Phytochemistry, 1978, Vol. 17, pp 183–186 Pergamon Press Printed in England

FLAVANONE SYNTHASE FROM CELL SUSPENSION CULTURES OF *HAPLOPAPPUS GRACILIS* AND COMPARISON WITH THE SYNTHASE FROM PARSLEY

NABIEL A. M. SALEH, HANSJÖRG FRITSCH, FRITZ KREUZALER and HANS GRISEBACH

Lehrstuhl für Biochemie der Pflanzen, Institut für Biologie II der Universität Freiburg, Schänzlestr. 1, D-7800 Freiburg i.Br., Germany

(*Received* 20 *June* 1977)

Key Word Index—*Haplopappus gracilis*; Compositae; *Petroselinum crispum*; Umbelliferae; cell culture; biosynthesis; flavonoids; flavanone synthase.

Abstract—Flavanone synthase was isolated and purified *ca* 62-fold from cell suspension cultures of *Haplopappus gracilis*. The enzyme preparation catalyzed the formation of naringenin from 4-coumaryl-CoA and malonyl-CoA with a pH optimum of *ca* 8. The same enzyme was also capable of synthesizing eriodictyol from caffeyl-CoA and malonyl-CoA; in this case the pH optimum lay between 6.5 and 7. The homogeneous flavanone synthase from cell suspension cultures of parsley showed the same dependence of the pH optimum on the nature of the cinnamyl-CoA. It can be concluded that both naringenin and eriodictyol are natural products of the synthase reaction.

INTRODUCTION

Flavanone synthase catalyzes the formation of naringenin from 4-coumaryl-CoA and malonyl-CoA [1]. This key enzyme of flavonoid biosynthesis has been purified from cell suspension cultures of parsley [1]. It was also found in cell cultures of *Haplopappus gracilis* [2] and in anthers of *Tulipa* cv. Apeldoorn [3]. Studies on the substrate specificity of the synthase from parsley cell cultures led us to conclude that naringenin is the only natural product of the synthase reaction [4]. At the pH optimum for naringenin formation (pH 8), only a very small amount of eriodictyol (5,7,3′,4′-tetrahydroxyflavanone) was formed with caffeyl-CoA as substrate, besides large amounts of 'release products' with only one or two C_2-units attached to the 4-coumaryl residue [4]. In contrast, it appeared that with the crude synthase from *Tulipa* a substantial amount of eriodictyol was formed from caffeyl-CoA, although quantitative data are not available [3].

In this paper we report on the substrate specificity of a partially purified synthase from cell cultures of *H. gracilis*. Since it was found that the pH optima for 4-coumaryl-CoA and caffeyl-CoA are different, we have reinvestigated the specificity of the parsley enzyme for these substrates.

RESULTS

Purification of the synthase from H. gracilis *cell cultures*

H. gracilis cells were extracted with potassium phosphate buffer and the crude extract was treated with Dowex ion-exchanger to remove phenolic compounds. The synthase was then partially purified by ammonium sulfate fractionation followed by chromatography on DEAE-cellulose. The purification procedure gave a preparation having a specific activity 62 times greater than that of the extract after Dowex treatment (Table 1). Because of the high instability of the enzyme (see below), further purification has so far not been possible.

Table 1. Partial purification of flavanone synthase. Specific activity was calculated from the amount of naringenin formed

Purification step	Protein (mg)	Specific activity (μkat/kg)	Purification (-fold)	Recovery (%)
Dowex 1 × 2 supernatant	326	0.22	1	100
$(NH_4)_2SO_4$ fractionation Sephadex G-25	20	3.0	14	82
DEAE-cellulose	0.7	13.8	63	13

Product identification

Authentic samples of naringenin, eriodictyol and homoeriodictyol (5,7,4′-trihydroxy-3′-methoxyflavanone) were used as unlabelled carriers in PC. The radioactivity corresponded in each case with the expected product in solvent systems 1–4. The substituted benzalacetones which appear as side products were also available as reference compounds [4]. For the styrylpyrones [4] reference samples were not available, but the compounds formed corresponded in R_f-values and fluorescence in UV light to those reported earlier for these compounds [4].

Stability of synthase from H. gracilis

In buffer at 4° the enzyme lost about half its activity after 40 hr. Addition of 10 vol.% glycerol or ethyleneglycol decreased enzyme activity. In contrast, bovine serum albumin at a concentration of 5 mg/ml enhanced

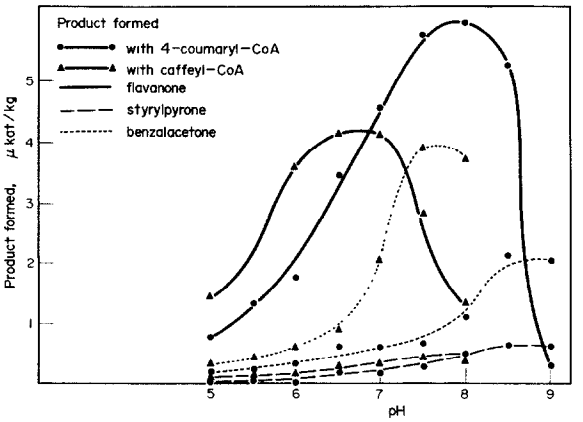

Fig. 1. Dependence of synthase activity on pH. Enzyme from *H. gracilis* cell cultures. Buffers used were: potassium phosphate–citric acid (pH 5–6); potassium phosphate (pH 6.5–8.5); glycine–NaOH (pH 9).

activity about two fold and gave good stabilization. In the presence of bovine serum albumin the enzyme could be stored for 12 days at −20° without loss of activity. At 4° about 50% of activity was lost during this time. Bovine serum albumin was therefore routinely added during enzyme purification and in the enzyme assays.

pH optimum

With *p*-coumaryl-CoA as substrate the pH optimum for naringenin synthesis was about 8. In contrast, when caffeyl-CoA was used as substrate the pH optimum for eriodictyol formation was between 6.5 and 7 (Fig. 1). With the same concentration of 4-coumaryl-CoA, naringenin formation at pH 8 was about 1.4 times higher than eriodictyol formation at pH 6.5–7.

Formation of release products

The ratio of flavanone formation in relation to the release products [4] is shown in Table 2. The absolute amount of homoeriodictyol formed with ferulyl-CoA as substrate was less than 10% of the yield of naringenin with 4-coumaryl-CoA under the same conditions. At pH values of *ca* 5–6.5, a new by-product was observed with all 3 cinnamyl-CoA esters. This unknown product had a slightly higher R_f (0.28) than eriodictyol (0.18) in 15% ethanol and showed some overlapping with naringenin in this solvent. It can be seen from Fig. 1 that benzalacetone formation with caffeyl-CoA as substrate was high at pH 7.5 but much less at pH 6.5, where eriodictyol synthesis is optimal.

Table 2. Relative amounts of release products of synthase from *H. gracilis*

Substrate	pH optimum	Ratio of flavanone/styrylpyrone/benzalacetone
4-Coumaryl-CoA	8.0	100:9:14
Caffeyl-CoA	6.5	100:11:40
Ferulyl-CoA	6.5	100:61:104

Table 3. Effect of products and of ascorbate on synthase from *H. gracilis*

Addition	Addition to enzyme assay (nmol)	Activity of control with *p*-Coumaryl-CoA*	Caffeyl-CoA†
None	—	100	100
Naringenin	1.8	72	58
	18	14	18
Eriodictyol	1.74	90	85
	17.4	84	38
Potassium ascorbate	115	100	121

*Enzyme assay at pH 8 (for details, see Experimental).
†Enzyme assay at pH 6.5.

Product inhibition and influence of ascorbate

The synthase reaction was inhibited by addition of the respective flavanones to the enzyme incubation (Table 3). Addition of potassium ascorbate enhanced the yield of eriodictyol slightly but had no effect on the yield of naringenin.

pH optima for synthase from parsley cell cultures

Product formation with 4-coumaryl-, caffeyl- and ferulyl-CoA esters was investigated at different pHs with a crude synthase preparation from parsley cell cultures and with an enzyme purified to apparent homogeneity [5]. The results, which were essentially similar with the two enzyme preparations, are shown in Fig. 2. As in the case of the *H. gracilis* enzyme eriodictyol formation from caffeyl-CoA was low at pH 8, the pH optimum for naringenin formation from 4-coumaryl-CoA, but increased toward lower pH values and showed a maximum around pH 6.5. The ratio of naringenin to eriodictyol at the corresponding pH optima was about 1.4. With ferulyl-CoA a small yield of a radioactive product was formed at lower pH values which had the same R_f in solvent systems 1–4 as homoeriodictyol. However, the release products also predominated at pH 6–6.5, as has been reported previously [4].

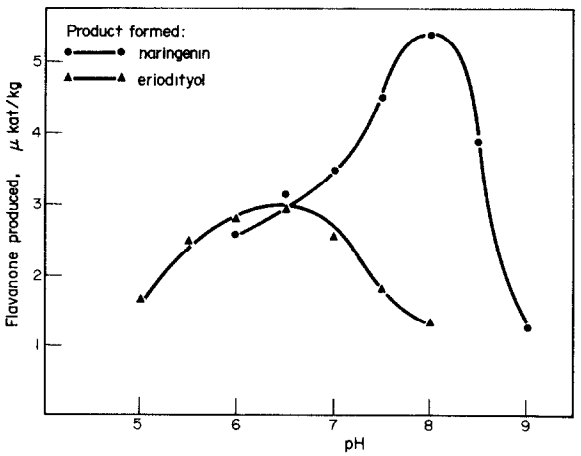

Fig. 2. Dependence of synthase activity on pH. Enzyme from parsley cell cultures. Buffers see Fig. 1.

DISCUSSION

The synthase from *H. gracilis* cell cultures is similar in its properties to the enzyme from parsley cell cultures. The relatively low activity in the *H. gracilis* cells and the lability of the synthase has so far prevented further purification. The release products identified with the parsley enzyme [4] were also formed with the *Haplopappus* synthase. The enzyme also showed strong product inhibition. Lineweaver–Burk plots were linear only over a small range and did not allow determination of Michaelis constants. This is not surprising since several products are formed in the *in vitro* reaction. K_m-values for 4-coumaryl-CoA (1.6 μM) and malonyl-CoA (35 μM) were obtained for the parsley synthase [4]. It could be estimated that the K_m-values for 4-coumaryl-CoA and caffeyl-CoA were also in the μmolar range for the *H. gracilis* enzyme. Essentially the same results were obtained with a cell-free extract of *H. gracilis* (Dowex supernatant) and with the partially purified synthase. Therefore there was no indication of more than one synthase in the *H. gracilis* cell cultures.

The most important finding is that the enzymes from both *H. gracilis* and parsley cell cultures have different pH optima for naringenin formation from 4-coumaryl-CoA and eriodictyol formation from caffeyl-CoA. Naringenin synthesis has a pH optimum at 8 and eriodictyol synthesis at about 6.5 to 7. The fact that naringenin formation exceeds eriodictyol synthesis at their respective pH optima only by a factor of about 1.4 indicates that eriodictyol is a physiological product at about neutral pH in the cell. Our earlier suggestion that naringenin is the only natural product of the synthase reaction [4] is therefore no longer tenable. One can rather conclude that both 4-coumaryl-CoA and caffeyl-CoA are physiological substrates for the synthase. Both CoA esters can be formed with the corresponding CoA-ligases from parsely [7] and *H. gracilis* cell cultures (unpublished results).

Besides concentration of the substrates, the pH at the site of synthase reaction could play a role in determining how much naringenin or eriodictyol is formed. In contrast, feruloyl-CoA seems to be not a physiological substrate, and it has already been pointed out that enzymes for methylation of flavanoids in the B-ring leading to the 3′-methoxy-4-hydroxy substitution pattern [4] are known.

A dependence of the pH optimum for phenylalanine ammonia lyase on the nature of the *p*-substituent of phenylalanine has recently been reported [6]. While the pH optimum for phenylalanine is 8.7, that for tyrosine, for example, is 7.7.

It would be interesting to see whether flavanone synthases from other plants have a similar pH-dependence for the conversion of cinnamyl-CoA substrates.

EXPERIMENTAL

Cell cultures. Cell suspension cultures of *H. gracilis* were grown in B-5 medium at 25–26° in the dark as previously described [8, 9]. Illumination was by a light field of light blue fluorescent tubes (Philips 40W/18) intensity *ca* 25000 lx. Seven-day-old cells (40 ml suspension) were transferred to fresh medium (400 ml in 2 l. flasks) and then kept in the light. Cells were harvested after 44–48 hr by filtration.

Chromatographic methods. PC was carried out on Schleicher Schüll 2043b using (1) 15% EtOH; (2) 8% HOAc; (3) 30% HOAc; (4) *n*-BuOH–HOAc–H_2O (4:1:5). For identification of the release products the solvents previously described were used [4].

Synthesis of CoA-thiol esters of substituted cinnamic acids. The CoA-thiol esters were synthesised with a butyryl-CoA synthetase from beef liver (EC 6.2.1.2) according to ref. [4].

Synthesis of benzalacetones. The benzalacetones were synthesised from the corresponding benzaldehydes by condensation with Me_2CO as in refs [4, 10, 11].

Materials. 2-^{14}C malonyl-CoA (21.4 mCi/mmol) (New England Nuclear, Boston), naringenin, 4-coumaric, caffeic and ferulic acids were from our laboratory collection. Eriodictyol was a gift from Dr. B. Janistyn, Freiburg, and homoeriodictyol a gift from Prof. Dr. H. Wagner, Munich.

Buffer solns. Buffer solns used were: (A) 100 mM K phosphate buffer, pH 7.6, containing 14.5 mM 2-mercaptoethanol, (B) 50 mM K phosphate buffer, pH 7.6, containing 1.45 mM 2-mercaptoethanol.

Enzyme purification. All steps were carried out at 4° *H. gracilis* cells (80 g wet wt) were filtered, washed with H_2O and homogenised in a mortar with 8 g PVP, 40 g quartz sand and 180 ml buffer A. The homogenised mixture was centrifuged for 20 min at 10000 *g*. To the supernatant liquid (160 ml), 16 g Dowex 1 × 2 (pre-equilibrated with buffer A) were added and stirred for 20 min. The ion-exchanger was filtered off under suction and the resultant liquid brought to 50% satn by the addition of $(NH_4)_2SO_4$ during 15 min. The mixture was stirred for 15 min at 0° and then centrifuged at 10000 *g* for 20 min. The supernatant was then brought to 75% satn with $(NH_4)_2SO_4$ and centrifuged at 10000 *g* for 20 min. The ppt. was dissolved in buffer B. This fraction was chromatographed on a column (3 × 30 cm) of Sephadex G-25 (fine), which had been previously equilibrated with buffer B. The protein fraction was applied to a DEAE-cellulose column (2 × 11 cm) which had been equilibrated with buffer B. After washing the column with buffer B, elution was continued with a linear 50–250 mM K phosphate gradient (120 ml), pH 7.6, containing 1.45 mM 2-mercaptoethanol. The flavanone synthase was eluted from the column after the phosphate buffer concentration had reached about 150 mM. The most active fractions were combined and used for the present study.

Enzyme assay. The assay mixture contained 1.5 nmol of 2-^{14}C malonyl-CoA (1.1 × 10^5 dpm), 1 nmol *p*-coumaryl-CoA, caffeyl-CoA or feruloyl-CoA, 25 μmol of K phosphate buffer of either pH 6.5 or 8, 115 nmol K ascorbate, 250 μg BSA and enzyme in a total vol. of 110 μl. The incubations were carried out at 30° for 30 min and then stopped by adding 10 μl HOAc followed by boiling for 10 min. Boiling minimized tailing effects on the chromatograms and converted the dihydropyrones to the corresponding benzalacetones [4]. Flavanones and benzalacetones were then added as carriers and PC was carried out with 15% EtOH.

Protein determination. The protein content of crude preps was determined by the biuret method, using crystalline BSA as standard [12]. After the DEAE-cellulose column, all protein determinations were made according to ref. [13].

Acknowledgements—This work was supported by the Deutsche Forschungsgemeinschaft (SFB 46) and by Fonds der Chemischen Industrie. One of us (NS) thanks the Alexander von Humboldt-Stiftung for the award of a Fellowship. We thank Mr. H. Müller for his skilled technical assistance and Prof. K. Hahlbrock for valuable discussions.

REFERENCES

1. Kreuzaler, F. and Hahlbrock, K. (1975) *European J. Biochem.* **56**, 205.
2. Wellmann, E., Hrazdina, G. and Grisebach, H. (1976) *Phytochemistry* **15**, 913.
3. Sütfeld, R. and Wiermann, R. (1976) *Z. Pflanzenphysiol.* **79**, 467.
4. Hrazdina, G., Kreuzaler, F., Hahlbrock, K. and Grisebach, H. (1976) *Arch. Biochem. Biophys.* **175**, 392.

5 Kreuzaler, F. and Hahlbrock, K., unpublished results.
6. Hanson, K. R. and Havir, E A (1977) *Arch. Biochem. Biophys.* **180**, 102.
7. Knobloch, K. H. and Hahlbrock, K. (1977) *Arch. Biochem Biophys.* **184**, 237.
8. Fritsch, H., Hahlbrock, K. and Grisebach, H (1971) *Z. Naturforsch.* **26b**, 581.
9. Fritsch, H. and Grisebach, H. (1975) *Phytochemistry* **14**, 2437.
10. McGookin, A. and Heilbron, I. M. (1924) *J. Chem. Soc.* 2099.
11. McGookin, A. and Sinclair, D. J. (1928) *J. Chem. Soc* 1170.
12. Lane, E. (1957) *Methods Enzymol.* **3**, 447
13. Warburg, O. and Christian, W. (1941) *Biochem Z.* **310**, 384.