Hydroxylation of the B-Ring of Flavonoids in the 3′- and 5′-Position with Enzyme Extracts from Flowers of *Verbena hybrida*

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Enzyme preparations from flowers of *Verbena hybrida* do not only catalyse hydroxylation of the B-ring of flavanones and dihydroflavonols in the 3′-position but also in the 5′-position. Enzyme activity for 3′,5′-hydroxylation was found to be localized in the microsomal fraction and required NADPH as cofactor. Evidence is provided that the formation of the 3′,4′,5′-hydroxylated flavanone (5,7,3′,4′,5′-pentahydroxyflavanone) and dihydroflavonol (dihydromyricetin), respectively, proceeds *via* the corresponding 3′,4′-hydroxylated compounds eriodictyol and dihydroquercetin, respectively, which are most probably formed by action of the same enzyme. Enzyme activity for 3′,5′-hydroxylation was found to be strictly correlated with the prescence of 3′,4′,5′-hydroxylated flavonoid compounds in the flowers.

Introduction

Recently, in flower extracts of *Matthiola incana* and *Antirrhinum majus* an NADPH-dependent microsomal 3′-hydroxylase activity could be demonstrated catalysing hydroxylation of naringenin (**1**) (Fig. 1) and dihydrokaempferol (**4**) in 3′-position to eriodictyol (**2**) and dihydroquercetin (**5**), respectively [1, 2]. In both plants a strict correlation between 3′-hydroxylase activity and the gene controlling the introduction of the hydroxy group in 3′-position of the B-ring of flavonoids was found. Thus, in *M. incana* and *A. majus* the 3′-hydroxy group is clearly introduced by hydroxylation of the flavonoid skeleton and not by incorporation of caffeic acid during synthesis of the flavonoid skeleton.

For further investigations on the determination of B-ring hydroxylation pattern of flavonoids we have now used flowers of pelargonidin- and delphinidin-producing strains of *Verbena hybrida.* Earlier chemogenetic studies have shown that in this plant the hydroxylation of the B-ring of anthocyanins in both 3′- and 5′-position is controlled by one gene [3, 4]. We now report on the first demonstration of an NADPH-dependent microsomal enzyme activity in flower extracts of *V. hybrida* which catalyses hydroxylation of naringenin and dihydrokaempferol in the 3′- and 5′-position and of eriodictyol and dihydroquercetin in the 5′-position. Enzyme activity was detected only in flower extracts of the delphinidin-producing strains.

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Material and Methods

Plant material

Because the genetically defined lines of *Verbena hybrida* originally described by Beale [3, 4] were not available, corresponding colour types were selected from commercial strains. The following strains were used: Blaze (scarlet), Flammenspiel (pink), Amethyst (blue), Sternenlicht (purple) and Azurella (blue). The plants were cultivated in a green house and during the summer month in the experimental garden of our institute.

The strains Blaze and Flammenspiel contain mainly pelargonidin and other 4′-hydroxylated flavonoid compounds whereas in the blue and purple flowering strains mainly 3′,4′,5′-hydroxylated flavonoids including delphinidin are present. In addition, in all strains investigated some flavonoid compounds with 3′,4′-hydroxylation pattern of the B-ring including cyanidin occur (Forkmann, unpublished).

Analytical methods

For the identification of the reaction products radioactive zones were scraped off from cellulose plates, eluted with methanol and evaporated to dryness under nitrogen. The residues were redis-

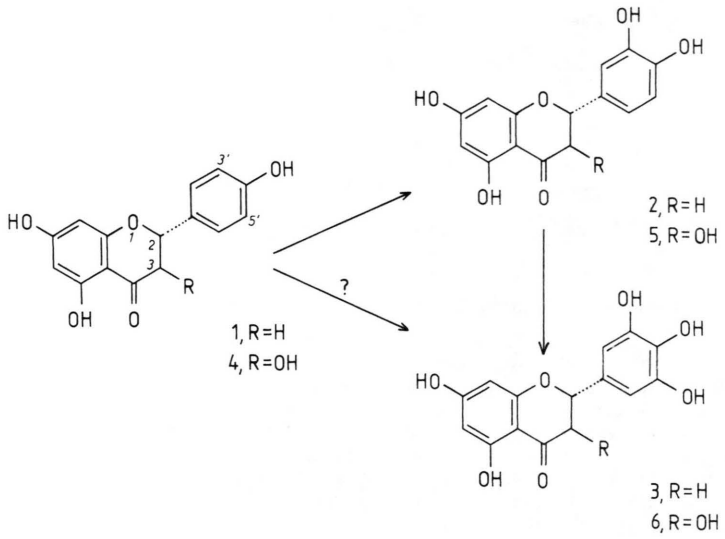

Fig. 1. Structural formulas of substrates and products. Naringenin (**1**); eriodictyol (**2**); 5,7,3′,4′,5′-pentahydroxyflavanone (**3**); dihydrokaempferol (**4**); dihydroquercetin (**5**); dihydromyricetin (**6**).

solved in methanol and co-chromatographed with authentic flavonoids in 3 different solvent systems. Eriodictyol and 5,7,3′,4′,5′-pentahydroxyflavanone were also identified by enzymatic 3-hydroxylation to the respective dihydroflavonols using enzyme preparations from flowers of *Matthiola incana* [1]. Dihydroquercetin and dihydromyricetin were further identified by oxidation with sodium metabisulfite to the respective flavonols [5]. The flavonols formed were identified on cellulose plates with solvent system 2 and 3 in succession.

Thin-layer chromatography of substrates and reaction products was performed on precoated cellulose plates (Schleicher & Schüll, Germany) with the following solvent systems: (1) chloroform/acetic acid/water (10:9:1), v/v/v; (2) 30% acetic acid; (3) acetic acid/HCl/water (30:3:10), v/v/v.

Flavonoids were detected on chromatograms under u.v.-light and after fuming with ammonia. Dihydroflavonols were also detected by the Zinc-HCl test [6] and flavanones by reduction with borohydride and subsequent exposure to HCl fumes [7].

Protein was determinated according to Bradford [8].

Chemicals and synthesis of substrates

Naringenin, dihydroquercetin, myricetin and quercetin were obtained from Roth (Karlsruhe, Germany). Eriodictyol, dihydrokaempferol and dihydromyricetin were from our labor collection. 5,7,3′,4′,5′-pentahydroxyflavanone was isolated from yellow flowering genotypes of *Antirrhinum majus* after treatment of the flower extracts with β-glucosidase (Forkmann, unpublished).

[2-^{14}C]Malonyl-CoA (60 Ci/mol) was obtained from Amersham Buchler and diluted to 26 Ci/mol with unlabelled material from Sigma. [4a,6,8-^{14}C]-Naringenin and [4a,6,8-^{14}C]eriodictyol (both 78 Ci/mol) were prepared enzymatically with crude chalcone synthase which includes chalcone isomerase [1, 9]. [4a,6,8-^{14}C]Dihydrokaempferol and [4a,6,8-^{14}C]-dihydroquercetin were prepared by incubation of labelled naringenin or eriodictyol, respectively, with crude flower extract of *Matthiola incana* [1].

Preparation of crude extract and microsomal fraction

5 g of the coloured parts of the flowers were homogenized at 4 °C in a prechilled mortar together with 2.5 g Dowex 1 × 2, 2.5 g quartz sand and 15 ml 0.1 M potassium phosphate buffer (pH 7.5) containing 28 mM 2-mercaptoethanol. After centrifugation for 20 min at 12000 × *g*, the clear supernatant served as curde extract and for preparation of the microsomal fraction.

Normally, the microsomal fraction was prepared by Mg^{2+}-precipitation [10]. The crude extract was mixed with 1 M $MgCl_2$-solution up to a final Mg^{2+}-concentration of 30 mM. The mixture was kept in an ice bath for 10 min and was than centrifuged for 20 min at 17000 × *g*. The microsomal pellet was suspended in 1/20 of the volume of the crude extract and homogenized in a glass homogenizer. In

addition, the microsomal fraction was prepared by ultracentrifugation of the crude extract for 75 min at 90000 × *g*.

Enzyme assay

The assay system contained in 200 µl total volume 20 µmol potassium phosphate buffer (pH 7.5), 0.4–2.5 µmol 2-mercaptoethanol, 0.1–0.3 nmol radioactive substrate (naringenin, eriodictyol, dihydrokaempferol or dihydroquercetin, respectively), 0.1 µmol NADPH and 20–60 µg protein (microsomal pellet or crude extract).

Incubation was carried out for 10–30 min at 30 °C and was terminated by addition of 20 µl methanol containing a mixture of authentic flavonoids. The phenolics were extracted twice with ethyl acetate (100 µl + 50 µl) and chromatographed on cellulose plates in solvent system 1. Radioactivity was localized by scanning the plates. The radioactive zones were scraped off and counted in Unisolve 1 in a scintillation counter.

Determination of the pH optimum

The enzyme assays were carried out in mixtures of 180 µl potassium phosphate buffer (between pH 6.5 and 8.0) and 20 µl of the microsomal fraction with dihydrokaempferol as substrate.

Results

After incubation of [^{14}C]naringenin (**1**) with enzyme preparations from delphinidin-producing flowers of *V. hybrida* in the presence of NADPH a range of radioactive reaction products were observed on radiochromatograms of the reaction mixture in solvent system 1. One product (R_f 0.55) was identified as eriodictyol (**2**) by co-chromatography with authentic **2** on cellulose plates in 3 different solvent systems (Table I) and by hydroxylation of the product in the 3-position to dihydroquercetin (**5**) with an enzyme extract from flowers of *M. incana* [1]. The other reaction products except for one were identified as flavones and dihydroflavonols by the methods described elsewhere (Stotz *et al.*, in preparation).

The remaining reaction product (R_f 0.25) was clearly localized behind luteolin and dihydroquercetin in solvent system 1 and separated also in other solvent systems from these compounds. A comparison of its chromatographic behaviour with that of flavanones and dihydroflavonols with different B-ring substitution pattern suggested that this product most probably is 5,7,3′,4′,5′-pentahydroxyflavanone (**3**) (Table I). This assumption could be confirmed by cochromatography with authentic **3** on cellulose plates in three solvent systems (Table I). Furthermore, enzymic 3-hydroxylation of this product using flower extracts of *M. incana* [1] yielded dihydromyricetin (**6**) which was identified as described below.

Table I. R_f-values (× 100) of substrates and products on cellulose TLC plates.

Compound	Solvent systems		
	1	2	3
Naringenin	83	61	91
Eriodictyol	60	56	81
5,7,3′,4′,5′-pentahydroxyflavanone	25	48	74
Dihydrokaempferol	64	70	86
Dihydroquercetin	35	67	79
Dihydromyricetin	13	62	71
Quercetin	–	08	41
Myricetin	–	04	26

Solvent systems see: Materials and Methods.

When [^{14}C]dihydrokeampferol (**4**) was used as substrate instead of naringenin the radiochromatogram of the reaction mixture showed two radioactive products (Fig. 2). These products were identified as dihydroquercetin (**5**) and dihydromyricetin (**6**), respectively, by co-chromatography with authentic samples (Table I) and by oxidation of **5** and

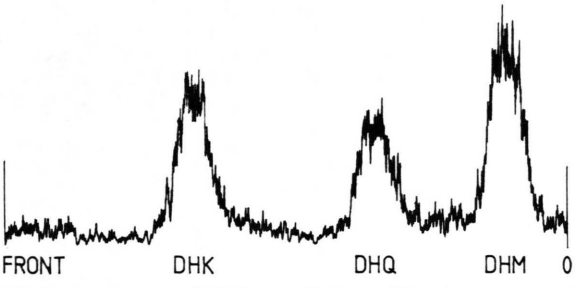

Fig. 2. Radioscan of TLC on cellulose with solvent system 1 from incubation of [^{14}C]dihydrokaempferol in the presence of NADPH with enzyme preparation from flowers of delphinidin-producing strains of *V. hybrida*. Dihydrokaempferol (DHK); dihydroquercetin (DHQ); dihydromyricetin (DHM).

6 with sodium metabisulfite [5] to the respective flavonols quercetin and myricetin.

With both naringenin and dihydrokaempferol as substrate, in no case the formation of 5,7,3′,4′,5′-pentahydroxyflavanone or dihydromyricetin alone was observed. The formation of these 3′,4′,5′-hydroxylated compounds was always accompanied by the formation of eriodictyol or dihydroquercetin, respectively, which are only hydroxylated in the 3′,4′-position. Both eriodictyol and dihydroquercetin were found to be substrates for hydroxylation in the 5′-position to 5,7,3′,4′,5′-pentahydroxyflavanone and dihydromyricetin, respectively.

After preparation of the microsomal fraction by Mg^{2+}-precipitation [10] or by ultracentrifugation the activity for 3′- and 5′-hydroxylation was present in the microsomal pellet (Table II). Hydroxylation in the 3′- and 5′-position was strictly dependent on NADPH. Substitution of NADPH by NADH strongly reduced the hydroxylation reactions (Table II). Besides the 3′,5′-hydroxylation activity, enzyme activity for flavone formation from flavanones was also found to be located in the microsomal fraction and required NADPH as cofactors (Stotz, unpublished). All efforts to exclude flavone formation by biochemical methods have so far failed. Therefore, at the further characterisation of the hydroxylation reaction in the 3′- and 5′-position dihydrokaempferol instead of naringenin was used as substrate. Highest conversion of dihydrokaempferol to dihydromyricetin was found between pH 7.0 and 7.5. The reaction was linear with protein concentration up to 40 µg protein. Linearity with time was only observed up to 10 min, a result which is obviously due to the temperature sensitivity of the microsomal fraction [1, 11]. Addition of EDTA, KCN, diethyldithiocarbamate, diethylpyrocarbonate and *p*-chloromercuribenzoate to the enzyme assay had no appreciable effect on the hydroxylation reaction. These reagents were found to be inhibitors of flavonoid 3′-hydroxylase and/or flavanone 3-hydroxylase in *M. incana* and *A. majus* [1, 2].

Tests with enzyme preparations from strains with different flower colour revealed that enzyme activity for 3′,5′-hydroxylation is only present in flower extracts of the delphinidin-producing strains (Amethyst, Sternenlicht, Azurella). In enzyme preparations from flowers of the scarlet or pink strain (Blaze or Flammenspiel) which contain mainly pelargonidin and other 4′-hydroxylated flavonoid compounds neither enzyme activiy for 3′-hydroxylation nor for 5′-hydroxylation was found.

Besides in *V. hybrida* the NADPH-dependent microsomal enzyme activity for hydroxylation in the 3′- and 5′-position could also be demonstrated in the flower extracts of *Petunia hybrida* and *Lathyrus odoratus*. As in *V. hybrida* enzyme activity for 3′- and 5′-hydroxylation of flavanones and dihydroflavonols was only found to be present in flower extracts of these strains which contain 3′,4′,5′-hydroxylated flavonoid compounds or methylated derivatives of them in the flowers (Stotz and Forkmann, unpublished).

Discussion

The determination of the substitution pattern of the B-ring of flavonoids belonged for a long time to

Table II. Subcellular localisation and cofactor requirement of 3′,5′-hydroxylation activity in the flowers of *Verbena hybrida*.

Enzyme source	Cofactor added	Substrate: naringenin cpm in 5,7,3′,4′,5′-pentahydroxyflavanone [a]	Substrate: dihydrokaempferol cpm in dihydromyricetin [a]
Crude extract	none	0	0
	NADPH	570	816
Supernatant of microsomal pellet	none	0	0
	NADPH	70 [b]	728 [c]
Microsomal pellet	none	0	0
	NADPH	1728 [b]	2712 [c]
	NADH	169 [b]	281 [c]

[a] Product formed with 50 µg protein.
[b] Microsomal pellet prepared by ultracentrifugation.
[c] Microsomal pellet prepared by Mg^{2+}-precipitation.

the unsolved questions in flavonoid biosynthesis [14]. In the last years, however, considerable progress has been made in elucidating this problem by the use of flowers of genetically defined plants as enzyme source. Thus, chemogenetic and enzymatic investigations on defined genotypes of *M. incana* and *A. majus* proved that the 3′-hydroxy group of the B-ring of flavonoids is clearly introduced at the flavanone and dihydroflavonol stage by action of the enzyme flavonoid 3′-hydroxylase [1, 2, 12, 13]. Recently, this enzyme was also found to be present in enzyme preparations from irradiated cell suspension cultures of parsley [9].

Using flower extracts of *Verbena hybrida* we could now demonstrate not only enzymatic hydroxylation of the B-ring of flavonoids in the 3′-position but also for the first time in the 5′-postition. Besides flavanones dihydroflavonols were found to be suitable substrates for both hydroxylation reactions. Furthermore, enzyme activity for 3′,5′-hydroxylation was strictly correlated with the presence of 3′,4′,5′-hydroxylated flavonoid compounds in the flowers. Thus, enzyme activity could be demonstrated in the flower extracts of the delphinidin-producing strains but not in the flower extracts of strains which lack 3′,4′,5′-hydroxylated flavonoid compounds. The absence of 3′,4′,5′-hydroxylated flavonoids in the latter strains is therefore due to the complete lack of the enzyme activity for 3′,5′-hydroxylation. These results show that the formation of 3′,4′,5′-hydroxylated flavonoid compounds in *V. hybrida* is obviously not achieved by incorporation of 3,4,5-trihydroxycinnamic acid into the flavonoid skeleton, but is due to hydroxylation of flavanones and dihydroflavonols in the 3′- and 5′-position by action of (a) specific hydroxylase(s).

Hydroxylation of naringenin and dihydrokaempferol in the 3′,5′-position was accompanied in each case by hydroxylation in the 3′-postition alone and the 3′,4′-hydroxylated flavonoid compounds eriodictyol and dihydroquercetin were found to be further hydroxylated in the 5′-position by the enzyme preparations. It can be assumed therefore that the formation of the 3′,4′,5′-hydroxylated compounds from naringenin and dihydrokaempferol, respectively, proceeds via the corresponding 3′,4′-hydroxylated compounds.

No separate enzyme activity for 3′-hydroxylation could be observed in the flower extracts of *V. hybrida* investigated, but enzyme extracts which lack activity for 3′,5′-hydroxylation also lack activity for 3′-hydroxylation. Furthermore, both hydroxylation reactions do not only agree with regard to subcellular localisation and cofactor requirements but they also behave similar towards several enzyme inhibitors. These results suggest that the hydroxylation in the 3′-position and the hydroxylation in the 3′,5′-position is most probably catalysed by the same enzyme. This assumption is also supported by the results of earlier chemogenetic studies which showed that in *V. hybrida* the hydroxylation of the B-ring of anthocyanins in both the 3′- and the 5′-position is controlled by only one gene [3, 4].

Future work on genetically defined lines of *V. hybrida* and defined genotype of other plants will be concerned with the further characterisation of the hydroxylation reactions.

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