Characterization, Development and Localization of "Flavanone Synthase" in Tulip Anthers

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1. "Flavanone synthase" was isolated from anthers of *Tulipa* cv. "Apeldoorn" and partially purified by $(NH_4)_2SO_4$ fractionation, gel chromatography and isoelectric focussing. The enzyme preparation was free of chalcone-flavanone isomerase activity.

2. *p*-Coumaroyl-CoA, caffeoyl-CoA and feruloyl-CoA were found to be efficient substrates of the synthase. The products formed were naringenin (5,7,4′-trihydroxyflavanone), eriodictyol (5,7,3′,4′-tetrahydroxyflavanone) and homoeriodictyol (5,7,4′-trihydroxy-3′-methoxyflavanone), respectively. Addition of thiol reagents at concentrations exceeding 10^{-3} M caused inhibition of the enzyme. "Release products", however, were not detectable. Although exclusively chalcones accumulate in the tulip anther, only flavanones but no chalcones were detectable in our *in vitro* system.

3. The apparent K_m values for *p*-coumaroyl-CoA, caffeoyl-CoA and feruloyl-CoA were 1.7×10^{-6} M, 1.6×10^{-6} M and 2.5×10^{-6} M, respectively. Similar data were observed for malonyl-CoA.

4. No cofactors are required for the synthase reaction. The enzyme is strongly inhibited by the reaction products flavanone and coenzyme A. Maximum enzyme activity was found at pH 8.0 and 30 °. The molecular weight was approx. 55,000.

5. Synthase activity develops in early postmeiotic stages of microsporogenesis. Highest specific activities of the enzyme coincide with a maximum in chalcone accumulation within the anthers.

6. The contents of anthers was separated into two fractions, pollen and tapetum. Highest specific activities were observed with tapetum fractions, while pollen fractions exhibited only very low activities. The high enzyme activity in the tapetum fraction points to the important role of the tapetum in the biosynthesis of flavonoids in the loculus of anthers.

Introduction

Previous studies [1, 2] have shown, that flavanone synthase represents the key enzyme in flavonoid biosynthesis. This enzyme catalyzes the formation of both naringenin (5,7,4′-trihydroxyflavanone) and eriodictyol (5,7,3′,4′-tetrahydroxyflavanone) from malonyl-CoA and *p*-coumaroyl-CoA or malonyl-CoA and caffeoyl-CoA, respectively [3, 4]. As yet no information is available on the *in vitro* formation of other than naringenin and eriodictyol. In anthers of *Tulipa* cv. "Apeldoorn", a temporary intensive accumulation of differently substituted chalcones occurs during specific developmental stages [5]. This system, therefore, seemed to be particularly well suited to investigate the enzymatic formation of flavonoids. In continuation of our previous studies [3] we have thus isolated the "flavanone synthase" from tulip anthers and characterized the enzyme with respect to its substrate specificity, localization and appearance during certain developmental stages of the anthers.

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Abbreviations: C-CoA, caffeoyl-CoA; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Fe-CoA, feruloyl-CoA; "FS", "flavanone synthase"; pC-CoA, *p*-coumaroyl-CoA; PCMB, *p*-chloromercuribenzoic acid.

Materials and Methods

Chemicals: [2-^{14}C]Malonyl-CoA (33 mCi/mmol) was obtained from N. E. N., Boston, Mass.. *p*-Coumaroyl-CoA was synthesized as described previously [3]. Sinapoyl-CoA was synthesized in the same manner, using the N-hydroxysuccinimide ester of sinapic acid. Its identity was established according to [6] and the yield determined as described [7]. Caffeoyl-CoA and feruloyl-CoA were generous gifts from Dr. J. Stöckigt, Bochum. We gratefully acknowledge a gift of bis-noryangonin from Dr. G. H. N. Towers, Vancouver.

Enzyme preparations: Whole anthers were homogenized in a mortar using liquid N_2. The powder and an aliquot of Polyclar AT (Serva, Heidelberg) were suspended in potassium phosphate buffer (0.1 M, pH 8.0) containing glucose (10%) and potassium ascorbate (20 mM). The suspension was

stirred for 15 min at 4 °C. Extraction of enzymes from tapetum and pollen fraction was carried out as described [8], using potassium phosphate buffer (0.1 M). The extracts were cleared by filtration using Whatman GFA filters and by centrifugation (15 min, 28,000 × *g*). To the supernatant solid $(NH_4)_2SO_4$ was added. The protein precipitating in the range between 30 to 80% saturation was centrifuged off and the pellet dissolved in potassium phosphate buffer (0.1 M, pH 8.0). Gel filtrations were carried out using Sephadex G-25 and G-200 columns, which were equilibrated with the same buffer. After gel chromatography on Sephadex G-200, fractions containing synthase activity were combined and passed through a Sephadex G-15 column, which was equilibrated with glycine (1%). Isoelectric focussing was performed using a sucrose-stabilized LKB 110 ml column and Ampholine carriers in pH-ranges between 3.5 to 10.0 and 4.0 to 6.0.

Assay of "flavanone synthase": The standard assay mixture for measuring "flavanone synthase" activity contained mercaptoethanol (72 nmol), glycylglycine (4.5 μmol), [2-^{14}C]malonyl-CoA (1.57 nmol, 50 nCi), hydroxycinnamoyl-CoA (0.7 nmol), potassium phosphate (4.5 μmol) and up to 100 μg protein in a total volume of 100 μl. Before incubation (20 min, 30 °C), the pH of the assay mixture was adjusted to 7.8 with potassium hydroxide solution (0.4 M). The reaction was terminated by acidification (0.3 ml, 0.1 M HCl).

Preliminary experiments had shown, that chalcones, flavanones and the styrylpyrone if present[9], were completely extractable into ether. Therefore the incubation mixture was vigorously shaken with diethylether (2 ml). The ether phase was evaporated to dryness, the products were dissolved in methanol (75 μl) and spotted on cellulose thin layer plates. Chromatography was performed as described previously [3]. The spots corresponding to the authentic flavanones were scrapped off. Scintillation spectrometry was performed after addition of methanol (1 ml) and a scintillation cocktail ("Premix 4" (0.4%) in toluene, total volume 20 ml; "Premix 4" was obtained from Zinsser, Frankfurt).

Assay of chalcone-flavanone isomerase: The assay was performed as described [10].

Estimation of molecular weights: Estimations were carried out using gel chromatography [11] and centrifugation on a linear sucrose gradient (10 – 25%) [12].

Protein estimation: Protein was measured by the biuret method [13].

Results

1. Characterization of "flavanone synthase"

1.1. Purification

When crude enzyme extracts or protein solutions obtained after $(NH_4)_2SO_4$ precipitations were used, enzyme activities were observed which were low and poorly reproducible. Addition of EDTA to the crude extract, or desalting of the $(NH_4)_2SO_4$ fraction, resulted in considerable stabilization and increased the specific activities (Table I). However, addition of EDTA to the desalted $(NH_4)_2SO_4$ fraction did not further increase the activity.

After $(NH_4)_2SO_4$ fractionation, the preparations were further purified using gel chromatography on Sephadex G-200. By this step, an almost complete separation of chalcone-flavanone isomerase from the "flavanone synthase" was achieved (Fig. 1 a). Further purification using isoelectric focussing achieved homogenity of the synthase (Fig. 1 b). The isoelectric point of the enzyme was about pH 5.3.

1.2. Stability

When enzyme preparations were stored in buffer at room temperature, about 50% of the initial enzyme activity was lost within 90 min. Highly purified preparations were stable at −20 °C for several weeks after an initial loss of about 30% of the activity within the first 14 days. Addition of glycerol, ethyleneglycol or bovine serum albumin did not affect the stability of the synthase.

Table I. Effects of EDTA and desalting on "flavanone synthase" activity using crude extracts and $(NH_4)_2SO_4$ fractionations. Desalting was performed using Sephadex G-25. Substrate: *p*-coumaroyl-CoA.

Enzyme preparation	Addition/ treatment	"FS" activity (nkat/kg prot.)
crude extract	—	100
crude extract	EDTA (3 mM)	160
30 – 80% $(NH_4)_2SO_4$ fract.	—	100
30 – 80% $(NH_4)_2SO_4$ fract.	desalting	330
desalted protein	EDTA (3 mM)	290

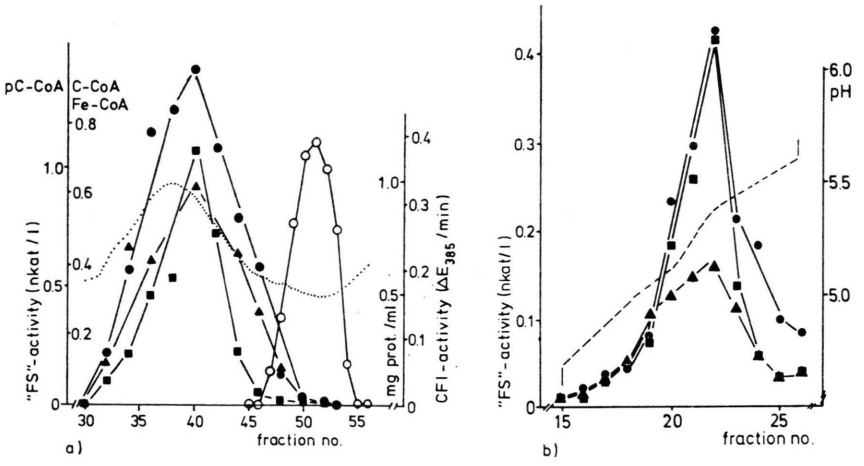

Fig. 1 a) Separation of "flavanone synthase" (black symbols) and chalcone-flavanone isomerase (open symbols) on a Sephadex G-200 column. (●—●), substrate *p*-coumaroyl-CoA; (■—■), substrate caffeoyl-CoA; (▲—▲), substrate feruloyl-CoA; (····), protein concentration.
Fig. 1 b) Fractionation of "flavanone synthase" by isoelectric focussing on a LKB 110 ml column. (– – –), pH-gradient; other symbols, see Fig. 1 a).

1.3 Linearity of the reaction with respect to time and to protein concentration

As shown in Fig. 2 a, b) (conversion of *p*-coumaroyl-CoA as example), the reaction of the synthase is linear with time over at least 20 min at protein concentrations of up to 10 mg/ml.

1.4. Dependence on pH

Maximum product formation was observed within a pH-range of 7.6 to 8.0 when either *p*-coumaroyl-CoA, caffeoyl-CoA or feruloyl-CoA were used as substrates (Fig. 3). Increasing the pH above 8.0 caused irreproducibility in the values obtained with caffeoyl-CoA and feruloyl-CoA.

1.5. Effect on temperature

Highest synthase activities were found at temperatures of about 30 °C. Incubations at 20 °C or between 50 °C and 60 °C caused a 50% decrease in enzyme activity.

1.6. Effects of bivalent cations

Addition of either Mg^{2+} or Ca^{2+} (10^{-3} M) caused a slight increase (10 to 20%) in enzyme activity. Mn^{2+} had no significant effect, whereas Zn^{2+} and Cu^{2+} decreased activity considerably (50%) at concentrations of above 10^{-3} M.

1.7. Effects of PCMB and sulfhydryl reagents

Synthase activity was greatly inhibited (50%) by addition of PCMB (2.5×10^{-5} M) (Table II). Complete inhibition was observed at a concentration of about 10^{-4} M. Addition of DTT caused an enzyme inhibition of 60%, whereas addition of mercaptoethanol at the same concentrations resulted in enhanced enzyme activities (40%). However, mer-

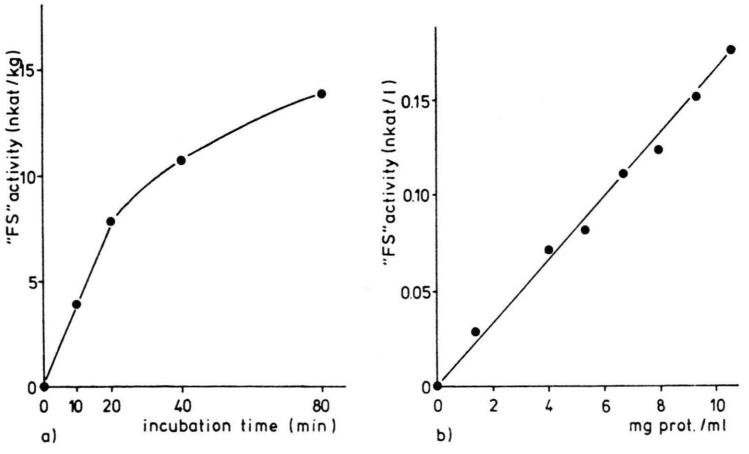

Fig. 2. Dependence of "flavanone synthase" activity from incubation time (a) and protein concentration (b).

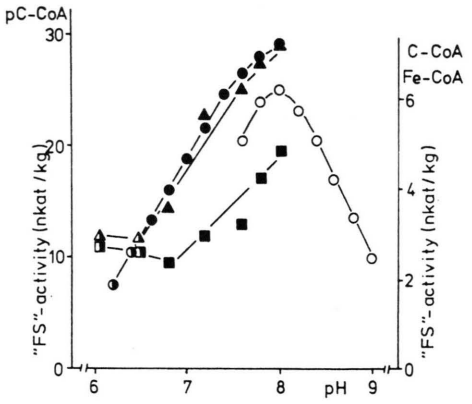

Fig. 3. Dependence of "flavanone synthase" activity on pH. Buffers used were citric acid — potassium phosphate (pH 6.0 to 6.5); glycylglycine — potassium phosphate (pH 6.5 to 8.0); Tris-HCl (pH 7.7 to 9.0); (◑, ●, ○), *p*-coumaroyl-CoA as substrate; (◧, ■), caffeoyl-CoA as substrate; (△, ▲), feruloyl-CoA as substrate.

captoethanol inhibited synthase activity at concentrations higher than 5×10^{-3} M.

1.8. Product inhibition

Both naringenin (25×10^{-6} M) and coenzyme A (50×10^{-6} M) inhibited the synthase to about 50%. Total inhibition was observed at concentrations of 10^{-4} M (naringenin) and 2.5×10^{-4} M (coenzyme A). Malonic acid showed no significant effect on the enzyme activity at concentrations up to 10^{-3} M.

1.9. Substrate specificity and kinetic data

The reaction products naringenin, eriodictyol and homoeriodictyol were identified as described [3]. When sinapoyl-CoA were used as substrate a product was formed the identity of which could not be established due to low amounts and lack of a reference sample.

Table II. Influence of PCMB and sulfhydryl reagents on "flavanone synthase" activity. Substrate: *p*-coumaroyl-CoA.

Addition	Concentn. (M)	"FS" activity (nkat/kg prot.)	effect (%) of control
none (control)	—	610	—
PCMB	2.5×10^{-5}	310	− 49
PCMB	1.0×10^{-4}	0	−100
DTT	1.5×10^{-3}	230	− 62
mercaptoethanol	2.4×10^{-3}	850	+ 39
mercaptoethanol	4.8×10^{-3}	500	− 19
mercaptoethanol	9.6×10^{-3}	460	− 24
mercaptoethanol	19.2×10^{-3}	190	− 69

The apparent K_m-values (Table III) were found to be essentially the same for *p*-coumaroyl-CoA, caffeoyl-CoA, feruloyl-CoA as well as malonyl-CoA. In the case of caffeoyl-CoA, a strong increase in specific activity of the enzyme was observed during successive purification (gel chromatography → isoelectric focussing) of the enzyme. Thus, the highest V_{max}/K_m ratios were reached for this substrate after isoelectric focussing.

1.10. Molecular weight

The twofold estimation of the molecular weight using a calibrated Sephadex G-200 column and a linear gradient centrifugation resulted in a value of approx. 55,000, with an experimental error of about ± 10%.

2. Development of "flavanone synthase" during microsporogenesis

As outlined in the introduction, a temporary intensive accumulation of chalcones occurs during specific postmeiotic stages of development. Therefore, we were interested in the development of synthase activity during these particular stages. The results (Fig. 4) show, that synthase activity is detectable in early postmeiotic stages. Highest specific activities appear in stages with the most intensive accumulation of chalcones.

3. Localization of "flavanone synthase"

When the combined contents of anthers were separated [8, 14] into a pollen containing fraction and a tapetum containing fraction and enzyme ac-

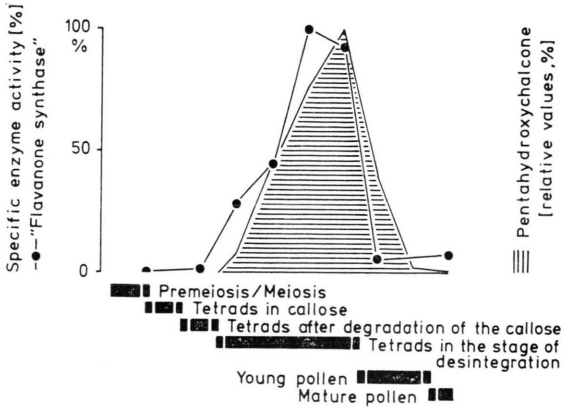

Fig. 4. Development of "flavanone synthase" and accumulation of pentahydroxychalcone during microsporogenesis of *Tulipa* cv. "Apeldoorn".

Table III. Kinetic data of "flavanone synthase" obtained from three linear transformation methods.

Substrate	K_m(substr.) [10^{-6} M]	K_m(mal-CoA) [10^{-6} M]	V/K_m * (mkat/kg) [10^{-6} M]	V/K_m ** (mkat/kg) [10^{-6} M]
*p*C-CoA	1.7	1.6	270	280
C-CoA	1.5	4.2	150	310
Fe-CoA	2.5	3.0	80	70

* Values obtained after gel chromatography.
** Values obtained after isoelectric focussing.

tivities measured, only 2% of the total activity was detected in the pollen fraction whereas 98% was in the tapetum fraction. Table IV shows a comparison between the localization of the synthase and values obtained from previous studies [8, 14] on the localization of the phenylalanine ammonia-lyase and hydroxycinnamate:CoA ligase.

Discussion

Flavanone synthase from cell suspension cultures of *Petroselinum hortense* and *Haplopappus gracilis* has been extensively studied [2, 4, 7, 9]. We have isolated this enzyme for the first time from intact tissue [3]. Some properties and observations related to the physiology of the enzyme are described in the present paper.

The K_m-values obtained for the three substrates, being in micromolar ranges, indicate that not only *p*-coumaroyl-CoA and caffeoyl-CoA, but also feruloyl-CoA is a physiological substrate for the enzyme of anthers (see also [4]). Thus, when compared to the enzyme from cell suspension cultures [4, 7] the synthase from anthers exhibits a wider substrate specificity. The high V_{max}/K_m ratio for caffeoyl-CoA is noteworthy in agreement with the fact, that pentahydroxychalcone, the product of malonyl-CoA and caffeoyl-CoA, is markedly accumulated in anthers [5]. Moreover, the substrate specificity of the hydroxycinnamate:CoA ligase [15] and the "flavanone synthase" are very much alike. In addition, the substitution pattern of the substrates of the "flavanone synthase" agrees with the substitution pattern of the chalcones accumulated in the natural system [5]. In the system of tulip anthers, accordingly, the enzymatic equipment exists for the transfer of three different substituted cinnamic acids into the subsequent (accordingly substituted) flavonoids. These data support the determination of the flavonoid substitution pattern at the cinnamic acid stage, as postulated in the "cinnamic acid starter hypothesis" [16].

Table IV. Distribution of activities of phenylalanine ammonia-lyase (PAL), hydroxycinnamate:CoA ligase and "flavanone synthase" in the tapetum and pollen fraction of tulip anthers (values in % of highest specific activities).

	Tapetum fraction rel. activity [%]	Pollen fraction rel. activity [%]
PAL	93	7
CoA-ligase	87	13
"FS"	98	2

The enzyme preparations from cell suspension cultures of *Petroselinum* and *Haplopappus* exhibit different pH optima for naringenin formation from *p*-coumaroyl-CoA and eriodictyol formation from caffeoyl-CoA [4]. Concerning the formation of naringenin, the pH optimum of the tulip enzyme is the same as the optimum of the enzyme from cell cultures. At lower pH (pH 6.0 to 7.0) the formation of eriodictyol does not increase in comparison to the enzyme of cell suspension cultures [4].

The apparent molecular weight of the synthase from anthers (55,000) is in the range of that of the subunits of the synthase from *Petroselinum* cell cultures (40,000; K. Hahlbrock, pers. communication). No criterion supported the existence of enzyme aggregations in anthers.

In accordance to [2], bivalent cations as well as EDTA at micromolar concentrations had no significant effects on the anther synthase activity. Effects on the enzyme activity occured at 10^{-3} M concentration. The effect of SH-reagents also corresponds well to previous observations [2, 9], concerning the stimulatory effect on enzyme activity at low concentrations and the inhibitory effect at high concentrations. Induction of "release products", as described for the enzymes of cell suspension cultures [4, 7, 9], did not succeed with enzyme preparations of tulip anthers, even when using high concentrations of mercaptoethanol.

Attempts to detect chalcones at reaction products of the synthase have so far been unsuccessful [2]. Even using extracts free of chalcone-flavanone isomerase activity, only flavanones could be detected as definite products of the synthase from anthers. If the findings previously reported [17] should eventually apply to the system of tulip anthers, the formation of flavanones could then be attributed

to the spontaneous cyclization of chalcones during the incubation. The fact that all chalcones in the loculus of tulip anthers are accumulated as aglyca [5], points to an endogenic, chalcone-stabilizing "factor" in the natural system. Investigations on this problem are in preparation.

The "flavanone synthase" from tulip anthers deserves attention with respect to the kinetics of accumulation of phenylpropanoids, and to the changes of activities of the enzymes responsible for flavonoid biosynthesis [15, 18, 19]. This is shown by the development of the synthase during microsporogenesis, and by the good correlation of its activity to the accumulation of chalcones in the contents of anthers. The localization of the synthase in the tapetal portion of the contents of anthers, together with other enzymes which are involved in phenylpropanoid metabolism [14], implicate the importance of the tapetum for the formation of flavonoid components in the loculus of anthers.

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