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Stilbene Synthase and Chalcone Synthase[1]

TWO DIFFERENT CONSTITUTIVE ENZYMES IN CULTURED CELLS OF *PICEA EXCELSA*

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ABSTRACT

Cultured cells of *Picea excelsa* capable of forming stilbenes and flavanoids have been established. Unlike needles of intact plants containing piceatannol (3,3′,4′,5-tetrahydroxystilbene) and stilbene glycosides the cultured cells converted phenylalanine and *p*-coumaric acid primarily into resveratrol monomethyl ether (3,4′-dihydroxy-5-methoxystilbene) and naringenin. Partially purified enzyme preparations were assayed for chalcone synthase as well as for stilbene synthase activity converting malonyl-CoA plus *p*-coumaroyl-CoA into 3,4′,5-trihydroxystilbene (resveratrol).

Although stilbene synthase and chalcone synthase use the same substrates and exhibit similar molecular properties, *i.e.* molecular weight and subunit molecular weight, they are two different proteins. This difference was demonstrated by gel electrophoresis and by means of monospecific antibodies.

Stilbene synthase is the key enzyme on the way to fungostatic stilbene derivatives characterized as phytoalexins (6, 8, 11). Chalcone synthase initiates the route to a flavanone and thus to other phytoalexins, *e.g* the one having a pterocarpan skeleton (7). Both enzymes function with the same substrates, malonyl-CoA and *p*-coumaroyl-CoA, and have in common mechanistic details of catalysis, *i.e.* nucleophilic attack of anion of acetyl-CoA at the ester group of the aromatic substrate (7, 12, 18). They fold, however, the intermediary polyketide chain topologically in a different way and apply different mechanisms of cyclization.

It was, therefore, of interest to compare the two enzymes when occurring in the same species. The primary purpose of the present study was to determine whether the two catalytic activities were due to a single protein modulated by specific cofactors or to two different proteins.

The data presented provide evidence that, although stilbene synthase and chalcone synthase resemble each other in several aspects, they are two proteins not related immunologically.

MATERIALS AND METHODS

Cell Cultures of *Picea excelsa*. Callus cultures from spruce (*Picea excelsa* L.) were obtained from germinating seeds. Stems of 2-week-old plants were excised, cut into pieces, and placed on B5 medium (4) supplemented with 0.7% (w/v) agar and 1 $\mu g\ g^{-1}$ 2,4-D. Some cultures were kept under continuous light at 26°C on the medium of Schenk and Hildebrandt (17) solidified with 0.7% agar.

Metabolism of Phenylpropanoids in Cell Cultures. Callus cultures (1.0 g fresh weight) were incubated under sterile conditions with 400 μl solution containing the precursor. After 5 h at 26°C the cells were extracted several times with ethyl acetate, the extract was concentrated *in vacuo* and separated by TLC with chloroform:ethyl acetate:HCOOH (5:4:1) as solvent system (2). Resveratrol was purified by chromatography in more than one well established solvent system (1, 9) and comparison with material isolated from *Veratrum album* (16). System 2 (for TLC), toluene:methanol (9:1); system 3 (for PC), acetic acid:water (1:2). ^{14}C-Labeled naringenin was purified by rechromatography according to (10) and by recrystallization after addition of nonlabeled authentic material, to constant specific radioactivity.

Enzyme Preparation. Twenty g of callus were homogenized in 25 ml of 20 mM HEPES-NaOH, pH 7.8, containing 1 mM DTT (referred to as extraction buffer) using an Ultraturrax homogenizer. The homogenate was centrifuged at 6000*g* for 20 min to remove insoluble cell debris. $(NH_4)_2SO_4$ was added to a final saturation of 90%. After stirring for 1 h, the precipitate was removed by centrifugation at 6000*g* for 30 min. The pellet was resuspended in 1.5 ml of extraction buffer and dialyzed against 500 ml of this buffer. The sample in the dialysis bag was concentrated by embedding the tubing in dry Sephadex G-100. This preparation was either used directly for enzyme studies or after separation according to mol wt, by zonal centrifugation or molecular sieving.

Molecular Sieving. The enzyme preparation was purified by gel filtration chromatography on Ultrogel AcA-34 (LKB, Bromma, Sweden). The native mol wt of stilbene synthase and chalcone synthase was determined by using a calibrated Ultrogel AcA-34 column (90 cm length, 70 ml bed volume) equilibrated with extraction buffer. Fractions of 3 ml were collected and assayed for protein and enzyme activities.

Zonal Centrifugation of Enzyme Fractions. A linear sucrose gradient ranging from 15 to 35% (w/w) was used to stabilize the solution in a 11-ml centrifuge tube (SW-40 rotor, Beckman). One hundred μl of partially purified enzyme mixture were applied and centrifuged at 40,000 rpm for 60 h. Standard proteins were run in parallel.

Stilbene Synthase and Chalcone Synthase Assayed *In Vitro*. Stilbene synthase (resveratrol-forming) was essentially assayed according to (16). Four kBq (0.11 μCi) [2-^{14}C]malonyl-CoA (2 GBq/mmol) were incubated with 33 μM *p*-coumaroyl-CoA (or other CoA-esters) in 280 μl enzyme preparation. After incubation at pH 7.8 in extraction buffer (total volume: 300 μl) at 30°C and 30 min, stilbenes and flavanones (as products of chalcone isomerization) were extracted with ethyl acetate and separated by TLC on silica gel (2) with chloroform:ethyl acetate:formic acid (5:4:1) as solvent system.

Immunoprecipitations. Antichalcone synthase antibodies were a gift of K. Hahlbrock; antistilbene synthase antibodies were from A. Schöppner (18). Enzyme fractions (100 μl) were incu-

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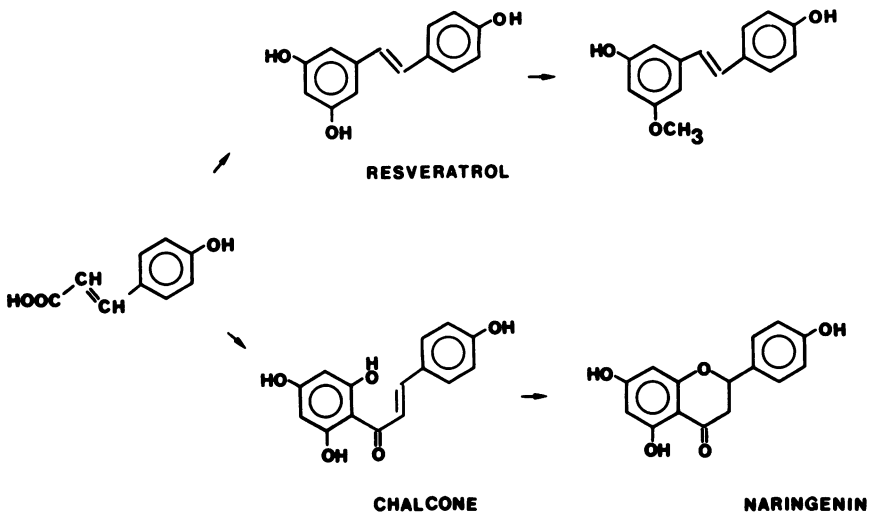

FIG. 1. Schematic view of the reactions taking place by administration of *p*-coumaric acid to spruce callus cultures. The figure shows the formula of resveratrol and naringenin, the products of the enzymic reactions proceeding from *p*-coumaroyl-CoA.

Table I. *Incorporation of Phenylpropanoids into Stilbenes and Flavanones*

1 g of callus was incubated with 400 μl water containing the precursor. After 5 h, aglycones and phenolic glycosides were extracted with ethyl acetate and analyzed by TLC. In solvent system 1, authentic naringenin had a R_F value of 0.85. Resveratrol monomethyl ether (R_F = 0.70) and resveratrol (R_F = 0.67) had to be resolved by rechromatography in another solvent system.

Precursor	Specific Radioactivity	Radioactivity	Product	Radioactivity
	GBq/mmol	*kBq*		*kBq*
L-[U-^{14}C]Phenylalanine	10	400	Resveratrol methyl ether	3.2
			Naringenin	1.5
[2-^{14}C]*p*-Coumaric acid	0.2	40	Resveratrol methyl ether	0.8
			Naringenin	0.5
[2-^{14}C]Ferulic acid	0.2	40	Stilbene (isorhapontigenin)	<0.01

Table II. *Enzymic Formation of Stilbenoids from [2-^{14}C]malonyl-CoA (specific radioactivity 2 GBq/mmol) and Various Nonlabeled Cinnamoyl-CoA Derivatives*

An enzyme preparation after molecular sieving was used. Phenols were extracted with ethyl acetate and analyzed by repeated chromatography.

Second Substrate	Product	Radioactivity
		Bq
Cinnamoyl-CoA	3,5-Dihydroxystilbene	1
p-Coumaroyl-CoA	3,4′,5-Trihydroxystilbene	50
m-Coumaroyl-CoA	3,3′,5-Trihydroxystilbene	1
Feruloyl-CoA	3,4′,5-Trihydroxy-3′-methoxystilbene	15
Caffeoyl-CoA	3,3′,4′,5-Tetrahydroxystilbene	3
Dihydro-*p*-coumaroyl-CoA	3,4′,5-Trihydroxybibenzyl	14
Dihydro-cinnamoyl-CoA	3,5-Dihydroxybibenzyl	1

bated with various amounts of antibodies for 2 h at 20°C in 100 mM Tris-HCl, pH 7.5, containing 1 mM DTT, 0.5% Triton X-100, and 150 mM NaCl (5). Immunoprecipitates were removed in the airfuge (Beckman) at 100,000*g* for 20 min. The enzyme activities were determined in the resulting supernatant fluid.

Other Procedures and Chemicals. Radioactivity (16) and protein concentrations (13) were determined as described elsewhere. Gel electrophoresis was carried out with enzyme samples obtained by zonal centrifugation according to the method of Maurer (14). Radioactive compounds were purchased or prepared as described elsewhere (2).

RESULTS AND DISCUSSION

Cell Cultures Capable of Synthesizing Stilbenes and Flavanoids. Callus cultures of *P. excelsa* were maintained either in the dark or under continuous white light. Upon illumination with white light, dark cultures became slightly green and could be kept in this stage during many transfers.

Both types of cell cultures, dark-grown or kept under continuous white light, synthesized stilbenes and flavanoids. We did not observe significant differences in quality and quantity of these compounds when cells were assayed during various phases of growth. Furthermore, the absence or presence of white light did not affect the level of stilbenes. Likewise, tests undertaken during a period of time immediately following transfer of cells to new agar plates did not reveal any induction phenomenon. In contrast to this behavior of *Picea* cultures, cultured cells of *Arachis hypogaea* had been found to react to such transfers by increased synthesis of stilbenes (15).

Unlike plant cells susceptible to induction of stilbene formation by UV light (3), or cells reacting, upon irradiation with UV light, by increasing chalcone synthesis (7), *P. excelsa* cells showed no significant induction of stilbene or flavanoid biosynthesis.

Within the groups of stilbenes and flavanoids the formation of two compounds dominated: resveratrol monomethyl ether

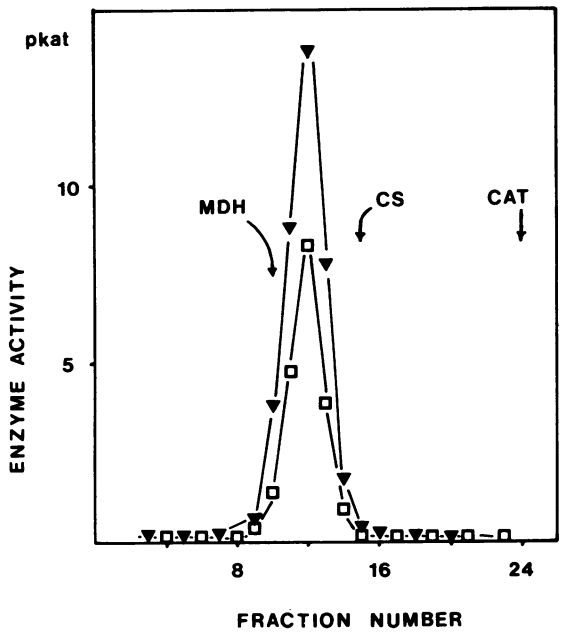

FIG. 2. Zonal centrifugation of stilbene synthase and chalcone synthase. An enzyme preparation with 30 pkat stilbene synthase activity (□) and 60 pkat chalcone synthase activity was applied to the sucrose gradient. Sedimentation is indicated from the left to the right. The positions of malate dehydrogenase (MDH, mol wt 70,000), citrate synthase (CS, mol wt 100,000), and catalase (CAT, mol wt 250,000) are designated.

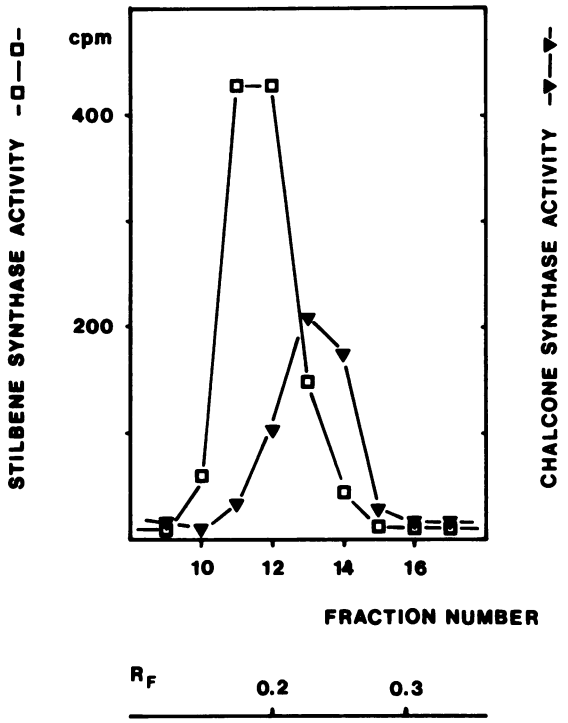

FIG. 3. Separation of stilbene synthase activity from chalcone synthase activity by electrophoresis of the native enzymes. Electrophoresis was on polyacrylamide gels at pH 7.5, in the absence of sodium dodecyl sulfate. Anode at the right.

and naringenin (Fig. 1). This was shown by administering radioactive phenylalanine and other phenylpropanoids to the cell cultures, preparing a total phenol fraction by ethyl acetate extraction, and chromatography by TLC (Table I). The phenolic fraction consisted of two peaks of radioactivity separated in solvent system 1. The compound with $R_F = 0.85$ was shown to be naringenin by elution of the zone and repeated recrystallization with authentic material. The second peak, at $R_F = 0.70$, was rechromatographed in system 2 and gave a sharp peak in the range of dihydroxy- and trihydroxystilbenes. A subsequent paper chromatography with system 3, affording good separation of dihydroxystilbene (pinosylvin), bibenzyls and trihydroxystilbene (resveratrol), showed that the unknown compound co-migrated with 3,4′-dihydroxy-5-methoxystilbene (resveratrol monomethyl ether). Permethylation of the compound yielded 3,5,4′-trimethoxystilbene as evidenced by comparison with authentic material and recrystallization to constant specific activity.

The results summarized in Table I and illustrated in Figure 1 show that the cell cultures, like spruce seedlings, contained the capacity of stilbene synthesis.

In spruce needles, 3,3′,4′,5-tetrahydroxystilbene (6) and flavonols, *i.e.* kaempferol and quercetin, have already been described. We verified this by studies using both intact needles from old trees and whole spruce seedlings (4-week-old intact plants). The latter were grown from the same seeds utilized for establishing the cell cultures. The data (not shown) demonstrated that intact plants contain primarily (a) stilbenes with four hydroxyl functions, and some glycosides, and (b) flavonols. Unlike cell cultures, intact plants did not contain resveratrol monomethyl ether or naringenin in significant amounts. Therefore, the products of phenylpropanoid metabolism in cultured cells differed in detail, but not in the main routes, from that of intact plants.

Proof of Stilbene Synthase and Chalcone Synthase Activity. Crude aqueous extracts from cultured cells, or extracts from an acetone powder, were tested prior to and after molecular sieving. Besides ^{14}C-labeled malonyl CoA as one substrate, CoA esters of phenylpropanoids were compared as to their function as the other substrate for stilbene synthase. As chalcone synthase and stilbene synthase use the same substrates, it was possible to determine both activities by a single incubation mixture, by separating the products and measuring the radioactivity of resveratrol as well as of 2′,4,4′,5′-tetrahydroxychalcone (assayed following isomerization, as the flavanone naringenin). This was true, also, for assays of enzyme preparations purified according to their mol wt, as both enzymes exhibit the same mol wt (see below).

In crude extracts from cell cultures, almost no resveratrol could be detected in the standard enzyme assay. On separation of the products by TLC radioactivity was found in the range containing hydroxystilbenes, but it was attributable almost entirely to a compound which was separable from resveratrol in system 2 and behaved like resveratrol monomethyl ether in system 3. When inactive resveratrol was added to the standard incubation mixture, we trapped labeled resveratrol. These findings indicated the role of resveratrol as intermediate and the presence of a very effective methylation system in the crude preparation. This could be demonstrated directly by incubating resveratrol and [methyl-^{14}C]*S*-adenosylmethionine with an enzyme preparation purified only by desalting over a Sephadex G-50 column. One μmol of resveratrol was almost quantitatively converted into the monomethyl ether after addition of 1.5 μmol of labeled *S*-adenosylmethionine and 280 μl enzyme preparation at the conditions of stilbene synthase assay (30 min, 30°C). Thus, the tests *in vitro* reflected rather exactly the metabolic situation *in vivo* in tissue culture (see above).

When using the enzyme preparation following chromatography on Ultrogel AcA-34, *O*-methylation was not interfering any more. The fraction containing the peak of stilbene synthase activity was utilized for further studies. The data observed with

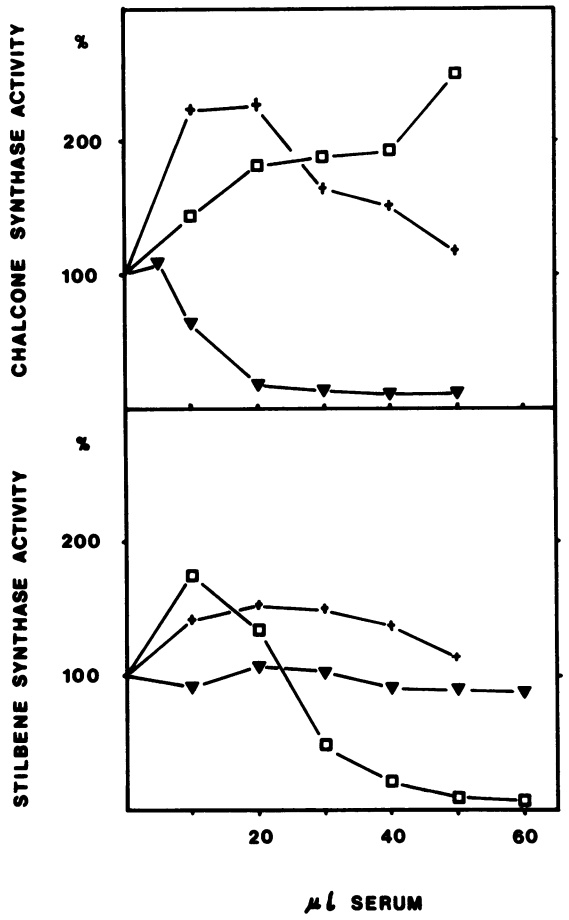

FIG. 4. Effect of increasing amounts of antibodies on the amount of enzyme remaining in the supernatant fluid after immunoprecipitation. A preparation purified by zonal centrifugation and containing 100 pkat stilbene synthase activity and 70 pkat chalcone synthase activity (the ordinate denotes these values as 100%) was used. The abscissa indicates µl of serum added to the enzyme mixture. Serum used: (□), antistilbene synthase antiserum; (▼), antichalcone synthase antiserum; (+), preimmune serum.

different substrates (Table II) indicate that *p*-coumaroyl-CoA is the optimal substrate and resveratrol the product of the stilbene synthase-catalyzed reaction.

Comparison of Stilbene Synthase and Chalcone Synthase. Zonal centrifugation of a partially purified fraction containing stilbene synthase and chalcone synthase activity demonstrated that both enzymes exhibited almost identical mol wt (approximately 6 S). By comparison with standard proteins, a value of 90,000 was estimated (Fig. 2).

A sample of the peak fraction obtained by zonal centrifugation was subjected to PAGE under nondenaturing conditions. Enzyme activities were tested using gel discs. By this means, profiles of stilbene synthase and chalcone synthase activities could be obtained, demonstrating that both activities were separable (Fig. 3).

The findings that both activities were attributable to different proteins was corroborated by immunological studies. A purified preparation obtained by zonal centrifugation and containing both enzymes stilbene synthase and chalcone synthase was incubated, in parallel, with antistilbene synthase, antichalcone synthase, or preimmune serum, respectively.

Figure 4 shows the per cent enzyme activities that remained in the supernatant of the immunoprecipitation upon increasing the amount of antibodies. The activity of stilbene synthase was selectively removed from the supernatant by antistilbene synthase antibodies. The chalcone synthase activity was not sedimentable by adding antistilbene synthase antibodies. Thus, it was evident that stilbene synthase and chalcone synthase were different antigens, and the antibodies did not cross-react.

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