Annals of Plant Sciences
ISSN: 2287-688X
Original Article

Succinic semialdehyde specific, NAD+ -dependent, Succinic semialdehyde Dehydrogenase from Sugarcane requires –SH group for activity and its gene resembles its rice counterpart

Gireesh Babu K[1]* and Naik GR[2]
[1]BioGenics, Veena Plaza, P.B. Road, Unkal, Hubli, Karnataka, India
[2]Department of Biotechnology, Gulbarga University, Gulbarga 585 106, Karnataka, India

Received for publication: March 15, 2013; **Accepted**: May 22, 2013.

Abstract: Succinic semialdehyde dehydrogenase (SSADH), oxidizes Succinic semialdehyde produced by first two enzymes of a γ-aminobutyric acid (GABA) shunt and links the shunt with TCA cycle. In the present study the biochemical properties of *Saccharum officinarum* L. (Var. Co 86032) were studied and the corresponding partial gene was isolated and sequenced. The sugarcane SSADH was purified by 6.9 folds and SDS-PAGE analysis revealed presence of ~45 kD protein, possibly a homomultimer. Biochemical characterization of Sugarcane SSADH is specific for SSA with 2.2 µM *Km* value. It displayed a broad activity range between pH 8.4-9.4 and found optimumly active at pH 8.8 and 9.0. Sugarcane SSADH was severely inhibited by thiol directed reagents and hence requires a SH group for catalytic activity and /or maintaining the active conformational structure of the enzyme. Isolation of SSADH gene through cDNA yielded 1425 bp stretch of sequence, resembling its counterpart from rice. Further analysis of deduced protein varied at characteristic positions of mature dehydrogenases, indicating a partial sequence

Keywords: cDNA, γ-aminobutyric acid, kinetic studies, *Saccharum officinarum,* Succinic semialdehyde dehydrogenase, purification.

Introduction

GABA is a non-protein ω-amino acid found ubiquitously (Satya Narayana and Nair, 1990, Metzer and Halpern, 1990, Tillakaratne et al, 1995) and metabolised by three enzymes of the GABA shunt. GABA is produced by the decarboxylation of L-glutamate by Glutamate decarboxylase (GAD, EC 4.1.1.15) (Baum et al, 1993). GABA-Transaminase (GABA-T, EC 2.6.1.19) converts GABA to Succinic semialdehyde (SSA) (Dixon and Fowden, 1961, Streeter and Thompson, 1972), which is then oxidized to succinate by Succinic semialdehyde dehydrogenase (SSADH, EC 1.2.1.16), coupled with NADH production (Bueitkruez and Shelp 1995).

Aside from its occurrence in a wide range of organisms, the GABA shunt has distinct physiological functions and regulatory mechanisms in different organisms. In plants, GABA is known to control the cytosolic pH, balance between carbon and nitrogen metabolism and adaptation to stress (Bown and Shelp, 1997, Snedden and Fromm, 1999) but very less is known about the regulation of the shunt in the plant. Two regulatory check points of the GABA shunt have been described in plants: positive regulation of GAD by Ca^{+2}/CaM in the cyosol and negative regulation of SSADH by ATP and NADH in mitochondrion (Busch and Fromm, 1999, Busch et al, 2000). The former is considered to be a mechanism involved in the activation of the enzyme in response to stress, whereas the latter is thought to control the GABA shunt by mitochondrial energy charge and reducing potential. Though, SSADH was purified and characterized in some plants (Yamaura et al, 1988, Satya Narayan and Nair, 1989, Busch and Fromm, 1999), the extent of literature available is less. Busch and Fromm (1999) cloned SSADH gene from Arabidopsis and characterized the recombinant enzyme. In the present study, as an objective of characterization of GABA shunt in sugarcane, we targeted the biochemical and molecular characterization of SSADH to understand the properties of the enzyme and the corresponding gene, for the first time in sugarcane.

Materials and Methods

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

***Corresponding Author:**
Dr. Gireesh Babu K,
BioGenics, Veena Plaza, P.B. Road,
Unkal, Hubli, Karnataka, India.

All the experiments were carried at 4^0C, unless stated. 500 g of leaf tissue collected from 40 days old greenhouse grown sugarcane Var. Co 86032 was ground in equivalent tissue weight of extraction buffer consisting 25 mM sodium phosphate buffer (pH 9.0), 1 mM DTT. 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.

Sugarcane SSADH was assayed according to Baush and Fromm, (1999). The 500 µl reaction mixture containing 0.1 M sodium phosphate buffer (pH 9.0), 1 mM DTT, 0.1 mM SSA, 0.5 mM NAD^+ and 50 µl enzyme extract at 25^0C. Measurements of the SSADH activity were recorded between 5 sec to 2 min at 340 nm. Total protein was estimated according to Bradford (1976).

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 8 h against dialysis buffer containing 5 mM sodium phosphate buffer (pH 9.0) and 1 mM DTT. 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 as 2 ml fractions with a linear gradient of 50-500 mM extraction buffer. The fractions containing SSADH were pooled and loaded onto Sephadex G-100 column pre-equilibrated with extraction buffer. The fractions (2 ml) were assayed for SSADH activity and separated on native and SDS-polyacrylamide gel (Laemmli, 1970).

The optimum pH for the partially purified sugarcane SSADH was determined by assaying at different pH using 0.1 M acetate buffer (pH 3.0-5.8), 0.1 M sodium phosphate buffer (pH 6.0-9.0), 0.1 M Tris buffer (pH 8.0-9.0) and 0.1 mM Glycine-NaOH buffer (pH 9.0-10.0).

The kinetic studies of sugarcane SSADH were carried by assaying the enzyme against different concentrations of SSA. The assays were carried in triplicates, as mentioned above.

Various amino acceptors, metal ions and inhibitors were analyzed for their effect on the enzyme activity (Table-II). The assays were carried by pre-incubating the enzyme at 4^0C for 20 min, in reaction mixture containing 0.5 mM concentrations of metal ions and inhibitors, brought to pH 9.0.

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

Purification step	Protein concentrations (mg/ml)	Specific activity U/mg protein	Yield (%)	Fold purity
Crude	4.2	0.7	100	1
Dialysis	3.7	0.85	88	1.21
DEAE-Cellulose	0.62	3.54	14.9	5.05
Sephadex-G75	0.39	4.83	9.3	6.9

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

Metal ion/inhibitor	% activity found
Control	100
Acetaldehyde	-80
Glyceraldehydes 3- phosphate	-80
GABA	-85
DTT	+600
Cysteine-HCL	+250
Proline	-85
PMSF	-30
$MnSO_4$	85
$MgSO_4$	100
$CuSO_4$	-72
$ZnSO_4$	90
$CoCl_2$	15
$FeCl_3$	90
$AgNO_3$	120
$HgCl_2$	0
1-10phenathroline	5
p-hydroxymercuribenzoate	-120
Dithionitrobenzoate	-105
$NADP^+$	-80

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 37^0C for 60 min. Subsequently double stranded cDNA was PCR amplified with 5´-ATGGAAGCTTTAATAGGAGCAGCAGCGCG-3´ and 3´-CGAACCCTCTA TACTTATCTGTTTCGAAGACT-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 (Altschul et al, 1990) search at NCBI. The homologous sequence hits were further analyzed with query

sequence by multiple alignments with ClustalW 1.7.

The two step purification of sugarcane SSADH yielded ~7 fold purified protein (Table-I). The elution pattern of the purification steps are depicted in figure 1 and 2. The specific activity of 4.9 units/mg protein is higher than the reported activity from barley (Yamura et al, 1988) and human (Chambliss and Gibson, 1992) but less than the activity reported in potato (Satya Narayan and Nair, 1989) or recombinant Arabidopsis (Busch and Fromm, 1999). The SDS- PAGE analysis (Fig. 4) showed the presence of a ~ 45 kD prominent protein along with 4 more less intense bands. The non-denaturing PAGE analysis (data not shown) did not show any difference suggesting that the enzyme was either a monomer or a polymer of identical subunits. The recombinant SSADH from Arabidopsis SSADH is a homotetramer of a 53 kD mitochondrial subunit (Busch and Fromm, 1999), but potato SSADH is either a monomer or a polymer of 35 kD subunits.

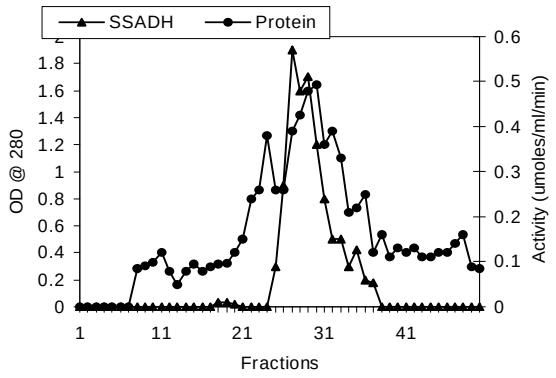

Fig.1: SSADH elution pattern from DEAE-Column

The sugarcane SSADH reaction was proportional to the enzyme concentration under all the assay conditions employed. The optimization of the pH was carried by using different buffer systems and the highest activity was observed in sodium phosphate buffer pH 8.8-9.0, though the activity range of the enzyme was between pH 8.4 -9.4. The figure 3 shows a typical bell shaped pH-activity curve of sugarcane SSADH. Among the other two buffer systems tested, sugarcane SSADH lost 28% and 88% activity in Glycine-NaOH and tris buffer, respectively, at pH 9.0. The present study confirms property of potato SSADH (Satya Narayan and Nair, 1989) on inhibition by tris.

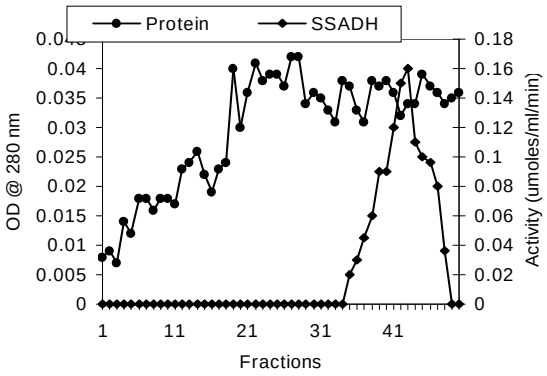

Fig.2: SSADH elution pattern from Sephadex G-100 column

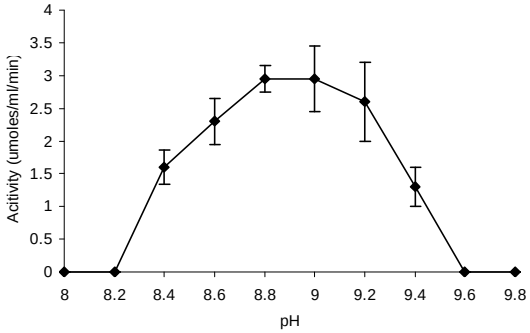

Fig.3: Graph showing the SSADH activity at various pH

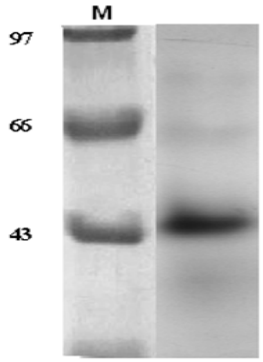

Fig.4: SDS-PAGE separation of partially purified sugarcane GAD (M-marker; lane 2-SSADH)

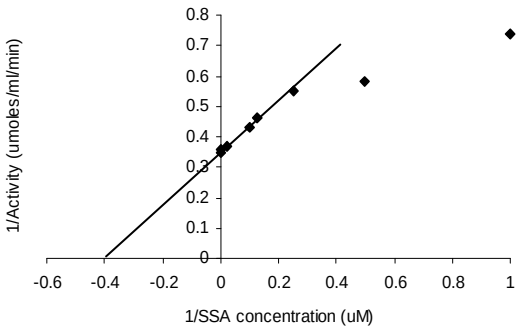

Fig.5: LB plot of SSADH against SSA

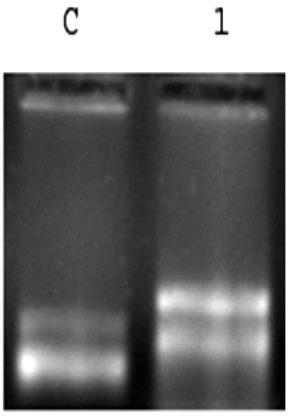

Fig.6: Total RNA isolated (C-control RNA; 1-Co 86032)

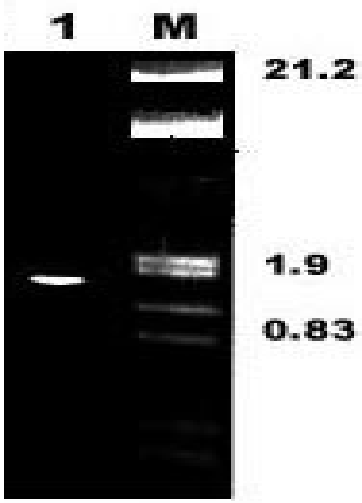

Fig.7: PCR amplification of sugarcane SSADH gene

The kinetic studies of sugarcane SSADH against 1µM 10 mM SSA concentration followed normal, Michaelis-Menton type of kinetics and found to have a *Km* value of 2.2µM (Fig 5), which is less than the corresponding values of potato (4.6µM, Satya Narayan and Nair, 1989) and recombinant arabidiopsis (500 µM, Busch and Fromm,1999). The enzyme was inhibited above 150µM SSA concentration. Sugarcane SSADH was specific to SSA and NAD^+ as it did not show activity in presence of other substrates tested [acetaldehyde and glyceraldehyde 3-phosphate (Table-II)] and could not utilize $NADP^+$ as cofactor. The recombinant Arabidopsis and potato SSADH (Satya Narayan and Nair, 1989, Busch and Fromm, 1999) carried the same properties.

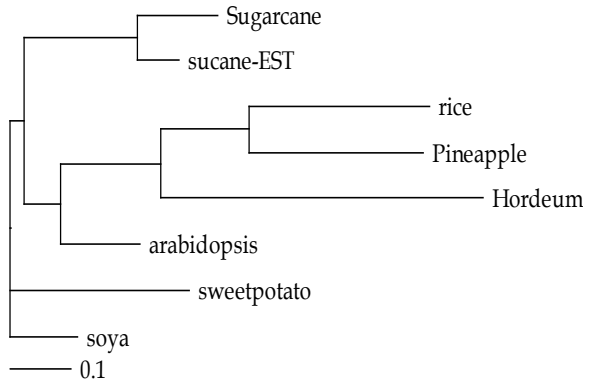

Fig. 8: Phylogram of Sugarcane SSADH gene with genes from other plants

The sugarcane SSADH was severely inhibited by thiol-directed reagents like p-Hydroxy mercuri benzoate and dithonitro benzoate (Table-II). It was also found that the heavy metal ions like Hg^{+2} and Cu^{+2} inhibited the activity, strongly suggesting sugarcane SSADH requires a SH group for catalytic activity and/or maintaining the active conformational structure of the enzyme. The activity at higher pH also suggests that the reaction involved a protein functional group with a pK_a value about 8.6, resembling that of a cysteinyl SH group.

Sequencing of PCR amplified product (Fig.7) yielded a 1425 bp stretch of DNA. The BLAST analysis at NCBI, hit the SSADH sequences across the species and those sequences were analyzed further by multiple alignment. The phylogram was generated (Fig.8) using gene sequences from other plants. Open reading frame of sugarcane SSADH was used to deduce the amino acid sequences of the protein and it was found to encode a 455 amino acid peptide. Though the translated BLAST hit the SSADH deduced proteins from other species, the present sugarcane SSADH varied at many characteristic positions of dehydrogenases. It varied at C-terminal conserved sequences (EEIFGP) found in most other alcohol dehydrogenases (Busch and Fromm, 1999) and LELGGNAP, the ADH Glu active site (Chambliss et al., 1995). From this analysis, it is clear that at least 100 more amino acids are missing in the deduced protein, compared to the mature protein from other species, as we could isolate a partial gene sequence.

Acknowledgement

We are thankful to Council of Scientific and Industrial Research, New Delhi for the

award of Senior Research Fellowship to Gireesh Babu K.

References

1. Satya Narayana V, Nair P.M. Metabolism, enzymology and possible roles of 4-aminobutyrate in higher plants, Phytochemistry. 1990, 29:367-375.

2. Metzer E, Halpern Y.S. In vivo cloning and characterization of gabCTDP gene cluster of *Eschericdhia coli* K-12. J. Bacteriol. 1990, 172: 3250-3256.

3. Tilakaratne JJK, Medina-Kauwe L, Gibson KM. γ-Aminobutyric acid (GABA) metabolism in mammalian neural and non-neural tissues. Comp Biochem Physiol. 1995 11: 247-263.

4. Baum G, Chen Y, Arazi T, Takatsuji H, Fromm H. A plant glutamate decarboxylase containing a calmodulin binding domain. Cloning, sequence and functional analysis. J Biol Chem. 1993 268 19610-19617.

5. Dixon ROD, Fowden L. Gamma-aminobutyric acid metabolism in plants. Part 2. Metabolism in higher plants. Ann Bot (London). 1961 5: 513-530.

6. Streeter JG. Thompson J.F. Anaerobic accumulation of gamma-aminobutyric acid and alanine in radish leaves (*Raphanus sativus* L.). Plant Physiol. 1972, 49:572-578.

7. Breitkruez K, Shelp BJ Subcellular compartmentation of 4-aminobutyrate shunt in protoplasts from developing soybean cotyledons. Plant Physiol. 1995, 108: 99-103.

8. Bown AW, Shelp BJ. The metabolism and function of γ-aminobutyric acid. Plant Physiol. 1997, 115: 1-5.

9. Snedden WA, Fromm H. Regulation of the γ-Aminobutyrate-synthesizing enzyme, glutamate decarboxylase by calcium-calmodulin: a mechanism for rapid activation in response to stress. *In*: Plant responses to Environmental Stresses: From Phytohormones to Genome Reorganization. Ed: Lerner H.R., Marcel Dekker, New York, 1999, pp. 549-574.

10. Bush KB, Fromm H. Plant Succinic Semialdehyde Dehydrogenase. Cloning, Purification, Localization in Mitochondria, and Regulation by Adenine Nucleotides. Plant Physiol. 1999, 121: 589-597.

11. Yamura I, Matsumoto T, Funatsu M, Shinohara T. Purification and some properties of Succinic semialdehyde dehydrogenase from barley seeds. Agric Biol Chem. 1988, 52: 2929-2930.

12. Satya Narayan V, Nair PM. Potato tuber succinate dehydrogenase: purification and characterization. Arch Biochem Biophys. 1989, 275: 469-477.

13. Bradford MM. A Rapid and sensitive method for quantification of microgram quantities of protein utilizing the principles of protein dye-binding. Ann Biochemistry 1976, 32: 248-253.

14. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970, 227: 680-685.

15. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990, 215 403-410.

16. Chambliss KL, Caudle DL, Hinson DD, Moomaw CR, Slaughter CA, Jakobs C, Gibson K.M. Molecular cloning of the mature NAD^+-dependent Succinic semialdehyde dehydrogenase from rat and human. J Biol Chem. 1995, 270: 461-467.

Source of support: Nil
Conflict of interest: None Declared