UDP-Glucose: Flavonol 7-O-glucosyltransferase Activity in Flower Extracts of *Chrysanthemum segetum*

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The yellow colour of *Chrysanthemum segetum* petals is due to the presence of the 7-O-glucosides of quercetin and particularly gossypetin (8-hydroxyquercetin). In petal extracts of *C. segetum* an enzyme was demonstrated which catalyzes the transfer of the glucosyl moiety of uridine 5'-diphosphoglucose (UDPG) to the 7-hydroxyl group of flavonols with gossypetin and quercetin as the best substrates. Besides flavonols flavanones and flavones were found to be glucosylated in the 7-position. The pH-optimum of the reaction highly depended on the substrate used. With quercetin as substrate, maximal enzyme activity occurred at a pH of 8.25 and a temperature of 25 °C, but 7-O-glucosylation also proceeded at low temperatures. Studies on temperature stability revealed, that there was no influence on the glucosylation reaction up to 40 °C. Higher temperatures led to a loss of enzyme activity. Using gossypetin as a substrate a similar course of temperature stability was observed. Addition of Mg^{2+}, Ca^{2+} and KCN slightly stimulated 7-O-glucosylation, whereas Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, p-hydroxymercuribenzoate and N-ethylmaleimide showed a strong inhibitory effect. Additional enzymatic studies were performed with the commercial strain "Stern des Orients" where gossypetin 7-O-glucoside is restricted to the inner parts of the petals. For enzyme extracts from both parts of the petals gossypetin was found to be the most attractive substrate. In comparison to quercetin (133.4 μkat / kg protein) an about three times higher specific activity of the 7-O-glucosyltransferase(s) was determined with gossypetin (382.1 μkat/kg protein) as substrate, indicating that hydroxylation of quercetin in 8-position to gossypetin precedes 7-O-glucosylation.

Introduction

Often the local concentration of UV absorbing pigments leads to the formation of "honey guides" attracting insects whose visual system is sensitive to this range of the spectrum. In *Asteraceae* flavonol glucosides, particularly flavonol 7-O-glucosides, are responsible for UV absorption. Thus, Geissman and Steelink (1957) reported the presence of quercetin 7-O-glucoside and gossypetin 7-O-glucoside in petals of *Chrysanthemum segetum*. Gossypetin 7-O-glucoside, which only differs from quercetin 7-O-glucoside by a hydroxyl group in the 8-position of ring A, is particularly responsible for the yellow colouration of the petals. Besides yellow coloured strains of this plant there are also commercial strains available, which show yellow colouration only in the inner part of the petals, whereas the outer parts are pale yellow coloured.

In the past few years it has been recognized that flowers are rich sources of flavonoid enzymes. This has resulted in successful elucidation of a number of steps in the biosynthetic pathway to the main flavonoid classes including flavonols (Heller and Forkmann, 1988; 1994). Biosynthesis of 8-hydroxylated flavonols and their glycosides, however, has still remained unknown. In this work we report for the first time on the characterization of a glucosyltransferase from petals of *Chrysanthemum segetum* which is responsible for glucosylation of several flavonols in position 7.

Material and Methods

Plant material

The investigations were performed on the commercial strains "Prado" (Austrosaat, Vienna,

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Austria) and "Stern des Orients" (Sperling & Co, Lüneburg, Germany) of *Chrysanthemum segetum*. The plant material was cultivated in the experimental garden of Schönbrunn during the summer period. Just opening buds were harvested.

Chemicals

Naringenin, eriodictyol, apigenin, luteolin, kaempferol, quercetin and myricetin were purchased from Roth (Karlsruhe, Germany). Gossypetin was a gift from W. Heller (Neuherberg, Germany). We also thank H. Greger (University of Vienna) for an authentic sample of kaempferol 7-O-glucoside and quercetin 7-O-glucoside. UDP-D-(U-^{14}C)-Glucose (12.0 GBq/mmol) was obtained from Amersham International (Great Britain).

Enzyme preparation

All steps were performed at 4 °C.

An aliquot of 1.0 g petals was homogenised together with 0.5 g quartz sand, 0.5 g polyvinylpyrrolidone (Serva, Germany) and 4 ml 0.1 M Tris/HCl buffer (pH = 7.5, containing 0.4% Na-ascorbate) in a precooled mortar. The homogenate was centrifuged for 15 min at 10000× *g* and the clear supernatant was centrifuged again for 10 min. 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 (Stich *et al.*, 1994). The average protein content obtained with this procedure was 10 µg protein / 10 µl enzyme extract.

Enzyme assay

The reaction mixture contained in a total volume of 100 µl: 80–88 µl 0.1 M Tris/HCl buffer (containing 0.4% Na-ascorbate), 2–10 µl enzyme extract (2–10 µg protein), 10 nmol of UDP-D-(U-^{14}C)-glucose (5170 Bq), 200 µg bovine serum albumin (BSA) and 30 nmol of the respective flavonoid substrate (dissolved in 5 µl ethylene glycol monomethyl ether). pH of the buffer was dependent on the actual substrate used (see Table III). Standard enzyme assays were carried out with quercetin as substrate.

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

All tests were run in triple. No measurable degradation of UDP-D-(U-^{14}C)-glucose could be observed during the enzyme assay. The measurements were confirmed by a second independent preparation of enzyme extracts.

Determination of the pH-optimum

Enzyme assays were carried out as described above, using 0.1 M Tris/HCl buffer (containing 0.4% Na-ascorbate) with pH between 6.5 and 8.8.

Temperature stability

The reaction mixture without UDPG was incubated for 10 minutes at the respective temperature (between 0 °C and 60 °C) followed by standard enzyme assay.

Kinetics

Kinetic constants were calculated from Lineweaver-Burk plots. Determination of the apparent Michaelis constant (K_m) and maximal velocity (V_{max}) for the respective flavonoid substrate was performed with a fixed concentration of UDP-glucose at 200 µM. At this concentration no substrate inhibition could be observed. All these values were determined at the respective pH optimum of the reaction.

Analytical methods

Standard procedures (Mabry *et al.*, 1970; Harborne, 1967; Stich *et al.*, 1994) were used for the analysis of the flavonoid floral constituents and for the identification of the enzymatically formed flavonoid glucosides.

TLC was performed on precoated cellulose plates (Merck, Germany), using the following

solvent systems: (1) 15% acetic acid; (2) 30% acetic acid; (3) chloroform/acetic acid/water (10:9:1); (4) *n*-butanol/acetic acid/water (6:1:2); (5) acetic acid/conc. HCl/ water (30:3:10).

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

The flavonoids present in the petals of *Chrysanthemum segetum* were identified spectrophotometrically and by thin-layer chromatography. The flower pigments of the commercial strain "Prado" were identified as quercetin 7-O-glucoside and gossypetin 7-O-glucoside, which are present in approximately equal amounts. Besides, small amounts (< 10% of the flavonol content) of a third compound were isolated and identified as kaempferol 7-O-glucoside. Additionally, luteolin 7-O-glucoside was detected in the petals. While all parts of the petals of the strain "Prado" are yellow coloured, only the inner part of the petals of the strain "Stern des Orients" had a yellow colour. The outer part of the petals had a pale yellow colouration. In both parts of this strain quercetin 7-O-glucoside was found to be present, while gossypetin 7-O-glucoside is restricted to the inner part of the petals.

Incubation of kaempferol, quercetin, myricetin or gossypetin with enzyme preparations from petals of *Chrysanthemum segetum* cv. "Prado" in the presence of UDP-D-(U-^{14}C)-glucose led to the formation of the respective flavonol 7-O-glucoside. The reaction products were identified by co-chromatography with authentic reference substances in five different solvent systems. Addition of bovine serum albumin (BSA) to the assay system caused a 2.5 times higher reaction rate. Thus, all experiments were performed in the presence of BSA. On standard assay conditions no formation of other glucosylated products could be detected.

Due to the sensibility of gossypetin to aerial oxidation and to the limited availability of pure gossypetin, properties of the enzyme(s) were determined using quercetin as flavonoid substrate, exept in a few cases, were it was interesting to compare the two substrates.

The pH optimum for the glucosylation of quercetin was about pH 8.25. Using gossypetin as a substrate the pH optimum was about pH 6.85. The flavonol 7-O-glucosyltransferase(s) exhibited a temperature optimum at 25 °C. But even at 0 °C quercetin 7-O-glucoside was formed in considerable amounts (Fig. 1). Studies on temperature stability using quercetin and gossypetin respectively as a substrate revealed, that there was no influence on the glucosylation reaction(s) up to 40 °C. Higher temperatures led to a loss of enzyme activity. Thus, no enzyme activity could be detected in the standard enzyme assay after the reaction mixture was incubated for 10 min at 60 °C without UDP-glucose (UDPG). The formation of quercetin 7-O-glucoside at pH = 8.25 and 25 °C was linear with protein up to 4 μg and with time for at least 35 min.

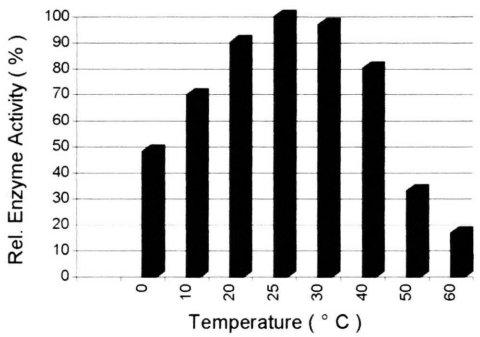

Fig. 1. Enzymatic formation of quercetin 7-glucoside in dependence on temperature.

The effect of some bivalent cations and potential enzyme inhibitors on the rate of reaction was studied (Table I). The addition of Mg^{2+}, Ca^{2+} and KCN showed weak but significant (three repetitions) stimulatory effects on enzymatic formation of quercetin 7-O-glucoside. Addition of heavy metal ions like Co^{2+}, Cu^{2+}, Fe^{2+} and Hg^{2+} clearly reduced the activity of the enzyme(s), whereas diethyldithiocarbamate inhibited to a minor extent. *p*-Hydroxymercuribenzoate and N-ethylmaleimide did affect the reaction strongly. Similar results were obtained when gossypetin instead of quercetin was used as a substrate (Table I). The influence of 2-mercaptoethanol (2-ME) on the inhibitory effect of p-hydroxymercuribenzoate and N-ethylmaleimide respectively was studied in detail (Table II). The presence of 14 mM 2-ME in the

Table I. Effect of various divalent ions and potential inhibitors on 7-O-glucosylation.

Additions	Quercetin as a substrate (%)	Gossypetin as a substrate (%)
None	100	100
1 mM $MgCl_2$	106	103
1 mM Ca Cl_2	104	n. d.
1 mM $MnSO_4$	89	n. d.
1 mM Co Cl_2	2	15
1 mM Cu Cl_2	1	n. d.
1 mM $FeSO_4$	2	n. d.
1 mM Hg Cl_2	1	n. d.
5 mM DDC	100	85
5 mM DPC	2	2
0.1 mM p-OHMB	0.7	n.d.
5 mM EDTA	93	n. d.
1 mM N-Ethylmaleimide	2	n. d.
5 mM KCN	119	n. d.

DDC, diethyldithiocarbamate; n. d.: not determined; DPC, diethylpyrocarbonate; *p*-OHMB, *p*-hydroxymercuribenzoate; EDTA, Ethylendiaminotetraacetic acid. Data in Table I are average values calculated from triple tests.

Table II. Influence of 2 – mercaptoethanol (2 – ME) on the inhibitory effect of *p*-hydroxymercuribenzoate and N-ethylmaleimide respectively.

Addition	Relative enzyme activity (%)
None	100
14 mM 2 – ME	94
1 mM N - ethylmaleimide	2
1 mM N - ethylmaleimide + 14 mM 2 – ME	92
1 mM N - ethylmaleimide + 14 mM 2 – ME*	1
0.1 mM *p*-hydroxymercuribenzoate	0.7
0.1 mM *p*-hydroxymercuribenzoate + 14 mM 2 – ME	94
0.1 mM *p*-hydroxymercuribenzoate + 14 mM 2 – ME*	45

* Addition after 15 minutes.

standard assay led only to a slight decrease of enzyme activity. No further inhibitory effect could be detected when 2-ME was added simultaneously with *p*-hydroxymercuribenzoate and N-ethylmaleimide respectively. Addition of 2-ME after 15 minutes cancelled the inhibitory effect of *p*-hydroxymercuribenzoate but not of N-ethylmaleimide.

No decrease of enzyme activity was detected, when the petals were frozen in liquid nitrogen and stored at -80 °C for several weeks.

For flavonol 7-O-glucosylation gossypetin was the most attractive substrate followed by quercetin, kaempferol and myricetin. *In vitro*, not only flavonols were glucosylated in the 7-position, but also the flavanones naringenin and eriodictyol and the flavones apigenin and luteolin. Specific activities, K_m- values and v_{max}/K_m – values of the substrates tested are summerized in Table III.

Table III. Kinetic data of the 7-O-glucosylation reaction with several substrates.

Substrate	pH – Optimum	Specific activity (μkat/kg)	K_m – value (μM)	v_{max}/K_m (μkat/μM.kg)
Kaempferol	7.8	44.1	29	2.15
Quercetin	8.25	133.4	49	3.60
Myricetin	8.10	48.4	67	1.01
Gossypetin	6.85	382.1	32	17.17
Naringenin	6.85	20.6	73	0.38
Eriodictyol	7.50	27.1	71	0.51
Apigenin	7.65	38.8	78	0.57
Luteolin	8.10	181.3	84	2.48

Additional enzymatic studies were performed with the commercial strain "Stern des Orients". With enzyme extracts prepared from the inner (yellow) and outer (pale yellow) parts respectively the substrate specificity of the 7-O-glucosyltransferase(s) was determined. In both preparations gossypetin was the most attractive substrate, although it is only present in the inner part (data not shown).

Discussion

In the petals of *Chrysanthemum segetum* gossypetin 7-O-glucoside and quercetin 7-O-glucoside were found in considerable amounts. This agrees with investigations of Geissman and Steelink (1957). They report on a further flavonol glucoside occurring in the plant in smaller amounts which was now identified as kaempferol 7-O-glucoside.

The biosynthetic pathway to kaempferol and quercetin respectively was studied in detail in *Petunia hybrida* (Forkmann *et al.*, 1986), *Matthiola incana* (Spribille and Forkmann, 1984) and *Dianthus caryophyllus* (Stich *et al.*, 1992 a). Biosynthesis of flavonol 3-O-glucosides was also extensively investigated in a number of different plants (Heller and Forkmann, 1988; 1994), but there exist only few reports dealing with the enzymatic formation of flavonol 7-O-glucosides (Sutter *et al.*, 1972; Cheng *et al.*, 1994).

Most properties of the flavonol 7-O-glucosyltransferase(s) occurring in *Chrysanthemum seg-*

etum are in accordance to those of several glycosyltransferases reported from the literature. A significant increase of enzyme activity by addition of BSA was also observed in the case of flavone 7-O-glucosyltransferase from parsley (Sutter *et al.*, 1972) and other glycosyltransferases (Saleh *et al.*, 1976 a, b). In vitro, the pH optimum for the reaction was strongly dependent on the respective substrate. Similar enzyme behaviour was observed by Jonsson *et al.* (1984) for the anthocyanidin/flavonol 3-O-glucosyltransferase from *Petunia hybrida* and is a result of different chemical properties of the respective substrate. Investigations on a wide range of plant material (Teusch *et al.*, 1986 a; Ruhnau and Forkmann, 1988; Teusch, 1986; Stich *et al.*, 1992 b) revealed at low temperatures relatively high reaction rates of various enzymes, involved in the flavonoid biosynthesis. This was also observed in our examinations and agrees with the fact that in autumn at low temperature formation of flavonol 7-O-glucosides in petals still takes place.

In agreement with other glucosyltransferases (Sutter *et al.*, 1972; Cheng *et al.*, 1994; Teusch 1986; Teusch *et al.*, 1986 b; Kamsteeg *et al.*, 1980) addition of KCN, Ca^{2+} or Mg^{2+} led to slight stimulation of the activity of the 7-O-glucosyltransferase(s), whereas the presence of heavy metal ions (Cu^{2+}, Fe^{2+} and Hg^{2+}) showed strong inhibitory effects. The activity of the 7-O-glucosyltransferase(s) was completely inhibited by addition of N-ethylmaleimide and *p*-hydroxymercuribenzoate respectively, indicating the presence of SH-groups at the active site of the enzyme(s). This is confirmed by the fact, that the inhibitory effect of N-ethylmaleimide which is known as an irreversible SH specific inhibitor (Dawson *et al.*, 1986) was not influenced by addition of 2-ME, whereas the inhibition of p-hydroxymercuribenzoate was nearly completely reversible with 14 mM 2-ME. Simultaneous addition of 2-ME and *p*-hydroxymercuribenzoate and N-ethylmaleimide results in a chemical reaction between the SH group of 2-ME with the SH specific inhibitor. Therefore no inhibitory effect could be observed in this case. The presence of SH groups in the active site of glucosyltransferases was also suggested by other authors (Larson, 1971; Ishikura and Yamamoto, 1990; Cheng *et al.*, 1994).

The results of the kinetic studies show clearly, that the 7-O-glucosyltransferase(s) preferred flavonols as glucose acceptors. The most attractive substrate was gossypetin followed by quercetin. In fact the main pigments in the petals were identified as gossypetin 7-O-glucoside and quercetin 7-O-glucoside. Thus, it may certainly be assumed that both compounds represent the natural substrates of the flavonol 7-O-glucosyltransferase(s). Apart from the flavonols gossypetin and quercetin, kaempferol and luteolin were glucosylated to a high extent. This is not surprising, since the 7-O-glucosides of kaempferol and luteolin are also present in the petals.

It is remarkable that glucosylation of gossypetin was also observed with enzyme preparations from the outer parts of the petals of "Stern des Orients", which do not contain gossypetin 7-O-glucoside. Thus, it may be concluded that one and the same glucosyltransferase is responsible for the glucosylation of quercetin and gossypetin. This is supported by the fact that the enzyme characterization with both substrates revealed largely corresponding data. Actually, definite elucidation of this question can only be obtained by studies on substrate specificity with the purified enzyme.

The fact that gossypetin was definitely found to be the most attractive substrate points out, that glucosylation is most probably the last step in the biosynthesis of gossypetin 7-O-glucoside and that the additional hydroxyl group is introduced at an earlier stage. Thus, the absence of gossypetin 7-O-glucoside in the outer parts of the petals of "Stern des Orients" would only be due to the lack of the up to now unknown 8-hydroxylase. At which level the introduction of the additional hydroxyl group in position 8 takes place will be the subject of further investigations.

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