Studies on Columnidin Biosynthesis with Flower Extracts from *Columnea hybrida*

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Columnidin, the characteristic 3-deoxyanthocyanidin of some *Columnea* species, possesses the 3′,4′-B-ring hydroxylation pattern of luteolinidin and an additional hydroxyl group at the A-ring, most likely in the 8-position. Studies on substrate specificity of chalcone synthase and flavanone 4-reductase and the demonstration of high flavonoid 3′-hydroxylase activity revealed that the 3′-hydroxyl group of the B-ring of columnidin is introduced at the flavanone stage by hydroxylation of naringenin to eriodictyol. Enzymatic hydroxylation of the A-ring, however, could neither be observed with soluble enzyme preparation nor with microsomal fraction. Most probably this step first occurs at the anthocyanidin level. Besides flavonoid 3′-hydroxylase the microsomal fraction of *Columnea* flower extracts contains flavone synthase II activity catalysing desaturation of flavanones to flavones with NADPH as co-factor. The presence of some apigenin, appreciable amounts of luteolin and of the 3′,4′-hydroxylated flavan-4-ol luteoforol in the flowers confirm the enzymatic data.

Introduction

Recently, we could demonstrate in flower extracts of *Sinningia* (syn. *Rechsteineria*) *cardinalis* the enzyme flavanone 4-reductase, which is specifically involved in the biosynthesis of the 3-deoxyanthocyanidins [1]. This rare type of anthocyanins mainly occurs in the new world species of the Gesneriaceae [2]. In general, glycosides of apigeninidin (3-deoxypelargonidin) and luteolinidin (3-deoxycyanidin) are found in these plants. But from flowers of some *Columnea* species a 3-deoxyanthocyanidin has been reported which has no counterpart in the series of the common anthocyanidins [2]. It is based on luteolinidin and possesses an additional hydroxyl group at the A-ring, most probably in the 8-position.

There are some examples for flavanones, flavones and flavonols which are hydroxylated in the 8-position of the A-ring [3]. Thus, the additional hydroxyl group can obviously be introduced very early in the biosynthetic pathway of flavonoids. For columnidin, however, it is unknown as yet at what stage of anthocyanin biosynthesis the hydroxylation in the 8-position occurs. Moreover, there is no information in what way the 3′,4′-hydroxylation pattern of the B-ring is established.

By measuring the reactions of the enzymes chalcone synthase, flavonoid 3′-hydroxylase and flavanone 4-reductase we could now show that the 3′,4′-hydroxylation pattern of the B-ring is introduced at the flavanone stage by action of flavonoid 3′-hydroxylase whereas the hydroxyl group of the A-ring in the 8-position is most probably introduced at the anthocyanidin level.

Materials and Methods

Plant material

The investigations were performed on flowers of the strain "Heklua" (HBLVA f. Gartenbau, Wien) of the Gesneriaceae *Columnea hybrida.* The plants were cultivated in a greenhouse.

Chemicals and synthesis of substrates

Naringenin, eriodictyol, dihydroquercetin, apigenin and luteolin were obtained from Roth (Karlsruhe, FRG). Dihydrokaempferol, luteolinidin, 4-coumaroyl-CoA and caffeoyl-CoA were from our laboratory collection. Apiforol and luteoforol were prepared from naringenin and eriodictyol, respectively, by borohydride reduction [4, 5]. Further work up of the reaction mixture was carried out as described earlier [1].

[2-^{14}C]Malonyl-CoA (2.22 GBq/mmol) was obtained from Amersham-Buchler (Braunschweig, FRG) and diluted to 1.03 GBq/mmol with unlabelled material from Sigma. [4a,6,8-^{14}C]naringenin (3.09 GBq/mmol) was prepared from [^{14}C]malonyl-CoA and 4-coumaroyl-CoA with enzyme preparation from parsley [6, 7]. Enzymatic transformation of labelled naringenin to labelled apigenin and eriodictyol (3.09 GBq/mmol each) was performed as described in ref. [6, 8, 9, 10].

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Preparation of enzyme extracts and enzyme assay

Crude extract and microsomal fraction were prepared from flowers and buds according to ref. [11, 12, 14]. Standard procedures were used for the demonstration and the measurement of the enzyme activity of chalcone synthase [7, 13], flavonoid 3′-hydroxylase [12, 14, 19], flavone synthase II [15, 18] and flavanone 4-reductase [1]. The assay systems for the latter three enzymes contained 0.06 nmol (167 Bq) labelled substrate (naringenin, eriodictyol or apigenin). All measurements were performed at the range of protein and time linearity.

Analytical methods

The determination of the protein content and the identification of substrates and products in the reaction mixture was performed as described in ref. [1, 11].

Results and Discussion

Columnidin possesses the 3′-4′-hydroxylation pattern of luteolinidin. At first, we therefore studied the question in what way the hydroxylation in the 3′-position is achieved.

In general, there are two different possibilities. The 3′-hydroxyl group is either introduced by the use of caffeoyl-CoA instead of 4-coumaroyl-CoA during synthesis of the flavonoid skeleton or by hydroxylation of flavonoid compounds in the 3′-position [11, 13]. The first reaction is catalysed by the key enzyme of flavonoid biosynthesis chalcone synthase, while the enzyme flavonoid 3′-hydroxylase governs the second possibility.

Our studies of both enzyme reactions led to the following results. When flower extracts from *Columnea* were incubated with [^{14}C]malonyl-CoA and 4-coumaroyl-CoA, the formation of one radioactive reaction product was observed, which was identified as naringenin by co-chromatography in four different solvent systems (Table I). Highest naringenin formation was found at pH 7.8 (Table II). When caffeoyl-CoA was used instead of 4-coumaroyl-CoA at this pH relatively small amounts of a reaction product could be observed. This product corresponded to eriodictyol in four solvent systems (Table I). Eriodictyol formation could be improved by incubation of the reaction mixtures at lower pH with a maximum at pH 6.8 (Table II). But even at this pH prod-

Table I. R_f-values (× 100) of substrates and products of the enzymatic reactions.

Compound	Solvent systems			
	CAW	H_2O	30% Acetic acid	BAW
Naringenin	85	15	60	94
Eriodictyol	64	8	55	88
Apigenin	78	–	20	87
Luteolin	44	–	11	81
Apiforol	46	22	–	–
Luteoforol	18	12	–	–

CAW, Chloroform/acetic acid/water (10:9:1, v/v/v).
BAW, *n*-Butanol/acetic acid/water (4:1:5, v/v/v, upper phase).
–, not used.

Table II. The conversion rate of suitable substrates to single products observed with the four enzymes studied.

Enzyme reaction	Substrate	Product	Specific activity* (μkat/kg protein)
Chalcone synthase with Chalkone isomerase	4-coumaroyl-CoA (pH 6.8)	naringenin	0.38
	4-coumaroyl-CoA (pH 7.8)	naringenin	0.42
	caffeoyl-CoA (pH 6.8)	eriodictyol	0.25
	caffeoyl-CoA (pH 7.8)		0.09
Flavonoid 3′-hydroxylase	apigenin	luteolin	3.10
Flavone synthase II	eriodictyol	luteolin	0.83
Flavanone 4-reductase	eriodictyol	luteolin	0.89

* All measurements were performed in triplicate. The S.E. values are < 0.01 μkat/kg protein.

uct formation from 4-coumaroyl-CoA was clearly higher than from caffeoyl-CoA (Table II). And in incubations at pH 6.8 containing a mixture (1:1) of both activated cinnamic acids only the formation of naringenin was observed. These results are similar to reports on substrate specificity of chalcone synthase from a range of other plants [11, 13, 16, 17]. In the most of these cases, the 3′-hydroxyl group of the B-ring was found to be introduced only in the presence of the enzyme flavonoid 3′-hydroxylase. This enzyme is known to be localized in the microsomal fraction and the reaction requires NADPH as cofactor [9, 12, 14, 16, 19]. Respective incubations of [^{14}C]naringenin with microsomal fraction from *Columnea* flower extracts in the presence of NADPH led to the formation of three radioactive products in approximately equal amounts. They were identified as the 3′,4′-hydroxylated flavanone eriodictyol and the flavones apigenin (4′-OH) and luteolin (3′,4′-OH) by co-chromatography with authentic samples in different solvent systems (Table I).

Thus, not only 3′-hydroxylation but also enzymatic conversion of flavanones to flavones occurred in the reaction mixture. The latter reaction is due to the presence of flavone synthase II activity, which has the same subcellular localization and co-factor requirement as flavonoid 3′-hydroxylase [15, 18]. From naringenin as substrate eriodictyol and apigenin are directly formed by action of flavonoid 3′-hydroxylase and flavone synthase II, respectively, whereas the formation of luteolin is due to both enzyme activities. The use of suitable substrates revealed that luteolin can be formed by 3′-hydroxylation of apigenin as well as by desaturation of eriodictyol. Moreover, apigenin and eriodictyol, respectively, as substrate allowed a separate measurement of both enzyme activities (Table II). In agreement with the pH-optima for the two enzymes from other plants [11, 12, 14, 15, 16, 18, 19] highest hydroxylation of apigenin to luteolin was found at pH 7.5, while the conversion of eriodictyol to luteolin was optimal at pH 7.0. Luteolinidin, the basic compound of columnidin, was recently found to be synthesized from eriodictyol *via* the flavan 4-ol luteoforol. An enzyme (flavanone 4-reductase) catalysing the reduction of flavanones to flavan 4-ols was first found in flower extracts of *Sinningia cardinalis* [1]. Now we could also demonstrate flavanone 4-reductase activity in the flower extracts of *Columnea*. As in *Sinningia*, the enzyme from *Columnea* is localized in the soluble fraction and the reaction needs NADPH as co-factor. Moreover, the flavanone 4-reductases from *Columnea* (pH 5.5) and *Sinningia* (pH 5.8) show similar low pH-optima. When [^{14}C]naringenin was incubated with crude flower extract in the presence of NADPH the formation of three radioactive products was observed. The products were identified as the flavan 4-ols apiforol (4′-OH) and luteoforol (3′,4′-OH), respectively, and as the flavanone eriodictyol (3′,4′-OH) by co-chromatography with authentic samples in different solvent systems (Table I) and in the case of the flavan 4-ols, by the conversion to the respective anthocyanidins as described earlier [1]. The three compounds are obviously formed by a combined action of flavanone 4-reductase and flavonoid 3′-hydroxylase, which is also active in crude flower extracts and at the low pH of the reaction mixture. But flavonoid 3′-hydroxylase activity could easily be excluded by the use of eriodictyol as substrate for the reduction reaction. In that case only the formation of luteoforol occurred (Table II).

The readily reduction of eriodictyol to luteoforol with *Columnea* flower extracts and the presence of relatively high flavonoid 3′-hydroxylase activity suggest that the 3′-hydroxyl group of columnidin is most likely introduced at the flavanone stage by the hydroxylation of naringenin to eriodictyol. A similar situation was found for the synthesis of luteolinidin in *Sinningia* flowers [11]. All our efforts, however, to demonstrate enzymatic hydroxylation of the A-ring of flavonoids failed. Such a hydroxylation reaction was neither observed with microsomal fraction and NADPH as co-factor nor with crude extracts in the presence of NADPH or of the co-factor system for dioxygenase reactions on flavonoids [10, 12, 6] 2-oxoglutarate, Fe^{2+} and ascorbate. In the latter case only the formation of dihydroflavonols from flavanones was observed. We therefore conclude that the hydroxyl group on the A-ring of columnidin is introduced at the level of 3-deoxyanthocyanidins.

Analytical work clearly supports the enzymatic results. Thus, besides some apigenin glycosides appreciable amounts of luteolin glycosides were found to be present in the flowers. But a luteolin derivative with an additional hydroxyl group at the A-ring could not be observed. Moreover, when ether extracts of the flowers were transferred to methanol, the ether evaporated and the methanol residue heated with 2 N HCl for 15 min the solution became cherry red. Both the spectral and the chromatographic data

of the cherry red compound corresponded to those for luteolinidin [1]. This result indicates the presence of luteoforol in the flower extracts, while the respective intermediate with the hydroxylation pattern of columnidin is obviously not present.

Further work will be concerned with the preparation of labelled luteolinidin and its use as substrate for the demonstration of a specific A-ring hydroxylase.

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