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Biocatalytic Synthesis of Butein and Sulfuretin by *Aspergillus alliaceus*

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Aspergillus alliaceus UI315 was examined for its potential to catalyze biotransformation reactions of chalcones that mimic plant biosynthetic processes. 3-(4″-Hydroxyphenyl)-1-(2′,4′-dihydroxyphenyl)-propenone (4,2′,4′-trihydroxychalcone, isoliquiritigein) (**1**) was efficiently transformed to two major metabolites that were isolated chromatographically and identified by spectroscopic methods as 3-(3″,4″-dihydroxyphenyl)-1-(2′,4′-dihydroxyphenyl)propenone (butein) (**7**) and 2-[(3,4-dihydroxyphenyl)methylene]-6-hydroxy-3(2*H*)benzofuranone (7,3′,4′-trihydroxyaurone, sulfuretin) (**10**). Inhibition experiments suggested that initial C-3 hydroxylation of **1** to **7** was catalyzed by a cytochrome P450 enzyme system. A second *A. alliaceus* enzyme, partially purified and identified as a catechol oxidase, catalyzed the oxidation of the catechol butein (**7**) likely through an *ortho*-quinone (**8**) that cyclized to the aurone product **10**. This work showed that *A. alliaceus* UI315 contains oxidative enzyme systems capable of converting phenolic chalcones such as **1** into aurones such as **10** in a process that mimics plant biosynthetic pathways.

KEYWORDS: Biotransformations; *Aspergillus alliaceus*; cytochrome P450; catechol oxidase; chalcone; aurone

INTRODUCTION

Flavonoids are a diverse group of natural products that play important roles in plant growth and development and in defenses against microorganisms and pests (*1*). Apart from their physiological roles in plants, flavonoids are important antioxidants in the human diet that can scavenge free radicals (*2*). Biosynthetically, flavonoids are derived from chalcone precursors that in turn are derived from the condensation of *p*-coumaroyl CoA and three malonyl CoAs by chalcone synthase (*1*). Aurones are plant flavonoids responsible for yellow colors in a variety of popular ornamental flowers such as snapdragons, cosmos, and dahlias. Although it has been suggested that aurones are derived from chalcones, few studies have confirmed such a biosynthetic pathway (*3*). A soy seedling peroxidase catalyzed the H_2O_2-dependent oxidation of chalcone **1** to an intermediate 2-(α-hydroxybenzyl) coumaranone (**2**) that underwent further enzymatic or nonenzymatic dehydration to form aurone **3** (**Figure 1A**) (*4*). More recently, snapdragon polyphenol oxidase known as aureusidin synthase transformed selected phenolic chalcones to aurones in a two step process involving initial hydroxylation of 3-(4″-hydroxyphenyl)-1-(2′,4′,6′-trihydroxyphenyl)propenone, (4,2′,4′,6′-tetrahydroxychalcone) (**4**) to **5** and subsequent cyclization of **5** to 2-[(3,4-dihydroxyphenyl)methylene]-4,6-dihydroxy-3(2*H*)benzofuranone (aureusidin) (**6**) (**Figure 1B**) (*3*, *5*−*7*). A tyrosinase from *Neurospora crassa* was also capable of catalyzing the overall cyclization reaction (*5*).

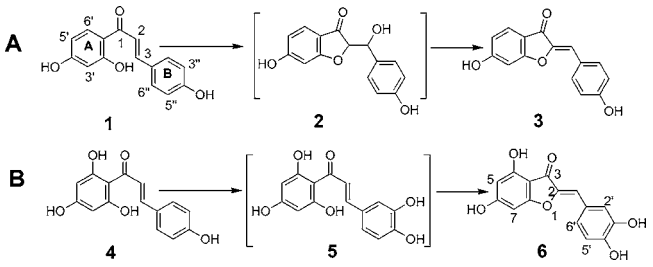

Figure 1. Proposed plant biosynthetic pathways for aurone biosynthesis. (**A**) Catalyzed by a H_2O_2-dependent peroxidase from soy seedlings (*4*). (**B**) Oxidative formation of aureusidin from 2′,4,4′,6′-tetrahydroxychalcone by aureusidin synthase from yellow snapdragon flowers (*6*).

We previously reported that *Aspergillus alliaceus* UI315 contained cytochrome P450 enzyme systems capable of biocatalytically transforming 3-(2″,3″-dimethoxyphenyl)-1-(2′-hydroxyphenyl)propenone (2′-hydroxy-2,3-dimethoxychalcone) to 3′,8-dihydroxy-2′-methoxyflavanone, 3′-hydroxy-2′-methoxyflavanone, and 2′,3′-dimethoxyflavanone plus modified chalcones (*8*). In the present study, we investigated the fungal hydroxylation of the chalcone isoliquiritigenin (**1**) and the cyclization of the hydroxychalcone product **7** to aurone **10** by *A. alliaceus*.

MATERIALS AND METHODS

Chemicals. 3-(4″-Hydroxyphenyl)-1-(2′,4′-dihydroxyphenyl)propenone (isoliquiritigein) (**1**), 3-(3″,4″-dihydroxyphenyl)-1-(2′,4′-dihydroxyphenyl)propenone (butein) (**7**), and 2-[(3,4-dihydroxyphenyl)-

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methylene]-6-hydroxy-3(2H)benzofuranone (sulfuretin) (**10**) were obtained from Indofine Chemical Company, Inc. (Somerville, NJ). 2-Diethylaminoethyl-2,2-diphenylvalerate (SKF 525A), phenylthiocarbamide (PTC), and tyrosine were obtained from Sigma Chemical Co. (St. Louis, MO). Metyrapone and 3-hydroxy-L-tyrosine (L-DOPA) were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Chromatography. Thin-layer chromatography (TLC) was performed on silica gel GF_{254} plates (Merck). Layers of 0.5 mm of thickness were used for analytical and 1 mm thickness for preparative TLC. Plates were developed using hexanes:acetone:CH_3COOH (55:45:1, v/v/v) as the solvent system. Developed plates were visualized under 254 and 360 nm UV light before being sprayed with Pauly's reagent (*9*). Pauly's reagent consisted of three separate solutions of 0.5% sulfanilic acid in 2% HCl, 0.5% $NaNO_2$, and 5% KOH in 50% ethanol. Equal volumes of $NaNO_2$ and sulfanilic acid solutions were mixed immediately prior to use, and plates were sprayed with this mixture and then with KOH before being warmed with a heat gun to give burnt-orange and red colors for phenolic compounds.

High-performance liquid chromatography (HPLC) was performed with a Shimadzu LC-6A dual pumping system connected to a Shimadzu SPD-6AV UV/vis detector and a Shimadzu SCL-6B system controller (Kyoto, Japan). Separations were carried out over a 250 mm × 4.6 mm i.d., 10 μm Econosil C18 column (Alltech Associates Inc., Deerfield, IL). The mobile phases consisted of CH_3CN:H_2O (10:90) containing 5% of HCOOH (A) and CH_3CN:H_2O (90:10) containing 5% of HCOOH (B). The gradient was 0−100% B over 65 min at a flow rate of 1 mL/min. UV absorbances of eluting peaks were recorded at 390 nm, and samples were resolved into three major peaks at Rv of 23, 29, and 32 mL for **10**, **7**, and **1**, respectively.

Quantitation of metabolites in biotransformation samples was based upon comparison of metabolite peak areas to standard curves obtained by duplicate injections of the following amounts of pure metabolites: 0.25−4 μg for **1**, 0.02−1 μg for **7**, and 25−250 ng for **10**.

Spectroscopic Analysis. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained with Bruker NMR 300 and 400 spectrometers (Bruker Instruments, Billerica, MA), operating at 75.47 and 100.62 MHz for ^{13}C, respectively. NMR spectra were obtained in methanol-d_4 and acetone-d_6 using tetramethylsilane (TMS) as internal standards, with chemical shifts expressed in parts per million (δ) and coupling constants (*J*) in Hertz.

Organism and Culture Conditions. *A. alliaceus* UI315 was maintained in the University of Iowa, College of Pharmacy culture collection and was grown and stored on Sabouraud maltose agar in sealed screw cap tubes at 4 °C. Organisms were cultivated in two stages in a medium composed of 20 g of dextrose, 5 g of soybean meal, 5 g of yeast extract, 5 g of NaCl, and 5 g of K_2HPO_4 in 1 L of distilled water, which was adjusted to pH 7 with 6 N HCl before autoclaving. Media were sterilized in an autoclave at 121 °C for 20 min. Cultures were grown in steel-capped DeLong culture flasks containing one-fifth of their volumes of culture medium. Incubations were conducted at 28 °C on a New Brunswick Scientific (Edison, NJ) Innova 5000, Gyrotory tier shaker, operating at 250 rpm. Stage one cultures were inoculated from fresh *A. alliaceus* slants and were grown as described above. A 10% v/v inoculum derived from a 72 h old, first-stage culture was used to initiate the second-stage culture, which was incubated as described above. After 24 h, **1** dissolved in *N*,*N*-dimethylformamide (DMF) (50 mg/mL) was added to second-stage cultures to a final medium concentration of 0.5 mg/mL. The progress of microbial transformation reactions was monitored by TLC or HPLC by the following sampling procedure. Samples (2 mL) were withdrawn from substrate-containing cultures. These were acidified to pH 2 with 6 N HCl and extracted with EtOAc (1 mL), and extracts were spotted (10 μL) onto TLC plates for analysis. For HPLC analysis, EtOAc extracts were dried over Na_2SO_4, evaporated to dryness, dissolved in 1 mL of HPLC grade MeOH (Fischer Scientific), and filtered through a 13 mm, 0.2 μm Nylon syringe filter (Gelman Laboratory) before injection.

Preparative Scale Biotransformation. Second-stage *A. alliaceus* cultures were grown in 1 L of medium held in 20 125 mL DeLong flasks. A total of 250 mg of **1** in 5 mL of DMF was evenly distributed among the 24 h old second-stage cultures. Substrate-containing cultures were incubated for 72 h; the fermentation beers were combined, and the cells and other solids were removed by filtration through cheesecloth. Solids containing only trace amounts of metabolites were discarded, and the filtrates were acidified to pH 2 with 6 N HCl and exhaustively extracted with EtOAc. The extract was dried over anhydrous Na_2SO_4 and evaporated to a brown oil. Compounds in the crude extract were purified by preparative TLC using the solvent system described above.

Preparation of Cell Free Extracts (CFEs). Cells from 72 h stage II cultures induced by adding 0.5 mg/mL of **1** were washed successively with 0.5% aqueous NaCl. Cells (10 g wet weight) suspended in 100 mL of pH 7 50 mM phosphate buffer containing 0.5% (v/v) Triton X-100, 1 mM PMSF, and 0.5 mM ascorbic acid at 4 °C were homogenized by making two passes through a SLM Aminco (SLM Instruments Inc., Urbana, IL) French pressure cell at 1100 psig (18000 psi/in^2). Homogenates were centrifuged at 30000*g* for 45 min at 4 °C, and the supernatant, or CFE, was concentrated 20-fold in an Amicon ultrafiltration cell equipped with a PM 10 membrane (10 kDa). Using the ultrafiltration cell, the CFE was washed three times with pH 8.3 5 mM Tris-HCl buffer.

Partial Purification of *A. alliaceus* Catechol Oxidase. The washed and concentrated CFE (1.5 mL, 11.2 mg/mL protein) was chromatographed over a 1.5 cm × 25 cm DEAE cellulose DE 52 (Whatman) column previously equilibrated with pH 8.3 5 mM Tris-HCl (*10*). The column was eluted at a flow rate of 0.8 mL/min with a linear gradient ranging from 0 to 1 M NaCl in the equilibration buffer, collecting a total of 100 mL in 2.5 mL fractions. Fractions with catechol oxidase activity were pooled and stored at 4 °C.

Enzyme Assays. Enzyme assays were conducted by adding aliquots of CFE or DEAE cellulose fractions to pH 7 50 mM phosphate buffer. Controls consisted of reaction blanks prepared in buffer without added enzyme. Assays for possible tyrosinase activity were conducted at pH 7 and 37 °C for 15−20 min by monitoring the oxidation of tyrosine (2 mM) to DOPA as evidenced by an increase in absorbance at 280 nm (*11*). One unit (U) of tyrosinase activity catalyzed the formation of 1 μmol of L-DOPA per min under these conditions. Assays for catechol oxidase activity were conducted at pH 7 and 37 °C for 15−20 min by monitoring the oxidation of L-DOPA (5 mM) as evidenced by an increase in absorbance at 475 nm (*12*). One unit (U) of catechol oxidase activity catalyzed the formation of 1 μmol of DOPAchrome per min under these conditions. Protein was determined by the Bio-Rad protein assay (*13*) using bovine serum albumin as standard.

In Vitro Enzymatic Transformation. To measure activity vs chalcone substrates, a 2 unit sample of catechol oxidase in 0.5 mL of pH 7 50 mM phosphate buffer was incubated at 30 °C with **1** (1.95 mM) or **7** (1.8 mM) while being shaken at 250 rpm. After 5 h, the reaction mixture was acidified to pH 2 with 6 N HCl and extracted with one volume of ethyl acetate. The extract was evaporated under vacuum and resuspended in 0.1 mL of MeOH for HPLC analysis. To confirm that reactions were enzymatically catalyzed, controls without substrate, CFEs, DEAE cellulose fractions, or heat denaturated or proteolyzed CFEs and fractions were used as described above with compounds **1** and **7**. For proteolysis of extracts, 18 μg of catechol oxidase (0.38 U/mL protein) was incubated for 2 h at 37 °C in 300 μL of pH 8 50 mM phosphate buffer containing 1 mM $CaCl_2$ and 15 U of trypsin. Catechol oxidase extracts incubated at pH 8 and 37 °C for 2 h without the addition of trypsin were used as controls.

Effects of Inhibitors on Chalcone Biotransformation. The effects of cytochrome P450 inhibitors on chalcone biotransformations by *A. alliaceus* were studied by incubating for 24 h stage II cultures containing 0.5 mg/mL of **1** or **7** and inhibitors for 72 h at 28 °C and 250 rpm. The inhibitors used were SKF 525A (8 and 17 mM) and metyrapone (27 and 54 mM). For catechol oxidase (polyphenol oxidase) activity, PTC (1.5 and 15 mM) was used as the inhibitor. Inhibitors were dissolved in 250 μL of DMF and filtered through 0.45 μm membranes before being added to cultures. Cultures with inhibitors were incubated for 10 min at 28 °C and 250 rpm prior to substrate addition. Control flasks consisted of cultures containing substrate without inhibitors. Reaction mixtures were analyzed by HPLC as described. All reactions were conducted in triplicate, and the results are the averages of three determinations for each case.

RESULTS AND DISCUSSION

Metabolite Isolation and Identification. *A. alliaceus* UI315 is a versatile microorganism that catalyzes aromatic and aliphatic hydroxylations and O-dealkylation of aryl ethers (*8*, *14*−*18*). We recently reported that this organism catalyzed aromatic hydroxylations, O-demethylations, and cyclizations of 2,3-dimethoxy-2′-chalcone to flavanones (8). On the basis of our earlier work, we were interested in determining whether products formed during *A. alliaceus* reactions with chalcones might be dependent upon the nature of aromatic ring substitutions. When we examined **1** as a substrate, two products were obtained. No products were obtained without *A. alliaceus*. Preparative scale incubations using 500 mg of **1** gave two products at 72 h that were isolated by extraction and subsequent preparative TLC. Product identifications were based on ^{1}H and ^{13}C NMR spectroscopic analysis and by comparison to values for similar compounds.

Biotransformation products **7** and **10** were isolated in 15 and 4% yields, respectively. Maximum yields were obtained at 72 h, after which time metabolite concentrations decreased. When **7** was used as a substrate, **10** was formed in a maximum of 10% yield in 72 h. Product **7** was identified as butein by NMR spectroscopic analysis (*19*). ^{1}H NMR at 300 MHz (methanol-d_4) of **7** showed a characteristic set of two doublets for chalcone olefinic protons at δ 7.73 (J = 15 Hz, H-3) and δ 7.53 (J = 15 Hz, H-2). Signals for ring A were similar to those for **1** with two doublets at δ 7.85 (J = 9 Hz, H-6′) and at δ 6.31 (J = 2.4 Hz, H-3′) and a doublet of doublets at δ 6.43 (J = 9/2.4 Hz, H-5′). However, signals for ring B were different from those for **1** due to the presence of a hydroxyl group at C-3″. Thus, two doublets at δ 7.19 (J = 2.1 Hz, H-2″) and δ 6.83 (J = 8.4, H-5″) and a doublet of doublets at δ 7.12 (J = 8.1/2.1 Hz, H-6″) were observed.

Aurone product **10** was apparently obtained by cyclization of **7**. The ^{1}H NMR at 400 MHz (acetone-d_6) of **10** showed no chalcone olefinic proton signals and a new singlet at δ 6.61 for a benzylidine proton showing that the aurone ring had been formed. Ring A proton signals were similar to those for **1** and **7** with two doublets at δ 7.59 (J = 8.3 Hz, H-4) and δ 6.82 (J = 1.3 Hz, H-7) and one doublet of doublets at δ 6.75 (J = 8.3/1.3 Hz, H-5). Ring B signals were similar to **7**, with two doublets at δ 7.56 (J = 1.9 Hz, H-2′) and δ 6.92 (J = 8.3 Hz, H-5′) and a doublet of doublets at δ 7.34 (J = 8.3/1.9 Hz, H-6′). The ^{13}C NMR spectrum at 100.62 MHz showed C-2 at δ 146, the carbonyl resonance at δ 182, and the exocyclic, olefinic, benzylidine methine at δ 111 ppm. These data support a *Z*-olefinic configuration for **10** (*20*). ^{1}H and ^{13}C NMR spectra were identical to those reported for 2-[(3,4-dihydroxyphenyl)-methylene]-6-hydroxy-3(2*H*)benzofuranone (3′,4′,7-trihydroxy aurone) (*21*).

Our results indicate that the *A. alliaceus* enzymatic system involved in cyclization reactions of chalcones requires phenolic hydroxyl groups at position-3″ of ring B for the synthesis of six-member rings. For example, 2,3-dimethoxy-2′-hydroxychalcone and 3-hydroxy-2′-hydroxychalcone were transformed to flavanones by *A. alliaceus* (*8*). The present work suggests that phenolic hydroxyl groups at position-4″ favor the synthesis of five-membered ring aurones such as **10**.

Effects of Cytochrome P450 Inhibitors on Chalcone Conversions. Although both *A. alliaceus* and aureusidin synthase catalyzed aurone synthesis from 4″-monohydroxy and 3″,4″-dihydroxy chalcones, the microbial process apparently involved two steps while only one is involved in the plant (*3*, *5*, *7*). In both biotransformations, the presence of a 3″,4″-dihydroxy B-ring chalcone (**7**) was crucial for the cyclization reaction to occur. However, unlike the plant process, *A. alliaceus* accumulated **7** as an intermediate product. This difference indicated that at least two *A. alliaceus* enzymes could have participated in the biotransformation sequence.

Table 1. Effects of Inhibitors on the Conversion of **1** to **7** and **10** by *A. alliaceus* UI315[a]

inhibitor concn (mM)	butein (**7**) (mg/L)	sulfuretin (**10**) (mg/L)
0	68.5 ± 3.53	22 ± 1.8
SKF525A		
8	20 ± 1.4	12.79 ± 0.92
17	13 ± 1.04	6.5 ± 0.53
PTC		
1.5	87 ± 4.35	12.17 ± 0.54
15	23 ± 1.31	3.5 ± 0.18

[a] Each result represents the mean of three independent analyses.

Previous efforts to directly demonstrate cytochrome P450 activities in *A. alliaceus* were unsuccessful (*8*). In fungi, the direct demonstration of cytochrome P450 catalysis in CFEs is rare and rendered difficult because of instability of these and related proteins (*22*). The indirect involvement of P450 enzymes in chalcone cyclizations to flavanones by *A. alliaceus* whole cells was shown with P450 inhibitors SKF525A and metyrapone (*8*). Preliminary experiments showed that both inhibitors blocked the synthesis of metabolites of **1** with *A. alliaceus* cultures. However, the high concentrations of metyrapone required in order to demonstrate inhibition greatly reduced the conversions of **1** to **7** and **10** and may have been toxic to *A. alliaceus*. Thus, SKF525A was examined more closely for its ability to inhibit the conversion of **1** to products.

Whole cell reactions containing **1** or **1** plus two different concentrations of SKF525A were evaluated by HPLC. As shown in **Table 1**, controls containing only organism and substrate but no inhibitor converted **1** into **7** and **10** in 65 and 22 mg/L, respectively. With 8 mM SKF 525A, yields of **7** and **10** were reduced to 20 and 14 mg/L, respectively while with 17 mM SKF525A gave the same compounds in 13 and 6.5 mg/L, respectively. The results suggest that SKF525A was more effective in blocking the hydroxylation of **1** to **7** than in blocking the cyclization of **7** to aurone **10**.

PTC is a catechol oxidase (polyphenol oxidase) inhibitor that interacts with the binuclear copper center at the enzyme active site where it competes for binding with *o*-diphenolic substrates (*6*, *23*). *A. alliaceus* cultures were incubated in the presence of **1** or **1** plus PTC and analyzed by HPLC (**Table 1**). With 1.5 mM PTC, yields of **10** from **1** were reduced to 12 vs 22 mg/L in controls. At the same time, yields of **7** were higher at 87 vs 68.5 mg/L in controls. With 15 mM PTC, the hydroxylation of **1** to **7** was reduced to 23 mg/L while levels of 10 were decreased to 3.5 mg/L. Although both hydroxylation and cyclization processes were blocked by PTC, the cyclization reaction was blocked to a greater degree. The slight increase in butein (**7**) levels observed in reactions containing 1.5 mM PTC suggests the accumulation of this intermediate while the cyclization of **7** to **10** was blocked.

These inhibition results pointed to the involvement of two different enzymes in the biotransformation sequence. Because C-3′ hydroxylation of **1** to **7** was inhibited by SKF525A, this reaction step is likely catalyzed by a cytochrome P450 enzyme system. The enzyme catalyzing the cyclization reaction was blocked more by PTC. PTC belongs to the thionosulfur-containing inhibitors that selectively blocked aureusidin synthase

and potato polyphenol oxidase (*6*, *23*). PTC may inhibit cytochrome P450 (*24*, *25*), and SKF 525A and metyrapone can block the activities of other metalloenzymes (*24*, *25*). Although no cytochrome P450 enzyme activity could be detected in *A. alliaceus* CFEs, it was possible to partially purify *A. alliaceus* catechol oxidase and to examine whether this enzyme could catalyze the cyclization reaction.

In earlier work with *Aspergillus nidulans*, others showed that an active, crude tyrosinase preparation could be reproducibly demonstrated only by removal of an inhibitory protein by ion exchange chromatography (*10*). Similarly, catechol oxidase activity in crude *A. alliaceus* CFEs was low, erratic, and frequently could not be detected. When *A. alliaceus* CFEs were subjected to DEAE cellulose DE52 column chromatography, enzyme activity was adsorbed at pH 8.3 and was eluted only when sodium chloride concentrations approached 1 M. In this way, 26 mU/mg protein of catechol oxidase activity were recovered. The low activity and the instability of the partially purified catechol oxidase to various concentration methods rendered the total purification of the protein difficult. The low protein and enzyme concentration and the self-destructive tendencies of catechol oxidases (*26*) could be responsible for the instability of the partially purified enzyme. Proteolysis of the active enzyme preparation with trypsin destroyed activity vs L-DOPA and in the conversion of **7** to **10**. Both L-DOPA oxidation and aurone formation were reduced by about 30% with 2 mM PTC, confirming the involvement of this enzyme in the cyclization process.

The partially purified enzyme was specific for diphenols such as L-DOPA and **7**. L-Tyrosine and **1** were not substrates for the enzyme. These results indicated that the partially purified *A. alliaceus* enzyme was a catechol oxidase. The optimal temperature for **7** transformation could not be measured because of the presence of contaminating enzymes in the partially purified enzyme that are active at 50 °C and transform **10** to more polar products. The optimal temperature for L-DOPA oxidation was 50 °C. Aurone synthetic activity was resistant to boiling temperatures and 4 M urea. *A. alliaceus* catechol oxidase retained 10% of its activity after being boiled in water for 10 min or in buffer containing 4 M urea.

Partially purified *A. alliaceus* catechol oxidase was used to catalyze the formation of aurone **10** from **7**. Reaction mixtures containing enzyme, and **1** or **7**, were analyzed by HPLC, and the results are shown in **Figure 2**. Results show that **1** was not a substrate (**Figure 2B**), while **10** was obtained from **7** (**Figure 2A**). To demonstrate that aurone biosynthetic activity was enzyme and not chemically mediated, a trypsinized sample of enzyme was inactived and found unable to convert **7** to **10**. Incubation of crude catechol oxidase with 2 mM PTC decreased conversion of **7** to **10** by 40% and activity vs L-DOPA by 30%.

Reactions with structurally different catechols butein and L-DOPA indicate that *A. alliaceus* catechol oxidase has a relatively broad substrate specificity. Plant-derived aureusidin synthase is specific for chalcones, with no activity vs L-DOPA (*6*). Catechol oxidases occur widely in animals, insects, plants, fungi, and bacteria. The biological roles of these enzymes are quite broad where they are implicated in the biosyntheses of melanin, lignins, bioflavonoids, tannins, adrenaline, and other compounds (*27*). Physiologically, the function of catechol oxidases in fungi is not yet fully understood (*27*).

Our results support a two-step process in *A. alliaceus* for aurone synthesis much like the plant biosynthetic pathway suggested by Nakayama (*6*) (**Figure 3**). In the first step, cytochrome P450 hydroxylates **1** at position-3 giving **7**, while

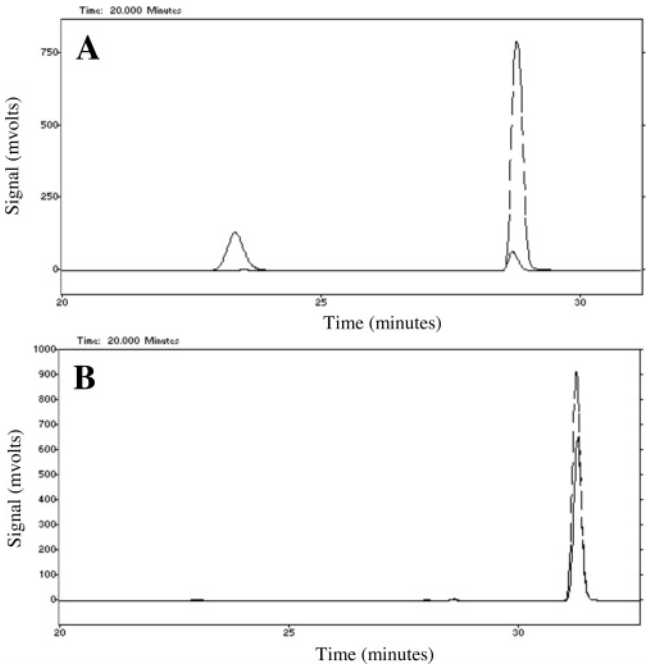

Figure 2. HPLC chromatograms from 5 h in vitro reactions with *A. alliaceus* catechol oxidase. (**A**) Chromatogram showing **10** (23 min) obtained from **7** (29 min). (**B**) Chromatogram showing no products obtained from **1** (32 min) as substrate.

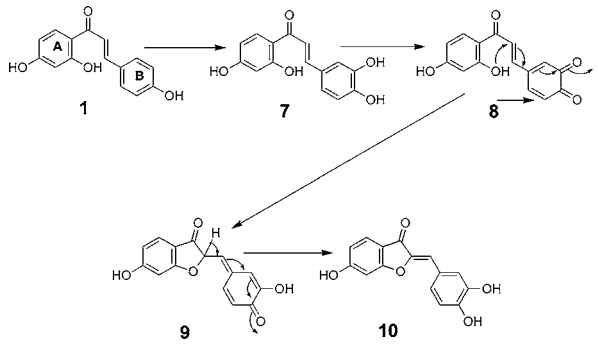

Figure 3. Proposed pathway for *A. alliaceus* biotransformation of **1** to butein (**7**) and aurone sulfuretin (**10**). Steps shown from **8** to **9** and **9** to **10** are considered to be nonenzymatic.

the ring-forming step that produces the aurone is catalyzed by a catechol oxidase, likely via an *o*-quinone intermediate (**8**). Intramolecular nucleophilic attack of the double bond by the 2-hydroxyl group would give **9** (unisolated), which then undergoes prototopic tautomerism to give **10** (*3*).

Although the precise metabolic roles of cytochromes P450 and catechol oxidase are unclear in *A. alliaceus*, our results show that this fungus is capable of mimicking plants in the synthesis of flavanoids (*8*) and aurones. The formation of one or another is dependent upon functionalities of the chalcone substrates used.

ABBREVIATIONS USED

PTC, phenylthiocarbamide; SKF 525A, 2-diethyl-aminoethyl-2,2-diphenylvalerate; L-DOPA, 3-hydroxy-L-tyrosine; CFE, cell free extract.

LITERATURE CITED

(1) Dixon, R.; Steele, C. Flavonoids and isoflavonoids a gold mine for metabolic engineering. *Trends Plant Sci*. **1999**, *4*, 394−400.
(2) Pietta, P.-G. Flavonoids as antioxidants. *J. Nat. Prod*. **2000**, *63*, 1035−1042.

(3) Nakayama, T. J. Enzymology of aurone biosynthesis. *Biosci. Bioeng*. **2002**, *94*, 487−491.
(4) Rathmell, W. G.; Bendall, D. S. The peroxidase-catalyzed oxidation of a chalcone and its possible physiological significance. *Biochem. J*. **1972**, *127*, 125−132.
(5) Nakayama, T.; Yonekura-Sakakibara, K.; Sato, T.; Kikichi, S.; Fukuki, Y.; Fukuchi-Mizutani, M.; Ueda, T.; Nakao, M.; Tanaka, Y.; Kusumi, T.; Nishino, T. Aureusidin synthase a polyphenol oxidase homologue responsible for flower coloration. *Science* **2000**, *290*, 1163−1166.
(6) Nakayama, T.; Sato, T.; Fukui, Y.; Yonekura-Sakakibara, K.; Hayashi, H.; Tanaka, Y.; Kusumi, T.; Nishino, T. Specific analysis and mechanism of aurone synthesis catalyzed by aureusidin synthase, a polyphenol oxidase homologue responsible for flower coloration. *FEBS Lett.* **2001**, *499*, 107−111.
(7) Sato, T.; Nakayama, T.; Kikuchi, S.; Fukui, Y.; Yonekura-Sakakibara, K.; Ueda, T.; Nishino, T.; Tanaka, Y.; Kusumi, T. Enzymatic formation of aurones in the extracts of yellow snapdragons flowers. *Plant Sci*. **2001**, *160*, 229−236.
(8) Sanchez-Gonzalez, M.; Rosazza, J. P. N. Microbial transformations of chalcones: Hydroxylations, *O*-demethylation, and cyclization to flavanones. *J. Nat. Prod*. **2004**, *67*, 553−558.
(9) Kirchner, G. J. Detection of colorless compounds. In *Techniques of Organic Chemistry: Thin Layer Chromatography*; Perry, E. S., Weissberger, A., Eds.; Interscience Publishers: New York, 1967; Vol. XII, pp 147−184.
(10) Bull, A. T.; Carter, B. L. A. The isolation of tyrosinase from *Aspergillus nidulans*, its kinetic and molecular properties and some consideration of its activity *in vivo*. *J. Gen. Microbiol*. **1973**, *75*, 61−73.
(11) Baldwin, M.; Ross, P.; Pate, J.; Tyeklar, Z.; Karlin, K.; Solomon, E. Spectroscopic and theoretical studies of an end-on peroxide-bridged coupled binuclear copper(II) model complex of relevance to the active sites in hemocyanin and tyrosinase. *J. Am. Chem. Soc*. **1991**, *113*, 8671−8679.
(12) Solano, F.; Garcia, E.; Perez de Egea, E.; Sanchez-Amat, A. Isolation and characterization of strain MMB-1 (CECT 4803), a novel melanogenic marine bacterium. *Appl. Environ. Microbiol*. **1997**, *63*, 3499−3506.
(13) Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **1976**, *72*, 248−254.
(14) Chien, M. M.; Rosazza, J. P. N. Microbial transformations of natural antitumor agents. VIII. Formation of 8- and 9-hydroxyellipticines. *Drug Metab. Dispos.* **1979**, *7*, 211−214.
(15) Chien, M. M.; Rosazza, J. P. N. Microbial transformations of natural antitumor agents: Use of solubilizing agents to improve yields of hydroxylated ellipticines. *Appl. Environ. Mirobiol.* **1980**, *40*, 741−745.
(16) Rosazza, J. P. N.; Kammer, M.; Youel, L. Microbial models of mammalian metabolism O-demethylations of papaverine. *Xenobiotica* **1977**, *7*, 133−143.
(17) Williamson, J.; Van Orden, D. E.; Rosazza, J. P. N. Microbiological hydroxylation of estradiol: Formation of 2- and 4-hydroxyestradiol by *Aspergillus alliaceus*. *Appl. Environ. Microbiol*. **1985**, *49*, 563−567.
(18) Williamson, J.; Van Orden, D. E.; Rosazza, J. P. N. O dealkylation and aliphatic and aromatic hydroxylation of 3-methoxy-17 beta-estradiol by Aspergillus alliaceus. *Appl. Environ. Microbiol*. **1989**, *55*, 3029−3031.
(19) Lin, C. N.; Lee, T. H.; Hsu, M. F.; Wang, J. P.; Ko, F. N.; Teng, C. M. 2′,5′-Dihydroxychalcone as a potent chemical mediator and cyclooxygenase inhibitor. *J. Pharm. Pharmacol*. **1997**, *49*, 530−536.
(20) Agrawal, P. K.; Bansal, M. Other flavonoids. In *Studies in Organic Chemistry: Carbon-13 NMR of Flavonoids*; Agrawal, P. K., Ed.; Elsevier: New York, 1989; Vol. 39, pp 236−282.
(21) Huke, M.; Görlitzer, K. Polyhydroxyaurones. 3. Systematic characterization by UV-spectroscopy and thin-layer chromatography. *Arch. Pharm*. **1969**, *6*, 423−434.
(22) van den Brink, H. J. M.; van Gorcom, R. F. M.; van den Hondel, C. A. M. J.; Punt, P. J. Cytochrome P450 enzyme systems in fungi. *Fungal Genet. Biol*. **1998**, *23*, 1−17.
(23) Klabunde, T.; Eicken, C.; Sacchettini, J. C.; Krebs, B. Crystal structure of a plant catechol oxidase containing a dicopper center. *Nat. Struct. Biol.* **1998**, *5*, 1084−1090.
(24) Testa, B.; Jenner, P. Inhibitors of Cytochrome P-450s and their mechanism of action. *Drug Metab. Dispos.* **1981**, *12*, 1−117.
(25) Zollner, H. *Handbook of Enzyme Inhibitors*; Wiley-VCH: Germany, 1991; Part A, pp 940−942.
(26) Kuwabara, T.; Katoh, Y. Involvement of the binuclear copper site in the proteolytic activity of polyphenol oxidase. *Plant Cell Physiol*. **1999**, *40*, 1029−1035.
(27) van Gelder, C. W. G.; Flurkey, W. H.; Wichers, H. J. Sequence and structural features of plant and fungal tyrosinases. *Phytochemistry* **1997**, *45*, 1309−1323.

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