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ENZYMATIC HYDROXYLATION OF 6′-DEOXYCHALCONES WITH PROTEIN PREPARATIONS FROM PETALS OF *DAHLIA VARIABILIS*

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Key Word Index—*Dahlia variabilis*; Compositae; biosynthesis; chalcone; 6′-deoxychalcone 3-hydroxylase (CH3H).

Abstract—Yellow colouration of *Dahlia variabilis* flowers is mainly based on isoliquiritigenin and butein 4′-malonylglucosides. Microsomal preparations from petals of the yellow strain 'Johann Nestroy' catalyse the enzymatic 3-hydroxylation of isoliquiritigenin to butein in the presence of NADPH. The reaction showed a pH optimum of 7.5, and was inhibited by *p*-hydroxymercuribenzoate and a number of cytochrome P450 specific inhibitors. These and further properties suggest that the 3-hydroxylation of isoliquiritigenin is mediated by a cytochrome P450-dependent monooxygenase. The apparent K_m value for isoliquiritigenin was 50 μM.

INTRODUCTION

Yellow colouration of *Dahlia* flowers is mainly due to the presence of 4′-malonylglucosides of the 6′-deoxychalcones isoliquiritigenin and butein (Fig. 1) [1–3] with usually larger amounts of the butein derivative. These chalcones co-occur with glucosides of the flavones apigenin and luteolin, and with anthocyanins, giving more or less yellow to orange coloured flowers. B-Ring hydroxylation of flavones and other flavonoids is due to the action of the well-known flavonoid 3′-hydroxylase (F3′H) [4, 5]. Up to now, however, it is an open question in which way the B-ring hydroxylation pattern of chalcones is established. This paper reports now on the occurrence of a microsomal NADPH-dependent monooxygenase which converts the 6′-deoxychalcone isoliquiritigenin to the respective 3-hydroxylated product, butein, with high efficiency.

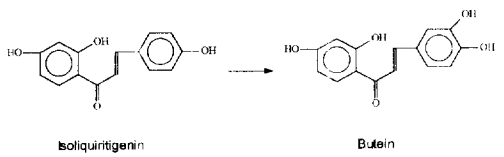

Fig. 1.

RESULTS AND DISCUSSION

Incubation of (^{14}C)-isoliquiritigenin with protein preparation from petals of *Dahlia variabilis* in the presence of NADPH led to the formation of one main product, identified as butein by co-chromatography with the authentic reference substance in four different solvent systems (Fig 1, Table 1). TLC on polyamide additionally revealed the formation of small amounts of a by-product, which was identified as the corresponding aurone sulfuretin. According to Harborne [3] aurones are naturally not present in *Dahlia* flowers, but are formed as artefacts by oxidation of chalcones during extraction. This may also apply to the sulfuretin traces found in our experiments. Using (^{14}C)-isoliquiritigenin 4′-glucoside as a substrate for hydroxylation, no reaction was observed. Furthermore, it has been reported that butein is a better substrate for the 6′-deoxychalcone 4′-glucosyltransferase from *Dahlia* compared to iso-

Table 1. R_fs (× 100) of substrate and product on TLC plates

Compound	Solvent systems* 1	2	3	4
Isoliquiritigenin	24	84	77	75
Butein	15	67	44	57
Sulfuretin	23	72	44	37

* For solvent key, see Experimental.

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Table 2. Subcellular localization and cofactor requirement of the 6′-deoxychalcone 3-hydroxylase in flowers of *Dahlia variabilis*

Enzyme source	Cofactor added	Enzyme activity (Bq in butein formed from 167 Bq isoliquiritigenin)*
Crude extract	none	0
	NADPH	39
Supernatant of microsomal pellet	NADPH	11
Microsomal pellet	none	0
	NADPH	56
	NADH	0

* Product formed in 15 min with 40 μg protein.

liquiritigenin [6]. These observations strongly indicate that hydroxylation of the chalcone precedes the glucosylation reaction.

The subcellular localization of the enzyme was investigated by preparing a microsomal protein fraction. Enzyme activity was increased by a factor of about 1.4 compared to the crude extract (Table 2). The enrichment was not as prominent as reported for most other microsomal enzymes of the flavonoid biosynthetic pathway, where factors of 3 or more were frequently found [7–9]. The reaction was strictly dependent on NADPH. No activity was detected when NADPH was substituted by NADH (Table 2).

The enzymatic formation of butein was studied in detail using the microsomal protein. The reaction showed an optimum at pH 7.5. Highest reaction rates were measured at 30°, where the formation of butein was linear with time up to 15 min and with protein concentration up to 15 μg protein in the standard assay. At 0°, the rate was about 15% of the maximum. Studies on temperature stability revealed that preincubation of the enzyme solution for 10 min at temperatures up to 30° had no influence on enzyme activity. Further increasing the temperature led to a reduction, and preincubation for 10 min at 60° to complete loss of the activity. When the microsomal fraction was frozen in liquid nitrogen and stored at −80° for several weeks, the enzyme activity remained unchanged. The values for K_m and V_{max} using isoliquiritigenin as the variable substrate were 50 μM and 41 μkat kg^{-1} protein as calculated from the Lineweaver Burk plot.

The course of the chalcone hydroxylating activity was studied during flower development. Under standard conditions, the activity was 4.9 μkat kg^{-1} protein in the first three developmental stages defined on the basis of petal size, and decreased gradually in later stages. Chalcone synthase catalysing the formation of isoliquiritigenin in co-action with chalcone polyketide reductase showed a similar course, as did 6′-deoxychalcone 4′-glucosyltransferase (data not shown).

The effect of some potential inhibitors on the enzymatic formation of butein from isoliquiritigenin is shown in Table 3. The presence of KCN, EDTA and *N*-ethylmaleimide led to weak inhibition, as did diethyldithiocarbamate (DDC) and diethylpyrocarbonate (DPC). However, *p*-hydroxymercuribenzoate strongly inhibited the reaction. Experiments were performed to specifically investigate the involvement of cytochrome P450. $NADP^+$, acting as a competitive inhibitor for NADPH in the cytochrome P450 reductase reaction [10] affected the reaction clearly, the degree of inhibition depending on the ratio of NADPH/$NADP^+$ as expected (Table 4). Additional studies were performed with a number of different cytochrome P450-specific inhibitors (Table 5). Ancymidol had no influence on the hydroxylation of isoliquiritigenin, whereas ketoconazole and tetcyclacis inhibited the reaction in a concentration-dependent manner. The triazole derivatives BAS 110..W, BAS 111..W and LAB 150978 inhibited to a lower extent.

Table 3. Effect of general inhibitors on the transformation of isoliquiritigenin to butein

Addition	Concentration [mM]	Relative Activity [%]
none		100
DDC	1	62
	5	30
DPC	1	62
	5	24
KCN	1	91
	5	91
EDTA	1	78
	5	74
N-Ethylmaleimide	1	92
p-Hydroxymercuribenzoate	0.1	57

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

Table 4. Effect of NADPH and $NADP^+$ on the enzymatic hydroxylation of isoliquiritigenin

NADPH [mM]	$NADP^+$ [mM]	$NADPH/NADP^+$	Relative Activity [%]
1.40	0		100
1.40	1.40	1.00	71
0.70	0.70	1.00	69
1.40	2.80	0.50	58
0.70	1.40	0.50	59
0.70	2.80	0.25	49
0.35	1.40	0.25	48

Differences in sensitivity of cytochrome P450-dependent enzymes towards a number of specific inhibitors has variously been reported [11–14]. Addition of cytochrome c, which interferes with the electron transport from the NADPH-cytochrome P450 reductase to the terminal oxidase [15], strongly inhibited the formation of butein (Table 5). Inhibition of the reaction by the oxygen-consuming glucose-6-phosphate oxidase system indicated the oxygen dependence of the hydroxylation. From these observations, it can be concluded that the enzyme belongs to the group of the cytochrome P450 mixed-function monooxygenases.

Flavonoid 3′-hydroxylase (F3′H), which has earlier been demonstrated in microsomal preparations from *Dahlia* flowers, is also a cytochrome P450-dependent monooxygenase catalysing B-ring hydroxylation of naringenin (flavanone), dihydrokaempferol (dihydroflavonol), apigenin (flavone) and kaempferol (flavonol), respectively, in position 3′ [11]. The question remained, whether chalcone 3-hydroxylation is catalysed by a novel enzyme activity or by the known F3′H. All attempts to separate the chalcone hydroxylating activity from F3′H activity by biochemical means including typical cytochrome P450 inhibitors failed so far. Moreover, genetic control has been described neither for F3′H nor for chalcone hydroxylation in *Dahlia*, which would have allowed the differentiation of the two activities using enzyme extracts from respective mutants. Therefore, we studied the substrate specificity of F3′H from plant species, e.g. *Matthiola incana* and *Dianthus caryophyllus*, that do not naturally form the 6′-deoxychalcones, for comparison. Genetic control of F3′H activity has unequivocally been demonstrated in these plants, and the enzyme has been thoroughly analysed [7, 16]. We have found that naringenin and dihydrokaempferol were hydroxylated in position 3′, as described, while isoliquiritigenin was not used as a substrate. This result indicates that the isoliquiritigenin-hydroxylating enzyme in *Dahlia* flower extracts might be separate from F3′H or, at least, an enzyme with a definitely different substrate specificity than F3′H from other plant species. The enzyme was therefore tentatively named chalcone 3-hydroxylase (CH3H). More detailed work will be required for final differentiation between the two possibilities.

Table 5. Effect of specific inhibitors on the transformation of isoliquiritigenin to butein

Addition	Concentration [μM]	Relative Activity [%]
Ancymidol	5	100
	50	100
Ketoconazole	5	84
	50	28
Tetcyclacis	5	65
	50	33
BAS 110..W	5	98
	50	87
BAS 111..W	5	86
	50	56
LAB 150978	5	99
	50	75
Cytochrome c	8	51
	16	45

EXPERIMENTAL

Plant material. The investigations were performed with petals of *Dahlia variabilis* cv. 'Johann Nestroy' (Dr Gärlnerei Wirth, Vienna, Austria). The plant material was collected in September and October 1995.

Chemicals. (^{14}C)-Isoliquiritigenin was synthesized according to Nabaei-Bidhendi and Bannerjee [17], using (^{14}C)-4-hydroxybenzaldehyde (7.6 mCi $mmol^{-1}$; Sigma). (^{14}C)-Naringenin was prepd as described [18, 19] using partially purified chalcone synthase and chalcone isomerase from parsley suspension cell cultures. Butein, sulfuretin, naringenin, eriodictyol, apigenin and luteolin were obtained from Roth (Karlsruhe, Germany). Isoliquiritigenin was from our laboratory collection. NADPH, cytochrome c, glucose oxidase, catalase and superoxide dismutase were purchased from Sigma. Cytochrome P450-specific inhibitors were a gift from BASF (Ludwigshafen, Germany).

Buffer solutions. Unless otherwise stated, the following buffer was used: 0.1 M KH_2PO_4–K_2HPO_4 (containing 0.4% Na-ascorbate), pH 7.50.

Enzyme preparation and protein determination. All steps were performed at 4°. Protein extraction and prepn of the microsomal frs by Mg^{2+} pptn from fresh flower tissue (stage 1–3) was performed as described in [20]. The microsomes were resuspended in buffer, shock frozen in liquid N_2 and stored at −80°. Protein was determined by a modified Lowry procedure [21], using crystalline BSA as standard.

Standard enzyme assays. Chalcone 3-hydroxylase (CH3H): The reaction mixt. (final vol. 100 μl) contained: 15 μl microsomal prepn, 80 μl buffer, 0.592 nmol (^{14}C)-isoliquiritigenin (133 Bq) and 140 nmol NADPH (in 5 μl H_2O). The reaction was started by the addition of NADPH. After 15 min at 30° the reaction was stopped with 10 μl HOAc. The phenolic compounds were extracted ×2 with EtOAc (100 + 50 μl), the extracts applied to a precoated cellulose plate (Schleicher and Schüll, Dassel, Germany) and chromatographed using solvent system 3. Radioactivity was detected and quantified by scanning the plates with TLC-linear analyser (Berthold, Wildbad, Germany).

Flavonoid 3′-hydroxylase (F3′H) assays were performed according to Stich *et al.* [11] and 6′-deoxychalcone 4′-glucosyltransferase assays as described earlier [6].

pH-Optimum determination. 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 buffers (containing 0.4% Na-ascorbate) with pH values between 6.75 and 8.0.

Oxygen dependence. Exclusion of O_2 from enzyme assay was carried out as described by Kochs and Grisebach [12].

Enzyme kinetics. Kinetic data were obtained from Lineweaver-Burk plots. Determination of the apparent Michaelis constant (K_m) and maximal reaction velocity (V_{max}) for isoliquiritigenin was performed using 1.4 mM NADPH. At the substrate concns chosen, no inhibition was observed.

Chromatography. Substrates and products were sepd and identified by TLC on precoated cellulose plates (Schleicher and Schüll, Dassel, Germany) using the following solvent systems: (1) 30% HOAc; (2) HOAc–conc. HCl–H_2O (30:3:10); (3) $CHCl_3$–HOAc–H_2O (10:9:1); and on precoated polyamide plates (Schleicher and Schüll, Dassel, Germany): (4) 2-butanone–MeOH (3:1).

Stages of flower development. The size of petals was used to define the various developmental stages. Stages 1 to 4 refer to petals with 10, 20, 30 and 40 mm length, respectively, stage 5 represents wilting petals.

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