isolated through its cDNA. The gel analysis clearly displayed intact RNA (Fig. 9) and the concentration of the isolated RNA was nearly 1 µg/µl.

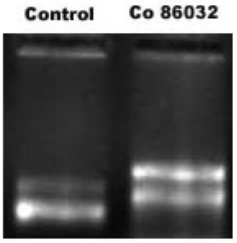


**Fig. 9- Total RNA isolated from sugarcane Var. Co 86032**

### PCR amplification of Sugarcane GABA-T

The first strand cDNA synthesized was used as the template for the amplification of GABA-T gene. The PCR conditions for the successful amplification were optimized by various trials. The amplification product was analyzed on agarose gel and the gel (Fig. 10) displayed a clear, single band of 1.5 kb fragment. The amplified product was eluted from the gel and sequenced.

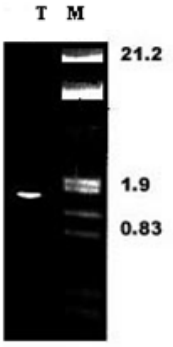


**Fig.10- PCR amplification of GABA-T gene**

### Sequence Analysis of Sugarcane GABA-T

The sequenced sugarcane GABA-T gene was 1468 b in length. The sequence obtained was initially analyzed to identify the sequenced stretch of DNA by BLASTn search. The BLASTn search hit the homologous GABA-T gene sequences isolated from different species. This confirmed that the sequence obtained was GABA-T gene from sugarcane. Further, we retrieved the homologous hits resulted from BLASTn and aligned them by ClustalW. The multiple alignment showed many conserved sequences across the species. The sequence was submitted to NCBI allotted with an accession no-DQ 518268. The sugarcane GABA-T gene was found to bear 6 ORFs, among them, ORF 4 and 6 beard the sorting signals for mitochondrial inter membrane space and ORF 5 beard the signal for plasma membrane. The GABA-T gene sequences for further analyzed for the CDs coding for the protein by submitting the sequence for tBLASTn search. This search matched with number of translated GABA-T protein sequences across the species. The 463 amino acid long deduced sugarcane GABA-T is presented in Seq.2. The homologous hits resulted from tBLASTn were retrieved and aligned with the help of ClustalW. The multiple alignment displayed number of conserved domains of varying length. The analysis for the GABA-T displayed a stretch of 26 amino acids specific to mitochondrial signal sequence, from 2-27 amino acids, though it varied at many positions.

## DISCUSSION

In the present study we have detected the presence of both GOT and GPT for the first time in sugarcane, in the ratio 3.0:3.7 and this is similar with the previous reports stating GABA-T appears to prefer pyruvate to 2-oxoglutarate (Shelp *et al.*, 1995; Van Cauwenberghe and Shelp, 1999) The tissue specific presence of GABA-T has been reported in many plants and the GPT to GOT ratio was found to vary among the plant species (Shelp *et al.*, 1995 Dixon and Fowden, 1961 Wallace *et al.*, 1984 Streeter and Thompson, 1972 Van

Cauwenberghe and Shelp, 1999 Palanivelu *et al*., 2003). The sugarcane GABA-T isoforms were partially purified by ~90 folds with ion-exchange and gel filtration chromatography. In all the fractions, GPT activity was more than GOT and this step of purification increased the purity of GOT and GPT by 2.8 and 5.4 folds respectively. But, interestingly, in the gel filtration fractions analyzed for GABA-T activity, GOT activity was found more than GPT and fold purification was also 2.3 folds more in GPT. It seems like sugarcane GPT is less stable than GOT. No report is available on the purification and characterization of plant GOT. Among the only two reports available on the purification of GABA-T, one states about 1530 fold purification of GPT from tobacco leaf tissue (Van Cauwenberghe and Shelp, 1999) and the other about 349 fold purification of GPT from anaerobically induced alanine amino transferase from barley roots (Good and Muench, 1992).

The PAGE gel showed the presence of three low molecular mass isoforms of sugarcane GABA-T. These three bands were confirmed as GABA-T by activity staining for GOT and GPT separately. But, here also, the unique presence of banding for either GOT or GPT were not distinguished. It again confirms that, the three bands of partially purified sugarcane GABA-T were active with both amino acceptors and no separate proteins were detected. Other plant aminotransferases have been reported to have native molecular masses ranging from 80-220 kD and are either dimers or tetramers with subunits ranging from 40-56 kD (De-Eknamkul and Esslis, 1987, Hoffman *et al*., 1986, Numazawa *et al*., 1989, Takada and Noguchi, 1985, Wightman and Forest, 1978). A 97 kD GPT was purified from barley roots (Good and Muench, 1992) with a subunit of 50 kD, indicating the possibility of a homodimer enzyme. Three and two GPT isoforms were found to be active in the leaves of *Atriplex spongiosa* (Hatch, 1973) *Panicum maximum* (Numazawa *et al*., 1989), respectively. The comparative analysis of our results indicates that we have characterized the subunits of sugarcane GABA-T.

The sugarcane GOT was optiumumly active at pH 8.2 but the range of active pH was less than that of GPT. The GPT displayed a broader range of pH for its activities and optimumly active at pH 6.2. Barley GPT was active at optimum pH 8.0 (Good and Muench, 1992) while, tobacco GPT was active at pH 8.2. This difference in the optimum activities at different pH indicates the unique functions carried by the same protein. The sugarcane GPT showed higher affinity for both GABA and pyruvate than that of GOT for GABA and 2-oxoglutarate. Both the forms of transaminases exhibited the substrate inhibition similar to the tendency observed for GPT in tobacco (Van Cauwenberghe and Shelp, 1999). The GABA-T isolated from barley roots has *Km* values of 500 μM for 2-oxtoglutarate and 60 μM and 1 mM for pyruvate and glutamate respectively (Good and Muench, 1992). As compared to these reports the sugarcane GABA-T isoforms displayed less affinity for substrate as well as the amino acceptors.

Like mammal GOT (John and Fowler, 1985), Beta alanine was found to bind sugarcane GOT irreversibly resulting in strong inactivation of the enzyme, unlike tobacco GPT (Van Cauwenberghe and Shelp, 1999), sugarcane GPT was found to prefer beta alanine and ornithine to pyruvate. SSA, the immediate product of transamination of GABA, inhibited both GABA-T forms, which keys the negative regulation on GABA-T and help in maintaining the higher GABA concentrations in adverse conditions. Aminooxyacetate interferes with the binding of coenzyme PLP (Kobayashi *et al.*, 1977, Yamaya and Matsumoto, 1985, Soper and Manning, 1985). The inhibition of sugarcane GABA-T by aminooxyacetate indicates that sugarcane GABA-T is a PLP dependent enzyme like other plants (Van Cauwenberghe and Shelp, 1999, Kwon *et al.*, 1992, Kim *et al.*, 1997) unlike barley GPT (Good and Muench, 1992). Among metal ions, $Cu^{+2}$ induced GOT sugarcane, but interestingly it strongly inhibited GPT activity. Though, GOT was moderately induced by $Fe^{+3}$, GPT is found to be $Fe^{+3}$ dependent enzyme.
The sequencing of the amplified product resulted in 1458 bp long stretch. The *Arabidopsis* GABA-T gene was 1794 bp in length (Van Cauwenberghe *et al*., 2002). The alignment showed many conserved domains in the sequences. The dendrogram generated showed that the sugarcane GABA-T gene sequence has larger similarities with that of rice and mouse. It was seen that non-plant GABA-T gene had more homology with plant GABA-T gene sequences. The CDs present on the gene were analyzed by ORF finder and sugarcane GABA-T had 6 such ORFs, among them ORF4 and ORF6 had sorting signals for mitochondria. It authenticates the site of GABA-T as mitochondria (Breitkruez and Shelp, 1995). The tBLASTn analysis yielded the translation product of the gene which was 463 amino acids long, which showed the presence of many conserved domains on the protein sequence.

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