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CHALCONE CYCLASE AND FLAVONOID BIOSYNTHESIS IN GRAPEFRUIT

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Key Word Index—*Citrus paradisi*; Rutaceae; grapefruit; chalcone; flavanone; enzyme; cyclase; biosynthesis.

Abstract—A chalcone cyclase (CC), which acts unidirectionally upon the chalcone–flavanone equilibrium reaction, was isolated from immature grapefruit. The enzyme required neohesperidose at C-4′ of the chalcone A-ring and a free, unhindered hydroxyl group at C-4 of the B-ring for activity. The CC bound, but did not cyclize, prunin chalcone ($K_i = 2.5 \times 10^{-5}$ M). The results suggest that the intermediates that form the B-ring of chalcones are hydroxylated prior to chalcone formation, that chalcones are glycosylated during their formation, and that methylation occurs after cyclization of the chalcones to flavanones.

INTRODUCTION

Grapefruit (*Citrus paradisi*) contain two classes of bitter compounds; the limonoids, triterpenoids which are present in the seeds and fruit tissue, and flavanone neohesperidosides. Naringin [1, 2], a member of the latter class, is distributed throughout the fruit tissue, and its concentration in young fruit can be as high as 41% on a dry wt basis [3]. The abundance of flavanone glycosides and the effect of bitterness on consumer acceptance of grapefruit prompted us to study the enzymes and intermediates involved in flavonoid biosynthesis in the fruit.

In earlier work at this laboratory, the principal phenolic aglycones of grapefruit were identified [4], as were enzymes in the sequence of reactions leading from phenylpyruvate to *trans*-cinnamate [5, 6], and *p*-coumarate [7]. The biosynthetic sequence leading from C-9 precursors to flavanone glycosides in grapefruit has not yet been demonstrated. However, there is good evidence that chalcones are enzymatically cyclized to flavanones. Naringin has the (2*S*) configuration in very young grapefruit and undergoes partial racemization as the fruit matures [8]. Chalcone flavanone isomerases have been demonstrated in a number of plant tissues [9–14], and a similar enzyme has been demonstrated in grapefruit [5].

We now report the isolation and characterization of a new chalcone cyclase from immature grapefruit and discuss its possible role in grapefruit flavonoid biosynthesis.

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RESULTS

Purification and general properties of the enzyme

Naringin was identified by TLC and UV as the sole product resulting from enzyme action of an acetone powder (AP) extract on naringin chalcone. When boiled for 5 min, the crude AP extract lost 80% of its initial activity; when boiled for 15 min, the extract showed no measurable activity.

The enzyme was purified *ca* 65-fold by DEAE- and CM-cellulose column chromatography (Table 1), and its properties were then studied. The purified enzyme preparation retained substantial activity for several weeks in the refrigerator.

The enzyme activity peaked sharply at pH 7, decreasing to less than 50% at 0.5 pH unit above and below the optimum. Naringin was not chalconized by the enzyme in the range of pH 4–10. Hence, the enzyme was considered to be a chalcone cyclase (CC) rather than a chalcone–flavanone isomerase (CFI). The CC had a MW of *ca* 53000, as determined by Sephadex G-200 chromatography.

Effects of inhibitors

The cyclase was unaffected by EDTA or sodium azide; direct addition of KCN to the assay mixture caused total inhibition. However, CC that had been incubated with KCN, then dialyzed for removal of the inhibitor, showed no loss of activity. Spectral examination of the chalcone-KCN blank indicated probable chemical reaction between them, yielding a nonflavanone product.

Exhaustive dialysis of the purified enzyme against deionized water did not decrease its activity. Hence, the

Table 1. Purification of chalcone cyclase

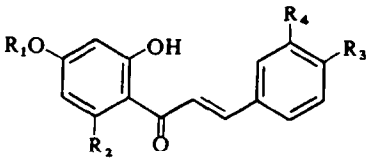
Treatment	Total vol. (ml)	Total protein (mg)	Sp. act. (unit/mg protein)	Recovery %	Relative activity
Extract (desalted, conc, × 7)	7	118	8150	100	1
DEAE-cellulose column	38	1.5	325000	51	40
CM-cellulose column (conc × 10)	39.5	0.7	540000	39	65

cyclase apparently did not require a metal cofactor. Nor was activity dependent on sulhydryl groups, as the cyclization rate was unaffected by either *p*-hydroxymercuribenzoate or Hg^{2+}.

Substrate specificity

Eleven chalcones, both as glycosides and as aglycones (**1–11**), differing widely in the substitution patterns of both aromatic rings were tested as substrates of the enzyme. The results (Table 2) showed that the cyclase was highly specific, cyclizing only glycosides **1**, **2**, **3**, and **5**.

The chalcone aglycones (**9–11**) were not cyclized by the enzyme, nor was prunin chalcone (**8**). Apparently, the enzyme required a disaccharide at C-4′ of ring A. Naringenin chalcone-4′*β*-rutinoside was unavailable, so we could not test the effect of the rhamnoside linkage on the enzyme.



(**1**) R_1 = Neo; R_2 = R_3 = OH; R_4 = H (naringin chalcone)
(**2**) R_1 = Neo; R_2 = OMe; R_3 = OH; R_4 = H
(**3**) R_1 = Neo; R_2 = OH; R_3 = OMe; R_4 = H (poncirin chalcone)
(**4**) R_1 = Neo; R_2 = R_3 = OMe; R_4 = H
(**5**) R_1 = Neo; R_2 = R_3 = OH; R_4 = OMe (homoeriodictyol chalcone neohesperidoside)
(**6**) R_1 = Neo; R_2 = R_4 = OH; R_3 = OMe (neohesperidin chalcone)
(**7**) R_1 = Rut; R_2 = R_4 = OH; R_3 = OMe (hesperidin chalcone)
(**8**) R_1 = Glc; R_2 = R_3 = OH; R_4 = H (prunin chalcone)
(**9**) R_1 = R_4 = H; R_2 = R_3 = OH (naringenin chalcone)
(**10**) R_1 = R_2 = R_4 = H; R_3 = OH (isoliquiritigenin)
(**11**) R_1 = H; R_2 = R_4 = OH; R_3 = OMe (hesperetin chalcone)

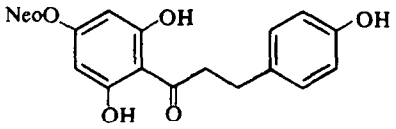
The presence of a OMe group at C-6′ (**2**) considerably decreased the rate of cyclization. This may be due in part to steric hindrance of the cyclase by the substituent. However, the inability of the substituted OH group to form a hydrogen bonded bridge with the carbonyl group may also be a factor, resulting in destabilization of the flavanone and a shift of the equilibrium to the left. This would lead to a slower observed net forward rate. Poncirin chalcone (**3**) and homoeriodictyol chalcone-4′*β*-neohesperidoside (**5**) were cyclized at only a fraction of the rate at which naringin chalcone (**1**) was cyclized; these results indicate that the enzyme required a free, unhindered OH group at C-4 of the B-ring for substantial activity. The indication was further supported by the inability of the enzyme to cyclize **4**, **6**, and **7**.

Table 2. Specificity of chalcone cyclase

Chalcone	Cyclization rate (ΔA 365 nm/hr/ml enzyme)	Relative rate (%)
1	2450	100
2	742	30
3	147	6
5	17	1

Kinetics of inhibition of chalcone cyclase

Since the CC was so specific, we tested it with two structurally related compounds, prunin chalcone (**8**) and naringin dihydrochalcone (**12**) for competitive inhibition. The K_m and V_{max} data (Table 3) indicate that naringin DHC had no affinity for the enzyme and did not cause inhibition. Prunin chalcone did competitively inhibit the enzyme and had a greater affinity for the enzyme (K_i = 2.5×10^{-5} M) than did naringin chalcone (K_m = 1.3×10^{-4} M).



(**12**)

Presence of other cyclases in grapefruit tissue

The crude acetone powder extract was tested for other cyclases (isomerases) with poncirin chalcone (**3**), neohesperidin chalcone (**6**), and isoliquiritigenin (**10**) as substrates. Only poncirin chalcone was cyclized. The relative rates at which **3** and **1** were cyclized was the same whether the crude extract or the purified CC was used. Hence, the activity of the crude preparation was not due to a separate cyclase.

DISCUSSION

The grapefruit has yielded one of the most complete complements of metabolic intermediates involved in flavonoid biosynthesis yet found in a single plant tissue [15]. Maier and Metzler [4] isolated a great number of simple phenolics and flavonoid aglycones from grapefruit tissues and proposed a metabolic grid with the chalcone–flavanone equilibrium placed at the center. Our results with the CC support their basic biosynthetic scheme, but suggest that glycosylation occurs during chalcone formation as discussed below.

Table 3. Kinetics of inhibition of chalcone cyclase

Substrate	K_m (M)	K_i (M)	V_{max} (units/mg protein)
Naringin chalcone	1.3×10^{-4}	—	4.5×10^4
Naringin chalcone + 10^{-4} M naringen dihydrochalcone	1.3×10^{-4}	—	4.5×10^4
Naringin chalcone + 10^{-5} M prunin chalcone	1.9×10^{-4}*	2.3×10^{-5}	4.5×10^4
Naringin chalcone + 3×10^{-5} M prunin chalcone	2.7×10^{-4}*	2.7×10^{-5}	4.5×10^4

* $K_{m_{app}}$

The initial steps of the flavonoid biosynthetic sequence in grapefruit have now been quite well established. Fisher [16] demonstrated the production of radioactive naringin in grapefruit fed phenylalanine-[^{14}C]. *trans*-Cinnamate is synthesized from phenylpyruvate and glutamate [7], and from phenylalanine by phenylalanine ammonia lyase [5]. Recently Hasegawa and Maier [6] also demonstrated that *p*-coumarate is synthesized in grapefruit tissue by hydroxylation of *trans*-cinnamate.

Caffeic acid synthesis in the grapefruit has not yet been studied. However, studies of other plant tissues [17–19] have shown that caffeic acid is formed by hydroxylation of *p*-coumarate. In grapefruit, the presence of *trans*-cinnamic, *p*-coumaric, caffeic, and ferulic acids [4] and of hydroxylating enzymes indicate that these compounds are involved in the formation of the B-ring of flavanones and are, thus, hydroxylated before flavanone formation. The almost total requirement of the CC for a free, unhindered B-ring OH group strongly supports this hypothesis.

However, further clarification is still needed in this area. A neoeriocitrin chalcone cyclase is yet to be found in the grapefruit. Eriodictyol is present in minute quantities in glycoside hydrolyzates [4]; and if it is like the major grapefruit flavanones, it should be present as both the neohesperidoside and rutinoside. Neoeriocitrin chalcone is very unstable and thus could not be studied with the CC. The formation of eriodictyol by enzymatic hydroxylation of naringenin has been reported in spinach beets [19] and must be considered to be possible in grapefruit.

Most notably absent in the series of substituted cinnamic acids described by Maier and Metzler [4] were isoferulic acid and *p*-methoxycinnamic acid. Their absence suggests that OH groups of the B-ring are methylated after formation of the chalcone or flavanone. Our results support the idea that methylation occurs after cyclization because the CC did not cyclize **6** and **7** which are derivatives of isoferulic acid and exhibited only slight activity with **3**, a derivative of *p*-methoxycinnamic acid.

The recent report by Ebel *et al.* [20] of an *o*-dihydric phenol *m*-*O*-methyl-transferase which catalyzed methylation of the *m*-OH group of luteolin and, to a lesser extent, eriodictyol and caffeic acid, indicates that in other plants methylation occurs after flavonoid formation.

The fact that eriodictyol is present in hydrolyzed extracts of grapefruit [4] and the fact that our crude CC extract did not cyclize neohesperidin chalcone indicate that neohesperidin and hesperidin, both of which are present in grapefruit tissue [21–23], probably arise from their corresponding eriodictyol glycosides by methylation and not via their chalcones.

The requirement of the CC for a β-linked neohesperidoside at C-4′ of the chalcone distinguishes it from the citrus 'flavanone syntheases' of Shimokoriyama [12] which cyclized isosakuranin chalcone, and from the chalcone–flavanone isomerases of *Datisca cannabina* [13], mung bean, garbanzo bean, and parsley [10], all of which were specific for chalcone aglycones.

This C-4′ requirement suggests that glycosylation precedes chalcone cyclization in the grapefruit. The suggestion is supported by the fact that neither free naringenin nor prunin has been found in grapefruit. Glycosylation of the free chalcone aglycones, however, is probably unlikely since the aglycones are much less stable than their respective glycosides and would probably cyclize spontaneously before glycosylation could occur.

Hahlbrock *et al.* [24] and Kruezaler and Hahlbrock [25] postulated an intermediate, enzyme-bound β-triketo acid which is transformed to the chalcone by a 'chalcone synthetase,' and demonstrated formation of naringenin from *p*-coumaroyl-CoA and malonyl-CoA by cell-free extracts from illuminated parsley cell cultures. A similar pathway in the grapefruit involving a β-neohesperidoside-specific 'chalcone synthetase' would be consistent with the specificity exhibited by the CC.

The strong competitive inhibition of the CC by prunin chalcone (**8**) in the cyclization of naringin chalcone may have implications in the control of flavonoid biosynthesis in grapefruit. The lack of measurable affinity between naringin DHC and the CC (Table 3) indicates that the chalcone double bond is necessary for binding by the CC.

The apparent CFI activity with isoliquiritigenin reported by Maier and Hasegawa [5] was not found in the fruit used for our study. The reason for the discrepancy in results is not known.

We have not yet conclusively demonstrated stereospecificity of the CC *in vitro*. Gaffield *et al.* [8] showed that naringin is present in young (10 g size) grapefruit almost exclusively as its (2*S*) isomer. The finding suggests that a stereospecific CC is present. Enzymatic stereospecific flavanone formation would be consistent with results reported for chalcone–flavanone isomerases in other plants [9, 24, 26–30].

Gaffield *et al.* [8] also demonstrated that as grapefruit increase in size, they become progressively richer in the (2*R*) isomer of naringin. At maturity, the ratio of (2*S*):(2*R*) isomers is about 3 : 2 and remains so thereafter. The fact that a racemate is never produced indicates that another enzyme-mediated reaction may maintain the predominance of the (2*S*) isomer. We have evidence of such an enzyme [31] and will report it elsewhere.

EXPERIMENTAL

Plant material. Immature grapefruit (*Citrus paradisi* Macfad. cv Marsh) ranging in wt from 2–10 g were obtained from several mature trees at the Shell Ranch and at the USDA Date and Citrus Station, Indio, California. The fruit were held at −20° until used.

Acetone powder (AP) preparation and extraction. Frozen fruits were ground × 3 at −50° in Me_2CO with a blender, the homogenate transferred to a filter pad and washed with Me_2CO at −50° until the filtrate was colorless. The resulting powder was dried *in vacuo* and stored at 0°. The AP was extracted as follows: 10 g of AP, 300 ml of cold 0.1 M Tris buffer (pH 8), and 20 g of hydrated insoluble PVP were mixed at 0° for 45 min. The extract was pressed through cheesecloth, and the solids were re-extracted with 150 ml of buffer. The combined extracts were centrifuged for 10 min at 25000 *g* and the pellets discarded. The supernatant was brought to 50% satn at 0° with solid $(NH_4)_2SO_4$ and centrifuged at 8500 *g* for 5 min; the pellets were discarded. The supernatant was brought to 90% satn with solid $(NH_4)_2SO_4$ and re-centrifuged. The supernatant was discarded, and the pellets were dissolved in 15 ml of 0.1 M Tris buffer (pH 8). The soln was then desalted on a 2.5 × 45 cm jacketed Sephadex G-25 column with the Tris buffer as an elutant. The active fractions were pooled and concd to 7 ml on an ultrafilter under 3.9 kg/cm^2 N_2 pres.

Cellulose column chromatography. The enzyme concentrate was dialyzed sequentially against deionized H_2O and 0.01 H Tris buffer (pH 8), then loaded onto a 2.5 × 19 cm jacketed DEAE-

cellulose column chilled to 3° and equilibrated with 0.01 M Tris buffer (pH 8). The column was washed with 70 ml of buffer, then with a 500 ml linear NaCl gradient. The mixer contained 250 ml of 0.01 M Tris buffer (pH 8), and the reservoir contained 250 ml of M buffered NaCl. The CC was eluted at a NaCl concn of *ca* 0.2 M; whereas the bulk of the proteins and all of the colored material were eluted at salt concns between 0.5 and 0.8 M. The fractions with CC activity were pooled and concd to 10 ml on the ultrafilter. The concentrate was dialyzed against deionized H_2O and loaded onto a 1.6 × 19 cm CM-cellulose column (3°) equilibrated with 0.01 M Pi buffer (pH 7). The column was eluted with a 400 ml linear NaCl gradient. the mixer containing 200 ml buffer and the reservoir 200 ml of M buffered NaCl. The CC was eluted at a NaCl concn of *ca* 0.1 M, whereas the major protein fraction was eluted at 0.2 M salt concn.

Chalcone cyclase assay and product analysis. CC activity was measured by the decrease in *A* at 365 nm and 25°. The assay mixture contained 0.1 M Pi buffer (pH 7), 8×10^{-5} M chalcone, and 100–200 units of enzyme in a total vol. of 1 ml. A blank soln was prepared as above with heat-inactivated enzyme. One unit of CC is defined as the amount of enzyme necessary to catalyze cyclization of 1 nmol of chalcone per min under these conditions. Protein was determined by the method of ref. [32]. For product identification, the assay mixture was extracted with 3 × 1 ml portions of EtOAc, the extracts combined and evapd to dryness. The residue was redissolved in a drop of MeOH, and 1 μl was chromatographed on a polyamide TLC strip with NO_2Me–MeOH (5:2) as solvent. The chromatogram was visualized as described below.

Tests for inhibition of CC activity. Known noncompetitive inhibitors were tested both by direct addition to the blank and assay mixtures to a final concn of 10^{-3} M and by assay of the enzyme after its dialysis first against 10^{-3} M inhibitor and then deionized H_2O. For competitive inhibition studies, naringin chalcone at 10, 5, 3.3, 2.5, and 2×10^{-5} M was assayed alone and in the presence of 10^{-5} M naringin DHC (**12**) or either 1 or 3×10^{-5} M prunin chalcone (**8**). The assay mixture contained substrate and inhibitor, 0.1 M Pi buffer (pH 7), and 105 units of enzyme in a total vol. of 1 ml. Blanks were determined in triplicate, and assays were the average of 7 determinations.

Substrates and analytical methods. Samples of known chalcones and flavanones were obtained from R. M. Horowitz and B. Gentili of this laboratory. Chalcones and flavanones were analyzed by TLC on polyamide strips with NO_2Me–MeOH (vols as indicated) as solvent. They were purified on preparative plates of Si gel G with $CHCl_3$–MeOH (vols as indicated) as solvent. For visualization of compounds, the chromatograms were sprayed with an $NaBH_4$–HCl [33] reagent and examined under long and shortwave UV light.

Preparation of substrates. 4′,5-Dimethoxy-7β-neohesperidosylflavanone was prepared by exhaustive methylation of naringin with CH_2N_2–Et_2O and was purified by preparative-TLC with $CHCl_3$–Me_2CO (3:2). Analyses: λ_{max}(EtOH) 278, 310 nm; no shifts with $AlCl_3$ or fused NaOAc indicated the absence of free OHs at either C-5 or C-7 [34]; TLC with NO_2Me–MeOH (7:1) gave R_f 0.23 (naringin 0.065; poncirin 0.16); negative ferric test; PMR 100 MHz ($CDCl_3$) of acetylated aglycone showed two 3-proton singlets at τ = 6.08 and 6.16 (OMe) and one 3-proton singlet at τ = 7.68 (OAc). For preparation of its chalcone, the flavanone was heated for 10 min in 25% NaOH. The reaction mixture was cooled to 0° and acidified to pH 3. The pptd chalcone (**4**) was recrystallized from EtOH containing a few drops of H_2O. Analyses: λ_{max}(EtOH) 362 nm; λ_{max}(+ NaOEt) 325 nm (38% loss in intensity). The spectra are characteristic of chalcones containing a free OH group only at C-2′ [35]. Mp 186–187°. Homoeriodictyol-7β-neohesperidoside (HN) was prepared from the chalcone formed by the condensation of vanillin with phloracetophenone-4β-neohesperidoside (PN) according to ref. [36]. The reaction mixture was acidified and the chalcone was cyclized by heating at 100° for 1 hr. Vanillin was removed by exhaustive extraction with Et_2O, and the aq. phase was concd *in vacuo* and chilled for 2 days to ppt. the PN. The mother liquor was transferred to a Sephadex G-10 column (2 × 40 cm), which was then sequentially eluted with H_2O and then with MeOH. The flavanone was eluted with MeOH. The HN soln was concd, seeded with pure HN crystals, and held 18 hr at 3°. The crystals were collected by suction filtration and washed with ice cold MeOH. Analyses: λ_{max}(EtOH) 283, 326 nm; λ_{max}(+ NaOAc) 283, 326 nm; λ_{max} (+ $AlCl_3$) 307, 376 nm; mp 262–262.5° (known HN, 261–262°); TLC with NO_2Me–MeOH (5:2) showed one spot, R_f 0.71 (known HN R_f 0.71). The flavanone was chalconized as described above yielding (**5**). Poncirin chalcone (**3**) was prepared by methylation of naringin chalcone with DMSO. The mole ratio of reactants was 1:1. Poncirin chalcone was partially purified by preparative-TLC, with $CHCl_3$–MeOH (7:2) as solvent. It was extracted from the adsorbent with H_2O, and the aq. soln was then extracted with EtOAc. Concentration of the EtOAc soln yielded a crystalline product. The chalcone was recrystallized from EtOAc. Analyses: λ_{max}(MeOH) 353 nm; λ_{max}(+ $AlCl_3$) 395 nm; TLC with NO_2Me–MeOH (5:2) showed one spot, R_f 0.49; PMR 100 MHz of the peracetylated chalcone ($CDCl_3$) showed one 3-proton singlet at τ = 6.18 (OMe), eight 3-proton singlets between τ = 7.62 and 8.02 (OAc). The chalcone was deacetylated and hydrolyzed and the aglycone was cyclized. Analyses: λ_{max} (MeOH) 280, 325 nm; λ_{max} (+ $AlCl_3$) 312, 378 nm. These maxima all indicated that the cyclized aglycone was isosakuranetin. The 6′-methoxyl derivative of PN was prepared by methylation with MeI. The mole ratio of PN to MeI was 2:1. The product was crystallized from EtOH. Analyses: TLC with NO_2Me–MeOH (3:2) showed one spot, R_f 0.59 (PN, R_f 0.45); λ_{max} (MeOH) 284, 318 nm; λ_{max} (+ $AlCl_3$) 307, 365 nm; PMR 100 MHz ($CDCl_3$) of the peracetylated derivative showed one 3-proton singlet at τ = 6.16 (OMe), one 3-proton singlet at τ = 7.54 (aromatic ring OAc), one 3-proton singlet at τ = 7.68 (acetophenone Me) and six 3-proton singlets between τ = 7.8 and 8.1; OAc/OMe = 7/1. The 6′-methoxyl derivative of naringin chalcone (**2**) was prepared by the condensation of 6′-methyl-PN with *p*-hydroxybenzaldehyde. The method was the same as that used for the preparation of HN. The chalcone was extracted from the aq. soln with EtOAc after removal of *p*-hydroxybenzaldehyde by exhaustive extraction with Et_2O. The chalcone was crystallized from the EtOAc soln after it was concd and chilled. The crystals were collected by filtration and washed with cold EtOAc. Upon contact with air, the crystals turned into an amorphous gum which was dried and ground in a mortar. The chalcone was recrystallized twice from EtOAc. Analyses: λ_{max} (MeOH) 366 nm; λ_{max} (+ $AlCl_3$) 420 nm; TLC with NO_2Me–MeOH (5:2) yielded one spot, R_f 0.32 (naringin chalcone, 0.14; poncirin chalcone, 0.587); PMR 100 MHz of the peracetylated derivative ($CDCl_3$) showed one 3-proton singlet at τ = 6.2 (OMe); eight 3-proton singlets between τ = 7.6 and 8.1 (OAc).

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