Formation of 6′-Deoxychalcone 4′-Glucosides by Enzyme Extracts from Petals of *Dahlia variabilis*

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Yellow colouration of *Dahlia variabilis* is mainly provided by isoliquiritigenin 4′-glucoside and butein 4′-glucoside. Incubation of petal extracts with uridin 5′-diphosphoglucose and isoliquiritigenin or butein led to the formation of one product which was identified as the respective 6′-deoxychalcone 4′-glucoside. Glucosylation of hydroxyl groups in other positions was not observed. Naringenin chalcone and eriodictyol chalcone were not accepted as substrates. The 4′-glucosylation of isoliquiritigenin and butein showed a broad pH optimum ranging from pH 7 to 8 and was stimulated by Mg^{2+}, Ca^{2+} and Mn^{2+}. N-Ethylmaleimide, *p*-hydroxymercuribenzoate, Cu^{2+}, Zn^{2+}, and Fe^{2+} clearly reduced the activity of the enzyme. The apparent K_m values for UDP-glucose, isoliquiritigenin and butein were 90, 26 and 276 μM respectively.

Introduction

Yellow colouration of flowers is mainly due to the presence of carotenoids (Harborne, 1988). Other classes of yellow pigments are betaxanthins, higher hydroxylated flavonols and the flavonoid-related deep yellow chalcones and aurones. In the petals of many plants, especially in some members of the Asteraceae, carotenoids co-occur with the latter compounds. But there are some cases, where chalcones and aurones are the only source of yellow colouration. Examples are *Anthirrhinum, Callistephus, Dahlia, Dianthus* and *Helichrysum* (Harborne, 1967).

Two types of chalcones are formed in flowers, the 6′-hydroxychalcones (*e.g.* naringenin chalcone), which are intermediates in the synthesis of the common 5-hydroxyflavonoids including the anthocyanins, and the 6′-deoxychalcones (*e.g.* isoliquiritigenin), which are involved in the 5-deoxy series of flavonoids. Consequently, the transformation of the chalcones to the respective flavanones is catalyzed by two different chalcone isomerases (CHI) (Heller and Forkmann, 1988). Both types of chalcones can be modified by further hydroxylation of the B-ring and glycosylation of hydroxyl groups at the ring A. Thus, the respective chalcones were found to be accumulated as glycosides in the flowers. This accumulation is particularly observed in genotypes lacking chalcone isomerase activity.

While the biosynthesis of flavonoids has already been well-established (Heller and Forkmann, 1988, 1994), our knowledge on the formation of the differently hydroxylated and glucosylated chalcones is still poor. Up to now, the respective hydroxylases and glucosyltransferases have not been characterized. Moreover, *in vitro* formation of 6′-deoxychalcones with enzyme preparations from flowers has not yet been demonstrated.

In flowers of *Dahlia variabilis* the biosynthesis of anthocyanins and other flavonoids has already been studied in detail (Koch, 1992; Fischer *et al.,* 1988; Stich *et al.,* 1988). As a rule, crude flower extracts exhibited high activity of all flavonoid enzymes measured. Thus, *D. variabilis* seems to be well-suited for the elucidation of the biosynthesis of different chalcone glucosides, too. Moreover, in yellow-flowering cultivars, colouration is caused by glucosides of the 6′-deoxychalcones isoliquiritigenin and butein (Nordström and Swain, 1956; Giannasi, 1975; Harborne, 1990). These chalcones co-occur with glycosides of the flavones apigenin

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and luteolin or even with anthocyanins, which leads to more or less yellow-orange flowers. Both the flavones and anthocyanins are synthesized *via* the 6′-hydroxychalcone (naringenin chalcone). The 6′-deoxychalcones isoliquiritigenin and butein are not used as intermediates.

Yellow flowers accumulate appreciable amounts of 6′-deoxychalcone glucosides but no naringenin chalcone glucoside. This is due to the fact that naringenin chalcone is completely isomerized to naringenin by CHI providing the substrate for flavone and anthocyanin formation. In contrast, the 6′-deoxychalcones isoliquiritigenin and butein are glucosylated but neither isomerized enzymatically nor chemically to the respective flavanones. This fact should allow the use of both 6′-deoxychalcones as stable substrates for the elucidation of the glucosylation reaction in flower extracts.

This paper reports for the first time on the occurrence of a glucosyltransferase converting 6′-deoxychalcones with high specificity to their respective 4′-glucosides.

Materials and Methods

Plant material

The investigations were performed on the yellow flowering commercial strain "Johann Nestroy" (Dr. Wirth) of *D. variabilis*, containing mainly isoliquiritigenin 4′-glucoside and butein 4′-glucoside as pigments in the flowers (Nordström and Swain, 1956; Giannasi, 1975). The plant material was cultivated in the "Bundesgärten Schönbrunn" during the summer period.

Chemicals

Naringenin, eriodictyol, apigenin, luteolin and their 7-glucosides as well as butein were purchased from Roth (Karlsruhe, Germany). Naringenin chalcone, eriodictyol chalcone, isoliquiritigenin, isoliquiritigenin 4′-glucoside and butein 4′-glucoside were from our laboratory collection. UDP-D-[U-^{14}C]glucose (12.0 GBq/mmol) was obtained from Amersham International (Great Britain).

Buffer solutions

Unless otherwise stated the following buffers were used: 0.1 M Tris/HCl (containing 0.4% Na-ascorbate), pH = 7.5 for assays with butein and pH = 8.00 for assays with isoliquiritigenin as substrates.

Enzyme preparation

All steps were performed at 4 °C. An aliquot of 1.0 g petals was homogenized together with 0.5 g quartz sand, 0.5 g polyvinylpyrrolidone (Serva, Germany) and 6 ml buffer in a precooled mortar. The homogenate was centrifuged for 10 min at 10000×*g*. To free the crude extract from phenolic compounds and other low molecular weight substances the extract was passed through a Sephadex G-50 (fine) column (bed volume = 1 ml).

Enzyme assay

The reaction mixture contained in a total volume of 100 µl: 50–82 µl buffer, 8–40 µl enzyme extract (8–40 µg protein), 70 nmol of UDP-D-[U-^{14}C]glucose (1600 Bq/70 nmol) and 30 nmol of the respective chalcone or other flavonoid substrates (dissolved in 5 µl ethylene glycol monomethyl ether). Standard enzyme assays were carried out with butein as substrate.

The reaction was started by the addition of UDP-D-[U^{14}C]glucose. After incubation for 15 min at 25 °C the reaction was stopped by the addition of 50 µl methanol. The mixture was chromatographed on paper (Schleicher & Schüll 2043 b, Germany) using 30% acetic acid. The zone containing the labelled product was localized with a TLC linear analyzer (Berthold, Germany), cut out and transferred to a scintillation cocktail (Ready Solve HP, Beckman, U.S.A.). Afterwards radioactivity was measured in a scintillation counter (LKB, Sweden).

Determination of the pH optimum

Enzyme assays for the determination of the pH optimum were carried out as described above, using 10 mM Tris/HCl buffer (pH 8.0, containing 0.4% Na-ascorbate) for homogenization and 0.2 M Tris/HCl buffer (containing 0.4% Na-ascorbate) with pH between 7.25 and 9.00 for the assay mixture.

Kinetics

Kinetic data were calculated from Lineweaver-Burk plots. Determination of the apparent

Michaelis constant (K_m) and maximal velocity (V_{max}) for the chalcones was carried out using a fixed UDP-glucose concentration of 700 μM. Determination of K_m and V_{max} for UDP-glucose were performed with a fixed concentration of butein at 300 μM. At these substrate concentrations no substrate inhibition could be observed.

Analytical methods

The enzymatically formed chalcone glucosides, flavanone glucosides and flavone glucosides were identified by TLC on precoated cellulose plates (Merck, Germany), using the following solvent systems: (1) 30% acetic acid; (2) chloroform/acetic acid/water (10:9:1); (3) *n*-butanol/acetic acid/water (6:1:2); (4) *t*-butanol/acetic acid/water (3:1:1). Radioactivity was localized by scanning the plates with a TLC linear analyzer (Berthold, Wildbad, Germany).

Protein was determined by a modified Lowry procedure (Sandermann and Strominger, 1972), using crystalline BSA as standard.

Results

Incubation of isoliquiritigenin and butein respectively with UDP-D-[U-^{14}C]glucose and enzyme preparations from yellow petals of *D. variabilis* led to the formation of a single product, which was identified as the respective chalcone 4′-glucoside by co-chromatography with authentic reference substances in four different solvent systems (Table I). Glucosylation of hydroxyl groups in other positions was not observed. Moreover, naringenin chalcone and eriodictyol chalcone did not serve as substrates. But the enzyme preparations glucosylated the flavanones naringenin and eriodictyol as well as the flavones apigenin and luteolin in the 7-position, which could be confirmed by co-chromatography with corresponding reference substances (Table I). The 4′-glucosylation of isoliquiritigenin and butein showed a broad optimum between pH 7 and 8.

Table I. R_f values (×100) of the enzymatically formed products.

Compound	Solvent system 1	2	3	4
Isoliquiritigenin 4′-glucoside	32	42	69	59
Butein 4′-glucoside	24	58	45	36
Naringenin 7-glucoside	75	74	86	89
Eriodictyol 7-glucoside	71	56	71	76
Apigenin 7-glucoside	38	60	67	71
Luteolin 7-glucoside	25	36	47	46

Because of the higher reaction rate (see below), the further characterization of the glucosyltransferase was carried out with butein as substrate. Addition of bovine serum albumin (BSA) did not cause any effect. At 25 °C the formation of butein 4′-glucoside was linear with protein concentration up to 20 μg protein per assay and with time for at least 60 min. The enzyme exhibited a temperature optimum at 50 °C. At 0 °C and 60 °C the reaction rate reached only 10% of the maximum.

The effect of some bivalent cations and potential enzyme inhibitors on product formation is shown in Table II. Addition of Mg^{2+}, Ca^{2+} and Mn^{2+} showed weak stimulatory effects, whereas heavy metal ions like Cu^{2+}, Fe^{2+} and Zn^{2+} clearly reduced enzyme activity. Moreover, *p*-hydroxymercuribenzoate and N-ethylmaleimide did strongly affect the reaction. Diethyldithiocarbamate (DDC) and diethylpyrocarbonate (DPC) mainly inhibited at higher concentrations. KCN and EDTA did not influence the reaction rate.

Using butein as substrate, a specific activity of 216 μkat/kg protein was measured, whereas with isoliquiritigenin as substrate only a specific activity of 13 μkat/kg protein was observed. The values for

Table II. Influence of divalent ions and inhibitors on 6′-deoxychalcone 4′-O-glucosyltransferase.

Addition	Concentration [mM]	CHGT activity [%]
None	–	100
Mg^{2+}	1	121
Ca^{2+}	1	108
Mn^{2+}	1	110
Cu^{2+}	1	0
Zn^{2+}	1	0
Fe^{2+}	1	10
DDC	1	78
	5	32
DPC	1	70
	5	0
KCN	1	97
EDTA	1	103
N-Ethylmaleimide	1	0
p-Hydroxymercuribenzoate	0.1	0

EDTA, ethylene diamine tetraacetate; DDC, diethyldithiocarbamate; DPC, diethylpyrocarbonate.

K_m and V_{max} calculated from the Lineweaver-Burk plot were 267 μM and 400 μmol/skg for butein and 26 μM and 13 μmol/skg for isoliquiritigenin, respectively. The ratios V_{max}/K_m were 1.5 for butein and 0.5 for isoliquiritigenin, confirming butein as the preferred substrate. Using butein as substrate, the K_m and V_{max} for UDP-glucose were 90 μM and 87 μmol/skg, respectively.

A loss of 30% of enzyme activity was observed, when the petals were frozen in liquid nitrogen. But no further decrease was detected during storage at −80 °C for several weeks.

Discussion

The glycosylation of flavonoids has been well studied in the case of flavones, flavonols and anthocyanidins (Heller and Forkmann, 1988, 1994). Up to now, however, there is little known about the enzymatic formation of chalcone glucosides. The only reports concern an enzyme preparation from *Citrus paradisii* seedlings, which was found to catalyze 7-O-glucosylation of flavanones but also glucosylates the 4′-position of naringenin chalcone (McIntosh *et al.,* 1990; McIntosh and Mansell, 1990).

Now in petal extracts of *D. variabilis,* we could demonstrate for the first time an enzyme which glucosylates 6′-deoxychalcones. Thus, *D. variabilis* flowers again proved to be a rich enzyme source. In agreement with the naturally present chalcone glucosides, the glucosyltransferase transfers the glucosyl moiety of UDP-glucose specifically to the 4′-position of isoliquiritigenin and butein. The enzyme preparation also catalyzed the glucosylation of flavanones and flavones in the 7-position. But the 6′-hydroxy compounds naringenin chalcone and eriodictyol chalcone were not accepted as substrates.

The kinetic data revealed that the glucosyltransferase shows high substrate specificity towards the 6′-deoxychalcones isoliquiritigenin and butein indicating that the enzyme is specifically involved in the biosynthesis of chalcone glucosides. Up to now, however, it cannot be excluded, that the glucosylation of 6′-deoxychalcones in the 4′-position and 5-hydroxyflavanones and -flavones in the related 7-position is catalyzed by one and the same enzyme.

The stimulatory effect of divalent ions on enzyme activity corresponds well with reported data (Larson and Lonergan, 1972; Kleinehollenhorst *et al.,* 1982; Teusch *et al.,* 1986; Cheng *et al.,* 1994). Surprisingly, however, the addition of BSA to the enzyme assay did not lead to a significant increase of enzyme activity as reported for other glucosyltransferases (Sutter *et al.,* 1972; Saleh *et al.,* 1976a, 1976b). The inhibitory effect of the heavy metal ions is most probably due to their interaction with SH groups of the enzyme. This assumption is confirmed by the fact, that the addition of SH group-binding reagents as *p*-hydroxymercuribenzoate and N-ethylmaleimide did affect the reaction strongly, too.

Maximal enzyme activity was observed at a surprisingly high temperature of 50 °C. Similar high values were found for the chalcone synthase (CHS) of *D. variabilis* (Forkmann, unpublished), which can be assumed to provide isoliquiritigenin and butein and thereby the substrates for the glucosylation reaction. Moreover, a very high temperature optimum was also found for the anthocyanidin 3-O-glucosyltransferase of *Matthiola incana* (Teusch *et al.,* 1986). Actually, the high optimum values measured *in vitro* are only of theoretical interest, since they are clearly not within near-physiological ranges. More surprising, however, is the strong reduction of enzyme activity at low temperatures, since yellow colouration of *D. variabilis* flowers is not reduced in autumn. Thus, biosynthesis of the pigments has to proceed even at low temperatures. Apparently, even a low glucosyltransferase activity is sufficient for the glucosylation reaction since the 6′-deoxychalcones are stable substrates and they are not converted by other enzymes. On the other hand, in several plant species a number of flavonoid enzymes were found to exhibit high activities also at low temperatures (Ruhnau and Forkmann, 1988; Teusch, 1986; Stich *et al.,* 1992). Studies in *Dendranthema grandiflora (Chrysanthemum morifolium)* which are typical "autumn flowers" also revealed that the reaction rate of a number of enzymes involved in biosynthesis of anthocyanins reached at 0 °C at least 50% of the optimum (Stich, unpublished).

The co-occurrence of isoliquiritigenin and butein glucosides in *D. variabilis* flowers raises the question, how the additional hydroxy group in ring B of butein is introduced. There are two possible ways. On the one hand, isoliquiritigenin could be

hydroxylated to butein by an up to now unknown chalcone 3-hydroxylase. On the other hand butein could be formed directly by the use of caffeoyl-CoA instead of *p*-coumaroyl-CoA during synthesis of the 6′-deoxychalcone skeleton. 6′-Deoxychalcone formation can be assumed to be catalyzed by the common chalcone synthase in coaction with an NADPH-dependent polyketide reductase (Welle and Grisebach, 1988). However, this reaction has not yet been demonstrated with enzyme preparations from flowers. Thus, further work will be concerned with the detailed elucidation of the biosynthesis of the 6′-deoxychalcones isoliquiritigenin and butein.

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