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THE OCCURRENCE OF UDPG-DEPENDENT GLUCOSYLTRANSFERASE SPECIFIC FOR SARSASAPOGENIN IN *ASPARAGUS OFFICINALIS*

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Key Word Index—*Asparagus officinalis*; Liliaceae; glucosyl-transferase; glucosylation of 3β-hydroxy steroids; sarsasapogenin glucoside; asparagoside biosynthesis.

Abstract—A number of 3β-hydroxy steroids including sterols and some steroid sapogenins can be glucosylated, with UDP-glucose (UDPG) as the sugar source, by lipid-depleted enzyme preparations ('acetone powders') obtained from shoots of two to three-week-old *Asparagus officinalis* plants. Different subcellular localizations of glucosyltransferase activities with various steroid acceptors and different sensitivities of individual reactions to a non-ionic detergent Triton X-100 indicate that at least two UDPG-dependent glucosyltransferases acting on steroids which exhibit entirely different specificity patterns are present in *A. officinalis*. Particulate fractions contain an enzyme which effectively glucosylates sterols and which is highly stimulated by Triton X-100. This glucosyltransferase seems to be similar to the well-known UDPG: sterol glucosyltransferase isolated earlier from several other higher plants. Another glucosyltransferase present in *A. officinalis* occurs in a soluble form and is distinctly inhibited by Triton X-100. This enzyme has little activity with sterols but efficiently glucosylates some spirostanol sapogenins. The best substrates are 5β-spirostanols: sarsasapogenin (the aglycone of steroid saponins present in *A. officinalis*) and its 25-epimer, smilagenin.

INTRODUCTION

Steroid saponins of the spirostane type occur in numerous plants, particularly in species belonging to the Dioscoreaceae, Solanaceae and Liliaceae; however, only sparse data are available on the biosynthesis of the sugar chains in these compounds [1, 2]. A frequent co-occurrence in the same plant of several structurally related glycosides which differ in the number of sugar residues suggests that oligosaccharide chains are formed by stepwise addition of monosaccharide moieties catalysed by specific glycosyltransferases. Recently we reported [3, 4] that oat leaves contain a soluble, specific enzyme, UDPG: nuatigenin glucosyltransferase, which catalyses glucosylation of nuatigenin (22,25-epoxy-20*S*,22*S*,25*S*-furost-5-ene-3β,26-diol) to its 3β-D-monoglucoside. We have suggested that this enzyme is specifically involved in the initiation of sugar-chain synthesis during the formation of oat saponins, avenacosides A and B, since nuatigenin β-D-monoglucoside can be regarded as an intermediate in the biosynthesis of avenacosides.

Asparagus officinalis leaves contain a series of spirostane glycosides in which the aglycone is sarsasapogenin (25*S*-5β-spirostan-3β-ol) [5, 6]. The simplest one of these glycosides is the 3β-D-monoglucoside [5, 6].

In this paper we give evidence that *A. officinalis* shoots contain a glucosyltransferase which is in some respects similar to the above mentioned UDPG: nuatigenin glucosyltransferase from oat leaves but seems to be specific for 5β-spirostanols as glucose acceptors.

RESULTS

Preliminary experiments with use of partially delipidated enzyme preparations, i.e. 'acetone powders' obtained from crude homogenates or from various subcellular fractions from shoots of *Asparagus officinalis* seedlings have shown that exogenous sitosterol (stigmast-5-en-3β-ol) and sarsasapogenin (25*S*-5β-spirostan-3β-ol) greatly stimulated the incorporation of [^{14}C]glucose from UDP-[U-^{14}C]glucose (UDPG) into components present in the *n*-butanolic extract, i.e. into the crude glycoside fraction. Labelled products migrated on TLC as expected for 3-monoglucosides of sitosterol or sarsasapogenin. Our further experiments have demonstrated that the stimulation of [^{14}C]glucose incorporation by sitosterol or sarsasapogenin is affected by Triton X-100 in clearly different ways. Triton X-100 (final conc. 0.32%) greatly enhanced (4–6 times) the synthesis of sitosteryl glucoside while it had a pronounced inhibitory effect (70–85%) on the glucosylation of sarsasapogenin. Moreover, the subcellular distribution of glucosyltransferase activities as measured with sitosterol or sarsasapogenin was clearly different (see Fig. 1). The particulate fraction obtained by centrifugation of crude homogenate at 105 000 *g* showed a very high glucosyltransferase activity with sitosterol but the activity with sarsasapogenin as the acceptor was hardly detectable. On the other hand sarsasapogenin was efficiently glucosylated by the cytosolic fraction (105 000 *g* supernatant) while this fraction had little activity with sitosterol as the acceptor. Data presented in Fig. 1 strongly suggest that the glucosylation of sitosterol and sarsasapogenin is catalysed by two different UDPG-dependent glucosyltransferases present in *A. officinalis* shoots, i.e. a membrane-bound enzyme, stimulated by Triton X-100 which seems to be similar to the well-known UDPG: sterol glucosyltransferase isolated earlier by several authors from a number of higher plants [7–9] and a soluble enzyme, inhibited by Triton X-100 which seems to

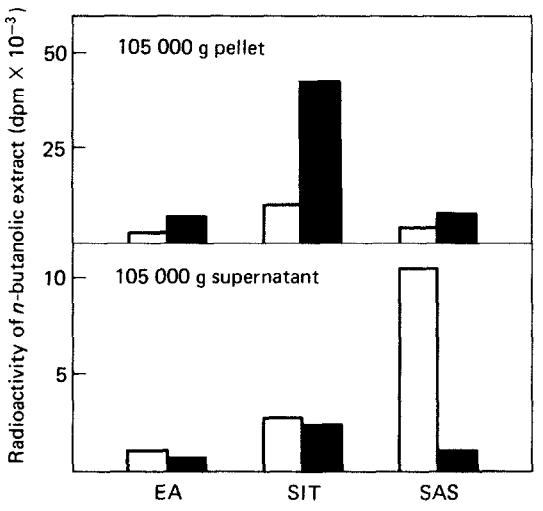

Fig. 1. Influence of Triton X-100 on glucosylation of endogenous acceptors (EA), sitosterol (SIT) or sarsasapogenin (SAS) by lipid-depleted membranous (105 000 *g* pellet) or soluble (105 000 *g* supernatant) fractions from *Asparagus officinalis* shoots. Reaction mixtures contained an enzyme preparation (0.25 mg), UDP-[^{14}C]glucose (2.2×10^5 dpm) and a steroid acceptor (25 nmol). Triton X-100 (a final conc. 0.32%) was included (black bars) or absent (white bars). Incubation time was 60 min. For other details see Experimental.

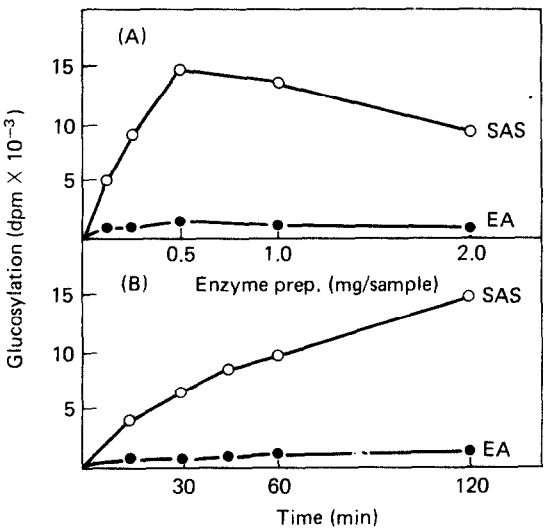

Fig. 2. Effects of enzyme concentration (A) or of incubation time (B) on glucosylation of sarsasapogenin (SAS) or endogenous acceptors (EA) by an acetone powder preparation obtained from the 105 000 *g* supernatant. Incubation time in A was 45 min. Enzyme conc. in B was 0.25 mg/sample. Triton X-100 was omitted.

be more or less specific for sarsasapogenin. Some properties of the latter glucosyltransferase were studied in our subsequent experiments.

Under incubation conditions specified in the Experimental, with lipid-depleted cytosol fraction and with sarsasapogenin as the acceptor, the formation of labelled glucoside was linear with time up to about 45 min (Fig. 2b). Prolonged incubations, up to eight hr, resulted in an about 20% incorporation of [^{14}C]glucose from UDPG into the *n*-butanolic extract. The glucosylation was proportional with enzyme preparation concentration within the range 0.05–0.5 mg/sample. However, at a higher enzyme concentration a rapid decrease of glucosylation rate was observed (Fig. 2a). The reason for this effect is not clear but it seems possible that an unknown, endogenous inhibitor is present in the enzyme preparation obtained from crude cytosol. The cytosolic glucosyltransferase exhibits a pH optimum at 9.6 (Fig. 3).

To investigate the specificity of the cytosolic enzyme a number of 3β-hydroxy spirostanol sapogenins structurally related to sarsasapogenin were tested as potential substrates (Table 1). Our results led to the conclusions that the best glucose acceptors are sarsasapogenin and its 25-epimer, smilagenin. These two sapogenins are almost equally good substrates. This indicates that the configuration of the C-25 methyl group is rather an unimportant factor for the enzyme. In contrast to all other spirostane sapogenins tested both sarsasapogenin and smilagenin are 5β-H compounds (*cis*-coupling of the A and B rings). Both 5α-H (A/B-*trans*) spirostanols such as tigogenin, hecogenin or chlorogenin and Δ^5-sapogenins such as diosgenin are much worse glucose acceptors. It is well known that the mode of coupling of the rings A and B has an effect on the overall shape of a steroid molecule. Compounds containing a double bond at C-5 or a 5α-H

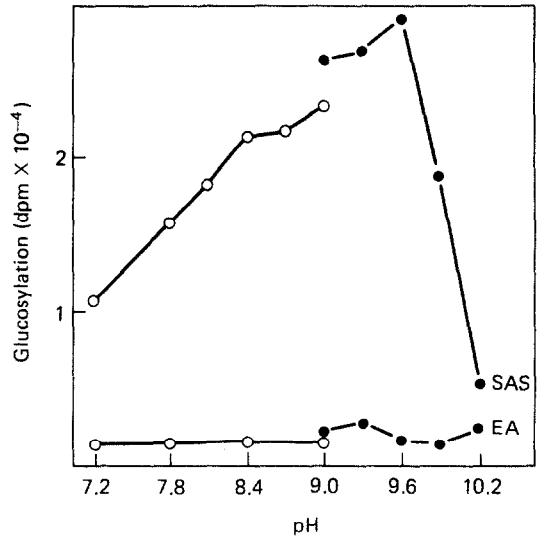

Fig. 3. Effect of pH on glucosylation of sarsasapogenin (SAS) or endogenous acceptors (EA) by the soluble glucosyltransferase. 0.2 M Tris–HCl, pH 7.2–9.0 (-o-) or 0.2 M glycine–NaOH, pH 9.0–10.2 (-●-) were used as buffers.

structure are relatively flat while 5β-H compounds are non-planar, i.e. the ring A lies in a different plane to the rings B–D. Our results indicate that the soluble glucosyltransferase preferentially utilizes compounds with the non-planar structure. This structural feature, however, is not sufficient for a high rate of glucosylation. Coprostanol which is a 5β-H sterol containing an identical steroid nucleus as in sarsasapogenin or smilagenin is very poor substrate for the enzyme. It may suggest that the presence of the heterocyclic ring system (rings E, F) in

Table 1. Specificity of the soluble glucosyltransferase from *Asparagus officinalis* shoots for some 3β-hydroxy steroids

Steroid acceptor (25 nmol/sample)	[^{14}C]Glucose incorporation into the *n*-butanol extract (dpm × 10^{-3})	
	−Triton X-100	+Triton X-100
Sarsasapogenin (25*S*-5β-spirostan-3β-ol)	10.36 (100)*	1.85
Smilagenin (25*R*-5β-spirostan-3β-ol)	10.52 (102)	1.72
Tigogenin (25*R*-5α-spirostan-3β-ol)	2.83 (27)	0.31
Diosgenin (25*R*-spirost-5-en-3β-ol)	2.01 (19)	0.29.
Hecogenin (25*R*-5α-spirostan-3β-ol-12-one)	3.85 (37)	0.45
Chlorogenin (25*R*-5α-spirostane-3β,6α-diol)	3.22 (31)	0.37
Sitosterol (stigmast-5-en-3β-ol)	2.40 (23)	2.16
Cholestanol (5α-cholestan-3β-ol)	1.84 (18)	2.02
Coprostanol (5β-cholestan-3β-ol)	1.62 (16)	0.21

* Relative activities (activity with sarsasapogenin = 100) are given in parentheses.

steroid sapogenins is essential for the proper interaction with the enzyme. It is also possible that the presence of a highly hydrophobic side chain in the sterol molecule exerts an unfavourable effect on the formation of the enzyme–substrate complex.

As can be deduced from data presented in Table 1 the non-ionic detergent, Triton X-100 distinctly inhibits glucosylation of all steroid sapogenins tested. On the other hand the glucosylation of sitosterol (a Δ^5-sterol) or cholestanol (a 5α-H sterol) is much less affected by this detergent. With sitosterol as the substrate there was only a slight inhibition by Triton X-100 while with cholestanol there was even a slight stimulatory effect. It seems that the glucosylation of these two sterols by the lipid-depleted cytosol fraction can be explained, at least in a part, by unavoidable contamination of the 105 000 *g* supernatant by small membrane fragments or/and by partial solubilization of the membranous UDPG : sterol glucosyltransferase during the isolation procedure. However, it should be noted that the glucosylation of coprostanol (a 5β-H sterol), like that of sarsasapogenin and other steroid sapogenins, is strongly inhibited by Triton X-100 which suggests that at least in this case the observed reaction can be attributed to the same enzyme which glucosylates sarsasapogenin.

Selected data on effects of some potential activators or inhibitors on the rate of sarsasapogenin glucoside formation are presented in Table 2. Like many other glucosyltransferases of plant origin [10–13] the soluble enzyme from *A. officinalis* shoots is activated by divalent metals. The highest stimulation (41% at 1 mM conc.) was found for Mn^{2+} ions. The requirement of the enzyme for divalent metals is confirmed by inhibitory effects of such metal chelators as EDTA (ethylenediaminetetraacetate) or EGTA [ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate]. With freshly prepared enzyme –SH reducing agents such as dithiothreitol, dithioerythritol or 2-mercaptoethanol were without effect within a wide concentration range of 10^{-5}–10^{-2} M. However, PCMB (*p*-hydroxymercuribenzoate) was a very potent inhibitor (over 90% inhibition at 10 μM conc.). This may indicate a requirement for free –SH groups in the enzyme for its activity. The glucosylation of sarsasapogenin was distinctly reduced in the presence of UDP and, to a lesser extent, by UMP which are rather common effects for many UDP-sugar-dependent plant glycosyltransferases [10, 14–16].

Table 2. Effects of some potential enzyme effectors on activity of UDPG: sarsasapogenin glucosyltransferase from *Asparagus officinalis* shoots

Additions	Concentration (mM)	Relative activity (%)
None (control)	—	100
$MnCl_2$	1	141
$MgCl_2$	1	128
$CaCl_2$	1	112
EDTA	1	57
	10	47
EGTA	1	71
	10	50
PCMB	0·01	6
UDP	0·1	34
	1	11
UMP	0·1	103
	1	49

DISCUSSION

The results presented in this paper furnish evidence that in addition to a membrane-bound, Triton-activated UDPG : sterol glucosyltransferase which seems to be similar to the sterol-specific glucosyltransferase isolated earlier from a number of higher plants [7–9], shoots of *A. officinalis* seedlings contain another UDPG-dependent glucosyltransferase specific for 5β-H spirostanols such as sarsasapogenin or smilagenin. In contrast to the UDPG: sterol glucosyltransferase the latter enzyme is a soluble one and its activity is distinctly inhibited by Triton X-100.

Although a full structure of the reaction product formed from sarsasapogenin and UDPG has not been unequivocally proven it is most likely to be sarsasapogenin 3β-D-monoglucoside. The latter compound is the simplest one of a series of structurally related sarsasapogenin glycosides (asparagosides) found in *A. officinalis* leaves [5, 6]. Thus, it can be assumed that the soluble glucosyltransferase from *A. officinalis* shoots is specifically involved in the initiation of sugar-chain formation at the C-3 hydroxyl group of the aglycone during the biosynthesis of asparagosides. This in turn indicate that the synthesis of oligosaccharide chains present in asparagosides proceeds step by step by sequential addition of monosaccharide moieties from nucleotide sugar precursors.

It is well known that in many higher plants [1, 2, 17] including *A. officinalis* [5, 6] spirostanol glycosides are accompanied by their furostanol analoques in which the ring F of the aglycone is open and an additional β-D-glucosyl moiety is attached to the primary hydroxyl group at C-26. The biogenetic relationship between spirostanol and furostanol glycosides is not quite clear [1, 17]. It is generally accepted [1, 2, 17–20] that cholesterol is a common precursor of all C_{27}-spirostanol sapogenins and that its conversion to sapogenins proceeds by stepwise introduction of oxygen functions at C-26 (or C-27), C-16 and C-22 followed by cyclization of the sterol side chain to form a spiroketal ring system (the rings E, F). Some authors believe [20–22] that the glucosylation at C-26 takes place at an early stage of the above described conversion preventing the closure of the ring F. Such furostanol 26-glucosides would be glycosylated at the C-3 hydroxyl group and only after that the glucosyl moiety bound at C-26 could be removed by a specific β-glucosidase with spontaneous cyclization of the ring F which leads to the formation of a corresponding spirostanol glucoside. The occurrence in *A. officinalis* of a specific glucosyltransferase which efficiently glucosylates free sarsasapogenin indicates that the presence of the glucosyl residue at C-26 is not essential for sugar-chain synthesis at C-3 of the aglycone. It may suggest that furostanol compounds glucosylated at C-26 are not obligatory intermediates in the biosynthesis of spirostanol glycosides, i.e. that furostanol and spirostanol glycosides can be formed on parallel routes.

Recently we have reported [3, 4] the occurrence in oat leaves of a UDPG-dependent glucosyltransferase specific for nuatigenin (a dihydroxy-Δ^5-furostenol sapogenin of a relatively rare type containing an additional five-membered epoxide ring). The nuatigenin-glucosylating enzyme from oat leaves and the sarsasapogenin-glucosylating enzyme described in this paper share several common features. Both these glucosyltransferases are soluble (cytosolic) enzymes, they are inhibited by Triton X-100 and show very little activity with typical sterols. These two enzymes, however, differ greatly in their specificity patterns with respect to the glucose acceptor. The UDPG : nuatigenin glucosyltransferase can glucosylate some Δ^5-spirostenol sapogenins such as, for example, diosgenin at a quite high rate [4] but show only very low activity (less than 10% as compared to nuatigenin) with 5β-H spirostanols such as sarsasapogenin or smilagenin (Kalinowska, M. and Wojciechowski Z. A., unpublished results).

Although the activity of UDPG : sarsasapogenin glucosyltransferase was found in the crude cytosol fraction, i.e, in the 105 000 *g* supernatant obtained from the crude homogenate, it can not be ruled out that *in vivo* this enzyme is located in some cell organellae, for example in vacuoles. This requires further studies.

EXPERIMENTAL

Plant material. Seeds of *Asparagus officinalis* L., cv. Mary Washington were germinated under light (*ca* 3000 lux, 16 hr/day) on several layers of wood-wool moistened with tap water.

Glucosyltransferase preparations. Whole shoots of 2 to 3-week-old plants were homogenized in a blender with cold 0.1 M Tris–HCl, pH 7.3 containing 10 mM 2-mercaptoethanol (1 ml/g fr. wt). After filtration through cheese-cloth, the crude homogenate was centrifuged at 2000 *g* during 10 min in order to remove cell debris. The supernatant was then recentrifuged at 105 000 *g* during 3.5 hr. The resulting pellet was resuspended in the homogenizing buffer, added dropwise, under vigorous stirring, to a 15-fold amount of cold (−20°) Me_2CO and allowed to stand at −20° for 20 min. The ppt. was then collected by centrifugation (10 000 g, 15 min), washed several times with cold dry Me_2CO and dried under red. pres. in a dessicator ('lipid-depleted membranous fraction'). The 105 000 *g* supernatant was treated with Me_2CO as described above for crude membranous fraction furnishing 'lipid-depleted cytosolic fraction'. Both partially delipidated enzyme preparations retained almost all glucosyltransferase activity for several weeks when kept at −20°.

Enzyme activity assays. Glucosyltransferase activities were measured essentially as described previously [3, 4]. The typical incubation mixture contained: delipidated enzyme preparation (usually 0.5 mg) in 0.25 ml of 0.2 M Tris–HCl, pH 8.7 containing 10 mM 2-mercaptoethanol; a steroid acceptor (25 nmol) in 0.01 ml EtOH and dist. H_2O (0.25 ml) or an equivalent amount of an enzyme effector solution in H_2O. All steroids used for incubations were carefully checked for their purity by TLC and/or GLC and repurified, if necessary, by prep. TLC and/or crystallization. After 10 min preincubation at 30°, the enzyme reaction was initiated by addition of UDP-[U-^{14}C]glucose, sp. act. 180 mCi/mmol (220 000 dpm) in 0.01 ml H_2O. The reaction was run at 30°, usually for 45–60 min and stopped by addition of 1 ml MeOH and boiling for 3 min.

Isolation of labelled products. The boiled reaction mixture was diluted with H_2O (1 ml). The labelled steroid glucosides were then seperated from unreacted UDPG or its degradation products by extraction with *n*-BuOH satd with H_2O (3 × 3 ml). The *n*-butanol extract was washed several times with H_2O saturated with *n*-BuOH and aliquots of the washed extract were taken for radioactivity counting or were analysed by TLC on silica gel ($CHCl_3$–MeOH, 4 : 1) followed by autoradiography.

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