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# SPONTANEOUS AND ENZYMIC REARRANGEMENT OF NARINGENIN CHALCONE TO FLAVANONE

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**Key Word Index**—*Petunia hybrida*; Solanaceae; flower petals; flavonoid pathway; chalcone isomerase; chalcone synthase.

**Abstract**—The isomerisation of 2',4,4',6'-tetrahydroxychalcone by the enzyme chalcone isomerase is difficult to assay accurately in view of the spontaneous cyclisation of this chalcone at the alkaline pH optimum of the enzyme. We report here that self-cyclisation of naringenin chalcone is dramatically reduced at pH-values ≤ 6.5 and in the presence of high concentrations of serum albumin (5–10 mg $ml^{-1}$). We have critically evaluated existing assay procedures of chalcone isomerase, utilizing the effects of a monospecific anti-(chalcone isomerase) serum to distinguish between spontaneous and enzymic cyclisation of chalcone. We conclude that the modifications listed above considerably facilitate the measurement of chalcone isomerase kinetics.

## INTRODUCTION

The enzyme chalcone isomerase (CHI, EC 5.5.1.6.) is the second key enzyme of flavonoid biosynthesis in higher plants and catalyses the stereospecific conversion of chalcones to their corresponding (−)-flavanones (Fig. 1) [1–5]. The enzyme activity is usually assayed using 2',4,4',6'-tetrahydroxychalcone (naringenin chalcone) as substrate, although the enzyme from certain sources, but not all those so far tested, can also act on 2',4,4'-trihydroxychalcone (isoliquiritigenin) [5, 6]. The substrate specificity of chalcone isomerase appears to reflect the A-ring hydroxylation pattern (6'-hydroxy or 6'-deoxy) of the flavonoid derivatives accumulating in the species from which the enzyme is isolated [7].

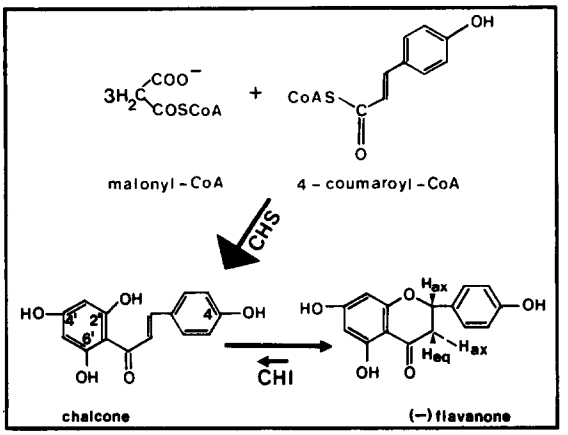


Fig. 1. Simplified scheme for the first two steps of the flavonoid glycoside pathway, CHS: chalcone synthase; CHI: chalcone isomerase.

Recently, considerable attention has been focussed on chalcone isomerase and other enzymes of phenylpropanoid metabolism in view of their inducibility by agents such as UV light, fungal pathogens and elicitor molecules isolated from fungal pathogens [7–11]. In *Petunia*, chalcone isomerase has been studied in relation to the gene *Po*. *Po*-recessive *Petunia* lines express chalcone isomerase in flower petals but not in pollen [3, 4], whereas *Po* dominant lines show activity in both petals and pollen.

The studies mentioned above require the availability of a reliable chalcone isomerase assay. In the procedures described for assay of enzymic cyclisation of 2',4,4',6'-tetrahydroxychalcone [2, 4], correction has to be made for the rapid spontaneous non-enzymic cyclisation of this chalcone substrate and precautions have to be taken to inhibit effectively the activity of peroxidase, which can catalyse extremely rapid oxidation of chalcones [12]. In order to optimize the measurement of chalcone isomerase activity in crude extracts of *Petunia*, we have studied the cyclisation of 2',4,4',6'-tetrahydroxychalcone in greater detail. We report on two improvements in the assay procedures which allow for accurate and unequivocal measurement of the stereospecific isomerase-mediated chalcone cyclisation in the near absence of random self-cyclisation.

## RESULTS AND DISCUSSION

In our initial experiments we used the assay conditions for chalcone isomerase as described by van Weely *et al.* [4]. We confirmed their (undocumented) observations that chemical cyclisation of 2',4,4',6'-tetrahydroxychalcone is linear with chalcone concentrations up to 25 μM, and that, as also shown by Sütfeld and Wiermann

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[13], the reaction kinetics depend critically on pH. We now document this pH effect in Fig. 2. At pH values $\leq 5$ hardly any spontaneous cyclisation is observed. The conversion rate peaks at about pH 7.5, and drops off again at higher pH values. The pH profile for a crude preparation of *Petunia* chalcone isomerase is also depicted in Fig. 2. Although the enzyme has a pH optimum at 7.5, it is difficult to measure the activity at this pH in view of the extensive spontaneous isomerisation of the chalcone. However, if the enzyme is assayed at pH 5.5–6.5, the self-cyclisation proceeds at 10% of its maximal rate whereas chalcone isomerase still exhibits 60% of the activity at its pH optimum (top panel, Fig. 2). Assay at lower pH therefore allows a more accurate measurement of enzyme kinetics.

Cyclisation of naringenin chalcone is generally assayed by the accompanying decrease in absorbance at 360–380 nm. High concentrations of KCN (approx. 50 mM) must be added to inhibit contaminating peroxidase activities which otherwise would interfere with isomerase measurement [5, 6]. To establish critically whether the observed decrease in absorbance was entirely due to the action of chalcone isomerase, the enzyme was specifically removed from a crude extract of *Petunia* flowers by indirect immunoprecipitation using a monospecific antiserum raised against *Phaseolus vulgaris* chalcone isomerase [11] and Protein A Sepharose (see Experimental). The residual supernatant after this treatment did not increase the initially monitored rate of self-cyclisation of naringenin chalcone, thus indicating that chalcone isomerase was completely neutralised by the antiserum and that no residual activities catalysing chalcone removal were detected (Fig. 3). Moreover, the spectral properties of the product matched those of the flavanone naringenin (not shown). We therefore conclude that $\Delta A_{365\,nm}$ in this case represents a true measure of chalcone isomerase activity.

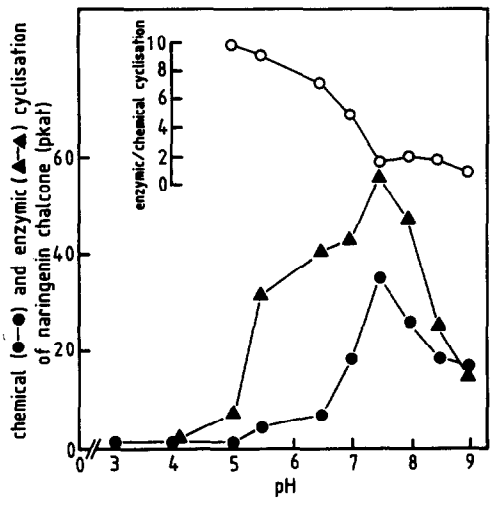


Fig. 2. The effect of pH on the chemical (●–●) and enzymic (▲–▲) cyclisation of naringenin chalcone. The spontaneous cyclisation of naringenin chalcone was monitored as described in Experimental and the enzymic conversion rate superimposed by the addition of 25 µl of crude *Petunia* V30 flower bud extract (= 50 µg protein).

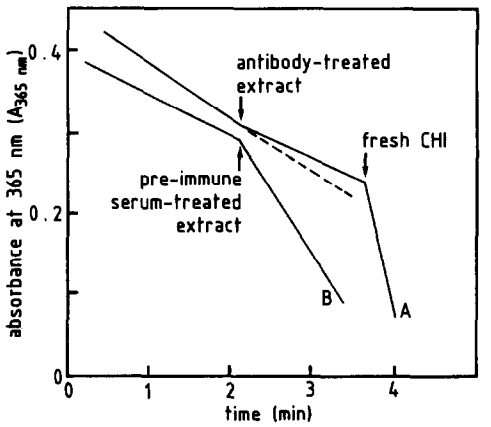


Fig. 3. Effects of removal of CHI from a crude *Petunia* flower bud extract followed by testing for residual enzyme activities which may cause a decrease in $A_{365\,nm}$. In curve (A), the rate of spontaneous chalcone cyclisation is measured for 2 min at pH 7.5 followed by addition (arrow) of a crude CHI extract from which the enzyme had been removed by treatment with 10 µl anti-(CHI) serum and protein A Sepharose. After 1 1/2 min, a fresh extract containing active CHI was added. In curve (B), a crude CHI extract treated with 10 µl pre-immune serum was added (arrow).

In control experiments using rabbit pre-immune serum, it was observed that the self-cyclisation reaction was consistently inhibited by serum concentrations of greater than 2% (v/v) at all pH values tested; at 5% the effect reached a plateau. This phenomenon is suggested by the results in Fig. 3, curve A, and is further documented in Table 1. The active component in the serum is most likely the albumin since the inhibitory effect could be mimicked by similar concentrations of bovine serum albumin.

In standard assay procedures for chalcone isomerase, corrections are made for spontaneous self-cyclisation. The protein effect reported above points to the possibility that isomerase itself or other proteins present in the extract may inhibit self cyclisation of chalcone in a concentration-dependent way, thus leading to errors in the estimation of enzymic rate. The results presented in Table 1 indicate that, at the protein concentration used (50–100 µg/ml), self-cyclisation is not inhibited. Furthermore, it follows that isomerase activity is not inhibited by the presence of 5% (v/v) of rabbit serum.

The above experiments therefore suggest two possible improvements in the assay procedures for chalcone isomerase. First, at slightly acid pH (5.5–6.5) the self-cyclisation of 2',4,4',6'-tetrahydroxychalcone proceeds extremely slowly, whereas the *Petunia* isomerase still exhibits half-maximal activity. Second, the presence of high concentrations of albumin severely depresses the self-cyclisation of chalcone at any pH value between 5.5 and 9.0. Under these conditions, the change in absorbance at 365 nm can be confidently assigned to stereospecific chalcone isomerisation. Both findings may be useful for studies which require (1) the accurate measurement of *Petunia* chalcone isomerase kinetics (acid pH and/or albumin), (2) the accurate measurement of chalcone isomerase from other plants which may have little activity at pH 5.5 (albumin), (3) the measurement of isomerase activity in non-denaturing gels (acid pH) and (4) the availability of pure stereoisomers of flavanones (acid pH and/or albumin).

Table 1. The effect of pre-immune serum on the cyclisation of naringenin chalcone

| Addition | Cyclisation of naringenin chalcone (pkat)* | | |
|---|---|---|---|
| | Spontaneous | Plus isomerase† | Isomerase (corr.) |
| None (pH 5.5) | 4.0 | 37.5 | 33.5 |
| 5% (v/v) serum‡ (pH 5.5) | <0.1 | 30 | 30 |
| None (pH 7.5) | 32 | 88 | 56 |
| 5% (v/v) serum (pH 7.5) | 3.0 | 63 | 60 |
| None (pH 9.0) | 18 | 40 | 22 |
| 5% (v/v) serum (pH 9.0) | 1.0 | 25 | 24 |

*Total naringenin chalcone present; 18.4 nmole. 100 pkat corresponds to 6 nmole naringenin converted $min^{-1}$.

†50 μl crude extract from *Petunia* flower buds.

‡Corresponding to 20 mg $ml^{-1}$ serum protein. Similar data are obtained using 5–10 mg/ml of bovine serum albumin.

## EXPERIMENTAL

*Chemicals.* Naringenin was obtained from Carl Roth (Karlsruhe). Naringenin chalcone (2',4,4',6'-tetrahydroxychalcone) was prepared according to Moustafa and Wong [14]. Bovine serum albumin was purchased from Organon Technica.

*Plant material.* The violet-flowering *Petunia hybrida* line V30 was grown in a greenhouse in normal daylight. Flower buds ranging in size from 30–50 mm were harvested and their apical parts frozen in liquid nitrogen until use.

*Enzyme preparation.* 4–5 buds were homogenized in a mortar with 1 g of Dowex 1 × 2–200 (Sigma) and a spoonful of acid-purified sand (BDH). Enzyme was extracted with 2 ml of 0.1 M sodium phosphate pH 8.0 containing 1.4 mM 2-mercaptoethanol. The homogenate was centrifuged for 5 min at maximum speed in an Eppendorf microcentrifuge and the supernatant was re-extracted twice with 0.5 g of Dowex to remove residual flavonoids. The final yellowish supernatant was used as enzyme source.

*Enzyme and immunological assays.* The composition of the basic chalcone isomerase reaction mixture was identical to that described by van Weely *et al.* [4]. At $t = 0$ min, 2',4,4',6'-tetrahydroxychalcone was added (final concentration 18.4 μM) and the non-enzymic cyclisation monitored, for 1–2 min, by the accompanying decrease in absorbance at 365 nm. The enzymic cyclisation was then estimated after addition of 10–100 μl crude extract (protein concentration 2 mg/ml; linear kinetics). The total conversion measured in this way was corrected for the non-enzymic conversion measured in the first 2 min [2–4].

For neutralization of *Petunia* chalcone isomerase activity by a monospecific antiserum raised against *Phaseolus vulgaris* chalcone isomerase [11], 25 μl of crude enzyme was incubated with increasing amounts of either pre-immune or anti- (CHI) sera (0–20 μl) plus 5 mg of Protein A-Sepharose beads (Pharmacia) on a rotary shaker at room temperature for 1 hr. After pelleting the beads in an Eppendorf centrifuge the supernatants were directly assayed for the presence of chalcone isomerase activity as described above. Protein was determined by the Biorad protein assay.

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