Bifunctional Polyphenol Oxidases: Novel Functions in Plant Pigment Biosynthesis

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Anyone who has observed a cut apple or a damaged banana notices the appearance of brown and black pigmentation from the formation of complex polyphenols. This color change is caused by a group of copper proteins, polyphenol oxidases (PPOs), in plants often described as latent thylakoid membrane-located enzymes.[1]

PPOs can be divided into tyrosinases, catechol oxidases, and laccases, all belonging to the copper type-3 proteins containing binuclear copper centers.[2] Tyrosinases are bifunctional PPOs, which catalyze hydroxylation of phenols to *o*-diphenols (EC1.14.18.1; monophenol: monooxygenase) and their subsequent oxidation to *o*-quinones (EC1.10.3.1; *o*-diphenol: oxygen oxidoreductase). Catechol oxidases catalyze only the latter reaction.[3] In contrast, laccases (EC1.10.3.2; *p*-diphenol: oxygen oxidoreductase) accept preferentially *p*-diphenols and a laccase-like enzyme is proposed to be involved in lignin biosynthesis.[4]

Tyrosinases play a pivotal role in the formation of complex polyphenols in bacteria, fungi, plants, and animals including humans. For example, in insects they are involved in cuticular melanization and sclerotization of the exoskeleton as well as in the defense against other organisms by encapsulating them in melanin. Tyrosinases are also active in the formation of the black colorant sepia in *Sepia officinalis* (octopus). In animals (including humans), these enzymes are responsible for skin and hair coloration (eumelanins, phaeomelanins); in plants they might have a role in defense reactions against insects and microbial pathogens, however, this has not yet been proven.

With the amino acid tyrosine as substrate, the activity of the enzyme tyrosinase leads from tyrosine via 3,4-dihydroxyphenylalanine (dopa) to dopaquinone. Dopaquinone then undergoes a spontaneous cyclization by Michael addition to form *cyclo*-dopa, which polymerizes following oxidation to dopachrome to form melanin. On the other hand the formation of dopa as an endproduct in various plant tissues by the hydroxylation activity of tyrosinase[5] or as an intermediate metabolite, for example, via dopamine in the biosynthesis of benzyltetrahydroisoquinoline alkaloids,[6] has been shown. However, with regard to their bifunctional properties, the plant PPOs are among those enzymes to which a function has not yet been assigned.[7] This situation has, however, recently changed. The involvement of both reactions, hydroxylation and oxidation, in the biosynthesis of low molecular weight products has been proven in two different pathways of plant pigment biosynthesis. A tyrosinase is involved in betalain biosynthesis[8] and a chalcone-specific PPO participates in aurone biosynthesis.[9]

The tyrosinase involved in betalain biosynthesis has been found in common portulaca (*Portulaca grandiflora*) and red beet (*Beta vulgaris*). Figure 1 shows a flowering common

Figure 1. A betacyanin-accumulating common portulaca plant and a corresponding callus culture, (photographs kindly provided by U. Steiner, University Freiburg, Germany).

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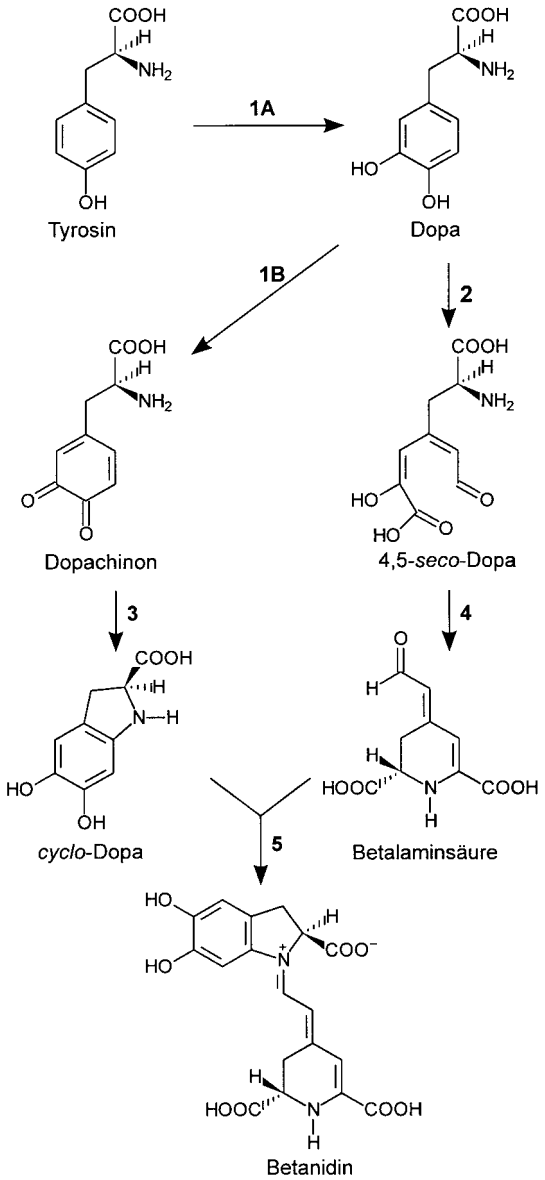
portulaca plant and a corresponding callus culture, which accumulate betacyanins, a class of red-violet betalains. Along with the yellow betaxanthins, betalains are characteristic water-soluble, nitrogen-containing pigments accumulating in flowers, fruits, and occasionally in vegetative tissues of plants of most families of the Caryophyllales.[10] These pigments take the place of the anthocyanins, a class of flavonoids, which occur in all other families of flowering plants. Scheme 1 shows



Scheme 1. Betacyanin biosynthesis involving two enzymes, the bifunctional tyrosinase (reaction **1A**, hydroxylation; reaction **1B**, oxidation) and the dopa 4,5-dioxygenase (reaction **2**). The tyrosinase acts bifunctionally as well as monofunctionally in the biosyntheses of *cyclo*-dopa and dopa, respectively. Reactions **3**, **4**, and **5** are considered to proceed non-enzymatically. The product is betanidin, the basic structure of glycosyl and acylglycosyl conjugates (pigments) in most families of the Caryophyllales.

the pathway of betacyanin biosynthesis in which only two enzymes, a tyrosinase and a dopa 4,5-dioxygenase are required. The bifunctional tyrosinase catalyzes the sequential formation of dopa and dopaquinone. The *cyclo*-dopa, spontaneously formed from dopaquinone, is protected in vivo against further oxidation and polymerization by an unknown mechanism. In vitro this protection can be achieved by the addition of reducing agents (e.g. ascorbic acid) in the tyrosinase assay mixture.[8]

Another unknown mechanism is how a significant portion of the tyrosine hydroxylation product, dopa, is protected against the second tyrosinase activity, the oxidation. This is absolutely essential, since dopa must be available as a substrate for the dopa 4,5-dioxygenase in betalain biosynthesis. The enzyme catalyzes the 4,5-extradiol cleavage of dopa to give 4,5-*seco*-dopa that recyclizes to betalamic acid, part of the betacyanin chromophore. Betalamic acid and *cyclo*-dopa enter a spontaneous condensation reaction. The product betanidin is the basic structure of various glycosyl and acylglycosyl conjugates.[10]

The betalain-specific tyrosinase was partially purified and characterized from common portulaca callus cultures[8] (Figure 1). The presence of a high salt concentration (500 mM NaCl), ascorbic acid, and $CuCl_2$ turned out to be essential for enzyme extraction. The highest hydroxylation activity was observed when ascorbic acid and catalase were added and the assay mixture saturated with O_2. The hydroxylation activity at a pH optimum of 5.7 was specific for L-tyrosine, the reaction rates with L-tyrosine and D-tyrosine are in a ratio of 1:0.2. Other monophenols tested were not accepted as substrates. The highest dopa oxidase activity was determined to be at pH 7.0. The enzyme appeared to be a monomer with an apparent molecular mass of around 53 kDa. Tyrosinase activity correlated with betacyanin accumulation in hypocotyls of developing red beet seedlings. These results showed for the first time that both tyrosinase reactions (hydroxylation and oxidation) are involved in the formation of low molecular weight plant products, the betacyanins.

A recent report on the involvement of a bifunctional PPO in the biosynthesis of another class of plant pigments, the aurones, is a true highlight.[9] The authors were able to demonstrate the role of this enzyme in the biosynthesis of aurones in snapdragon (*Antirrhinum majus*) flowers (Figure 2). Besides chalcones, flavanones, and dihydroflavonols, aurones belong to the group of "minor flavonoids".[11] Based upon 2-benzylidene-coumaran-3-one[12] their structures lack the typical flavonoid skeleton, but they are biosynthetically directly derived from chalcones, the pivotal precursor of all flavonoids.[13] Aurones are gold-colored (latin aurum = gold) pigments occurring as glycosides in petals of some members of the Asteraceae (e.g. *Cosmos*, *Coreopsis*), Scrophulariaceae (e.g. snapdragon), Cyperaceae, and Fabaceae. The 6-*O*-glucosides of aureusidin and bracteatin are the main pigments in snapdragon petals.

For almost 30 years an oxidative cyclization of chalcones catalyzed by a peroxidase and a subsequent spontaneous dehydration of the intermediate was postulated.[14] Contrary to this, it has been demonstrated[9] with an enzyme from snapdragon buds that both the hydroxylation and the oxidative cyclization of chalcones are catalyzed by a single enzyme (called aureusidin synthase), a PPO. The purified enzyme catalyzes at a pH optimum of 5.4, in the presence of

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Figure 2. Aurone-accumulating snapdragon flowers, (photograph kindly provided by T. Nakayama, Tohoku University, Sendai, Japan).

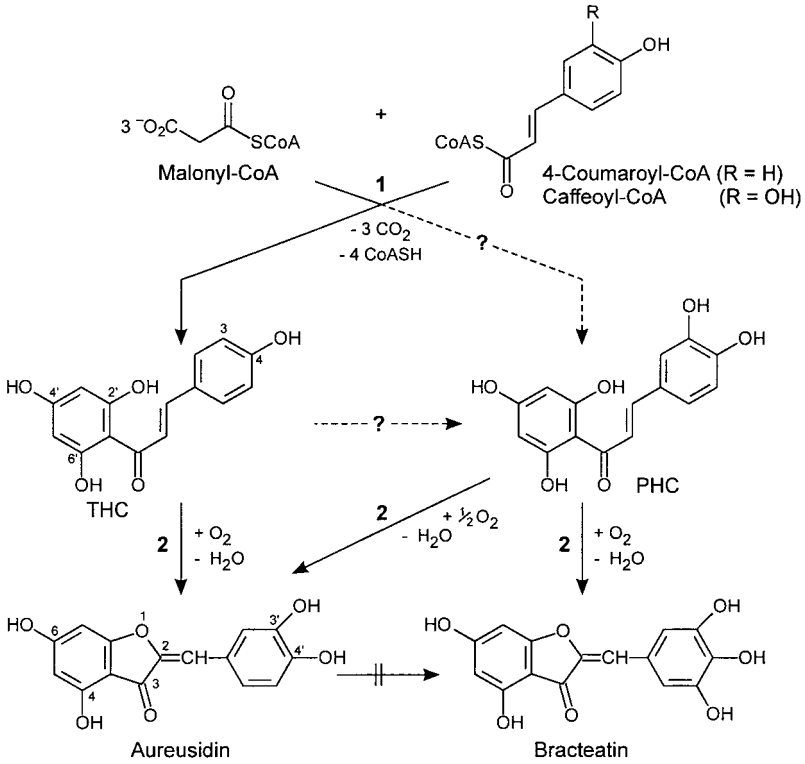
H_2O_2, the 3-hydroxylation and oxidative cylization of 4,2′,4′,6′-tetrahydroxy-chalcone (THC) to yield aureusidin as the only product (Scheme 2). The enzyme was found to be monomeric with an apparent molecular mass of 39 kDa. Aureusidin formation proceeds with the equimolar consumption of oxygen, but not of H_2O_2, which may work as an enzyme activator.[15] 3,4,2′,4′,6′-Pentahydroxychalcone (PHC) was a much better substrate than THC (relative activity 2210%) for the enzyme to produce aureusidin and bracteatin in a molar ratio of 6:1. In this case, H_2O_2 was also not required for enzyme activity but rather inhibited the reaction. These results suggest that aureusidin can be produced either from THC or PHC. Bracteatin is not produced through the 5′-hydroxylation of aureusidin but arises solely from PHC. Not only the free chalcones (THC, PHC) were good substrates in vitro, but also the corresponding 4′-*O*-glucosides, with relative activities of 220 and 2490%, respectively.

With regard to the mechanism of the chalcone-specific PPO activity, it has been proposed[16] that both THC and PHC are converted into a 3,4-*o*-quinone derivative which cyclizes by nucleophilic attack of the activated double bond by the 2′-hydroxyl group leading, after subsequent hydrogen transfer, to the final aureusidin (Scheme 3). However, for naturally occurring aurones lacking a 3,4-dihydroxy substitution pattern, clearly a different reaction mechanism has to be postulated.

The amino acid sequences of proteolytic fragments of aureusidin synthase were found to be identical to stretches of the deduced amino acid sequence of a clone that was specifically expressed in the pigment-containing snapdragon petals and obtained by substractive hybridization. A cDNA (*AmAS1*) was isolated from a petal cDNA library using this clone as a probe. The cDNA encoding the precursor of aureusidin synthase contained an open reading frame of 1686 base pairs encoding 562 amino acids. That the AmAS1 protein is a binuclear copper enzyme, expected for a bifunctional PPO, was demonstrated by identification of two histidin-rich Cu-binding domains and by atomic absorption spectroscopic analysis.

The AmAS1 protein showed significant sequence similarity to other plant PPOs, such as those from apple fruit (*Malus* x *domestica*) (identity, 51%), grape berry (*Vitis vinifera*) (47%) as well as to two PPO cDNAs from pokeberry (*Phytolacca americana*) (48 and 49%). Increase of the transcript levels of the pokeberry PPOs correlated with the betacyanin formation in the fruits.[17]

The identification of aureusidin synthase as a PPO homologue gave a reason to test *Neurospora crassa* tyrosinase for aureusidin synthase activity,[9] and indeed, aureusidin was



Scheme 2. Aurone biosynthesis in snapdragon flowers. The chalcone-specific PPO (reactions **2**) catalyzes the hydroxylation and/or oxidation of the chalcones THC and PHC in the formation of aureusidin and bracteatin. It is unknown whether PHC derives from chalcone synthase (reaction **1**) acting towards caffeoyl-CoA[19, 20] or alternatively from a putative B-ring hydroxylase[19] accepting THC. On the other hand, the hydroxylation activity of the aureusidin synthase towards THC to yield PHC (in a reducing milieu) is conceivable, analogous to the role of the betalain-specific tyrosinase (see Scheme 1).

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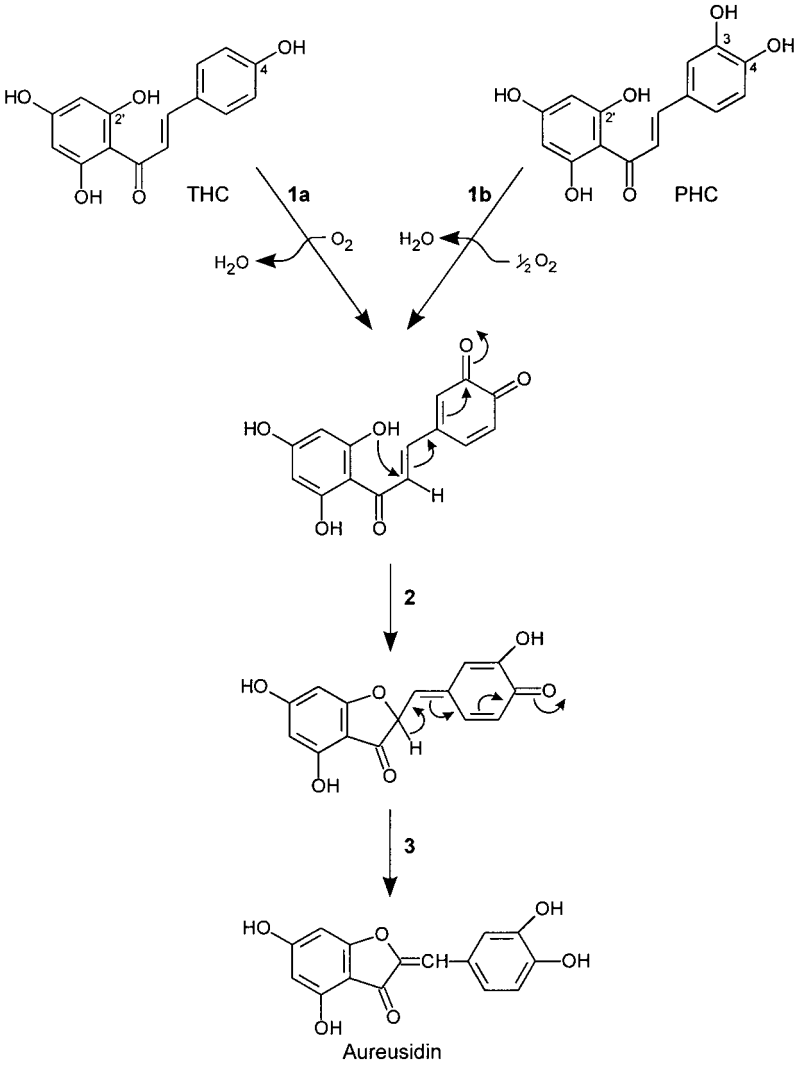

Scheme 3. Proposed mechanism (redrawn from ref.[16]) of aureusidin-synthase-catalyzed aurone formation from THC (reaction **1a**) or PHC (reaction **1b**). Hydroxylation and oxidation is followed by a two-step non-enzymatical cyclization (reactions **2** and **3**).

formed from THC, thus establishing the general ability of PPO to catalyze aurone synthesis from chalcones. In contrast to other PPOs, the aureusidin synthase showed virtually no 3′,4′-dehydrogenation activity towards aureusidin and very low L-dopa-oxidizing activity.[16] Finally, the spatial and temporal expression of the *AmAS1* gene analyzed by RNA gel blots revealed a strict correlation with the aurone formation during flower development.

In the light of these topical results, which demonstrate novel and surprising functions of bifunctional PPOs in the biosynthesis of low molecular weight plant products, it is now a priority to address the question of the molecular mechanism of these enzyme activities, a question that has been raised recently in this journal by Decker et al.[18]

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