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Purification and characterization of a novel glucosyltransferase specific to 27β-hydroxy steroidal lactones from *Withania somnifera* and its role in stress responses

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Abstract

Sterol glycosyltransferases catalyze the synthesis of diverse glycosterols in plants. *Withania somnifera* is a medically important plant, known for a variety of pharmacologically important withanolides and their glycosides. In this study, a novel 27β-hydroxy glucosyltransferase was purified to near homogeneity from cytosolic fraction of *W. somnifera* leaves and studied for its biochemical and kinetic properties. The purified enzyme showed activity with UDP-glucose but not with UDP-galactose as sugar donor. It exhibited broad sterol specificity by glucosylating a variety of sterols/withanolides with β-OH group at C-17, C-21 and C-27 positions. It transferred glucose to the alkanol at C-25 position of the lactone ring, provided an α-OH was present at C-17 in the sterol skeleton. A comparable enzyme has not been reported earlier from plants. The enzyme is distinct from the previously purified *W. somnifera* 3β-hydroxy specific sterol glucosyltransferase and does not glucosylate the sterols at C-3 position; though it also follows an ordered sequential bisubstrate reaction mechanism, in which UDP-glucose and sterol are the first and second binding substrates. The enzyme activity with withanolides suggests its role in secondary metabolism in *W. somnifera*. Results on peptide mass fingerprinting showed its resemblance with glycuronosyltransferase like protein. The enzyme activity in the leaves of *W. somnifera* was enhanced following the application of salicylic acid. In contrast, it decreased rapidly on exposure of the plants to heat shock, suggesting functional role of the enzyme in biotic and abiotic stresses.

Keywords: Glucosyltransferase; Substrate specificity; Withanoside; Stress response; Saponin; Kinetic mechanism; Salicylic acid signal

1. Introduction

Glycosylation of bioactive secondary metabolites, including sterols is implicated in improving their bioavailability and pharmacokinetics [1–3]. 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 [4]. Sterols are structurally diversified through a variety of cellular transformations. Among them, cytochrome P_{450}-dependent oxidations and glycosylations are most predominant [5]. Glycosylation not only stabilizes the products but also modulates their physiological activities and governs intracellular distribution [6–9]. Transcriptional analysis using microarray comprising a large set of genes, including 109 secondary product glycosyltransferases suggest the role of glycosylation in defense response of *Arabidopsis thaliana* [10]. The exogenously added cholesterol glucosides induce heat shock pathway in human fibroblast cells [11].

Saponins are glycosylated triterpenoids or steroidal alkaloids found in many plant species [12–15]. They are mainly characterized by an oligosaccharide chain, consisting of sugars attached at the C-3 position. Some saponins also have a glucose chain attached at C-26 or C-28 positions. Saponins are important

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components of a number of herbal medicines [15–17] and known for a wide range of bioactivities like allelopathic, antimicrobial, anticholesterolemic, anti-cancer, adjuvant, immunomodulatory, antioxidant and hemolytic activities [18–27]. The saponins, avenacosides A and B are glucosylated at C-3 and C-26 positions. The avenacosides are biologically inactive, but are converted into antifungal compounds upon tissue damage or pathogen invasion by glucosidase specific for the C-26 position [28,29].

The studies in which glucosyltransferases are involved in saponin biosynthesis with glucose at C-3 position of steroidal sapogenins and steroidal alkaloids have been reported [30–32]. The enzymes with specificity towards glycosylation of sterols at higher positions are limited. Though, molecules like osladin-26-*O*-glucoside from *Polypodium vulgare* [33], avenacosides (3-*O*-oligosaccharide-26-*O*-glucopyranoside nuatigenin) from *Avena sativa* [34] and sitoindosides IX and X (27-*O*-glucopyranoside withanolides) from *Withania somnifera* [26,35] have been identified. The glycosyltransferases involved in their biosynthesis are not known. A novel UGT73C5 was reported from *Arabidopsis* cDNA library, whose over expression in transgenic *Arabidopsis* conferred enhanced tolerance by glucosylating fungal deoxynivalenol [36]. Interestingly, this enzyme glucosylates the 23β-OH position of brassinosteroids (BR's) also, and resulted in BR-deficient phenotypes [37]. However, its specificity for steroidal lactones hydroxyl groups present at higher position was not studied.

W. somnifera (L) Dunal (family *Solanaceae*) has been used extensively in Indian traditional system of medicine and is equated with ginseng in its health related applications. It is especially attractive for studying the enzymes involved in steroidal transformations like glycosylation because it is a rich source of a variety of glycosylated steroidal lactones called withanosides, in roots [24,38] and leaves [39]. Withanosides have been reported to possess neuroregenerative, adaptogenic, anticonvulsant, immunomodulatory and antioxidant activities [24,26,27,35,38,39]. These withanosides mainly comprise of withanolides with one or more glucose units attached to C-3 or C-27 positions. A sterol glucosyltransferase specific to 3β-hydroxy position has been purified from the leaves of *W. somnifera*. Its substrate specificity for both phytosterols and steroidal sapogenins has been reported recently by our group [40]. This enzyme shows a low level of activity for flavonoid and isoflavonoid substrates also, as in the case of *Medicago truncatula* glucosyltransferase (Q5IFH7) enzyme [32]. A gene that codes for sterol glucosyltransferase transfers glucose to C-3 position of sterols has been cloned from *W. somnifera* and characterized [41]. No enzyme for the glycosylation of sterols/withanolides at positions other than C-3 has been reported, though several 27-*O*-glucosylated pharmacologically important metabolites, like sitoindosides IX and X are known from *W. somnifera* [26,34,39]. In animals, multiple forms of sterol glucuronyltransferases which can glucuronate β-OH present at C-3 and C-17 positions have been studied in details [42–44]. The present study reports the purification and characterization of a novel 27β-hydroxy glucosyltransferase from *W. somnifera,* which can conjugate glucose to C-27 position in the withanolide lactone ring and to higher positions in sterols. The enzyme is suggested to function *in vivo* in defense response.

2. Materials and methods

2.1. Chemicals

Chemically pure withaferin A, 17α-OH withaferin A, withanone, 5α,6β,17α,27β-tetrahydroxywithanolide, 17α-OH-27-dehydroxywithaferin A, withanolide A were isolated from the leaves or roots of *W. somnifera* . General chemicals and reagents were obtained from Sigma Aldrich unless otherwise stated. The 21β-hydroxyprogesterone was obtained from Chromadex. Chromatographic matrices, 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 in the botanical garden, following standard cultivation practices. The leaves sampled from the 90 days old plants were directly pulverized in liquid nitrogen and stored at −80 °C until used.

2.3. Buffers

Compositions of different buffers used during purification were 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 (vinyl-polypyrrolidone); 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) to remove debris and 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 pre-equilibrated with Buffer B. The column was washed with Buffer B (five volumes) at a flow rate of 4 ml min^{-1}. The bound proteins were then eluted with Buffer C (4 ml min^{-1}). The eluate containing the enzyme activity was collected and subjected to ammonium sulphate precipitation (80% w/v saturation).

2.6. Ion exchange chromatography

The enzyme preparation from the Cibacron Blue column was dissolved in minimum volume of Buffer B, desalted on Sephadex G-25 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 by using testosterone as aglycone substrate. 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 and resolved on a finer molecular sieve, using Superdex 75 (26/60) column as mentioned 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.

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

The standard reaction mixture (100 μl) contained 0.06 μg of the purified enzyme preparation, 1.5 μM UDP-[U-^{14}C] glucose (specific activity 304 mCi/mmol), 0.15 mM sterol/withanolide (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 2.5 h. The assay exhibited linearity till 6 h. At end of the reaction, the reaction contents were extracted with 800 μl of ethyl acetate. The organic phase was collected after centrifugation (13,000×*g*, 2 min). Radioactivity in the organic phase was monitored in liquid scintillation counter using Optiphase 'Hisafe 3" as scintillant.

2.9. Product identification by TLC

Formation of the glucosylated products in the catalytic reaction was ascertained also 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 G60) and run using methanol: chloroform (1.2: 8.8, 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/withanolides as glucose acceptor substrates 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. The values of K_{cat} were calculated by assuming that the molecular mass of pure enzyme was 52 kDa and each reaction contained 60 ng of pure enzyme.

2.11. Denaturing polyacrylamide gel electrophoresis

Samples from each step of the purification were analyzed by SDS-PAGE (10%), using discontinuous buffer system [45]. 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.

2.12. 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.

2.13. Protein identification by peptide mass fingerprinting

After SDS-PAGE, the band was visualized by the Coomassie Stain. The 52-kDa 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% trifluoroacetic acid (TFA) in 50% acetonitrile (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).

2.14. 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 product inhibition studies, the reaction products UDP or testosterone glucuronoside were added prior to the initiation of reaction. Concentration-dependent role of different metals, NTPs and irreversible inhibitors was studied by adding these, prior to the initiation of reaction.

2.15. Stress responses of the 27β-hydroxy glucosyltransferase

W. somnifera leaves on 3rd to 8th node from 90 days old plants in vegetative phase were used for studying defense stress response of the glucosyltransferase, using salicylic acid as the elicitor. 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 the enzyme 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 80% ammonium sulphate precipitation. The preparation was desalted on Sephadex G-25 column and monitored for the glucosyltransferase activity by using testosterone as glucosyl acceptor.

3. Results and discussion

A novel cytosolic 27β-hydroxy glucosyltransferase that catalyzed conjugation of a glucose moiety from UDP-glucose to sterols/withanolides with β-OH group at higher positions was purified to near homogeneity. An enzyme with such substrate specificity for steroidal lactones has not been reported from plants so far. It has been characterized in detail with respect to its physico-kinetics, steady state kinetics to discern reaction mechanism and substrate specificity. The enzyme's response to stress and stress signal molecule (salicylic acid) has been profiled to study its putative *in vivo* functionality.

3.1. Purification of the cytosolic glucosyltransferase

W. somnifera leaf 150,000×*g* supernatant prepared under isotonic conditions had glucosyltransferase activity for testosterone; a structure with OH group at C-17 position. The enzyme was purified to near homogeneity by a series of chromatographic steps. It was bound to Cibacrone Blue and eluted as a broad peak at high salt concentration (2M NaCl). This step provided a considerable (38 fold) enrichment of the specific activity and eliminated pigments (Table 1). Further purification of the enzyme was achieved by ion exchange and gel filtration Superdex 200 column chromatographies. The testosterone glucosyltransferase activity was eluted as a single peak in each of these steps. Finally, a fine gel filtration chromatography on Superdex 75 gave purification to near homogeneity (2946

Table 1
Summary of 27β-hydroxy glucosyltransferase enzyme 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	0.0003	17.8	100	1
Blue Sepharose eluted with 2 M NaCl	701 mg	0.0125	8.8	49.3	38
Mono Q (16/10) anion exchange chromatography	14 mg	0.23	3.2	36.8	705
Hiload Superdex 200 (26/60) gel filtration chromatography	8 mg	0.25	2	62.4	769
Hiload Superdex 75 (26/60) gel filtration chromatography	0.18 mg	1	0.18	9	2946

fold). Following the above chromatographic protocols, the overall recovery was about 1% that accounted for about 0.0003% of the total soluble leaf protein (Table 1). Silver stained SDS-PAGE profile showing stage wise purification of the enzyme is shown in Fig. 1. The estimated molecular mass of 52 kDa as determined by denaturing SDS-PAGE and gel filtration (Superdex 75) suggested that the enzyme was a monomer. The optimum temperature for catalytic activity was about 30 °C (Supplementary data Fig. 1S-A) and the enzyme had optimum activity at pH 8.0, with half maximal activities at 7.0 and above 9.0 (Supplementary data Fig. 1S-B). Apparent pI value of the enzyme as determined by chromatofocusing was 4.8. The purified enzyme was highly sensitive to Triton X-100 detergent (Supplementary data Fig. 1S-C).

3.2. Substrate specificity

Different steroidal substrates used to examine substrate specificity of the purified enzyme are given in Fig. 2. Chemically pure withaferin A, 17α-OH withaferin A, withanone, 5α,6β,17α,27β-tetrahydroxywithanolide, 17α-OH-27-dehydroxywithaferin A, withanolide A were isolated by us from *W. somnifera* [46]. The purified enzyme was highly specific to sterols and showed no activity with non steroidal structures such as salicylic acid and indole acetic acid (data not included). It transferred glucose to β-OH present at C-17, C-21 and C-27 positions in sterols/withanolides (Fig. 2). In contrast to the previously reported 3β-OH enzyme from *W. somnifera* [40], it did not transfer glucose to the C-3 β-OH in any of the tested sterol substrates. Further, the enzyme did not require A–B planar ring system in substrates for glucosylation. This 27β-hydroxy glucosyltransferase glucosylated various natural sterols obtained from *W. somnifera,* like 17α-OH withaferin A, 27β-OH withanone, 5α,6β,17α,27β,-tetrahydroxywithanolide, withanolide A and withanolide U (Fig. 2). It transferred glucose to C-27 β-OH group of the lactone ring, provided a C-17 α-OH was present in the steroidal skeleton. This is true in the case of 27β-OH withanone, 17α-OH withaferin A and 5α,6β,17α,27β-tetrahydroxywithanolide. In the absence of α-OH at C-17, as in the case of withaferin A and 5α,27-dihydroxy-6,7α-epoxy-2,24-dien withanolide, the glucosylation at C-27 β-OH was not observed. The results on withaferin A, withanone and 17α-OH-27-dehydroxywithaferin also suggest that the glucosylation does not occur at C-17 α-OH, C-4 β-OH and C-5 α-OH positions by this enzyme (Fig. 2). In the presence of α-methyl at C-17, as in the case of 17α-methyl testosterone, glucosylation at C-17 β-OH was not observed (Fig. 2). The C-17 α-OH group is important for glucosylation of C-27 hydroxyl group in lactone ring of withanolides. However, in sterols without the lactone ring, the C-17 α-methyl group hindered glucosylation. These observed results suggest role of the chemical group present at C-17 α position in glucosylation activity of this enzyme. The result with hydrocortisone and 21β-hydroxy progesterone substrates establishes that the enzyme also glucosylates C-21 β-OH position (Fig. 2). The enzyme did not show any activity with C-27 acetoxy group present in 27-acetoxy withanone (Fig. 2) and therefore, shows strict specificity for hydroxyl group. The enzyme activity with withanolide A and withanolide U suggests its possible glucosylation site at C-20 β-OH position (Fig. 2). Sitoindoside IX and X are the pharmacologically active 27-*O*-glucosylated withanolides reported from *W. somnifera* [26,34]. The non glucosylated structures of these sitoindosides resemble 17α-OH withaferin A (Fig. 2), which could be its possible precursor *in vivo*.

UDP-galactose could not replace UDP-glucose (data not shown) as sugar donor. Similar specificity for the donor sugar has been reported for some solanaceae glucosyltransferases [30,40,41]. Specific amino acids at the active sites have been identified as critical to the sugar specificity of the glucosyltransferases [47,48]. However, more detailed engineering study

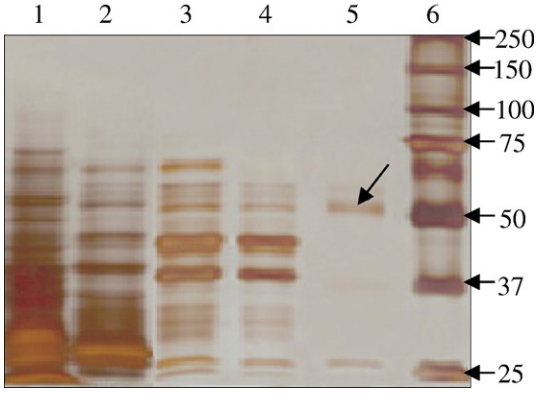
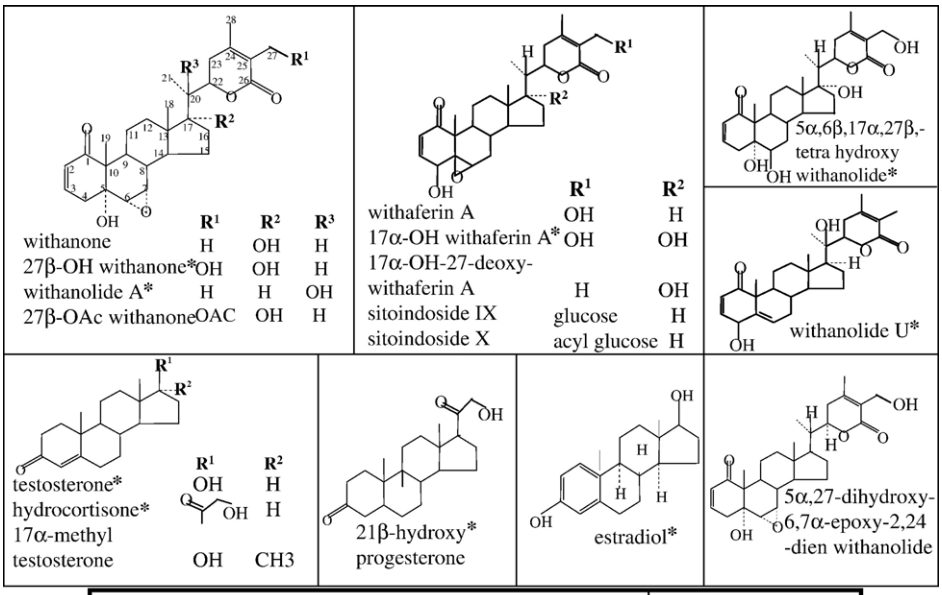


Fig. 1. The silver stained 10% SDS-PAGE of *W. somnifera* 27β-hydroxy glucosyltransferase at different stages of purification. Lane 1, crude leaf extract; lane 2, Cibacron Blue; lane 3, Mono Q; lane 4, Superdex 200 (26/60); lane 5, Superdex 75 (26/60) eluates and lane 6, molecular mass standards shown in kDa. The purified enzyme appeared as a single major band on gel, as indicated by arrow at 52 kDa.



Sterol substrate	% Activity
testosterone*	100
estradiol*	41 ± 1.2
withanolide A*	21 ± 0.9
withanolide U*	17 ± 1
5α,6β,17α,27 β –tetrahydroxywithanolide*	23 ± 0.6
27β -OH withanone*	15 ± 0.5
17α -OH withaferinA*	15 ± 0.7
Hydrocortisone*	11 ± 0.3
21β-OH progesterone*	13 ± 1
withaferin A	0
withanone	0
5α,27-dihydroxy-6,7α-epoxy-2,24-dien withanolide	0
17α -OH- 27-dehydroxywithaferin A	0
27β -OAcwithanone	0
17α-methyl testosterone	0

Fig. 2. Structures of various substrates used to examine substrate specificity of *W. somnifera* 27β-hydroxy glucosyltransferase. Asterisks (*) indicate the accepted substrates for 27β-hydroxy glucosyltransferase. Enzyme activity was measured at 1.5 μM UDPG and 0.15 mM of the respective sterol. The activity with testosterone substrate was considered as 100%. Values are the means±standard deviation for three separate experiments.

of glycosyltransferases responsible for steroid saponin biosynthesis in solanaceous plants suggested that the differences in the amino acid residues in SaGT4A and StSGT do not simply reflect their distinct sugar donor specificities [49].

The reaction products of a few representative sterol substrates resolved on the TLC are shown in Fig. 3. The glucosylated products migrated to positions similar to the standard β-sitosterol glucoside. The R_f values of the resulting reaction products were between 0.4 and 0.6. Some of the withanosides reported from *W. somnifera* have more than one glucose or have acylated glucose [24,26,34,39]. Resolution of the enzymatic products on TLC suggested that the purified enzyme transferred single glucose to the sterol substrates, as in the case of previously reported enzymes from *Solanum* species [30,40,41]. Hence, the further oligosaccharide extension steps may be undertaken by some other type of glucosyltransferase(s).

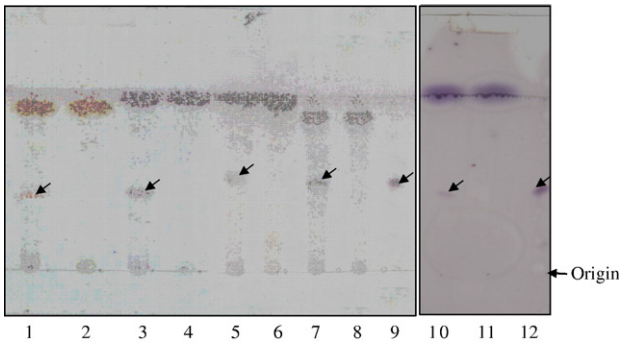


Fig. 3. TLC resolution of reaction products glucosylated by purified enzyme. Aliquots extracted from reaction after incubation, and at 0 min respectively are shown, using different sterols as substrates, lanes 1 and 2, estradiol; lanes 3 and 4, testosterone; lanes 5 and 6, 17α-OH withaferin A; lanes 7 and 8, withanolide A; lane 9, standard β-sitosterol glucoside; lanes 10 and 11, 21β-OH progesterone; and lane 12, standard β-sitosterol glucoside. Arrows indicate position of the respective product.

Table 2
Kinetic measurements of the purified 27β-hydroxy glucosyltransferase from *W. somnifera* leaves

Sugar acceptor/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})
17α-OH withaferin A	0.08±0.01	0.17±0.03	0.07	0.41
27β-OH withanone	0.05±0.01	0.11±0.01	0.04	0.40
Withanolide A	0.04±0.00	0.11±0.01	0.03	0.30
5α,6β,17α,27β-tetrahydroxywithanolide	0.02±0.00	0.01±0.00	0.02	1.9
Withanolide U	0.03±0.00	0.05±0.00	0.03	0.57
Testosterone	1.05±0.02	0.17±0.01	0.91	5.5
Estradiol	0.15±0.01	0.04±0.00	0.13	3.1
21β-OH progesterone	0.06±0.04	0.07±0.01	0.05	0.7
UDP-glucose	1.06+0.10	0.02+0.00	0.92	42

The values of K_{cat} were calculated assuming that the molecular mass of purified enzyme is 52 kDa. Each reaction contained 60 ng of the pure enzyme. Values are the means±standard deviation for two separate experiments.

3.3. Physico-kinetic study

The K_m value (0.022 mM) of enzyme for UDP-glucose was comparable to those reported for other sterol glucosyltransferases [40,41,50]. Depending upon the acceptor substrates, several fold differences were noticed in the V_{max} as well as K_m values for sterols. The catalytic efficiency (K_{cat}/K_m) of the purified enzyme was fairly similar for the tested withanolides, except with 5α,6β,17α,27β,-tetrahydroxywithanolide and withanolide U. Both of these structures lack epoxy group in the A–B ring, which may influence the affinity of the enzyme for the withanolides. Among the five natural substrates, 5α,6β,17α,27β-tetrahydroxywithanolide showed the highest affinity (K_m=0.009) for the enzyme (Table 2). This enzyme showed highest catalytic efficiency for testosterone in spite of high K_m value (low affinity) for this substrate (Table 2).

The enzyme showed different patterns of inhibition by metal ions, NTPs, and irreversible inhibitors. Like previously reported 3β-hydroxy glucosyltransferase from *W. Somnifera* [40], divalent heavy metal ions (Hg^{2+}, Ni^{2+}, $Co^{2+}$and Zn^{2+}) inhibited the glucosyltransferase activity (Supplementary data Table 1S). However, 27β-hydroxy glucosyltransferase was several folds more sensitive in all the cases. The other metal ions Mg^{2+}, Mn^{2+}, Ca^{2+} and Co^{2+} did not show any effect (data not shown). Strong and irreversible inhibition by the thiol-directed reagents para-chloromercuribenzoate, N-methyl maleimide and iodoacetamide suggested the requirement of free –SH groups for the catalytic activity. Inhibitory effect of UTP and GTP was less prominent as compared to UDP (Supplementary data Table 1S).

3.4. Protein identification by peptide mass fingerprinting

The protein was digested with trypsin. The peptides were separated and subjected to MS analysis. The spectrum was acquired using Flex Control™ 2.2 software. The MS data were subjected to searches against non-redundant protein sequence database (MSDB) using the program Mascot (Matrix Science, London, UK; www.matrixscience.com). It showed similarity with Q6V9S8, which is a glycuronosyltransferase like protein. The MS analyses resulted in 4 peptides (Table 3) giving a total of 52 amino acids. The *m/z* peptides 1209 (QVDGTVVHEVK), 1256 (LKGIPPEECSK), 1379 (TGIMYRHLVFK) and 2224 (ENFTDPEAELNHQRNVALK) showed similarity to Q6V9S8. The spectrum analysis report of the MS peptides is shown in supplementary data Fig. 2S. The swiss-prot sequences of the reported secondary metabolite specific glucosyltransferases (Q2Q478, Q5H861, Q9ZQ94, Q5IFH8 and Q5IFH7) upon NCBI blastp search showed similarity with UDP-glucose glucosyltransferase and glucuronyltransferase enzymes. The 27β-hydroxy glucosyltransferase also showed similarity with glycuronosyltransferase like protein. Hence, it was classified with secondary metabolite glucosyltransferses family.

Table 3
The purified 27β-hydroxy glucosyltransferase MS peptides (1209,1256,1379 and 2224) showed similarity with cotton glycuronosyltransferase like protein by peptide mass fingerprint search

Mass Mr	Range[a] Start–End	Deviation[b]	Missed[c] cleavages	Sequence
1209.592	99–109	−0.043	0	QVDGTVVHEVK
1256.616	328–338	−0.028	1	LKGIPPEECSK
1379.788	168–178	0.049	1	TGIMYRHLVFK 171: Oxidation (M)
2224.106	179–197	0.019	1	ENFTDPEAELNHQRNVALK

[a] Inclusive numbering of the amino acid residues, starting with 1 for the N-terminal residue of the protein.
[b] Difference (error) between the experimental and calculated masses.
[c] Number of missed enzyme cleavage sites.

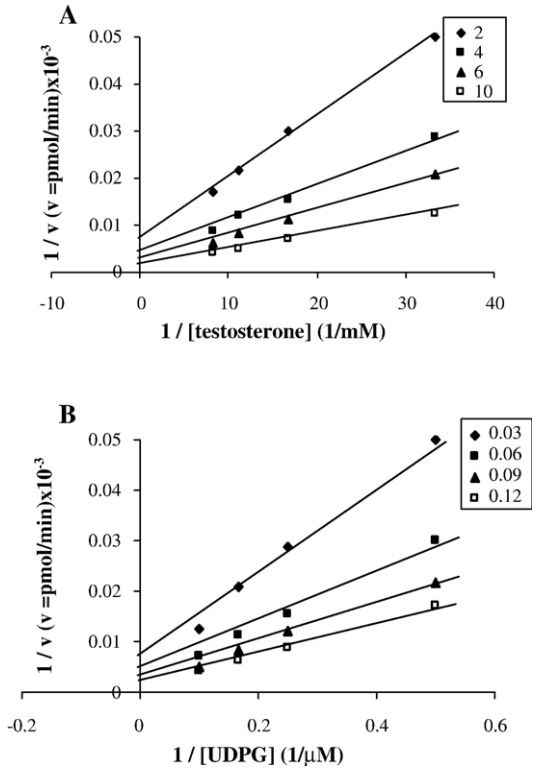


Fig. 4. Kinetic mechanism of purified 27β-hydroxy glucosyltransferase from *W. somnifera*. Representative Lineweaver–Burk plots of initial rates as a function of (A) UDP-glucose (2–10 μM) (B) testosterone (0.03–0.12 mM). Data points represent the means of duplicate measurements.

3.5. Steady state kinetic mechanism

Analysis of the kinetic mechanism of the purified enzyme was performed under varying non-saturating concentrations of testosterone 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. 4A and B). The lines in 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 i.e. both the UDP-glucose and the sterol substrates are required to bind to the enzyme at the same time to catalyze the reaction [51]. This ternary complex mechanism is in agreement with the limited work reported out on other glucosyltransferases [50,52]. 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.

Specific kinetic mechanisms like random order ternary complex [43,53] or compulsory order ternary complex [39,54,55] were examined earlier through product inhibition studies in various glucuronyl and glucosyltransferases. The catalytic reaction products, UDP and testosterone glucuronide were tested to study product inhibition kinetics (Fig. 5). UDP was found to be a competitive inhibitor, when analyzed under varying concentrations of UDP-glucose at fixed testosterone concentration (Fig. 5A). It acted as a mixed inhibitor with respect to varying concentrations of testosterone at fixed UDP-glucose concentration (Fig. 5C). However, the second product testosterone glucuronide, was found to be a mixed inhibitor in both the situations (Fig. 5B 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 enzyme is distinct from the previously characterized 3β-hydroxy specific sterol glucosyltransferase from *W. somnifera* [40], though both of the enzymes followed similar kinetic mechanisms of reaction. The product UDP was a strong competitive inhibitor with the first binding substrate (UDP-glucose). It suggested a possible role of cellular UDP in *in vivo* enzyme activity.

3.6. Stress response of the 27β-hydroxy glucosyltransferase

Plant defense pathways triggered by pathogen infection are known to utilize salicylic acid as a signal molecule [56]. Hence, response to exogenous application of salicylic acid was studied. Following incubation of excised leaves with salicylic acid, the enzyme activity increased in the leaves of *W. somnifera*. The net enhancement in activity reached its maximum at about 10 h and then, began to decline, reaching negligible level after 20 h (Fig. 6A). In contrast, the excised control leaves without salicylic acid exhibited a slow decline in the enzyme activity till 20 h duration. Since excised leaves were used in the salicylic acid treatment, the increase in enzyme activity may be a combined response of wounding and salicylic acid activated biotic stress related pathways. The response of 27β-hydroxy glucosyltransferase to salicylic acid is similar to that of the previously reported 3β-hydroxy glucosyltransferases from *W. somnifera* [40,41]. The wound induced expression of solanidine [57] and tomato [58] glucosyltransferases has been reported. However, specific functional role of such changes is not understood. Altered distribution

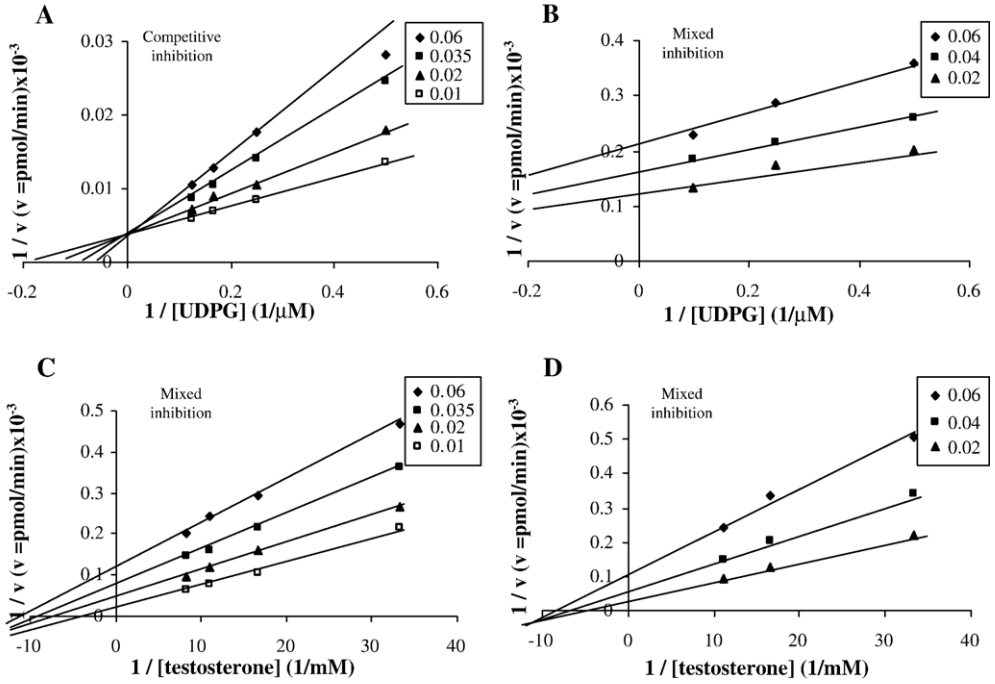


Fig. 5. *W. somnifera* 27β-hydroxy glucosyltransferase kinetic mechanism by product inhibition. Double reciprocal plots of variable UDP-glucose concentration versus testosterone glucoside formation in the presence of 0.20 mM testosterone and different concentrations of (A) UDP (0.01–0.06 mM) and (B) testosterone glucuronide (0.02–0.06 mM) as the products. Double reciprocal plots of variable testosterone concentration versus testosterone glucoside formation in the presence of 30 μM UDP-glucose and different concentrations of (C) UDP (0.01–0.06 mM) and (D) testosterone glucuronide (0.02–0.06 mM) as the products.

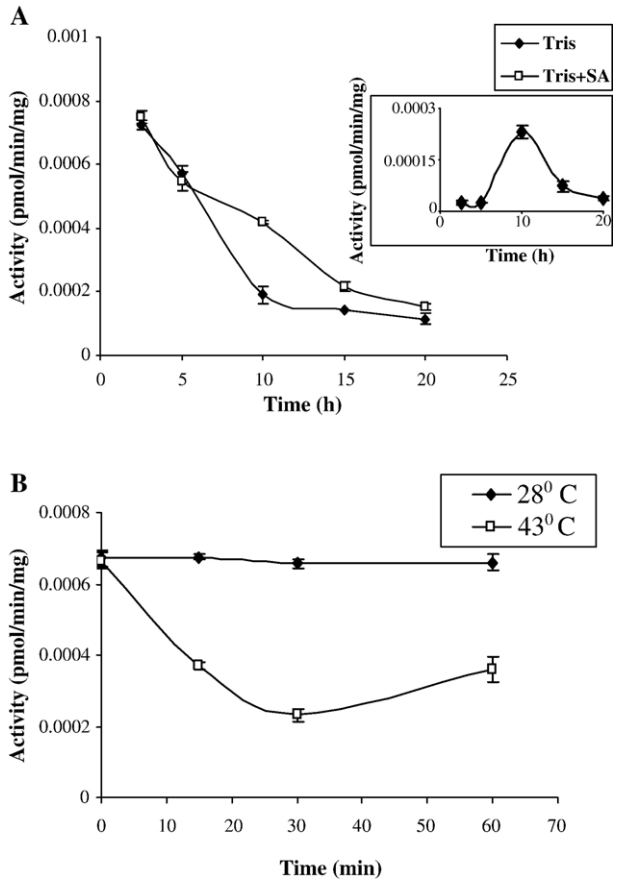


Fig. 6. Effect of salicylic acid and heat stress on *W. somnifera* leaf 27β-hydroxy glucosyltransferase. (A) The effect of salicylic acid treatment by incubating the excised leaves as immersed in Tris–HCl, pH 8.0 with (–□–) and without (–◆–) 1 mM salicylic acid. The inset shows net differential of the two activities. (B) Effect of temperature seen by incubating potted plants at 28 °C (–◆–) and 43 °C (–□–). Testosterone was used as sugar acceptor substrate in these experiments.

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 decline in the enzyme activity within 15 min and then rose to maintain at a level lower than the control. The control plants, exposed to 28 °C showed no change in enzyme activity (Fig. 6B). The contrasting temperature stress response of this enzyme to the previously reported mammalian, plant and protozoan enzymes [11,40,59] suggests different roles of sterol glucosides in plants.

4. Conclusion

This study identifies a novel 27β-hydroxy glucosyltransferase, distinct from the previously identified secondary metabolite glucosyltransferases reported from plants. The substrate specificity of this enzyme towards β-OH at higher carbon positions in withanolides and sterols can be valuable for generating new steroidal molecules with modified biological activities. Characterization of novel sterol glucosyltransferases with contrasting substrate specificities with a wide range of metabolites will provide the basis for genetic manipulation of the genes for these enzymes. Such studies are desirable to elucidate the function of different classes of glycosylated sterols in plants, especially under conditions of biotic and abiotic stresses.

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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.2007.06.015.

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