Hydrolytic Polyketide Shortening by Ayg1p, a Novel Enzyme Involved in Fungal Melanin Biosynthesis*

The pentaketide 1,3,6,8-tetrahydroxynaphthalene (T4HN) is a key precursor of 1,8-dihydroxynaphthalene-melanin, an important virulence factor in pathogenic fungi, where T4HN is believed to be the direct product of pentaketide synthases. We showed recently the involvement of a novel protein, Ayg1p, in the formation of T4HN from the heptaketide precursor YWA1 in Aspergillus fumigatus. To investigate the mechanism of its enzymatic function, Ayg1p was purified from an Aspergillus oryzae strain that overexpressed the ayg1 gene. The Ayg1p converted the naphthopyrone YWA1 to T4HN with a release of the acetoacetic acid. Although Ayg1p does not show significant homology with known enzymes, a serine protease-type hydrolytic motif is present in its sequence, and serine-specific inhibitors strongly inhibited the activity. To identify its catalytic residues, site-directed Ayg1p mutants were expressed in Escherichia coli, and their enzyme activities were examined. The single substitution mutations S257A, D352A, and H380A resulted in a complete loss of enzyme activity in Ayg1p. These results indicated that the catalytic triad Asp352-His380-Ser257 constituted the active-site of Ayg1p. From a Dixon plot analysis, 2-acetyl-1,3,6,8-tetrahydroxynaphthalene was found to be a strong mixed-type inhibitor, suggesting the involvement of an acyl-enzyme intermediate. These studies support the mechanism in which the Ser257 at the active site functions as a nucleophile to attack the YWA1 side-chain 1'-carbonyl and cleave the carbon-carbon bond between the naphthalene ring and the side chain. Acetoacetic acid is subsequently released from the Ser257-O-acetoacylated Ayg1p by hydrolysis. An enzyme with activity similar to Ayg1p in melanin biosynthesis has not been reported in any other organism.

Aspergillus fumigatus, a ubiquitous fungus, causes allergies, noninvasive colonization, or life-threatening invasive pulmonary aspergillosis (1, 2). Its bluish green conidial pigmentation was reported to be an important virulence factor that resulted from polymerization of a pentaketide similar to 1,8-dihydroxynaphthalene (DHN)-melanin synthesis in black fungi (3, 4). In brown and black fungi, DHN is a pentaketide biosynthesized from the precursor 1,3,6,8-tetrahydroxynaphthalene (T4HN) by successive reduction and dehydration (5). T4HN was determined to be a product of the pentaketide synthase PKS1 in the black fungus Colletotrichum lagenarium (6, 7). In A. fumigatus, genetic and biochemical investigations showed that its conidial polyketide melanin biosynthesis required a six-gene cluster that included the genes abr1, abr2, alb1, arp1, arp2, and ayg1 (4). The products of these genes are referred to as Abr1p, Abr2p, Alb1p, Arp1p, Arp2p, and Ayg1p. A homology search suggested that arp2 and arp1 code for T4HN reductase and scytalone dehydratase, respectively, and that abr1 and abr2 encode multi-copper oxidases. Heterologous expression of alb1 in Aspergillus oryzae demonstrated that the Alb1p polyketide synthase synthesized the heptaketide naphthopyrone YWA1 (8), which was identified as a precursor of the green spore pigment in Aspergillus nidulans (9). The ayg1 gene in the biosynthetic gene cluster, however, showed no significant homology with known genes in the data base.

Genetic analyses suggested that Ayg1p catalyzes the biosynthetic step subsequent to the Alb1p polyketide synthase reaction. In our previous study, we reported that the Ayg1p cell-free extracts prepared from an A. oryzae transformed with pTA-ayg1 could convert the heptaketide YWA1 to the pentaketide T4HN (10). This suggested that the novel protein Ayg1p is involved in T4HN formation by chain-length shortening of a heptaketide YWA1 in A. fumigatus. (Fig. 1)

In this report, we purified and characterized the Ayg1p from the fungal transformant that overexpressed the protein. Using the homogeneously purified Ayg1p, we identified acetoacetic acid as the in vitro reaction product derived from the YWA1 side chain. This result indicated the hydrolytic cleavage of the carbon-carbon bond between the naphthalene nucleus and the side-chain 1'-carbonyl of the YWA1 open form. From the kinetic studies and site-directed mutagenesis analyses, we identified the catalytic domain of Ayg1p as being responsible for the enzymatic activity and proposed an unusual mechanism of hydrolytic polyketide shortening for T4HN formation.

**EXPERIMENTAL PROCEDURES**

Materials—YWA1 was obtained from the culture extract of an A. oryzae transformant with pTA-alb1 (11). 2-Acetyl-1,3,6,8-tetrahy-
droxynaphthalene (ATHN) was prepared from the culture extract of an A. oryzae transformed with pTA-sw-B (11). Benzamidine and 3,4-dichloroisoucainum were purchased from Sigma. All other reagents were purchased from Wako (Tokyo, Japan).

**Ayg1p Enzyme Assay—**Ayg1p enzyme activity was assayed spectrophotometrically as follows. Fifty microliters of 1 mM YWA1 dissolved in ethylene glycol monomethyl ether and 50 μl of the enzyme solution were added to 900 μl of degassed assay buffer (50 mM potassium phosphate buffer, pH 6.5). The decrease of A_{406} was then monitored at 30 °C. Reaction velocities were calculated based on the A_{406} molar absorption coefficient of YWA1 (ε = 1.1 × 10⁴).

**Purification of Ayg1p—**All purification procedures were carried out at 4 °C. Unless stated otherwise, 20 mM Tris-HCl buffer, pH 7.5, was used throughout the purification. For the purification of Ayg1p, 270 g of induction culture mycelia of the A. oryzae strain transformed with pTA-ayg1 (10) were homogenized with a Waring blender in 600 ml of the buffer containing 100 mM benzamidine. One-tenth weight of Polyclar AT (Gokyo Sangyo, Tokyo, Japan) was added to the homogenate and placed on ice for 30 min. After filtering through four-layered gauze, the homogenate was centrifuged at 10,000 × g for 20 min. The supernatant was used as a crude extract for further purification. Precipitates between 30 and 80% ammonium sulfate saturation were dissolved in the buffer and dialyzed overnight against 5 liters of the buffer containing 100 μM benzamidine and 1.1 mM ammonium sulfate. After centrifugation, the supernatant was applied onto a Butyl-Sepharose 4 Fast Flow (Amersham Biosciences) column (2.2 × 6 cm) equilibrated with the buffer containing 1.1 mM ammonium sulfate. The column was then washed with the equilibration buffer. Active Ayg1p was eluted with the buffer containing 0.8 M ammonium sulfate. The active fraction pooled (92 ml) was dialyzed against 5 liters of the buffer containing 100 μM benzamidine and then concentrated by ultrafiltration using Centriprep YM-30 (Amicon). Ayg1p was purified on Mono Q HR 10/10 anion exchange column (Amersham Biosciences) equilibrated with the buffer using the AKTA explorer system (Amersham Biosciences). Ayg1p was eluted at ~70 m2 NaCl. The pooled active fraction was concentrated with Centiprep YM-30 and purified by gel filtration on Superose 12 HR 10/30 column (Amersham Biosciences) equilibrated with the buffer containing 0.2 M NaCl. From 270 g of mycelia, 15 mg of the purified Ayg1p was obtained with 10% recovery of activity. The result of purification is summarized in Table I.

**Derivatization of Acetoacetic Acid—**Fifty microliters of substrate YWA1 solution (2 mM dissolved in ethylene glycol monomethyl ether) and 50 μl of the purified Ayg1p (0.4 nmol) were added to 900 μl of 50 mM potassium phosphate buffer, pH 6.5. The reaction mixture was incubated at 30 °C for 20 min. Five mg of O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (12) and 1 ml of H₂O₂ were then added to the reaction mixture. The reaction mixture was adjusted to pH 2–3 with 0.1 M H₂SO₄ and left at room temperature for 2 h. After extraction with n-hexane, the product was desiccated with N₂ gas and redisolved in 50 μl of methanol.

**Analysis by Liquid Chromatography-coupled Mass Spectrometry (LC-MS)—**The methanol solution of the Ayg1p reaction product derivatized with O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine was analyzed by an ion trap mass spectrometer (LC/ThermoQUEST) with atmospheric pressure chemical ionization (APCI) coupled to a TSK-Gel ODS-80Ts column (Toosoh, Japan; 4.6 × 15 mm). The HPLC conditions were a gradient system of 25–100% (v/v) methanol in water (each containing 0.5% (v/v) acetic acid) within 30 min at a flow rate of 0.8 ml/min at 40 °C.

**HPLC Analysis of Enzymatic Products from Substrate Analogs—**To investigate the substrate specificity of Ayg1p, the aryl alkyl ketone compounds were reacted with Ayg1p under standard assay conditions. After incubation with the purified Ayg1p at 30 °C for 15 min, 50 μl of the reaction mixture was analyzed by HPLC on an ODS-80Ts column (4.6 × 150 mm) with a gradient system of 5–100% (v/v) acetonitrile in water (each containing 0.5% (v/v) acetic acid) within 30 min at a flow rate of 0.8 ml/min at 40 °C. The formation of T4HN and flavinol was analyzed by LC-APCI-MS coupled to an ODS-80Ts column (4.6 × 150 mm) with the same gradient conditions.

**Expression of the Wild-type and Mutated Ayg1 Genes in Escherichia coli—**The full-length ayg1 cDNA was amplified by AmpliTaq Gold DNA polymerase (Applied Biosystems) from the plasmid pGC31, which contains cDNA lacking the N-terminal 15 bp of the ayg1 coding sequence, using the forward primer 5′-ATCATGGGGCAGCCACCCGCCCCCA-CACCCCAACCCGCGGCTCGGTATGACGACGAAAGCCACCG-TGATCTCTGAGACAGTCTC-3′ and the reverse primer 5′-TCAGA-TCTGCTCCTCCTC-3′. The Ncol site is tagged, and the enterokinase recognition amino acid sequence (DDDDK) were designed in the forward primer, and the BglII site was in the reverse primer. The resulting 1.3-kbp PCR product was purified by the Wizard PCR Prep kit (Promega) and subcloned with pT7 Blue T-vector (Novagen). After sequence confirmation, the correct insert was cleaved by NcoI and BglII digestion and ligated into NcoI/BamH1-digested pET-3d to construct pET-ayg1Ht. E. coli BL21(DE3)pLysE transformed with pET-ayg1Ht was grown at 37 °C to an A₆₀₀ of 0.5. The culture was cooled to 30 °C, and expression was induced with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside by incubating at 30 °C for 3 h. The cells were harvested by centrifugation (10,000 × g for 10 min) and resuspended in ice-cold 20 mM Tris-HCl buffer, pH 7.5, containing 100 μg/ml lysozyme and incubated at 30 °C for 15 min. After sonication, the soluble fraction was obtained by centrifugation (14,000 × g for 10 min) which showed YWA1 conversion activity. No activity was detected in the control preparation obtained from E. coli BL21(DE3)pLySE harboring.

**TABLE I**

| Purification step for Ayg1p | Volume | Protein | Total activity | Specific activity | Recovery |
|-----------------------------|--------|---------|----------------|------------------|---------|
| Crude extract              | 600    | 5200    | 300,000        | 58               | 100     |
| Amm. Sulf. Ppt. (30–80%)    | 100    | 1460    | 200,000        | 140              | 66      |
| Butyl-Sepharose            | 2.8    | 100     | 110,000        | 1100             | 37      |
| Mono Q                     | 0.98   | 22      | 39,000         | 1500             | 13      |
| Superose 12                | 0.13   | 15      | 31,000         | 2100             | 10      |

*Ammonium sulfate precipitation.
the pET-3d empty vector. Among the induction conditions tested (26–30 °C for 3–16 h), the highest specific activity was observed when induction was carried out at 30 °C for 3 h.

Mutagenesis was performed with the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions on the plasmid pET-ayg1Ht with a pair of primers as follows (mutated codons are underlined): T185V (5’-CATCGTGCTATGCTGGGCCTAGCAGGTA-3’ and 5’-TACCGCTTAGCCACCATGATCCGACGACGATG-3’); E212Q (5’-CTGTCGTCGCCCAGATCCCCGGC-3’ and its antisense); S257A (5’-GGTGTGGGGACTGGCGGCGGGTTACT-3’ and its antisense); R271L (5’-CCGCGATACGCACCTGGACCGGTGCTGC-3’ and 5’-GCAGCCGGTCCAGGTGCGTATGCGC-3’); D352A (5’-TGGGGTCGACGCTGGCGTCGTGC-3’ and its antisense); H380A (5’-CTACAAGGGGGCTCCGGCAATGGGATATCCCAACAGT-3’ and its antisense). Mutations were verified by sequencing.

The plasmids containing mutated ayg1 genes were transformed into E. coli BL21(DE3)LysE, and mutant ayg1 genes were expressed in the same way as described for the expression of the wild-type ayg1. Purification of these wild-type and mutated Ayg1p was carried out by Ni2+-affinity using Probond resin (Invitrogen) or a nickel-nitrilotriacetic acid spin column (Qiagen) following the manufacturer’s instructions. Further purification of the wild-type and S257A-mutated Ayg1p were performed on Mono Q HR 5/5 anion exchange column on the A¨KTA Explorer system.

Other Methods—Protein concentrations were determined by the Bradford method (13). The concentration of purified enzyme was determined by the UV absorption method. SDS-PAGE was performed on 11% acrylamide gels by the method of Laemmli (14). Gels were stained with GelCode Blue Stain Reagent (Pierce). N-terminal amino acid sequencing of the purified Ayg1p was carried out by APRO Life Science Institute, Inc. (Tokushima, Japan). CD spectra were recorded by Shimadzu AVIV CD 202.

RESULTS

Purification of Ayg1p—A cell-free extract was prepared from 270 g of the mycelia of A. oryzae transformed with pTA-ayg1, which had been cultured in a starch-containing induction medium for 3 days. Ayg1p was purified in four steps, i.e. ammonium sulfate fractionation, Butyl-Sepharose hydrophobic chromatography, Mono Q anion exchange chromatography, and Superose 12 size exclusion chromatography. Fifteen milligrams of purified Ayg1p was obtained from 270 g mycelia as summarized in Table I.

The purified Ayg1p eluted as a single peak at 90 kDa on Superose 12 chromatography and appeared as a single band on SDS-PAGE at 45 kDa as shown in Fig. 2, indicating that Ayg1p is a dimer in its native form. N-terminal amino acid sequence analysis revealed a single sequence, Pro-Arg-Trp-Ile-Leu, which is identical with the N-terminal sequence of Ayg1p as deduced from its gene sequence, excluding the initial methionine. These results clearly proved the homogeneity and identity of the purified Ayg1p.

Identification of Reaction Products—In a previous paper we reported the formation of the pentaketide T4HN from the heptaketide YWA1 by reaction with the crude cell-free extract containing Ayg1p (10). To definitely confirm the reaction products, YWA1 was reacted with the homogeneously purified Ayg1p. As shown in Fig. 3, HPLC analysis of the reaction mixture showed two products with retention times of 15.4 and 18.3 min. These products were identified as T4HN and flavio- llin, respectively, by LC-MS analysis (15). Flaviolin was formed by the autooxidation of T4HN.

As T4HN was identified to be the product from YWA1, diketide acetoacetic acid was proposed to be the another prod-

FIG. 2. SDS-PAGE analysis of Ayg1p purification fractions. Lane 1, crude extract; lane 2, ammonium sulfate precipitation; lane 3, Butyl-Sepharose; lane 4, Mono Q; lane 5, Superose 12. Arrow indicates the position of the 45-kDa protein.

FIG. 3. HPLC analysis of the Ayg1p reaction product. HPLC analysis of the Ayg1p reaction products from YWA1. I, incubation for 0 min; II, incubation for 15 min. The peaks at retention times of 15.4, 18.3, and 20.7 min are T4HN, flavio- llin, and YWA1, respectively, as determined by LC-MS analysis.

FIG. 4. Derivatization of acetoacetic acid to acetoacetic acid oxime.
FIG. 5. LC-APCI-MS analysis of the acetoacetic acid oxime. A, selected ion monitoring at m/z 298. I, enzyme reaction for 0 min; II, enzyme reaction for 20 min; III, standard acetoacetic acid oxime. B, MS/MS analysis of the precursor ion at m/z 298 of the peak at a retention time of 27.8 min. I, the enzyme reaction for 20 min; II, standard acetoacetic acid oxime.
uct from the side chain of YWA1. Because of the instability of acetoacetic acid to heat and/or acid, identification of acetoacetic acid was carried out after derivatization. It is known that 3-keto acids can react with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride to give stable oxime derivatives with benzyl chromophore (12). The Ayg1p reaction mixture was treated with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine, and the oxime product formed was analyzed by LC-MS. Mass chromatogram analysis at m/z 298, which corresponds to the [M+H]⁺ of acetoacetic acid oxime, showed a single peak at a retention time of 27.8 min. MS/MS analysis of this parent ion m/z 298 gave ions of m/z 284 ([M-COOH+CH₂OH]⁺), m/z 270 ([M-COOH+H₂O]⁺), and m/z 252 ([M-COOH]⁺). Because these LC-MS/MS data were identical to those of standard acetoacetic acid and no m/z 298 ion was detected in the reaction mixture at zero time, we concluded that acetoacetic acid is formed by hydrolytic cleavage of the YWA1 side chain. (Figs. 4 and 5)

**Effect of Chemical Reagents on Ayg1p Activity**—The effect of chemical reagents on Ayg1p activity was examined as shown in Table II. The metal chelating reagent EDTA did not affect the enzyme activity of Ayg1p. Also, sulphydryl reagents like iodoacetic acid and N-ethylmaleimide showed no effect. On the other hand, serine protease inhibitors strongly inhibited Ayg1p activity. Especially, 3,4-dichloroisocoumarin (16) showed a 32% inhibition of Ayg1p activity even at 2 μM. These results suggested that serine residues have a critical role in Ayg1p reaction as proposed previously (10).

**Substrate Specificity**—The heptaketide naphtopyrone YWA1 is a natural substrate of Ayg1p. Because YWA2, a dehydrated derivative of YWA1, did not react with Ayg1p, Ayg1p is considered to react with the side-chain open form of YWA1. Thus, aryl alkyl ketone compounds were tested for their reactivity with Ayg1p. (Fig. 6) Of the compounds tested, only ATHN reacted with Ayg1p. The conversion of norsolorinic acid, ethyl coumarin-3-carboxylate, phenyl butanedione, and acetoephones could not be catalyzed by Ayg1p. Although the reactivity of ATHN with Ayg1p was only 5% of the reactivity of YWA1, T4HN was identified by HPLC and LC-MS analysis as the product from ATHN (data not shown).

**Kinetic Analysis of Ayg1p Reaction**—Ayg1p reaction velocities were determined using 2.5 pmol of purified Ayg1p in the standard assay condition with variable concentrations of YWA1 from 5 to 100 μM. From the Eadie-Hofstee plot of these data, Kₘ and kₗ values for YWA1 were determined to be 44 μM and 4.6 s⁻¹, respectively. From the substrate specificity studies, ATHN was found to be a substrate of Ayg1p, although its reactivity was very low. In contrast, ATHN was found to be a strong inhibitor against the conversion of YWA1 by Ayg1p. Dixon plot analysis of ATHN inhibition kinetics indicated that ATHN acts as a mixed-type inhibitor with a Kᵢ value of 1.7 μM. (Fig. 7)

**Site-directed Mutagenesis**—Ayg1p converts YWA1 to T4HN and acetoacetic acid formally by adding one molecule of H₂O. Although Ayg1p does not show significant homology with functionally characterized enzymes in the data base, Ayg1p has a serine protease-type motif (-kmVVwGlS257AGGYyA-) that suggests hydrolytic cleavage of the YWA1 side chain by Ayg1p. A conserved domain search indicated the presence of a dipeptidyl aminopeptidase/acylaminoacyl-peptidase domain (DAP2, COG1506) and/or a esterase/lipase domain (Aes, COG0657) (www.ncbi.nlm.nih.gov:80/Structure/cdd/cdd.shtml). Alignment of Ayg1p with Aes family proteins indicated the amino acid residues around Ser²⁵⁷ to be highly conserved. Alignment with DAP2 family proteins also indicated the conserved Asp²⁵² and His²⁸⁰ near the C terminus of Ayg1p. Of these conserved amino acid residues, Thr²⁸⁵, Glu²³², Ser²⁵⁷, Arg²⁷¹, Asp²⁵², and His²⁸⁰ were chosen as target amino acids to introduce mutations. The wild-type Ayg1p with an His₉ tag expressed in E. coli showed YWA1 conversion activity to have kinetics similar to those of Ayg1p expressed in A. oryzae. The S2⁵⁷A-mutated

![Fig. 6. Compounds tested as the substrate of Ayg1p.](image-url)
Ayg1p completely lost its YWA1-converting activity. Inactivation of the S257A Ayg1p did not result from secondary structure alteration, because the CD spectra of Mono Q-purified S257A and wild-type Ayg1p were identical (data not shown). The Ser257, therefore, is considered to be a critical residue of the active site involved in the Ayg1p reaction. In addition, D352A- and H380A-mutated Ayg1p also had no detectable activities. In contrast, T185V- and R271V-mutated Ayg1p retained ~30% activities compared with the wild-type Ayg1p. (Fig. 8)

**DISCUSSION**

The novel enzyme Ayg1p was purified and characterized for analysis of its catalytic mechanism. Ayg1p catalyzes the carbon-carbon bond cleavage of the heptaketide naphthopyrone YWA1 to release the pentaketide T4HN and the diketide acetoadic acid. In polyketide biosynthesis, basic carbon skeletons of polyketides are generally determined by polyketide synthases alone. Although tailoring enzymes modify the basic skeletons by hydroxylation, alkylation, oxidation, etc., such modifications are not so drastic, and the basic carbon skeletons are usually retained in their final products (17). Thus, the polyketide-shortening reaction catalyzed by Ayg1p is highly unusual in polyketide biosynthesis.

The naphthopyrone YWA1 is considered to be a precursor of green spore pigment in aspergilli such as *A. nidulans* (9). T4HN has been established as the precursor of DHN-melanin in black fungi (5). In contrast to these fungi with green or black conidial pigmentation, *A. fumigatus* shows the characteristic bluish green pigmentation. In the biosynthetic gene cluster responsible for conidial pigmentation in *A. fumigatus*, there are two copper oxidase genes, *abr1* and *abr2* (4). If these two oxidases could polymerize DHN for black DHN-melanin and YWA1 for green spore pigments, respectively, *A. fumigatus* could use the single gene cluster for the biosynthesis of two different pigments. Thus, Ayg1p may play a critical role in providing the appropriate substrates required for the two biosynthetic pathways, and the combination of two different pigments may result in bluish green conidia in *A. fumigatus*.

The identification of acetoadic acid as a product in the Ayg1p reaction with YWA1 indicated that the carbon-carbon bond between the naphthalene aromatic ring and the side-chain carbonyl is cleaved hydrolytically. There are only a few enzymes that catalyze the hydrolytic carbon-carbon bond cleav-
These enzymes are classified by the Enzyme Commission on Nomenclature as the hydrolase group (E.C. 3.7.1. X), which includes fumarylacetoacetate hydrolase (18), kynureninase (19), and \( \beta \)-diketone hydrolase (20). Fumarylacetoacetate hydrolase is a typical \( \beta \)-ketio acid hydrolase that converts fumarylacetoacetate to fumarate and acetoacetate. The structure of fumarylacetoacetate hydrolase, which was resolved recently, suggested that the substrate carbonyl carbon atom is attacked by a nucleophilic water molecule that is activated by histidine imidazole as a general base and that the \( \text{Ca}^{2+} \) ion, as a cofactor, serves for substrate recognition (18). Oxo-camphor hydrolase is a \( \beta \)-diketone hydrolase that converts 6-oxo-camphor to \( \text{a} \)-campholinic acid. The enzyme from \textit{Rhodococcus} sp. showed significant homology to the superfamily of enzymes known as crotonases (21). Based on the mechanism of enoyl-CoA hydrolase, a typical crotonase, it was proposed that cleavage of 6-oxo-camphor carbon-carbon bond proceeds via nucleophilic attack at one carbonyl by a water molecule activated by an acidic residue in the active site of the enzyme. From the structure determined by x-ray crystallography, the Glu\textsuperscript{244} or Asp\textsuperscript{154} residue is considered to be responsible for the activation of a water molecule to attack the substrate’s carbonyl carbon (22).

In addition to the \( \beta \)-diketone hydrolases mentioned above, there is a group of hydrolases that are involved in the degradation of various aromatic compounds including xylene and toluene. These are, e.g. \textit{Pseudomonas putida} TodF (23) and XylF (24), \textit{Pseudomonas azelaica} HbpD (25), \textit{Rhodococcus} sp. EthDs (26) and BphD (27), \textit{Pseudomonas fluorescens} CumD (28, 29), and \textit{E. coli} MhpC (30). Their substrates are ketonic ring fission products of aromatic compounds and are hydrolytically converted to smaller molecules. These proteins belong to the \( \alpha/\beta \)-hydrolase family and they all contain a catalytic triad composed of a serine, an aspartate, and a histidine residue. These residues are borne on a series of loops that are the best conserved structural features of the fold.

MhpC is 2-hydroxy-6-keto-nona-2,4-diene 1,9-dioic acid 5,6-hydrolase involved in the \textit{E. coli} phenylpropionate catabolic pathway, which is a dimeric protein that requires no cofactors for activity. The catalytic mechanism of carbon-carbon bond cleavage by MhpC was studied in detail, and the \( \text{gem} \)-diol intermediate was proposed instead of the acyl enzyme intermediate in which the active site serine works as a general base to assist water in attacking carbonyl (31). MhpC is active at low pH, a behavior that is consistent with the formation of a \( \text{gem} \)-diol intermediate. Flemming \textit{et al.} (31) also suggested that a general base mechanism would be a realistic alternative in \( \alpha/\beta \)-hydrolase family reactions (Fig. 9).

By 3D-PSSM (www.sbg.bio.ic.ac.uk/~3dssm/), a web-based program for protein fold recognition, significant matches indicated that Ayg1p also belongs to the \( \alpha/\beta \)-hydrolase family. In contrast to MhpC, Ayg1p lost its activity at a pH \( \leq 5.5 \) (data not shown). In addition, 3,4-dichloroisocoumarin and ATHN were found to inhibit Ayg1p activity. The former is known as a mechanism-based serine protease inhibitor that forms covalently acylated enzyme (16). The latter, a substrate analog with 5% reactivity of the natural substrate YWA1, could function as an inhibitor against the Ayg1p activity. The former is known as a mechanism-based serine protease inhibitor that forms covalently acylated enzyme (16). The latter, a substrate analog with 5% reactivity of the natural substrate YWA1, could function as an inhibitor against the Ayg1p activity. The former is known as a mechanism-based serine protease inhibitor that forms covalently acylated enzyme (16). The latter, a substrate analog with 5% reactivity of the natural substrate YWA1, could function as an inhibitor against the Ayg1p activity. The former is known as a mechanism-based serine protease inhibitor that forms covalently acylated enzyme (16). The latter, a substrate analog with 5% reactivity of the natural substrate YWA1, could function as an inhibitor against the Ayg1p activity. The former is known as a mechanism-based serine protease inhibitor that forms covalently acylated enzyme (16). The latter, a substrate analog with 5% reactivity of the natural substrate YWA1, could function as an inhibitor against the Ayg1p activity. The former is known as a mechanism-based serine protease inhibitor that forms covalently acylated enzyme (16). The latter, a substrate analog with 5% reactivity of the natural substrate YWA1, could function as an inhibitor against the Ayg1p activity. The former is known as a mechanism-based serine protease inhibitor that forms covalently acylated enzyme (16). The latter, a substrate analog with 5% reactivity of the natural substrate YWA1, could function as an inhibitor against the Ayg1p activity. The former is known as a mechanism-based serine protease inhibitor that forms covalently acylated enzyme (16). The latter, a substrate analog with 5% reactivity of the natural substrate YWA1, could function as an inhibitor against the Ayg1p activity. The former is known as a mechanism-based serine protease inhibitor that forms covalently acylated enzyme (16). The latter, a substrate analog with 5% reactivity of the natural substrate YWA1, could function as an inhibitor against the Ayg1p activity. The former is known as a mechanism-based serine protease inhibitor that forms covalently acylated enzyme (16). The latter, a substrate analog with 5% reactivity of the natural substrate YWA1, could function as an inhibitor against the Ayg1p activity. The former is known as a mechanism-based serine protease inhibitor that forms covalently acylated enzyme (16). The latter, a substrate analog with 5% reactivity of the natural substrate YWA1, could function as an inhibitor against the Ayg1p activity. The former is known as a mechanism-based serine protease inhibitor that forms covalently acylated enzyme (16). The latter, a substrate analog with 5% reactivity of the natural substrate YWA1, could function as an inhibitor against the Ayg1p activity. The former is known as a mechanism-based serine protease inhibitor that forms covalently acylated enzyme (16). The latter, a substrate analog with 5% reactivity of the natural substrate YWA1, could function as an inhibitor against the Ayg1p activity. The former is known as a mechanism-based serine protease inhibitor that forms covalently acylated enzyme (16). The latter, a substrate analog with 5% reactivity of the natural substrate YWA1, could function as an inhibitor against the Ayg1p activity. The former is known as a mechanism-based serine protease inhibitor that forms covalently acylated enzyme (16).
by releasing acetic acid from an acetylated enzyme at a far slower rate than the release of acetoacetic acid from acetoacetylated enzyme. This results in the release of acetic acid from an acetylated enzyme at a far slower rate than the release of acetoacetic acid from acetoacetylated enzyme. These results favor the catalytic mechanism in which the active site serine residue of Ayg1p works not as a general base but as a nucleophile to attack the side-chain 1’-carbonyl of YWA1.

Ser257 hydroxy anion of Ayg1p attacks the site with the side-chain open form, that is, 2-acetoacetyl T4HN. The proposed as follows. Naphthopyrone YWA1 is in equilibrium with the side-chain open form of YWA1. Thus, the mechanism of the Ayg1p reaction is described as follows. Naphthopyrone YWA1 is in equilibrium with the side-chain open form of YWA1. Then, the carbon-carbon bond is cleaved between the naphthalene aromatic ring and the side chain when the oxyanion intermediate backs to carbonyl to give T4HN and acetoacetylated Ayg1p. The acetoacetylated Ayg1p is then hydrolyzed to acetoacetic acid and free Ayg1p as depicted in Fig. 10. As far as we are aware, an enzyme with activity similar to that of Ayg1p has not been reported in melanin biosynthesis in any other organism.

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Hydrolytic Polyketide Shortening by Ayg1p, a Novel Enzyme Involved in Fungal Melanin Biosynthesis

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