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Enzymes of B-Ring-Deoxy Flavonoid Biosynthesis in Elicited Cell Cultures of "Old Man" Cactus (*Cephalocereus senilis*)[1]

Qin Liu,* Maureen S. Bonness,* Ming Liu,* Espanta Seradge,*
Richard A. Dixon,†,[2] and Tom J. Mabry*

**Department of Botany, The University of Texas at Austin, Austin, Texas 78713; and †Plant Biology Division, The Samuel Roberts Noble Foundation, P.O. Box 2180, Ardmore, Oklahoma 73402*

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Elicited cell cultures of the cactus *Cephalocereus senilis* produce a group of flavonoids with unsubstituted B-rings, including an aurone which represents a new class of phytoalexin. Preliminary enzymological studies indicated that the chalcone synthase (CHS) and chalcone isomerase (CHI) from cultures of *C. senilis* were active with cinnamoyl-CoA and 2′,4′,6′-trihydroxychalcone, respectively, probable intermediates for synthesis of flavonoids with unsubstituted B-rings. We now demonstrate that the cultures contain two isoforms of CHI, both of which are induced by elicitor treatment and are active with both 2′,4,4′,6′-tetrahydroxy- and 2′,4′,6′-trihydroxychalcone. (Hydroxy)-cinnamate:CoA ligase in the cactus cultures was active with cinnamic, 4-coumaric, caffeic, ferulic, and 4-methoxycinnamic acids, but not sinapic acid. A single form of CoA ligase, as resolved by chromatofocusing analysis, was active against both cinnamate and 4-coumarate. Cinnamic acid 4-hydroxylase (CA4H) activity was induced by elicitor treatment. Thus, elicited cultures contain the necessary enzymatic activities for synthesis of B-ring-hydroxy and -deoxy flavonoids. Synthesis of only the deoxy class in response to elicitation may result from some form of metabolic compartmentation through which the CA4H reaction is bypassed, leading to formation of cinnamoyl CoA which may then be incorporated into B-ring deoxy flavonoids via nondiscriminating CHS and CHI activities.

Key Words: **flavonoid biosynthesis; enzyme induction; cinnamic acid 4-hydroxylase; (hydroxy)-cinnamate:CoA ligase; chalcone isomerase; elicitation.**

Phytoalexins have long been proposed to be important factors in resistance to plant pathogens, and it has recently been demonstrated that transgenic plants producing a novel phytoalexin have enhanced disease resistance (1). The isoflavonoid phytoalexins of leguminous crop plants such as alfalfa, soybean, and bean have been extensively studied, at both enzymological and molecular genetic levels (2–5). We have developed *Cephalocereus senilis* ("old man" cactus) as a model system for phytoalexin research on drought-adapted plants (6). Cell cultures of this cactus have an interesting response to microbial infection and elicitor treatment, producing a series of unusual flavonoid derivatives. Chemical analysis of cultures elicited with chitin led to the discovery of a group of flavonoids lacking a hydroxyl group at the 4′ position of the B-ring (Fig. 1) (7–10). One of these flavonoids was characterized as a new aurone, cephalocerone, representing a new class of phytoalexins. For comparison, we examined the flavonoids produced in stem tissues of old man cactus plants and found that these possess the usual 4′-hydroxyl group (11). The lack of flavonoids in unelicited cultures and the appearance of the B-ring unsubstituted compounds following elicitation indicate that a novel biosynthetic pathway is induced by elicitors.

The biosynthetic origin of flavonoids with unsubstituted B-rings is not fully understood. 4-Coumaric acid was thought to be the exclusive precursor for plant flavonoids, except for a few cases where caffeic acid was utilized (12). However, Paré *et al.* (6) demonstrated that both 4-coumaroyl-CoA and cinnamoyl-CoA were

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[2] To whom correspondence should be addressed at Plant Biology Division, The Samuel Roberts Noble Foundation, P.O. Box 2180, Ardmore, OK 73402. Fax: (405) 221-7380.

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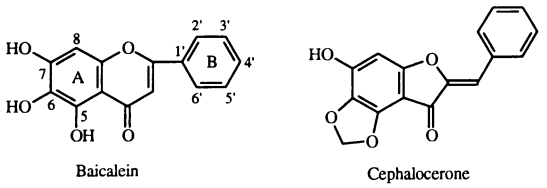


FIG. 1. Examples of B-ring unsubstituted flavonoids isolated from elicited cultures of *C. senilis*.

substrates for the chalcone synthase (CHS)[3] in elicited old man cactus cultures and that both 2′,4,4′,6′-tetrahydroxychalcone and 2′,4′,6′-trihydroxychalcone were substrates for the subsequent enzyme, chalcone isomerase (CHI) (Fig. 2). However, it was not clear whether this dual substrate specificity reflected the presence of single CHS and CHI forms that were active on both classes of substrate or the presence of isoforms that could discriminate between the different B-ring substitution patterns. CHS activity with cinnamoyl-CoA was recently reported to be present in Scots pine (*Pinus sylvestris*) (13).

In this paper we show that elicited cultures of *C. senilis* contain all the enzymatic activities necessary for the formation of both B-ring hydroxy and B-ring deoxy flavonoids, according to the pathways shown in Fig. 2. Specifically, the cultures contain two forms of CHI, but both are capable of converting chalcone with or without the B-ring 4-hydroxyl group into flavonoids. The enzyme (hydroxy)-cinnamate:CoA ligase (generally referred to as 4-coumarate:CoA ligase, 4CL) usually shows a strong preference for hydroxy- or methoxy-substituted cinnamic acids; the cactus enzyme is able to efficiently activate unsubstituted cinnamic acid to its CoA thiol ester. We propose that flux into the B-ring-unsubstituted flavonoid pathway in elicited cells occurs as a result of the presence of this unusual (hydroxy)-cinnamate CoA ligase activity along with some novel form of metabolic compartmentation which bypasses the cinnamic acid 4-hydroxylase (CA4H) reaction, thus leading to the formation of cinnamoyl CoA, the key substrate for the formation of B-ring deoxy flavonoids.

MATERIALS AND METHODS

Plant material. Cell suspension cultures derived from stem tissue of *C. senilis* were grown as described previously (6, 7). Cells were elicited by addition of autoclaved chitin to a final concentration of 0.25 mg chitin per ml culture (9).

[3] Abbreviations used: PAL, phenylalanine ammonia-lyase; CA4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; NMR, nuclear magnetic resonance; PVPP, polyvinylpolypyrrolidone; Bis–Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

Young plants (3–4 years old) of *C. senilis* and other cacti were purchased from Living Desert Nursery (Austin, TX). *Petunia hybrida* was obtained from a local plant nursery. Cut parsley was purchased from a local grocery store. A voucher of *C. senilis* is deposited in the Plant Resources Center, The University of Texas at Austin.

Preparation of chalcones. 2′,4,4′,6′-Tetrahydroxychalcone and 2′,4′,6′-trihydroxychalcone were prepared from the flavanones naringenin (Sigma, St. Louis, MO) and pinocembrin (Indofine Chemical Company, Belle Mead, NJ), respectively, according to Moustafa and Wong (14), except that the alkali treatment of pinocembrin was carried out at 70°C instead of 100°C. The chalcones were purified over a silica gel column eluted with CH_2Cl_2–MeOH containing increasing amounts of MeOH. The structures of the chalcones were confirmed by ^{1}H-NMR and uv spectroscopy. The molar extinction coefficient of 2′,4,4′,6′-tetrahydroxychalcone at 400 nm, pH 8, was 33,113 (15) and that of 2′,4′,6′-trihydroxychalcone in 60 mM KH_2PO_4, pH 8, containing 50 mM KCN (the assay buffer for CHI), was determined to be 16,982.

Extraction and separation of CHI isoforms by chromatofocusing. All of the following procedures for enzyme extraction and purification were carried out at 4°C unless otherwise noted.

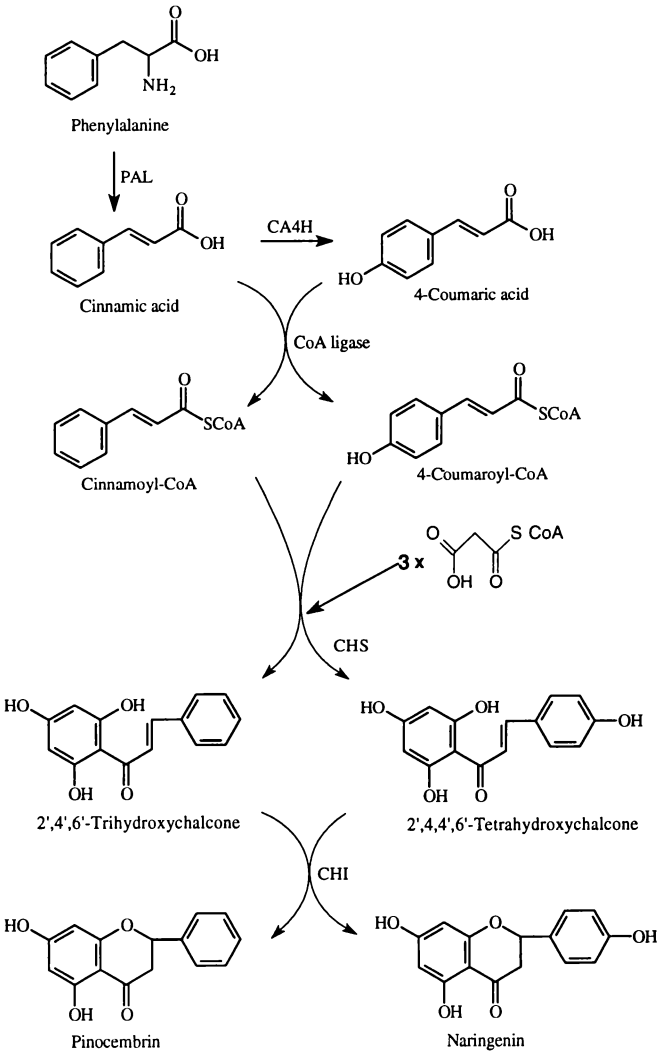


FIG. 2. Biosynthetic pathways leading to B-ring unsubstituted and substituted flavonoids in *C. senilis*.

CHI was extracted by homogenizing frozen cactus culture cells (20 g) in 40 ml of 50 mM Tris–HCl, pH 8.5, containing 1.4 mM 2-mercaptoethanol in the presence of 5% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was squeezed through four layers of cheesecloth, centrifuged at 25,000*g* for 20 min, and the supernatant subjected to ammonium sulfate fractionation. The pellet which precipitated between 40 and 80% saturation was dissolved in 5 ml of homogenization buffer and desalted through two PD-10 columns (Pharmacia, Uppsala, Sweden). A sample containing 10.58 mg protein was applied to a chromatofocusing column (1 × 20 cm) packed with polybuffer exchanger 94 (Pharmacia) which had been equilibrated with 150 ml of 25 mM Bis–Tris/HCl, pH 6.3, containing 2 mM dithiothreitol. The column was eluted with 7% polybuffer 74, pH 4, containing 2 mM dithiothreitol. Ninety fractions (2 ml each) were collected. For examining CHI in H_2O-treated (control) cactus cultures, 10.46 mg of protein material was loaded onto the column and the collection volume was 3 ml.

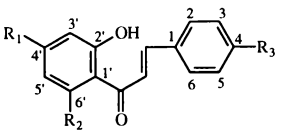
CHI was extracted from chlorenchyma tissue of 3- to 4-year-old *C. senilis* plants by peeling off the epidermis and excising the thin underlying dark green layer of tissue. This layer was chosen as it contained markedly less mucilage than stem cross-sections. A sample from the chlorenchyma tissue containing 10.60 mg protein was analyzed by chromatofocusing as above, but collecting 5.5-ml fractions. The fractions with CHI activity were combined and subjected to a second round of chromatofocusing, with 1-ml fractions being collected.

CHI assay. CHI activity was determined according to Edwards and Kessmann (15). The kinetics of the reaction were monitored by measuring the decrease in absorbance at 400 nm for 2′,4,4′,6′-tetrahydroxychalcone and at 366 nm for 2′,4′,6′-trihydroxychalcone. The control decrease of absorbance in the absence of enzyme extracts was subtracted.

Extraction and partial purification of (hydroxy)cinnamate:CoA ligase by chromatofocusing. Cactus culture cells (150 g) harvested 24 h after elicitation were homogenized in a mortar in 300 ml of 0.1 M Tris/HCl, pH 7.8, 14 mM 2-mercaptoethanol, and 30% (v/v) glycerol in the presence of 5% (w/v) PVPP. After centrifugation at 25,000*g* for 20 min, the supernatant was treated with 5% (w/v) Dowex-1 ion exchange resin which had been preequilibrated with the same buffer and then subjected to ammonium sulfate fractionation. Proteins which precipitated between 40 and 80% saturation were collected and desalted on PD-10 columns in the buffer (25 mM Bis–Tris/HCl, pH 6.5, 2 mM dithiothreitol, 30% glycerol) in which the chomatofocusing column had been equilibrated. A sample containing 52.95 mg of protein was loaded onto the column which was eluted as for CHI except that the buffer contained 30% glycerol. Fraction size was 1.25 ml.

(Hydroxy) cinnamate:CoA ligase assay. The activity of CoA ligase was determined using a direct spectrophotometric assay (16). Formation of CoA esters was monitored at the relevant wavelengths, and the amount of product formed was calculated using previously reported extinction coefficients. In controls, CoASH was replaced with Tris buffer.

Preparation of microsomal fractions. Microsomal fractions were prepared from cultured cactus cells (5–10 g cells per time point) essentially as described by Edwards and Kessmann (15). Cells were homogenized in extraction buffer (0.1 M KH_2PO_4, pH 7.5, 0.4 M sucrose, 28 mM 2-mercaptoethanol) (1 g cells/ml buffer) in a mortar in the presence of Dowex-1 (200–400 mesh preequilibrated in the same buffer) and acid-washed sand. Homogenates were centrifuged at 8000*g* for 15 min, filtered through Miracloth, and the supernatants centrifuged at 135,000*g* for 80 min. Pellets were washed with 1 ml resuspension buffer (0.1 M KH_2PO_4, pH 7.5, 0.4 M sucrose, 3.5 mM 2-mercaptethanol) and resuspended in 350-μl resuspension buffer for CA4H assays. Microsomes were assayed for the marker enzyme NADH-dependent cytochtome C reductase by the method of Wray and Filner (17).



Chalcones	R_1	R_2	R_3
2′,4,4′,6′-tetrahydroxy	OH	OH	OH
2′,4′,6′-trihydroxy	OH	OH	H
2′-hydroxy	H	H	H
2′-hydroxy-4-methoxy	H	H	OMe
2′,4′-dihydroxy-4-methoxy	OH	H	OMe
2′,4,4′-trihydroxy	OH	H	OH

FIG. 3. Structures of the chalcones tested on *C. senilis* CHI.

CA4H assay. The activity of CA4H was determined by a modification of the HPLC method described by Edwards and Kessmann (15). Fifty microliters of microsomal preparation was preincubated with 480 μl 0.1 M KH_2PO_4, pH 7, and 20 μl cinnamic acid (2 mM in CH_3CN) for 5 min at 30°C. The reaction was initiated by adding 50 μl of 20 mM NADPH and incubating for 60 min at 30°C. The reaction was stopped by adding 40 μl 6 N HCl and the resulting solution was partitioned twice with 0.6 ml EtOAc (water-saturated), removing 0.45 ml of the organic phase after each extraction. The EtOAc extract was evaporated under vacuum. The residue was redissolved in 100 μl CH_3CN:H_2O (1:1) and the resulting solution analyzed by HPLC using an Altex Ultrasphere-octyl column or Econosphere column (250 × 4.6 mm, 5 μm). The mobile phase was a mixture of CH_3CN and 1% aqueous H_3PO_4 (40:60) with a flow rate of 0.8 or 1.0 ml/min. Peaks were monitored with a HP1040M diode array detector. UV spectra of peaks were compared to the spectrum of authentic 4-coumaric acid. A calibration curve for 4-coumaric acid (0–40 nmol) was established. Controls consisted of incubations in which the reaction was stopped at zero time or carried out in the absence of either NADPH or cinnamic acid.

Protein determination. Protein concentrations were determined by the BioRad dye-binding assay according to the manufacturer's method, using bovine serum albumin as a standard.

RESULTS

Substrate Specificity and Induction of Cactus CHI

The substrate specificity of the CHI activity in crude extracts from chitin-elicited cell cultures was examined with six chalcones: 2′,4,4′,6′-tetrahydroxychalcone, 2′,4′,6′-trihydroxychalcone, 2′-hydroxychalcone, 2′-hydroxy-4-methoxychalcone, 2′,4′-dihydroxy-4-methoxychalcone, and 2′,4,4′-trihydroxychalcone (Fig. 3). Only 2′,4,4′,6′-tetrahydroxychalcone and 2′,4′,6′-trihydroxychalcone were found to be substrates for the enzyme.

The utilization of 2′,4′,6′-trihydroxychalcone by CHI has not, to the best of our knowledge, been previously reported in any other system (6). In order to determine whether cactus CHIs are unique in their substrate specificity, we examined the substrate specificity of CHI in crude extracts from five cactus species as well as petunia and parsley. CHI from the two latter species is known to catalyze the isomerization of 2′,4,4′,6′-tetrahydroxychalcone (18). All the cell extracts examined

TABLE I

Activity of CHI in Crude Extracts from Various Sources with 2′,4,4′,6′-Tetrahydroxychalcone or 2′,4′,6′-Trihydroxychalcone as Substrates

Enzyme source	Specific activity (μkat/g protein)	
	2′,4,4′,6′-Tetrahydroxychalcone	2′,4′,6′-Trihydroxychalcone
Cactus species		
Cephalocereus senilis stem	2.86	4.52
C. senilis culture (NE)	9.34	28.58
C. senilis culture (E)	18.2	46.69
Echinocactus horizonthalonius stem	0.41	0.76
E. horizonthalonius culture (NE)	1.52	6.08
E. horizonthalonius culture (E)	3.13	9.22
Stenocereus thurberi stem	Not detected	Not detected
Opuntia spp. stem	0.53	0.95
Myrtillocactus geometrizans stem	0.62	1.81
Noncactus species		
Parsley—whole plant	1.26	2.99
Petunia—flower	2.74	6.48

Note. E, elicited; NE, nonelicited.

were more active against the 4-deoxy chalcone than the tetrahydroxychalcone (Table I). It is not known whether the other cactus species accumulate B-ring deoxy flavonoids.

The question of whether the isomerase activity with 2′,4,4′,6′-tetrahydroxychalcone and 2′,4′,6′-trihydroxychalcone is due to dual substrate specificity of a single enzyme or to two distinct enzymes was addressed by chromatofocusing analysis (Fig. 4A). Although two isoforms of CHI with p*I*s of 5.0 and 4.8 were revealed by chromatofocusing, activities against the two substrates were not separable. Both forms of CHI from *C. senilis* exhibited dual specificity.

To determine if either of the two isoforms is specifically induced by elicitation, the pattern of CHI isoforms from H_2O-treated (control) cultures was also analyzed (Fig. 4B). Nonelicited cultures also contained two isoforms of CHI. Comparison of Figs. 4A and 4B indicates that both forms of CHI are induced by chitin treatment.

The activities of the two isoforms of CHI as a function of various substrate concentrations were determined for 2′,4,4′,6′-tetrahydroxychalcone and 2′,4′,6′-trihydroxychalcone. The two peaks of CHI activity, partially purified by chromatofocusing, were used for the assays. The K_m values for 2′,4,4′,6′-tetrahydroxychalcone as determined by Lineweaver–Burk double reciprocal plots were 16.2 μM for isoform I and 11.9 μM for isoform II. For 2′,4′,6′-trihydroxychalcone, the K_m was 4.0 μM for isoform I and 7.2 μM for isoform II.

Chemical investigation of flavonoids from intact plants of old man cactus has established that these contain a B-ring 4′-hydroxyl group and are thus typical of the type found in most plants (11). The activity of CHI from cactus stems was examined. This CHI was also able to use both 2′,4,4′,6′-tetrahydroxychalcone and 2′,4′,6′-trihydroxychalcone as substrates. As in cactus cell cultures, the activities of cactus stem CHI against 2′,4,4′,6′-tetrahydroxychalcone and 2′,4′,6′-trihydroxychalcone were not separable by chromatofocusing (Fig. 5A). However, only one form of CHI was found in cactus stems. To improve the resolution of the chromatofocusing analysis, fractions containing CHI activity were combined and subjected to a second round of chromatofocusing with smaller fraction volumes being collected. Again only one peak of CHI activity was resolved (Fig. 5B).

Substrate Specificity and Induction of C. senilis (Hydroxy) Cinnamate:CoA Ligase

The substrate specificity of CoA ligase in crude extracts from both elicited and unelicited cactus cells was determined using various cinnamic acid derivatives as substrates. With the exception of sinapic acid, all (hydroxy) cinnamic acids tested were substrates for the CoA ligase activity (Table II). Highest activity was seen for caffeic acid, but cinnamic acid was also a good substrate (73% of the activity observed for caffeic acid). The relative activities against cinnamic and 4-coumaric acids were almost identical.

The induction time courses of CoA ligase activity with both 4-coumaric and cinnamic acids as substrates were determined for chitin-elicited and unelicited cactus cultures (Fig. 6). Activities against both substrates showed similar increases within 12 h after elicitation and reached a maximum after 24 h. Unelicited cultures showed no increase in CoA ligase activity against either substrate.

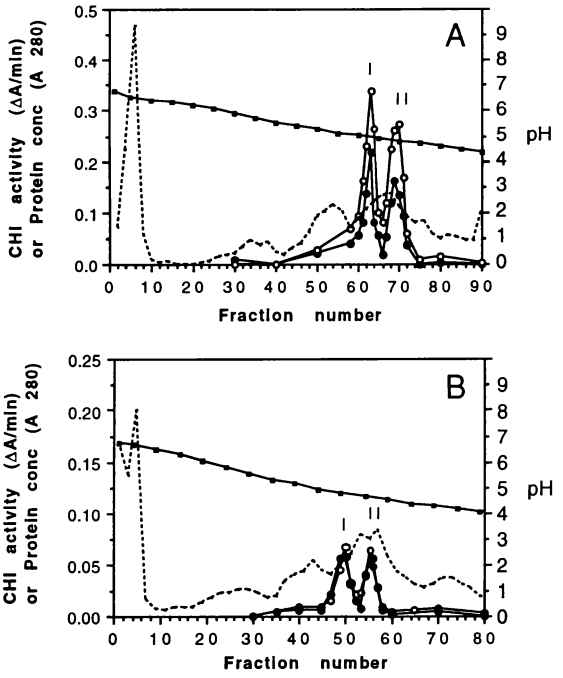


FIG. 4. Separation of two CHI isoforms (I and II) by chromatofocusing of extracts from chitin-elicited (A) and nonelicited (B) cell cultures of *C. senilis*. Enzyme activities were measured with 2′,4,4′,6′-tetrahydroxychalcone (●) or 2′,4′,6′-trihydroxychalcone (○) as substrates. Activity represents the activity of 25 μl of each fraction. The columns were eluted with polybuffer to give a pH gradient (■) from 7 to 4. Fraction size was 2 ml in A, 3 ml in B. Protein concentration (-----) was determined by measuring A_{280}.

Cinnamic acid is not commonly an effective substrate for (hydroxy) cinnamate:CoA ligase. It was therefore important to determine whether there were two or more CoA ligases in the elicited cactus cultures or one enzyme with multiple substrate specificity. Chomatofocusing revealed only a single form of (hydroxy) cinnamate:CoA ligase in the cactus cells (Fig. 7), with apparent p*I* of 4.6. This was active against both cinnamic and 4-coumaric acids.

Elicitation of CA4H Activity

Having established that the elicited cactus cultures contain both cinnamate and coumarate CoA ligase activities, the simplest explanation for the origin of B-ring deoxy flavonoids in the elicited cactus cultures would be a lack of induction of CA4H activity, leading to formation of cinnamate with no conversion to 4-coumarate. CA4H activity was therefore determined in microsomal preparations of both nonelicited and elicited cactus cultures. As support for the above explanation would be critically dependent on our not underestimating the CA4H activity, we tested many parameters to optimize the assay of the enzyme in cactus microsomes, based on the procedure of Edwards and Kessmann (15), and repeated the experiment on several independent batches of elicited cactus cultures. We found that it was essential to use freshly harvested rather than frozen cells, although microsomal preparations could themselves be frozen without significant loss of activity. The cactus CA4H was more active at pH 7.5 than at pH 8.0, and dithiothreitol could not be substituted for 2-mercaptoethanol. NADH-dependent cytochrome C reductase activity was measured as a microsomal marker enzyme.

A typical time course for changes in the activities of CA4H, NADH-dependent cytochrome C reductase and PAL is shown in Fig. 8. CA4H activity was clearly induced, from an undetectable basal level. The induction kinetics were broadly similar to those of PAL (Fig. 8) and the cinnamate CoA ligase (Fig. 6), with a major increase in activity between 8 and 16 h postelicitation.

DISCUSSION

Chemical analysis of chitin-elicited cactus cultures has led to the identification of a series of induced flavonoids with unsubstituted B-rings (8–10), whereas,

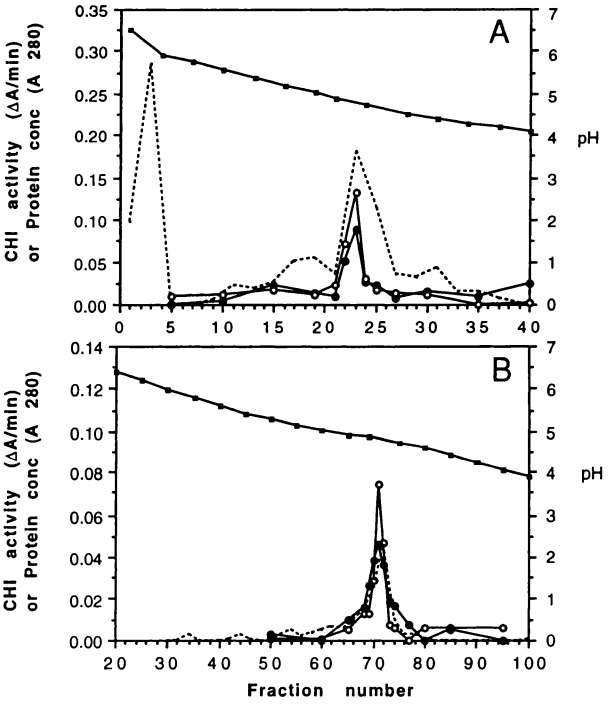


FIG. 5. Chromatofocusing analysis of CHI from stems of *C. senilis*. The peak of CHI activity collected from the first column run (A, 5.5-ml fractions) was rechromatographed (B, 1-ml fractions). Enzyme activities were measured with 2′,4,4′,6′-tetrahydroxychalcone (●) or 2′,4′,6′-trihydroxychalcone (○) as substrates. Activity represents the activity of 25 μl of each fraction. The columns were eluted with polybuffer to give a pH gradient (■) from 7 to 4. Protein concentration (-----) was determined by measuring A_{280}.

TABLE II

Substrate Specificity of the (Hydroxy)-cinnamate: CoA Ligase from Cell Suspension Cultures of *C. senilis*

Substrate	Specific activity (μkat/g)		Relative activity (%)	λnm for CoA esters	ε (cm^2/mol)
	Elicited	Nonelicited			
4-Coumaric acid	0.226	0.034	74	333[a]	23×10^6
Cinnamic acid	0.223	0.018	73	311[a]	22×10^6
Caffeic acid	0.306	0.061	100	363[a]	13×10^6
Ferulic acid	0.272	0.017	89	345[a]	19×10^6
Sinapic acid	0.000	0.007	0	352[b]	20×10^6
4-Methoxy-cinnamic acid	0.216	0.007	71	339[a]	27×10^6

Note. Data are the average of duplicate measurements by a spectrophotometric assay at the long-wave absorption maxima of the corresponding CoA thiol esters. Reported long-wave absorption maxima and molar extinction coefficients (ε) for CoA esters of cinnamic acid derivatives are summarized.

[a] Gross and Zenk (19).

[b] Lüderitz *et al.* (20).

in unchallenged intact plants of *C. senilis,* the more usual B-ring hydroxylated flavonoids accumulate (11). The purpose of the present study was to describe the enzymatic activities present in elicited cell cultures in relation to the accumulation of B-ring deoxy flavonoids. As elicited cultures only produce trace amounts of one B-ring hydroxy flavonoid (10), an efficient mechanism must be present to produce cinnamoyl CoA as substrate for chalcone synthase leading to 2′,4′,6′-trihydroxychalcone, the first C15 precursor of the B-ring deoxy compounds (Fig. 2).

Activity of *C. senilis* CHI was observed with 2′,4,4′,6′-tetrahydroxychalcone and 2′,4′,6′-trihydroxychalcone, but not 2′,4,4′-trihydroxychalcone, reflecting the substitution patterns of the known flavonoids of *C. senilis;* all possess 5- and 7-hydroxyl groups which arise as the 4′- and 6′-hydroxyl groups of the corresponding chalcones and are either unsubstituted on the B-ring (and therefore derived from 2′,4′,6′-trihydroxychalcone) or hydroxylated at the 4′ position (derived from 2′,4,4′,6′-tetrahydroxychalcone). Activity with 2′,4,4′-trihydroxychalcone is observed with legume CHIs, which accumulate 5-deoxy flavonoid derivatives as phytoalexins (18). As parsley and petunia

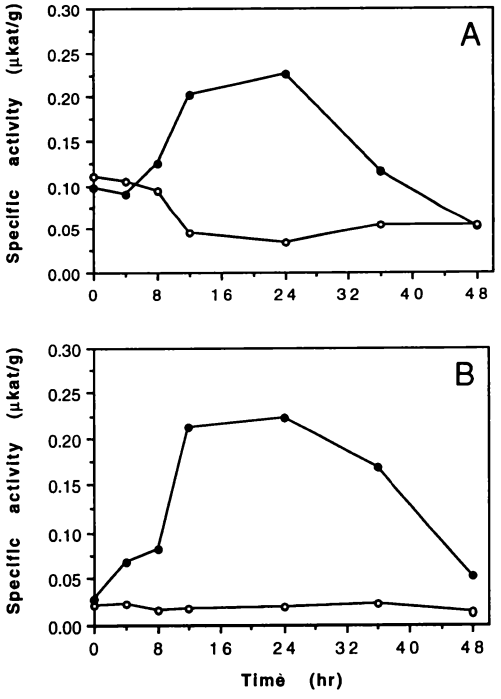


FIG. 6. Induction of (hydroxy)-cinnamate:CoA ligase activity in elicited (●) and H_2O-treated (○) cultures of *C. senilis.* Specific activities (μkat/g protein) were determined with 4-coumaric acid (A) and cinnamic acid (B) as substrates.

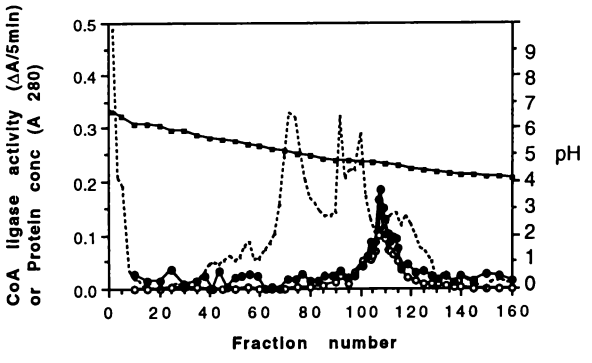


FIG. 7. Chromatofocusing analysis of (hydroxy)-cinnamate:CoA ligase from elicited cell cultures of *C. senilis.* Enzyme activities were measured with 4-coumaric acid (●) or cinnamic acid (○) as substrates. The column was eluted with polybuffer to give a pH gradient (■) from 7 to 4. Protein concentration (-----) was determined by measuring A_{280}.

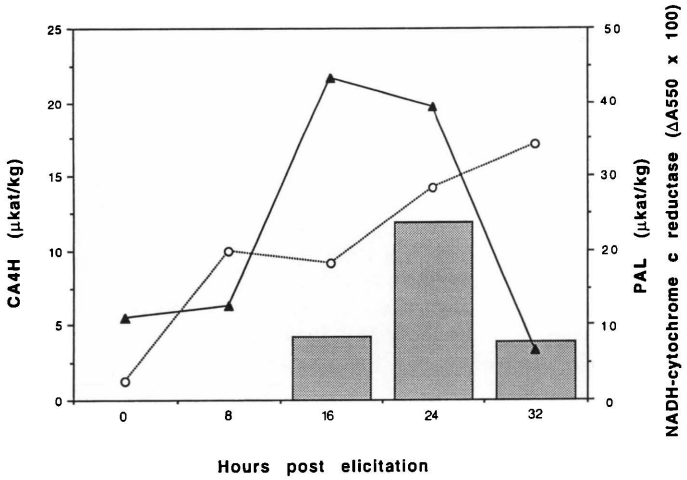


FIG. 8. Activities of PAL (▲), CA4H (bars), and NADH-dependent cytochrome C-reductase (○) in elicitor-treated cell cultures of *C. senilis*. CA4H and cytochrome C reductase were measured in microsomal preparations. No CA4H activity was observed in unelicited cultures.

CHIs were active against both 2′,4,4′,6′-tetrahydroxy- and 2′,4′,6′-trihydroxychalcones but neither plant has been reported to accumulate B-ring deoxy flavonoids, it would appear that a 4′-hydroxy group is not required for the action of chalcone isomerase.

Although two isoforms of CHI were identified in the cactus cultures, both were active against 2′,4,4′,6′-tetrahydroxychalcone and 2′,4′,6′-trihydroxychalcone, and both were induced by chitin treatment. Isoforms of CHI have been previously purified from mung bean (*Phaseolus aureus*) and garbanzo bean seedlings (*Cicer arietinum*), and have also been detected in parsley (*Petroselinum hortense*) (21). Although isoform I of *C. senilis* CHI has a somewhat higher affinity for 2′,4′,6′-trihydroxychalcone than isoform II, and isoform II has a somewhat greater affinity for 2′,4,4′,6′-tetrahydroxychalcone than isoform I, our data indicate that accumulation of B-ring deoxy flavonoids in the elicited cactus cultures is unlikely to be associated with the specific induction of a novel form of CHI.

If the unsubstituted aromatic ring of cinnamic acid is to be incorporated into the B-rings of the elicitor-induced flavonoids, the (hydroxy)-cinnamate:CoA ligase in cactus cultures must be able to activate cinnamic acid to cinnamoyl-CoA. Crude extracts from the cactus cultures were indeed active with cinnamic acid, as well as 4-coumaric, ferulic, caffeic, and 4-methoxycinnamic acids, but not, however, sinapic acid. Lack of activity against sinapic acid suggests that the lignin of the mature cactus might lack syringyl units, and thus be similar to that of softwood gymnosperms. In most plants, the CoA ligase which activates 4-coumaric acid exhibits little or no activity against cinnamic acid (22–25), although enzymatic activation of cinnamic acid to the CoA thiol ester has been reported in crude enzyme extracts from sugar beet (26), and a partially purified soybean coumarate:CoA ligase has been shown to exhibit approximately 10% of the activity against cinnamic acid as observed with 4-coumaric acid (27).

Activities of the CoA ligase versus cinnamic and 4-coumaric acids were coinduced and were not separable by chromatofocusing, suggesting that the cactus cultures may have a single (hydroxy) cinnamate:CoA ligase with multiple substrate specificity. Although the resolution of this analysis prevents us from completely ruling out the possibility of a cinnamate-specific CoA ligase isoform, these data nevertheless strongly suggest that the B-ring hydroxylation pattern of the elicitor-induced flavonoids is not regulated at the level of CoA ligase, although the presence of a CoA ligase with an unusually high activity against cinnamic acid is clearly of central importance for the synthesis of B-ring deoxy flavonoids. Substrate availability (i.e., cinnamate vs coumarate) for the CoA ligase would therefore appear to be the factor responsible for the accumulation of flavonoids with unsubstituted B-rings in the elicited cactus cultures, as the cultures contain all the enzymatic activities necessary for formation of both B-ring deoxy- and hydroxy-intermediates. Essentially, this could be controlled by two distinct mechanisms: (i) lack of induction of CA4H and therefore high cinnamate to coumarate ratios leading to preferential activation of cinnamate by CoA ligase, (ii) metabolic compartmentation of the PAL and CoA ligase reactions such that CA4H is bypassed in the elicited cultures. A third alternative could be the existence of novel enzymatic processes for the deoxygenation of coumarate or some later intermediate in the pathway, but this seems extremely unlikely, particularly in view of the strong activity of the CoA ligase for cinnamate.

CA4H activity was strongly induced by elicitor treatment, the specific activity being approximately half that found in fungally infected wheat leaves which accumulate lignin via the PAL and CA4H reactions (25) and approximately equal to that reported in elicited alfalfa cell cultures, which accumulate B-ring hydroxy flavonoid derivatives (28). Furthermore, the ratio of induced PAL to CA4H activities was lower in the cactus than in the alfalfa cultures. Thus, it is very unlikely that lack of utilization of coumarate for flavonoid synthesis results from lack of enzymatic capacity for hydroxylation of cinnamate. We are therefore left with the possibility that, although CA4H is induced as normally observed in cell cultures producing phenylpropanoid-derived phytoalexins (29), its activity is somehow bypassed. This would imply metabolic compartmentation, at least at the level of the PAL, CA4H, and CoA ligase reactions. Chaneling of phenylalanine through the PAL and CA4H reactions has been previously reported (30).

Our data suggest either a disruption of this process in elicited cells or an establishment of a new direct channel between the elicited PAL and CoA ligase enzymes, leading directly to the formation of cinnamoyl CoA. Further compartmentation of the subsequent reactions may occur, but this would not appear to be essential.

Although we now understand many details of the transcriptional regulation of phenylpropanoid/flavonoid biosynthesis (5, 29), our understanding of how the pathways are organized in the cell is virtually nonexistent. Evidence for metabolic compartmentation in phenylpropanoid synthesis has come primarily from precursor feeding experiments and attempts to immunolocalize enzymes such as PAL and CHS (30). Elicited *C. senilis* cultures may be a good model system for future studies on metabolic compartmentation, as they appear to feature a mechanism for avoiding a key reaction of phenylpropanoid synthesis. The recent availability of techniques for determining physical interactions between proteins *in vivo* (31) should now make it possible to analyze directly the molecular basis of metabolic compartmentation in plant secondary product biosynthesis.

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