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ENZYMATIC SYNTHESIS OF NUATIGENIN 3β-D-GLUCOSIDE IN OAT (*AVENA SATIVA*) LEAVES

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Key Word Index—*Avena sativa*; Gramineae; glucosyltransferases; nuatigenin glucoside; steryl glucosides.

Abstract—Crude homogenates or acetone powder preparations from oat leaves efficiently catalyse the glucosylation of a steroidal sapogenin, nuatigenin [22,25-epoxy-(20*S*)(22*S*)(25*S*)-furost-5-en-3β,26-diol], using UDP–glucose as the sugar donor. The reaction product was identified as nuatigenin 3β-D-monoglucoside. In contrast to the glucosylation of phytosterols, which is also catalysed by enzyme preparations from oat leaves, the formation of nuatigenin glucoside is not stimulated by Triton X-100. This result suggests that glucosyltransferases with different specificity patterns are involved in sterol and nuatigenin glucosylation in oat leaves. Enzymatic acylation of nuatigenin glucoside to its monoacyl derivative with the use of an endogenous acyl source was also observed with a crude homogenate or a crude membranous fraction as the enzyme preparation.

INTRODUCTION

Young oat leaves contain two steroid glycosides, avenacosides A and B, which are derivatives of nuatigenin [22,25-epoxy-(20*S*)(22*S*)(25*S*)-furost-5-en-3β,26-diol]. Avenacoside A is 3-*O*-[α-L-rhamnopyranosyl-(1 → 4)]-[β-D-glucopyranosyl-(1 → 2)]-β-D-glucopyranoside, the 26-*O*-β-D-glucopyranoside of nuatigenin while avenacoside B is 3-*O*-[α-L-rhamnopyranosyl-(1 → 4)]-[β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl-(1 → 2)]-β-D-glucopyranoside, the 26-*O*-β-D-glucopyranoside of nuatigenin [1–3]. These glycosides, which are localized in vacuoles [4], are regarded as preformed chemical protectants against fungal infections [5]. It is known that oat leaves contain a very active specific β-glucosidase (so-called avenacosidase) which immediately converts avenacosides A and B into the antifungally active 26-desgluco derivatives if cells are damaged [5–8]. *In vivo* studies with [2-^{14}C]mevalonate as the precursor have shown that a very rapid synthesis of avenacosides occurs in young oat seedlings [9]. In this paper we report on the formation of nuatigenin 3β-D-monoglucoside by cell-free preparations from leaves of oat seedlings. To our knowledge, this is the first report on *in vitro* glycosylation of a steroid sapogenin of the furostanol or spirostanol type. Most probably the above reaction constitutes the first step in the biosynthesis of the sugar chains of avenacosides.

RESULTS AND DISCUSSION

Nuatigenin glucosylation by crude homogenates

Preliminary experiments with crude homogenates from the leaves of 7-day-old oat seedlings incubated with UDP–[^{14}C]glucose have shown their ability to glucosylate endogenous or exogenous sterols and to acylate steryl monoglucosides (SG) with the formation of 6′-*O*-acyl steryl monoglucosides (ASG) using endogenous acyl donors. This is consistent with the results obtained earlier with crude subcellular fractions from various tissues of several higher plants (for a review, see ref. [10]).

Under similar experimental conditions the incubation of the crude homogenate from oat leaves (or a membranous fraction obtained from the homogenate by centrifugation at 105 000 *g*) with UDP–[^{14}C]glucose and nuatigenin led to the formation of a labelled compound which was slightly more polar than SG, as would be predicted for nuatigenin monoglucoside (Table 1). The addition of nuatigenin to the reaction mixture also resulted in a marked increase in the formation of a labelled compound with the chromatographic properties of ASG. This result suggested that nuatigenin glucoside could be partly converted to the corresponding 6′-*O*-acyl derivative, which is analogous to the conversion of SG into ASG.

The above supposition was confirmed directly by the incubation of labelled nuatigenin glucoside (isolated by TLC from several samples incubated with UDP–[^{14}C]glucose and nuatigenin as above) with a fresh portion of the membranous fraction (Table 1). In this experiment, Triton X-100 was not included in the reaction mixture since it had been shown that it inhibits the formation of acylated derivatives. The reaction product was alkali-labile. It could be quantitatively converted back to nuatigenin glucoside by treatment with 1% potassium hydroxide in methanol (80°, 1 hr).

The results of the above experiments clearly indicate that the crude enzyme preparations from oat leaves can catalyse glucosylation of both phytosterols and nuatigenin with the use of UDP–glucose (UDPG) as the sugar donor as well as the subsequent conversion of steryl or nuatigenin monoglucosides into the corresponding monoacyl derivatives with the use of endogenous acyl donors. In contrast to the glucosylation of phytosterols the formation of nuatigenin glucoside is observed only with exogenous nuatigenin. This is probably due to a low level of free nuatigenin in oat leaves. In fact, TLC analysis of a diethyl ether extract from oat leaves could not

Table 1. Formation of glucoside derivatives of nuatigenin or sitosterol by a crude membranous fraction from the leaves of oat seedlings*

Substrates	Radioactivity (dpm × 10^{-3})		
	Nuatigenin glucoside (R_f = 0.50)	Steryl glucoside (R_f = 0.55)	Acylated glucosides (R_f = 0.73)
UDP-[^{14}C]glucose, endogenous acceptors only	0	12.2	3.7
UDP-[^{14}C]glucose, nuatigenin	24.6	11.5	5.9
UDP-[^{14}C]glucose, sitosterol	0	16.4	3.9
Nuatigenin [^{14}C]glucoside, endogenous acyl donors	85	0	170

*Samples incubated with labelled UDPG (2.2 × 10^5 dpm) contained 0.3% Triton X-100. In the experiment with labelled nuatigenin glucoside (2.6 × 10^5 dpm), Triton X-100 was omitted. The reaction was run for 30 min (experiments with labelled UDPG) or 120 min (experiment with labelled nuatigenin glucoside). Labelled products were separated by TLC on silica gel with CH_3Cl–MeOH (4 : 1). For other details, see Experimental.

demonstrate the presence of any detectable amounts of free nuatigenin.

Experiments with acetone powder preparations

Further experiments were carried out with the homogenate partly depleted of lipid with acetone (acetone powder preparations—see Experimental). Such preparations retained the glucosyltransferase activity with both nuatigenin and phytosterols as the acceptors. However, they showed a much reduced activity with endogenous glucose acceptors and they almost completely lost the ability to catalyse the conversion of the glucosides into the corresponding acyl derivatives.

With acetone powder preparations, in the presence of 0.3% Triton X-100 the rates of sitosterol and nuatigenin glucosylation were rather similar. In both cases, the amount of radioactive glucoside formed was proportional to the time, within 0–80 min (Fig. 1). Prolonged incubations (up to 24 hr) did not lead to any destruction of the synthesized compounds. With respect to the labelled UDPG used for the incubation, the yield of nuatigenin glucoside ranged from 15 to 30% (for 90 min incubations).

The ability of enzyme preparations from oat leaves to glucosylate nuatigenin was confirmed using another incubation variant, i.e. the incubation of [^{14}C]nuatigenin (obtained biosynthetically from [1-^{14}C]acetate—see Experimental) with an excess amount of unlabelled UDPG (Fig. 2). After acid hydrolysis of the glucoside obtained in this way, the sole labelled product was a nuatigenin–isonuatigenin mixture.

Preliminary experiments on the properties of glucosyltransferase from oat leaves with nuatigenin as the acceptor showed a pH optimum at 7.5–8.5. Among the three buffers tested (0.1 M Tris–maleate, 0.1 M Tris–HCl and 0.1 M sodium phosphate), the best results were obtained with Tris–HCl. It could not be shown that divalent cations are necessary for the glucosylation. Ca^{2+}, Mn^{2+} and Mg^{2+} (at 5×10^{-3} M) had no effect on the rate of nuatigenin glucosylation. The activation of plant UDPG:sterol glucosyltransferase by divalent cations has been observed by some authors [11, 12].

Identification of nuatigenin glucoside

It is well known that the leaves of oat seedlings contain a β-glucosidase which selectively splits off the glucose moiety bound to C-26 of nuatigenin in avenacosides A and B without affecting the sugar chains at C-3 [5–8]. We have shown that enzyme preparations used in this work (both crude homogenate and acetone powder preparations) are characterized by a high activity of this β-glucosidase and they can rapidly hydrolyse avenacosides A and B to their 26-desgluco derivatives. As has been pointed out above, labelled glucoside formed from nuatigenin and UDP-[^{14}C]glucose was not degraded to any perceptible extent during prolonged incubation with the

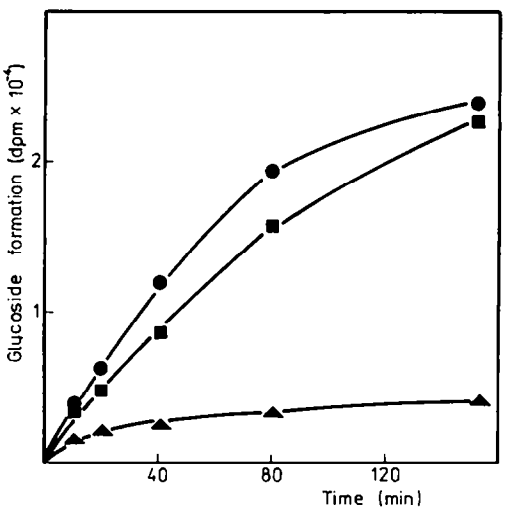

Fig. 1. Time course of nuatigenin and sitosterol glucosylation by an enzyme preparation from oat leaves. An acetone powder preparation (2.5 mg) was incubated with UDP-[^{14}C]glucose (2.2 × 10^5 dpm) and 10 μg of nuatigenin (●) or 10 μg of sitosterol (■). In control samples (▲), only endogenous glucose acceptors were present. Triton X-100 (final concentration 0.3%) was included in the reaction mixtures. For other details, see Experimental.

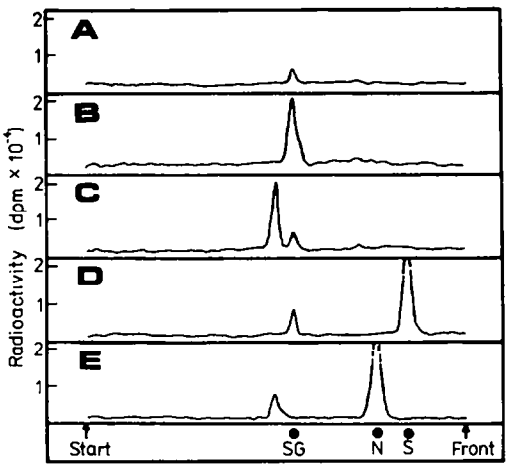

Fig. 2. Thin-layer chromatography of labelled products synthesized from UDP-[^{14}C]glucose, [^{14}C]nuatigenin or [4-^{14}C]cholesterol by lipid-depleted enzyme preparations from oat leaves. The following substrates were used: (A) labelled UDPG, endogenous acceptors; (B) labelled UDPG, unlabelled sitosterol; (C) labelled UDPG, unlabelled nuatigenin; (D) unlabelled UDPG, labelled cholesterol; (E) unlabelled UDPG, labelled nuatigenin. The incubation time was 30 min (A–C) or 120 min (D, E). Labelled products extracted with *n*-butanol were separated on silica gel with CH_3Cl–MeOH (4:1) as the solvent. Sitosteryl β-D-glucoside (SG), nuatigenin (N) and sitosterol (S) were used as the standards.

enzyme preparation. It therefore seems unlikely that it may be nuatigenin 26-glucoside but rather nuatigenin 3-glucoside, which is formed in the enzyme-catalysed reaction.

Unequivocal identification of the product synthesized enzymatically from UDPG and nuatigenin was achieved by comparing this product with the products obtained by mild acid hydrolysis of avenacosides A and B (see Experimental). Such hydrolysis gave, apart from a small amount of free aglycone, a number of products of incomplete hydrolysis. Three of these products (I–III, with respect to increasing polarity) which contained glucose as the only sugar component were isolated and analysed in detail. Product I, which migrated on thin-layer chromatograms slightly faster than enzymatically formed nuatigenin glucoside, was completely hydrolysed with a crude preparation of oat β-glucosidase to yield free nuatigenin and it was therefore identified as nuatigenin 26-monoglucoside. The slightly more polar product II had chromatographic mobility, in several solvent systems, identical to that of labelled nuatigenin glucoside formed enzymatically. It is likely that product II is the 3-monoglucoside. However, it is known that splitting off the sugar present at position C-26 in nuatigenin glycosides by acid hydrolysis leads to reversible acid-catalysed isomerization of the F-ring in nuatigenin with the formation of isonuatigenin [1, 13]. Therefore, product II is most probably a mixture of nuatigenin 3-monoglucoside and isonuatigenin 3-monoglucoside. Thus, we focused our attention on the more polar product III which was expected to be nuatigenin 3,26-diglucoside. Indeed, enzymatic hydrolysis of product III with oat β-glucosidase gave a compound which was chromatographically identical to the glucoside formed enzymatically (as well as to product II). Its identity with nuatigenin 3β-D-monoglucoside was confirmed by mass spectrometry and FAB mass spectrometry (see Experimental). Co-crystallization of the above compound with labelled product formed from nuatigenin and UDP-[^{14}C]glucose by the acetone powder preparation from oat leaves without any decrease in the specific radioactivity (Table 2) proved that the labelled product was really nuatigenin 3β-D-monoglucoside.

Effect of Triton X-100 on glucosylation of nuatigenin and sitosterol

It is known that Triton X-100 at low concentrations (0.1–0.5%) stimulates the activity of plant UDPG:sterol glucosyltransferase distinctly. For example, several-fold activation of sterol glucosylation has been observed with enzyme preparations from *Calendula* leaves [14], etiolated maize coleoptiles [15], white mustard seedlings [16] and potato tubers [17, 18].

The results of our studies with an acetone powder preparation from oat leaves have shown that Triton X-100 exerts entirely different effects on the glucosylation of sitosterol and nuatigenin (Fig. 3). Glucosylation of sitosterol was markedly stimulated by all the concentrations of Triton X-100 used (0.25–1.0%), which is in accordance with the above-cited data for UDPG:sterol glucosyltransferase preparations obtained from other plants. Maximum stimulation (*ca* 4.5-fold) was found with 0.25% Triton X-100. On the other hand, Triton X-100 inhibited the glucosylation of nuatigenin. The observed entirely different effects of Triton X-100 on the rate of glucosylation of both acceptors, i.e. sitosterol and nuatigenin, may indicate the presence of two distinct UDPG-dependent glucosyltransferases with different specificity patterns in oat leaves. Attempts to separate sterol- and nuatigenin-specific glucosyltransferases are now being undertaken. Preliminary experiments employing fractionation of cell components of oat leaves (M. Kalinowska and Z. A. Wojciechowski, unpublished results) demonstrated that UDPG:sterol glucosyltransferase activity occurs almost exclusively in membranous fractions, while a large part of the UDPG:nuatigenin glucosyltransferase activity is found in the cytosol.

Table 2. Co-crystallization of the radioactive product formed enzymatically from nuatigenin and UDP-[14C]glucose with carrier nuatigenin 3β-D-monoglucoside*

Crystallization No.	Weight (mg)	Specific activity (dpm x 10^{-3}/mg)
1	30.6	41.7
2	27.0	42.0
3	25.9	48.1
4	21.9	41.4
5	18.8	46.3

*The radioactive product isolated by TLC (*ca* 1.5×10^6 dpm) was diluted with authentic nuatigenin 3β-D-glucoside (*ca* 40 mg). The mixture was acetylated with acetic anhydride–pyridine, purified by TLC on silica gel with CH_3Cl–MeOH (49:1) as the solvent and crystallized from aqueous ethanol.

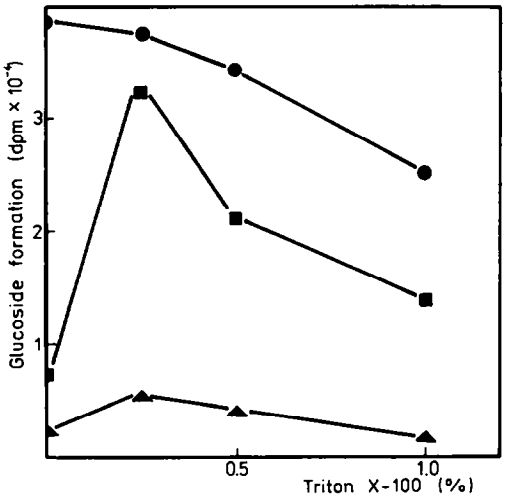

Fig. 3. Effect of Triton X-100 on the enzymatic glucosylation of nuatigenin (●), sitosterol (■) and endogenous acceptors (▲). The reaction was run for 30 min. Other incubation conditions were as described in the legend to Fig. 1.

EXPERIMENTAL

Plant material. Oat seeds, *Avena sativa* L. cv. Rumak, were allowed to germinate in the dark for 5–6 days and then exposed to light for another 2 days.

Enzyme preparations. Leaves (80 g fr. wt) were homogenized with 240 ml of cold 0.1 M Tris–HCl buffer, pH 7.3. The homogenate was filtered through cheesecloth and the resulting filtrate was added dropwise to a 15-fold amount of cold (−20°) Me_2CO. The mixture was allowed to stand at −20° for 15 min. The ppt. was collected by centrifugation at 4000 *g* for 10 min, washed several times with dry, cold Me_2CO and dried *in vacuo*. This preparation (the 'acetone powder'), stored at −20°, retained almost all glucosyltransferase activity for several weeks. In some expts a crude membranous fraction obtained from the homogenate by centrifugation at 105 000 *g* (3 hr) was used.

Glucosyltransferase assays. The standard reaction mixture contained the following in a total vol. of 0.52 ml: filtered homogenate (0.5 ml) or crude membranous fraction (equivalent to 0.5 ml homogenate) or acetone powder preparation (2.5 mg), 50 nmol Tris–HCl (pH 7.3), 10 μg nuatigenin or sitosterol in 0.01 ml EtOH and UDP–[^{14}C]glucose (2.2×10^5 dpm; 0.555 nmol) in 0.01 ml 50% EtOH. In some expts, samples additionally contained Triton X-100 (final concn 0.25–1.0%). Once the labelled UDPG was added, the reaction was run at 30°, usually for 15–120 min, and stopped by addition of 1 ml MeOH and heating for 3 min on a boiling water bath. Subsequently samples were extracted with 4 ml *n*-BuOH and the *n*-BuOH extract was washed several times with H_2O saturated with *n*-BuOH. Aliquots of the *n*-BuOH extract were taken for radioactivity measurements or TLC. On silica gel plates with CH_3Cl–MeOH (4:1) as the solvent, 3β-D-monoglucosides of sitosterol and nuatigenin had R_f values of 0.55 and 0.50, respectively. Labelled compounds were localized by autoradiography and eluted from silica gel with MeOH. Incubations with labelled glucose acceptors were carried out as described above using [4-^{14}C]cholesterol (2.2×10^5 dpm; 1.85 nmol) or [^{14}C]nuatigenin (2.2×10^5 dpm; 310 nmol—see below) and unlabelled UDPG, disodium salt (4 μmol).

Isolation of avenacosides and nuatigenin. Avenacosides A and B were isolated from a MeOH extract of 7-day-old oat seedlings leaves [4] by CC on silica gel using a MeOH gradient in CH_3Cl and purified by prep. TLC on silica gel with CH_3Cl–MeOH–H_2O (70:30:5.5) as solvent. Total hydrolysis was carried out with 5% HCl in MeOH at 80° for 2.5 hr. Nuatigenin and its isomerization product isonuatigenin (which is an artefact formed from nuatigenin by acid-catalysed isomerization) were separated after acetylation according to ref. [13]. After deacetylation and crystallization from aq. EtOH, pure nuatigenin and isonuatigenin were obtained and their identity was confirmed by MS. Nuatigenin, *m/z* (rel. int.): 430 $[M]^+$ (0.6), 412 (0.6), 399 (35.9), 381 (1.2), 345 (1.8), 342 (8.3), 324 (1.8), 300 (11.4), 285 (5.7), 282 (16.4), 271 (40.6), 267 (7.8), 253 (12.8), 155 (100.0). Isonuatigenin, *m/z* (rel. int.): 430 $[M]^+$ (1.5), 412 (0.6), 399 (15.9), 381 (1.3), 345 (5.3), 342 (41.8), 324 (2.5), 300 (17.4), 285 (7.5), 282 (29.1), 271 (38.4), 267 (11.6), 253 (13.6), 155 (100.0).

Labelled nuatigenin. Excised leaves of 7-day-old etiolated oat seedlings (5.5 g fr. wt) were supplied with [1-^{14}C]acetate (2.75×10^9 dpm; 6.23 μmol) in 1.0 ml H_2O. After the soln was taken up, the leaves were kept in H_2O in the light for 24 hr. Avenacosides were extracted with boiling MeOH, purified and hydrolysed as described above. Labelled nuatigenin obtained in this way had an approx. spec. act. of 320 μCi/mmol.

Nuatigenin 3β-D-monoglucoside. Avenacosides A and B were hydrolysed under mild conditions (1.5% HCl in 95% MeOH, at 80° for 1.5 hr). TLC examination of the *n*-BuOH extract from the hydrolysate on silica gel with CH_3Cl–MeOH (17:3) as the solvent revealed the presence of several products of incomplete hydrolysis. The product with R_f 0.34 was isolated by prep. TLC and treated with a crude preparation of oat β-glucosidase (see below). The resulting compound, which had an identical chromatographic mobility to that of the labelled glucoside obtained enzymatically from nuatigenin and UDP–[^{14}C]glucose, was purified by TLC on silica gel with CH_3Cl–MeOH (4:1) as the solvent. Its identity with nuatigenin 3β-D-monoglucoside was confirmed by MS and FAB-MS. Direct MS exhibited the fragmentation pattern of the aglycone (prominent ions at *m/z* 399, 342, 282, 271, 253, 155). Negative ion FAB-MS in polyethylene glycol 200 gave the abundant quasi-molecular peak ($[M-H]^-$) at *m/z* 591 and a peak at *m/z* 427 ($[M-H-\text{hexose}-2H]^-$). The overall yield of nuatigenin 3β-D-monoglucoside from avenacosides A and B was 1.2%.

Enzymatic deglucosylation at position C-26. Selective deglucosylation of nuatigenin glycosides at position C-26 was performed by incubation of a given glucoside (80 mg) with the acetone powder preparation (100 mg) from oat leaves (see above) in 26 ml H_2O–EtOH (10:3) at 30° for 24 hr. The reaction products were extracted with *n*-BuOH. Under the above conditions, practically complete deglucosylation at position C-26 was observed.

Other methods. Radioactivity was assayed by liquid scintillation counting as described earlier [16]. Sugars were identified by TLC on cellulose plates using C_6H_6–*n*-BuOH–C_5H_5N–H_2O (1:5:3:3; upper phase) as the solvent.

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