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Purification and physico-kinetic characterization of 3β-hydroxy specific sterol glucosyltransferase from *Withania somnifera* (L) and its stress response

Bhaskara Reddy Madina [a], Lokendra Kumar Sharma [a], Pankaj Chaturvedi [a], Rajender Singh Sangwan [b], Rakesh Tuli [a,*]

[a] *National Botanical Research Institute, Rana Pratap Marg, Lucknow-226001, (U.P.) India*
[b] *Central Institute of Medicinal and Aromatic Plants, Lucknow-226015, India*

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Abstract

Sterol glycosyltransferases catalyze the synthesis of diverse glycosteroids in plants, leading to a change in their participation in cellular metabolism. *Withania somnifera* is a medically important plant, known for a variety of pharmacologically important withanolides and their glycosides. In this study, a cytosolic sterol glucosyltransferase was purified 3406 fold to near homogeneity from *W. somnifera* leaves and studied for its biochemical and kinetic properties. The purified enzyme was active with UDP-glucose but not with UDP-galactose as sugar donor. It exhibited broad sterol specificity by glucosylating a variety of sterols and phytosterols with 3β-OH group. It showed a low level of activity with flavonoids and isoflavonoids. The enzyme gave maximum K_{cat}/K_m value (0.957) for 24-methylenecholesterol that resembles aglycone structure of pharmacologically important sitoindosides VII and VIII from *W. somnifera*. The enzyme follows ordered sequential bisubstrate mechanism of reaction, in which UDP-glucose and sterol are the first and second binding substrates. This is the first detailed kinetic study on purified plant cytosolic sterol glucosyltransferases. Results on peptide mass fingerprinting and substrate specificity suggested that the enzyme belongs to the family of secondary metabolite glucosylating glucosyltransferases. The enzyme activity exhibited a rapid in vivo response to high temperature and salicylic acid treatment of plants, suggesting its physiological role in abiotic and biotic stress.

Keywords: Heat stress; Salicylic acid signal; Sterol glucoside; Sterol glucosyltransferase; Stress response; Substrate specificity; Withanosides

1. Introduction

Enzymatic glycosylation involves the transfer of sugar from an activated donor (nucleotidediphosphate sugar) to an aglycone substrate. Sterol glycosyltransferases in plants catalyze glycosylation of phytosterols and related compounds to generate their glyco-conjugates. Sterol glycosyltransferases play an important regulatory role in the activity of sterols in higher organisms ranging from molds and plants to insects and mammals [1–3]. A group of oxidized sterols, called brassinosterols, function as plant growth regulators and influence growth and development in plants [4]. Mutations in sterol transformation pathway deviates the normal balance of different sterol metabolites and lead to defects in embryonic and post-embryonic development and flower morphogenesis [5].

In plant cells, sterols are synthesized primarily in endoplasmic reticulum using mevalonate pathway of isoprenogenesis by generating prenyl precursors from cytosol. Some contribution of the plastid localized DOXP pathway of isoprenogenesis has also been suggested [6]. These isoprene units lead to the biosynthesis of 2,3-oxidosqualene, which serves as the common progenitor of different classes of sterols. Most of the higher plant sterols possess β-OH group at C-3 position and largely occur in free form. However, some of them are structurally diversified through a variety of catalytic transformations including desaturation, chain-elongation, cyclisation, esterification, epoxidation, hydroxylation and glycosylation. Amongst

* Corresponding author. Tel./fax: +91 522 2205698 (Direct); tel.: +91 522 2205849x324 (Off), +91 522 2789499, 2789535 (Res.); fax: +91 522 2205836, 2205839.
E-mail address: rakeshtuli@hotmail.com (R. Tuli).

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them, cytochrome P_{450} dependent oxidations and glycosylations represent the most predominant transformations [7]. Glycosylation not only stabilizes the products but also modulates their physiological activities and governs intracellular distribution [8]. Glycosylation enhances water solubility of otherwise lipophilic membrane sterols and therefore can lead to a change in cellular mobility, fluidity, permeability, hydration and phase behavior [9–11]. Glycosylation of bioactive secondary metabolites including sterols is considered as a promising option for improving their bioavailability and pharmacokinetics [12,13].

Sterol glycosides and acyl sterol glycosides mainly comprise Δ^5-sterols and are believed to be synthesized and located in plasma membrane [14]. They are rapidly induced in cells from molds to humans by exposure to environmental stresses [3,15]. The exogenously added cholesterol glucosides induce heat shock pathway in human fibroblast cells [1]. Transcriptional analysis using microarray comprising a large set of genes including, 109 secondary product glycosyltransferases [16] suggested the role of glycosylation in defense response of *Arabidopsis thaliana*. In *Solanum aculeatissimum*, the expression of a gene for 3-*O*-glycosylation of steroidal sapogenins was reported to decrease systemically in response to the wounding of leaf [17]. The involvement of glycosylated sterols in sensitivity to fungal pathogens has been reported in oat [18]. In spite of these examples, suggesting the importance of sterol glycosides, no work has been reported on the role of plant sterol glucosyltransferases in stress response.

Some purified sterol glucosyltransferases that glucosylate C-3 hydroxy group of sterols have been reported from oat shoots [19], *Solanum melongena* [20] and *Panax ginseng* [21]. The oat shoot sterol glucosyltransferase is membrane-bound while the egg plant and *Panax* enzymes have been reported to be cytosolic. However, unlike the animal enzymes [22], a detailed kinetic study on purified plant cytosolic sterol glucosyltransferases has not been reported. *W. somnifera* (L) Dunal (*Solanaceae*) is used extensively in Indian traditional system of medicine and is equated with ginseng in its therapeutic benefits. It is especially attractive for studying the enzymes involved in steroidal transformations since it is a rich source of a variety of steroidal compounds [23]. Characteristically, it contains a variety of glucosylated steroids called withanosides in roots [24,25] and leaves [26]. Withanosides have been reported to possess neuroregenerative, adaptogenic, anticonvulsant, immunomodulatory and antioxidant activities [24–27]. As a part of our efforts to examine glycosylation of steroid secondary metabolites (withanolides) and their role in *W. somnifera*, this study reports purification and detailed physicokinetic characterization of a cytosolic sterol glucosyltransferase and its possible role in stress response of the plant.

2. Materials and methods

2.1. Chemicals and reagents

General chemicals and reagents were obtained from Sigma-Aldrich, unless otherwise stated. The 24-methylenecholesterol and withanoside V were obtained from Chromadex. Chromatographic matrices, silver staining kit, uridine diphospho-D-[U-^{14}C] glucose and uridine diphospho-D-[U-^{14}C] galactose were obtained from Amersham Biosciences. Optiphase Hisafe 3 scintillant was from Wallac. Pre-coated TLC plates were from Merck.

2.2. Plant material

W. somnifera was grown at the National Botanical Research Institute, following standard cultivation practices. The leaves sampled from the 90-day-old plants were directly pulverized in liquid nitrogen and stored at −80 °C until used.

2.3. Buffers

Composition of different buffers used during purification was as follows. Buffer A: 100 mM Tris–HCl, pH 8.0, 150 mM sorbitol, 12.5 mM β-mercaptoethanol, 1 mM PMSF, 500 μl/l plant protease inhibitor cocktail and 2% (w/v) poly (vinylpolypyrrolidone); Buffer B: 20 mM Tris–HCl, pH 8.0, 2.5 mM β-mercaptoethanol, 1 mM PMSF; Buffer C: Buffer B + 2 M NaCl; Buffer D: Buffer B + 500 mM NaCl and Buffer E: Buffer B + 150 mM NaCl.

2.4. Enzyme extraction and purification

The enzyme extraction and purification steps were carried out at 4 °C unless specified otherwise. Biologic Duo flow FPLC system (Bio-Rad) was used for purification of the enzyme. Leaves were pulverized in liquid nitrogen using a grinder-mixer. Powdered material was suspended in Buffer A and homogenized in a Polytron homogenizer. The homogenate was filtered through three layers of cheesecloth followed by nylon cloth. The filtrate was centrifuged at 5000×*g* (10 min) followed by 150,000×*g* (75 min) to sediment the membrane fraction. The supernatant (cytosolic preparation) was collected and processed for the purification of sterol glucosyltransferase.

2.5. Cibacron Blue chromatography

The supernatant was loaded on Cibacron Blue column (2.5 × 25 cm, 300 ml) pre-equilibrated with Buffer B. The column was washed with Buffer B (five volumes) at a flow rate of 4 ml min^{-1}. The column was then eluted with Buffer C (4 ml min^{-1}). The eluate contained the enzyme activity. It was collected and subjected to ammonium sulphate precipitation (80% saturation).

The membrane preparation (150,000 g pellet) solubilized in Buffer A containing 0.5% Triton X-100 also contained enzyme activity. It was also retained by the Cibacron Blue column, as in the case of cytosolic enzyme. The bound membrane enzyme was eluted with Buffer C and desalted on Sephadex G-25 column. It was purified and compared with the soluble cytosolic enzyme in few experiments on substrate specificity. Detailed kinetic studies were conducted on the cytosolic enzyme and are reported in this manuscript.

2.6. Ion exchange chromatography

The cytosolic preparation from the Cibacron Blue column was dissolved in minimum volume of Buffer B, desalted on a Sephadex G-25 (1.3 × 10 cm, 53 ml) and loaded on a Mono Q anion exchange column (20 ml), pre-equilibrated with Buffer B. After washing with 3 column volume of the same buffer, the bound proteins were eluted using a linear gradient of NaCl from 0.1 M to 0.5 M at a flow rate of 2 ml min^{-1}. Fractions (2.0 ml) were monitored for protein (A_{280}) and tested for the enzyme activity. Active fractions were pooled and concentrated on BioMax-10 K NMWL (Millipore).

2.7. Gel filtration chromatography

The 5.0 ml concentrate from ion-exchange column was injected into Superdex 200 (26/60) column and eluted at 2.0 ml min^{-1} using Buffer E. Active fractions were pooled and concentrated to 1.0 ml as above and resolved on a finer molecular sieve, using Superdex 75 (26/60) column as above except that the flow rate was 1.0 ml min^{-1}. Fractions (1.5 ml) were monitored for protein (A_{280}) and tested for the enzyme activity.

2.8. Sterol glucosyltransferase (SGT) assay

SGT activity was monitored radiometrically using UDP-[U-^{14}C] glucose as the radioactive substrate in the following reaction.

$$\text{Sterol} + \text{UDP-[U-}^{14}\text{C]Glucose} \xrightarrow{\text{SGT}} \text{UDP} + [^{14}\text{C}]\text{Sterolglucoside}$$

The standard reaction mixture (100 μl) contained 0.05 μg of the purified enzyme preparation, 1.5 μM UDP-[U-^{14}C] Glucose (specific activity 304 mCi/m mol), 0.15 mM sterol (dissolved in ethanol) and 20 mM Tris–HCl, pH 8.0. The reaction was initiated by addition of the enzyme and the reaction mixture was incubated at 30 °C for 50 min. The assay exhibited linearity till 70 min. At end of the reaction, the contents were extracted with eight volumes (800 μl) of ethyl acetate and the organic phase was collected after centrifugation (13,000×*g*, 2 min). Radioactivity in the organic phase was monitored in liquid scintillation counter (Wallac) using Optiphase 'Hisafe 3' as scintillant.

2.9. Product identification by TLC

Formation of the glucosylated sterols in the catalytic reaction was also ascertained on the basis of mobility shift on TLC by using non-radioactive UDP-glucose in the assay mixture. The reaction product extracted in the organic phase was collected and evaporated to dryness at room temperature. The samples were re-dissolved in methanol and spotted on a TLC plate (Silica gel G 60) and run using methanol: chloroform (1.5: 8.5, v/v) as the mobile phase. The plates were developed with vanillin sulfuric acid (1%, w/v in 50% sulfuric acid) as spray reagent and chromogenically visualized after heating at 110 °C.

2.10. Substrate specificity

The purified enzyme was tested for substrate specificity using various sterols as acceptor aglycones and UDP-[U-^{14}C] glucose or UDP-[U-^{14}C] galactose as glycosyl donors. Specificity constants (K_{cat}/K_m) for the substrates were calculated by using V_{max} and K_m values. Ethanolic (25%) leaf and root extracts of *W. somnifera* were used to establish the presence of endogenous substrates for glycosylation by the enzyme. Extracts were prepared from different chemotypes (details to be published elsewhere) of *W. somnifera* and examined for possible differences in the levels of aglycones that could be glucosylated by the cytosolic enzyme.

2.11. Deglycosylation of withanoside V

Withanoside V is a glycosylated derivative of 20S, 22R-1α, 3β-dihydroxy-witha-5, 24-dienolide with two glucose units attached at the C-3 position. Partial hydrolysis of withanoside V was done with β-glucosidase to obtain 3-0-β-D-glucopyranosyl derivative and the aglycon product. Each of these was separately used as substrate to examine glucosylation. The hydrolytic reaction contained 0.1 M citrate phosphate buffer (pH 4), 0.4 mg withanoside V and the purified β-glucosidase preparation (380 IU) from *W. somnifera*. The reaction was performed at 30 °C for 5 h and the reaction products were extracted 4 times with 2 ml chloroform. The chloroform phase was concentrated and its constituents were resolved on preparative TLC by using chloroform:ethylacetate:methanol:toluene (70:4:8:24 v/v) as mobile phase. The complete and partially deglucosylated products scratched from TLC were used as substrates to examine their glucosylation by the sterol glucosyltransferase.

2.12. Denaturing polyacrylamide gel electrophoresis

Samples from each step of the purification were analyzed by SDS-PAGE (10%), using discontinuous buffer system [28]. The protein gel was silver stained using Amersham Pharmacia kit. Protein concentration was determined using Bio-Rad Bradford dye reagent and BSA as the standard.

2.13. Chromatofocusing

The purified enzyme in 25 mM Tris–HCl pH 7.0 was applied onto Mono P chromatofocusing column and eluted with 100 ml of diluted polybuffer-74 (Amersham Biosciences) using a linear pH gradient of 7.0 to 4.0 as per manufacturer's instructions. Fractions (1.0 ml) were collected in tubes containing 50 μl 1 M Tris–HCl, pH 9 (to minimize the polybuffer inhibitory effect) and each fraction was screened for the enzyme activity.

2.14. Effect of Triton X-100

The effect of Triton X-100 on enzyme activity was examined by conducting the assay with and without Triton X-100. In one case, the enzyme was pre-incubated in buffer supplemented with 0.03% Triton X-100 for different intervals of time. Aliquots were drawn and diluted into the assay mixture. The final concentration of Triton X-100 during the assay was 0.0015%. Activity of the enzyme pre-incubated in 0.03% Triton X-100 was compared with the enzyme activity assayed directly in the presence of 0.0015% Triton X-100 (i.e., without the pre-incubation step). A separate assay was also performed with the enzyme pre-incubated in the presence 0.3% Triton X-100.

2.15. Protein identification by peptide mass fingerprinting

After SDS-PAGE, the band was visualized by the Coomassie Stain. The band was excised from the gel, cut into small pieces and put into 0.5 ml micro tubes. In-gel digestion was performed using 1.0 mM modified trypsin at 37 °C for 12 h. The gel plugs were extracted with 100 μl of 20 mM NH_4CO_3 for 20 min followed by 100 μl of 1% TFA in 50% ACN for 20 min and 100 μl of 100% ACN for 20 min. All the extracts were dried in speed vacuum. The recovered peptides were dissolved in 0.1% TFA. A saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile/0.1% TFA was used as a MALDI matrix. An aliquot of the purified peptides (1 μl) and 1 μl of the matrix solution were premixed in a test tube. Then the mixture was pipetted on the target plate and allowed to dry at ambient temperature. The analysis was carried out in MALDI-TOF/TOF mass spectrometer (Bruker Ultraflex). The spectrum was acquired using Flex Control™ 2.2 software. Database searches were performed against non-redundant protein sequence database MSDB using the program Mascot (Matrix Science, London, UK; www.matrixscience.com).

2.16. Physico-kinetic measurements and steady state kinetic mechanism

The standard reaction mixture composition and assay conditions were altered as explained under Results and discussion. For all steady state kinetic assay measurements, 0.03% Triton X-100 were included in the assay mixture as it considerably enhanced the enzyme activity. For product inhibition studies, the reaction products UDP or β-sitosterol glucoside were added prior to the initiation of reaction. Concentration dependent role of different metals, NTP's and irreversible inhibitors was studied by adding these, prior to the initiation of reaction.

2.17. Stress responses of the sterol glucosyltransferase

W. somnifera leaves on 3rd to 8th node from 90 days old plants in vegetative phase were used for studying stress response of the cytosolic sterol glucosyltransferase. The effect of salicylic acid treatment was studied by incubating the excised leaves as immersed in Tris–HCl, pH 8.0 with and without 1 mM salicylic acid at 28 °C under 16 h photoperiod and 60 μmol m^{-2} s^{-1} white fluorescent light. To monitor response to temperature stress, the experimental and control potted plants were kept at 43 °C and 28 °C respectively in light. Leaves were excised at different time intervals and pulverized immediately in liquid nitrogen. Each sample, powered and dissolved in Buffer A was spun at 5000×*g* and then at 150,000×*g* to get a clear supernatant. This was subjected to 70% ammonium sulphate precipitation. The preparation was desalted on a Sephadex G-25 column and monitored for the sterol glucosyltransferase activity by using a number of substrates, including stigmasterol and solasodine as glucosyl acceptors.

3. Results and discussion

In this study, a cytosolic sterol glucosyltransferase that catalyzed the transfer of a glucose moiety from UDP-glucose to sterols and related compounds was purified from *Withania somnifera* leaves to near homogeneity. It was characterized with respect to its steady-state kinetics to discern reaction mechanism, substrate specificity, subunit composition and peptide mass fingerprinting. The enzyme response to stress and a stress elicitor (salicylic acid) was examined to study its in vivo function.

3.1. Purification of the cytosolic sterol glucosyltransferase

The 150,000 g supernatant prepared from *W. somnifera* leaves under isotonic conditions had sterol glucosyltransferase activity. The enzyme was bound to Blue Sepharose 6B and eluted as a broad peak at high salt concentration (2 M NaCl). This step provided a considerable (37 fold) enrichment of the specific activity and eliminated pigments (Table 1). Partial purification of the enzyme was achieved by ion exchange (Mono Q) and gel filtration Superdex 200 (26/60) column chromatographies. The sterol glucosyltransferase activity eluted as a single peak in these two steps. Finally, the fine gel filtration chromatography Superdex 75 (26/60) resulted in purification (3406 fold) of the enzyme to homogeneity with an overall recovery of about 1% (Table 1). Considering its 1% recovery in pure form, it accounts for about 0.03% of the total soluble leaf protein. The results suggest that the glucosyltransferase is a minor protein in plant cells, as also reported earlier for enzymes that use secondary metabolites as substrates [29,30]. Silver stained SDS-PAGE profile displaying the pattern of the cytosolic enzyme during the course of purification is shown in Fig. 1A. The SDS-PAGE results together with the estimated native molecular mass of 37 kDa (through a calibrated gel filtration column of Superdex G-75) suggested that it is a monomeric enzyme with subunit molecular mass of 37 kDa. The molecular mass is similar to the other known sterol glucosyltransferases that range from 40 to 60 kDa [19–21]. Temperature optimum for catalytic activity was about 30 °C (Supplementary Fig. 2S-A) and the enzyme had optimum pH for activity around 8.0 with half maximal activities at 7.0 and above 9.0 (Supplementary Fig. 2S-B). The slightly alkaline pH for optimal activity is similar to the other reported cytosolic [20] and membrane associated [19] sterol glucosyltransferases. Apparent pI value of the enzyme, as determined by chromatofocusing was 3.8.

The 150,000 g pellet also showed sterol glucosyltransferase activity with substrates containing β-OH at C-3 position. The membrane bound enzyme was solubilized in Buffer A containing of 0.5% Triton X-100. The membrane bound enzyme was purified, as described briefly in legend to Fig. 1. It is a larger (54 kDa) protein (Fig. 1B) than the cytosolic enzyme (Fig. 1A). Detailed physico-kinetic parameters and purification of this enzyme will be published elsewhere.

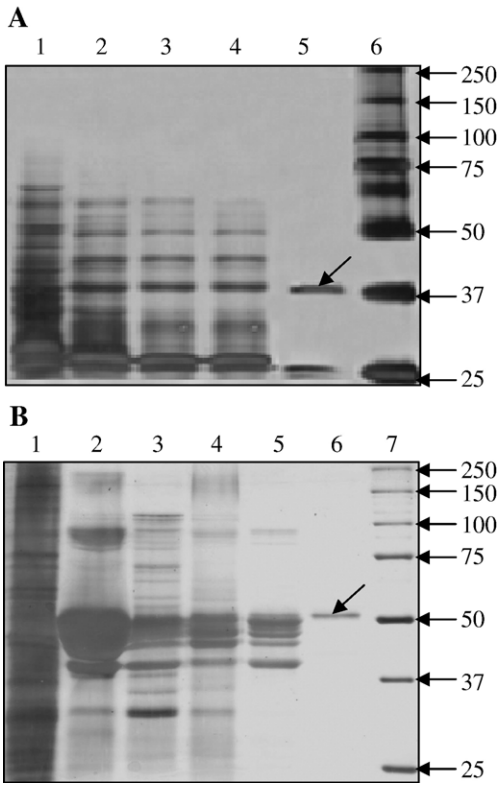

Fig. 1. The silver-stained 10% SDS-PAGE of *W. somnifera* cytosolic and membrane-bound sterol glucosyltransferases. (A) Cytosolic preparation at different stages of purification. Lane 1, crude leaf extract; lane 2, Cibacron Blue; lane 3, Mono Q; lane 4, Superdex 200 (26/60); lane 5, Superdex 75 (26/60) eluates and lane 6, molecular mass standards shown in kDa. The purified enzyme appeared as a single major band on gel, as indicated by arrow at 37 kDa. (B) Membrane preparation at different stages of purification. Lane 1, crude leaf extract; lane 2, Q-Sepharose Fast Flow; lane 3, Cibacron Blue; lane 4, Mono Q; lane 5, Superdex 200 (26/60); lane 6, Superdex 75 (26/60) eluates and lane 7, molecular mass standards. The enzyme appeared as single major band on the gel, as indicated by the arrow at 54 kDa.

3.2. Substrate specificity

Structures of different sterol substrates used to examine substrate specificity of the purified cytosolic enzyme are

Table 1
Summary of sterol glucosyltransferase purification from the cytosolic fraction of *W. somnifera* leaves

Step	Total protein	Specific activity (pmol/min/mg)	Total activity (pmol/min/total protein)	Recovery (From preceding step) %	Fold enrichment
Crude 150,000 g supernatant	54.6 g	0.0005	29.7	100	1
Blue Sepharose eluted with 2 M NaCl	701 mg	0.02	14.4	48	37
Mono Q (16/10) anion exchange column	14 mg	0.39	5.6	39	728
Hiload Superdex 200 (26/60) gel filtration column	8 mg	0.46	3.8	67	857
Hiload Superdex 75 (26/60) gel filtration column	0.2 mg	1.85	0.4	11	3406

given in the Supplementary figure (Fig. 1S). The enzyme from *W. somnifera* is highly specific to sterols (Table 2) and showed no activity with non steroidal substrates like salicylic acid and indole acetic acid (data not shown). Amongst the sterols, the enzyme was found to be position specific and worked only with C-3 hydroxyl sterols like, stigmasterol, sitosterol, diosgenin, deacetyl-16-DPA, hecogenin, tigogenin etc. However, the estradiol C-3 hydroxyl group was not glucosylated, apparently because it is not an alcoholic 3β-OH as in case of the other positive substrates tested. Instead, it harbors a phenolic OH, which is chemically different. The observed limitation of the substrate range suggested that the enzyme may require a planar ring system i.e. 5α-H (as in case of hecogenin and tigogenin) or 5-en (as in β-sitosterol, deacetyl-16-DPA etc.) structures, as proposed previously [31]. Furthermore, sterols with A-B ring 5-en (deacetyl-16-DPA, β-sitosterol etc.) were glucosylated more efficiently than those with 5α-H (hecogenin and tigogenin) (Table 2).

The enzyme did not show any activity with 17β-OH-testosterone (Table 2). Methyl group at C-4 position hindered glucosylation, as was noticed when euphol or ursolic acid were used as substrates. The importance of an alkyl group at C-24 in the enzyme activity was observed when 24-methylenecholesterol, cholesterol and β-sitosterol were compared (Table 2). The 24-methylenecholesterol, a ubiquitously occurring plant sterol gave the highest turnover number $K_{cat}/K_m = 0.957$ (Table 3). The sitoindosides VII and VIII have been reported from *W. somnifera*

Table 2
Substrate specificity of the purified cytosolic and membrane-bound sterol glucosyltransferases from leaves of *W. somnifera*

Sterol substrate	% Activity	
	Cytosolic enzyme	Membrane-bound enzyme
24-methylene cholesterol	100	100
Dehydroepiandrosterone	75±0.7	367±1
Stigmasterol	70±0.1	265±1
β-sitosterol	50±0.2	414±1
Deacetyl-16-DPA	44±0.7	577±0.6
Diosgenin	31.5±0.5	2±0.3
Solasodine	20.3±0.3	0
Pregnenolene	14.1±0.7	319±0.8
16,17α-epoxy pregnenolene	8.9±0.5	0
Cholesterol	7.2±0.3	124±0.7
Hecogenin	4.9±0.2	0
20S, 22R-1α, 3β-dihydroxy-witha-5, 24-dienolide (P_1 in Fig. 4)	5.2±0.2	0
Tigogenin	4.8±0.2	0
Brassicasterol	63±3.9	391±1.2
Quercetin	3.7±0.1	0
Daidzein	2.1±0.1	0
Euphol	0	0
Ursolic acid	0	0
Testosterone	0	0
Estradiol	0	0
Single glucose withanolide (P_2 in Fig. 4)	0	0

Activity at 5 μM UDPG and 0.15 mM of respective sterol. 24-methylene cholesterol activity was considered as 100%. Values are the means±standard deviation for three separate experiments.

Table 3
Kinetic measurements of the purified cytosolic sterol glucosyltransferase from leaves of *W. somnifera*

Sugar acceptor and donor substrates	$V_{max} \times 10^{-3}$ (pmol/min)	K_m (mM)	$K_{cat} \times 10^{-3}$ (min^{-1})	$K_{cat}/K_m \times 10^{-3}$ (min^{-1}/mM^{-1})
24-methylene cholesterol	33±1	0.024±0.003	22.9	957
Deacetyl-16-DPA	0.77±0.05	0.006±0.00	0.56	93
Pregnenolene	0.38±0.02	0.005±0.00	0.28	56
β-sitosterol	0.30±0.01	0.005±0.00	0.22	44
Diosgenin	0.11±0.01	0.002±0.00	0.14	70
Stigmasterol	0.72±0.08	0.125±0.003	0.53	4.3
Dehydroepiandrosterone	0.58±0.02	0.119±0.06	0.43	3.6
UDP-glucose	0.71±0.02	0.014±0.00	0.52	37

The values of K_{cat} were calculated assuming that the molecular mass of enzyme is 37 kDa. Each reaction contained 50 ng of the pure enzyme. Values are the means±standard deviation for two separate experiments.

[27] and their aglycones resemble with 24-methylenecholesterol (Supplementary Fig. 1S). The glucosylation (P_1 in Fig. 3) of 20S, 22R-1α, 3β-dihydroxy-witha-5, 24-dienolide (Table 2) suggested the involvement of the sterol glucosyltransferase in the biosynthesis of pharmacologically active 3β-hydroxy glucosylated withanolides [25]. The steroidal sapogenins (diosgenin, hecogenin, tigogenin) and steroidal alkaloids (solasodine) were also glucosyltaed by the purified cytosolic enzyme (Table 2). In this respect, it was similar to *Solanum melongena* [32] and *Solanum aculeatissimum* [17] glucosyltransferases. The purified enzyme also gave low activity for flavonoid (quercetin) and isoflavonoid (daidzein) structures (Table 2). The reactivity of the cytosolic enzyme with a broad range of aglycone substrates suggests its regiospecificity. Broad regiospecificity has been reported for other enzymes, like the triterpenoid (UGT71G1) cytosolic glucosyltransferase from *M. truncatula* [33,34] that catalyses glucosylation of both triterpenoids and flavonoids. However, the relative substrate specificity of the purified membrane-bound enzyme from *W. somnifera* is different from the cytosolic enzyme (Table 2). The membrane enzyme is more specific towards phytosterols and the steroidal hormone brassicasterol. It showed no activity towards secondary metabolites e.g., diosgenin, solasodine, hecogenin, tigogenin and withanolides. It does not show any activity with flavonoids and isoflavonoids (Table 2). The cytosolic enzyme showed differential activity with withanolide extracts of different *W. somnifera* chemotypic variants. The results suggested large variation in the level of endogenous substrates in different chemotypes (Supplementary Table 1S). As a noticeable example, RSS35 extract had distinctly higher level of the substrates both in the leaves and roots.

The reaction products of a few representative sterol substrates resolved on the TLC are shown in Fig. 2. Resolution of the products on TLC suggested that the enzyme transferred single glucose to the sterol substrates. The TLC resolution gave single spot and their R_f values were similar with β-sitosterol mono glucoside (standard). It suggested that the enzyme catalyzed transfer of single glucose to the sterol substrates (Fig. 2). Some of the withanosides isolated from *W. somnifera* leaves and roots,

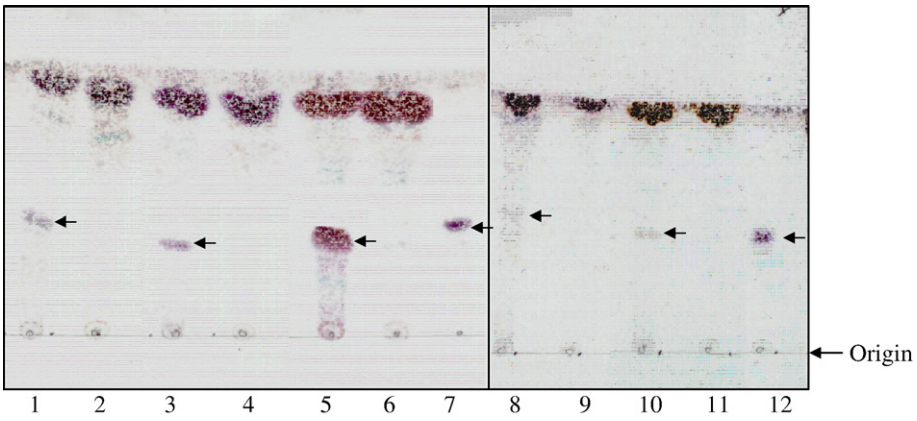

Fig. 2. TLC resolution of reaction products glucosylated by the cytosolic enzyme. Aliquots extracted from reaction after incubation, and at 0 min, respectively are shown, using sterols as substrates, lanes 1 and 2, stigmasterol; lanes 3 and 4, β-sitosterol; lanes 5 and 6, deacetyl-16-DPA; lane 7, standard β-sitosterol glucoside; lanes 8 and 9, pregnenolene; lanes 10 and 11, diosgenin; lane 12, standard β-sitosterol glucoside. Arrows indicate respective products.

possess multiple glucose moieties conjugated in series [25–27]. Therefore, the presence of a single glucosylated product in the catalytic reaction indicated that further extension of glucose chain may be carried out by recruitment of other glucosyltransferase(s). The inability of the enzyme to catalyze multiple glucosylations in series was further confirmed when a slow migrating partial deglucosylated product (P_2 in Fig. 3) of withanoside V could not serve as a substrate for glucosylation (Table 2). UDP-galactose could not replace UDP-glucose (data not shown). Similar specificity for the donor sugar has been reported for SaGT4A and StSGT [17,20] enzymes. Specific amino acids at the active sites have been identified as critical to the sugar specificity of the glucosyltransferases [34,35].

3.3. Physico-kinetic study

The K_m value (0.014 mM) for UDP-glucose was comparable with the reported sterol glucosyltransferases [19,38]. The V_{max} and K_m values varied several fold for different sterols (Table 3). It gave the highest activity with 24-methylene cholesterol as compared to other closely related molecules, for example β-sitosterol, stigmasterol, dehydroepiandrosterone, deacetyl-16-DPA etc. showed several fold lower activity. In these cases, V_{max} was reduced substantially. This happened in spite of several fold lower K_m for many of these substrates as compared to the 24-methylenecholesterol (Table 3).

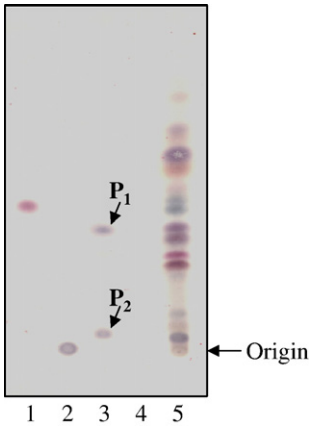

Fig. 3. Deglucosylated products of withanoside V. Lane 1, withaferin A standard; lane 2, purified, native glycosylated withanolide, withanoside V; lane 3, $CHCl_3$ phase of withanoside V hydrolytic reaction showing the partially glucosylated P_2 (3-0-β-D-glucopyranosyl derivative) and the aglycone P_1 (20S, 22R-1α, 3β-dihydroxy-witha-5, 24-dienolide); lane 4, aqueous phase of the hydrolytic reaction showing no withanolide; lane 5, 25% ethanolic extract showing the presence of several withanolides in leaves of *W. somnifera*.

Inhibitory effect of UTP and GTP was less prominent as compared to UDP (Table 4). Strong and irreversible inhibition by thiol-directed reagents like para chloromercuribenzoate, N-methyl maleimide and iodoacetamide suggested the requirement of free-SH groups for the catalytic activity. Divalent heavy metal ions like Hg^{2+}, Ni^{2+}, Co^{2+} and Zn^{2+} strongly inhibited the sterol glucosyltransferase activity (Table 4) whereas Mg^{2+}, Mn^{2+}, Ca^{2+} and Co^{2+} did not show any effect (data not shown).

At low concentration, Triton X-100 included in the assay mixture enhanced the catalytic activity of the enzyme (Supplementary Fig. 2S-C). There was no change in the enzyme activity (Fig. 4) upon its pre-incubation in Triton X-100 (0.03%). In the absence of Triton X-100, the enzyme showed lower activity. The increase in enzyme activity up to 0.03% Triton X-100 in the assay mixture was because of increase in sterol substrate

Table 4
Effect of metal ions, nucleotides and irreversible inhibitors on cytosolic sterol glucosyltransferase activity

Inhibitor	IC_{50} (mM)
Hg^{2+}	0.5±0.05
Ni^{2+}	1.2±0.2
Zn^{2+}	0.04±0.01
Co^{2+}	1.3±0.05
UDP	0.07±0.00
UTP	0.17±0.00
GTP	2.0±0.2
n-ethylmaleimide	0.9±0.02
Iodoacetamide	1.5±0.05
p-chloromercuribenzoate	2.5±0.05

Values are the means±standard deviation for two separate experiments.

solubility and not because of enzyme activation. At higher level (0.3%), Triton X-100 inactivated the enzyme, giving 10% of the original activity after 30 min pre-incubation (Fig. 4).

3.4. Steady state kinetic mechanism

Analysis of the kinetic mechanism of the purified enzyme was performed under varying non-saturating concentrations of β-sitosterol in the presence of several suboptimal concentrations of UDP-glucose and *vice versa*. Results of the initial velocity are shown as Lineweaver–Burk plots (Fig. 5A and B). The lines in the plots converged to the left of the vertical axis intersecting in the second quadrant, which is the characteristic of an enzyme that acts via the formation of a ternary complex [36]. Thus, both the UDP-glucose and the sterol substrates are required to bind to the enzyme at the same time to catalyze the reaction. Ternary complex formation is entirely in agreement with the theory that UDP glucuronyltransferases possess two major functional domains; a theory proposed as a result of structural and sequence homology studies made on these enzymes [37]. Each of these domains is thought to contain a binding site for one of the two substrates, enabling both substrate molecules to be bound to the enzyme at the same time. This ternary complex sequential mechanism is in agreement with the limited work carried out on other enzymes [2,38]. Kinetic constants obtained in this study by using secondary plot analysis are similar to the values obtained when one substrate was kept at saturating concentration while concentration of the other substrate was varied.

The catalytic reaction products, UDP and β-sitosterol glucoside were tested to study product inhibition kinetics (Fig. 6). UDP was found to be a competitive inhibitor, when analyzed under varying concentrations of UDP-glucose at fixed β-sitosterol concentration (Fig. 6A) and it acted as a mixed inhibitor with respect to varying concentrations of β-sitosterol at fixed UDP-glucose concentration (Fig. 6C). However, the second product β-sitosterol glucoside, was found to be a mixed

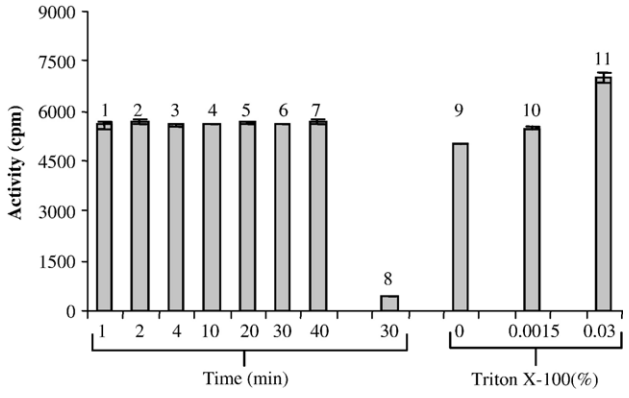

Fig. 4. Effect of Triton X-100 on cytosolic sterol glucosyltransferase. The enzyme activity following pre-incubation for different time intervals (shown in minute at the base of bars 1–8) in the presence of 0.03% (bars 1–7) or 0.3% (bar 8) or the absence (bars 9–11) of Triton X-100. In case of bars 9 to 11, increasing Triton X-100 was added in assay mixture at concentrations shown at the base of bars.

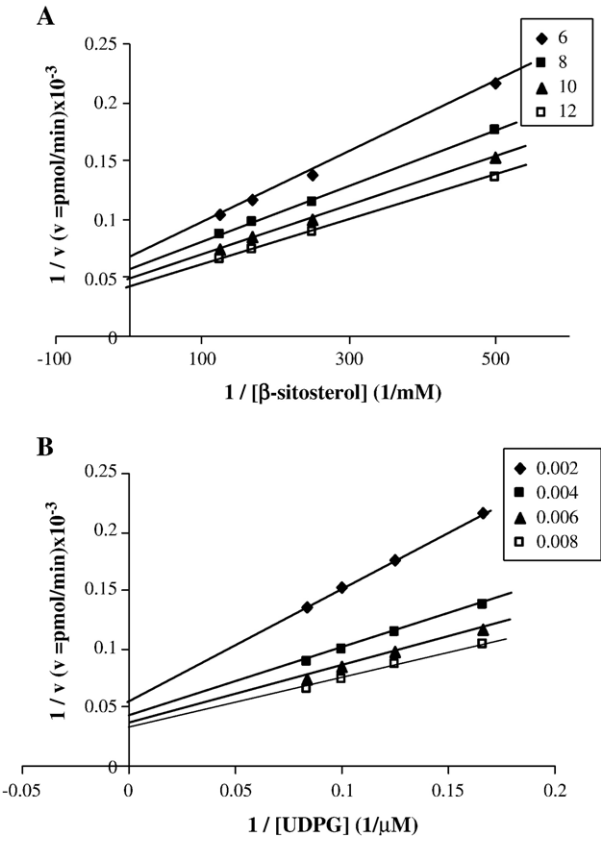

Fig. 5. Enzyme kinetics of *W. somnifera* cytosolic sterol glucosyltransferase. Sample Lineweaver–Burk plots of initial rates as a function of either (A) UDP-glucose (6–12 μM) or (B) β-sitosterol (0.002–0.008 mM) concentrations. Data points represent the means of duplicate measurements.

inhibitor in both the situations (Fig. 6B and D). The inhibition pattern suggested that the reaction followed a compulsory order ternary complex mechanism in which UDP-glucose was the first binding substrate. This is the first detailed kinetic study on purified plant cytosolic sterol glucosyltransferases. The kinetic mechanism is similar to partially purified membrane sterol glucosyltransferase reported from maize [38]. Specific kinetic mechanisms like random order ternary complex [22,39] or compulsory order ternary complex [37,40] have been examined through product inhibition studies. The product UDP was a strong competitive inhibitor with first binding substrate (UDP-glucose). It suggested the role of cellular UDP for in vivo enzyme activity.

3.5. Protein identification by peptide mass fingerprinting

The protein was digested with trypsin. The peptides were separated and subjected to MS analysis. The MS data on the cytosolic SGT was subjected to searches against non-redundant protein sequence database (MSDB) using the program Mascot (Matrix Science, London, UK; www.matrixscience.com). Raw *m/z* data on the peptides are available from the authors upon request. The Mascot peptide mass fingerprint search showed similarity with accession number Q5K5A2 on the first hit list, which is a wheat glucosyltransferase fragment (Supplementary

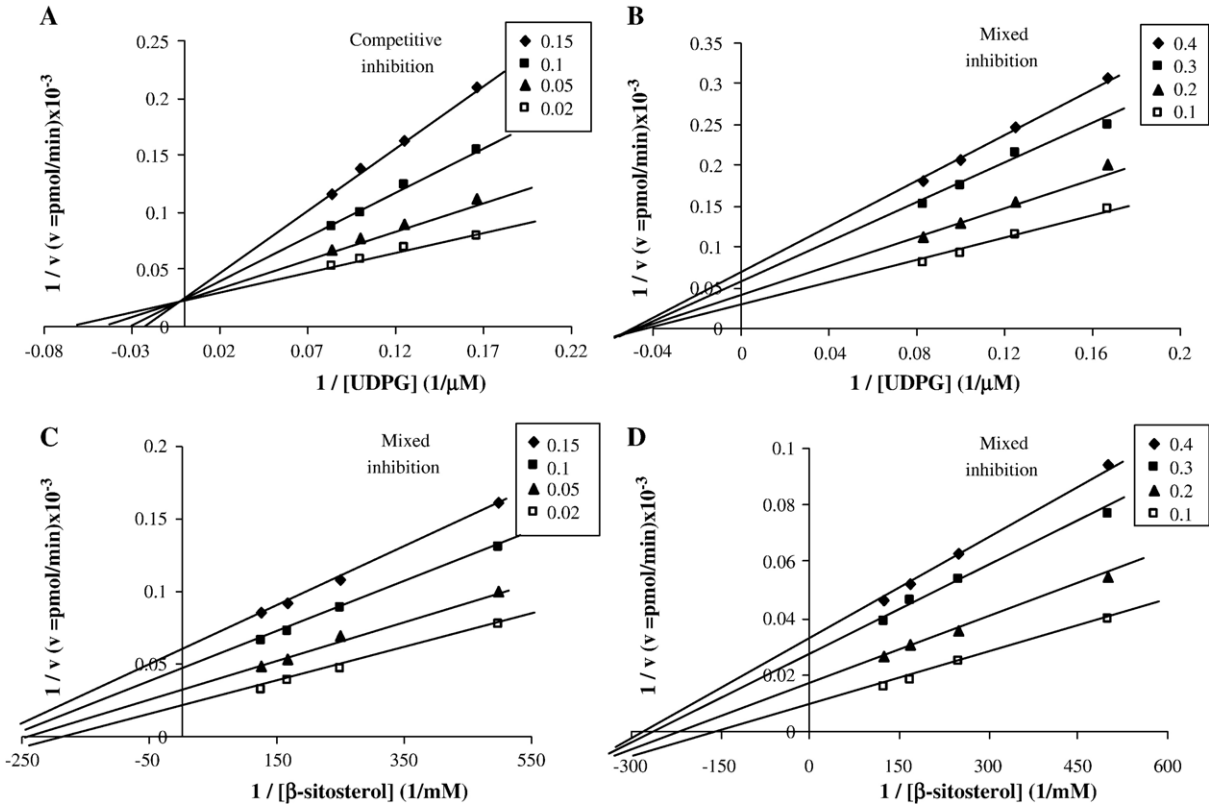

Fig. 6. *W. somnifera* cytosolic sterol glucosyltransferase kinetic mechanism by product inhibition study. (A) Double reciprocal plots of variable UDP-glucose concentration versus β-sitosterol glucoside formation in the presence of 0.008 mM β-sitosterol and different concentrations of UDP (0.02–0.15 mM) as product. (B) Double reciprocal plots of variable UDP-glucose concentration versus β-sitosterol glucoside formation in the presence of 0.008 mM β-sitosterol and different concentrations of β-sitosterol glucoside (0.10–0.40 mM) as product. (C) Double reciprocal plots of variable β-sitosterol concentration versus β-sitosterol glucoside formation in the presence of 22 μM UDP-glucose and different concentrations of UDP (0.02–0.15 mM) as product. (D) Double reciprocal plots of variable β-sitosterol concentration versus β-sitosterol glucoside formation in the presence of 22 μM UDP-glucose and different concentrations of β-sitosterol glucoside (0.10–0.40 mM) as product.

Fig. 4S). The MS analyses resulted in 3 peptide sequences (Supplementary Table 2S) covering a total of 61 amino acids. The 1262, 2120 *m*/*z* peptides (VPCVLFWTASACGYIGYR and FLMQEGIAPLK) showed similarity with accession number Q5K5A2. The 3217 *m*/*z* peptide (LNEEAEASGGALPPVTCVVADSVMSFGLRAAR) showed similarity with Q8GSR9 by individual search in mascot program (Supplementary Fig. 5S), which is also a wheat glucosyltransferase fragment. It was partially similar to Q5K5A2. The CLUSTAL W alignment of MS peptides with Q5K5A2 and Q8GSR9 (Fig. 7) showed more than 70% similarity. The swiss-prot fasta format sequence of Q5K5A2 query was used for NCBI protein–protein BLAST (blastp) search. It showed similarity with UDP-glucose glucosyltransferase (score245), glucuronosyltransferase (score228) and solanidine glucosyltransferase (score 51.6).

The swiss-prot sequences of the reported *Solanum tuberosum* solanidine glucosyltransferase (Q2Q478), *Solanum aculeatissimum* UDP-glucose glucosyltransferase (Q5H861) and two *Medicago truncatula* triterpenoid glucosyltransferases (Q5IFH8, Q5IFH7) were also used in NCBI protein–protein BLAST (blastp) search. They also matched with UDP-glucose glucosyltransferase and glucuronyltransferase enzymes rather sterol glucosyltransferases. These enzyme sequences were highly variable by CLUSTAL W alignment and are different from sterol glucosyltransferases (O23649, O22678). Hence, the *Withania* cytosolic SGT is to be classified with secondary metabolite glucosylating UDP-glucose glucosyltransferses.

3.6. Stress response of the cytosolic sterol glucosyltransferase

Plant defense pathways triggered by pathogen infection are known to utilize salicylic acid as a signal molecule [41]. Hence, response to exogenous application of salicylic acid was studied. Incubation of the leaves of *W. somnifera* in the presence of salicylic acid led to a rise in the sterol glucosyltransferase activity measured by using stigmasterol as the glucosyl acceptor. It reached its maximum at about 10 h and then began to decline, reaching negligible level after 20 h (Fig. 8A). In contrast, the leaves without salicylic acid exhibited a slow decline in the enzyme activity till 20 h duration. Potato solanidine glucosyltransferase [42] and tomato glucosyltransferase [43] induction by wounding have been reported. However, specific functional role of such changes are not understood. Altered distribution of different types of glucosylated lipids may generate early signals in response to pathogen attack or wounding.

The plants subjected to 43 °C showed a sharp increase in the enzyme activity within 15 min and then maintained at a level

```
Q8GSR9|Q8GSR9_WHEAT      ------------------------------------------------------------
MS                       ------------------------------------------------------------
Q5K5A2|Q5K5A2_WHEAT      RGRENMEMKKPHAVIVPLPTQGHVTPMLKLAKLLHCKGFHITFVNTEYNHRRLVRSRGDA 60
                              : :     ::: :     :  :: :  ::    : : ::        ::
                                             3217
Q8GSR9|Q8GSR9_WHEAT      -----P--RFKELIVKLN-EEAEASG---------------------GAL----PPVTCV 27
MS                       ----------------LN-EEAEASG---------------------GAL----PPVTCV 18
Q5K5A2|Q5K5A2_WHEAT      AVEGLPDFRFATIPDGLPPSDADATQDIPSLCYSTMTTCLPPLKRLLGELNRVGPPVTCV 120
                          :  :  : :  :    *  .:*:*:  :  :  :  :  :  ::* *  : ******
                                                 2120, 1262
Q8GSR9|Q8GSR9_WHEAT      VADSVMSFGLRAARELGLRCATLWTASACGYMGYCHYKDLLDRGLFPLKEEAQLSNGYLD 87
MS                       VADSVMSFGLRAARVP---CVLFWTASACGYIG---YRFLMQEGIAPLK----------- 61
Q5K5A2|Q5K5A2_WHEAT      VADNVMSFSVDAAAEIRVPCVLFWTASACGYIGYRNFRFLMQEGIAPLKDEAQLSNGYLD 180
                         ***.****.: **    : *. :********:*   :: *::.*: ***    :    :

Q8GSR9|Q8GSR9_WHEAT      TTIDWIPAMPKDIRLRDLPTFLRATDPDDIMFNFFVHETAAMSQASAVVINTWDELDSPL 147
MS                       ------------------------------------------------------------
Q5K5A2|Q5K5A2_WHEAT      TPVAQAPGMSRHMRLRDFPSFICT------------------------------------ 204
                           :     :   : :  :  ::

Q8GSR9|Q8GSR9_WHEAT      LDAMSKLL 155
MS                       --------
Q5K5A2|Q5K5A2_WHEAT      --------
```

Fig. 7. Multiple sequence alignment performed by CLUSTAL W. MS refers to the *Withania* sterol glucosyltransferase peptides by peptide mass fingerprinting. These peptides show more than 70% similarity to wheat glucosyltransferase fragments Q5K5A2 and Q8GSR9.

higher than control. While in the control plants, identically exposed at 28 °C there was no change in enzyme activity and was maintained almost at steady level (Fig. 8B). The heat shock induction of sterol glucosyltransferases activity is not reported in plants. However, under heat shock conditions increased poriferasterol glucosyltransferase in the protozoan; *Physarum polycephalum* [3,44] and cholesterol glucosyltransferase activity in human fibroblast cell lines [1] have been reported. In *Physarum polycephalum,* the production of poriferasterol glucoside is induced quickly (2–5 min) by heat stress and is followed the synthesis of heat shock proteins (HSP-70). An increase in the sterol glucosyl transferase activity of *W. somnifera* leaves in response to temperature stress suggests a possible role of specific glucosterols in early stages of signal transduction. Glucosylation of sterols may alter the distribution of 3β-OH sterols in membrane rafts and this may be involved in the transmission of heat shock signals in cells by change in membrane functions like fluidity, phase transition, etc. The membrane rafts rich in 3β-OH sterols have been reported in plants [45]. The response to temperature was faster (within 15 min) than that in the case of salicylic acid. In some experiments, we used solasodine also as glucosyl acceptor because it is highly specific to the cytosolic enzyme and does not give any activity with the membrane enzyme (Table 2). The patterns of activity obtained by both the substrates i.e., solasodine (Supplementary Fig. 3S) and stigmasterol (Fig. 8B) were similar. The results suggest a role of the cytosolic enzyme in the stress response.

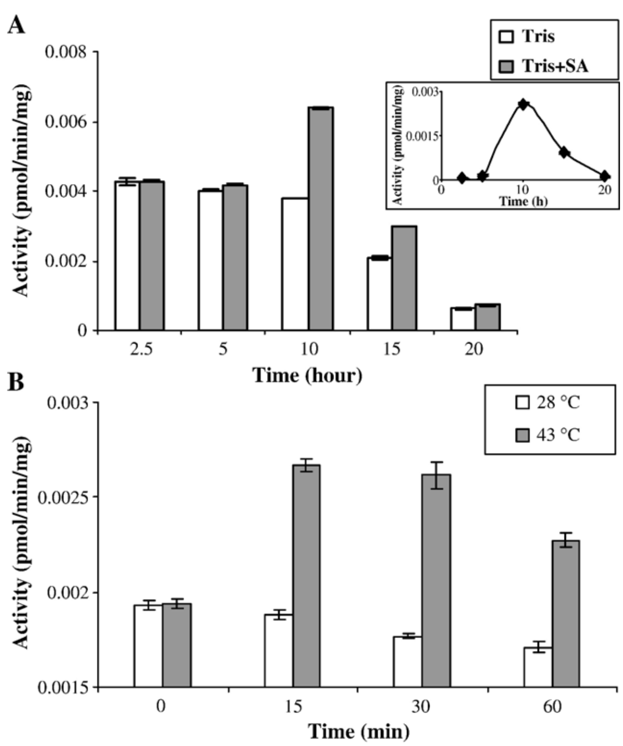

Fig. 8. Effect of salicylic acid and heat stress on *W. somnifera* leaf sterol glucosyltransferase. (A) The effect of salicylic acid treatment by incubating the excised leaves as immersed in Tris–HCl, pH 8.0 with (■) and without (□) 1 mM salicylic acid. The inset shows net differential (–◆–) of the two activities. (B) Effect of temperature seen by incubating potted plants at 28 °C (□) and 43 °C (■). Stigmasterol was used as sugar acceptor substrate in the stress experiments.

In summation, the regiospecific 3β-hydroxy UDP glucose sterol glucosyltransferase in the cytosol of *W. somnifera* leaves is a multi-functional enzyme that participates in physiological processes ranging from secondary metabolite transformation to stress response.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbapap.2006.12.009.

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