

Purification of Chalcone Synthase from Tulip Anthers and Comparison with the Synthase from *Cosmos* Petals

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Chalcone synthase was isolated from both anthers of *Tulipa* cv. "Apeldoorn" and petals of *Cosmos sulphureus* Cav. After certain prepurification steps, the enzymes were further purified using gel chromatography on Sephadex G-200 followed by repeated hydroxylapatite absorption chromatography. Both the enzymes showed the same chromatographic properties. After gel chromatography as well as after the first hydroxylapatite fractionation, the reaction products appeared as flavanones. However, after the second hydroxylapatite step, production of chalcones was observed. Like the enzyme from tulip anthers, the synthase from *Cosmos* petals produced the correspondingly substituted chalcones when *p*-coumaroyl-CoA, caffeoyl-CoA and feruloyl-CoA, respectively, were used as substrates. In both the cases, the ratios of the different chalcones produced were found to be about the same. The appearance of chalcone synthesis in this *in vitro* assay is caused by the complete elimination of chalcone isomerase in the purification procedure. The importance of the isomerase for flavonoid biosynthesis, particularly in plant systems which are accumulating chalcones, is discussed.

Introduction

The condensation of activated hydroxycinnamic acids and malonyl-CoA has been described as the key reaction of flavonoid biosynthesis [1, 2]. With the first successful enzymatical work on parsley cell suspension cultures [3] it seemed that the synthase catalyzed the production of flavanones [2, 4–6]. However, with the enzyme of tulip anthers, we suggested [7] and could later demonstrate [8], using a special biphasic assay, that chalcones rather than flavonones were produced. Unlike the substrate specificity of the flavanone synthase reported from cell suspension cultures [2–4, 6], *p*-coumaroyl-CoA, caffeoyl-CoA and also feruloyl-CoA were good substrates for the enzyme from tulip anthers [7, 8], in keeping with the accumulation of the chalcone aglycones with different substitution pattern which are accumulated in the loculus of the anther [9]. Very recently, chalcone synthesis also was demonstrated with highly purified enzyme preparations from parsley cell suspension cultures [10]. Using a similar method (*cf.* [6]), we have now purified the synthase from tulip anthers in order to establish its identity with the previously described "flavonone-

synthase" [7] and to confirm the results obtained from experiments with the biphasic assay [8]. For comparison, the same investigations were performed on another intact plant system, petals of *Cosmos sulphureus*.

Materials and Methods

Chemicals: [2-¹⁴C]Malonyl-CoA (33 mCi/nmol) was obtained from N.E.N., Boston, Mass. Hydroxycinnamoyl-CoA esters were synthesized as described [8, 11].

Enzyme preparations: To prepare tapetum fractions of tulip anthers, the material of 160 tulip bulbs was squeezed into 0.1 M potassium phosphate buffer, pH 6.8, containing 10% sucrose (w/v) and 20 mM of potassium ascorbate. The pollen was filtered off, as previously described [7, 10, 12].

Petals from about 100 *Cosmos* plants, stored for a few weeks in glycerol at –70°, were homogenized in a mortar after thawing and removing the glycerol, using the same buffer as above. Insoluble residues were removed by filtration on Whatman GFA filters and by subsequent centrifugation, as previously described [7].

For both the enzyme sources, the same purification procedure was used. This consisted of:

- (NH₄)₂SO₄ precipitation (80% saturation);
- dissolving of the precipitate in 0.1 M potassium phosphate buffer, pH 6.8 (= buffer A);

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- c) prepurification of the protein on a Sephadex G-25 column, equilibrated with buffer A;
- d) fractionation on a Sephadex G-200 column equilibrated with buffer A;
- e) collecting of fractions with maximum synthase activity;
- f) $(\text{NH}_4)_2\text{SO}_4$ precipitation (80% saturation);
- g) dissolving of the precipitate in 0.001 M potassium phosphate buffer, pH 6.8 (= buffer B);
- h) desalting the protein on a Sephadex G-25 column, equilibrated with buffer B;
- i) fractionation on a LKB Ultrogel HA column; starting buffer, buffer B; gradient: increasing PO_4^{3-} concentrations up to 1.0 M, pH 6.8;
- j) collecting of fractions with maximum synthase activity;
- k) desalting the protein as described (h);
- l) fraction as described (i).

Enzyme assays: Chalcone isomerase activity was measured spectrophotometrically, as previously described [8].

The synthase incubation mixture was slightly modified according to Sütfeld *et al.* [8] and Heller *et al.* [10]: 130 μl incubation mixture contained 72 nmol of mercaptoethanol in 55 μl of buffer A, 1.57 nmol (50 nCi) of $[2-^{14}\text{C}]$ malonyl-CoA in 5 μl hydro-

chloric acid (pH 3.5) and 0.7 nmol of hydroxycinnamoyl-CoA in 10 μl of 0.1 M potassium phosphate buffer, pH 7.0. After addition of 60 μl enzyme solution the mixture (pH 6.8) was incubated for 10 minutes at 30°. The reaction was stopped by addition of 5 μl methanol (containing reference substance) and 200 μl ethylacetate. The subsequent isolation, qualitative and quantitative analysis of the reaction products has been described elsewhere [8].

Results

Table I shows the yield of chalcone and flavanone obtained with successive purification of the synthase from tulip anthers and from *Cosmos* petals. Furthermore, the elimination of the chalcone isomerase activity, as determined by spectrophotometry is also shown. The synthase from *Cosmos* showed exactly the same elution behaviour on Sephadex G-200 as was described for the tulip anther synthase, exhibiting a molecular weight of about 55,000 [7]. Like the chalcone isomerase from tulip anthers [13], the isomerase from *Cosmos* petals was eluted within a minor molecular weight range (*cf.* [7]). In each case, the enzyme activities could be

Table I. Purification of the synthase from *Tulipa* and *Cosmos*. Enzyme assays with *p*-coumaroyl-CoA as substrate.

Enzyme preparation ^b	Purification step	Synthase yield on products (%) ^a				Chalcone isomerase activity ($\Delta E_{385}/\text{min}$)	
		<i>Tulipa</i>		<i>Cosmos</i>		<i>Tulipa</i>	<i>Cosmos</i>
		Ch.	Fl.	Ch.	Fl.		
Total protein	Crude extract, 1 st $(\text{NH}_4)_2\text{SO}_4$ precipitate, G-25 protein, 2 nd $(\text{NH}_4)_2\text{SO}_4$ precipitate	0	100	0	100	> 0.1	> 0.1
Fractions in m.w. range at about 50,000 ^c	Fractionation on Sephadex G-200	0	100	0	100	< 0.01	< 0.01
Fractions eluting at about 0.4–0.5 M $[\text{PO}_4^{3-}]$ ^d	1 st Fractionation on hydroxylapatite	0	100	0	100	< 0.005	< 0.005
Fractions eluting at about 0.4–0.6 M $[\text{PO}_4^{3-}]$ ^e	2 nd Fractionation on hydroxylapatite	56	44	55	45	0 ^f	0 ^f

^a Sum of radioactivity of total products = 100%.

^b For detailed information, see under "Methods".

^c For elution pattern on Sephadex G-200, see (7). The protein from *Cosmos* petals exhibited the same chromatographic elution pattern.

^d See Fig. 1, 2.

^e See Fig. 3, 4.

^f Chalcone isomerase activity not measurable spectrophotometrically.

Abbreviations used: Ch. = chalcone; Fl. = Flavanone produced; m.w. = molecular weight

drawn as symmetrical plots; for both the systems, no indications of the existence of synthases or isomerase within higher or lower molecular weight ranges were observed. At this stage of purification, fractions with the highest synthase activity contained barely measurable chalcone isomerase activity ($\Delta E_{385} < 0.01/\text{min}$). However, the reaction product of the synthase appeared as a flavanone. The

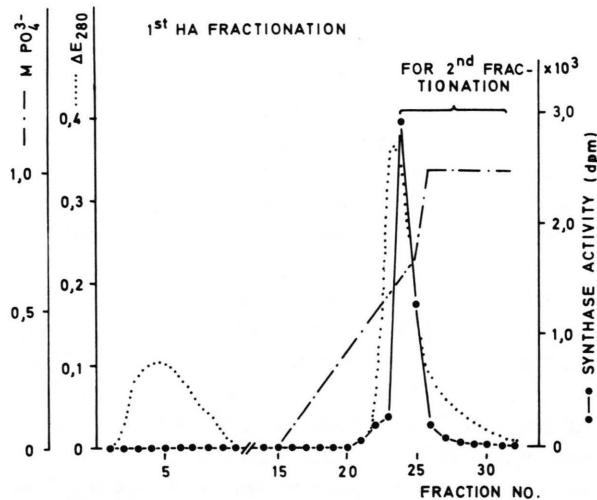


Fig. 1. 1st hydroxylapatite fractionation of pre-purified enzyme preparation from tulip anther tapetum. Chalcone isomerase present in fraction No. 22 and 23 (●—●: product formed; flavanone).

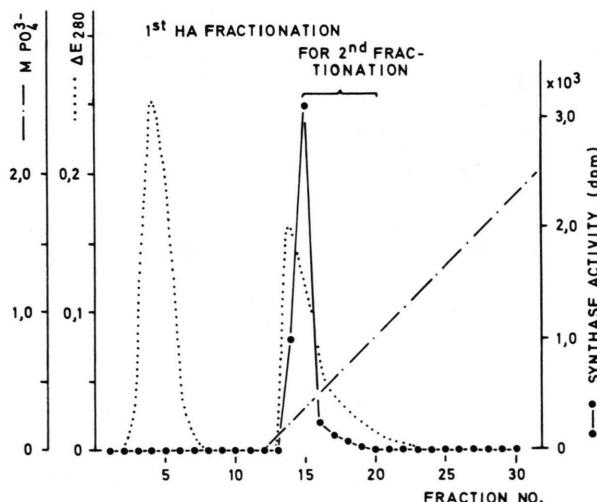


Fig. 2. 1st hydroxylapatite fractionation of pre-purified enzyme preparation from *Cosmos* petals. Chalcone isomerase present in fraction No. 14 (●—●: product formed; flavanone).

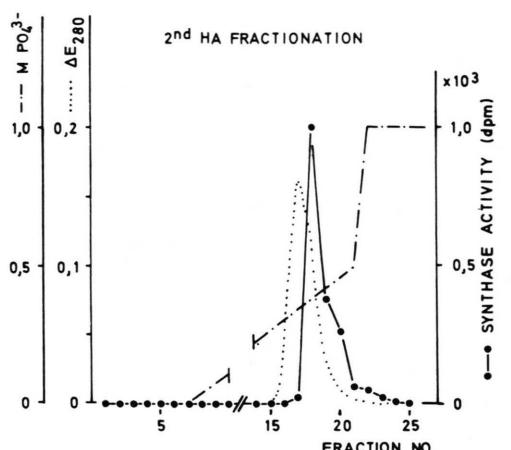


Fig. 3. 2nd hydroxylapatite fractionation of fractions marked in Fig. 1. No chalcone isomerase detectable (●—●: product formed; chalcone and flavanone).

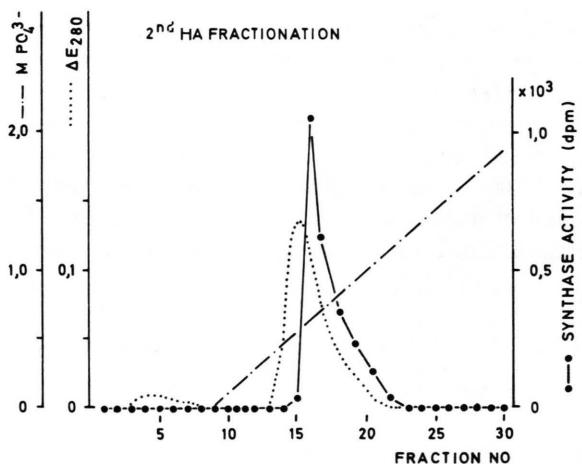


Fig. 4. 2nd hydroxylapatite fractionation of fraction marked in Fig. 2. No chalcone isomerase detectable (●—●: product formed; chalcone and flavanone).

same result was obtained after the next purification step, namely absorption chromatography on hydroxylapatite (Figs. 1, 2). In this case, only traces of chalcone isomerase were measurable ($\Delta E_{385} < 0.005/\text{min}$). With the second fraction on hydroxylapatite (Figs. 3, 4), however, isomerase activity was no longer detectable. Synthase activity was eluted as shown before, and the major reaction product was now found to be a chalcone. The specific activities of the synthases from both *Tulipa* and *Cosmos*, calculated as nkat per kg protein were in about the same ranges.

Table II. Yields of total product (a) and chalcone/flavanone ratios (b) using differently substituted hydroxycinnamoyl-CoA esters as substrates and the most highly purified enzyme preparations from tulip anthers.

Substrates	Substitution pattern of substrates	Total product (%) ^a	Formation of (%) ^b	
			chalcone	flavanone
<i>p</i> -coumaroyl-CoA	4-OH	100	46	54
caffeooyl-CoA	3,4-OH	31	58	42
feruloyl-CoA	3-OCH ₃ , 4-OH	18	29	71
sinapoyl-CoA	3,5-OCH ₃ , 4-OH	0	—	—

^a Total product with *p*-coumaroyl-CoA = 100%.

^b Total product with the respective hydroxycinnamoyl-CoA ester = 100%.

Using differently substituted hydroxycinnamoyl-CoA esters as substrates, and the highest purified enzyme preparation from both the systems, *p*-coumaroyl-CoA was found to be the best substrate, followed by caffeooyl-CoA and feruloyl-CoA. Sinapoyl-CoA was not converted enzymatically. Table II shows the results obtained with the synthase from tulip anthers: a) values calculated for total product formation related to the amount of total production obtained with *p*-coumaroyl-CoA as substrate; b) values calculated for chalcone/flavanone ratio related to the amount of the total product for each the hydroxycinnamoyl-CoA ester as substrate. Whereas the chalcone/flavanone ratio obtained with *p*-coumaroyl-CoA and caffeooyl-CoA ranged at about 1/1, the amount of chalcone produced with feruloyl-CoA appeared to be considerably lower.

For the enzyme from *Cosmos* petals, similar values were obtained, except that the amount of total product with feruloyl-CoA as substrate was generally slightly higher (about 25% related to 100% total product with *p*-coumaroyl-CoA as substrate). The chalcone/flavanone ratio was about the same as shown for the tulip anther preparation (Table II).

Dicussion

This is the first report about the isolation, purification and comparison of chalcone synthase from very different intact plant systems. Chalcone production can only be demonstrated by use of enzyme preparations which are totally free from chalcone isomerase activity (Table I). A similar result was recently shown with the enzyme from parsley cell suspension cultures [10]. Corresponding to these results, it was demonstrated that the chalcone/flavanone ratio after 10 minutes of incubation also

reaches values of about 1/1, with *p*-coumaroyl-CoA as substrate. The ratios reported here with caffeooyl-CoA and feruloyl-CoA, of about 3/2 and 3/7, respectively, may reflect other reaction courses than it was postulated for the formation of tetrahydroxychalcone [10].

In agreement with previous investigations [8, 10, 14, 15], the results reported here confirm the necessity for chalcone isomerase in order to cyclize chalcones to their isomeric flavanones leading to further steps in flavonoid biosynthesis. This enzyme could play an important role in the regulation of flavonoid metabolism, particularly in systems which are accumulating chalcones like tulip anther system [9]. However, in tulip anthers, it could be demonstrated that the most of chalcone isomerase is localized in the pollen fraction in contrast to all the other investigated enzymes of phenylpropanoid and flavonoid metabolism which occur mainly in the tapetum fraction of anthers [16].

Furthermore, the results obtained with the tulip anther system confirm, that the described "flavanone synthase" [5, 7] is really a chalcone synthase and that chalcone synthesis demonstrated with a special biphasic assay [8] is really attributed to the action of the same enzyme which is localized in the tapetum fraction of the anthers.

In this connection, there is once more a good correlation between the substitution pattern of chalcone aglycones and flavonol glycosides accumulated in the tulip anthers [9, 16] and the substrate specificity of the enzymes involved in their synthesis [7, 8, 17].

Whether such correlations are also applicable to the system from *Cosmos* petals, cannot yet be decided because of a lack of other enzymological work. Also there are at present only a few analytical

data about the occurrence of flavonoids in distinct organs of this plant species [18].

The similarity of the chalcone synthase from *Tulipa* and from *Cosmos* in their chromatographic behaviour and substrate specificity is most surprising. It would be interesting to see whether chalcone synthases from other plant species have similar properties.

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