Anthocyanin Biosynthesis in Flowers of *Matthiola incana* Flavanone 3- and Flavonoid 3′-Hydroxylases

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Enzyme preparations from flowers of defined genotypes of *Matthiola incana* contain two different hydroxylases for hydroxylation of naringenin in the 3- and 3′-position, respectively. The 3-hydroxylase is a soluble enzyme and requires as cofactors 2-oxoglutarate, Fe^{2+} and ascorbate. Besides naringenin eriodictyol is a substrate for the 3-hydroxylase. The 3′-hydroxylase is localized in the microsomal fraction and requires NADPH as cofactor. Naringenin and dihydrokaempferol but not 4-coumarate or 4-coumaroyl-CoA are substrates for this enzyme. 3′-Hydroxylase activity is present only in genetic lines of *M. incana* with the wild-type allele b^+.

Introduction

Radioactive tracer work and experiments with flower mutants of several plants have shown that dihydroflavonols (3-hydroxyflavanones) are intermediates in anthocyanin biosynthesis [1]. These findings have been corroborated by results of inhibition experiments [2]. Hydroxylation of the flavanone naringenin (**1**) (Fig. 1) in the 3-position to dihydrokaempferol (**3**) and in the 3′-position to eriodictyol (**2**) as well as of dihydrokaempferol to dihydroquercetin (**4**) was found with permeabilized cells and with a microsomal fraction from *Haplopappus gracilis* cell cultures [3]. Enzyme activity of these preparations was variable and the results did not give an answer to the question whether hydroxylation in the 3- and 3′-position is catalyzed by separate enzymes.

For further investigations on these hydroxylation reactions we have now used flowers from defined genotypes of *Matthiola incana.* Analytical investigations and supplementation experiments had shown that the locus b is responsible for hydroxylation of the B ring of flavonoids in the 3′-position [4, 5]. It was further concluded that introduction of the 3′-hydroxyl group probably occurs at the dihydroflavanol stage [5]. We now report on the first successful correlation between a genotype of *M. incana* and enzymatic 3′-hydroxylation. An NADPH-dependent microsomal 3′-hydroxylase activity was detected only in flowers with the wild-type allele b^+. Furthermore, a soluble enzyme is described which catalyses hydroxylation of naringenin in the 3-position. 2-Oxoglutarate, Fe^{2+} and ascorbate are cofactors for the latter reaction.

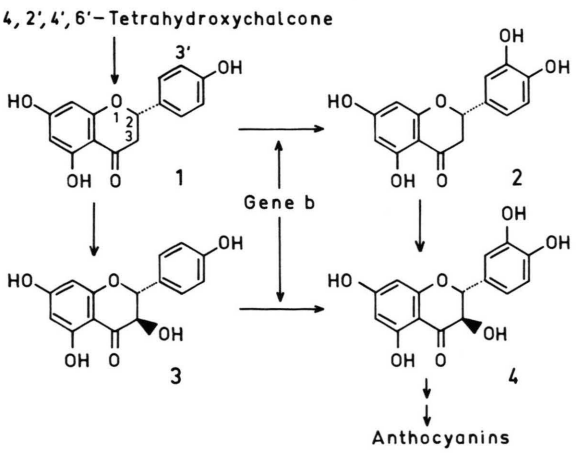

Fig. 1. Structural formulas of substrates and products. Naringenin, (**1**); eriodictyol, (**2**); dihydrokaempferol, (**3**); dihydroquercetin, (**4**).

Abbreviations: K-Pi, potassium phosphate buffer; PVP, polyvinylpyrrolidone.

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Materials and Methods

Plant material

The white flowering mutant lines 17 (genotype: ee f^+f^+ bb) and 18 (genotype: e^+e^+ ff b^+b^+) of *M. incana* were used. The action of the genes f and e interferes with the anthocyanin pathway before chalcone synthesis (recessive f) and after dihydroflavonol formation (recessive e). In both lines the conversion of flavanones to dihydroflavonols is not impaired [6]. Gene b is responsible for the hydroxylation of the B ring in 3′-position. The plants were cultivated in a greenhouse.

Chemicals and synthesis of substrates

[2-^{14}C]Malonyl-CoA (59 Ci/mol) obtained from Amersham Buchler was diluted to 21 Ci/mol with unlabelled material from Sigma. [4a,6,8-^{14}C]Naringenin and [4a,6,8-^{14}C]eriodictyol (both 64 Ci/mol) were prepared by incubation with pure chalcone synthase (1.4 µg) [7, 8] in presence of partially purified chalcone isomerase [8]. The incubation mixture containing 1.5 nmol [^{14}C]malonyl-CoA, 0.7 nmol coumaroyl-CoA and 1.0 nmol caffeoyl-CoA, respectively, 10 nmol K-P_i (pH 7.5) and 0.28 nmol 2-mercaptoethanol in a total volume of 110 µl was incubated for 1 h at 30 °C. After extraction with ethyl acetate aliquots of approx. 20000 dpm (0.2 nmol) were evaporated directly before use. [4a,6,8-^{14}C]Dihydrokaempferol (64 Ci/mol) was prepared enzymatically from labelled naringenin as described below. [3-^{14}C]-4-Coumaric acid and [3-^{14}C]-4-coumaroyl-CoA (both 2 Ci/mol) and authentic flavonoids were from our collection.

Preparation of crude extract and microsomal fraction

10 g of completely opened flowers were homogenized at 4 °C in a prechilled mortar together with 5 g PVP (or Dowex 1 × 2), 5 g quartz sand and 20 ml 0.1 M K-P_i buffer (pH 7.5) containing 28 mM 2-mercaptoethanol. After centrifugation for 20 min at 12000 × *g*, the clear supernatant (S1) served as enzyme source and for preparation of the microsomal fraction.

The microsomal fraction was prepared by Mg^{2+}-precipitation [9]. 20 ml of S1 were mixed with aqueous 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 supernatant (S2) or the microsomal pellet suspended in 2 ml of the K-P_i buffer and homogenized in a glass homogenizer served as enzyme source.

In addition, a microsomal fraction was prepared by ultracentrifugation of S1 for 90 min at 90000 × *g*.

Assay for flavonoid-3′-hydroxylase

The assay system contained in 200 µl total volume 20 µmol K-P_i (pH 7.5), 0.5–2 µmol 2-mercaptoethanol, 0.2 nmol radioactive substrate, 0.2 µmol NADPH and 5–15 µg (microsomal pellet) or 50–150 µg (S1) protein. Incubation was carried out for 10–30 min at 30 °C and was terminated by addition of each 5–10 µg naringenin, dihydrokaempferol, and dihydroquercetin in 10 µl methanol. The phenolics were extracted with ethyl acetate and chromatographed on a cellulose plate with solvent system 1. The plate was scanned for radioactivity. The radioactive zones were scraped off and counted in 0.5% PPO in toluene in a scintillation counter.

Assay for flavanone 3-hydroxylase

The assay system contained in 200 µl total volume 20 µmol Tris-HCl (pH 7.5), 0.28 µmol 2-mercaptoethanol, 0.2 nmol radioactive substrate, 50 nmol 2-oxoglutaric acid, 10 nmol ferrous sulfate, 1 µmol sodium ascorbate and S1 (50–200 µg protein) or S1 after equilibration with the incubation buffer on Sephadex G-50. After incubation for 10–60 min at 30 °C the mixture was worked up as described above.

Identification of reaction products

Radioactive zones corresponding to dihydrokaempferol and dihydroquercetin were eluted with methanol and evaporated to dryness under nitrogen. The residues were oxidized with sodium metabisulfite to the respective flavonols kaempferol and quercetin [10]. The products were identified on cellulose plates with solvent systems 1 and 3 in succession.

Dependence of reaction on pH

S1 was prepared containing 10 mM K-P_i (pH 7.5), 28 mM 2-mercaptoethanol and 10% sucrose. Enzyme assays were carried out in 0.15–0.2 M K-P_i.

Analytical methods

Protein was determined by a modified Lowry procedure [11] after precipitation of the protein with trichloroacetic acid in presence of deoxycholate [12]. Thin-layer chromatography was performed on precoated cellulose plates (E. Merck) with the following solvent systems (1) chloroform/acetic acid/water (10:9:1, v/v/v); (2) 15% acetic acid; (3) *n*-butanol/acetic acid/water (6:1:2, v/v/v). Flavonoids were detected under UV-light. Dihydroflavonols were also detected by the Zinc-HCl test [13] and flavanones by reduction with borohydride and subsequent exposure to HCl fumes [22].

Results

For preliminary experiments line 18 of *M. incana* was especially well suited, because recessive alleles of gene f interrupt the anthocyanin pathway early in this line and the flowers, therefore, largely lack flavonoids. The investigations showed that flower extracts from line 18 catalysed hydroxylation of naringenin in the 3- and 3′-position. To characterize the 3-hydroxylase reaction flowers of line 17 were used. This line is homozygous recessive for the gene b [6] and was therefore expected to lack flavonoid 3′-hydroxylase activity.

Flavanone 3-hydroxylase

Incubation of [^{14}C]naringenin (**1**) (Fig. 1) with crude flower extract (S 1) prepared in the presence of PVP led to the formation of a new radioactive product. This product was identified as dihydrokaempferol (**3**) by co-chromatography with authentic **3** on cellulose plates in 3 different solvent systems (Table I) and by oxidation of **3** to kaempferol with sodium metabisulfite [10]. With [^{14}C]eriodictyol (**2**) as substrate the reaction product was identified as dihydroquercetin (**4**) in the same manner. When the crude extract was prepared in the presence of Dowex 1X2 instead of PVP no or only very low conversion of **1** to **3** was found. Hydroxylase activity disappeared completely when the crude extract was subjected to gel filtration on Sephadex G-50 (Table II). In both cases enzyme activity could be restored by addition of heat inactivated extract (PVP-preparation). These results suggested that the reaction depends on a low molecular anionic cofactor(s) which can be removed by Dowex 1X2 or gel filtration.

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

Compound	Solvent system		
	1	2	3
Naringenin	80	39	–
Eriodictyol	60	32	–
Dihydrokaempferol	65	53	92
Dihydroquercetin	33	51	88
Kaempferol	–	04	87
Quercetin	–	02	75

Solvent systems see: Materials and Methods.

Of various cofactors tested, NADPH showed a stimulatory effect on enzyme activity in PVP preparations but could not restore enzyme activity after treatment with Dowex or after gel filtration. However, extensive restoration of enzyme activity was possible by addition of a combination of 2-oxoglutarate, ferrous ions and ascorbate. No effect was found upon addition of any of these cofactors alone (Table II).

The reaction was linear with protein concentration up to 100 µg protein in the assay and with time up to at least 60 min. The pH optimum with naringenin was found to be at about pH 7.5. After preparation of the microsomal fraction by Mg^{2+}-precipitation [9] or by ultracentrifugation the activity for 3-hydroxylation was exclusively found in the supernatant (Table II).

Substantial inhibition was observed by addition of KCN, EDTA or diethyldithiocarbamate to the enzyme assay. *p*-Chloromercuribenzoate and diethylpyrocarbonate had no appreciable effect (Table III). The latter reagent, which specifically carbethoxylates histidyl residues, was found to be an inhibitor of chalcone isomerase [23].

Flavonoid 3′-hydroxylase

When a crude extract from flowers of line 18 with PVP (S 1) was incubated with [^{14}C]naringenin the radiochromatograms of the reaction mixture showed at least two products in solvent system 1. One product was identified as dihydroquercetin (**4**). The second broad peak (R_f 0.5–0.7) could be further resolved into two products by rechromatography in 15% acetic acid. These products were identified as

Table II. Cofactor requirement and subcellular localisation of hydroxylating activities in flowers of *Matthiola incana*.

Enzyme source	Cofactor added	3-Hydroxylation (cpm in dihydrokaempferol) [a]	3′-Hydroxylation (cpm in eriodictyol) [a]
Crude extract with PVP (S 1) [b]	none	3900	0
	NADPH	4200	2200
Supernatant of microsomal pellet (S 2)	NADPH	4800	100
Microsomal pellet	none	–	0
	NADPH	0	8700
	NADH	–	1100
S 1 after gel filtration [c]	ascorbate, or 2-oxoglutarate, or Fe^{2+}	0	–
	ascorbate and 2-oxoglutarate	2900	–
	ascorbate and 2-oxoglutarate and Fe^{2+}	3400	–

Conditions see enzyme assays. [a] Product formed with 100 µg protein; [b] in K-phosphate buffer; [c] in Tris-HCl buffer.

Table III. Effect of inhibitors on 3- and 3′-hydroxylation of naringenin.

Additions [mM]	3-Hydroxylation [%]	3′-Hydroxylation [%]
none	100	100
EDTA (1)	25	–
EDTA (2)	–	65
KCN (5)	0	0
Diethyldithiocarbamate (2)	0	90
Diethylpyrocarbonate (0.5)	85	5
p-Chloromercuribenzoate (0.1)	95	85

Additions were made to the enzyme assay.

dihydrokaempferol (**3**) and eriodictyol (**2**) by the methods mentioned above. Separate investigation of 3′-hydroxylation was possible by treatment of the extract with Dowex 1×2, which eliminates the cofactors for 3-hydroxylation. Furthermore, after Mg^{2+}-precipitation [9] or after centrifugation at 90000 × *g*, 3′-hydroxylase activity was present in the microsomal pellet whereas 3-hydroxylase activity remained in the supernatant (Table II).

Hydroxylation in the 3′-position was dependent on NADPH. Substitution of NADPH by NADH gave only about 12% of the activity with NADPH (Table II). The reaction was linear with protein concentration up to 12 µg microsomal protein per assay. Linearity with time was not observed, a result which seems to be due to the temperature sensitivity of the enzyme preparation. For naringenin as substrate the pH optimum was determined to be about pH 7.2. The reaction was strongly inhibited by potassium cyanide and diethylpyrocarbonate. Partial inhibition was also observed with EDTA (Table III). Besides naringenin dihydrokaempferol was hydroxylated in the 3′-position. In contrast, 4-coumaric acid and 4-coumaroyl-CoA were not transformed to caffeic acid and caffeoyl-CoA, respectively.

Enzyme activity for 3′-hydroxylation could be demonstrated not only in flower extracts of line 18 but also in those of other lines of *M. incana* with wild-type alleles at the locus b. In contrast, in enzyme preparations from line 17 and other lines with recessive alleles of b, 3′-hydroxylation could be found neither in the crude extract nor in the microsomal fraction.

Discussion

As had been predicted earlier [5], flowers of *M. incana* proved to be a valuable source of hydroxylating enzymes involved in anthocyanin biosynthesis. With enzyme preparation from defined genotypes it was for the first time possible to prove that hydroxylation of naringenin in the 3 and 3′-position is catalyzed by two different enzymes (Fig. 1). The 3-hydroxylase is a soluble enzyme and belongs according to its cofactor requirement to the 2-oxo-

glutarate-dependent dioxygenases [14]. The locus controlling 3-hydroxylation in *M. incana* is not yet known, but in *Antirrhinum majus*, the gene *inc* seems to control the conversion flavanone → dihydroflavonol [15]. In this plant a correlation between genotype and 3-hydroxylase should therefore be possible.

The 3′-hydroxylase proved to be an NADPH dependent microsomal enzyme. It can therefore be assumed that this enzyme is a heme-containing monooxygenase [16] like cinnamate 4-hydroxylase [17]. 3′-Hydroxylation was observed with flavanone (**1**) and dihydroflavonol (**3**). Earlier work had suggested that 3′-hydroxylation occurs at the dihydroflavonol stage [5]. However, it remains to be elucidated whether a "metabolic grid" [18] is present under physiological conditions. In confirmation of the genetic experiments [4, 5], a strict correlation between 3′-hydroxylase activity and the wild-type allele b^+ was found.

4-Coumarate and 4-coumaroyl-CoA did not serve as substrates for the 3′-hydroxylase. The situation is therefore similar to that in *Petunia hybrida*, where it was concluded from complementation experiments that the gene Ht1 is responsible for hydroxylation of dihydrokaempferol or its 7-glucoside in the 3′-position [21]. In contrast, genetical and biochemical investigations with petals of *Silene dioica* indicate that in this plant the hydroxylation pattern of the B-ring is determined at the 4-coumaroyl-CoA stage [19, 20].

Future work will be concerned with purification and characterization of these hydroxylases.

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