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UV-INDUCED BIOSYNTHESIS OF QUERCETIN 3-*O*-β-D-GLUCURONIDE IN DILL CELL CULTURES

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Key Word Index—*Anethum graveolens*; Apiaceae; dill; biosynthesis; UV-B effect; flavonol glycoside; quercetin 3-*O*-β-D-glucuronide; UDP-glucuronic acid; glucuronosyltransferase.

Abstract—In dill (*Anethum graveolens*) cell suspension cultures, quercetin 3-*O*-β-D-glucuronide is formed selectively as the predominant flavonoid in response to UV-B irradiation. Flavonoid synthesis is regulated via UV-induction of the enzymes involved. UV-Dependent appearance of a UDP-glucuronosyltransferase is shown. UV-Regulated flavonoid accumulation as an active protective mechanism is indicated.

INTRODUCTION

Regulation of the flavonoid pathway has been investigated extensively in cell suspension cultures of parsley [1, 2]. The enzymes involved in this pathway are known to be regulated at the level of messenger RNA synthesis [3] in response to irradiation with UV light [4]. This effect showed maximum quantum efficiency at around 295 nm and a linear fluence relationship [5]. In addition, phytochrome [6] and a blue light receptor [7] are involved in this response. In dill cell suspension cultures, a similar UV effect on the formation of phenylpropanoids has been observed. Only one quercetin glycoside is accumulated in response to UV [8] while in parsley cell cultures the pattern of induced flavonoids is complex, including both flavones and flavonols [9].

The UV-induced flavonoid from dill cell suspension cultures has now been identified and UV regulation of UDP-glucuronosyltransferase and of further enzymes leading to this compound could be demonstrated.

RESULTS AND DISCUSSION

The UV-induced flavonoid from the dill cell cultures was identified as quercetin 3-*O*-β-D-glucuronide using such standard methods such as paper chromatography and cellulose TLC in different solvent systems, and UV spectroscopy (including characteristic shifts by NaOAc–H_3BO_3 and NaOMe [10]). Further support for a glucuronide came from high voltage paper electrophoresis, where the compound moved towards the cathode ($R_{\text{picric acid}} = 0.214$). Enzymatic cleavage with β-glucuronidase yielded quercetin as the aglycone.

Our previous finding that arabinose is the glycosidic moiety [8] can be explained by the fact that it is an artefact formed during hydrolysis in the presence of methanol. The compound formed under mild acid conditions could be identified as quercetin 3-*O*-β-D-glucuronide methyl ester [11]. After strong acid hydrolysis arabinose was detected, presumably resulting from glucuronic acid after decarboxylation and isomerization at C-4 [12]. Thus reports on arabinosides solely identified as acid hydrolysis products should be reconsidered.

Further proof for the structure of the 3-glucuronide came from ^{1}H NMR and ^{13}C NMR spectroscopy at ambient temperature. Comparison of the spectrum of the glucuronide with those of its methyl ester and 4-nitrophenol-β-D-glucuronide as a reference compound (data not shown) allowed unequivocal identification of the compound. In particular, the signal for H-5″ could not be directly observed in the spectrum of the 3-glucuronide. It must occur at relatively high field between 3.4 and 3.6 ppm together with those for H-2″, H-3″ and H-4″. The signals for the respective protons of the methyl ester and 4-nitrophenol-3-β-D-glucuronide, on the other hand, appeared as a doublet at 3.745 and 4.063 ppm, respectively. Similarly, in the ^{13}C NMR spectrum C-5 of the glucuronic acid residue of the 3-glucuronide gave rise to a signal at 74.62 ppm which is 1.3 ppm at higher field compared to literature data [13]. Again this signal appeared at 75.61 and 75.37 ppm in the spectra of the methyl ester and 4-nitrophenol-β-D-glucuronide, respectively.

Quercetin 3-glucuronide represents one of the main flavonoids in leaves of adult dill plants [14]; in addition, the presence of an arabinoside was shown [15]. The total phenylpropanoid fraction in dill cell cultures was found to be specifically UV-induced with optimal effectiveness in the UV-B spectral range ($\lambda < 320$ nm) [8]. The selective UV effect on 3-glucuronide synthesis is shown in Table 1. As in parsley cell cultures [16], the UV effect can be measured at the enzyme level. A strong UV-B effect on three representative enzymes of the flavonoid pathway could be shown (Table 2). For the first time, induction of a glucuronosyltransferase could be shown in a higher plant system (Table 3) although the enzyme has previously been described as occurring at very low levels in French bean leaf extracts [17]. In dill cell cultures UDP-glucuronic acid, as the donor for the glycosidic compound, is only transferred to quercetin by enzyme preparations from UV-irradiated cell cultures. No enzyme activity could be detected in dark-grown cells. On the other hand, a low

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Table 1. UV-induction of quercetin 3-glucuronide by irradiation for 3 hr with white light varied by UV cut-off filters*

Waveband (nm)	Qu 3 GlcA (A_{360})
Dark	0.020
$\lambda > 420$	0.025
$\lambda > 280$	0.410

*The isolated glycoside from 0.5 g cells (fr. wt) was measured at 360 nm.

Table 2. UV waveband responsible for induction of PAL, CHS and CHI*

Waveband (nm)	PAL (μkat/kg)	CHS (μkat/kg)	CHI (μkat/kg)
Dark	22	0.04	39
$\lambda > 360$	25	0.04	78
$\lambda > 320$	60	0.24	292
$\lambda > 280$	152	1.01	1654

*Irradiation was for 6 hr with white light varied by UV cut-off glasses. After a further 14 hr of darkness, enzyme activities were measured.

UDP-glucosyltransferase activity is present in the dark whose activity is increased 3-fold on UV-irradiation (Table 3). However, on the basis of crude extracts, it cannot be excluded that the UV-induced glucosyltransferase activity is a side activity of the glucuronosyltransferase. No UV effect could be detected on UDP-glucose dehydrogenase activity (Table 3). Induction of an isoenzyme responsible for the flavonoid pathway might still, however, be possible.

A relatively high activity of an isoenzyme which would be responsible for synthesis of cell wall precursors might cover activity changes related to flavonoid synthesis. The dill cell culture turned out to be a useful system not only for the study of enzymatic reactions leading to quercetin 3-glucuronide formation, but also for detailed analysis of a selective UV photoresponse. As in cell cultures, seedling roots [8] and hypocotyls (B. Möhle and E. Wellmann, unpublished results) respond with flavonoid formation to UV-B fluences corresponding to natural radiation conditions. The UV-induced pigment absorbs effectively in the UV spectral range and therefore may function as a screen against potential UV-radiation damage. Such active UV-protective mechanisms are widespread in plants [4] and their regulation and capacity require further investigation.

EXPERIMENTAL

Cell cultures from dill (*Anethum graveolens*) were obtained, grown and used for experiments as described previously [8].

Light sources. White light (50 W/m²) was obtained from a modified Zeiss projector fitted with an Osram XBO 450 W xenon arc and quartz optics. The UV spectral range was varied by means of a series of glass filters with a sharp transmission cut-off at the shorter wavelengths (Schott, Mainz, F.R.G.). Ultraviolet (UV) light was obtained from Osram 40 W/73 lamps (λ_{max} = 350 nm, fluence rate 7.8 W/m²). This light source provides sufficient UV-B (below 320 nm) to induce the UV-B specific responses.

Pigment assay and characterization. Cells (0.5 g) were extracted with 1.5 ml 100% EtOH for 30 min at 75°. Clear extracts (10000 *g* for 5 min) were separated by descending PC (paper No. 2043, Schleicher & Schüll, Dassel, F.R.G.) in *t*-BuOH–HOAc–H_2O (3:2:1) and 15% HOAc. The 3-glucuronide spots were eluted in 2 ml 100% MeOH and measured at 360 nm. For hydrolysis, equal amounts of flavonoid soln and 0.5% (mild hydrolysis) or 6% HCl (strong hydrolysis) were incubated for 30 min at 95°. Standard methods were used for UV spectroscopy including characteristic shifts by NaOMe and NaOAc–H_3BO_3 [10].

For NMR spectroscopy the flavonoids were purified by CC on Sephadex LH-20 (190 ml bed vol.) with MeOH as solvent. The purity of the components was tested by RP-HPLC (Li-Chrosorb RP18, 5 μm, 0.9 × 25 cm, 2.5 ml/min) with 1% HOAc in MeOH–H_2O (1:1). ^{1}H NMR (300 MHz, CD_3OD, 33°): 3-glucuronide: δ3.41–3.65 (*m*, H-2″, H-3″, H-4″, H-5″), 5.354 (*d*, *J* = 7.4 Hz, H-1″), 6.197 (*d*, *J* = 2.2 Hz, H-6), 6.388 (*d*, *J* = 1.9 Hz, H-8), 6.856 (*d*, *J* = 8.5 Hz, H-5′), 7.485 (*dd*, *J* = 2.2, 8.5 Hz, H-6′), 7.974 (*d*, *J* = 2.1 Hz, H-2′); 3-glucuronide methyl ester: δ3.344 (*s*, MeO–), 3.440 (*t*, *J* = 8.2, 8.7 Hz, H-3″), 3.514 (*t*, *J* = 7.6, 8.9 Hz, H-2″), 3.561 (*t*, *J* = 8.8, 9.4 Hz, H-4″), 3.745 (*d*, *J* = 9.6 Hz, H-5″), 6.194 (*d*, *J* = 1.9 Hz, H-6), 6.380 (*d*, *J* = 2.1 Hz, H-8), 6.841 (*d*, *J* = 9.9 Hz, H-5′), 7.57 (*m*, H-6′), 7.58 (*m*, H-2′). ^{13}C NMR (75.47 MHz, DMSO-d_6, 33°): 3-glucuronide: δ71.62 (C-4″), 74.55 (C-2″), 74.62 (C-5″), 76.50 (C-3″), 93.67 (C-8), 98.84 (C-6), 102.86 (C-1″), 103.79 (C-10), 115 34 (C-2′), 117.89 (C-5′), 120.53 (C-1′/C-6′), 133.89 (C-3), 144.83 (C-3′), 148.42 (C-4′), 156.45* (C-2), 157.55* (C-9), 161.03 (C-5), 164.39 (C-7), 171.10 (C-6″), 177.5 (C-4); methyl ester: δ51.70 (MeO–), 71.29 (C-4″), 73.69 (C-2″), 75.61 (C-2″/C-5″), 75.61 (C-3″), 93.51 (C-8), 98.69 (C-6), 101.37 (C-1″),

Table 3. UV-induction of enzymes related to glucuronic acid metabolism in extracts of dark-grown and 20 hr UV-irradiated cell cultures.

Detected enzyme	Incorporation into product	Treatment	Enzyme activity (μkat/kg) (%)
UDP-Glucuronosyl-transferase	Qu 3GlcA	Dark	0.05 (0.4)
		UV	12.16 (100)
UDP-Glucosyl-transferase	Qu 3Glc	Dark	0.36 (30)
		UV	1.20 (100)
UDP-Glucose-dehydrogenase	UDP-GlcA	Dark	7.20 (90)
		UV	7.96 (100)

103.85 (C-10), 115.08 (C-12'), 116.03 (C-5'), 120.84 (C-1'), 121.58 (C-6'), 133.12 (C-3), 144.80 (C-3'), 148.52 (C-4'), 156.24* (C-9), 156.39* (C-2), 161.12 (C-5), 164.17 (C-7), 168.78 (C-6''), 177.06 (C-4).

Enzyme assays. Standard procedures were used for preparing crude cell extracts and measuring the activities of PAL [18], CHS [19] and CHI [20] with the exception of using a mortar for extraction instead of thawing frozen cells.

Preparation of crude extracts. Cells (1.0 g) were homogenized at 0° in a prechilled mortar with 0.2 g quartz sand and 2 ml 0.1 M Tris–HCl buffer (pH 8.0) containing 4 mM DTE. The homogenate was stirred for 15 min at 0° with 0.2 g Dowex 1 × 2 and centrifuged for 10 min at 8000 *g*. 0.5 ml supernatant was centrifuged through an equilibrated 5 ml column with Sephadex G-50 coarse for 3 min at 4000 *g*. The eluate was used for the enzyme tests.

UDP-glucuronosyltransferase assay. The reaction mixture contained in a total vol. of 50 μl: 2 μmol Tris–HCl (pH 8.0), 80 nmol DTE, 50 nmol NAD, 2.5 nmol UDPGlcA, 0.2 nmol UDP[^{14}C]GlcA (321 Ci/mol), 5 nmol Qu and 25 μl protein extract. The reaction mixture was incubated for 1 hr at 30°, stopped by the addition of 2 nmol Qu 3 GlcA and 30 μl MeOH, applied on a cellulose plate and chromatographed in 15% HOAc. The 3-glucuronide spot was detected under UV, scraped off and counted in 0.5% PPO in toluene in a scintillation counter.

UDP-Glucose dehydrogenase assay. The reaction mixture contained in a total vol. of 50 μl: 2 μmol Tris–HCl (pH 8.0), 80 nmol DTE, 50 nmol NAD, 2.5 nmol UDPGlc, 0.2 nmol UDP[^{14}C]Glc (312 Ci/mol) and 25 μl protein extract. The reaction mixture was incubated for 1 hr at 30°, stopped by the addition of 10 μl HOAc, heated at 96° for 30 min, and together with carriers Glc (5 μg) and GlcA (5 μg) applied on a cellulose plate and chromatographed in EtOAc–C_5H_5N–HOAc–H_2O (36:36:7:21). Radioactive compounds were detected by scanning and the zone corresponding to GlcA—detected by comparison with a cochromatographed reference substance—was scraped off and counted in a toluene cocktail.

UDP-Glucosyltransferase assay. The reaction mixture contained in a total vol. of 50 μl: 2 μmol Tris–HCl (pH 8.0), 80 nmol DTE, 5 nmol Qu, 2 5 nmol UDPGlc, 0 2 nmol UDP[^{14}C]Glc (312 Ci/mol) and 25 μl protein extract. Incubation was for 1 hr at 30°, stopped by 2 nmol Qu 3-glucuronide, 2 nmol Qu 3-glucoside and 30 μl MeOH. Chromatography on cellulose was first in H_2O (half of maximal run)—the glucuronide and glucoside were separated—and then in 15% HOAc—UDP compounds were separated from flavonoids. The labelled Qu 3 Glc was scraped off and counted in toluene cocktail.

Protein content was estimated by the biuret method [21].

*Values may be interchanged.

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