



THE FORMATION OF BETALAMIC ACID AND MUSCAFLAVIN BY RECOMBINANT DOPA-DIOXYGENASE FROM *AMANITA*

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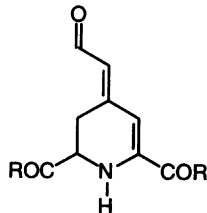
Key Word Index—*Amanita muscaria*; basidiomycete; betalains; muscaflavin; DOPA-dioxygenase; reaction products.

Abstract—DOPA-dioxygenase from *Amanita muscaria* is known to catalyse the conversion of 3-(3,4-dihydroxyphenyl)alanine (DOPA) to betalamic acid in the key reaction of betalain biosynthesis. In this work, we re-examined the reactivity of DOPA-dioxygenase using a cDNA clone encoding active DOPA-dioxygenase the kinetic parameters of which were comparable to those of the native enzyme. Using L-DOPA as a substrate, the enzyme catalysed the formation of two products. In addition to betalamic acid, the enzyme also catalysed the formation of muscaflavin, a compound that occurs naturally in *A. muscaria* and in mushrooms of the *Hygrocybe* family but not in the betalain-containing plants of the order Caryophyllales. Muscaflavin arises by a 2,3-extradiol cleavage of DOPA, whereas betalamic acid is the product of a 4,5-cleavage. Our results indicate that the recombinant enzyme has both 2,3- and 4,5-dioxygenase activity, and do not support the prevailing view that the two compounds are produced by two distinct enzymes. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

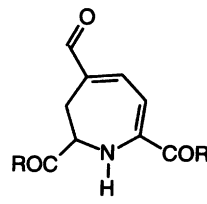
Betalains are pigments found in the plant order Caryophyllales and in some fungi [1, 2]. All betalains contain a betalamic acid (**1a**) moiety, which condenses with an amino acid or amine to form yellow betaxanthins, or with a cyclo-DOPA derivative to form violet betacyanins. The betalains found in the mushroom *Amanita muscaria* [3] and their enzymology have been studied in detail [4–6]. A DOPA-4,5-dioxygenase characterized from this mushroom [5] catalyses the 4,5-ring opening of a 3-(3,4-dihydroxyphenyl)alanine (DOPA) molecule, which leads to the formation of 4,5-seco-DOPA [6]. The non-enzymic rearrangement of 4,5-seco-DOPA then yields betalamic acid (**1a**).

It has been noted that, in addition to betalains, *A. muscaria* also contains muscaflavin (**2a**) [7], a yellow compound thought to arise by a 2,3-extradiol cleavage of DOPA and therefore biosynthetically related to betalamic acid (**1a**). The predominant view has been that there are two distinct enzymes catalysing the 2,3- and the 4,5-ring opening reactions [5, 6], particularly because betalamic acid, but not muscaflavin, is found in plants of the order Caryophyllales, whereas the mushrooms of the *Hygrocybe* family accumulate high levels of muscaflavin and traces of betalains and betalamic acid. However, due to the similarities in their



1

1a: R=OH
1b: R=OCH₃



2

2a: R=OH
2b: R=OCH₃

biosynthesis, it is possible that the synthesis of both betalamic acid and muscaflavin are catalysed by the same enzyme. This led us to reconsider the reaction products of *A. muscaria* DOPA-dioxygenase. In this work, recombinant DOPA-dioxygenase from *A. muscaria* was used to study the products of the reaction with DOPA as substrate, using HPLC and GC-mass spectrometry techniques.

RESULTS AND DISCUSSION

Screening of a cDNA expression library prepared from the coloured cuticle of *A. muscaria* with an antibody raised against DOPA-dioxygenase permitted the isolation of several clones encoding active DOPA-dioxygenase. The recombinant β -galactosidase fusion

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protein (M_r 27 000) was visible as a single band on a Western blot. The fact that the native protein has a M_r of 25 000 suggested that the cDNA was not complete and that *ca* 20 amino acids were missing at the amino terminus. However, the substrate specificity and kinetic parameters of the recombinant enzyme were found to be close to those of the native enzyme: the recombinant enzyme had a K_m value for DOPA of 4.5 mM, compared with 3.9 mM for the native enzyme, and the K_m of the substrate dopamine was 8.3 mM compared with 6.3 mM. The v_{max} for dopamine relative to DOPA was 2.2 for the recombinant enzyme and 2.7 for the native enzyme [5]. In addition, the initial rates of activity in a crude enzyme preparation using L-DOPA, dopamine and catechol have been reported [6]. The results for the recombinant enzyme, expressed relative to L-DOPA, were in good agreement with these data: L-DOPA, 1.0 (1.0 [6]), dopamine 1.62 (1.69) and catechol 0.20 (0.19). This suggests that the results obtained with the recombinant enzyme are also valid for the native enzyme.

The choice of reaction buffer was important because betalamic acid is unstable due to reactive groups that can interact with the buffer and yield side products. In particular, betalamic acid can form Schiff bases with amines, therefore excluding the use of Tris in the reaction buffer, as well as some other commonly used buffers. We found that betalamic acid is stable in EDTA, and all subsequent reactions were carried out in EDTA buffer (100 mM EDTA, pH 8.6, 5 mM DTT, 5 mM DOPA). HPLC analysis [8] of the reaction products indicated the formation of two products with absorption maxima of *ca* 420 nm and retention times of 10.1 and 11.9 min. The first peak, at 10.1 min, was readily identified as betalamic acid (**1a**), because its spectrum ($\lambda_{max} = 415$ nm in 20% acidified methanol, 424 nm in potassium phosphate buffer, pH 4.2) and elution time were identical to authentic betalamic acid. Incubation of the reaction product with an excess of amino acids under acidic conditions yielded betaxanthins, the spectra and retention times of which corresponded to their natural counterparts. The spectrum of the second peak, at 11.9 min ($\lambda_{max} = 409$ nm in 20% acidified methanol, 415 nm in potassium phosphate buffer, pH 4.2) closely matched that of muscaflavin (**2a**) [6]. The elution time of peak 2 was compared with a sample of *Hygrocybe* extract, the main pigment in which is muscaflavin. The elution time of peak 2 and the muscaflavin peak of the extract and their spectra were identical. To corroborate the identity of the two products further, they were analysed using GC-mass spectroscopy, following desalting and conversion into their more stable methyl esters (**1b** and **2b**). Betalamic acid methyl ester (**1b**) and muscaflavin methyl ester (**2b**) had slightly different retention times in the gas chromatograph (14.4 and 13.9 min, respectively). The mass spectrograms closely matched those of betalamic acid and muscaflavin methyl esters reported in the literature (see Experimental section) [7, 9]. Betalamic acid methyl ester gave a double peak with identical

mass spectrograms which probably represented the *cis/trans* isomers. The surface ratio of the peaks (4.75:3) was very close to the 5:3 ratio observed for the two isomers at equilibrium, as shown in NMR studies [9]. The observation that the two compounds are produced by the same enzyme contrasts with previous results suggesting that betalamic acid and muscaflavin are produced by two distinct enzymes [5, 6]. Our results do not exclude the existence of a separate enzyme with a DOPA-2,3-dioxygenase activity, but this appears unlikely considering that DOPA-dioxygenase catalyses both reactions.

DOPA-2,3 and 4,5-dioxygenase activities have also been reported in legumes, but they catalyse the formation of stizolobinic and stizolobic acid [10]. The 2,3- and 4,5-opening reactions in legumes are catalysed by two distinct enzymes, which exhibit a high specificity for DOPA. Stizolobinic and stizolobic acid have also been identified in *A. muscaria*; stizolobic acid forms the betalain pigment muscaurin II when it condenses with betalamic acid [3]. Under the reaction conditions used here, neither stizolobinic nor stizolobic acid were detected as reaction products of DOPA-dioxygenase from *A. muscaria*. The enzyme probably does not produce any, or only very small amounts, of these compounds. Another enzyme may exist for the synthesis of stizolobic and stizolobinic acid in *A. muscaria*.

Muscaflavin has not been identified in the plants of the order Caryophyllales [11]. Thus the plant enzyme probably lacks the DOPA-2,3-dioxygenase activity that is associated with the *A. muscaria* enzyme. Western blot analysis of protein extracts from *Beta vulgaris* and *Portulaca grandiflora* using antibodies directed against the *A. muscaria* DOPA-dioxygenase exhibited no detectable signals. However, other betalain and muscaflavin producing *Amanita* and *Hygrocybe* species contained cross-reactive proteins (unpublished results). These results indicate that the enzymes involved in the biosynthesis of betalains in fungi and in plants are different in structure and reactivity, and probably share no common evolutionary origin.

EXPERIMENTAL

Cloning of cDNA. A cDNA library of coloured *A. muscaria* cuticle was prepared according to the manufacturer's instructions (Pharmacia) and cloned into λ ZAPII (Stratagene). The library was screened using anti-DOPA-dioxygenase antisera. Positive clones were isolated and assayed for activity. *In vivo* excision of the positive clones yielded pBS-Dod.

Production of recombinant enzyme. The *Escherichia coli* harbouring pBS-Dod were grown overnight in 3 ml on selective medium (Luria-Broth (LB) with 50 μ g ml⁻¹ ampicillin). The cultures were then diluted 1/100 in 100 ml selective medium and grown to satn. After centrifugation, the cells were resuspended in 0.1 M Tris buffer, pH 8.5. Tween and lysozyme were added to a final concn of 0.1% Tween and 1 mg ml⁻¹, respec-

tively, and incubated for 1 hr. The suspension was centrifuged and washed $2 \times$ with 0.1 M EDTA pH 8.5, and finally resuspended in 10 ml of the same buffer. This suspension was used without further purification.

DOPA-dioxygenase reaction. The reaction was carried out in 1 ml of reaction mixture containing 0.1 M EDTA pH 8.5, 5 mM L-DOPA, 5 mM DTT and 100 μ l of the enzyme suspension and incubated for 4 hr at room temp.

Measurement of kinetic parameters. Initial rates of reaction were measured in 0.5 M EDTA, 5 mM DTT and with the following substrate concns: 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 mM. The kinetic parameters were determined using Lineweaver-Burk plots.

HPLC analysis of reaction products. Before analysis, the samples were acidified to pH 3 with H_3PO_4 and centrifuged. The supernatant was analysed by HPLC [8] on a reverse phase column (Hypersil ODS C18 3 μ m). The gradient started with 100% phase A (50 mM KP_i , 2.5 mM triethylamine, pH 4.2) and rose to 30% phase B (40% CH_3CN) in 15 min, to 60% B after 20 min and to 80% B after 25 min and maintained at 80% B until 30 min. The column was then washed for 10 min with 100% A to prepare for the next injection. The peaks were detected using a diode array detector.

HPLC purification of reaction products. For the collection of peaks a simplified HPLC protocol was used: isocratic 100% phase A (2% HOAc) for 10 min, then a gradient in 2 min to 100% phase B (20% MeOH in 2% HOAc), and finally 100% phase B for 20 min. The two products absorbing at *ca* 420 nm eluted at 20 and 24 min, respectively.

Derivatization. The products were esterified according to the method in ref. [12]. The dried pellets were resuspended in 120 μ l MeOH, to which 900 μ l CH_2CN_2 in MTBE were added and incubated 30 min at room temp., and dried under N_2 .

GC-MS analysis of reaction products. The samples were resuspended in 100 μ l hexane, of which 1 μ l was injected, corresponding to *ca* 100 ng product. The GC-MS was carried out with a 30 m \times 0.25 mm HP-5MS column, an ionization energy of 70 eV and a total He flow rate of 50 ml min^{-1} . The injector temp. was 250 $^\circ$ and the temp. profile was programmed from 100 $^\circ$ to 280 $^\circ$ at 10 $^\circ$ min^{-1} . Results for betalamic acid and muscaflavin obtained as reaction products from

DOPA-dioxygenase, and previously published values for reference—Betalamic acid: 239 $[M]^+$ (31.0), 180 $[M-CO_2CH_2]^+$ (79.9), 148 $[180-CH_3OH]^+$ (58.6), 120 $[148-CO]^+$ (100.0), 92 $[120-CO]^+$ (16.8). Betalamic acid according to ref. [9]: 239 $[M]^+$ (36.6), 180 $[M-CO_2CH_2]^+$ (77.0), 148 $[180-CH_3OH]^+$ (63.5), 120 $[148-CO]^+$ (100.0), 92 $[120-CO]^+$ (23.4). Muscaflavin: 239 $[M]^+$ (34.1), 180 $[M-CO_2CH_2]^+$ (100.0), 148 $[180-CH_3OH]^+$ (74.0), 120 $[148-CO]^+$ (43.0), 92 $[120-CO]^+$ (21.5) Muscaflavin according to ref. [7]: 239 $[M]^+$ (44.0), 180 $[M-CO_2CH_2]^+$ (100.0), 148 $[180-CH_3OH]^+$ (77.3), 120 $[148-CO]^+$ (56.0), 92 $[120-CO]^+$ (29.7).

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