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Enzymatic reduction of (+)-dihydroflavonols to flavan-3,4-*cis*-diols with flower extracts from *Matthiola incana* and its role in anthocyanin biosynthesis

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Abstract. A cell-free extract from flowers of *Matthiola incana* catalyzes a NADPH-dependent stereospecific reduction of (+)-dihydrokaempferol to 3,4-*cis*-leucopelargonidin (5,7,4′-trihydroxyflavan-3,4-*cis*-diol). The pH-optimum of this reaction is around 6. The rate of reaction with NADH was about 50% of that found with NADPH. (+)-Dihydroquercetin and (+)-dihydromyricetin were also reduced by the enzyme preparation to the corresponding flavan-3,4-*cis*-diols. Correlation between the genotype of *M. incana* and the presence of dihydroflavonol 4-reductase is strong evidence that this enzyme is involved in anthocyanin biosynthesis.

Key words: Anthocyanin biosynthesis – Dihydroflavonol reductase – Flavan-3,4-diol – *Matthiola*.

Introduction

Supplementation experiments with genetically defined acyanic flowers of *Matthiola incana* R.Br. furnished strong evidence for the role of flavan-3,4-diols as intermediates in anthocyanidin biosynthesis (Heller et al. 1985). Feeding of leucopelargonidin to line 17 (blocked between dihydroflavonols and anthocyanins) and line 18 (blocked in the chalcone synthase gene) led to formation of the corresponding anthocyanidin 3-O-glucosides, whereas supplementation of line 19 (blocked between dihydroflavonol and anthocyanin in a locus other than that blocked in line 17) did not result in anthocyanin synthesis.

Dihydroflavonols have also been established as intermediates in this pathway (Grisebach 1982), and their enzymatic reduction to flavan-3,4-diols has recently been reported in connection with proanthocyanidin biosynthesis in cell cultures of Douglas-fir needles (Stafford and Lester 1982, 1984). We therefore assumed that such a reductase is also involved in anthocyanidin formation.

In this paper we report on the enzymatic reduction of (+)-dihydroflavonols to flavan-3,4-*cis*-diols with an enzyme preparation from flowers of *Matthiola incana*. Correlations of genotype with enzyme activity were used to evaluate the role of this enzyme in the anthocyanidin pathway.

Materials and methods

Plant materials. Plant lines used for the investigations included the cyanic lines 10 (genotype: $e^+e^+f^+f^+g^+g^+bb$), 09 and 11 through 16 (genotype: *bb*), which produce pelargonidin derivatives in the flowers, lines 04 and 08 (genotype: b^+b^+), which produce cyanidin derivatives, and the acyanic flowering lines 17b (genotype: $ee f^+f^+g^+g^+bb$), 18b (genotype: $e^+e^+ff g^+g^+bb$) and 19b^+ (genotype: $e^+e^+f^+f^+gg\ b^+b^+$) (Seyfert 1983). The action of genes *e* and *g* interferes with the anthocyanin pathway after dihydroflavonol formation (Forkmann 1977), whereas gene *f* correlates with the activity of chalcone synthase (Spribille and Forkmann 1981). Gene *b* controls the activity of flavonoid 3′-hydroxylase, which catalyses hydroxylation of naringenin to eriodictyol and dihydrokaempferol to dihydroquercetin with NADPH as cofactor (Forkmann et al. 1980). Plant material was cultivated in a greenhouse.

Chemicals. [2-^{14}C]Malonyl-CoA (2.15 GBq mmol^{-1}) was from Amersham-Buchler (Braunschweig, FRG). (+)-Dihydroflavonols and 4-coumaroyl-CoA were from our laboratory collection. (+)-3,4-*trans*-Leucocyanidin and (+)-3,4-*trans*-leucopelargonidin were prepared as described (Heller et al. 1985). (+)-3,4-*trans*-Leucodelphinidin was a gift from H. Outtrup, Valby, Denmark. The 3,4-*cis* isomers of leucoanthocyanidins were prepared according to Stafford and Lester (1984).

Labeled substrates. [4a,6,8-^{14}C]Naringenin (2.34 GBq mmol^{-1}) was prepared from [2-^{14}C]malonyl-CoA (0.78 GBq mmol^{-1})

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and 4-coumaroyl-CoA with enzyme preparations from parsley as described (Britsch et al. 1981). Hydroxylation of labeled naringenin to (+)-dihydrokaempferol was achieved with a partially purified enzyme preparation from *Petunia hybrida* cv. "Red Titan" (Benary, Hann. Münden, FRG) (unpublished). Labeled (+)-dihydrokaempferol was transformed to (+)-dihydroquercetin and (+)-dihydromyricetin by microsomal preparations from blue flowers of *Petunia hybrida* cv. "Blue Titan" (Benary, Hann. Münden) containing 3′,5′-hydroxylating activity (Stotz 1983). Separation of labeled dihydroflavonols was achieved by thin-layer chromatography (TLC) on cellulose plates with solvent system 2. After extensive drying, radioactive zones were eluted with methanol and the eluates stored in the dark at +6° C.

Buffers. The following buffers were used: A, 0.1 mol l^{-1} 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-HCl, pH 7.5, with 2.8 mmol l^{-1} 2-mercaptoethanol and 10% (v/v) glycerol; B, as A but with 0.05 mol l^{-1} Tris-HCl, pH 7.0; C, McIlvaine (0.037 mol l^{-1} citrate, 0.126 mol l^{-1} orthophosphate) pH 6.0 with 2.8 mmol l^{-1} 2-mercaptoethanol and 10% (v/v) glycerol; D, McIlvaine, double concentrated, pH 4.5–7.5; E, 0.5 mol l^{-1} Tris-HCl, pH 7.0–9.0. Buffers were made oxygen free by boiling for 5 min and subsequent cooling in an ice bath under a nitrogen atmosphere before addition of 2-mercaptoethanol.

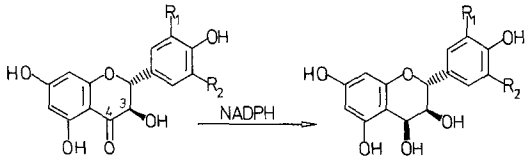
Preparation of crude extract. All steps were carried out at +4° C. Buds and flowers (1.0 g; stages 2–5; Dangelmayr et al. 1983) were homogenized in a prechilled mortar together with 0.5 g Dowex 1X2, 0.5 g quartz sand and 5 ml buffer A. The homogenate was centrifuged twice for 5 min each at 10000 *g*. The supernatant of the second centrifugation served either directly as enzyme source for assays at pH 7.5 or was subjected to buffer exchange by gel filtration on Sephadex G-50 with buffer C.

Standard enzyme assay. The incubation mixture contained in a total volume of 50 µl: radioactive substrate (167 Bq), 50 nmol unlabeled substrate, 250 nmol NADPH in 10 µl water, and 15–50 µg protein in buffer C. Incubation was carried out for 20 min at 25° C. The mixture was immediately extracted twice (50 µl and 30 µl) with ethylacetate and the extract chromatographed on a cellulose plate with solvent system 2. The plates were scanned for radioactivity and enzyme activity was determined by integration of the peak areas of the dihydroflavonol used as substrate and the respective leucoanthocyanidin formed.

Dependence of reaction on pH. Buffer of the crude extract was exchanged against buffer B on Sephadex G-50. This gave a concentration of 20 mmol l^{-1} Tris-HCl, pH 7.0 in the assay. Incubations were carried out with either additional buffer D or E.

Analytical methods. Protein was determined by a modified Lowry procedure (Sandermann and Strominger 1972) after precipitation of the protein in the presence of deoxycholate (Bensadoun and Weinstein 1976) and using citrate instead of tartrate in the colour reaction. Protein was also determined according to Bradford (1976). For both methods bovine serum albumin was used as a standard.

Thin-layer chromatography was performed on precoated cellulose plates (E. Merck, Darmstadt, FRG) in: 1) 6% acetic acid; 2) chloroform/acetic acid/water (10:9:1, by vol.); 3) *n*-butanol/acetic acid/water (6:1:2, by vol.); and 4) *n*-butanol saturated with 0.01 mol l^{-1} phosphate buffer, pH 6.8. Dihydroflavonols and leucoanthocyanidins were detected under UV-light or by spraying the plates with 0.1% aqueous fast blue B salt and subsequent exposure to ammonia vapors. Dihydroflavonols were also detected by treatment of the plates with zinc dust followed by spraying with 6 mol l^{-1} HCl (Barton 1968). Radioactivity was localized either by scanning the plates (TLC analyzer, Berthold, Wildbad, FRG) or by autoradiography (Kodak X-Omat XR-5).



I II

Fig. 1. Reaction of (+)-dihydroflavonol 4-reductase. (+)-Dihydrokaempferol, *I*, $R_1=R_2=H$; (+)-dihydroquercetin, *I*, $R_1=OH$, $R_2=H$; (+)-dihydromycricetin, *I*, $R_1=R_2=OH$; 3,4-*cis*-leucopelargonidin, *II*, $R_1=R_2=H$; 3,4-*cis*-leucocyanidin, *II*, $R_1=OH$, $R_2=H$; 3,4-*cis*-leucodelphinidin, *II*, $R_1=R_2=OH$

Table 1. R_f-values of dihydroflavonols and flavan-3,4-diols on cellulose plates

Compound		Solvent system			
		1	2	3	4
Dihydrokaempferol		0.39	0.71	0.93	0.95
Dihydroquercetin		0.39	0.44	0.87	0.89
Dihydromyricetin		0.35	0.19	0.75	0.78
Leucopelargonidin	*cis*	–	–	–	0.67
	trans	0.53	0.30	0.80	0.81
Leucocyanidin	*cis*	–	–	–	0.45
	trans	0.53	0.14	0.63	0.61
Leucodelphinidin	*cis*	–	–	–	0.28
	trans	0.48	0.04	0.50	0.44

Results

Reduction of (+)-dihydrokaempferol and identification of reaction products. Flowers from lines of *M. incana* with recessive alleles of the gene *b* were used as enzyme source, since such lines completely lack flavonoid 3′-hydroxylase activity (Forkmann et al. 1980). Cell-free extracts of flowers were prepared from early flower stages which were found to exhibit maximum activity of other enzymes involved in anthocyanin biosynthesis (Dangelmayr et al. 1983). When enzyme preparations from flowers of line 10 were incubated with (+)-[^{14}C]dihydrokaempferol in the presence of NADPH, a new radioactive compound was detected on thin-layer chromatography plates with solvent systems 1–3 which comigrated with (+)-leucopelargonidin (Fig. 1; Table 1). With these solvent systems it was not possible to decide whether the 3,4-*trans* or the 3,4-*cis* isomer had been formed, because the two

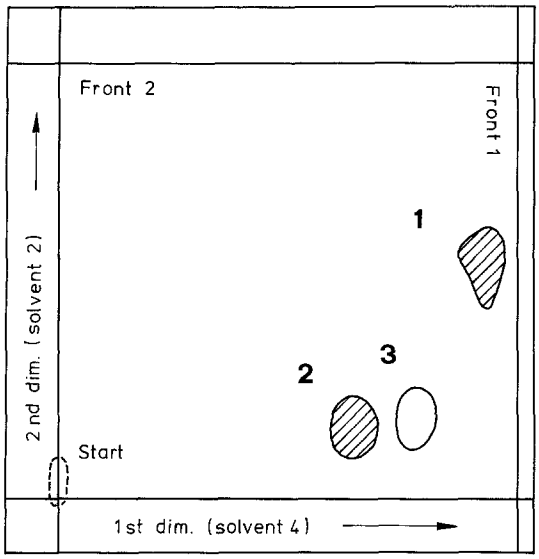


Fig. 2. Two-dimensional thin-layer chromatogram of extract from incubations of (+)-[^{14}C]dihydrokaempferol (*1*) with an enzyme preparation from flowers of *Matthiola incana* in the presence of NADPH. Before chromatography a mixture of 3,4-*cis*-(*2*) and 3,4-*trans*-(*3*) leucopelargonidin was added to the extract. *Shaded areas* show presence of radioactivity as detected by autoradiography

isomers were not separated sufficiently. A clear separation of the isomers was achieved by two-dimensional chromatography with solvent 4 in the first and solvent 2 in the second dimension (Table 1). Under these conditions the radioactive product comigrated exactly with synthetic 5,7,4′-trihydroxyflavan-3,4-*cis*-diol (Fig. 2).

Properties of reductase preparation. (+)-Dihydrokaempferol was used as substrate in these studies. Because this substrate is so valuable, suboptimal concentrations (approx. $3[S]^1_{0,5}$) were used under conditions which did not give more than 20% transformation of the substrate. Linearity of the reaction with time up to 30 min and with protein concentration up to 50 µg protein in the enzyme assay was observed at 25° C. The reaction had a broad pH-optimum between 5.5 and 6.5 with half-maximal activities at pH 5.1 and 7.2. At substrate saturation, maximal activities in extracts were between 0.2 and 0.4 mkat kg^{-1}. For (+)-dihydrokaempferol in desalted crude extracts $[S]_{0,5}$ was 0.36 mmol l^{-1}.

Flower extracts containing 10% (v/v) glycerol could be frozen in liquid nitrogen and stored at −70° C for several weeks without appreciable loss of enzyme activity. When flowers were stored under the same conditions a loss of about 50% of extractable enzyme activity was observed.

1 Substrate concentration at half-maximal velocity

Cofactor dependence. Reductase activity was dependent on NADPH. The rate with NADH (5 mmol l^{-1}) was about 50% of the rate observed with NADPH at the same concentration. Flavine-adenine dinucleotide, flavin mononucleotide and 6,7-dimethyl-5,6,7,8-tetrahydropterine either in the presence or absence of NADPH had no effect on the reaction. $[S]_{0,5}$ for NADPH in desalted crude extracts was 1.6 mmol l^{-1}.

Substrate specificity. Besides (+)-dihydrokaempferol, both (+)-dihydroquercetin and (+)-dihydromyricetin (Fig. 1) served as substrates for dihydroflavonol 4-reductase. The 3,4-*cis* isomers of (+)-leucocyanidin and (+)-leucodelphinidin (Fig. 1) were identified as products of the enzyme reaction by two-dimensional cochromatography with synthetic substances as described for leucopelargonidin. However, extractable radioactivity decreased markedly when (+)-dihydroquercetin or (+)-dihydromyricetin were used as substrate instead of (+)-dihydrokaempferol. Chromatography of the aqueous phases of the incubation mixtures on cellulose plates with solvent system 1 or 2 showed the presence of further radioactive products of yet unknown structure.

Correlation between genetic constitution of M. incana and reductase activity. Dihydroflavonol 4-reductase activity could be demonstrated not only in flower extracts of line 10 but also in all other pelargonidin-producing lines of *M. incana* (09 and 11 through 16) as well as in the cyanidin-producing lines tested (04 and 08). Moreover, enzyme activity was found to be present in enzyme preparations from the white-flowering line 18b, which is blocked in the chalcone-synthase reaction by recessive alleles of gene *f* (Spribille and Forkmann 1981). In contrast, enzyme preparations from the acyanic lines with recessive alleles of gene *e* (line 17b) or of gene *g* (line $19b^+$) were found to lack this enzyme activity completely. In contrast, activities of other enzymes involved in flavonoid biosynthesis are present in flower extracts of these two lines (Spribille and Forkmann 1981). In mixtures containing dihydroflavonol 4-reductase activity and enzyme preparations from flowers of line 17b or line $19b^+$ which lack this activity, no inhibition of reductase activity was observed. Thus, the absence of dihydroflavonol 4-reductase activity in extracts of lines 17b and $19b^+$ is not caused by an inhibitor.

Discussion

After we had shown that flavan-3,4-diols lead to anthocyanin synthesis in acyanic flowers of *M. in-*

cana (Heller et al. 1985), it was gratifying to find that extracts of such flowers do contain an enzyme for synthesis of flavan-3,4-*cis*-diols from (+)-dihydroflavonols. As has already been discussed, it has not yet been clarified whether further transformation to anthocyanidins takes place with the *cis*- or *trans*-diols (Heller et al. 1985). The properties of the (+)-dihydroflavonol 4-reductase from flowers of *M. incana* are similar to those of the enzyme from cell-suspension cultures of Douglas fir (*Pseudotsuga menziesii;* Stafford and Lester 1982, 1984). In both cases a stereospecific, NADPH-dependent reduction to flavan-3,4-*cis*-diols is observed. Whereas for the enzyme from Douglas fir a pH-optimum of 7.4 was reported, a pH-optimum around 6 was found for the enzyme from *M. incana*. In addition to (+)-dihydrokaempferol, both (+)-dihydroquercetin and (+)-dihydromyricetin (Fig. 1) were reduced to the corresponding flavan-3,4-*cis*-diols. We have recently shown that administration of (+)-dihydromyricetin to white flowers of line 18 of *M. incana* results in synthesis of delphinidin glycosides (data not shown). The in vivo incorporation of (+)-[^{14}C]dihydroquercetin into 3,4-*cis*-leucocyanidin has recently been found in mutants of barley (Kristiansen 1984).

Strong support for the role of the dihydroflavonol 4-reductase in anthocyanidin biosynthesis comes from the correlation of enzyme activity with the genotype of *M. incana*. All lines of *M. incana* with cyanic flowers contained this enzyme. In contrast, flower extracts of line 17 (recessive *ee*) contained no reductase activity, which is in agreement with the finding that such flowers could be induced to form anthocyanins by supplementation with flavan-3,4-diols but not with dihydroflavonols (Heller et al. 1985). We can therefore conclude that gene *e* is the gene for the 4-reductase. Unexpectedly, line 19 with dominant alleles of gene *e* but recessive alleles of gene *g* also lacked reductase activity. This result can be rationalized by the assumption that gene *g* exerts a regulatory function on gene *e*. Such an assumption is supported by the existence of a third allele *g'*, which leads to intermediate expression of the anthocyanin phenotype (Kappert 1957). In addition, the activity of flavonoid 3-O-glucosyltransferase is considerably reduced in the presence of recessive alleles of gene *g* and moderately reduced in genotypes with the *g'*-allele (Seyffert 1982).

Further work is concerned with purification of the reductase, the function of gene *g* as well as with the elucidation of the steps between flavan-3,4-diols and anthocyanins.

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Note added in proof: With the partially purified enzyme K_M-values for (+) dihydrokaempferol and NADPH are in the micromolar range (K. Stich).