Specificity analysis and mechanism of aurone synthesis catalyzed by aureusidin synthase, a polyphenol oxidase homolog responsible for flower coloration

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Abstract Aureusidin synthase, which plays a key role in the yellow coloration of snapdragon flowers, is a homolog of plant polyphenol oxidase (PPO). The enzyme specifically acted on chalcones with a 4-monohydroxy or 3,4-dihydroxy B-ring to produce aurones, for whose production the oxidative cyclization of chalcones must be preceded by 3-oxygenation. However, it exhibited virtually no PPO activity toward non-chalcone phenolics. The enzyme was competitively inhibited by phenylthiourea, a specific PPO inhibitor. These results led us to propose a mechanism of aurone synthesis by aureusidin synthase on the basis of known PPO-catalyzed reactions and conclude that the enzyme is a chalcone-specific PPO specialized for aurone biosynthesis.

Key words: Aurone; Aureusidin synthase; Bracteatin; 2′,4,4′,6′-Tetrahydroxychalcone; 2′,3,4,4′,6′- Pentahydroxychalcone; Polyphenol oxidase; Tyrosinase

1. Introduction

Aurones (Fig. 1A, a) are plant flavonoids that provide a yellow flower color to a variety of popular ornamental flowers such as snapdragon, cosmos, and dahlia [1,2]. Although it has been suggested that aurones are closely related to chalcones (Fig. 1A, b) in their biosynthesis [3], the biosynthetic pathway of aurones remained a mystery until recently. In 2000, we analyzed the biosynthesis of aurones from chalcones in yellow snapdragon (*Antirrhinum majus*) flowers and established that a single enzyme, aureusidin synthase, catalyzes dual chemical transformations, i.e. 3-hydroxylation and oxidative cyclization (2′,α-dehydrogenation) of 2′,4,4′,6′-tetrahydroxychalcone (THC) to produce aureusidin (Fig. 1B, scheme I) [4,5]. Aureusidin synthase is a binuclear copper enzyme with sugar chain(s) and was identified as a homolog of plant polyphenol oxidase (PPO) [4]. PPOs occur ubiquitously in higher plants and catalyze the oxidation of mono- and *o*-diphenols to produce *o*-diphenols and/or *o*-quinones (Fig. 1B, schemes IIa and IIb) [6,7]. Although aureusidin synthase was an unequivocal example of a PPO homolog participating in flower pigment biosynthesis, the mechanistic details of the enzymatic formation of aurones from chalcones remain to be clarified. In soy seedlings, it has been proposed that aurones were synthesized in a two-step pathway, in which the H_2O_2-dependent oxidation of chalcones yields a 2-(α-hydroxybenzyl)-coumaranone derivative, a hydrated form of aurone, followed by dehydration of the intermediate (Fig. 1B, scheme III) [8,9]. In the case of aureusidin synthase-catalyzed formation of aurones from chalcones, however, this mechanism is unlikely [4,5].

In this work, we have extensively analyzed the substrate and product specificities of aureusidin synthase to propose the mechanism of aurone synthesis, which could be described on the basis of PPO-catalyzed reactions.

2. Materials and methods

2.1. Chemicals

THC and 2′,3,4,4′,6′- pentahydroxychalcone (PHC) were prepared from naringenin and eriodictyol (each from Nacalai Tesque, Kyoto, Japan), respectively, as described previously [10]. 6-Glucosides of aureusidin and bracteatin and 4′-glucosides of THC and PHC were extracted from petals of *A. majus* cv. Yellow Butterfly and were purified by HPLC as described previously [5]. Aureusidin and bracteatin were purified by HPLC [5] after digesting their 6-glucosides with β-glucosidase (from almond; Toyobo, Osaka, Japan). PHC 3-glucoside was isolated from sepals of *Limonium sinuatum* Mill. cv. Gold Coast. 4,4′,6-Trihydroxyaurone was prepared according to the method of Varma and Varma [11]. Isoliquiritigenin was purchased from Funakoshi, Tokyo, Japan. Butein, 2′-hydroxychalcone, 4′-hydroxychalcone, 2′,6′-dihydroxy-4,4′-dimethoxychalcone, and sulfuretin were products of Extrasynthése Co., Geny, France. Phenylthiourea, 3,4-dihydroxy-L-phenylalanine (L-DOPA), caffeic acid, coumaric acid, and H_2O_2 (34% by vol. in water) were from Nacalai Tesque, Kyoto, Japan.

2.2. Enzyme purification and assays

Aureusidin synthase was purified from the crude extract of yellow snapdragon flowers as described previously [4]. Aureusidin synthase activity was assayed by reversed-phase HPLC as previously described [5]. Sodium acetate, pH 6.6, was used as a buffer component for

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Abbreviations: THC, 2′,4,4′,6′-tetrahydroxychalcone; PHC, 2′,3,4,4′,6′- pentahydroxychalcone; L-DOPA, 3,4-dihydroxy-L-phenylalanine; PPO, polyphenol oxidase

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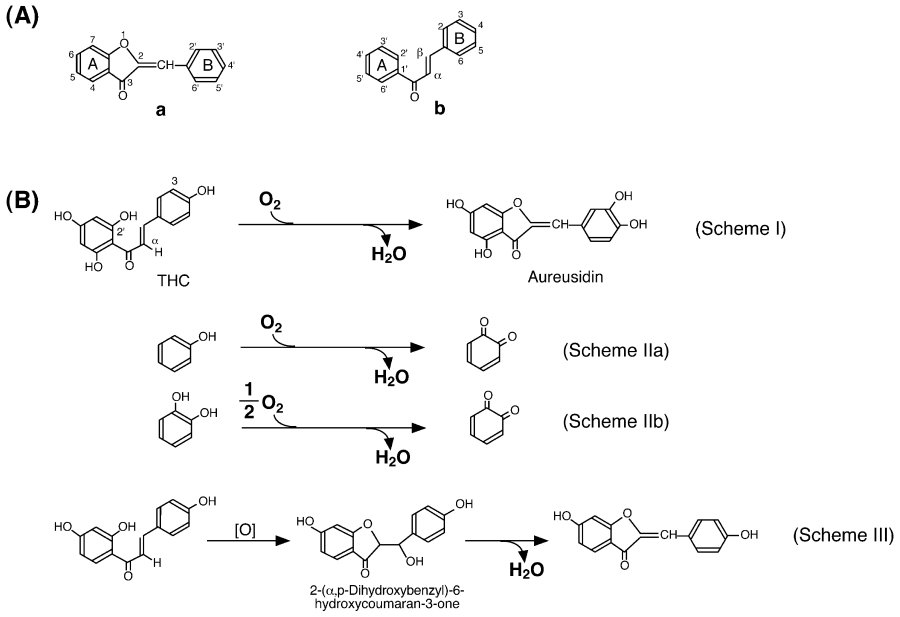

Fig. 1. A: General structures of aurones (a) and chalcones (b). Note that the positional numbering in the aurones and chalcones is different. A and B shown in the aromatic rings indicate A- and B-rings in the flavonoid structures, respectively. B: (Scheme I) Oxidative formation of aureusidin from THC catalyzed by aureusidin synthase from yellow snapdragon flowers [4,5]. (Scheme II) PPO-catalyzed oxidation of phenol (Scheme IIa, tyrosinase activity) and catechol (Scheme IIb, DOPA oxidase activity) [7]. (Scheme III) A proposed pathway of aurone biosynthesis in soy seedling [9].

assaying with PHC and PHC 4′-glucoside, and H_2O_2 was omitted when the substrate was THC 4′-glucoside, PHC, butein, or PHC 4′-glucoside. Analytical HPLC was performed using a Gilson 305 system which was equipped on-line with a Rainin auto-sample injector (model AI-3): column, YMC *J*′sphere ODS M80 (4.6×150 mm; YMC Co., Ltd., Kyoto, Japan); flow rate, 0.7 ml min^{-1}; solvent A, 0.1% (by vol.) trifluoroacetic acid in H_2O; solvent B, 0.1% trifluoroacetic acid in a 9:1 (by vol.) mixture of acetonitrile and H_2O. After injection (100 μl) onto the column that was equilibrated with 20% B (by vol.), the column was initially developed isocratically with 20% B for 3 min, followed by a linear gradient from 20% B to 60% B in 10 min. The column was then washed isocratically with 60% B for 7 min, followed by a linear gradient from 60% B to 20% B in 1 min. There was a 10-min delay before the next injection to ensure re-equilibration of the column. The chromatograms were obtained with detection at both 290 nm and 405 nm. Peak identification of each component was confirmed post-run by photodiode-array spectroscopic analysis from 200 to 600 nm using the Shimadzu SPD M6A system. Retention times (in min) of aurones, chalcones, and related flavonoids under these HPLC conditions are as follows: aureusidin, 13.1; aureusidin 6-glucoside, 7.3; bracteatin, 10.6; bracteatin 6-glucoside, 4.6; THC, 16.6; THC 4′-glucoside, 13.6; PHC, 15.2; PHC 4′-glucoside, 12.3; naringenin, 16.9; and eriodictyol, 15.3. The amounts of pigments were determined from peak integrals using authentic samples that were used for calibration. The rate of oxygen consumption was monitored using the Hansatech DW1/CB1D oxygen electrode system (Hansatech Instruments, Ltd., Norfolk, UK) [5]. K_m values for chalcone substrates were calculated from double-reciprocal plots of the data obtained from HPLC assays.

3. Results and discussion

3.1. Specificity of enzymatic aurone synthesis

We extensively analyzed the ability of the enzyme to synthesize aurones from a variety of chalcones and related flavonoids (Fig. 2). A chalcone lacking the 4-hydroxy function (Fig. 2, compound **13**) could not serve as a substrate for the enzyme. Furthermore, a chalcone lacking the 2′-hydroxy function (**14**) did not yield any aurone product(s) as monitored by photodiode-array spectrophotometry during HPLC analysis. On the other hand, THC (**1**), THC 4′-glucoside (**2**), and isoliquiritigenin (**3**), which share a common 4-monohydroxy B-ring as well as the 2′-hydroxy function, yielded a single aurone product having 3′,4′-dihydroxy B-ring. Thus, the presence of hydroxy functions at both 2′- and 4-positions of chalcone is essential for aureusidin synthase-catalyzed formation of aurones. Hydrogen peroxide (5 mM, final concentration) was needed for the maximum activation of the enzyme when reacted with isoliquiritigenin, as was the case for the reaction with THC [4,5].

PHC (**4**), PHC 4′-glucoside (**5**), and butein (**6**), which share a 3,4-dihydroxy B-ring, showed more than 10 times higher reactivity than the corresponding chalcones with a 4-monohydroxy B-ring (i.e. **1**, **2**, and **3**, respectively), giving rise to two aurone products (entries d, e, and f). One of these products was an aurone with a 3′,4′-dihydroxy B-ring, which should simply arise from the oxidative cyclization, whereas the other product was an aurone with a 3′,4′,5′-trihydroxy B-ring, which should result from the 5-oxygenation and oxidative cyclization of the substrate. The former product was produced in a 5–23 times larger amount than the latter product. Hydrogen peroxide was not required for the enzyme activity when reacted with the substrates with a 3,4-dihydroxy B-ring.

Glucosylation of the A-ring at the 4′-position of the substrate rather enhanced the affinity and reactivity of chalcones to the enzyme (entries b and e). On the other hand, the modification of B-ring hydroxyl functions of the substrate chalcones caused a loss of reactivity as substrate for the enzyme, judging from the fact that PHC 3-glucoside (**15**) and 2′,6′-dihydroxy-4,4′-dimethoxychalcone (**16**) could not serve as substrates for the enzyme.

Aureusidin synthase did not catalyze the 3′-oxygenation of 4,4′,6-trihydroxyaurone (**19**), as shown by monitoring aureusidin formation by HPLC and assaying by the O_2-electrode method. Other aurones (aureusidin and sulfuretin) could not

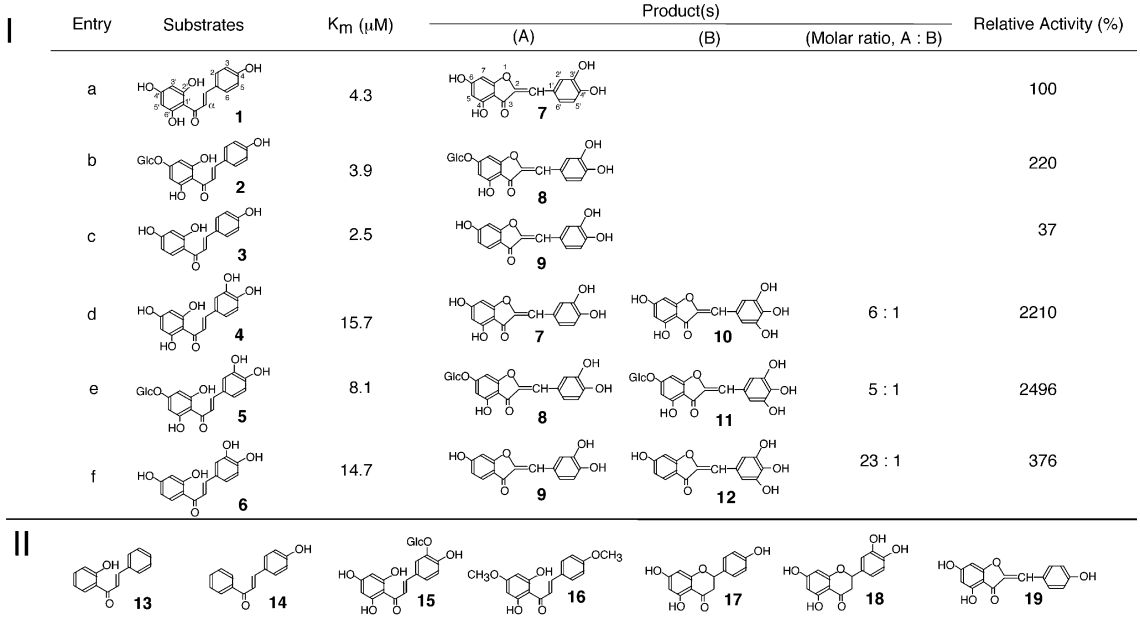

Fig. 2. Substrate and product specificities of aureusidin synthase. (I) Aurone-producing chalcones are listed with their K_m values, structure(s) of product aurone(s), and relative activities. (II) Flavonoids which were inert as substrates for aurone synthesis by aureusidin synthase. To avoid possible confusions, positional numberings of chalcone and aurone are labeled on **1** and **7**, respectively. The rates of aurone formation and oxygen consumption were measured by HPLC and oxygen electrode assays, respectively, as described [4,5]. The relative activities were compared at a substrate concentration of 10 μM with the rate of aureusidin formation (specific activity, 578 nmol min^{-1} mg^{-1}; [4]) from **1** taken to be 100%. The relative activities of **4**, **5**, and **6** were based on the sum of the amounts of aurones or their glucosides produced, assuming that their extinction coefficients are the same as that of **7**. Relative activities of **1**, **2**, **4**, and **5** were quoted from [4]. Names of flavonoids are as follows: **1**, THC; **2**, THC 4′-glucoside; **3**, isoliquiritigenin; **4**, PHC; **5**, PHC 4′-glucoside; **6**, butein; **7**, aureusidin; **8**, aureusidin 6-glucoside; **9**, sulfuretin; **10**, bracteatin; **11**, bracteatin 6-glucoside; **12**, 3′,4′,5′,6-tetrahydroxyaurone; **13**, 2′-hydroxychalcone; **14**, 4-hydroxychalcone; **15**, PHC 3-glucoside; **16**, 2′,6′-dihydroxy-4,4′-dimethoxychalcone; **17**, naringenin; **18**, eriodictyol; and **19**, 4,4′,6-trihydroxyaurone. Glc, β-D-glucopyranoside.

undergo further 5′-oxygenation either. In addition, flavanones (naringenin (**17**) and eriodictyol (**18**)) were inert as substrates for the enzymatic aurone synthesis.

3.2. PPO activity of aureusidin synthase

We recently identified the aureusidin synthase as a homolog of PPO and also showed that aurone synthesis is also catalyzed by *Neurospora crassa* tyrosinase, which is a PPO distantly related to aureusidin synthase [4]. These results led us to examine whether aureusidin synthase has PPO activity (Fig. 1B, schemes IIa and IIb) toward phenolics using an O_2-electrode assay. Virtually no O_2 consumption was observed with the following (relative activities, less than 1% of rate of O_2 consumption with THC): L-tyrosine, L-DOPA, 4-coumaric acid, caffeic acid, naringenin, eriodictyol, 4,4′,6-trihydroxyaurone, and aureusidin. Although a very slow enzymatic formation of DOPAchrome from L-DOPA could be observed by a spectrophotometric assay [12] using a large amount of aureusidin synthase, such 'DOPA oxidase' activity was too weak, compared with aureusidin synthase activity, to exactly estimate its relative activity by an O_2-electrode assay.

3.3. Inhibition of aureusidin synthase by a PPO inhibitor

Recently, the stereo structure of potato PPO complexed with phenylthiourea, a specific inhibitor, has been elucidated to show that the bound inhibitor interacts with a binuclear copper center at the active site of PPO; the inhibitor competes with the *o*-diphenolic substrates for the active-site binuclear copper [13]. This led us to analyze whether phenylthiourea also inhibits the reaction with PHC of aureusidin synthase, which is also a binuclear copper enzyme [4]. The results showed that phenylthiourea competitively inhibited aureusidin synthase with a K_i value of 1.0 μM.

3.4. Mechanistic considerations

The specificity of aureusidin synthase presented in Section 3.1 indicates that aureusidin synthase is essentially an internal monooxygenase that seemingly catalyzes the 3-hydroxylation of the B-ring and the oxidative cyclization (2′,α-dehydrogenation) of a variety of chalcones having hydroxy functions at both 2′- and 4-positions (Fig. 1B, scheme I). Aurone formation from chalcones having a 4-monohydroxy B-ring (e.g. THC) must be accompanied by the oxygenation of the B-ring, whereas the aurone formation from chalcones with a 3,4-dihydroxy B-ring (e.g. PHC) was not necessarily accompanied by B-ring oxygenation. These results indicate that the 3-oxygenation of the B-ring is a prerequisite for the oxidative cyclization of THC into aureusidin; in other words, the oxidative cyclization of THC must be preceded by the 3-oxygenation of the B-ring. This was further confirmed by the fact that the enzyme could not produce aureusidin from 4,6,4′-trihydroxyaurone by 3′-oxygenation. Importantly, the mechanism that was previously proposed for aurone synthesis assuming a 2-(α-hydroxybenzyl)-coumaranone intermediate (Fig. 1B, scheme III; [9]) cannot explain the dual chemical transformations.

These results, along with the facts that (i) aureusidin synthase shares a very similar primary structure with plant PPOs [4]; (ii) the enzyme was competitively inhibited by a specific PPO inhibitor; and (iii) a fungal tyrosinase distantly related to

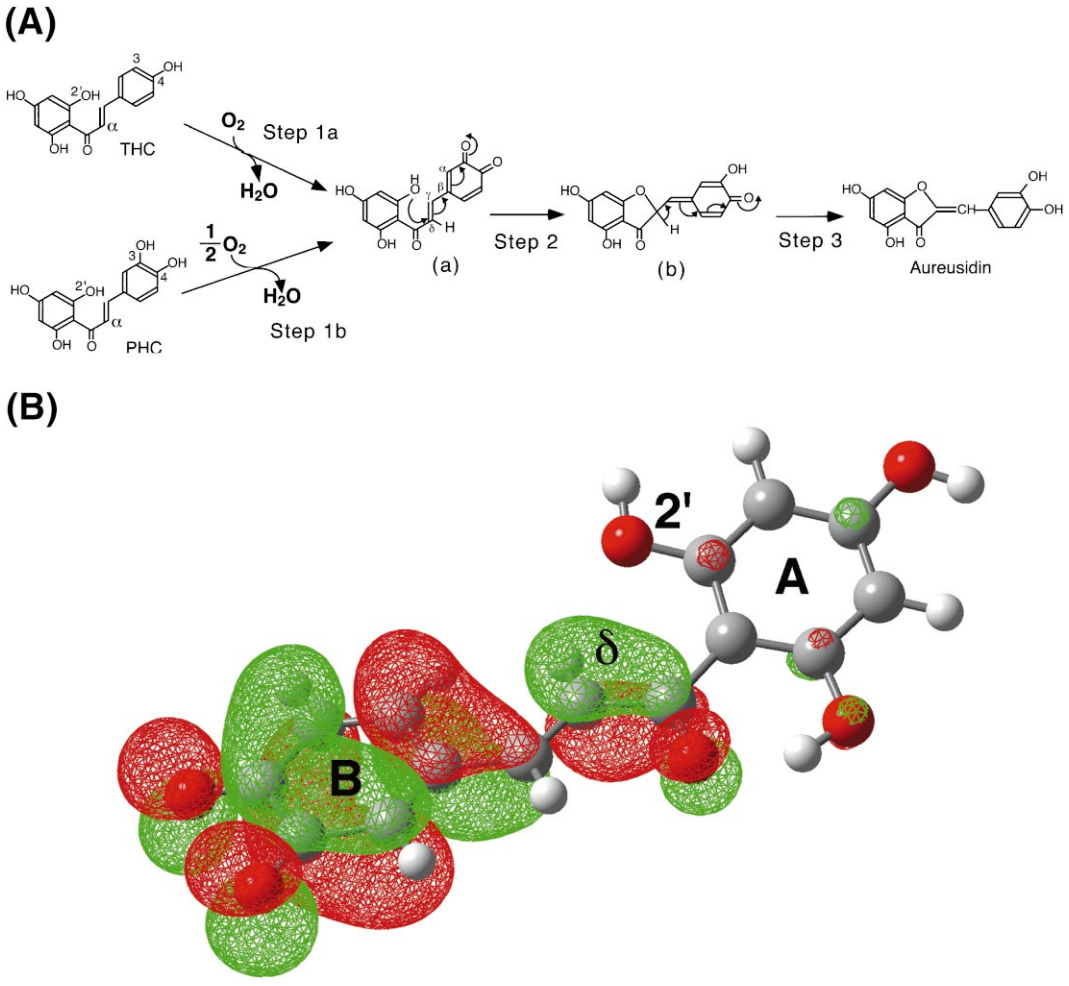

Fig. 3. A: Proposed mechanism of aurone synthesis from THC or PHC catalyzed by aureusidin synthase. Steps 1a and 1b are the PPO-catalyzed processes, whereas Steps 2 and 3 may take place non-enzymatically. It should be mentioned that two alternative mechanisms have been proposed for the PPO-catalyzed oxidation of monophenols (Step 1a). According to one mechanism, PPO catalyzes the hydroxylation of monophenol to yield *o*-diphenol which is then dehydrogenated to *o*-quinone [15]. The other mechanism does not involve the hydroxylation of monophenol [17]. It remains to be clarified which is the case for the aureusidin synthase-catalyzed oxidation of THC. (B) LUMO of intermediate (a). The structure of the intermediate (a) was optimized at the restricted Hartree–Fock level of theory with the 6-31G(d) basis set using Gaussian 98 package [18]. The molecular orbitals were calculated at the B3LYP/6-31G(d) level. The A-ring is twisted relative to the conjugated system by 18 degrees around the bond C1′-C(carbonyl), allowing 2′-hydroxyl group to appropriately position for its nucleophilic attack on the δ carbon.

aureusidin synthase could also catalyze the formation of aureusidin from THC [4], allowed us to propose the aureusidin synthase mechanism on the basis of PPO catalysis (Fig. 3A). The enzyme catalyzes the production of a chalcone with an *o*-quinone form of the B-ring (intermediate (a), see Fig. 3A) from a chalcone with a mono- or diphenolic B-ring. The α,β-, γ,δ-unsaturated carbonyl substructure of the resultant intermediate should induce the Michael-type addition of 2′-hydroxyl on the δ carbon to yield the intermediate (b). Molecular orbital calculations using Gaussian software (Fig. 3B) indicate that the calculated distribution of the lowest unoccupied molecular orbital (LUMO) in the intermediate (a) is consistent with the electrophilic nature of the δ carbon. Subsequent deprotonation from the intermediate (b) allows the aromatization of the B-ring as well as full conjugation of double bonds throughout the molecule, producing aurone. Steps 2 and 3 may take place non-enzymatically because PHC could be non-enzymatically oxidized to aureusidin at alkaline pHs in aqueous solutions without transient formation of any intermediates detectable by HPLC (T. Sato and T. Nakayama, unpublished results). When PHC is used as a substrate, there are two possible ways, one of which (frequency, 14%) starts with the B-ring oxidation analogous to Step 1a which allows the introduction of a third oxygen atom into the B-ring, while the other (86%) starts with 3,4-dehydrogenation (Step 1b), respectively, producing bracteatin and aureusidin in a molar ratio of 1:6 (see Fig. 2, entry d). Activation of aureusidin synthase by H_2O_2 during reaction with THC and isoliquiritigenin (i.e. substrates having monophenolic B-ring, see Section 3.1) is also consistent with the nature of PPO catalysis with monophenolic substrates (Fig. 3, Step 1a) [15].

This mechanism states that aureusidin synthase does not, in reality, catalyze the 2′,α-dehydrogenation but it does catalyze the oxidation of phenolic B-ring of chalcones, as do the known PPOs. This does not contradict our observation that aureusidin synthase shows no or only negligible PPO activity toward a variety of phenolics, because the substrate specificity

of plant PPOs significantly differs with the source of the enzyme; for example, PPO from the glandular trichomes of the wild potato, *Solanum berthaultii*, is known to exhibit a substrate preference for chlorogenic acid and essentially no tyrosinase or DOPA oxidase activity [14]. Thus, aureusidin synthase from yellow snapdragon flowers is a PPO homolog specifically acting on chalcones and should be specialized for aurone biosynthesis during flower coloration. In this context, aureusidin synthase does not catalyze 3′,4′-dehydrogenation of aureusidin and such strict substrate specificity is of great importance for aurone synthesis in the flower. Because a putative mature form of aureusidin synthase (residues 61–416, see [4]) shows high sequence similarities to known plant PPOs along the entire polypeptide chain (identity, more than 40%; [4]), it would be interesting to examine which amino acid residue(s) in the enzyme is responsible for the unique substrate specificity of aureusidin synthase. Moreover, subcellular localization of aureusidin synthase in the cells of yellow snapdragon flowers may be distinct from those (plastids) which have previously been known for plant PPOs [4,5], and this may also allow this enzyme to uniquely fulfil a role in flower coloration.

It must also be mentioned that, according to this PPO-catalyzed mechanism of aurone synthesis, the product aurone must have a 3′,4′-dihydroxy or 3′,4′,5′-trihydroxy B-ring. Consistently, almost all aurones found so far in nature possess the 3′,4′-dihydroxy or 3′,4′,5′-trihydroxy B-ring moiety or their *O*-substituted derivatives, rather than 4′-monohydroxy B-ring [16]. However, some aurones have been reported to have a B-ring moiety with no hydroxyl function or only one [16] (e.g. hispidol found in soy seedling [8]), suggesting that these may be produced through an alternative mechanism [8,9] which would require further examination.

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