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DIFFERENTIATION OF METABOLIC PATHWAYS IN THE UMBEL OF *DAUCUS CAROTA*

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Key Word Index—*Daucus carota*; Apiaceae; anthocyanin; chalcone isomerase; chalcone synthase; PAL.

Abstract—The extractable activities of PAL, chalcone synthase and chalcone isomerase of white and coloured petals of the inflorescence of the umbel of wild carrot (*Daucus carota*) were compared. PAL showed no difference in both types of petals, whereas the activity of chalcone synthase, the enzyme responsible for the synthesis of the flavonoid skeleton, was nearly absent in white flowers. By contrast, the coloured flowers from the centre of the inflorescence contained very high activities of this enzyme. Chalcone isomerase, which catalyses the subsequent reaction in this pathway, was present in appreciable activities in both types of flowers, but in white flowers the activity was clearly lower. These results suggest that chalcone synthase is rate limiting for the synthesis of cyanidin in white flowers of the umbel. If caffeoyl-CoA was offered as a substrate instead of 4-coumaroyl-CoA significant incorporation into eriodictyol was observed; the pH optimum was 6.3. Both activities were strongly dependent on the presence of bovine serum albumin in the assay. The umbel of the wild carrot is an interesting system for studying differentiation at a metabolic level.

INTRODUCTION

The umbel of the wild carrot displays a morphological, anatomical, and metabolic differentiation. The flowers of secondary umbels from the margin differ in their petal morphology from the flowers of the secondary umbel in the centre of the inflorescence. Concomitantly, an anatomical differentiation can be observed: the petals of the central flowers are more fleshy and possess larger vacuoles compared to the marginal flowers. In addition to this morphological and anatomical differentiation, a metabolic differentiation takes place. In the centre of the inflorescence one or a few flowers are coloured dark violet by anthocyanins. The only aglycone present in these flowers is cyanidin [1]. One prerequisite for the synthesis of anthocyanins is the production of phenylpropane compounds; the key enzyme of this pathway is phenylalanine ammonia-lyase (for review see [2]). The formation of the skeleton of flavonoids is catalysed by chalcone synthase which represents the key enzyme [3] of flavonoid biosynthesis. The isomerisation of chalcones to flavanones is catalysed by chalcone isomerase [4].

In the present investigation, enzymatic studies were carried out in order to investigate this differentiation at the level of extractable catalytic activities. The enzyme activities from anthocyanin-free and anthocyanin-containing flowers were compared. In white flowers from the margin of the inflorescence no chalcone synthase activity was detectable, whereas in anthocyanin-containing flowers from the centre high activities of CHS were present. The other enzymes, i.e. PAL and CHI, were present in both types of petals. On the basis of these data the regulatory role of chalcone synthase during this metabolic differentiation of the carrot inflorescence is discussed.

RESULTS

Extractable activity of PAL

The extractable activity of PAL was determined in both types of flowers: anthocyanin-free ones from the margin of the inflorescence and anthocyanin-containing ones from the secondary umbel in the centre. As shown in Fig. 1, there was no marked difference in terms of the extractable specific activity from the two types of flower, although it was slightly higher in the case of the white petals.

Extractable activity of CHS

In extracts from anthocyanin-containing flowers, CHS had a high specific activity (0.2–1.0 μkat/kg) when 4-coumaroyl-CoA and [^{14}C]malonyl-CoA were used as substrates. In contrast, the activity in extracts from anthocyanin-free flowers was hardly detectable (Fig. 1). For these determinations two methods were applied [3], the total radioactivity in the ethyl acetate phase was determined and compared with the radioactivities of the flavanone-spots after chromatography on TLC-plates (Table 1). The isomeric chalcones could not be ascertained due to presence of CHI and spontaneous cyclization [5]. The difference between the two methods results from products which are not flavanones and the self-absorbance by cellulose during liquid scintillation counting. Under standard conditions no additional products

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Abbreviations: PAL, phenylalanine ammonia-lyase (EC 4.3.1.5.); CHS, chalcone synthase; CHI, chalcone isomerase (EC 5.5.1.6.); BSA, bovine serum albumin.

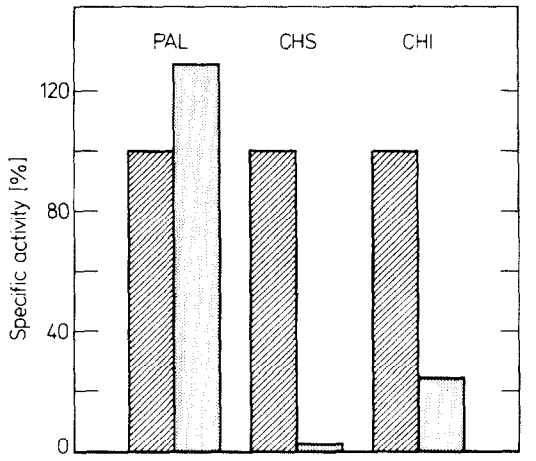

Fig. 1. Relative amounts of extractable specific enzyme activities in flowers from *D. carota*. ▨, white flowers from the margin of the inflorescence; ▩, anthocyanin-containing flowers from the centre (100%). PAL (100%) = 55.6 μkat/kg; CHS (100%) = 0.62 μkat/kg; CHI (100%) = 437 ΔA_{385}/sec · kg.

Table 1. Product formation in a standard assay for CHS under various conditions using extracts from anthocyanin-containing flowers with a specific activity of 0.2 μkat/kg

Assay conditions	Total radioactivity (cpm)	Naringenin (cpm)
Complete	3400	1587
Boiled extract	20	n.d.*
-BSA	145	27
-2-Mercaptoethanol	3055	1930
-4-Coumaroyl-CoA	170	n.d.

*Not detectable.

besides flavanones were detectable on the radiochromatograms. Therefore, for calculations of the specific activity of CHS only the radioactivity incorporated into flavanone was used. With boiled extracts or in the absence of cinnamoyl-CoA no activities could be measured (Table 1). The activity of CHS strongly depended on the presence of BSA in the assay mixture. The data in Table 1 also show that 2-mercaptoethanol, in the concentrations used, had only slight effects on the activity and the nature of the main product, but it seemed to promote the incorporation of label into side products which are not flavanones.

Substrate specificity of CHS

These experiments were carried out with extracts derived from anthocyanin-containing flowers. Using 4-coumaroyl-CoA as a substrate, the main product had R_f values which were identical to those of naringenin (Table 2). Maximum naringenin (5,7,4'-trihydroxyflavanone) production was obtained at pH 7.5. This pH-optimum was very sharp and similar with both methods (data not shown). If caffeoyl-CoA was present in the assay

Table 2. R_f values of products formed during incubation with CHS. TLC on cellulose developed in different solvent systems

Compound	R_f-values in solvent system I	II	III
Naringenin	0.22	0.57	0.72
Eriodictyol	0.19	0.52	0.43

instead of 4-coumaroyl-CoA, a compound was synthesized with an R_f value which corresponded to that of eriodictyol (Table 2). Caffeoyl-CoA and eriodictyol (5,7,3',4'-tetrahydroxyflavanone) possess the same substitution pattern with respect to cyanidin which is the only aglycone present in flowers of *Daucus carota*. The pH-optimum of this reaction differs significantly from that of naringenin synthesis *in vitro* and was found to be 6.3 as determined with both methods.

Sometimes under optimum conditions for eriodictyol production an additional labelled compound appeared after chromatography in solvent system III, contrary to what had been found during naringenin synthesis. This compound was not identified. In Fig. 2, CHS activities with 4-coumaroyl-CoA and caffeoyl-CoA as substrates were compared at pH's of 6.3 and 7.5. From this it was clear that the substrate specificity of the enzyme depended on the pH of the assay. At pH 7.5, CHS had a high specificity for 4-coumaroyl-CoA, whereas at pH 6.3, both substrates were accepted by CHS with similiar but lower specific activity.

With extracts from anthocyanin-free petals under all assay conditions tested, no significant incorporation could be detected on the radiochromatograms. A very low incorporation of radioactivity into a spot corresponding

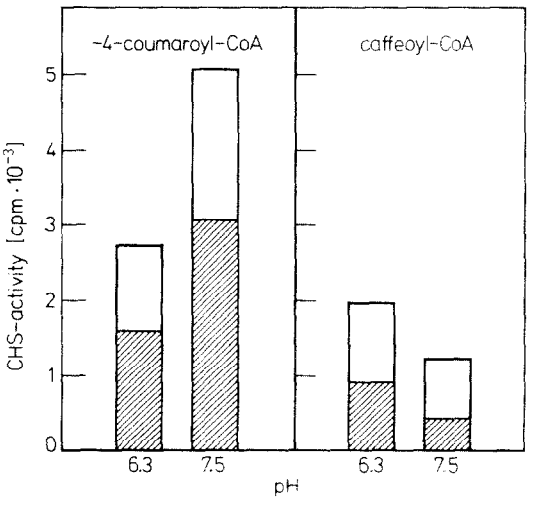

Fig. 2. Comparison of extractable CHS activities with 4-coumaroyl-CoA and caffeoyl-CoA as substrates at pH 6.3 and 7.5 respectively. □, total radioactivity extracted with EtOAc; ▨, radioactivity incorporated into flavanone.

with naringenin was found only at very high protein concentrations. The maximum specific activity of 12 nkat/kg (Fig. 1) seemed to be beyond the limit of determination. In summary, the experiments clearly demonstrated that anthocyanin-free flowers do not contain any appreciable CHS activity (Fig. 1).

Extractable activity of CHI

The incubation of extracts with malonyl-CoA and either 4-coumaroyl-CoA or caffeoyl-CoA led to the formation of naringenin and eriodictyol respectively. The isomerization of chalcones to flavanones was primarily the result of the chalcone isomerase present in these enzyme preparations. Therefore, the extractable activities of this enzyme from white and coloured flowers were compared with respect to CHI. The enzyme was present in both types of flowers; however, the specific activity of CHI in anthocyanin-free cells was about 24% of the activity determined in anthocyanin-containing cells (Fig. 1).

In Fig. 1, all these data are summarized: PAL showed no prominent differences if extracts from white and coloured flowers were compared with respect to their specific activities. However, CHI had lower activities in extracts derived from white flowers. In contrast, chalcone synthase seemed to be absent in anthocyanin-free petals or at least its activity was reduced dramatically.

DISCUSSION

Based on results of isotope-labelling experiments it [5, 6], has been assumed that the formations of chalcones as the primary C_{15} intermediates play an important role in flavonoid biosynthesis. This initial step is catalysed by chalcone synthase, which has been frequently referred to as a key enzyme of this pathway. The inflorescence of wild carrots exhibit a differentiation in anthocyanin accumulation; the flowers in the centre are often coloured by anthocyanins. This observation prompted us to compare the CHS activity in anthocyanin-free and anthocyanin-containing petals from the umbel of *D. carota*. The activity of CHS is extremely different in both types of petals. In flowers from the centre, high specific activities for CHS are observed, whereas in anthocyanin-free petals from the margin of the inflorescence, the activity of CHS is at the limit of determination.

Under the assay conditions used in the present work, only flavanones are produced. This is due either to spontaneous isomerization of chalcones to flavanones or to the activity of chalcone isomerase present in the protein extracts [5]. When caffeoyl-CoA is used as a substrate instead of 4-coumaroyl-CoA, eriodictyol is formed. The activity is only 30% of that obtained with 4-coumaroyl-CoA. In *Haplopappus gracilis* and parsley cell cultures, caffeoyl-CoA is accepted to a higher degree [7]. The pH-optima of both reactions are different with respect to the incorporation of the two substrates. Enzyme extracts from cell cultures of parsley and *Haplopappus* [7] also exhibit different pH-optima as well as preparations from flowers of *Dianthus caryophyllus* [8]. In flowers of *Matthiola incana* [9] and *Antirrhinum majus* [10] only 4-coumaroyl-CoA is accepted by the enzyme at any pH. By contrast, in tulip anthers [11] 4-coumaroyl-CoA, caffeoyl-CoA and feruloyl-CoA are all incorporated into the corresponding flavanone. It is not yet clear whether caffeoyl-CoA or other cinnamoyl-CoA thioesters are physiological substrates of CHS in general.

Bovine serum albumin has a stabilizing effect on CHS [3]. The CHS activity is enhanced by the presence of BSA in the assay as already described for extracts from parsley and *Haplopappus* [7]. This stabilizing effect may be due to lowered protease activity as well as the protection of the catalytic centre of the enzyme. The carrot inflorescence also exhibits a differentiation on a metabolic level. For the most part mariginal petals of adult umbels do not accumulate anthocyanins, and the extractable activity of CHS, referred to as the key enzyme of the flavonoid pathway, is extremely low. On the contrary, PAL, the key enzyme of the general phenylpropanoid pathway, is present in both types of petals in similar activities. The very low CHS activity in white petals seems to be rate limiting for the biosynthesis of anthocyanins. In callus cells from an anthocyanin-producing carrot root, a similar situation can be induced after treatment of the cells with gibberellic acid [1]. In both cases anthocyanin biosynthesis seems to be controlled on a regulatory level, perhaps by regulation of gene expression. At least in the case of the carrot inflorescence, the different CHS activities cannot be explained by different structural genes for the enzyme. The inflorescence of *Daucus carota* is an interesting example for differentiation at a metabolic level.

EXPERIMENTAL

Plant material. Flowers of wild carrots (*D. carota L.*) were collected near Tübingen. Coloured flowers from the centre and white flowers from the margin of the inflorescence were removed, frozen in liquid N_2, and stored at $-70°$.

Materials. [2-^{14}C]Malonyl-CoA (59 mCi/mmol) was purchased from Amersham Buchler (Braunschweig). It was diluted with unlabelled malonyl-CoA from Sigma (München) to a final sp. act. of 29.5 mCi/mmol. 4-Coumaroyl-CoA and caffeoyl-CoA were synthesized according to [12] using hydroxysuccinimide-esters as intermediates. The resulting cinnamoyl-CoA-thioesters were separated by TLC on cellulose layers and identified by their UV spectra. 4-Coumaric acid, caffeic acid, naringenin (5,7,4'-trihydroxyflavanone) and eriodictyol (5,7,3',4'-tetrahydroxy-flavanone) were obtained from Roth (Karlsruhe). Naringenin-chalcone (4,2',4',6'-tetrahydroxy-chalcone) was a gift from Prof. Hahlbrock (Freiburg).

Buffers. The following buffers were used: (A) 100 mM KPi, pH 8; (B) 100 mM KPi containing 1.4 mM 2-mercaptoethanol, pH 8; (C) 100 mM KPi containing 1.4 mM 2-mercaptoethanol and 1% (w/v), BSA, pH 7.5; (D) 100 mM H_3BO_3–$Na_2B_4O_7$, pH 8.8.

Enzyme preparations. All steps were carried out at 4°. PAL was prepared with buffer (D) as described in [13]. CHS preparation was based on [9]. 0.5 g frozen flowers were homogenized in a mortar together with 2 g Dowex 1 × 2, 3 g quartz sand and 8 ml of buffer (B). The ion exchanger was equilibrated with buffer (A). Centrifugation at 25 000 *g* for 15 min and filtration through glass wool yielded the enzyme extract for CHS assays.

CHI was tested after precipitation with $(NH_4)_2SO_4$ (0–85% saturation). After centrifugation at 38 000 *g* for 15 min the pellet was washed and diluted in buffer (B). The resulting protein soln was used to test CHI activity.

Enzyme assays. PAL activities were determined as described in [13]. CHS was assayed with some modification according to [3]. Two methods were applied. The assay mixture contained in a total vol. of 210 μl: 150 μl buffer (C), 50 μl protein extract (18–45 μg protein), 5 μl 4-coumaroyl-CoA or caffeoyl-CoA (1 nmol) and 5 μl [2-^{14}C]malonyl-CoA (2.12 nmol,

139 000 dpm). After incubation for 60 min at 30° the reaction was stopped by adding 10 μg naringenin or eriodictyol dissolved in 10 μl MeOH. The reaction products were separated from malonyl-CoA by extraction with 250 μl EtOAc. An aliquot of 50 μl was counted in 4 ml Unisolve 1 (Zinsser, Frankfurt) for radioactivity. In order to determine the incorporation of label into flavanones exactly, aliquots (150 μl) of the EtOAc phase were subjected to TLC (Cellulose, Merck, Darmstadt) in different solvent systems: (I) 15% (w/v) EtOH, (II) 30% (w/v) HOAc, (III) $CHCl_3$–HOAc–H_2O (10:9:1). The flavanone spots were detected by UV light (336 nm) and by scanning with a radiochromatogram scanner (Berthold, Wildbad, W. Germany). Labelled spots corresponding to authentic flavanones were scraped off and counted for radioactivity in Unisolve 1 (4 ml). The sp. act. of CHS was calculated only on the basis of radioactivity incorporated into flavanone.

The CHI assay was performed and slightly modified as described in [14]. The reaction mixture contained in a total vol. of 1 ml: 970 μl of buffer (B), 10 μl naringenin-chalcone dissolved in ethyleneglycolmonomethylether (1 mg/ml) and 20 μl protein extract (15–30 μg protein). The decrease in substrate was determined at 385 nm within 1 min at 30°. The values were corrected for non-enzymatic reaction, determined without protein extracts.

Protein determination. Protein concs were determined according to [15].

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