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Phytochemistry 67 (2006) 1080–1087
PHYTOCHEMISTRY
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An NADPH and FAD dependent enzyme catalyzes hydroxylation of flavonoids in position 8

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Received 2 December 2005; received in revised form 1 March 2006
Available online 6 May 2006

Abstract

Yellow flavonols contribute to flower pigmentation in Asteraceae. In contrast to common flavonols, they show additional hydroxyl groups in position 6 and/or 8 of the aromatic A-ring in addition to the basic 5,7-hydroxylation pattern. An enzyme introducing a hydroxyl group in position 8 of flavonols and flavones was demonstrated for the first time with enzyme preparations from petals of *Chrysanthemum segetum*. Flavanones, dihydroflavonols and glucosylated flavonols and flavones were not accepted as substrates. The enzyme was localized in the microsomal fraction and uses NADPH and FAD as cofactors. Experiments with carbon monoxide/blue light and with antibodies specific for cytochrome P450 reductase did not indicate the involvement of a classical cytochrome P450 dependent monooxygenase in the reaction. Thus, the flavonoid 8-hydroxylase represents a novel type of hydroxylating enzyme in the flavonoid pathway. Apart from flavonoid 8-hydroxylase activity, the presence of all enzymes involved in the formation of flavonoid 7-*O*-glucosides in *C. segetum* was demonstrated. The pathway leading to 8-hydroxyflavonoids in *C. segetum* has been derived from enzyme activities and substrate specificities observed.

Keywords: *Chrysanthemum segetum*; Asteraceae; Flavonoids; A-Ring hydroxylation; Flavonoid 8-hydroxylase; FAD; Higher hydroxylated flavonols; Gossypetin; 8-Hydroxyluteolin

1. Introduction

Flavonoids have a common, basic C6–C3–C6 skeleton structure consisting of two aromatic rings (A and B) and a heterocyclic ring (C) containing one oxygen atom (Fig. 1). The most common flavonoids show a basic 5,7,4′- or a 5,7,3′4′-hydroxylation pattern. Furthermore, most flavonoid classes possess hydroxyl groups in ring C. Yellow flavonols such as quercetagetin (6-hydroxyquercetin), gossypetin (8-hydroxyquercetin) and their derivatives owe their color to the presence of an extra hydroxyl group in position 6 or 8 of the aromatic A-ring (Fig. 1) (Harborne, 1967). Whereas members of common flavonols are wide-spread and may be found in nearly all plant families, the occurrence of such higher hydroxylated flavonols seems to be much more restricted, but their methylated and/or glucosylated derivatives occur in Asteraceae, Malvaceae and Leguminosae (Harborne, 1967). In flowers of Asteraceae, 6-hydroxyflavonols are part of the pigmentation pattern fulfilling important physiological functions as honey guides (Thompson et al., 1972; Halbwirth et al., 2000). In addition, the main pigments of cotton flowers are based on gossypetin, and gossypetin derivatives also contribute to yellow flower color in *Chrysanthemum segetum*, *Lotus corniculatus*, *Hibiscus tiliaecus* and *Primula vulgaris* (Geissman and Steelink, 1957; Harborne, 1967).

Abbreviations: CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; FHT, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F8H, flavonoid 8-hydroxylase; F6H, flavonol 6-hydroxylase; F7GT, Flavonoid 7-*O*-glucosyltransferase; FNS II, flavone synthase II.

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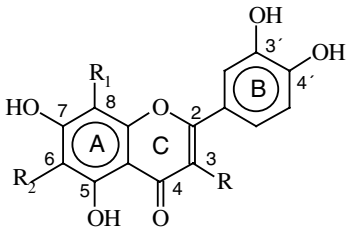
doi:10.1016/j.phytochem.2006.03.008



R=OH, R_1=H, R_2=H: 5,7,3′,4′-Tetrahydroxyflavonol (Quercetin)
R=OH, R_1=OH, R_2=H: 5,7,8,3′,4′-Pentahydroxyflavonol (8-Hydroxyquercetin)
R=OH, R_1=H, R_2=OH: 5,6,7,3′,4′-Pentahydroxyflavonol (Quercetagetin)
R=H, R_1=H, R_2=H: 5,7,3′,4′-Tetrahydroxyflavone (Luteolin)
R=H, R_1=OH, R_2=H: 5,7,8,3′,4′-Pentahydroxyflavone (8-Hydroxyluteolin)
R=H, R_1=H, R_2=OH: 5,6,7,3′,4′-Pentahydroxyflavone (6-Hydroxyluteolin)

Fig. 1. Chemical structures of flavonoids showing additional hydroxyl groups in ring A.

In the flavonoid pathway, hydroxyl groups in the A-, B- and C-rings are introduced at various biosynthetic stages. As a rule, the enzymes catalyzing these reactions are either 2-oxoglutarate-dependent dioxygenases such as flavanone 3-hydroxylase (FHT) or cytochrome P450 dependent monooxygenases such as flavonoid 3′-hydroxylase (F3′H), flavonoid 3′,5′-hydroxylase, and chalcone 3-hydroxylase (Forkmann and Heller, 1999; Wimmer et al., 1998). However, particular hydroxyl groups can be also obtained by reducing of oxo-groups (introduction of the hydroxyl group in position 4) or by the action of a bifunctional polyphenol oxidase (hydroxylation during aurone formation) (Forkmann and Heller, 1999; Strack and Schliemann, 2001).

During the past few years, hydroxylation in position 6 has been studied in more detail. Interestingly, two different enzyme systems were shown to catalyze this reaction in the flavonoid pathway. Anzellotti and Ibrahim (2000, 2004) were able to show a novel 2-oxoglutarate-dependent dioxygenase, flavonol 6-hydroxylase (F6H), to be responsible for the 6-hydroxylation of the partially methylated flavonols in *Chrysosplenium americanum* leaves. In contrast, we have previously shown another F6H of petals of *Tagetes* species, which could be classified as cytochrome P450 dependent monooxygenase (Halbwirth et al., 2004), and which is specifically involved in flower pigment formation. Furthermore, Latunde-Dada et al. (2001) reported a cytochrome P450 dependent flavonoid 6-hydroxylase from elicitor treated soybean, which seems to be specifically involved in the biosynthesis of 6-hydroxyisoflavones.

Hydroxylation in position 8 had not been studied before, but a flavonol 7-*O*-glucosyltransferase (F7GT) catalyzing the glucosylation of gossypetin in position 7 was already characterized in detail from petals of *C. segetum* (Stich et al., 1997). We report on the occurrence of a microsomal oxidoreductase dependent on NADPH and FAD in the petals of *C. segetum* catalyzing the 8-hydroxylation of flavonols and flavones with very high efficiency and high specificity.

2. Results and discussion

2.1. Introduction of the hydroxyl group in position 8

Incubation of [^{14}C]quercetin with crude enzyme preparations from petals of *C. segetum* in the presence of NADPH led to the formation of one [^{14}C]-labeled product (Fig. 2A), which was identified as gossypetin by TLC with the authentic reference substance in four different solvent systems. Furthermore, the identity of the [^{14}C]-labeled product was confirmed by HPLC co-chromatography with the authentic reference substance using a photodiode array detector coupled with a radioactivity detector. In the presence of 2-oxoglutarate, ascorbate and Fe^{2+}, no formation of gossypetin could be observed. The enzyme seems to be membrane-bound, since a more than 2-fold enrichment of F8H activity was obtained in the microsomal protein fraction compared to the crude extract, whereas only 10% of the activity remained in the supernatant after the final precipitation of the microsomal fraction (Table 1). No enzyme activity was observed, when preparations from leaves instead of petals were used. Therefore, all further enzymatic studies were performed with the microsomal fraction from petals. To investigate the potential involvement of oxygen in the reaction, an oxygen consuming system consisting of glucose, glucose oxidase and catalase was used according to Kochs and Grisebach (1987) (Table 2). Interestingly, exclusion of oxygen did not lead to a total loss of enzymatic activity as described for other hydroxylating enzymes (Wimmer et al., 1998; Halbwirth et al., 2004). However, the most striking observation during these investigations was the distinct enhancement of the enzyme activity (almost 3-fold activity compared to the control) in the presence of heat-inactivated catalase and glucose oxidase (Table 2). One possible explanation was the release of FAD from the active center of glucose oxidase after enzyme denaturation during boiling. Therefore, we studied the involvement of further potential cofactors in the reaction.

Addition of FAD up to a concentration of 0.01 mM led to a distinct increase in product formation (Fig. 3), but

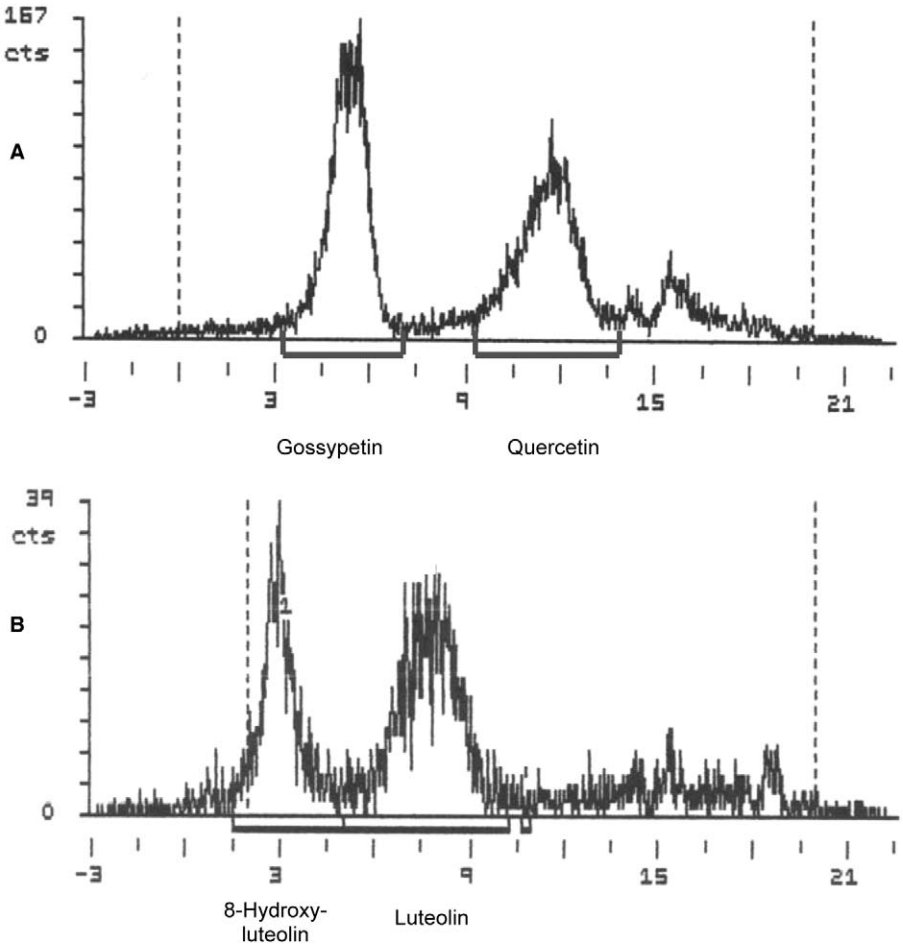


Fig. 2. Radioscan of TLC on cellulose from incubation of enzyme preparations from *C. segetum* in the presence of NADPH and FAD with (A) [^{14}C]quercetin as a substrate (solvent system 1) (B) [^{14}C]luteolin as a substrate (solvent system 2).

Table 1
Dependence on cofactors and subcellular localization of the flavonoid 8-hydroxylase

Enzyme source	Additions	Relative activity (%)
Crude extract	None	0
Crude extract	500 μM 2-oxoglutarate, 100 μM $FeSO_4$	0
Crude extract	480 μM NADPH	100
Supernatant	480 μM NADPH	10
Microsomal fraction	480 μM NADPH	220
Microsomal fraction	480 μM NADH	50
Microsomal fraction	480 μM NADPH, 0.1 μM FAD	498
Microsomal fraction	480 μM NADH, 0.1 μM FAD	51
Microsomal fraction	0.1 μM/100 mM FAD	0/0

All activities were expressed per kg of total protein and relative activities were calculated compared to the activity obtained with crude extract in the presence of 480 μM NADPH. 100% correspond to 1.4 μkat/kg protein. The effect of various potential cofactors was tested using microsomal preparations.

Table 2
Dependence of the flavonoid 8-hydroxylase on oxygen

Additions	Relative activity (%)
480 μM NADPH	100
50 mM glucose, 5 U glucose oxidase, 10 U catalase	48
50 mM glucose	101
50 mM glucose, 5U boiled glucose oxidase, 10 U boiled catalase	280
480 μM NADPH, 0.1 μM FMN	98
480 μM NADPH, 0.1 μM Haem	91

All activities were expressed per kg of total protein and relative activities were calculated compared to the activity of the microsomal fraction obtained in the presence of 480 μM NADPH. 100% correspond to 1.3 μkat/kg protein.

stimulated the reaction only in the presence of NADPH (Table 1). FMN and Haem (prosthetic group of catalase) were not able to affect the reaction in the same way (Table 2). Substitution of NADPH by NADH led to a pronounced loss of enzyme activity of approximately 75%. In the presence of NADH, however, addition of FAD did not result in enhanced enzyme activities (Table 1). Thus, FAD and NADPH were added as cofactors for all further studies. The subcellular localization in the microsomal fraction and the dependence on oxygen could also be confirmed in assays containing FAD and NADPH as cofactors (data not shown).

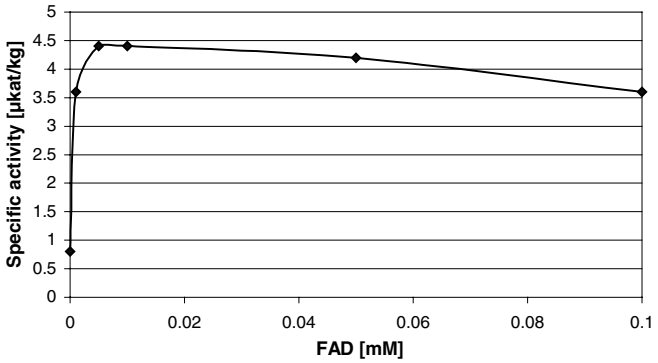


Fig. 3. Influence of FAD on flavonoid 8-hydroxylase activity.

Beside quercetin, the flavone luteolin was converted to a great extent to the corresponding 8-hydroxyluteolin (Fig. 2B). Thus, the F8H shows a broader substrate specificity than the F6H from *Tagetes* sp., which clearly preferred flavonols and hydroxylated flavones only to a very small extent (Halbwirth et al., 2000). When kaempferol was used as a substrate, no 8-hydroxykaempferol could be detected, but the formation of gossypetin was observed. In case of apigenin as substrate, only 8-hydroxyluteolin and small amounts of luteolin were formed. This may be easily explained by the high F3′H activity, which is also present in the microsomal preparations of *C. segetum* petals. No reaction products were detected, when quercetin 3-*O*-glucoside, quercetin 7-*O*-glucoside or luteolin 7-*O*-glucoside were incubated under the same conditions. Flavanones, dihydroflavonols and 6′-deoxychalcones were not accepted as substrates either.

The reaction showed an optimum at pH 7.0 for the hydroxylation of quercetin and at pH 6.75 for the hydroxylation of luteolin. Fifty percent of the maximum were still observed at pH 6.0 and 8.5, respectively. Thus, the reaction takes place within a rather broad pH range. A more detailed characterization was performed with quercetin as a substrate. Highest reaction rates were measured at 30 °C, where the formation of gossypetin was linear with time for up to 30 min and with protein concentration up to 2.5 µg protein in the standard assay. At 5 °C and 40 °C, the rate was approximately 50% of the maximum. Studies on temperature stability revealed that pre-incubation of the enzyme solution for 10 min at up to 30 °C had no influence on enzyme activity. Further increases in temperature led to a marked reduction of enzyme activity. When the microsomal fraction was frozen in liquid nitrogen and stored at −80 °C, no loss of activity could be observed over two years. The values for apparent K_m and V_{max} were 9 µM and 133 µkat/kg protein for quercetin ($V_{max}/K_m = 14.8$) and 10 µM and 72 µkat/kg for luteolin ($V_{max}/K_m = 7.2$), respectively. Since V_{max}/K_m provides an indication of substrate specificity, quercetin seems to be the preferred substrate for F8H. The F8H activity was studied during the development of buds and flowers (Fig. 4). The highest specific activities were measured in enzyme preparations from closed buds and buds just opening (stages 1–3), where the specific activity was 2.9 µkat/kg protein under standard conditions. However, relatively high activities could be still observed in open flowers (stages 4 and 5, Fig. 4).

The data obtained raised the question, whether F8H is a cytochrome P450 dependent monooxygenase as many other hydroxylating enzymes in the flavonoid pathway (Forkmann and Heller, 1999). These enzymes are mem-

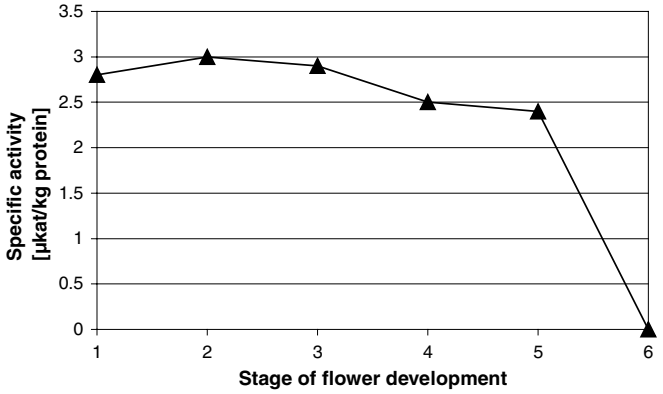


Fig. 4. Dependence of flavonoid 8-hydroxylase activity on the stage of flower development.

brane-bound haem proteins with a minimal catalytic system constituted from the cytochrome P450 and the membrane-bound flavoprotein NADPH-cytochrome P450-reductase (Bolwell et al., 1994). Subcellular localization in the microsomal fraction and NADPH dependence are typical for this enzyme class. The use of FAD is rather unusual, but could be explained if the NADPH-cytochrome P450-reductase from *C. segetum* easily looses FAD. However, in contrast to F8H, well-known cytochrome P450 dependent monooxygenases from the flavonoid pathway such as F3′H and FNS II were not activated by addition of FAD. Therefore, we investigated the effects of potential inhibitors on the enzymatic formation of gossypetin (Table 3). Addition of EDTA and cytochrome c did not influence the enzyme reaction, whereas *p*-hydroxymercuribenzoate, 2,2′-dipyridyl or *N*-ethyl maleimide were able to inhibit the reaction to a moderate extent. The cytochrome P450 specific inhibitors ancymidol and BAS 111 W had only a weak influence, whereas the cytochrome P450 specific inhibitors ketoconazole and tetcyclacis clearly reduced enzyme activity. However, the influences observed were rather moderate compared to other cytochrome P450 dependent monooxygenases (Stich et al., 1988; Halbwirth et al., 2004; Wimmer et al., 1998).

The most specific reactions for the identification of cytochrome P450 monooxygenases are the photoreversible inhibition of carbon monoxide and the addition of cytochrome P450 reductase specific antibodies (Benveniste et al., 1989; Nielsen and Møller, 1999; Halbwirth et al., 2004). The hydroxylation reaction was partially inhibited when the microsomal preparation was pre-incubated with carbon monoxide (CO) (Table 3). Pre-incubation with nitrogen did not have a comparable effect. However, the CO inhibition could not be reversed by exposition of the enzyme assay to blue light (Table 3). The addition of a crude fraction of cytochrome P450 reductase specific antibodies led to a moderate loss of F8H activity, whereas the cytochrome P450 dependent monooxygenase F3′H, which was also present in the same preparations, was drastically affected. A control assay with flavanone 3-hydroxylase (FHT) revealed no influence of the antibodies on the 2-oxoglutarate dependent dioxygenase (Table 4). These results clearly indicate that the enzyme does not belong to the classical cytochrome P450 dependent monooxygenases commonly found in the flavonoid pathway. However, the nature of the enzyme still remains unclear. There are many reports on FAD dependent monooxygenases (Wagner et al., 1990; Eppink et al., 2000; Luying and Sandvik, 2000). Some are even localized in the microsomal fraction (Tynes and Hodgson, 1984; Nagase et al., 1992), but most of them have been found in micro-organisms and they seem to be involved in catabolic processes rather than in the biosynthesis of secondary compounds. In contrast, the enzyme from *C. segetum* seems to be specifically involved in the 8-hydroxylation of flavonols and flavones.

The fact that the cytochrome P450 reductase specific antibodies (Benveniste et al., 1989) inhibited F3′H but not F8H, allowed us to study whether 4′-hydroxylated flavonoids are accepted as substrates by F8H. In the presence of the antibodies, the formation of 8-hydroxykaempferol from kaempferol and 8-hydroxyapigenin from apigenin was observed. Thus, a 3′,4′-hydroxylation pattern is not a precondition for the introduction of an additional hydroxyl group in position 8.

Table 3
Effect of CO and other potential inhibitors on the flavonoid 8-hydroxylase activity from *C. segetum*

Assay condition	Relative activity (%)
Control	100
Control with blue light	98
CO	76
CO with blue light	73
N_2	101
N_2 with blue light	99
EDTA (5 mM)	100
*p*OHMB (0.1 mM)	31
N-Ethyl maleimide (5 mM)	53
2,2′-Dipyridyl (1 mM)	67
Cytochrome *c* (0.5 mM)	100
Ancymidol (0.05 mM)	90
Ketoconazole (0.05 mM)	30
Tetcyclacis (0.05 mM)	58
BAS 111 W (0.05 mM)	87

Relative activities were calculated compared to a control. 100% correspond to 4 μkat/kg protein. The data represent an average of three independent experiments.

2.2. Biochemical pathway leading to gossypetin 7-*O*-glucoside in *C. segetum*

Besides F8H activity, the presence of all enzymes involved in the formation of flavonoid 7-*O*-glucosides could be demonstrated in preparations from *C. segetum* petals: Chalcone synthase/chalcone isomerase (CHS/CHI), flavone synthase II (FNS II), FHT, flavonol synthase (FLS), F3′H and flavonoid 7-*O*-glucosyltransferase (F7GT). Dihydroflavonol 4-reductase (DFR) activity could not be detected, thus providing an explanation for the absence of anthocyanins in the yellow cultivar Helios. The pathway leading to gossypetin 7-*O*-glucoside is shown in Fig. 5. Chalcone synthase (CHS) clearly preferred *p*-coumaroyl-Co A as a substrate. Activities were only two times higher than those observed with caffeoyl-CoA, but when *p*-coumaroyl-CoA and caffeoyl-CoA were present in the assay in equimolar concentrations, 90% naringenin (derived from *p*-coumaroyl-CoA) and 10% eriodictyol (derived from caffeoyl-CoA) were formed. F3′H showed very high activity converting naringenin, apigenin, dihydrokaempferol and kaempferol to the respective 3′,4′-hydroxylated products. Thus, the 3′,4′-hydroxylation pattern seems to be introduced at the flavonoid level by F3′H rather than by CHS using caffeoyl-CoA as a substrate. Since flavanones and dihydroflavonols were not hydroxylated in position 8, it is obvious that the formation

Table 4
Influence of cytochrome P450 reductase specific antibodies on selected enzymes of the flavonoid pathway

Enzyme tested	Type of enzyme	Relative activities (%) in the presence of 10 μg	
		Cyt. P450 red. specific antibodies	Boiled Cyt. P450 red. specific antibodies
F8H	NADPH, FAD dependent oxidoreductase	85	100
F3′H	Cytochrome P450 dependent monooxygenase	0	100
FHT	2-Oxoglutarate dependent dioxygenase	100	100

Enzyme activities were measured in the presence of 10 μg cytochrome P450 reductase specific antibodies. Relative activities were calculated compared to a control.
Abbreviations. F8H: Flavonoid 8-hydroxylase, F3′H: Flavonoid 3′-hydroxylase, FHT: Flavanone 3-hydroxylase, cyt.: cytochrome, red.: reductase.

of flavonols and flavones has to precede A-ring hydroxylation. However the hydroxyl group is introduced at the aglycone level, since flavone and flavonol glycosides are not accepted as substrates. This corresponds well with the substrate preference of F7GT for gossypetin (Stich et al., 1997) in *C. segetum*.

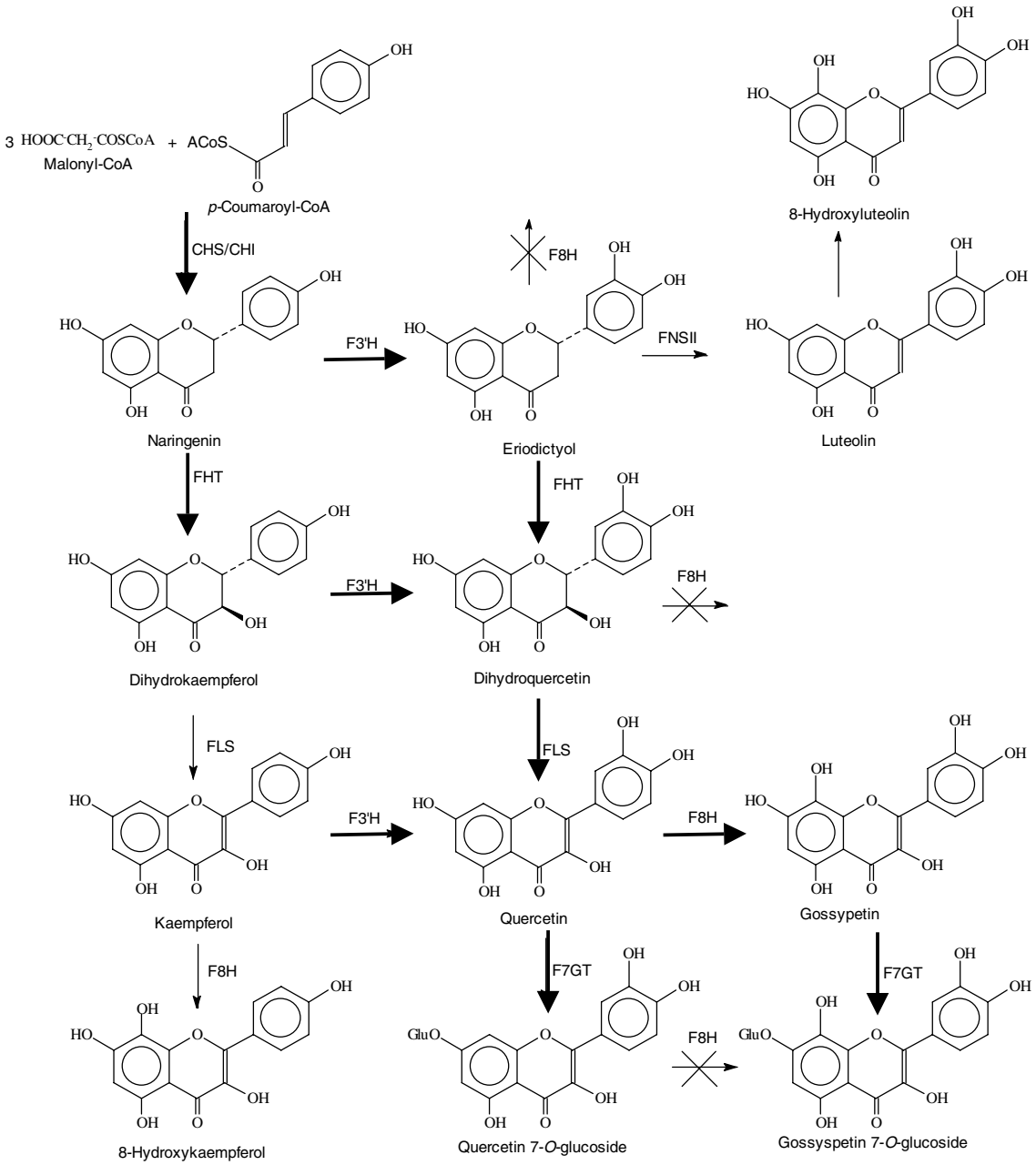


Fig. 5. Biochemical pathway leading to gossypetin 7-*O*-glucoside in *C. segetum*. (Bold arrows indicate putative main routes derived from enzyme activities and substrate specificities observed).

It may be assumed that 8-hydroxyluteolin is formed also in vivo in the *C. segetum* petals, since high FNS II activity was observed and luteolin represents an excellent substrate for the F8H. However, the presence of 8-hydroxyluteolin in *C. segetum* has not been reported so far although the flavonoids in this plant have been investigated in detail (Geissman and Steelink, 1957). Possible explanations could be the preference of F8H for quercetin and the competition of FHT and FNS II for common substrates. However, possible involvement of multi-enzyme complexes and substrate channelling should also be considered. Further work on this topic is in progress.

3. Experimental

3.1. General experimental procedures

TLC was performed on Merck pre-coated cellulose (without fluorescence indicator, 1.0571.001, Merck, Darmstadt, Germany). Radiolabeled substances were detected with a TLC Linear Analyzer (Berthold LB 2842, Wildbad, Germany). HPLC analysis was performed on a Perkin–Elmer Series 200 system equipped with a photodiode array detector coupled with a 500TR Flow Scintillation Analyzer for the detection of radiolabeled substances (Perkin–Elmer, Vienna, Austria).

3.2. Plant material

The investigations were performed on the commercial variety 'Helios' (Meisert Samenzucht, Hannover, Germany) of *C. segetum*. The plant material was cultivated in the municipal parks of Vienna and collected during the summer periods 1997–2000. *Dahlia variabilis* cv. Rubens was purchased from Wirth (Vienna, Austria).

3.3. Chemicals

UDP-D-[U-^{14}C]Glucose (12.0 GBq/mmol) and [2-^{14}C]malonyl-coenzyme A (2 GBq/mmol) were puchased from Amersham International (UK). [^{14}C]Naringenin, [^{14}C]eriodictyol, [^{14}C]kaempferol, [^{14}C]dihydrokaempferol, [^{14}C]dihydroquercetin, [^{14}C]quercetin and [^{14}C]quercetin 7-*O*-glucoside and [^{14}C]quercetin 3-*O*-glucoside were prepared as previously described (Halbwirth et al., 2004, 2006). [^{14}C]Luteolin was synthesized from [^{14}C]naringenin using microsomal preparations from *D. variabilis* cv. Rubens for flavone formation and introduction of a 3′-hydroxyl group (Stich et al., 1988). The cytochrome P450 reductase specific antiserum from Jerusalem artichoke was a gift from F. Durst and I. Benveniste (CNRS, Strasbourg, France). Quercetin, quercetagetin, kaempferol, apigenin, luteolin and eriodictyol were purchased from Extrasynthese (Genay, France), gossypetin from Apin Chemicals (Abingdon, UK). 8-Hydroxyluteolin came from W. Heller (GSF, Munich, Germany). NADPH, cytochrome *c*, glucose oxidase and catalase were obtained from Sigma (Vienna, Austria). Cytochrome P450 specific inhibitors were a gift from BASF (Limburgerhof, Germany).

3.4. Enzyme preparations and protein determination

Enzyme preparations from petals were obtained as described (Halbwirth et al., 2002), using 0.1 M KH_2PO_4/K_2HPO_4 (containing 0.4% ascorbate, pH 7.0). Microsomal preparations from *C. segetum* and *D. variabilis* were obtained according to Wimmer et al. (1998). The microsomal fraction was resuspended in buffer, shock frozen in liquid nitrogen and stored at −80 °C. Protein content was determined by a modified Lowry procedure (Sandermann and Strominger, 1972) using crystalline bovine serum albumin as a standard.

3.5. Enzyme characterization

All data represent an average of at least three independent experiments. Subcellular determination was carried out according to Halbwirth et al. (2000). Determination of the pH optimum was carried out as described for the standard assay, but using 0.2 M KH_2PO_4–K_2HPO_4 buffer (containing 0.4% sodium ascorbate) with pH values between 6.0 and 8.5. Exclusion of oxygen from the enzyme assay was carried out as described (Wimmer et al., 1998) using an oxygen consuming system consisting of glucose, glucose oxidase and catalase. Carbon monoxide inhibition assays were performed according to Nielsen and Møller (1999) with the exception that a gas mixture composed of carbon monoxide/oxygen (9:1, v/v) was used. Kinetic data were calculated from Lineweaver–Burk plots. Determination of the apparent Michaelis constant (K_m) and maximum reaction velocity (V_{max}) for quercetin were performed with fixed concentrations of 480 μM NADPH and 0.01 μM FAD.

3.6. Standard enzyme assay

The reaction mixture contained in a final volume of 100 μl: 2 μl microsomal preparation (2.5 μg protein), 88 μl 0.1 M KH_2PO_4/K_2HPO_4 (containing 0.4% ascorbate pH 7.0), 0.048 nmol [^{14}C]quercetin (108 Bq), 0.001 nmol FAD and 48 nmol NADPH (each dissolved in 5 μl H_2O). The reaction was started by addition of NADPH. After 15 min at 30 °C the reaction was stopped with 10 μl glacial acetic acid. The phenolic compounds were extracted twice with EtOAc (70 + 50 μl, respectively), the organic phases were applied to a precoated cellulose plate and chromatographed using solvent system 1. Radioactivity was detected and quantified by scanning the plates with a TLC-linear analyzer (Berthold, Wildbad, Germany).

3.7. Stages of flower development

Morphological criteria were used for subdividing the developmental process into six different stages. Stages 1 and 2 refer to closed buds of 5 and 7 mm length, respectively, stage 3 are buds just opening with a petal length 7 mm. Stages 4 and 5 represent open flowers 25 and 35 mm in diameter, respectively, and stage 6 wilting flowers.

3.8. Chromatography

Substrates and products were separated and identified by TLC on precoated cellulose using the following solvent system: (1) *n*-butanol/acetic acid/water (6:1:2, v/v/v); (2) chloroform/acetic acid/water (10:9:1, v/v/v); (3) *t*-butanol/acetic acid/water (3:1:1,v/v/v); (4) 30% acetic acid. Product identification was performed by HPLC analysis according to Vande Casteele et al. (1982) (R_t quercetin: 24.66 min; R_t gossypetin: 21.62 min; R_t luteolin: 25.74 min; R_t 8-hydroxyluteolin: 22.71 min).

Acknowledgements

These investigations were supported by a grant from the Austrian FWF (Project 13020). We are grateful to F. Durst and I. Benveniste for their gift of cytochrome P450 reductase specific antiserum. We would like to thank W. Rademacher for gifts of various cytochrome P450 specific inhibitors. Finally, we are indebted to E. Meggeneder for critically reading the manuscript.

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