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# THE 3-*O*-GLUCOSYLATION OF STEROIDAL SAPOGENINS AND ALKALOIDS IN EGGPLANT (*SOLANUM MELONGENA*); EVIDENCE FOR TWO SEPARATE GLUCOSYLTRANSFERASES

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**Key Word Index**—*Solanum melongena*; Solanaceae; steroidal alkaloids and sapogenins; glucosylation; glucosyltransferases.

**Abstract**—UDP-glucose:diosgenin and UDP-glucose:solasodine glucosyltransferase are present in leaves, stems, roots and ripening seeds of garden eggplant (*Solanum melongena*). These two enzymes share several common properties: i) they occur mainly in the soluble proteins fraction; ii) they co-purify during purification procedure involving ammonium sulphate precipitation, ion-exchange chromatography on Q-Sepharose and gel filtration on Sephadex G-100; iii) they exhibit native $M_r$ values of *ca* 55 000; iv) they absolutely require reduced -SH groups; and v) they are strongly inhibited by some UDP-glucose analogues such as UDP, UDP-2′,3′-dialdehyde and UDP-mannose. However, the above mentioned enzyme activities can be clearly distinguished using some other effectors. Low concentrations of several non-ionic detergents including Tween 80, Triton X-100 and Tyloxapol almost completely abolish glucosylation of diosgenin but have a slight stimulatory effect on glucosylation of solasodine. Synthetic diosgenin 3-*O*-β-D-glucopyranoside strongly inhibits glucosylation of diosgenin and some related spirostanols but not that of solasodine. Cholesterol is a potent competitive inhibitor of diosgenin glucosylation ($K_i = 1.15\ \mu M$) but it has little effect on the glucosylation rate of solasodine. The above data strongly suggest that 3-*O*-glucosylation of steroidal sapogenins and alkaloids in eggplant leaves is catalyzed by two similar though separate UDP-glucose-dependent glucosyltransferases.


## INTRODUCTION

Steroidal saponins and glycoalkaloids are widely distributed in vascular plants including a number of economically important crop plants. Their importance for plant protection against microbial pathogens or herbivores has been suggested [1–3]. The frequently observed antiviral [4], fungitoxic [1, 5], cytostatic [4, 6] and hypocholesterolemic [7, 8] activities of these glycosides have aroused for years an interest of plant biochemists and physiologists. Despite the fact that the occurrence, chemical structure and various biological activities of steroidal saponins and glycoalkaloids have been studied by numerous authors, a relatively small body of information has been published concerning their biosynthesis, especially at the enzyme level.

Garden eggplant (*Solanum melongena* L.) is an interesting model for studies on the biosynthesis of steroidal saponins and glycoalkaloids since this plant may synthesize both these types of glycosides. Steroidal saponins, so-called melongosides, are oligosides of spirostane-type sapogenins: diosgenin and its 5,6-dihydro derivative, i.e. tigogenin, and occur mainly in mature seeds [9, 10] while steroidal glycoalkaloids contain solasodine, a steroidal alkaloid of the spirosolane type, as the aglycone and accumulate in the pulp of unripe fruits [11, 12].

In previous papers [13–15] we have reported that crude, partly delipidated enzyme preparations ("acetone powders") obtained from eggplant leaves efficiently glucosylated, in the presence of UDP-glucose (UDPGlc), diosgenin as well as solasodine. The products of these reactions may be regarded as intermediates in the biosynthesis of melongosides and solasodine-based glycoalkaloids produced in eggplant. We have shown [14] that good substrates for the above enzyme preparations consisted of, apart from diosgenin and solasodine, several other $\Delta^5$ or 5α-H spirostanols related to diosgenin, i.e. tigogenin, yamogenin or hecogenin, as well as tomatidine which is a spirosolane-type alkaloid related to solasodine. However, the enzyme preparation isolated from eggplant

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Table 1. Distribution of UDPGlc:diosgenin Glc-Tase (A) and UDPGlc:solasodine Glc-Tase (B) activities in lipid-depleted cytosolic fractions obtained from various organs of eggplants of different age

| Plant organ | Plant age (weeks) | Glc-Tase specific activity (nmol mg protein$^{-1}$ hr$^{-1}$) | | Specific activity ratio (A/B) |
|---|---|---|---|---|
| | | A | B | |
| Leaves | 3 (whole shoots) | 3.82 | 2.93 | 1.3 |
| | 6 | 3.91 | 2.79 | 1.4 |
| | 9 | 4.41 | 2.95 | 1.5 |
| | 11 | 4.29 | 3.01 | 1.4 |
| Roots | 6 | 3.17 | 2.26 | 1.4 |
| | 9 | 3.15 | 2.99 | 1.1 |
| Stems | 9 | 2.57 | 1.37 | 1.9 |
| Fruits | 22 (unripe, *ca* 3 cm long, whole) | 0.88 | 0.86 | 1.0 |
| | 23 (mature, *ca* 30 cm long): | | | |
| | pericarp parenchyma | traces | traces | — |
| | ripening seeds | 1.79 | 2.45 | 0.7 |

was completely inactive towards typical sterols and displayed only very limited activity with the solanidane-type steroidal alkaloids such as solanidine or demissidine. In this respect the glucosyltransferase (Glc-Tase) present in eggplant leaves greatly differs from the enzyme isolated from potato [16, 17, 18]. Partially purified UDPGlc-dependent Glc-Tase from potato sprouts has been reported [18] to be highly active with both solanidane- and spirosolane-type steroidal alkaloids but it was virtually inactive towards spirostanol sapogenins such as diosgenin.

The present study was aimed at partial purification and comparative characterization of UDPGlc:diosgenin and UDPGlc:solasodine Glc-Tase activities which occur in eggplant. We expected that this study would help to answer the question whether the parallel glucosylation of spirostane-type sapogenins and spirosolane-type alkaloids by crude enzyme preparations from this plant is catalyzed by a single enzyme or by two separate Glc-Tases specific for spirostane sapogenins and spirosolane alkaloids, respectively.

## RESULTS

Data on the presence of UDPGlc:diosgenin and UDPGlc:solasodine Glc-Tase activities in various organs of eggplant are summarized in Table 1. Both these activities were high in leaves, roots, stems and ripening seeds, lower in unripe fruits and very low (if any) in pericarp parenchyma of fully mature fruits. In all cases 70–95% of the enzyme activities originally present in crude homogenates was recovered in the fractions of soluble proteins (105 000 *g* supernatant). Evidently, there is no correlation between the level of Glc-Tase activities and accumulation of steroidal saponins and glycoalkaloids in individual organs. For instance, leaves of plants of different age exhibit very high activities of UDPGlc:diosgenin and UDPGlc:solasodine Glc-Tase but contain no significant amounts of melongosides and only very low concentrations of solasodine-based glycoalkaloids (Kintia, P. K., personal communication). These results suggest that the accumulation of steroidal glycosides is not regulated by the activity of corresponding Glc-Tase but rather by the rate of aglycone biosynthesis. It appears noteworthy, however, that the ratio of UDPGlc:diosgenin Glc-Tase to UDPGlc:solasodine Glc-Tase was significantly different in enzyme preparations obtained from various organs as it ranged from 0.7 (ripening seeds) to 1.9 (stems).

Results of our attempts to purify the Glc-Tase (Glc-Tases?) are shown in Table 2. The procedure applied for purification gave *ca* 50-fold increase in the specific activities of both UDPGlc:diosgenin and UDPGlc:solasodine Glc-Tase, however, the activity ratio did not alter significantly throughout purification steps. During ion-exchange chromatography on Q-Sepharose and gel filtration on Sephadex G-100 both enzyme activities eluted exactly in the same fractions as single activity peaks. It is remarkable that the total activities of both UDPGlc: diosgenin and UDPGlc:solasodine Glc-Tase increased significantly (*ca* 2-fold) during fractionation with ammonium sulphate and subsequent chromatography on Q-Sepharose. Such a rise in the total enzyme activity was frequently noticed during early steps of purification of several plant Glc-Tases, e.g. Glc-Tase involved in DDT metabolism from soybean cell cultures [19] or UDPGlc:dolichol phosphate Glc-Tase from mung bean hypocotyls [20], and it has been explained by removal of endogenous inhibitors and/or enzyme destabilizing factors, e.g. proteinases. In contrast to the starting material, i.e. crude delipidated cytosol, UDPGlc: diosgenin and UDPGlc:solasodine Glc-Tase activities present in the partially purified preparation were rather unstable and decreased by *ca* 55% when the latter enzyme preparation was kept at 4° for 24 hr. Similar losses of both enzyme activities were found when the purified

Table 2. Purification of UDPGlc:diosgenin Glc-Tase (A) and UDPGlc:solasodine (B) Glc-Tase from eggplant leaves

| Step | Protein (mg) | Total activity (nmol $hr^{-1}$) A | B | Specific activity (nmol mg $protein^{-1}$ $hr^{-1}$) A | B | Purification (fold) A | B |
|---|---|---|---|---|---|---|---|
| Delipidated cytosol | 72.0 | 172.8 | 194.4 | 2.4 | 2.7 | 1.0 | 1.0 |
| Buffer extraction | 49.3 | 187.3 | 152.8 | 3.8 | 3.1 | 1.6 | 1.1 |
| $(NH_4)_2SO_4$ precipitation | 14.5 | 253.7 | 277.0 | 17.5 | 19.1 | 7.3 | 7.1 |
| Q-Sepharose | 6.4 | 339.2 | 364.2 | 53.0 | 56.9 | 22.1 | 21.1 |
| Sephadex G-100 | 1.4 | 198.1 | 186.8 | 131.5 | 133.4 | 54.8 | 49.4 |

enzyme preparation was stored in 20–50% glycerol at −20°, irrespective of the presence or absence of thiol reagents (2-mercaptoethanol, dithiothreitol) or proteinase inhibitors (e.g. PMSF). In order to obtain more purified enzyme preparations and, eventually, to separate UDPGlc:diosgenin and UDPGlc:solasodine Glc-Tase activities, we tried to apply several techniques which have been successfully used for high purification of other plant Glc-Tases, e.g. hydrophobic chromatography on Phenyl- and Octyl-Sepharose [19, 21, 22] or affinity chromatography on UDP-Hexanolamine-Agarose [23]. Our attempts, however, were unsuccessful. Both Glc-Tases studied were not retained on Phenyl-Sepharose and UDP-Hexanolamine-Agarose. On Octyl-Sepharose they were readily bound, however, they could not be recovered from the column in active forms. Another serious problem in purification of UDPGlc:diosgenin Glc-Tase and UDPGlc:solasodine Glc-Tase was created by a very potent inhibitory effect of increased ionic strength on these two enzyme activities (see below).

With the partially purified enzyme preparation, the apparent native $M_r$s of UDPGlc:diosgenin and UDPGlc:solasodine Glc-Tase activities were estimated to be near 55000, based on gel filtration on a Sephacryl S-200 HR column calibrated with proteins of known molecular weights [21]. The $M_r$ of the eggplant Glc-Tase falls within the range of reported $M_r$ of a number of soluble plant Glc-Tases, e.g. UDPGlc:flavonoid Glc-Tase from strawberry fruit [24], UDPGlc:salicylic acid Glc-Tase from oat roots [25], UDPGlc:betanidin Glc-Tase from cell suspension cultures of *Dorotheanthus bellidiformis* [22] or UDPGlc:cyanidin Glc-Tase from grape cell suspension cultures [26]. The kinetic studies showed that apparent $K_m$ for diosgenin (0.33 μM) was several fold higher than that for solasodine (0.04 μM). The apparent $K_m$ for UDPGlc was 2.1 μM, regardless of the steroidal substrate used for the enzyme assay. The pH optimum for glucosylation of both diosgenin and solasodine was in the alkaline range (7.5 to 8.5) but it was strongly dependent on the buffer used for enzyme assay (see Fig. 1). The activities of both UDPGlc: diosgenin and UDPGlc:solasodine Glc-Tase were the highest in Tris-HCl and HEPES buffers but quite low in Tris-maleate and hardly detectable in KPi buffer (data not shown). Both Glc-Tase activities were stable at 30° for at least 1–2 hr but at a temperature of 40° or greater they were lost very quickly. They were not significantly affected by the divalent cations such as $Mg^{2+}$, $Ca^{2+}$ or $Mn^{2+}$ (0.1–10 mM) as well as by divalent metal chelators (EDTA and EGTA) indicating that, unlike several other plant Glc-Tases (see e.g. [27, 28, 29]), UDPGlc: diosgenin and UDPGlc: solasodine Glc-Tases had no requirement for metal cofactors. In contrast, ions of heavy metals such as $Zn^{2+}$, $Cu^{2+}$ or $Hg^{2+}$ strongly inhibited both enzyme activities (more than 80% inhibition at 1 mM concentration).

In order to gain some information concerning the specificity of eggplant Glc-Tase (Glc-Tases?) with respect to the sugar moiety source, partly purified enzyme preparation was incubated with diosgenin or solasodine, UDP-[$^3$H]Glc and excess amounts of various cold nucleotide sugars (see Fig. 2). Under experimental conditions in which the presence of 75 μM cold UDPGlc caused *ca* 80–90% reduction in the synthesis of labelled diosgenin or solasodine glucoside,

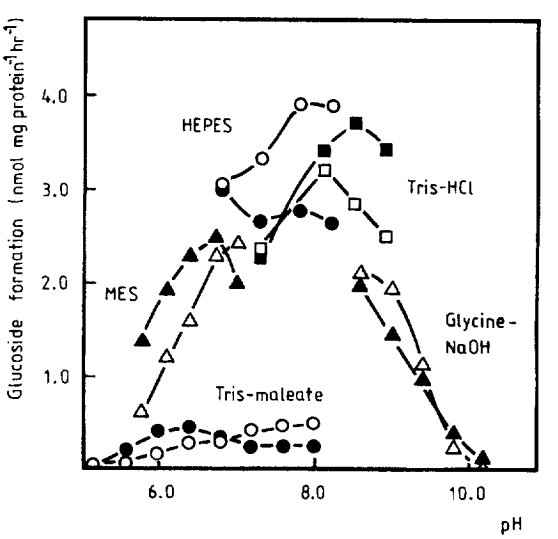


Fig. 1. Effects of pH and various buffers on the UDPGlc:diosgenin (open symbols) or UDPGlc:solasodine (filled symbols) Glc-Tase activities. All buffers were 50 mM and contained 5 mM 2-mercaptoethanol.

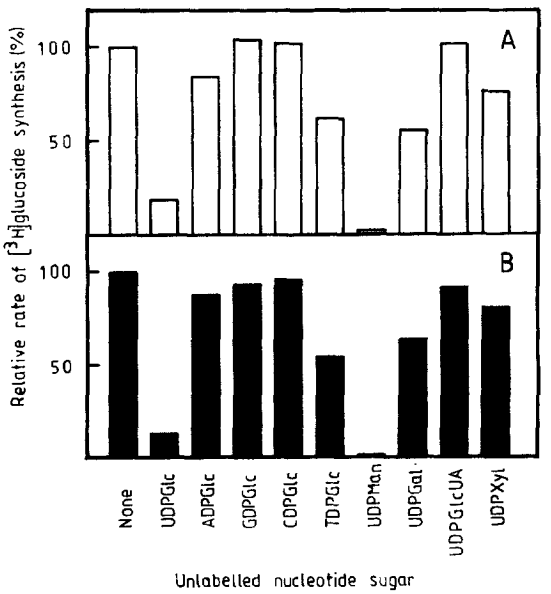


Fig. 2. Influence of excess amounts of unlabelled nucleotide sugars (75 $\mu$M) on the synthesis of labelled diosgenin (A) or solasodine (B) glucosides from UDP-[$^3$H]Glc.

Table 3. Synthesis of labelled tigogenin glucoside from [$^3$H]tigogenin and unlabelled nucleotide sugars

| Nucleotide sugar | Synthesis of the [$^3$H]tigogenin glucoside (dpm $\times 10^{-3}$ per sample) | Relative glucosylation rate (%) |
|---|---|---|
| None | 0.19 | — |
| UDPGlc | 31.69 | 100.0 |
| ADPGlc | 0.39 | 0.6 |
| GDPGlc | 0.17 | 0.0 |
| TDPGlc | 2.33 | 6.8 |
| CDPGlc | 0.38 | 0.6 |
| UDPGal | 0.75 | 1.8 |
| UDPMan | 0.47 | 0.9 |
| UDPGlcUA | 0.59 | 1.4 |
| UDPXyl | 0.32 | 0.4 |

the addition of 75 $\mu$M ADPGlc, GDPGlc or CDPGlc had no significant effect on the formation of labelled glycosidic products. However, inhibition (40–50%) was found using cold TDPGlc. UDP-glucuronate (UDPGlcUA) and UDPXyl had no pronounced effect on the formation of labelled glucosides of either diosgenin or solasodine but the addition of cold UDPGal and, particularly, UDPMan strongly decreased the incorporation of [$^3$H]Glc from UDP-[$^3$H]Glc. The above results suggest that TDPGlc, UDPGal and UDPMan can effectively compete with UDPGlc for binding with the catalytic site of the investigated enzyme (enzymes). However, the question remains open whether these nucleotide sugars can serve as sugar donors for glycosylation of diosgenin or solasodine. A partial answer to this question was furnished by another experiment in which [$^3$H]tigogenin and various cold nucleotide sugars were used for incubations. The results presented in Table 3 clearly demonstrate that among nucleotide sugars tested, only TDPGlc can substitute for UDPGlc as sugar donor for tigogenin glucosylation. This is not surprising since TDPGlc is the closest structural analogue of UDPGlc. It has been frequently reported that various UDPGlc-dependent Glc-Tases of plant origin showed some activity with TDPGlc; examples are UDPGlc: coniferyl alcohol Glc-Tase from cambial sap of spruce [30], UDPGlc:sterol Glc-Tase from cotton fibres [31] or UDPGlc:flavonol Glc-Tase from Norway spruce needles [32]. In the presence of all other nucleotide sugars tested, including UDPMan and UDPGal, no visible synthesis of labelled tigogenin glycoside could be observed. Most probably, at least in case of spirostanol glucosylation, UDPMan and UDPGal can bind to the catalytic site of the investigated enzyme blocking interactions of the enzyme with its proper substrate, i.e. UDPGlc, but they cannot serve as alternative sugar donors.

Some selected data on inhibitory effects of various chemicals on glucosylation of diosgenin or solasodine by enzyme preparations from eggplant leaves are summarized in Table 4. Both enzymic activities were strongly inhibited by PCMB (*p*-chloromercuribenzoate). At 0.02 mM concentration of PCMB glucosylation of diosgenin and solasodine was almost completely arrested. However, the inhibitory effect of PCMB was nearly completely reversible with 10 mM 2-ME (2-mercaptoethanol). This indicates an absolute requirement for reduced cysteine residues in the active site of the enzyme (or enzymes?) for its (their) activity. The glucosylation of diosgenin, as well as that of solasodine, was distinctly reduced by UDP and, especially, by its periodate oxidized analogue, oUDP (UDP-2′,3′-dialdehyde). Feedback inhibition by UDP and/or its analogues is a rather common effect observed with various UDPGlc-dependent Glc-Tases of plant origin [33–35]. Another common feature of the two Glc-Tase activities studied was strong inhibition by various salts added to the incubation medium. It should be noted that inhibitory effects of similar magnitude were found using NaF, KF, NaCl, KCl, $NH_4Cl$, KBr, $Na_2SO_4$, $(NH_4)_2SO_4$, NaOAc and $NH_4OAc$. This indicates that the above described inhibition results rather from the unfavorable effect of increased ionic strength on the catalytic efficiency of the Glc-Tase than it is caused by a specific interaction of the enzyme with some particular ions. The inhibitory effect of various salts could be reversed in part by dialysis against dilute Tris-HCl or HEPES buffers.

Further studies showed that several other enzyme effectors exerted entirely different effects on the two Glc-Tase activities. Several non-ionic detergents including Tween 20, Tween 80, Triton X-100, reduced

Table 4. Effects of some inhibitors on UDPGlc:diosgenin Glc-Tase and UDPGlc:solasodine Glc-Tase activities

| Enzyme inhibitor | Inhibitor concentration for half maximal activity ($I_{50}$) | |
|---|---|---|
| | UDPGlc:diosgenin Glc-Tase | UDPGlc: solasodine Glc-Tase |
| PCMB | 6.0 μM | 10 μM |
| UDP | 80 μM | 68 μM |
| oUDP | 2.0 μM | 2.5 μM |
| Tween 80 | 230 μM | n.i.* |
| Triton X-100 | 460 μM | n.i. |
| Sodium deoxycholate | 140 μM | 190 μM |
| Zwittergent 3–16 | 3.0 μM | 22 μM |
| NaF | 250 mM | 175 mM |
| KCl | 170 mM | 100 mM |
| Sodium acetate | 280 mM | 225 mM |
| Diosgenin β-D-glucopyranoside | 2.0 μM | n.i. |
| 3-Ketotigogenin | 4.1 μM | n.i. |
| Cholesterol | 2.2 μM | n.i. |
| Sitosterol | 2.6 μM | n.i. |
| Phosphatidylcholine (hen egg) | 42 μM | n.i. |
| Phosphatidylethanolamine (*E. coli*) | 75 μM | n.i. |

* No significant inhibition (or even slight stimulation) at inhibitor concentration for half maximal activity of the second Glc-Tase studied

Triton X-100 and Tyloxapol used at very low concentrations (0.01–0.05%) strongly decreased the rate of diosgenin glucosylation. At these concentrations of detergents glucosylation of solasodine was even slightly stimulated. We were unable to find any significant inhibition of solasodine glucosylation even at 1% concentrations of the above listed detergents (this detergent concentration caused almost full inhibition of diosgenin glucosylation!). In contrast to non-ionic detergents, anionic detergent, deoxycholate, as well as zwitterionic detergents such as Zwittergent 3–8, 3–10, 3–14 and 3–16 were potent inhibitors of both UDPGlc:diosgenin and UDP:solasodine Glc-Tase activities. Effects of increasing concentrations of selected detergents (Tween 80, Triton X-100 and deoxycholate) are presented in Fig. 3.

As shown in Fig. 4 the synthetic diosgenin 3-β-D-glucopyranoside, i.e. the product of diosgenin glucosylation by the investigated enzyme preparation, was a very potent inhibitor of UDPGlc:diosgenin Glc-Tase activity having at the same time only a slight effect on glucosylation of solasodine. It is remarkable that inhibition of diosgenin glucoside formation in the presence of diosgenin glucoside, resulting most probably from feedback inhibition of the glucosylation by the reaction product, was evident already at concentrations of diosgenin glucoside exceeding only a few times the concentration of diosgenin in the incubation mixture! It is noteworthy that a similar strong inhibition of the glucosylation reaction by diosgenin glucoside was found also when some spirostanol sapogenins structurally related to diosgenin, such as tigogenin or yamogenin were used for incubation.

Significant differences were also found studying effects of some analogues of steroidal substrates of the investigated Glc-Tase (Glc-Tases?). Synthetic 3-ketotigogenin was potent inhibitor of diosgenin glucosylation but not that of solasodine (see Fig. 5).

The present studies with partially purified enzyme preparation confirmed our earlier observations [14] that free sterols could not be glucosylated by soluble enzyme (enzymes?) present in eggplant leaves. However, we found that an addition of some sterols, e.g. cholesterol or sitosterol, to the incubation medium clearly decreased glucosylation of diosgenin but was without effect on glucosylation of solasodine (see

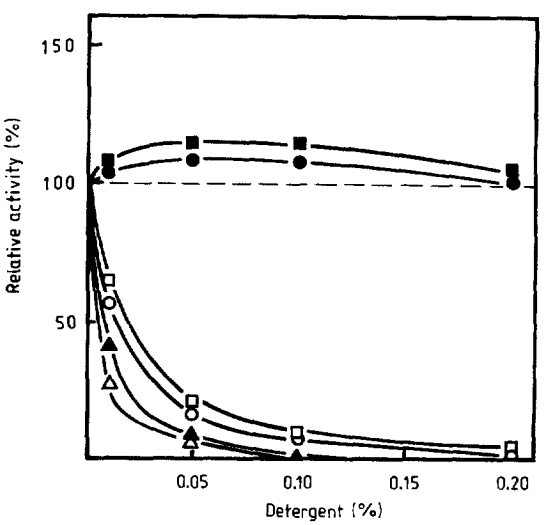


Fig. 3. Effects of increasing concentrations of Tween 80 (○, ●), Triton X-100 (□, ■) and sodium deoxycholate (△, ▲) on glucosylation of diosgenin (open symbols) or solasodine (filled symbols).

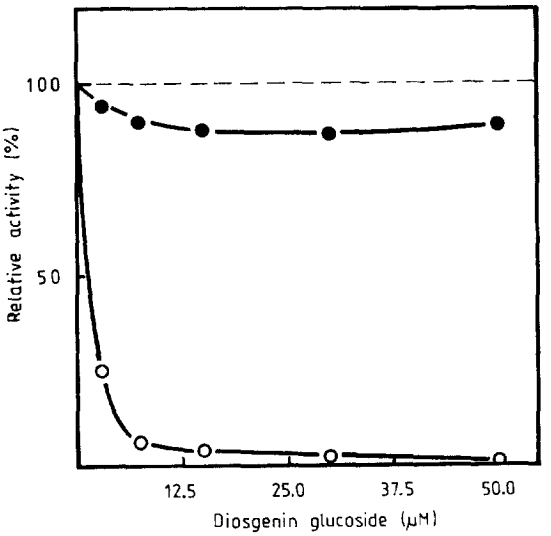


Fig. 4. Effect of diosgenin 3-*O*-β-D-glucopyranoside on glucosylation rate of diosgenin (○) and solasodine (●) by eggplant Glc-Tase preparation.

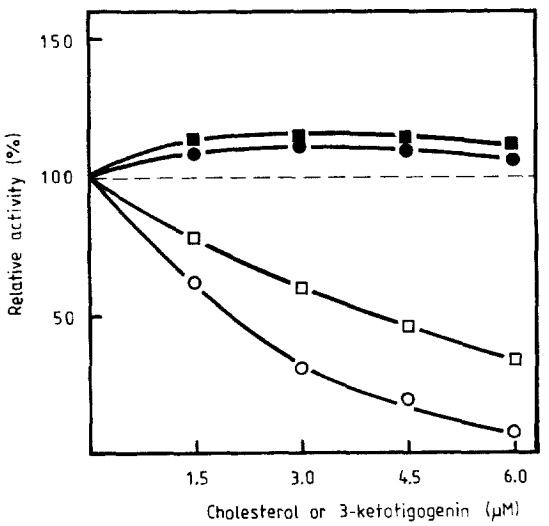


Fig. 5. Effects of cholesterol (○, ●) or 3-ketotigogenin (□, ■) on glucosylation of diosgenin (open symbols) and solasodine (filled symbols).

Table 4 and Fig. 5). The results of the kinetic studies (data not presented) showed clearly that cholesterol was a competitive inhibitor with respect to diosgenin with $K_i = 1.15\,\mu M$. This suggests that inhibition of diosgenin glucosylation by cholesterol (and probably by other sterols) may be explained on the assumption that it can successfully compete with diosgenin (but not with solasodine) for the active site of the Glc-Tase.

Furthermore, differentiating effects of some glycerophospholipids including phosphatidylcholine (PC) and phosphatidylethanolamine (PE) on the two Glc-Tase activities studied could be observed (see Table 4). Again, in the presence of these phospholipids, glucosylation of diosgenin was clearly inhibited. At PC or PE concentrations which caused 50% reduction of diosgenin glucosylation the synthesis of solasodine glucoside was significantly (20–40%) stimulated.

## DISCUSSION

Although we were unable to achieve physical separation of UDPGlc:diosgenin Glc-Tase from UDPGlc:solasodine Glc-Tase, several lines of evidence strongly suggest that these two activities reside in two closely related but separate enzyme proteins, specific for spirostane sapogenins and spirosolane alkaloids, respectively. Though UDPGlc:diosgenin and UDPGlc:solasodine Glc-Tase activities co-occur in various organs of eggplant the activity ratio is significantly different in individual organs. Moreover, various chemicals affect glucosylation of diosgenin and solasodine by partially purified enzyme in remarkably different fashion. Low concentrations of nonionic detergents, such as Tween 20, Tween 80, Triton X-100, reduced Triton X-100 or Tyloxapol, almost completely inhibit glucosylation of diosgenin but not that of solasodine. Some glycerophospholipids, such as PC or PE, significantly enhance glucosylation of solasodine but strongly decrease the formation of diosgenin glucoside. Free sterols, 3-ketotigogenin and synthetic diosgenin 3-*O*-β-D-glucopyranoside are potent inhibitors of diosgenin glucosylation but have little effect on glucosylation of solasodine. Assuming that glucosylation of diosgenin and solasodine is catalyzed by the same enzyme protein, it is very difficult to find a reasonable explanation how these structurally diverse compounds may affect the glucosylation of diosgenin and solasodine in such different ways. A possibility that eggplant contains separate UDPGlc:diosgenin Glc-Tase and UDPGlc:solasodine Glc-Tase is not surprising, since there are many reports on the occurrence in plants of multiple Glc-Tase isoforms which differ in their aglycone specificity. For instance, it has been shown that 2″-*O*-glucosylation of vitexin and isovitexin in petals of *Silene alba* is catalyzed by two different enzymes which are governed by different genes [28]. Three isoforms of UDPGlc:betanidin 5-*O*-Glc-Tase and one isoform of UDPGlc: betanidin 6-*O*-Glc-Tase which differ in their isoelectric points have been reported in *Dorotheanthus bellidiformis* [22]. Evidence has been presented that seedlings and suspension-cultured cells of alfalfa (*Medicago sativa*) contain at least two closely related UDPGlc-dependent Glc-Tases with activities directed toward flavonoids and isoflavonoids [21]. Five different antraquinone-specific Glc-Tases were detected in *Cinchona succirubra* cell suspension cultures [35]. The presence of four Glc-Tases differing in their specificity toward various flavonoid aglycones and/or in their apparent $M_r$, pI or pH optimum values have been described in grapefruit (*Citrus paradisi*) seedlings [36].

In our previous paper [14] we have demonstrated that crude, partially delipidated cytosol fraction obtained from eggplant leaves, apart from its ability to synthesize, in the presence of UDPGlc, monoglucosides of diosgenin or solasodine, can catalyze, in the presence of UDPGal, the formation of diosgenin or solasodine monogalactosides. Though the latter activity was several times lower than the former one, it was easily detectable. When assayed by TLC, the labelled products formed upon incubation of crude enzyme preparations with spirostanols or spirosolane alkaloids and UDP-[$^{14}$C]Gal showed somewhat lower mobilities as compared to the products formed in the presence of UDP-[$^{14}$C]Glc and could be easily distinguished [14]. In the present study, using partially purified enzyme preparation, we were unable to detect any formation of spirostane or spirosolane galactosides. These results suggest that the Glc-Tase activity present in crude enzyme preparations is either separated from Glc-Tase activity during purification steps or it is much less stable and loses its activity in the course of purification. It is of interest to point out that a similar situation has been found in potato. It has been shown [16–18] that in crude enzyme preparations obtained from potato leaves, tubers or sprouts UDPGlc:solanidine Glc-Tase activity is accompanied by UDPGal:solanidine Gal-Tase activity but the latter activity vanished during the initial steps of purification [18].

## EXPERIMENTAL

### *Plant material*

Eggplant (*cv* Black Beauty) seeds were sown in pots containing garden soil-sand (1:1) and germinated in darkness at 26°. After one week pots were transferred into a greenhouse. In most experiments fresh leaves were used as a source of enzyme preparations.

### *Glucosyltransferase purification*

The purification procedure was essentially similar to that described earlier for partial purification of UDPGlc:solasodine Glc-Tase activity [15]. All enzyme extraction and purification steps were carried out at 0–4° and the enzyme activity was assayed in parallell using diosgenin or solasodine as substrates. Crude lipid-depleted enzyme preparations ("acetone powders") were obtained from crude cytosol ($1.05 \times 10^5 g$ supernatant) of 8-week-old eggplant leaves exactly as described previously [14, 37]. These enzyme preparations could be stored at −20° with no loss of the UDPGlc:diosgenin and UDPGlc:solasodine Glc-Tase activities for several months. TLC/autoradiography [14] of the glycosidic fraction formed upon incubations of crude enzyme preparations with labelled UDPGlc and diosgenin or solasodine showed the presence of main reaction products (>95%) with the $R_f$ values of 0.51 or 0.20, respectively, i.e. identical to those of synthetic diosgenin 3-β-D-glucopyranoside or solasodine 3-β-D-glucopyranoside, and small amounts (<5%) of slightly more polar products ($R_f$ 0.47 or 0.16, respectively) with chromatographic properties of diosgenin or solasodine 3-β-D-galactopyranosides [14]. In the absence of exogenously added diosgenin or solasodine the formation of radioactive glycoside was negligible. In a typical purification procedure crude, delipidated enzyme preparation (0.7 g, 0.72 mg protein) was stirred for 15 min in 50 ml of buffer A (0.05 M Tris-HCl, pH 7.3, containing 10 mM 2-mercaptoethanol) and then centrifuged at $2 \times 10^4 g$ for 20 min. The insoluble fraction (*ca* 30% protein present in the starting material) was discarded as it contained only traces of UDPGlc:diosgenin and UDPGlc:solasodine Glc-Tase activities and the clear supernatant was fractionated with solid $(NH_4)_2SO_4$. Material precipitating between 45 and 80% $(NH_4)_2SO_4$ saturation was collected by centrifugation, resuspended in a small amount of buffer A, and dialyzed overnight against the same buffer. The dialysate was immediately applied to a Q-Sepharose column (6 cm, i.d. 15 mm) equilibrated with buffer A. Initially, elution was carried out using buffer A (15 ml) and then with 0.20, 0.35 and 0.50 M Tris-HCl buffers, pH 7.3 (20, 13 and 13 ml, respectively). Fractions eluting between 25 and 32 ml were pooled and proteins precipitated with solid $(NH_4)_2SO_4$ (up to 75% saturation). The precipitate was dissolved in 1 ml of buffer A and applied to a Sephadex G-100 column (40 cm, i.d. 10 mm). Elution was carried out with buffer A and fractions eluting between 17 and 22 ml were pooled (partially purified enzyme preparation). During column chromatography protein absorbance was continuously monitored at 280 nm. Incubations of the purified enzyme preparation with labelled UDPGlc and diosgenin or solasodine gave a single radioactive product with chromatographic properties of diosgenin or solasodine 3-β-D-glucopyranoside, respectively.

### *Assays for UDPGlc:diosgenin and UDPGlc:solasodine Glc-Tase activities*

Routine enzyme assays were performed virtually as described earlier [14, 37]. Unless otherwise stated, a typical incubation mixture contained in a total volume of 0.52 ml: 0.25 ml of the enzyme preparation (30 μg or 0.6 μg protein for crude and purified enzyme, respectively) in 0.1 M Tris-HCl, pH 8.5 (buffer B) or in 0.1 M HEPES, pH 8.0 (buffer C); solution of diosgenin or solasodine (0.4 nmol) in 0.01 ml of EtOH; solution of UDP-[$^3$H]Glc (1.5 nmol, $2 \times 10^5$ dpm) in 0.01 ml of 50% EtOH and 0.25 ml of $H_2O$ or an equivalent amount of enzyme effector solution in $H_2O$. Water-insoluble effectors, e.g. diosgenin glucopyranoside, cholesterol or phospholipids, were added in 0.01 ml of EtOH; in this case control samples contained equivalent amount of pure EtOH. In the above described incubation variant, saturating con-

centrations of both substrates, i.e. labelled UDPGlc and the steroidal acceptor, were used. In some experiments UDP-[$^{14}$C]Glc was used instead of UDP-[$^{3}$H]Glc at a non-saturating concentration (6.8 pmol, $2 \times 10^5$ dpm). The reaction was initiated by the addition of labelled UDPGlc and carried out usually at 30° for 30 min. The synthesized labelled glucosides were separated from unreacted UDPGlc and its degradation products by extraction with *n*-butanol and aliquots of the organic phase were counted in a liquid scintillation counter exactly as described earlier [38]. Under the above described conditions the reaction was time, substrate and protein dependent. Specificity of the enzyme preparation for nucleotide sugar was studied using [$^{3}$H]-tigogenin, 0.5 nmol, $3.5 \times 10^5$ dpm (see below) and various unlabelled sugar nucleotides (2.0 nmol); other incubation conditions were as above.

*Determination of kinetic parameters*

Apparent Michaelis constants for the steroidal acceptors were determined by measuring the initial reaction velocities at different concentration of diosgenin or solasodine (0.02–0.60 $\mu$M) while maintaining the concentration of labelled UDPGlc at a saturating level (3.0 $\mu$M). Apparent $K_m$ values for UDPGlc were studied using fixed concentrations of diosgenin (0.8 $\mu$M) or solasodine (0.24 $\mu$M) and varying concentration of UDPGlc (0.03–0.40 $\mu$M). The $K_m$ and $V_{max}$ were calculated from Lineweaver-Burk plots. The manner of inhibition of diosgenin glucoside synthesis by cholesterol and the corresponding $K_i$ value was determined from the results of incubations carried out at a fixed concentration of UDP-[$^{3}$H]Glc (3.0 $\mu$M), two fixed concentrations of cholesterol (2.4 and 4.8 $\mu$M) and varying concentration of diosgenin (0.2–0.8 $\mu$M).

*Other analytical methods*

Commercial preparations of diosgenin, tigogenin and solasodine (from Sigma) were additionally purified by recrystallization from EtOH immediately before use. Native $M_r$ values of UDPGlc:diosgenin and UDPGlc:solasodine Glc-Tases were determined by gel filtration on a Sephacryl S-200 HR column calibrated with proteins of known molecular weights [21]. Protein was measured using the dye-binding assay [39] with a protein assay kit (Bio-Rad). [$^{3}$H]-tigogenin was obtained by oxidation of tigogenin with $CrO_3$ under mild conditions [40] and subsequent reduction of the resulting 3-oxo derivative with $NaB^3H_4$ (spec. act. 296 GBq/mmol). The reduction product was diluted with a small amount of authentic cold tigogenin and crystallized twice from EtOH. The resulting preparation of labelled tigogenin had spec. act. 11.7 GBq/mmol and was radiochemically pure when analysed by TLC/autoradiography.

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