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SUBCELLULAR LOCALIZATION OF UDPG:NUATIGENIN GLUCOSYLTRANSFERASE IN OAT LEAVES

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Key Word Index—*Avena sativa*; Gramineae; nuatigenin 3β-D-glucoside; steryl 3β-D-glucosides; enzymatic glucosylation.

Abstract—Cell-free enzyme preparations from oat leaves effectively catalyse the conversion of both phytosterols and nuatigenin (a furostanol sapogenin) to the corresponding 3β-D-glucosides, with UDPG acting as a sugar donor. Subcellular fractionation has shown that UDPG:sterol glucosyltransferase activity is present almost exclusively in the membranous fraction (105 000 *g* pellet) while a large part (*ca* 70%) of UDPG:nuatigenin glucosyltransferase activity occurs in the cytosol (105 000 *g* supernatant). The results obtained indicate clearly that oat leaves contain at least two UDPG-dependent glucosyltransferases catalysing glucosylation of 3β-hydroxysteroids which are localized in different cell compartments and exhibit different specificity patterns.

INTRODUCTION

Many tissues of higher plants have been found to contain UDPG:sterol glucosyltransferase, which catalyses the formation of steryl 3β-D-monoglucosides, using various phytosterols and UDPG as substrates. This membrane-bound enzyme was solubilized, partially purified and its properties were studied in detail by several authors (for reviews, see refs. [1–3]).

In contrast, only sparse data are available on enzymes involved in the glycosylation of other types of plant steroids such as steroid sapogenins, steroid alkaloids, kardenolides, bufadienolides, etc., which occur frequently in higher plants as glycosides. So far, there have been only a few reports on this subject. The formation of solasodine 3β-D-glucoside from solasodine and UDPG by extracts from *Solanum laciniatum* was reported by Liljegren [4]. Lavintman *et al.* [5] have described *in vitro* glucosylation of solanidine by crude enzyme preparations from potato tubers.

Recently we have reported [6] that crude cell-free preparations from oat leaves effectively glucosylate both phytosterols and a steroid sapogenin—nuatigenin [22,25-epoxy-20*S*,22*S*,25*S*-furost-5-ene] in the presence of UDPG as the glucose source. The product formed enzymatically from nuatigenin and UDPG was identified as nuatigenin 3β-D-glucoside. The above reaction is probably the first step in the synthesis of sugar chains of oat saponins—avenacosides A and B, which are tetra- and pentaglycosyl derivatives of nuatigenin, respectively (for the structures of avenacosides, see refs. [7, 8]).

This paper presents evidence that two different UDPG-dependent glucosyltransferases are involved in the glucosylation of phytosterols and nuatigenin in oat leaves.

RESULTS

Subcellular distribution of UDPG:nuatigenin and UDPG:sitosterol glucosyltransferase activities in oat leaves

As we have previously shown, the presence of a non-ionic detergent, Triton X-100, has entirely different effects on the glucosylation of nuatigenin and sitosterol by the crude homogenate of oat leaves. Triton X-100, at low concentrations, stimulates several-fold the formation of sitosteryl 3β-D-glucoside but has a pronounced inhibitory effect on nuatigenin 3β-D-glucoside synthesis [6]. For this reason, in our present study on subcellular localization of glucosyltransferase(s) involved in steryl glucoside and nuatigenin glucoside formation parallel measurements of glucosylation rates in the presence of 0.3% Triton X-100 and its absence were made.

The data in Table 1 show the glucosyltransferase activities of acetone powder preparations from the cell membrane fraction (105 000 *g* pellet) and from the cytosol

Table 1. Conversion of nuatigenin or sitosterol to their 3β-D-glucosides by lipid-free subcellular fractions from oat leaves*

Additions		Glucoside formation (pmol/mg/hr)	
Glucose acceptor	Triton X-100 (0.3%)	Cellular membranes	Cytosol
None†	–	1	1
	+	3	1
Nuatigenin	–	38	34
	+	32	28
Sitosterol	–	7	1
	+	30	2

*The reaction mixtures contained an acetone powder preparation (2.5 mg) obtained from the membranous fraction (105 000 *g* pellet) or from the cytosol (105 000 *g* supernatant), UDP-[U-^{14}C]glucose (2.2×10^5 dpm) and nuatigenin or sitosterol (10 μg) added in 0.01 ml of ethanol. For other details, see the Experimental. The reaction time was 30 min and the temperature was 30°. The radioactivities of the *n*-butanol extracts were taken as a measure of glucoside formation.

†Endogenous acceptors only.

fraction (105000 *g* supernatant) obtained by differential centrifugation of the crude homogenate. These results clearly indicate that the membranous fraction catalysed the glucosylation of both acceptors, i.e. nuatigenin and sitosterol, at similar rates. In contrast, the cytosol fraction, which is highly active in the synthesis of nuatigenin glucoside, shows very little activity with sitosterol as the acceptor.

The glucosyltransferase activities presented in Table 1 are expressed per mg of lipid-free enzyme preparations and therefore they do not illustrate precisely the distribution of total glucosyltransferase activities between the two fractions studied. Taking into account the yields of the lipid-free enzyme preparation from a given amount of fresh oat leaves (see Experimental), it can be deduced that the cellular membranes contain 33% of the total UDPG:nuatigenin glucosyltransferase activity and 93% of the UDPG:sterol glucosyltransferase activity while the cytosol fraction contains 67% of the total activity of nuatigenin glucosylation and only 7% of the total activity of sitosterol glucosylation (see also Table 2). Washing the crude cell membrane fraction with fresh portions of buffer with recentrifugation at 105000 *g* did not result in a noticeable change in the relative ratio of sitosterol and nuatigenin glucosylation. Moreover, attempts to separate the UDPG:sterol and UDPG:nuatigenin glucosyltransferase activities present in the 105000 *g* pellet by successive centrifugation of the homogenate at 3000, 15000 and 105000 *g* were unsuccessful. The lipid-free preparations obtained from all these pellet fractions had rather similar activity ratios with the two substrates used.

It should be stressed that the differential effects of Triton X-100 on sterol and nuatigenin glucosylation observed by us earlier [6] in experiments with the crude homogenate can be clearly seen with lipid-free subcellular fractions. 0.3% Triton X-100 distinctly stimulates sterol glucosylation by both membranous and cytosol fractions while *ca* 25–30% inhibition of nuatigenin glucosylation is observed in both these subcellular fractions.

Table 2. Distribution of UDPG:nuatigenin and UDPG:sterol glucosyltransferase activities between cellular membranes (105000 *g* pellet) and the cytosol (105000 *g* supernatant) in various plants*

Plant material	Glucose acceptor†	Total glucosylation activity (%) Cell membranes	Cytosol
Oat (*Avena sativa*)			
Leaves	N	33	67
Roots	S	93	7
	N	99	1
	S	99	1
Pea (*Pisum sativum*)			
Leaves	N	95	5
	S	93	7
White mustard (*Sinapis alba*)			
Cotyledons	N	98	2
	S	98	2

*The reaction mixtures were as described in the legend to Table 1 but Triton X-100 (final concentration 0.3%) was present in all samples. The incubation time was 60 min and the temperature was 30°.

†N, nuatigenin; S, sitosterol.

Some properties of the cytosolic glucosyltransferase

The above results clearly demonstrate that oat leaves contain a soluble (cytosolic) glucosyltransferase which effectively glucosylates nuatigenin but has little affinity for plant sterols. Some of the results of preliminary studies on the properties of this enzyme are summarized below.

The glucosyltransferase activity of an acetone powder preparation from the cytosol fraction of oat leaves was stable for several months when stored at −20°. The time courses of nuatigenin and sitosterol glucosylation by this preparation are shown in Fig. 1. In spite of the presence of 0.3% Triton X-100 in this experiment the rate of sitosterol glucosylation is many times lower than that of nuatigenin glucosylation.

Figure 2 shows the effects of increasing the concentration of Triton X-100 (0–1%) on the glucosyltransferase activity with the two acceptors used. Although the level of sitosteryl glucoside synthesis is low, a stimulatory effect of Triton X-100 can clearly be seen (300% at 0.1% Triton X-100). In contrast, the formation of nuatigenin glucoside is distinctly inhibited by this detergent within the whole concentration range.

The effect of pH on the synthesis of nuatigenin 3β-D-glucoside was studied with 0.1 M Tris–maleate, pH 5.3–8.5. With this buffer, strong glucosylation of nuatigenin occurred within the range pH 6.5–8.5. No pronounced activity maximum was observed. Below pH 6.5, the activity decreased sharply (50% activity at pH 5.8). However, at pH 7.3, the activity in the 0.1 M Tris–HCl buffer was *ca* 50% higher than that in 0.1 M Tris–maleate. The enzyme had the highest activity at 30°. Above 35°, the activity decreased rapidly. At 39°, the rate of nuatigenin glucosylation was about seven times lower than that at 30°. Divalent metals (Mg^{2+}, Ca^{2+} and Mn^{2+}), which stimulate many plant glycosyltransferases, had no effect (at 10^{-4}–10^{-3} M) on the enzyme studied. Zn^{2+} and Hg^{2+}

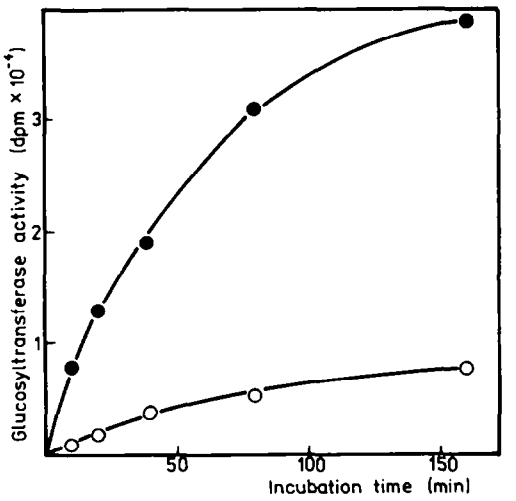

Fig. 1. Time courses of nuatigenin (●) and sitosterol (○) glucosylation by an acetone powder preparation from the cytosol fraction of oat leaves. Reaction mixtures were as for Table 1 but Triton X-100 was always present.

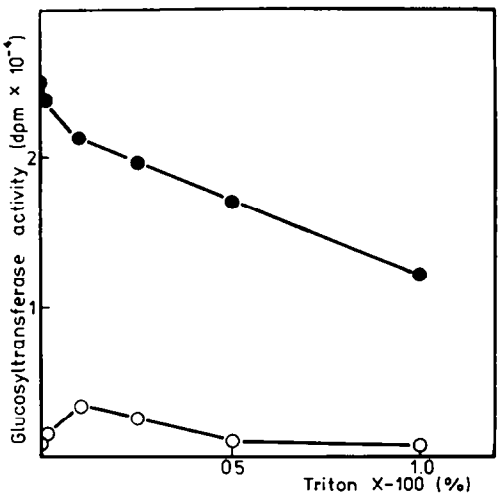

Fig. 2. Influence of increasing Triton X-100 concentration on nuatigenin (●) and sitosterol (○) glucosylation by an acetone powder preparation from the cytosol fraction of oat leaves. Reaction mixtures were as described in the legend to Table 1. Incubation time was 30 min and the temperature was 30°.

were inhibitory (50% inhibition at 8×10^{-6} and 3×10^{-6} M concentration, respectively).

The results of preliminary studies on the specificity of the cytosolic glucosyltransferase are given in Fig. 3. Spirostanol sapogenins structurally related to nuatigenin, i.e. isonuatigenin (20*S*,22*S*,25*S*-spirost-5-ene-3β,25-diol) and diosgenin (25*R*-spirost-5-en-3β-ol), are glucosylated at distinctly lower rates than nuatigenin but still at a much higher rate than sitosterol. It should be stressed that Triton X-100 affects the glucosylation of isonuatigenin and diosgenin in a similar way to the case of nuatigenin but differently as compared with sitosterol.

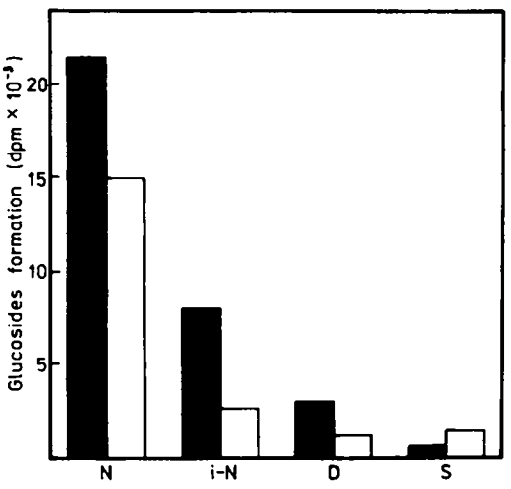

Fig. 3. Glucoside formation catalysed by an acetone powder preparation from the cytosol fraction of oat leaves with nuatigenin (N), isonuatigenin (i-N), diosgenin (D) and sitosterol (S) as glucose acceptors. Reaction mixtures contained lipid-free enzyme preparation (2.5 mg), UDP-[U-^{14}C]glucose (2.2×10^{-5} dpm) and glucose acceptor (10 μg). 0.3% Triton X-100 was included (white bars) or absent (black bars). Incubations were carried out for 30 min at 30°.

PHOTO 26/2-C

Glucosylation of nuatigenin by subcellular fractions from some other plants

As pointed out above, in contrast to the cytosol fraction, the membranous fraction from oat leaves catalyses parallel glucosylation of nuatigenin and sitosterol at similar rates. It cannot be excluded that the glucosylation of both substrates by the membranous fraction is catalysed by the same membrane-bound enzyme, which is identical or similar to UDPG:sterol glucosyltransferase isolated from many other higher plants [1–3]. It is known that the above-mentioned enzyme exhibits a rather broad specificity pattern with respect to glucose acceptor and in addition to typical phytosterols it can glucosylate a number of sterol-like substances [9–11]. For this reason, we decided to carry out some experiments similar to those described above for oat leaves with other plant tissues such as white mustard cotyledons and pea leaves. These plants are rich in UDPG:sterol glucosyltransferase activity [10, 12, 13] but do not synthesize nuatigenin or any structurally related steroid sapogenins.

In fact, acetone powder preparations obtained from white mustard cotyledons and pea leaves catalysed glucosylation of nuatigenin at appreciable rates. The identity of the reaction product with nuatigenin 3β-D-glucoside was confirmed using TLC and autoradiography (see Experimental). However, it is evident (Table 2) that in white mustard, as well as in pea, virtually all the glucosyltransferase activity, both with nuatigenin and sitosterol as the substrate, is present in cell membranes sedimenting at 105 000 *g*. In contrast to oat leaves, neither white mustard cotyledons nor pea leaves contain the cytosolic, nuatigenin-specific glucosyltransferase. It is interesting that similar results were obtained with oat roots where only traces of glucosyltransferase activity could be detected in the cytosol fraction. This is not surprising as it is known [14] that avenacosides are formed and accumulated exclusively in the leaves of oat plants.

DISCUSSION

In an earlier paper [6] we reported on the synthesis of nuatigenin 3β-D-glucoside from free nuatigenin and UDPG catalysed by cell-free preparations from oat leaves. Under similar experimental conditions these preparations could also catalyse glucosylation of sterols. On the grounds of entirely different sensitivities of the above-mentioned glucosylation reactions to Triton X-100 we suggested that oat leaves contain two different enzymes specific towards phytosterols and nuatigenin, respectively.

The present results confirm this suggestion, furnishing direct evidence that at least two UDPG-dependent glucosyltransferases localized in different cell compartments are present in oat leaves. One is a soluble, cytosolic, enzyme which can glucosylate nuatigenin and the other is a tightly membrane-bound enzyme which can glucosylate both nuatigenin and phytosterols. The cytosolic enzyme seems to be a highly specific UDPG:nuatigenin glucosyltransferase since it glucosylates nuatigenin at a much higher rate than some structurally related steroid sapogenins such as isonuatigenin and diosgenin. The affinity of the cytosolic glucosyltransferase towards typical plant sterols is very low (if any). Slight glucosylation of

sitosterol observed with the lipid-free cytosol (less than 7% of the total UDPG:sterol glucosyltransferase activity present in oat leaves) can be easily explained by unavoidable contamination of the cytosol fraction with small membrane fragments formed during the homogenization procedure.

With reference to sterol glucosylation, our results are consistent with data obtained for many other higher plants—among others, for pea seedlings [12, 13], white mustard cotyledons [10], maize coleoptiles [11, 16–18], potato tubers [15, 19] and tobacco seedlings [20]. In all these plants, UDPG:sterol glucosyltransferase is tightly bound to the cell membranes and is activated considerably by Triton X-100. Lipid-free membranous fractions from oat leaves can glucosylate both sitosterol and nuatigenin. There are several possible explanations for the above fact.

It can be assumed that the ability of cell membrane fractions to glucosylate both substrates is due to the presence of two different UDPG-dependent enzymes with different specificity patterns. One of them might be a membrane-bound form of the nuatigenin-specific glucosyltransferase present in the cytosol, and the second one might be UDPG:sterol glucosyltransferase identical or similar to the enzyme found in many other plants. This assumption is substantiated by our observations on the effects of Triton X-100 on glucosyltransferase activities present in cell membranes. In contrast to sterol glucosylation, which is greatly enhanced in the presence of Triton X-100, the synthesis of nuatigenin glucoside is inhibited by this detergent at an extent very similar to that observed for the cytosolic enzyme. However, another explanation for the different effects of Triton X-100 is still possible. Sterols are much less polar than nuatigenin. Therefore, it seems possible that Triton X-100 affects in different ways the availability of both substrates for the enzyme.

It can also be assumed that the concurrent synthesis of sitosterol and nuatigenin glucoside by the membranes fraction from oat leaves is catalysed by one enzyme of relatively low specificity towards the glucose acceptor. Studies with partially purified preparations of UDPG:sterol glucosyltransferase from *Digitalis purpurea* [9], white mustard [10] and maize [11] indicate that the specificity patterns of these enzymes are rather broad. In addition to typical plant sterols, various other structurally related steroid compounds can also be glucosylated provided that they contain a hydroxyl group at C-3 in the β-configuration, a flat ring system (5α-H or Δ^5), and there are no additional methyl groups at C-4. The structure of the side chain at C-17 is less important [10]. Nuatigenin fulfils all the above requirements and it seems quite possible that it can be glucosylated by UDPG:sterol glucosyltransferase. Our experiments with enzyme preparations from white mustard and pea (Table 2) strongly support such a possibility. Membranous fractions from these plants can glucosylate nuatigenin at a fairly high rate but cytosol fractions are practically devoid of glucosyltransferase activity. There is no information on the presence of nuatigenin or of any related steroid sapogenins in these plants. Very similar results are obtained with oat roots. This is not surprising since oat roots, in contrast to leaves, do not accumulate glucosyl derivatives of nuatigenin, i.e. avenacosides [14].

Irrespective of possible explanations for the concurrent glucosylation of sterols and nuatigenin by membranous fractions, it is evident that oat leaves contain a soluble glucosyltransferase which definitely differs from UDPG:sterol glucosyltransferase occurring in many higher plants. There are strong indications that *in vivo* the cytosolic enzyme takes part in the initiation of the synthesis of sugar chains of oat saponins, i.e. avenacosides A and B. The occurrence of separate UDPG:nuatigenin and UDPG:sterol glucosyltransferases differing in their subcellular localization in oat leaves is not surprising. The biological functions of steryl glucosides and avenacosides are different. Steryl glucosides are components of cellular membranes [1–3] while avenacosides are accumulated in vacuoles [21] and are regarded as performed chemical protectants against fungal infections [22].

EXPERIMENTAL

Plant material. Oat seeds (*Avena sativa* cv. Rumak) were sown on several layers of moistened wood-wool and germinated in darkness at 20°. After 5–6 days of germination, seedlings were transferred into light (*ca* 3000 lx, 16 hr/day). Leaves or roots of 7 to 8-day-old plants were used in most expts. Cotyledons of 7-day-old white mustard (*Sinapis alba*) or leaves of 10-day-old pea (*Pisum sativum*) were also used in some expts.

Enzyme preparations. Fresh plant material was homogenized in a blender with cold 0.1 M Tris–HCl, pH 7.3 (3 ml/g fr. wt). The homogenate was filtered through cheese cloth and centrifuged at 2000 *g* to remove cell debris. Cytosolic and membranous fractions were then obtained by centrifugation at 105000 *g* (3 hr). The membranous fraction was suspended in the initial amount of buffer and purified by recentrifugation as above. Lipid-free enzyme preparations (Me_2CO powders) were obtained as described earlier [10]. In a typical expt, 100 g of fresh oat leaves yielded 0.97 g (0.097 g protein) of lipid-free cell membranes and 2.14 g (0.222 g protein) of lipid-free cytosol fraction. All operations were performed at 0–4°.

Glucosyltransferase assays. Incubations were carried out at 30° usually for 30–60 min with the following assay mixtures: 0.5 ml enzyme preparation (0.2–0.5 mg protein) in 0.1 M Tris–HCl, pH 7.3; 0.01 ml UDP-[U^{4}-^{14}C]glucose (2.2×10^5 dpm; 0.555 nmol); a steroid acceptor (10 μg) in 0.01 ml EtOH. The enzymatic reaction was terminated by addition of MeOH (1 ml) and boiling for 3 min. Subsequently the reaction mixture was extracted with *n*-BuOH (4 ml) and the extract was washed with H_2O (5 × 3 ml). Aliquots of the *n*-BuOH extract were taken for direct radioactivity measurements [10] or were analysed by TLC and autoradiography.

Thin-layer chromatography. Reaction products were separated on silica gel using CH_3Cl–MeOH (4:1) as the solvent. Authentic sitosteryl 3β-D-glucoside (R_f 0.55), nuatigenin 3β-D-glucoside (R_f 0.50) and nuatigenin 26β-D-glucoside (R_f 0.53) were used as the standards. Labelled products obtained enzymatically with nuatigenin, isonuatigenin, diosgenin and sitosterol as the [^{14}C] glucose acceptor had R_f values 0.50, 0.45, 0.52 and 0.55, respectively.

Other methods. Protein was measured by the method of Bradford [23] using BSA as the standard. Nuatigenin, isonuatigenin, nuatigenin 3β-D-glucoside and 26β-D-glucoside were obtained as descibed earlier [6].

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