Conversion of Dihydroflavonols to Flavonols with Enzyme Extracts from Flower Buds of *Matthiola incana* R. Br.

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Soluble enzyme preparations from flower buds of *Matthiola incana* catalysed the conversion of dihydrokaempferol to kaempferol and of dihydroquercetin to quercetin. The reaction required 2-oxoglutarate, ascorbate and Fe^{2+} as cofactors and had a pH-optimum at about 7.0. Highest enzyme activity was already present in the youngest buds followed by a rapid decline during bud and flower development. Furthermore, a substantial correlation was observed between the enzyme activity for flavonol formation and the flavonol content of the buds and flowers.

Introduction

Recently, a soluble enzyme preparation from cell suspension cultures of parsley was shown to catalyse not only the hydroxylation of flavanones in 3-position to dihydroflavonols but also the conversion of the latter compounds to flavonols. Both reactions required 2-oxoglutarate, ascorbate and Fe^{2+} as cofactors [1].

3-Hydroxylation of flavanones to dihydroflavanols have also been demonstrated with enzyme preparations from flowers of *Matthiola incana* [2]. However, no further conversion of dihydroflavonols to flavonols could be observed with these enzyme extracts, although flavonols are known to be present in the flowers [3]. Thus, either the enzyme activity for flavonol formation is present at other stages of flower development or the reaction is not (as with parsley) catalysed by a soluble 2-oxoglutarate-dependent dioxygenase but by a different enzyme. The latter phenomenon has been reported for a similar reaction namely the conversion of flavanones to flavones. In parsley, this reaction was found to be catalysed by a soluble dioxygenase, whereas in flowers of several plants an NADPH-dependent microsomal enzyme activity was shown to be responsible [1, 4].

We investigated these possibilities and could now demonstrate *in vitro* conversion of dihydroflavonol to flavonol with enzyme preparations from *M. incana*. Enzyme activity was found to be present in extracts from small buds but not in extracts from unfolded flowers. Furthermore, as with parsley, the reaction is catalysed by a soluble enzyme and requires 2-oxoglutarate, ascorbate and Fe^{2+} as cofactors.

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Material and Methods

Plant material

The investigation included the lines 01, 04, and 08 (genotype b^+b^+) which produce cyanidin derivatives in the flowers and the lines 09 and 16 (genotype *bb*) which contain pelargonidin derivatives [5]. The gene *b* controls the activity of flavonoid 3′-hydroxylase (Fig. 1) which catalyses hydroxylation of naringenin to eriodictyol and of dihydrokaempferol to dihydroquercetin [2]. The plant material was cultivated in a greenhouse and during the summer months in the experimental garden of our institute.

Chemicals and synthesis of substrates

Naringenin, dihydroquercetin, kaempferol and quercetin were obtained from Roth (Karlsruhe, Germany). Dihydrokaempferol and eriodictyol came from our laboratory collection.

[2-^{14}C]Malonyl-CoA (2.22 GBq/mmol) was obtained from Amersham Buchler and diluted to 1.03 GBq/mmol with unlabelled material from Sigma. [4a,6,8-^{14}C]Naringenin, [4a,6,8-^{14}C]eriodictyol, [4a,6,8-^{14}C]dihydrokaempferol and [4a,6,8-^{14}C]di-

hydroquercetin (all 3.09 GBq/mmol) were prepared enzymatically [1, 2].

Enzyme preparation

All steps were carried out at 4 °C. 1.0 g buds or flowers were homogenized in a pre-chilled mortar together with 1.0 g Dowex (1 × 2), 0.5 g quartz sand and 3.0 ml 0.1 M potassium phosphate buffer (pH 7.0), containing 28 mM 2-mercaptoethanol. The homogenate was transferred to Micro Test Tubes (Eppendorf) and centrifugated for 5 min at about 10000 × *g*. The supernatants were pooled and centrifugated as described above. The clean supernatant of the second centrifugation served as enzyme source for the conversion of dihydrokaempferol.

Enzyme assay

The assay system contained in 200 µl total volume 20 µmol potassium phosphate buffer (pH 7.0), 0.28 µmol 2-mercaptoethanol, 0.2 nmol radioactive substrate (naringenin, eriodictyol, dihydrokaempferol or dihydroquercetin, respectively), 50 nmol 2-oxoglutarate, 10 nmol ferrous sulfate, 1 µmol sodium ascorbate and crude enzyme extract (3–11 µg protein) or enzyme extract after equilibration with the preparation buffer on Sephadex G-50. Incubation was carried out for 5–20 min at 30 °C and was terminated by addition of 10 µl methanol containing a mixture of authentic flavonoids. The phenolics were immediately extracted with ethylacetate 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 150 µl buffer (between pH 6.0 and 8.5) and 10 µl crude extract.

Analytical methods

For the determination of protein the method of Bradford [6] was used.

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

Flavonoids were detected on chromatograms under UV-light and after fuming with ammonia. Dihydroflavonols were also detected by treatment with zinc dust and subsequent spraying with 6 M HCl [7]. For the identification of the reaction products radioactive zones were scraped off from the cellulose plates and eluted with methanol. After concentration the eluates were co-chromatographed with authentic samples in the 3 solvent systems mentioned above.

Results

Investigations on the course of flavanone 3-hydroxylase activity during the development of buds and flowers [8] revealed the presence of an as yet not observed enzyme activity in enzyme preparations of *M. incana*. When crude extracts prepared from small buds were incubated with [^{14}C]naringenin (**1**) (Fig. 1) and the co-factors for the 3-hydroxylase reaction 2-oxoglutarate, ascorbate and Fe^{2+}, the radiochromatograms of the reaction mixture in solvent system 1 showed besides dihydrokaempferol (**3**) the formation of a further product (Fig. 2). When, however, flower extracts were used as enzyme source only the formation of dihydrokaempferol could be observed. Furthermore, the relative amounts of dihydrokaempferol and the new product depended clearly on the incubation time. With short periods of incubation, dihydrokaempferol was the main product (Fig. 2a). But with longer incubation times, the dihydrokaempferol amount was found to be reduced in favour of the other product (Fig. 2b), indicating that the latter compound is formed from naringenin *via* dihydrokaempferol (Fig. 1). In confirmation of these results, incubation of [^{14}C]dihydrokaempferol with enzyme preparations from buds and the three co-factors mentioned above led also to the formation of the new product. The compound was identified as the flavonol kaempferol (**5**) (Fig. 1) by co-chromatography with an authentic sample in three different solvent systems (Table I).

After preparation of the microsomal fraction by Mg^{2+}-precipitation [9] the enzyme activity for flavonol formation remained in the supernatant.

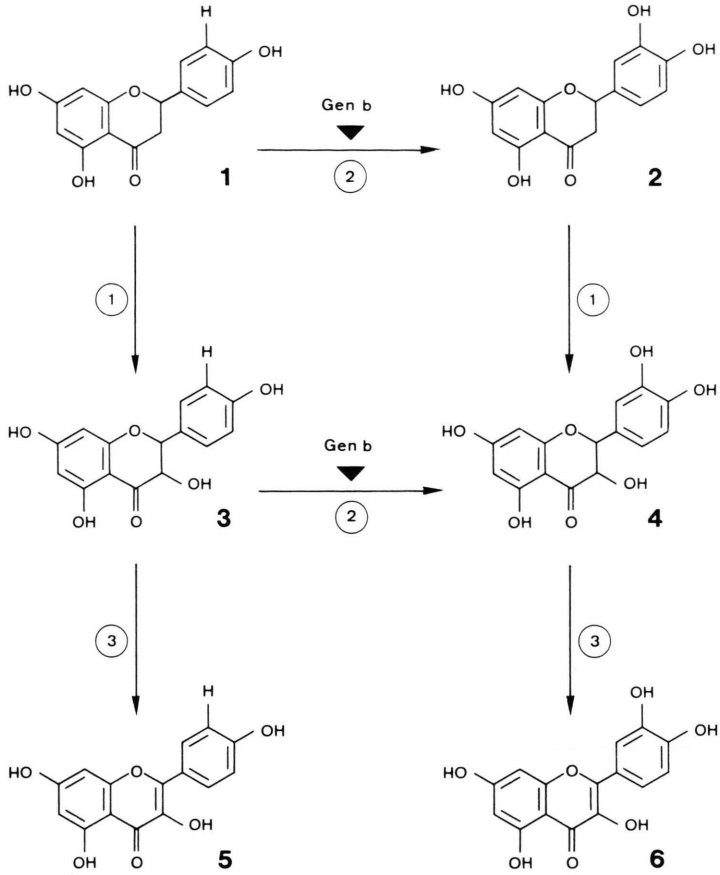

Fig. 1. Structural formulas of substrates and products and some enzymes of flavonol biosynthesis in *Matthiola incana*. Naringenin (**1**); eriodictyol (**2**); dihydrokaempferol (**3**); dihydroquercetin (**4**); kaempferol (**5**); quercetin (**6**); 3-hydroxylase ①; 3′-hydroxylase ②: enzyme activity for flavonol formation ③.

Furthermore, the reaction was strictly dependent on the three co-factors 2-oxoglutarate, ascorbate and Fe^{2+} (Table II). When low molecular weight substances were removed by gel filtration (Sephadex G-50), no enzyme activity could be observed. Addition of any of the co-factors alone or of a combination of two co-factors led only to a small restoration of enzyme activity, whereas high restoration was found upon addition of all three co-factors. NADPH and NADH had no pronounced effect (Table II).

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

Compound	Solvent system		
	1	2	3
Naringenin	61	85	91
Eriodictyol	56	60	81
Dihydrokaempferol	72	65	87
Dihydroquercetin	67	37	80
Kaempferol	19	70	56
Quercetin	14	36	40

For conversion of dihydrokaempferol to kaempferol a pH optimum around 7.0 was found (Fig. 3). The reaction was linear with protein concentration up to 11 µg protein per assay. Linearity with time was observed for about 10 min. Strong inhibition was found with EDTA and diethyldithiocarbamate. Furthermore, substantial inhibition was observed after addition of KCN or *p*-chlormercuribenzoate (Table III). These four compounds also had an inhibitory effect on the conversion of dihydrokaempferol in parsley [1].

Incubation of [^{14}C]dihydroquercetin (**4**) with bud extracts and the three co-factors mentioned above also led to the formation of a radioactive product. It was identified as quercetin (**6**) (Fig. 1) by co-chromatography with an authentic sample in three different solvent systems (Table I). Furthermore, in

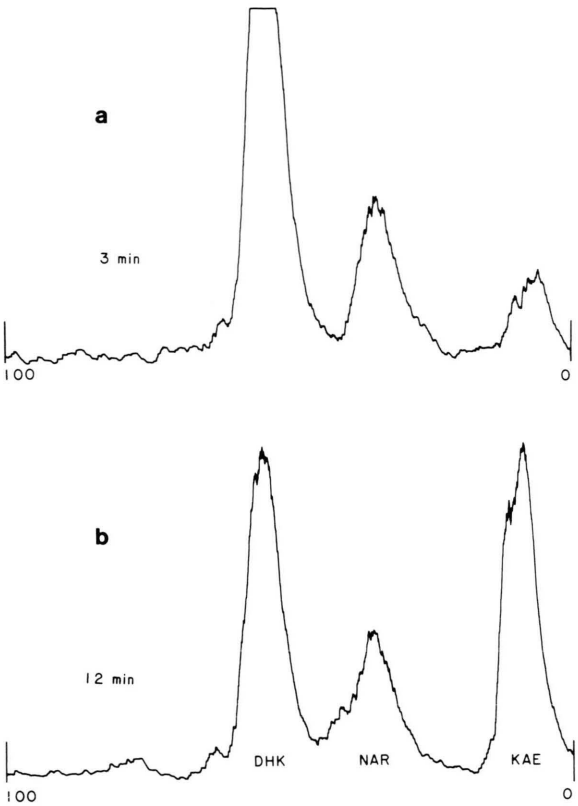

Fig. 2. Radioscan of TLC on cellulose with solvent system 1 from incubations of [^{14}C]naringenin in the presence of 2-oxoglutarate, ascorbate and Fe^{2+} with enzyme preparations from buds of *Matthiola incana*. a: 3 min incubation time; b: 12 min incubation time. Naringenin (NAR); dihydrokaempferol (DHK); kaempferol (KAE).

incubations with [^{14}C]eriodictyol as substrate, dihydroquercetin and quercetin were found to be formed.

Dihydrokaempferol and dihydroquercetin were converted to the corresponding flavonols by enzyme preparations from both pelargonidin- and cyanidin-producing lines. But in each case, the formation of quercetin from dihydroquercetin was clearly lower (approx. 65%) than the conversion of dihydrokaempferol to kaempferol. Furthermore, in incubations which contained both substrates, quercetin formation was even reduced to about 50% in comparison to kaempferol formation.

The enzyme activity for flavonol formation as well as the flavonol content were studied during the development of buds and flowers. Morphological

Table II. Co-factor requirement of enzyme activity for flavonol formation.

Enzyme source	Co-factor added	Enzyme activity (cpm in kaempferol) [a]
Crude extract	none	0
	ascorbate and 2-oxoglutarate and Fe^{2+}	4110
Crude extract after gelfiltration (G50)	none	0
	ascorbate	497
	2-oxoglutarate	588
	Fe^{2+}	473
	ascorbate and 2-oxoglutarate	524
	ascorbate and Fe^{2+}	633
	2-oxoglutarate and Fe^{2+}	716
	ascorbate and 2-oxoglutarate and Fe^{2+}	2605
	NADPH	380
	NADH	390

[a] Product formed with 50 µg protein.

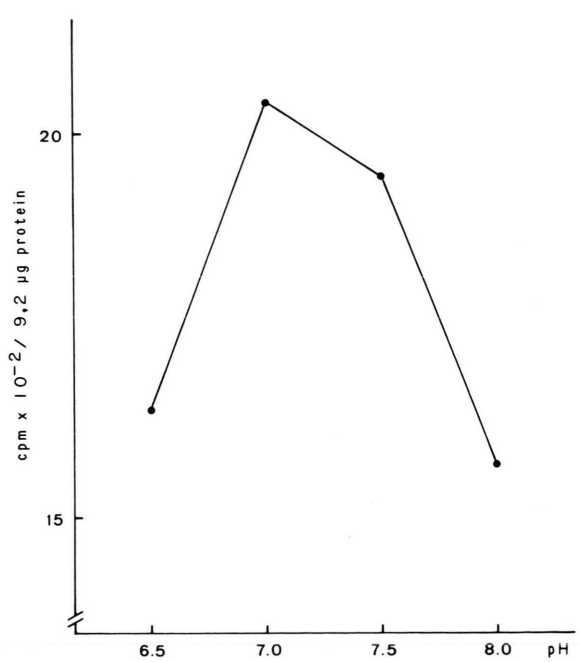

Fig. 3. Dependence of kaempferol formation from dihydrokaempferol on pH.

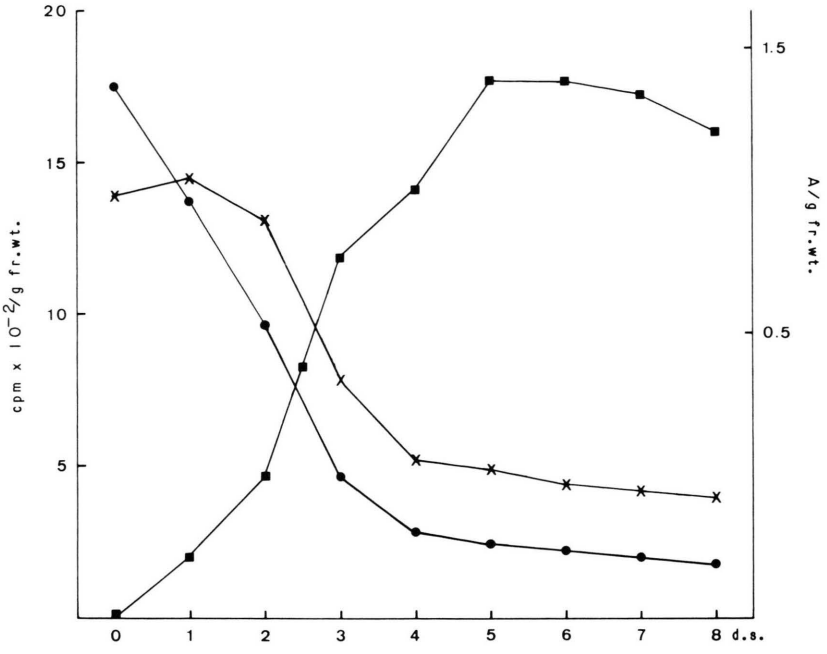

Fig. 4. The course of flavonol content, anthocyanin accumulation and enzyme activity for flavonol formation during bud and flower development (d. s.). (●—●) Enzyme activity; (■—■) anthocyanin accumulation (A = 525 nm); (×—×) flavonol content (A = 366 nm).

criteria were used to divide the developmental process into nine significantly different stages. In addition to the stages 1 to 8 described earlier [8], very small buds (stage 0) were also included in the investigations. The buds of stage 0 are significantly smaller than those of stage 1. The buds are completely closed and the petals are not coloured by anthocyanins.

The amount of flavonols present in the nine developmental stages of buds and flowers is shown in Figure 4. The flavonol content is already high in the smallest buds (stage 0), reaches a maximum value at stage 1 and decreases in the following stages at first slowly than progressively to a rather low level. For comparison the course of anthocyanin accumulation during bud and flower development is also shown in Figure 4. Beside the flavonol amounts the type of aglycone was determined. As expected, pelarginidin-producing lines (genotype *bb*) only contained kaempferol. However, the cyanidin-producing lines (genotype b^+b^+) did not contain quercetin instead of kaempferol. On the contrary, here as well kaempferol alone was present in all stages. The data for enzyme activity (Fig. 4) were calculated on a fresh weight basis, since the protein content of the enzyme preparations decreased rapidly and continuously with the growth of the buds and flowers [8]. The enzyme activity for flavonol formation did not increase during bud development as observed for other enzymes involved in flavonoid biosynthesis of *M. incana* [8] but had already the highest value in extracts from stage 0. In enzyme preparations of the following stages, the enzyme activity decreased rapidly to values near zero. Because smaller buds than those of stage 0 do not allow an exact enzyme preparation, the stage of maximal enzyme activity for conversion of dihydroflavonols to flavonols could not be determined.

Table III. Effect of various inhibitors on enzyme activity.

Additions	Enzyme activity [%]
none	100
5 mM KCN	45
0.25 mM EDTA	7
0.5 mM DPC	116
2 mM DDC	18
0.1 mM *p*-CMB	48
1 mM $MgCl_2$	103

DPC, diethylpyrocarbonate; DDC, diethyldithiocarbamate; *p*-CMB, chloromercuribenzoate.

Discussion

In vitro conversion of dihydroflavonols to flavonols was first observed with enzyme preparations of parsley cell suspension cultures [1]. We could now also demonstrate such an enzyme activity in extracts from buds of *Matthiola incana*. Thus, this plant proved, once again to be a valuable source for enzymes involved in flavonoid biosynthesis.

Surprisingly, the highest enzyme activity was found in the youngest buds. This was followed by a rapid decline in the older bud stages. Thus, the course of enzyme activity for flavonol formation during organ development differed markedly from the course of other enzyme activities involved in flavonoid biosynthesis [8]. On the other hand, a substantial correlation was found between flavonol content and enzyme activity for flavonol formation. This indicates that the synthesis of flavonols is obviously restricted to that period of bud development, where no or only a low amount of anthocyanin is present. As soon as the anthocyanin content rapidly increases the formation of flavonols stops.

In agreement with the result reported for parsley [1], the reaction is catalysed by a soluble enzyme which belongs according to its co-factor requirement to the 2-oxoglutarate-dependent dioxygenases [10]. Thus, the double bond between the C atom 2 and 3 of dihydroflavonols is probably introduced by hydroxylation in 2-position with subsequent elimination of water. A similar mechanism is discussed for the formation of flavones from flavanones [1, 4]. The postulated 2-hydroxylated flavonoids formed as intermediates were as yet not observed in enzyme assays and could also not be demonstrated during our investigations. But they have been found naturally and were shown to convert easily to flavones or flavonols, respectively, throught loss of water [11, 12]. In the presence of flavonoid 3′-hydroxylation activity (genotype b^+b^+) (Fig. 1) cyanidin instead of pelargonidin is formed in the flowers, but kaempferol is not replaced by quercetin. It was expected therefore that dihydrokaempferol is the only suitable substrate for flavonol synthesis. Beside dihydrokaempferol, however, dihydroquercetin was found to be converted to the respective flavonol quercetin. The absence of quercetin *in vivo* can be rationalized by a synergistic effect of two findings. Firstly, dihydrokaempferol was, in all lines investigated, a better substrate for flavonol formation than dihydroquercetin. Secondly, in the presence of high enzyme activity for flavonol formation (stages 0 and 1) mainly dihydrokaempferol is available as substrate, because flavonoid 3′-hydroxylase activity catalysing the synthesis of dihydroquercetin is still rather low [8]. The latter enzyme only reaches maximal activity at the stages 3 to 4, where enzyme activity for flavonol formation has already declined to a low level.

The gene controlling the conversion of dihydroflavonols to flavonols in *M. incana* has not yet been identified, but in *Petunia hybrida*, the gene *Fl* seems to control the conversion dihydroflavonol → flavonol [13].

In the latter plant, a correlation between genotype and enzyme activity for flavonol formation which definitely proves that the enzyme activity measured *in vitro* also catalyses this reaction *in vivo* should therefore be possible.

Corresponding work is in progress.

Acknowledgements

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