Oxidation of Flavanones to Flavones with Flower Extracts of *Antirrhinum majus* (Snapdragon)

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Enzyme preparations from flowers of *Antirrhinum majus* catalysed the oxidation of naringenin to apigenin and of eriodictyol to luteolin. Enzyme activity was found to be localized in the microsomal fraction. The reaction required NADPH as cofactor and had a pH optimum of about 7.0. The NADPH-dependent microsomal enzyme activity was also present in flower extracts of other flavone-producing plants, whereas flower extracts of plants which lack flavones were found to lack also this enzyme activity.

Introduction

Of the various classes of naturally occuring flavonoid compounds, the flavones are one of the most abundant group. They occur not only in flowers and leaves but also in many other parts of higher plants. However, little information is available as yet about their formation.

Radioactive tracer work and enzymatic investigations on the biosynthesis of flavones were only performed with parsley (*Petroselinum hortense*). They have shown that flavones are most probably formed from flavanones by an oxidation reaction. Thus, the flavanone naringenin (**1**) (Fig. 1) was found to be a suitable precursor for the corresponding flavone apigenin (**3**) on tracer studies [1]. The enzymatic investigations revealed that cell-free extracts from very young primary leaves of parsley plants catalyse the oxidation of naringenin to apigenin. Furthermore, enzyme activity was found to be localized in the supernatant after ultracentrifugation and the reaction required oxygen, ferrous ions and a not identified heat stable cofactor [2].

For further investigations on the formation of flavones we have now used flowers from defined genotypes of *Antirrhinum majus* (snapdragon). Analytical work had shown that in the flowers of this plant besides anthocyanins and other flavonoids the flavones apigenin (**3**), luteolin (**4**) and chrysoeriol (3′-methyl ether of luteolin) occur [3]. Furthermore, flower extracts of *A. majus* proved to be a valuable source of enzymes involved in flavonoid biosynthesis [4]. We now report on an enzyme in flower extracts of *A. majus* which catalyses the oxidation of naringenin (**1**) to apigenin (**3**) and of eriodictyol (**2**) to luteolin (**4**) (Fig. 1). The enzyme activity was found to be localized in the microsomal fraction and the reaction required NADPH as cofactor.

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Materials and Methods

Plant material

The investigations on *Antirrhinum majus* included a pink flowering line (genotype: *Inc/Inc eos/eos*), a magenta flowering line (genotype: *Inc/Inc Eos/Eos*) and a line with ivory flowers (genotype: *inc/inc Eos/Eos*). The plant material was cultivated in a greenhouse and during the summer months in the experimental garden of our institute.

The gene inc is known to control the hydroxylation of flavanones in 3-position to dihydroflavonols [5]. In presence of recessive alleles (*inc/inc*), no activity of flavanone 3-hydroxylase is detectable in the flower extracts [4]. Therefore, the ivory line contains only flavanones, flavones and some aurones in the flowers, whereas in the flowers of lines with wild-type alleles (*Inc/Inc*) anthocyanins and flavonols are also formed.

The gene *eos* governs the B-ring hydroxylation of flavonoids in 3′-position. The enzyme flavonoid 3′-hydroxylase which catalyses the hydroxylation is only active in genotypes with the wild-type allele *Eos* [4]. Therefore, in flowers of recessive genotypes (*eos/eos*) only 4′-hydroxylated flavonoids like apigenin, kaempferol and pelargonidin are present, whereas in genotypes with one or two wild-type alleles the corresponding 3′,4′-dihydroxy compounds were formed.

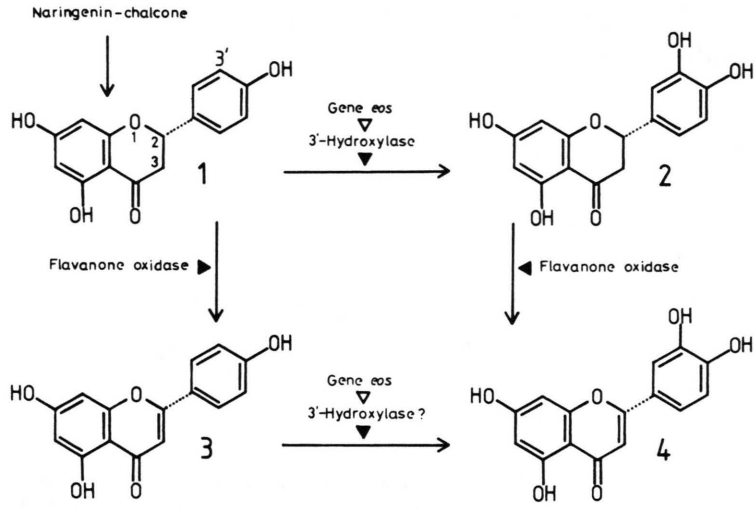

Fig. 1. Structural formulas of substrates and products. Naringenin (**1**); eriodictyol (**2**); apigenin (**3**); luteolin (**4**).

Chemicals and synthesis of substrates

Naringenin, apigenin and luteolin were obtained from Roth (Karlsruhe). Eriodictyol was prepared by demethylation of hesperitin (Roth) by a BBr_3 treatment (Kho, personal communication). [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 [6] using partially purified chalcone synthase and chalcone isomerase from parsley. [4a,6,8-^{14}C]Apigenin (78 Ci/mol) was prepared enzymatically from labelled naringenin as described below.

Enzyme preparation

10 g flowers without anthers and pistils were used. The preparation of the crude extract and the preparation of the microsomal fraction by Mg^{2+}-precipitation and by ultracentrifugation was performed according to ref. [6] with the exception that the microsomal pellet was suspended in 1 ml of the potassium phosphate (K-Pi) buffer.

Assay for flavanone oxidase

The assay system contained in 200 µl total volume 20 µmol K-Pi buffer (pH 7.5), 0.8–1.4 µmol 2-mercaptoethanol, 0.1 nmol radioactive naringenin and 0.05 nmol radioactive eriodictyol, respectively, 0.1 µmol NADPH and 15–30 µg protein (microsomal pellet or crude extract). Incubations were carried out for 15–30 min at 30 °C and were terminated by addition of 10 µl methanol containing 10 µg of each naringenin, eriodictyol, apigenin and luteolin. The phenolics were twice extracted with ethyl acetate (100 µl and 50 µl) and separated on a cellulose TLC plate using solvent system 2. The plate was scanned for radioactivity. The radioactive zones were scraped off and counted in Unisolve in a scintillation counter.

Separation and identification of the reaction products

TLC was performed on precoated cellulose plates (Schleicher & Schüll). The following solvent systems were used: (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); (4) *tert.*-butanol/acetic acid/water (3:1:1, v/v/v).

Radioactive zones corresponding to apigenin, luteolin and eriodictyol were eluted with methanol and evaporated to dryness under nitrogen. The residues were redissolved in methanol and chromatographed together with authentic samples.

Flavonoids were detected under UV-light and after fuming with ammonia. Flavanones were also detected by reduction with borohydride and subsequent exposure to HCl fumes [7].

Determination of the pH optimum

The enzyme assays were carried out in mixtures of 180 µl K-Pi buffer (between pH 6.5 and 8.0) and 20 µl of the microsomal fraction.

Determination of protein

The method of Bradford [8] was used for both, crude extract and microsomal fraction.

Results

Preliminary experiments showed that flower extracts of *A. majus* catalyse not only the hydroxylation of naringenin (**1**) in 3- and 3′-position but also its oxidation to flavones. Separate investigation of the oxidation reaction was achieved by use of defined genotypes which are known to lack flavanone 3-hydroxylase activity (genotype: *inc/inc*) and flavonoid 3′-hydroxylase activity (genotype *eos/eos*), respectively [4]. With regard to 3′-hydroxylation the use of genetically defined lines proved to be especially advantageous because no biochemical methods have been found so far to separate 3′-hydroxylase activity from the enzyme catalysing flavone formation. In contrast, 3-hydroxylase activity was also excluded by treatment with Dowex 1 × 2 which eliminates the cofactors of this reaction [4, 6] and by enzyme preparation with K-Pi buffer [4].

When a crude extract prepared with Dowex 1 × 2 from pink flowers (genotype: *Inc/Inc eos/eos*) was incubated with [^{14}C]naringenin in presence of NADPH, the formation of a new radioactive product was observed. This product was identified as apigenin (**3**) by co-chromatography with authentic **3** on cellulose TLC plates in three different solvent systems (Table I). Incubations of [^{14}C]naringenin with crude extracts from magenta flowers (genotype: *Inc/Inc Eos/Eos*) or from ivory flowers (genotype *inc/inc Eos/Eos*) led to the formation of three radioactive products in presence of NADPH. One product was identified as eriodictyol (**2**) by the methods described earlier [4]. The other two products corresponded on co-chromatography with authentic samples to apigenin (**3**) and luteolin (**4**), respectively (Table I).

With naringenin as substrate the formation of luteolin besides apigenin was only observed with enzyme preparations containing 3′-hydroxylase activity (genotype: *Eos/Eos*). The question was investigated whether the formation of luteolin proceeds *via* apigenin or *via* eriodictyol. When [^{14}C]-apigenin was incubated with flower extracts containing 3′-hydroxylase activity, no reproducible formation of luteolin could be observed. In contrast, [^{14}C]eriodictyol as substrate was clearly converted to luteolin. With this substrate the reaction was found to be independent on the genetic state of the gene *eos*. Thus the formation of luteolin most probably proceeds *via* eriodictyol which is first formed from naringenin by action of the 3′-hydroxylase [4].

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

Compound	Solvent systems			
	1	2	3	4
Naringenin	83	61	–	90
Eriodictyol	60	50	78	86
Apigenin	76	21	76	–
Luteolin	43	12	58	82

Solvent systems see: Materials and Methods.

Table II. Subcellular localization and cofactor requirement of the flavanone oxidase activity in flowers of *Antirrhinum majus*.

Enzyme source	Cofactor added	cpm in Apigenin [a]
Crude extract	none	0
	NADPH	11 985
Supernatant of microsomal pellet	NADPH	1 042
Microsomal pellet	none	0
	NADPH	31 138
	NADH	4 484

[a] Apigenin formed with 100 µg protein.

Table III. Effect of several additions on flavanone oxidase activity.

Additions	Flavanone oxidase activity [%]
none	100
2 mM EDTA	100
5 mM KCN	68
0.5 mM Diethylpyrocarbonate	53
0.1 mM Chloromercuribenzoate	76
2 mM Diethyldithiocarbamate	89
0.1 mM *o*-Phenanthroline	86
1 mM Fe^{2+}	89

The subcellular localization of the enzyme was investigated by preparation of the microsomal fraction from the crude extract. After both Mg^{2+}-precipitation or centrifugation at 90 000 × *g*, the enzyme activity was found in the microsomal pellet (Table II). The reaction was strictly dependent on NADPH. Substitution of NADPH by NADH strongly reduced

the enzyme activity (Table II). No flavone formation could be observed with ascorbate and/or 2-oxoglutarate instead of NADPH. Furthermore, addition of ferrous ions showed no stimulatory effect but reduced the enzyme activity (Table III). 2-Oxoglutarate, ascorbate and Fe^{2+} are cofactors for the formation of flavones in parsley described in the following paper [9]. The reaction was partially inhibited by diethylpyrocarbonate, KCN and *p*-chloromercuribenzoate. Addition of EDTA, diethyldithiocarbamate and *o*-phenanthroline had no appreciable effect (Table III). *O*-phenanthroline was found to inhibit almost completely the oxidation of naringenin to apigenin at a concentration of 0.1 mM in cell-free extracts from young parsley leaves [2]. For naringenin as substrate the pH optimum was determined to be at about pH 7.0. Apigenin formation increased linear with protein concentration up to about 20 µg microsomal protein per assay. Linearity with time was not observed. Preincubations of the microsomal fraction revealed that only about 50% of the enzyme activity remained after 10 min at 30 °C. The non-linearity with time therefore is obviously due to the temperature sensitivity of the enzyme.

Besides in *A. majus* the NADPH-dependent microsomal flavanone oxidase activity could also be demonstrated in the flower extracts of *Verbena hybrida* and *Taraxacum officinale* but not in the flower extracts of *Matthiola incana, Petunia hybrida* and *Dianthus caryophyllus* (Stotz and Forkmann, unpublished results). *Verbena* and *Taraxacum* are known to produce flavones in the flowers, whereas in the flowers of *Matthiola, Petunia* and *Dianthus* no flavones occur naturally [10].

Discussion

Enzyme preparations from flowers of *A. majus* catalysed not only the oxidation of naringenin to apigenin but also the hydroxylation of naringenin in 3-position to dihydrokaempferol and in 3′-position to eriodictyol. Therefore, an exclusion of the hydroxylating enzymes was necessary especially because the 3′-hydroxylase and the flavanone oxidase behaved similarly with regard to subcellular localisation, cofactor requirement and towards several inhibitors [4]. The use of genetically defined mutants which lack the hydroxylating enzyme activities [4] allowed a separate characterisation of the oxidation reaction and an unequivocal identification of the reaction products apigenin and luteolin.

It has been in question for a long time whether the chalcone or the isomeric flavanone serves as substrate for the formation of flavones [11, 12]. At the enzymatic studies on flavone formation with parsley plants, it has been shown by using substrates with a resorcinol-type of ring A that the flavanone is the actual substrate for the oxidation to flavone [2]. Investigations on chalcone-accumulating mutants of *Callistephus chinensis* proved that the biosynthesis of flavones and other flavonoids with a phloroglucinol-type of ring A also proceeds via the flavanone which is formed from the chalcone by action of the chalcone isomerase [13]. Thus, there is no question that the flavanones used in the present investigation were the actual substrates for the enzymatic formation of apigenin and luteolin.

Flavones differ from flavanones only by a double bond between the C atoms 2 and 3 (Fig. 1). The enzyme catalysing this oxidation reaction in *A. majus* was found to require NADPH as cofactor and it proved to be localized in the microsomal fraction. These results suggest that this enzyme belongs to the mixed-function oxidases (mono-oxygenases) [14] like flavonoid 3′-hydroxylase [4, 6] and cinnamate 4-hydroxylase [15]. Furthermore, the flavonoid 3′-hydroxylase and the flavanone oxidase of *A. majus* behaved similarly towards several inhibitors [4]. Thus, the double bond between the C atoms 2 and 3 of flavanones is obviously introduced by a hydroxylation reaction with subsequent elimination of water. A similar mechanism is discussed for the introduction of *cis*-double bonds into fatty acids by action of desaturases [16]. But the hydroxylated compounds formed as intermediates during introduction of the double bond were neither found at the synthesis of unsaturated fatty acids [16] nor at our investigations on flavone formation.

In case of the flavone synthesis in *A. majus* it seems to be unlikely that the intermediate is hydroxylated in 3-position. This hydroxylation gives dihydroflavonols and the reaction is known to be catalysed by an enzyme which belongs to the 2-oxoglutarate-dependent dioxygenases [4]. Thus the hydroxylation most probably occurs in 2-position and the resulting intermediates could be 2-hydroxyflavanone or 2-hydroxydibenzoylmethane. Both hydroxy compounds have been naturally found [17–19] and they are also known as intermediates of the

chemical synthesis of flavones [20]. Because tracer experiments with labelled 2-hydroxydibenzoylmethane gave no incorporation of radioactivity into flavone (Schill and Grisebach, unpublished results), the 2-hydroxyflavanone rather than the 2-hydroxydibenzoylmethane could be the actual intermediate. Further work will be required for a precise characterisation of the mechanism involved in the oxidation reaction.

It could not be exactly proved whether the NADPH-dependent microsomal enzyme activity found in our *in vitro* experiments actually catalyses the formation of flavones *in vivo* because the gene which governs the conversion of flavanones to flavones in *A. majus* is not yet known. However the enzyme activity proved to be present also in flower extracts of other flavone-producing plants, whereas flower extracts of plants which lack flavones were found to lack also this enzyme activity. These results clearly support the significance of the NADPH-dependent microsomal enzyme activity for the formation of flavones *in vivo*.

In contrast, the enzyme catalysing flavone formation in parsley plants was found to be localized in the $150000 \times g$ supernatant and the reaction required not NADPH but ferrous ions and (an)other not identified cofactor(s) [2]. Recent studies on parsley cell cultures confirmed not only the subcellular localisation of the enzyme in the supernatant but led also to the identification of 2-oxoglutarate, ascorbate and ferrous ions as cofactors for the oxidation reaction [9]. It must be assumed therefore that in flowers of *A. majus* and of other flavone-producing plants and in leaves and cell cultures of parsley the formation of flavones is catalysed by different enzymes.

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