Plant Physiol. (1996) 110: 791–799

Aromatic Polyketide Synthases[1]

Purification, Characterization, and Antibody Development to Benzalacetone Synthase from Raspberry Fruits

Wlodzimierz Borejsza-Wysocki and Geza Hrazdina*

Institute of Food Science, Cornell University, Geneva, New York 14456–0462

***p*-Hydroxyphenylbutan-2-one, the characteristic aroma compound of raspberries (*Rubus idaeus* L.), is synthesized from *p*-coumaryl-coenzyme A and malonyl-coenzyme A in a two-step reaction sequence that is catalyzed by benzalacetone synthase and benzalacetone reductase (W. Borejsza-Wysocki and G. Hrazdina [1994] Phytochemistry 35: 623–628). Benzalacetone synthase condenses one malonate with *p*-coumarate to form the pathway intermediate *p*-hydroxyphenylbut-3-ene-2-one (*p*-hydroxybenzalacetone) in a reaction that is similar to those catalyzed by chalcone and stilbene synthases. We have obtained an enzyme preparation from ripe raspberries that was preferentially enriched in benzalacetone synthase (approximately 170-fold) over chalcone synthase (approximately 14-fold) activity. This preparation was used to characterize benzalacetone synthase and to develop polyclonal antibodies in rabbits. Benzalacetone synthase showed similarity in its molecular properties to chalcone synthase but differed distinctly in its substrate specificity, response to 2-mercaptoethanol and ethylene glycol, and induction in cell-suspension cultures. The product of the enzyme, *p*-hydroxybenzalacetone, inhibited mycelial growth of the raspberry pathogen *Phytophthora fragariae* var *rubi* at 250 μM. We do not know whether the dual activity in the benzalacetone synthase preparation is the result of a bifunctional enzyme or is caused by contamination with chalcone synthase that was also present. The rapid induction of the enzyme in cell-suspension cultures upon addition of yeast extract and the toxicity of its product, *p*-hydroxybenzalacetone, to phytopathogenic fungi also suggest that the pathway may be part of a plant defense response.**

The characteristic aroma of raspberries is caused by the compound *p*HPB, also referred to as the "raspberry ketone" (Schinz and Seidel, 1957; Braun and Hieke, 1977; Larsen et al., 1991). Recent investigations in our laboratory have shown that raspberry ketone is synthesized in the berries by a two-step biosynthetic pathway that is reminiscent of the pathways involving CHS (Hrazdina et al., 1976) and STS (Schöppner and Kindl, 1984). In the first step, malonyl-CoA is condensed with *p*-coumaryl-CoA to form the polyketide intermediate *p*-hydroxybenzalacetone (*p*-hydroxyphenylbut-3-ene-2-one). This reaction is carried out by an enzyme, BAS. In the second step, the intermediate product, *p*-hydroxybenzalacetone, is reduced to *p*HPB, the raspberry ketone, by the enzyme benzalacetone reductase, which uses NADPH as the proton donor (Borejsza-Wysocki and Hrazdina, 1994a).

BAS, the first enzyme of the pathway, is interesting for a number of reasons. Its reaction mechanism shows similarity to the other aromatic polyketide synthases such as CHS and STS (Schröder and Schröder, 1990); however, the condensation of the malonate unit with *p*-coumaryl-CoA is followed by decarboxylation that leads to a chain shortening in the molecule. Like CHS in flavonoid metabolism and STS in that of stilbenes, BAS appears to be a key enzyme in the biosynthesis of the raspberry ketone. Figure 1 illustrates the similarities as well as the differences in the reactions catalyzed by CHS, STS, and BAS.

In earlier work (Hrazdina et al., 1976) we reported the formation of benzalacetone derivatives with a purified CHS preparation from parsley cell-suspension cultures in the presence of 2-ME. At that time, it was thought that this compound was the "early release product" of the CHS reaction, because of interference by 2-ME with the active center of the enzyme. Our investigation of the biosynthesis of the raspberry aroma compound *p*HPB showed that the production of benzalacetone is not an artifact but that this compound is the product of a specific enzyme (Borejsza-Wysocki and Hrazdina, 1994a). In that work we also reported the biosynthetic steps leading to the formation of the raspberry ketone and the detection of activities of the enzymes involved. We have continued this investigation and report here the partial purification and characterization of BAS from raspberry fruits, development of its antibodies, and induction of the enzyme in raspberry cell-suspension cultures upon treatment with yeast extract.

MATERIALS AND METHODS

Reagents

[2-^{14}C]Malonyl-CoA was from New England Nuclear; *p*-coumaryl-, cinnamyl-, caffeyl-, ferulyl-, 5-hydroxyferu-

[1] This research was supported by funds from the Kraft General Foods Co., Inc. (Tarrytown, NY) and the Cornell Biotechnology Program, which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, the U.S. Army Research Office, and the National Science Foundation.

* Corresponding author; e-mail gh10@cornell.edu; fax 1–315–787–2397.

Abbreviations: BAS, benzalacetone synthase; CHS, chalcone synthase; 2-ME, 2-mercaptoethanol; *p*HPB, *p*-hydroxyphenylbutan-2-one; STS, stilbene synthase; TBST, Tris-buffered saline plus Tween 20.

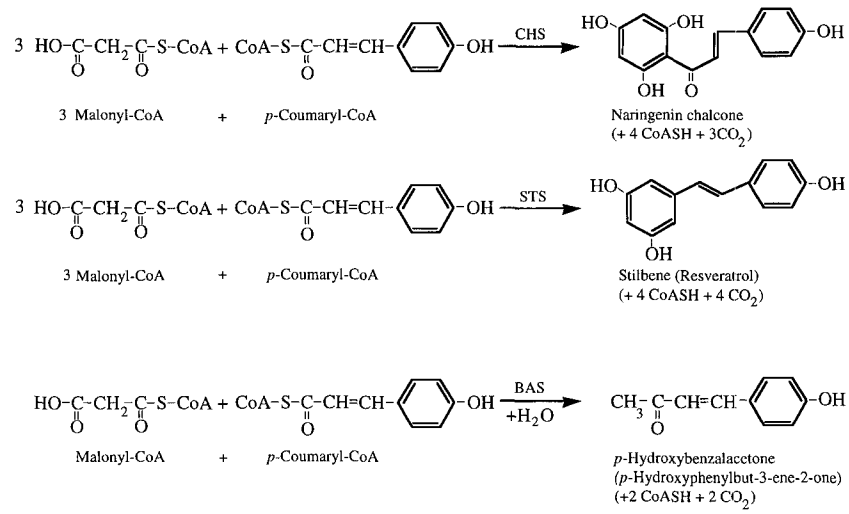

Figure 1. Reaction products of CHS, STS, and BAS in aromatic polyketide synthesis. CoASH, Free CoA.

lyl-, and sinapyl-CoAs were synthesized as previously described (Hrazdina et al., 1976). Ultrogel AcA-44 was from IBF Biotechnics (Villeneuve la-Garenne, France); Phenyl-Sepharose CL-4B and PBE 94 were from Pharmacia; DEAE Bio-Gel A was from Bio-Rad. Molecular weight markers for calibration of the AcA-44 column were from Pharmacia; SDS-PAGE standards were from Bio-Rad; anti-rabbit IgG alkaline phosphatase conjugate and 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium color development substrate were from Promega. Acrylamide/bis-acrylamide, ammonium persulfate, Trizma base, 2-ME, and 2,5-diphenyloxazole were from Sigma; and *N,N,N',N'*-tetramethylethylenediamine was from Bio-Rad. All chemicals for tissue culture (i.e. basal salt mixture, growth regulators, vitamins, and *myo*-inositol) were from Sigma. Bacto yeast extract was obtained from Difco Laboratories (Detroit, MI), and 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene was from Packard (Downers Grove, IL).

Buffers

The following buffer solutions were used: A, 0.2 M potassium phosphate (pH 8.0) containing 0.4 mM Suc and 1 mM PMSF; B, 0.2 M potassium phosphate (pH 8.0); C, 0.2 M potassium phosphate containing 40% (w/v) ammonium sulfate; D, 0.2 M potassium phosphate (pH 8.0) containing 20% (w/v) ammonium sulfate and 20% (v/v) ethylene glycol; E, 0.2 M potassium phosphate (pH 8.0) containing 40% (v/v) ethylene glycol; F, 20 mM potassium phosphate (pH 8.0); G, 8-fold diluted Pharmacia Polybuffer 74 (pH 4.0); H, 20 mM imidazole buffer (pH 7.4); I, 1.0 M Tris-HCl (pH 8.5); J, Tris/Gly/SDS for PAGE (Kodak); K, Tris/Gly for protein transfer to immunoblots (Kodak); L, 1.59 g of Na_2CO_3, 2.93 g of $NaHCO_3$, and 0.2 g of NaN_3 in 1 L of H_2O (pH 9.4); M, 50 mM Mes (pH 5.5–6.5); N, 50 mM Tris-HCl (pH 7.0–8.5); O, 50 mM Gly (pH 9.0–10.0). Buffers from A to H also contained 6 mM 2-ME.

Plant Material

Raspberry plants (*Rubus idaeus* L. cv Royalty) were grown in the experimental plots of Cornell University's New York State Agricultural Experiment Station (Geneva). Mature berries were harvested and used immediately or frozen in liquid nitrogen and stored at −90°C.

Raspberry cell-suspension cultures were established from callus culture and propagated in a modified Anderson's basal salt medium supplemented with 2,4-D, indole-3-butyric acid, and 6-(dimethylallylamino)-purine (Borejsza-Wysocki and Hrazdina, 1994b). Cells were harvested in the linear phase of growth, and 3-g samples were transferred to 125-mL Erlenmeyer flasks containing 20 mL of medium. After 7 d of growth, 10 mL of medium (control) or 10 mL of medium containing Suc or yeast extract were added to a final concentration of 0.5 M (Suc) and 3 g L^{-1}(yeast extract). Cultures were incubated in a shaking incubator at 150 rpm in darkness and at 25°C. Cells were harvested by filtration in 12-h intervals, frozen in liquid N_2, and stored at −90°C for later use.

Purification of BAS

All procedures were carried out at 4°C. Fresh or frozen berries were homogenized in a Waring blender with 1 volume of buffer A, in the presence of 10% (w/v) PVP. The homogenate was squeezed through a fine nylon mesh and centrifuged for 20 min at 20,000*g*. Dowex-1 (Sigma) (22 g L^{-1}) was added to the supernatant, stirred for 60 min, and removed by filtration. The filtrate was used for enzyme purification.

Harvested cells were ground in a chilled mortar (0°C) with buffer B (1:2, w/v) and centrifuged for 5 min at 11,500*g*. Saturated ammonium sulfate was added to the crude extract to 30% saturation and stirred for 30 min. The precipitate was removed by centrifugation at 20,000*g* for 20 min. The ammonium sulfate concentration of the supernatant was increased stepwise to 40, 50, and 80% saturation, the pH of the solution was adjusted to 8.0 and stirred for an additional 30 min, and the precipitate was removed by centrifugation as above. The protein pellet obtained from the 80% ammonium sulfate saturation was resuspended in 30 mL of buffer B. This protein solution was applied to an Ultrogel AcA-44 column (2.5 × 87 cm) previously equilibrated with buffer B and eluted with the same buffer, and

6.5-mL fractions were collected. BAS-containing fractions were pooled, brought to 40% ammonium sulfate saturation, and loaded onto a Phenyl-Sepharose CL-4B column (1.5 × 8 cm) that was equilibrated with buffer C. The column was washed with 100 mL of buffer C and eluted by stepwise addition of 100 mL of buffer D and 100 mL of buffer E. Fractions (5 mL) were collected and assayed. The fractions containing the highest BAS activity were pooled and dialyzed overnight against two changes of buffer F. The dialyzed protein extract was loaded onto a DEAE Bio-Gel A column (2.5 × 7 cm) that was equilibrated with buffer F. The column was washed with 150 mL of buffer F and eluted with 400 mL of a linear gradient (20 mM buffer F to 200 mM buffer B). Fractions (6.5 mL) containing BAS activity were pooled and concentrated to 5 mL in a concentrator (PM-10 membrane; Amicon, Beverly, MA). The concentrated protein was diluted (1:10, v/v) in buffer G and applied to a PBE 94 column (1.5 × 8 cm) equilibrated in the same buffer. Unabsorbed protein was removed with 50 mL of buffer G, and the column was eluted with 400 mL of buffer H. Fractions of 6.5 mL were collected, and every fifth fraction was used for measuring pH. For enzyme assays, 0.5 mL of buffer I was added to the fraction to adjust the pH to 8.0. Fractions with the highest BAS activity were pooled and concentrated as described above.

Protein was determined by the method of Bradford (1976) using BSA as standard.

Enzyme Assays

Enzyme activity was determined as previously described (Borejsza-Wysocki and Hrazdina, 1994a). The reaction mixture (110 μL) contained 100 μL of extract or eluate, 1 nmol of *p*-coumaryl-CoA (in 5 μL of 1 mM acetic acid), and 5 nmol of [2-^{14}C]malonyl-CoA (in 5 μL of aqueous hydrochloric acid solution) and was incubated at 30°C for 30 min. The reaction was stopped by adding 20 μL of glacial acetic acid and 50 μg of *p*-hydroxybenzalacetone and 50 μg of naringenin in 5 μL of ethanol. Reaction products were extracted for 5 min with 250 μL of ethyl acetate and separated on Whatman No. 1 paper strips (4 × 40 cm) in a mixture of water:acetic acid:*n*-butanol (11:1:8, v/v/v). The area containing the trapped reaction products was detected under UV light (350 nm) and cut out, and radioactivity was determined by liquid scintillation spectrometry in 20 mL of toluene cocktail containing 2,5-diphenyloxazole (2.5 g L^{-1}) and 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (0.1 g L^{-1}). Enzyme activity was expressed as nkat kg^{-1} protein.

Effect of Reducing Agents and Ethylene Glycol on Enzyme Activity

The activity of BAS and CHS as a function of 2-ME and glutathione concentration in the reaction mixture was determined with a crude protein fraction precipitated at 80% ammonium sulfate saturation. The precipitate from a 1-mL sample was removed by centrifugation at 12,000*g* for 20 min, and the protein pellet was resuspended in 1 mL of buffer B containing 0 to 10 mM 2-ME or glutathione.

For experiments with ethylene glycol, protein was collected from the Phenyl-Sepharose CL-4B column and dialyzed overnight against two changes of buffer F. Fifty microliters of buffer F containing different amounts of ethylene glycol (0–9.0 M) were added to 50 μL of dialyzed protein extract (20 μg of protein), which was mixed and incubated for 5 min at 4°C before starting the enzymic reaction by addition of substrates. Enzyme activity was measured as described above.

Enzyme Characterization

The M_r of the native enzyme was estimated by gel filtration on an AcA-44 column (2.5 × 87 cm) equilibrated with buffer B. The column was calibrated with Blue Dextrane 2000 (Pharmacia) (M_r 2,000,000), aldolase (M_r 158,000), BSA (M_r 66,000), and chymotrypsinogen A (M_r 25,000). A BAS preparation purified through the Phenyl-Sepharose CL-4B step was used for the M_r determination. The M_r of enzyme subunits was also determined using SDS-PAGE as described by Laemmli (1970) with unstained protein standards (Bio-Rad Low Range M_r Calibration Kit) as markers.

The pH optimum of the enzyme was determined in the range 5.5 to 10.0, using 50 mM buffers M, N, and O. The pI of the enzyme was determined during chromatofocusing as described above.

A BAS preparation purified through the DEAE step was used in the Michaelis-Menten kinetic experiments. The reaction mixtures contained 35 μg of protein dissolved in 70 μL of 50 mM phosphate buffer, pH 8.0 (6 mM 2-ME), and varying concentrations of malonyl-CoA (0.25–40.0 μM) and *p*-coumaryl-CoA (0.1–50.0 μM) in a total volume of 100 μL. Reaction mixtures were incubated at 30°C for 30 min, and enzyme activity was measured as described above.

The substrate specificity of BAS and CHS was determined in standard incubations with a partially purified enzyme using *p*-coumaryl-, cinnamyl-, caffeyl-, feruloyl-, 5-hydroxyferulyl-, and sinapyl-CoAs (1–10 μM). The activity of the enzyme using *p*-coumaryl-CoA was designated 100%.

Electrophoresis and Immunoblotting

Gel electrophoresis was carried out in 10% denaturing polyacrylamide gels as described by Laemmli (1970) in a Bio-Rad Mini-Protean II cell using buffer J. Two-dimensional gel electrophoresis was performed as described by O'Farrell (1975). Protein was detected by staining with Coomassie brilliant blue.

Immunoblotting was carried out basically as described by Towbin et al. (1979). Protein from SDS gels was electroblotted in buffer K for 1 h (83- × 55- × 0.75-mm gels) or overnight (142- × 110- × 3-mm gels) at 150 mA. Membranes were washed three times (for 10 min each time) with TBST containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20 and blocked for 30 min with 0.5% and for 3 h with 5% nonfat dry milk in TBST. Membranes were incubated overnight with anti-BAS antibody (1:5000 diluted with TBST) and washed four times (for 15 min each time) with TBST, and antibody complexes were detected

with anti-rabbit IgG alkaline phosphatase conjugate according to Promega's method.

Immunological Experiments

Polyclonal antibodies to BAS were developed by injecting protein purified through a final SDS-PAGE step in Freund's complete adjuvant in a New Zealand White rabbit. The initial injection contained approximately 300 μg of protein. Two additional booster injections (200 μg of protein each) were given at 2-week intervals. Blood collection started 35 d after the initial injection, and the rabbit was bled at 10-d intervals. After the highest antibody titer was reached, the rabbit was exsanguinated. Sera were frozen and stored at −20°C.

The IgG fraction was purified by chromatography on an Affi-Gel Protein A column (1 × 6.5 cm) using an antibody purification system (Affi-Gel Protein A Maps II, Bio-Rad). Fractions (1 mL) were collected, and the presence of BAS antibodies was monitored by ELISA as described by Engvall and Perlmann (1972).

The effect of the antibodies on enzyme activity was tested as described by Hrazdina et al. (1986) with a slight modification for BAS. Varying amounts of the purified antibody (0.7–16.6 μg) dissolved in 100 μL of eluting buffer were added to 100 μL of an enzyme preparation (21 μg of protein) that was purified through the Phenyl-Sepharose step. Reaction mixtures were incubated for 1 h at 37°C and overnight at 4°C. After incubation, the mixtures were centrifuged for 30 min at 15,000*g* and 100 μL of supernatant was used for the enzyme assay. Controls contained preimmune rabbit serum.

Measurement of Fungitoxicity

Fungitoxicity of the reaction product was determined with *Phytophthora fragariae* (narrow host range) and *Botrytis cinerea* (wide host ranges). Cultures of *P. fragariae* NY 334 designated later as *P. fragariae* var *rubi* (Wilcox, 1991) were grown as described by Wilcox (1989). *B. cinerea* was grown on Difco potato dextrose agar medium (39 g L^{-1}). Fungal colonies were incubated at 24°C in darkness. A 9-mm-diameter medium plug with mycelia was removed from the inoculum with a sterile cork borer and placed on the surface of the medium containing *p*-hydroxybenzalacetone or *p*HPB (0, 0.125, 0.250, 0.500, 0.750, or 1.0 mM) in 100- × 15-mm plastic Petri dishes. Experiments were carried out in quadruplicate. Changes in colony diameter were measured during incubation. Fungitoxicity is expressed as percentage of growth rate inhibition of mycelium.

RESULTS

Purification and Molecular Characterization of BAS

Because of the relatively low protein content of raspberry fruits, a large amount of plant material was used for the isolation of the enzyme. The purification consisted of removal of the viscous polysaccharidic material by stepwise addition of ammonium sulfate to 50% saturation and removal of the precipitate by centrifugation. This step removed the majority of the polysaccharide fraction that interfered with the chromatographic separations. The enzyme was precipitated in the 50 to 80% ammonium sulfate saturation range and purified in four column chromatographic steps (Fig. 2). These included gel filtration (Fig. 2A), hydrophobic chromatography (Fig. 2B), ion-exchange chromatography (Fig. 2C), and chromatofocusing (Fig. 2D). The purification of BAS as monitored by SDS-PAGE in 10% gels is shown in Figure 3. This purification process resulted in a 170-fold enrichment of enzyme activity. The final enzyme preparation contained approximately 3 mg of protein and had 17% of the total enzyme activity present in the crude homogenate (Table I). CHS was co-purified with

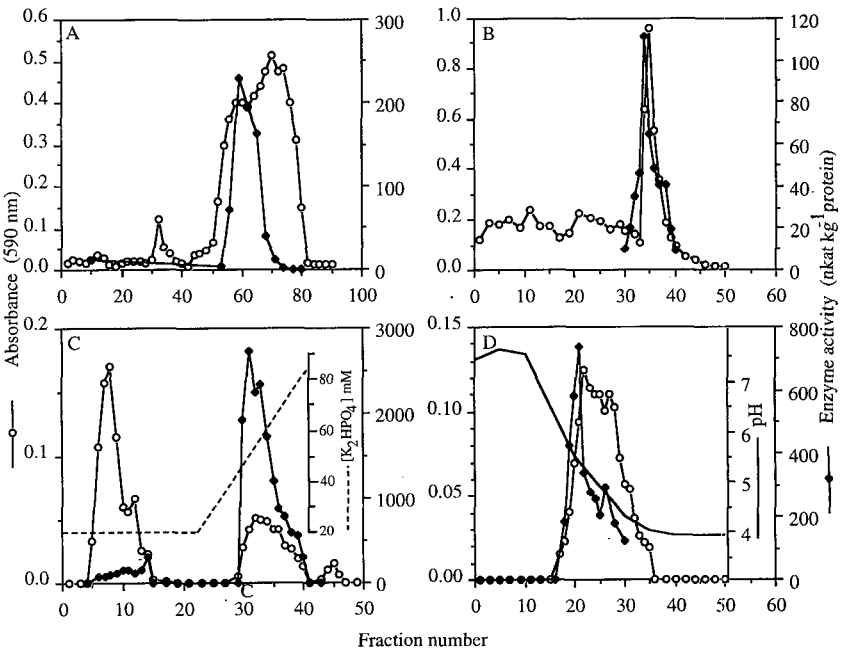

Figure 2. Purification of BAS from ripe raspberries cv Royalty. A, Gel filtration on Ultragel AcA-44; B, hydrophobic chromatography on Phenyl-Sepharose CL-4B; C, ion-exchange chromatography on DEAE Bio-Gel A; D, chromatofocusing on a PBE 94 column.

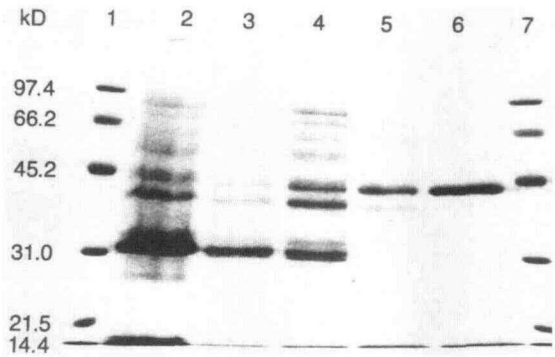

Figure 3. SDS-PAGE of the BAS during purification. Lanes contain 10 μg of protein per well. The purification steps were ammonium sulfate fractionation (lane 2), gel filtration on Ultrogel AcA-44 (lane 3), hydrophobic chromatography on Phenyl-Sepharose CL-4B (lane 4), ion-exchange chromatography on DEAE Bio-Gel A (lane 5), and chromatofocusing on PBE 94 (lane 6). Lanes 1 and 7 contained protein markers. The gel was stained with Coomassie brilliant blue.

BAS in all chromatographic steps. However, whereas BAS was purified 172-fold, CHS was co-purified only 14-fold (data not shown).

The molecular characteristics of BAS closely resembled those of CHS and STS, two other aromatic polyketide synthases that catalyze similar reactions and that occur widely in the plant kingdom. BAS had an approximate M_r of 83,000 (±5,000) as determined by molecular sieving, and it was composed of two identical or similar subunits each with an M_r of 41,500 (±1,500). The pH optimum of the enzyme was approximately 8.0, and the pI was approximately 5.3, as determined by chromatofocusing on a polybuffer exchange column. In Michaelis-Menten experiments the enzyme showed half-saturation (K_m) at 1.0 μM with malonyl-CoA and 3 μM with *p*-coumaryl CoA (Table II).

Substrate specificity experiments using other aromatic CoA derivatives as second substrates were carried out, and the results were compared with those for CHS. The results of these experiments are shown in Table III. Use of *p*-coumaryl-CoA as substrate in both the BAS and CHS reactions was set as 100%, and utilization of other aromatic CoA derivatives was calculated as a fraction or multiple of this. For CHS, the highest incorporation of aromatic CoA derivatives was with *p*-coumaryl-CoA, followed by cinnamyl-CoA. BAS used feruloyl-CoA as substrate most efficiently, followed by *p*-coumaryl-CoA, 5-hydroxyferulyl-CoA, cinnamyl-CoA, caffeyl-CoA, and sinapyl-CoA. These experiments clearly indicate that the two enzymes are distinctly different in their substrate specificity.

The presence of 2-ME, glutathione, and ethylene glycol in the reaction mixture further confirmed differences in activities of BAS and CHS. The results of these experiments are shown in Figure 4. The presence of 2 mM 2-ME reduced CHS activity by 43% and 10 mM 2-ME in the reaction mixture resulted in an 80% inhibition of the enzyme. Under the same conditions the activity of BAS increased to approximately 35% in the presence of 4 and 6 mM 2-ME. Higher concentrations of 2-ME did not change the activity of BAS significantly (Fig. 4A).

Glutathione (2 mM) markedly decreased (40%) the activity of both enzymes. However, the activity of BAS was significantly higher than CHS at all glutathione concentrations tested (Fig. 4B).

A different effect on the activities of BAS and CHS was observed upon addition of ethylene glycol during the purification step on the Phenyl-Sepharose CL-4B column. Enzyme activity containing fractions before dialysis had a BAS/CHS ratio of 13, decreasing to 2 after dialysis. The presence of ethylene glycol at different concentrations affected the activities of BAS and CHS differently (Fig. 4C). Although the activity of BAS increased steadily up to 6 M ethylene glycol in the solution, the activity of CHS showed a steady decrease at these concentration levels.

Preparation and Properties of the Anti-BAS Antibody

The protein preparation after chromatofocusing (1.0 mg) contained a minor contaminating band (Fig. 3) that had to be removed for the antibody preparation. Therefore, the protein preparation was further subjected to SDS-PAGE. The protein band corresponding to BAS (approximately 700 μg) was visualized with the Rapid Reversible Stain (Diversified Biotech, Newton Center, MA), excised from the gel, and used for development of polyclonal antibodies. The anti-BAS serum was affinity purified and used in the immunoblotting and immunoprecipitation experiments. Two-dimensional immunoblotting of the BAS preparation showed the presence of a narrow band with an approximate M_r of 41,500 that was located in the pH 5.6 to 6.2 region (data not shown). The shape of the band indicated the presence of differently charged protein species in the BAS preparation with similar or identical M_rs.

The purified antibody precipitated the activity of BAS from a protein preparation that was purified through the hydrophobic chromatography step (Fig. 5). Although the antibody also precipitated the activity of CHS, its effectiveness in inhibiting enzyme activity was less than with BAS. This result could be interpreted as indicating a similarity in

Table I. *Purification of BAS*

Fraction	Total Protein	Total Activity	Specific Activity	Purification	Yield
	mg	*pkat*	*μkat kg⁻¹ protein*	*fold*	*%*
Crude extract	2656	5.91	0.002	1	100
$(NH_4)_2SO_4$ fractionation and AcA 44 filtration	92	4.42	0.048	24	75
Hydrophobic chromatography	28	4.39	0.157	78	74
DEAE chromatography	14	3.12	0.223	111	53
Chromatofocusing	3	1.03	0.345	172	17

Table II. *Molecular properties of BAS*

Property	Value
M_r	83,000 ± 5,000
M_r subunit	41,500 ± 1,500
pH optimum	8.0
pI	5.3
K_m	
Malonyl-CoA	1 μM
p-Coumaryl-CoA	3 μM

epitopes between BAS and CHS. However, we cannot rule out the possibility that two distinct enzymes gave rise to two sets of antibodies.

Induction of BAS and CHS Activity in Cell-Suspension Cultures

To further investigate the relationship between BAS and CHS, we carried out enzyme induction experiments with cell-suspension cultures in the presence of Suc and yeast extract (Fig. 6). Addition of fresh medium (10 mL) to 20 mL of raspberry cell-suspension culture (control) changed BAS activity slightly without measurable effect on the activity of CHS. Addition of Suc (0.5 M) to the cells resulted in a more than 2-fold induction in the activity of BAS, but it had no measurable effect on the activity of CHS. Treatment of the raspberry suspension cultures with a yeast extract resulted in a rapid, transient increase of BAS activity (5-fold) that reached a maximum approximately 50 h after treatment. During this time the activity of CHS in the cell-suspension cultures remained unchanged at a basal level.

In immunoblots a protein band with an approximate M_r of 41,500 was recognized (data not shown). Although protein bands with higher M_rs were also recognized by the antibody preparation and gave stronger staining, they were not related to either BAS or CHS. The cross-reactivity of these bands was apparently caused by recognition of carbohydrate residues by the antibody. When blots were subjected to periodate oxidation to remove carbohydrate residues (Woodward et al., 1985), only the BAS/CHS band was recognized by the antibody preparation (data not shown). However, periodate treatment also diminished the intensity of the immunostaining significantly.

Inhibition of Mycelial Growth

A 40% inhibition of mycelial growth was suggested in the literature as the value at which to classify a fungus as sensitive or tolerant to a phytoalexin (Delserone et al., 1992). *p*-Hydroxybenzalacetone showed higher toxicity to *P. fragariae* var *rubi* and to *B. cinerea* than *p*HPB at all concentrations tested (Fig. 7). The compound showed a 40% inhibition at 250 μM concentration (40 μg mL^{-1}) and completely inhibited mycelial growth at 1 mM (162 μg mL^{-1}) of *P. fragariae* var *rubi*, the most frequently isolated fungus from infected raspberry plants (Wilcox, 1991). *p*-Hydroxybenzalacetone had markedly less effect on *B. cinerea* (Fig. 7A). Both fungi were tolerant to *p*HPB; however, mycelial growth of *P. fragariae* var *rubi* was inhibited more than 50% in the presence of 1 mM *p*HPB (Fig. 7B).

Table III. *Substrate specificity of a partially purified enzyme preparation in the BAS and CHS reactions*

Substrate	BAS	CHS
p-Coumaryl-CoA	100	100
Cinnamyl-CoA	36	57
Caffeyl-CoA	15	2
Feruloyl-CoA	300	6
5-Hydroxyferulyl-CoA	60	2
Sinapyl-CoA	8	1

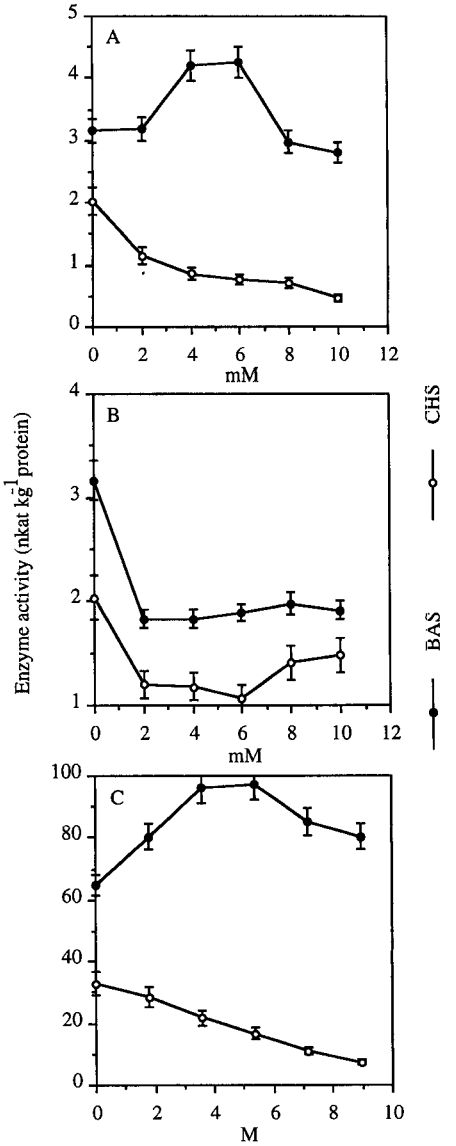

Figure 4. Effect of 2-ME (A), glutathione (B), and ethylene glycol (C) on the activities of BAS and CHS in standard enzyme assays. Bars indicate ±SE.

DISCUSSION

Polyketide synthases are multifunctional enzymes that catalyze repeated decarboxylative condensations between

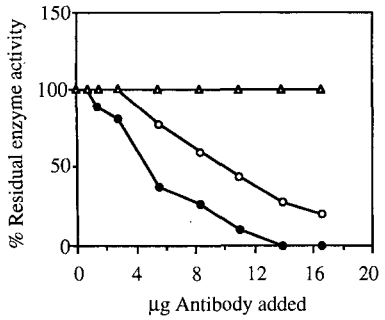

Figure 5. Inhibition of BAS (●) and CHS (○) activities by the purified anti-BAS antiserum. The preimmune serum (△) had no inhibitory effect on the activities of either enzyme.

acyl thioesters (McDaniel et al., 1994). CHS is a key enzyme in the biosynthesis of flavonoid compounds that condenses three malonyl-CoA with one *p*-coumaryl-CoA to form an aromatic polyketide (Hrazdina et al., 1976). This polyketide is cyclized to naringenin chalcone, from which most flavonoid compounds derive. It has been reported that hydroxystilbenes are synthesized by a similar reaction mechanism (Rolfs and Kindl, 1984; Schöppner and Kindl, 1984; Fliegmann et al., 1992) and that CHS and STS are closely related enzymes both in structure and function. Evolutionary trees based on the amino acid sequences do not place the two enzyme types into separate groups, and differences in the amino acid sequences of the two enzymes are not confined to certain regions of the proteins (Tropf et al., 1994). Both enzymes catalyze the stepwise addition of acetate units to *p*-coumaryl-CoA or cinnamyl-CoA and contain a family signature box of 12 amino acids that has been strictly conserved (Bairoch, 1992). Although the condensation reaction of both enzymes is similar, the products of their reaction arise from different ring closure mechanisms. This requires a different folding of the enzyme-bound tetraketide intermediate (Schröder and Schröder, 1990). In addition to condensing acetate with *p*-coumaryl-CoA or cinnamyl-CoA, STS also performs a decarboxylation reaction, shortening the polyketide chain by one carbon atom (Fliegmann et al., 1992). Lanz and co-workers (1991) have

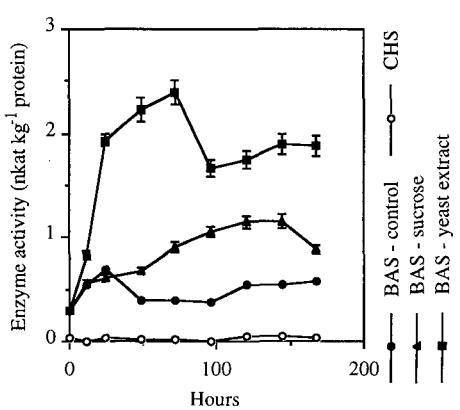

Figure 6. Changes in the activity of BAS and CHS in raspberry tissue cultures in the presence of 0.5 M Suc, or yeast extract (3 g L^{-1}). Bars indicate ±SE.

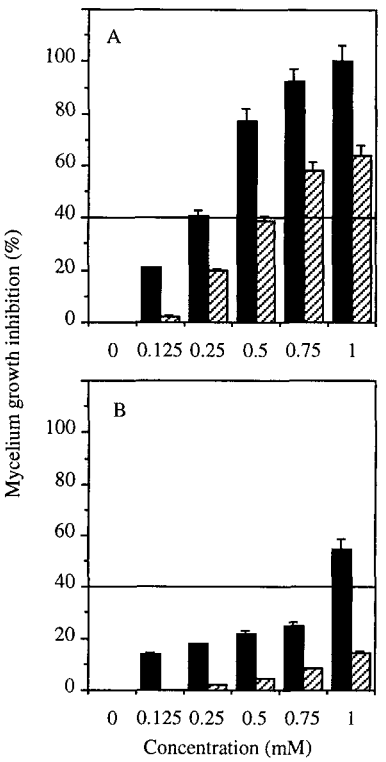

Figure 7. Effect of *p*-hydroxybenzalacetone (A), and *p*HPB (B) on mycelium growth of *P. fragariae* var *rubi* (■) and *B. cinerea* (▨). Bars indicate ±SE.

shown that Cys^{169}, which is strictly conserved in CHS and STS proteins, is essential for enzyme activity and that this Cys represents the active site for the condensing reaction. A single change of His to Gln at position 166 changed the substrate preference of the enzyme (Schröder and Schröder, 1992; Schröder et al., 1993). With the exception of position 166, the amino acids surrounding the essential Cys at 169 are strongly conserved in all known CHSs and STSs. Investigations by co-expression of active site mutants with differently mutated, inactive proteins showed that the heterodimers synthesized the end products, indicating that each subunit performed all three condensation steps (Tropf et al., 1995). Both enzymes expressed as a single species in *Escherichia coli* and synthesized both products, which were also obtained with a CHS heterodimer containing a single active site. Therefore, deducing the amino acid sequence of BAS will be of importance in understanding not only the reaction mechanism of the aromatic polyketide synthases but also their structural similarities.

As shown in Figure 1, BAS carries out a reaction similar to those carried out by CHS and STS. Although some molecular properties of the three enzymes appear to be similar, the reaction catalyzed by BAS differs partially from the others. To illustrate both the similarities and the differences, we have listed the molecular properties of BAS below and compared it to CHS where appropriate. The M_r of BAS and CHS are similar or identical. BAS has a M_r of approximately 83,000 and is composed of two subunits with similar M_rs of 41,500 each. CHS has been reported to

have a M_r of 83,000 in buckwheat (Hrazdina et al., 1986) with a subunit M_r of 41,500. Since CHS activity was present in the purified BAS preparation, albeit to a lesser extent, the M_rs of BAS and CHS in raspberry fruits seem to be identical. Two-dimensional immunoblot analysis resulted in the recognition of a single narrow band. Since the purified BAS preparation also contained some CHS activity, we do not know presently whether this protein band represents a multifunctional polyketide synthase or a mixture of two enzymes. The anti-BAS serum had significantly less effect on CHS than on BAS (Fig. 5). The pH optimum of both enzymes falls in the same range, i.e. 7.5 to 8.5, as does the pI (pH 5.2 for CHS, 5.3 for BAS). K_m values for both enzymes are within the 1 to 10 μM range, showing equally high substrate affinity. However, BAS and CHS showed different substrate specificities (Table III). CHS in raspberries used *p*-coumaryl-CoA exclusively as substrate, whereas BAS utilized feruloyl-CoA three times more efficiently than *p*-coumaryl-CoA. It is not known presently whether the preferential use of feruloyl-CoA is the result of in vitro conditions, since a purified CHS preparation from parsley also produced the corresponding benzalacetone derivative (Hrazdina et al., 1976). Neither the corresponding benzalacetone derivative nor its reduction product have been reported in raspberries or other plant genera. 5-Hydroxyferuloyl-CoA was also used by BAS as substrate, albeit at a much lesser extent.

Further evidence that BAS is a different enzyme from CHS came from the activities of these two enzymes in stressed raspberry cell cultures and in the presence of 2-ME or ethylene glycol (Fig. 4). When raspberry cell-suspension cultures were exposed to 0.5 M Suc in the medium and the activities of both enzymes were determined, BAS showed a 2-fold increase in activity (Fig. 6). When raspberry cultures were treated with a yeast extract to mimic the effect of fungal invasion, the activity of BAS increased 5-fold, reached a maximum 50 h after treatment, and declined thereafter. During the same time course the activity of the CHS remained steady at a basal level and did not show significant differences between the control and stressed raspberry cells. Since BAS and CHS were assayed under conditions in which both BAS and CHS are active, this is a clear evidence for the preferential induction of BAS upon yeast extract treatment. A similar response was shown in carrot cell-suspension cultures treated with fungal elicitors (Marinelli et al., 1994). Changes in the activities of BAS and CHS in raspberry cell-suspension culture were similar to activities observed for STS and CHS in peanut cell-suspension cultures (Rolfs et al., 1987). In both systems activity of CHS was lower than the activities of the BAS or STS.

Additional evidence that CHS and BAS may be different enzymes came from inhibition experiments with 2-ME and ethylene glycol. Whereas BAS activity increased in the 4 to 6 mM 2-ME concentration range, CHS activity decreased under the same conditions. Susceptibility of CHS to increasing concentration of 2-ME was reported earlier by Kreuzaler and Hahlbrock (1975). However, whereas an increasing concentration of 2-ME in the reaction mixture affected the formation of naringenin/naringenin chalcone and bis-noryangonin, it had no visible effect on the formation of *p*-hydroxybenzalacetone. The presence of ethylene glycol in the enzyme preparation markedly increased the activity of BAS but inhibited the activity of CHS by 80% (Fig. 4C). A similar effect of ethylene glycol on enzyme activity (phosphofructokinase) was observed by Podestá et al. (1994) in potato. Therefore the reaction mechanism, substrate specificity, effect of antibody, and the analysis of induction kinetics in stressed raspberry tissue cultures argue that BAS is an independent enzyme.

CHS and STS are closely related plant-specific polyketide synthases that are key enzymes in the biosynthesis of flavonoid and stilbene phytoalexins (Rolfs and Kindl, 1984; Lanz et al., 1991; Schröder et al., 1993). The toxic effect of flavonoid (Skipp and Bailey, 1977; Hahn et al., 1985; Delserone et al., 1992) and stilbene phytoalexins (Hart, 1981; Dercks and Creasy, 1989) on pathogenic fungi is well documented. Isoflavonoid phytoalexins have been reported in a number of plant species, although to date the work has mainly been limited to members of the *Leguminosae* (Dixon, 1980; Ingham, 1982). In the *Rosacea*, phytoalexins have been identified in apple (Kuc, 1976) and strawberry cultivars (Mussell and Staples, 1970). Postinfection accumulation of phenolics in the bordering zone of resistant raspberry cultivars has also been reported (Kozlowska and Krzywanski, 1993a, 1993b). Therefore, the inhibitory effect of the *p*-hydroxybenzalacetone on *P. fragariae* var *rubi* and *B. cinerea* in the micromolar to millimolar range (Fig. 7) suggests that the raspberry ketone biosynthetic pathway may also be involved in the defense mechanism of the genus *Rubus* toward pathogenic fungi. BAS has also been detected in pea, apple, grape, and tobacco tissue cultures (W. Borejsza-Wysocki and G. Hrazdina, unpublished data) and its pathway product or the corresponding glucosylated form (Pabst et al., 1990) has been found in rhubarb roots (Murakami and Tanaka, 1972), in European cranberries (Hirvi et al., 1981), in the fruits of sea buckthorn (Hirvi and Honkanen, 1984), in *Scutellaria* species (Lin and Chou, 1985), and in needles of pine (*Pinus contorta*) (Bauer et al., 1955; Higuchi and Donnelly, 1977). These data indicate that this pathway may not be restricted to *Rubus* only.

ACKNOWLEDGMENTS

The authors wish to thank Ms. Diane F. LaPoint, College of Veterinary Medicine, Cornell University, for developing the antibodies, and Mrs. E. Borejsza-Wysocka for help with the tissue culture experiments, Dr. J. Sanford for the grape and tobacco tissues culture, and Drs. W. Wilcox and R. Pearson for the *Phytophthora* and *Botrytis* cultures.

Received September 13, 1995; accepted December 19, 1995.

LITERATURE CITED

Bairoch A (1992) PROSITE: a dictionary of sites and patterns in proteins. Nucleic Acids Res **20:** 2013–2018

Bauer L, Birch AJ, Ryan AJ (1955) Studies in relation to biosynthesis. VI. Rheosmin. Aust J Chem **8:** 534–538

Borejsza-Wysocki W, Hrazdina G (1994a) Biosynthesis of *p*-hydroxyphenylbutan-2-one in raspberry fruits and tissue culture. Phytochemistry **35:** 623–628

Borejsza-Wysocki W, Hrazdina G (1994b) Establishment of callus and cell suspension cultures of raspberry (*Rubus idaeus* L.) cv. Royalty. Plant Cell Tissue Org Cult **37:** 213–216

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Braun G, Hieke E (1977) Analysis of aroma components in foods. Dtsch Lebensm Rundsch **73:** 273–278

Delserone LM, Matthews DE, VanEtten HD (1992) Differential toxicity of enantiomers of maackiain and pisatin to phytopathogenic fungi. Phytochemistry **31:** 3813–3819

Dercks W, Creasy LL (1989) The significance of stilbene phytoalexins in the *Plasmopara viticola*-grapevine interaction. Physiol Mol Plant Pathol **34:** 189–202

Dixon RA (1980) Plant tissue culture methods in the study of phytoalexin induction. *In* DS Ingram, JP Helgeson, eds, Tissue Culture Methods for Plant Pathologists. Blackwell Scientific, Oxford, UK, pp 185–196

Engvall E, Perlmann E (1972) Enzyme linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme labeled antiimmunoglobulin in antigen coated tubes. J Immunol **109:** 129–135

Fliegmann J, Schröder G, Schanz S, Britsch L, Schröder J (1992) Molecular analysis of chalcone and dihydropinosylvin synthase from Scots pine (*Pinus sylvestris*), and differential regulation of these and related enzyme activities in stressed plants. Plant Mol Biol **18:** 489–503

Hahn MG, Bonhoff A, Grisebach H (1985) Quantitative localization of the phytoalexin glycelollin I in relation to fungal hyphae in soybean roots infected with *Phytophthora megasperma* f. sp. *glycinea*. Plant Physiol **77:** 591–601

Hart JH (1981) Role of phytostilbenes in decay and disease resistance. Annu Rev Phytopathol **19:** 437–458

Higuchi R, Donnelly DMX (1977) Glycosides from *Pinus contorta* needles. Phytochemistry **16:** 1587–1590

Hirvi T, Honkanen E (1984) The aroma of the fruit of Sea Buckthorn, *Hippophae rhamnoides*, L. Z Lebensm Unters Forsch **179:** 387–388

Hirvi T, Honkanen E, Pyysalo T (1981) The aroma of cranberries. Z Lebensm Unters Forsch **172:** 365–367

Hrazdina G, Kreuzaler F, Hahlbrock K, Grisebach H (1976) Substrate specificity of flavanone synthase from cell suspension cultures of parsley and structure of release products *in vitro*. Arch Biochem Biophys **175:** 392–399

Hrazdina G, Lifson E, Weeden NF (1986) Isolation and characterization of buckwheat (*Fagopyrum esculentum* M.) chalcone synthase and its polyclonal antibodies. Arch Biochem Biophys **247:** 414–419

Ingham JL (1982) Phytoalexins from the Leguminosae. *In* JA Bailey, JW Mansfield, eds, Phytoalexins. John Wiley and Sons, New York, pp 21–80

Kozlowska M, Krzywanski Z (1993a) The possible role of phenolic compounds in red raspberry resistance to *Didymella applanata*. International Symposium on Natural Phenols in Plant Resistance, September 13–17, 1993, Vol I. Freising-Weihenstephon, Germany, p 26

Kozlowska M, Krzywanski Z (1993b) Lignin deposition and its role in restriction of the penetration of *Didymella applanata* in red raspberry canes. *In* B Fritig, M Legrand, eds, Mechanisms of Plant Defense Responses. Kluwer Academic, Dordrecht, The Netherlands, p 357

Kreuzaler F, Hahlbrock K (1975) Enzymatic synthesis of aromatic compounds in higher plants. Formation of bis-noryangonin (4-hydroxy-6[4-hydroxystyryl]2-pyrone) from *p*-coumaroyl-CoA and malonyl-CoA. Arch Biochem Biophys **169:** 84–90

Kuc JA (1976) Phytoalexins. *In* R Heitefuss, PH Williams, eds, Physiological Plant Pathology. Springer-Verlag, New York, pp 632–652

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lanz T, Tropf S, Marner FJ, Schröder J, Schröder G (1991) The role of cysteines in polyketide synthases. Site-directed mutagenesis of resveratrol and chalcone synthases, two key enzymes in different plant-specific pathways. J Biol Chem **266:** 9971–9976

Larsen M, Poll L, Callesen O, Lewis M (1991) Relations between the aroma compounds and the sensory evaluation of 10 raspberry varieties (*Rubus idaeus* L.). Acta Agric Scand **41:** 447–454

Lin YL, Chou CJ (1985) Studies on the constituents of aerial parts of *Scutellaria rivularis* wall. Chemical Abstracts **102:** 92951

Marinelli F, Ronchi VN, Salvadori P (1994) Elicitor induction of enzyme activities and 6-methoxymellein production in carrot cell suspension culture. Phytochemistry **35:** 1457–1460

McDaniel R, Ebert-Khosla S, Fu H, Hopwood DA, Khosla C (1994) Engineered biosynthesis of novel polyketides: influence of a downstream enzyme on the catalytic specificity of a minimal aromatic polyketide synthase. Proc Natl Acad Sci USA **91:** 11542–11546

Murakami T, Tanaka K (1972) Neue phenolische Glykoside in *Rhizoma rhei*. Tetrahedron Lett **29:** 2965–2968

Mussell HW, Staples C (1970) Phytoalexin like compounds apparently involved in strawberry resistance to *Phytophthora fragariae*. Phytopathology **61:** 515–517

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem **250:** 4007–4021

Pabst A, Barron D, Adda J, Schreier P (1990) Phenylbutan-2-one β-D-glucosides from raspberry fruit. Phytochemistry **29:** 3853–3858

Podestá FE, Moorhead GBG, Plaxton WC (1994) Potato tuber pyrophosphate-dependent phosphofructokinase: effect of thiols and polyalcohols on its intrinsic fluorescence, oligomeric structure, and activity in dilute solutions. Arch Biochem Biophys **313:** 50–57

Rolfs CH, Kindl H (1984) Stilbene synthase and chalcon synthase. Two different constitutive enzymes in cultured cells of *Picea excelsa*. Plant Physiol **75:** 489–492

Rolfs CH, Schön H, Steffens M, Kindl H (1987) Cell-suspension culture of *Arachis hypogea* L.: model system of specific enzyme induction in secondary metabolism. Planta **172:** 238–244

Schinz H, Seidel CF (1957) Nachtrag zu der Arbeit Nr. 194. Helv Chim Acta **40:** 278

Schöppner A, Kindl H (1984) Purification and properties of stilbene synthase from induced cell suspension cultures of peanut. J Biol Chem **259:** 6806–6811

Schröder G, Schröder J (1992) A single change of histidine to glutamine alters the substrate preference of stilbene synthase. J Biol Chem **267:** 20558–20560

Schröder J, Schröder G (1990) Stilbene and chalcone synthase: related enzymes with key function in plant-specific pathways. Z Naturforsch **45c:** 1–8

Schröder J, Schanz S, Tropf S, Kärcher B, Schröder G (1993) Phytoalexin biosynthesis: stilbene synthase and co-action of a reductase with chalcone synthase. *In* B Fritig, M Legrand, eds, Mechanisms of Plant Defense Responses. Kluwer Academic, Dordrecht, The Netherlands, pp 257–267

Skipp RA, Bailey JA (1977) The fungitoxicity of isoflavanoid phytoalexins measured using different types of bioassay. Physiol Plant Pathol **11:** 101–112

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA **76:** 4350–4354

Tropf S, Kärcher B, Schröder G, Schröder J (1995) Reaction mechanisms of homodimeric plant polyketide synthases (stilbene and chalcone synthases). J Biol Chem **270:** 7922–7928

Tropf S, Lanz T, Rensing SA, Schröder J, Schröder G (1994) Evidence that stilbene synthases have developed from chalcone synthases several times in the course of evolution. J Mol Evol **38:** 610–618

Wilcox WF (1989) Identity, virulence, and isolation frequency of seven *Phytophthora* spp. causing root rot of raspberry in New York. Phytopathology **79:** 93–101

Wilcox WF (1991) Phytophthora root rot. *In* MA Ellis, RH Converse, RN Williams, B Williamson, eds, Compendium of Raspberry and Blackberry Diseases and Insects. American Phytopathological Society Press, St. Paul, MN, pp 34–36

Woodward MP, Young WW Jr, Bloodgood RA (1985) Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. J Immunol Methods **78:** 143–153