STUDIES IN CELL SUSPENSION CULTURES OF CASSIA DIDYMOBOTRYA. PART II. THE BIOTRANSFORMATION OF CHALCONES TO FLAVANONES

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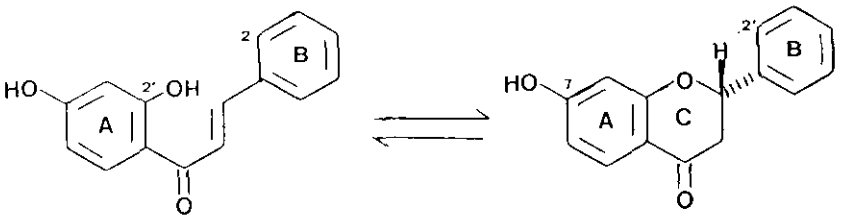
Abstract - An enzyme system derived from cell cultures of Cassia didymobotrya showed, inter alia, a chalcone - flavanone isomerase which revealed a maximum activity when isolated from 22 day old cultures. Its substrate specificity was demonstrated by using sixteen chalcones featuring different substitutions patterns in rings A and B. The highest yields of flavanone formation were observed with various ring A functionalyzed chalcones lacking functionality in ring B. The chalcones having an isoprenyl side chain in the 3'- position of ring A were not transformed. The presence of a free hydroxyl group at C-4 of ring B stimulated flavanone formation relative to other ring B substrates.
Some comments concerning the possible mechanism of the chalcone - flavanone isomerase reaction are provided.

Chalcones are widely distributed among plants[1] and are considered the key precursors in the biosynthesis of flavonoids.[2] The isomeric flavanones represent, very likely, the first intermediates in the biosynthetic pathway.[3] Chalcone-flavanone isomerase [E.C. 5.5.1.6.], the enzyme responsible for the chalcone (a) - (-) 2S-flavanone (b) interconversion (Scheme 1), has been isolated from many plants.[4-8]

Scheme 1



a b

We now report the isolation from tissue cultures, derived from seedlings of Cassia didymobotrya Fres of an enzymes mixture which catalyzes the isomerization of chalcones to flavanones.

Preliminary experiments with cell free extracts (C.F.E.) of Cassia didymobotrya revealed a biotransformation as shown in Scheme 1.

A series of experiments were performed to establish the variation of the enzymatic activity at different ages of the cultures. The cell suspensions, grown in 250 ml Erlenmayer flasks, were harvested at different times of the transition period between the early lag phase and late stationary phase, specifically at 1, 15, 22 and 28 days of growth. The cultures were homogenized in a buffer solution and, after centrifugation, the activity of the supernatant was tested with 2',4'-dihydroxychalcone (see Experimental) as a substrate. As shown in Table 1, the highest activity was found in the crude extracts from 22 day old cultures. These extracts afforded 75% of the corresponding flavanone after a 1.5 h incubation. It should also be noted (Table 1) that other enzymes occurring in older cells (28 days) are competitive with the chalcone-flavanone isomerase. For example, in experiment d, 50 % conversion of substrate was noted, but only 10 % represented flavanone. C.F.E. from younger cultures did not give any other unexpected product, that is, conversion of substrate resulted only in flavanone formation (see expts a and b).

Table 1

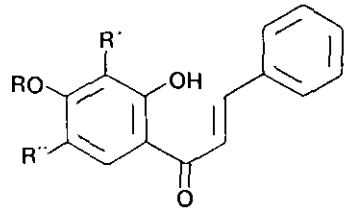
Biotransformation of 2',4'-dihydroxychalcone

Expt	Age of cells (days)	Overall substrate conversion (%)	% of flavanone	Reaction time (hours)
a	0	10	10	8
b	15	40	40	4
c	22	75	75	1.5
d	28	50	10	8

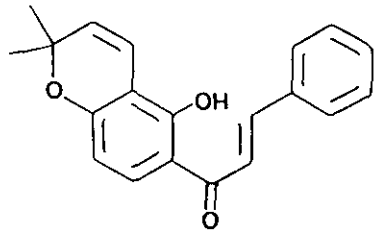
optimum pH = 7.7 ~ 8.0; temperature = 37 °C

The specificity of the chalcone - flavanone isomerase system was then studied with chalcone substrates featuring different substitution patterns in the A ring (Scheme 2).

Scheme 2



	R	R'	R''
1	H	H	H
2	Pr	H	H
3	H	Pr	H
5	Me	H	H
6	Me	H	Pr
7	Me	Pr	H
8	H	H	Pr



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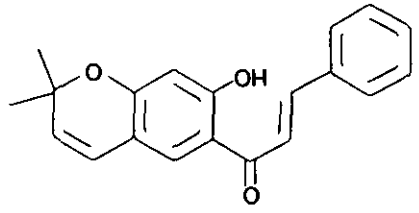

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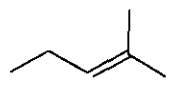
Pr ≡ 

The results, as summaryzed in Table 2, reveal that a prenyl side chain at the 3'-position inhibits conversion of substrate to the corresponding flavanone (see lack of conversion of substrates 3 and 7). On the other hand, chalcones possessing a prenyl side chain at the 5'- position (see conversion of 6 and 8) does not inhibit conversion. Perhaps the 3'- prenyl function prevents enzymatic conversion due to steric hindrance as a result of its close proximity to the chelated hydroxyl group. As noted in the studies with the substrates 2 and 5, various oxygenated side chains can be tolerated by the enzyme.

Although the cell cultures normally produce flavonoids having the phloroglucinol substitution pattern (2', 4' and 6'), the isolated enzyme system in this study is capable of utilizing other substitution patterns, as shown in substrates 1, 2, 5, 6, 8 and 9 to form the corresponding flavanones. It thus appears that a rather broad substrate specificity exists within our enzyme systems.

Table 2

Chalcone substrate specificity (Ring A) *

Substrate	Reaction time (hours)	Overall substrate conversion (%)	% of flavanone	$[\alpha]_D$ (solvent)
1	1.5	75	75	-28 (MeOH)
2	1.5	50	50	-43 ($CHCl_3$)
3	1.5	—	—	—
4	1.5	48	48	-81 ($CHCl_3$)
5	1.5	30	30	-4 ($CHCl_3$)
6	1.5	75	75	-15 ($CHCl_3$)
7	1.5	—	—	—
8	1.5	45	45	-25 (MeOH)
9	0.5	45	45	-47 ($CHCl_3$)

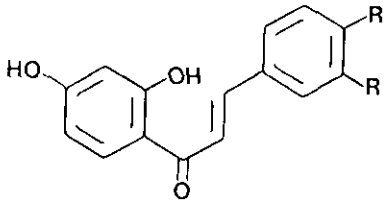
* The enzymes were extracted from 22 day old cell cultures

The next goal was to study the enzyme specificity for precursors having different substitution patterns in ring B. It would be desirable to maintain the "normal" phloroglucinol type pattern in ring A. However, in our study, it was shown in controlled experiments (no enzyme and buffer at pH 7.7 - 8.0), that such precursors

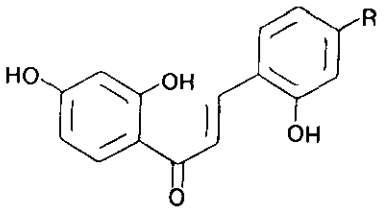
rapidly converted to the corresponding flavanones and we therefore turned our attention to the more stable precursors with the 2',4' - (resorcinol type) substitution patterns (Scheme 3).

The chalcone 10, prepared according to the published procedure,[10] was incubated with the C.F.E. obtained from the 22 day old cell cultures. An interesting difference in product formation was observed. Although the substrate 10 was utilized by the enzyme to an extent of 60% in a 3 h incubation time, only a 45% yield of the expected flavanone was obtained. The other products, as characterized in the accompanying publication, were obtained in 15% yield.

Scheme 3



	R	R
10	OH	H
11	OMe	H
12	H	OH
14	OMe	OH
16	OH	OMe



	R
13	H
15	OMe

As will be noted from Table 3, flavanone formation occurs to a significant extent with substrate 10, bearing only a C-4 hydroxyl function but is retarded when an additional C-3 methoxyl group is present (see substrate 16). Other substituents in ring B, for example substrates 11-15 inclusive, are similarly detrimental to enzyme-catalyzed flavanone formation. Comparison of the data in Table 3 with those of Table 2, the latter involving substrates lacking any substituents in ring B, reveales highest yields of flavanone formation when no substitution is present in ring B. It thus appears that the "steric bulk" of ring B substituents prevents appropriated association of the substrates with the active sites of the enzyme.

Table 3

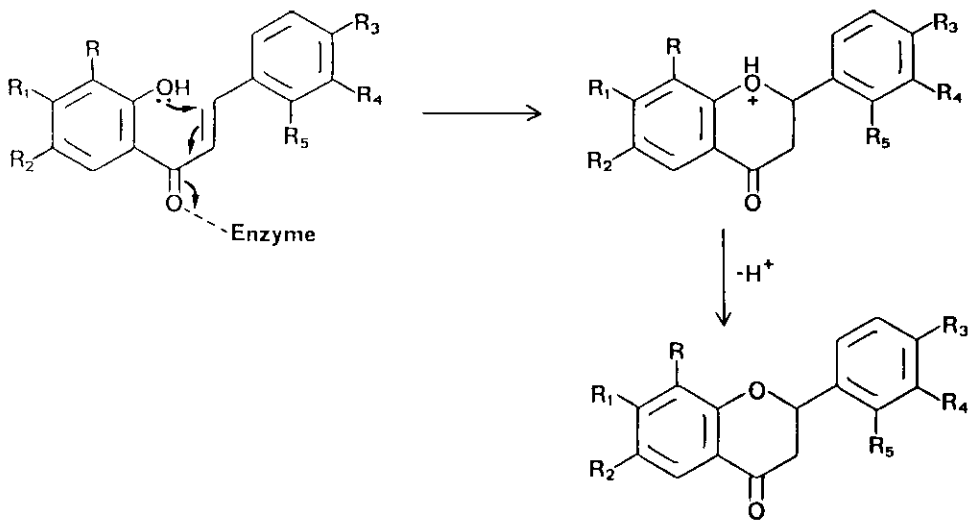
Chalcone substrate specificity (Ring B) *

Substrate	Reaction time (hours)	Overall substrate conversion (%)	% of flavanone	$[\alpha]_D$ (solvent)
10	3	60	45	-12 (MeOH)
11	3	—	—	—
12	3	—	—	—
13	3	—	—	—
14	3	—	—	—
15	3	—	—	—
16	1	100	16	-9 ($CHCl_3$)

* The enzymes were extracted from 22 day old cell cultures

Considerations of the possible mechanisms which could be involved in flavanone formation are of interest in view of the above results. A plausible mechanism involves "enzymatic activation" of the enone system as shown in Scheme 4.[10] Such activation implies a subsequent nucleophilic attack by the C-2' hydroxyl function in a conjugated manner.

Scheme 4



In this instance R_3, as for example, for substrate 10 (C-4 hydroxyl) is expected to impart resonance stabilization to the carbocationic species prior to cyclization

and therefore enhanced formation of flavanone. When R is a bulky group as in substrates 3 and 7 (Table 2) it is reasonable to assume steric hindrance toward cyclization. Substituent R_5 (C-2 hydroxyl group in substrates 13 and 15) may inhibit, via a steric factor, the coordination of the enone with the active site of the enzyme. Substituents R_1, R_2, and R_4 would have an insignificant effect on the above-noted cyclization.

In summary, we consider that one of the most important criteria in the chalcone - flavanone conversion is the overall "bulk" of the substrate and its ability to fit into the active site of the enzyme.

Further results relating to the above study are provided in the accompanying publication.

EXPERIMENTAL

Cell suspension cultures

Growth and optimization of the suspended cell cultures have been already published.

Cell free extraction procedure (C.F.E.)

The filtered cells, at the appropriate age, were homogenized with phosphate buffer (50 mM, pH=7.2, 0-4°C) in a ratio of 1 g of cells (fresh weight) per 1 ml of buffer. The homogenate was centrifuged at 2,500 rpm for 20 min, and the supernatant was used for incubations, which were carried out for different time (see Tables 1, 2 and 3) at 37°C. The protein contents were measured by Lowry assay.[12]

C.F.E. assays. Standard method.

Assays were done in tris-HCl (pH 7.7-8.0, 50 mM) at 37°C.

The optimum pH was determined by using potassium phosphate buffer (50 mM) up to pH 7.5 and tris-HCl up to pH 8.5.

In both cases EDTA was added to a final conc. 200 mM.

For incubations, enzyme preparations (1 mg of proteins / 1 ml) were diluted to 2 ml with buffer and 1 mg of chalcones (1-16) dissolved in 2-methoxyethanol.

The enzyme activity was not dependent on cofactors.

The conversion of the chalcones to flavanones was monitored by C-18 reverse phase hplc, checking with uv detector at two different wavelenghts (320 nm for the chalcones and 290 nm for the flavanones, respectively).

The conversion of both the chalcones and the obtained flavanones was monitored by hplc and calculated by using the internal standard (chalcone and flavanone respectively) for purposes of calibration.

The structures of the obtained flavanones were confirmed by spectral analysis (uv absorptions and typical ABX systems in the ^{1}H nmr spectra) and by hplc coinjection with standard samples.

The eluting system used was MeOH-H_2O (65-35) enriched with 1% of TFA and the flow rate was 1 ml/min.

Large scale experiments were carried out with 30 mg of substrate. Solvent, C.F.E. and buffer were added proportionally to standard method.

The reaction mixtures were purified by chromatography on silica gel columns with various eluting systems.

Synthesis of chalcones (1-16)

Chalcones 1-16 were prepared according to the literature.[9,11]

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