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CHARACTERIZATION OF A TYROSINASE FROM *AMANITA MUSCARIA* INVOLVED IN BETALAIN BIOSYNTHESIS

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Key Word Index—*Amanita muscaria*; Basidiomycete; tyrosine hydroxylation; DOPA; pigment.

Abstract—A tyrosinase was characterized from the betalain-containing pileus of the mushroom *Amanita muscaria*. The enzyme was located exclusively in the coloured parts of the mushroom and hydroxylated tyrosine to 3-(3,4-dihydroxyphenyl)-alanine (DOPA), suggesting that it is involved in betalain biosynthesis. The tyrosinase was not specific for tyrosine, and in addition to the monophenolase activity, the enzyme also oxidized diphenols to *o*-quinones (diphenolase activity). The native enzyme appeared to be a heterodimer of two subunits with M_rs of 27 000 and 30 000, which is unusual for tyrosinases. Typical tyrosinase inhibitors such as tropolone, 2-mercaptobenzothiazole and benzoic acid strongly inhibited the enzyme.

INTRODUCTION

Tyrosinases (EC 1.14.18.1 and EC 1.10.3.1), also termed polyphenoloxidases (PPOs), have been characterized from sources as diverse as bacteria, fungi, plants and animals and are usually associated with the biosynthesis of brown melanin pigments [1–4]. Tyrosinases catalyse two reactions: (1) the hydroxylation of mono- to di-phenols, called the cresolase or monophenolase reaction, and (2) the oxidation of diphenols to *o*-quinones, which is referred to as the catecholase or diphenolase reaction. When tyrosine is used as a substrate, it is first hydroxylated to form 3-(3,4-dihydroxyphenyl)alanine (DOPA) and then oxidized to dopaquinone, an unstable compound which polymerizes spontaneously to give rise to melanin pigments (Fig. 1).

The betalains are a class of pigments that occur in the plant order Caryophyllales and in certain basidiomycetes such as *Amanita muscaria* [5]. The first step in betalain biosynthesis is also the hydroxylation of tyrosine [6], but very little attention has been paid to the enzymology of this reaction. This is in part due to the difficulty in obtaining sufficiently active preparations from plants, where pigments accumulate very slowly and the levels of betalain producing enzymes are low. Even though very sensitive assays exist for the measurement of tyrosine hydroxylating enzymes [7, 8], it is often not possible to measure reliably the tyrosine hydroxylating activity in extracts from betalain producing plants.

In the mushroom *A. muscaria*, however, betalain pigments accumulate rapidly during the growth of the mushroom, making it an ideal system for characterizing enzymes involved in betalain biosynthesis [9]. The only enzyme from the betalain pathway to be characterized to date is the DOPA-4,5-dioxygenase from *A. muscaria* [10, 11]. Moreover, protein extracts have been described that contain acyl- and glucosyl-transferases involved in the synthesis of betacyanins in *Chenopodium rubrum* and *Dorotheanthus bellidiformis* [12, 13]. In this paper, we report the purification and characterization of a tyrosinase from the coloured parts of *A. muscaria*.

RESULTS AND DISCUSSION

Tissue-specific expression

In intact *A. muscaria* mushrooms, only the cuticle of the pileus is coloured red, but upon wounding, a yellow coloration appeared underneath the cuticle. In this zone, betalain producing enzymes seemed to be present, but lacked either substrate or oxygen. The remaining tissue was white, and no brown coloration appeared that would indicate the formation of melanin-like pigments. The tissue specificity of tyrosinase expression was tested by assaying tyrosinase activity in the different parts of the mushroom: cuticle, tissue just beneath the cuticle, and stalk tissue. The extracts were analysed (25 μg of protein) on native polyacrylamide gel electrophoresis (PAGE) with subsequent staining for activity (Fig. 2). Tyrosinase activity was present in the coloured cuticle of the pileus, as well as in the tissue just beneath. No signals were detected in extracts from stalk tissue, suggesting that tyrosinase activity was confined exclusively to coloured tissue.

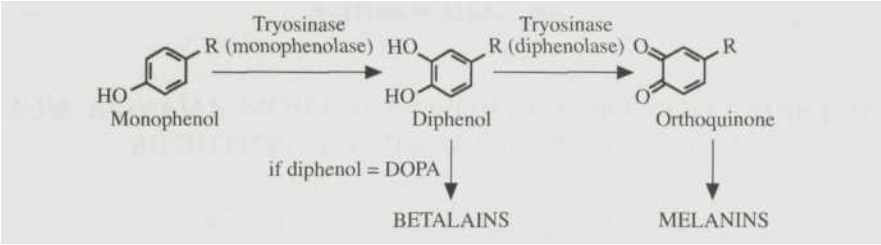

Fig. 1. The biosynthetic relationship between betalain and melanin pigments. A monophenolic substrate (tyrosine) is converted into a diphenol by the monophenolase activity of tyrosinase. In the biosynthesis of betalains, this intermediate is converted into betalamic acid by the action of another enzyme, DOPA-4,5-dioxygenase. In the biosynthesis of melanins, the diphenolic intermediate is further oxidized by the diphenolase activity of tyrosinase to yield o-quinones, which polymerize to form melanin-like pigments.

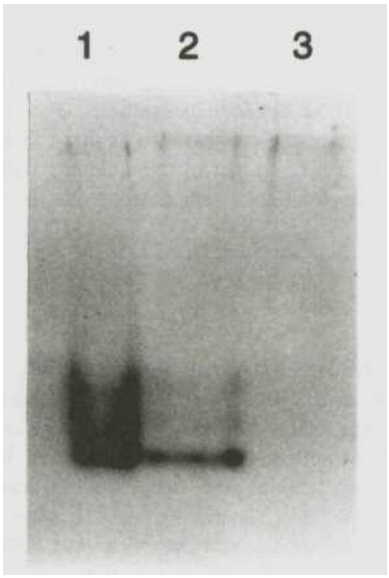

Fig. 2. The tissue-specific expression was assayed by analysing extracts from different parts of the mushroom on native PAGE with subsequent staining for activity as described in the Experimental. Activity was found in the coloured cuticle (lane 1) and in the tissue just beneath the coloured cuticle (lane 2). Stalk extracts were devoid of tyrosinase activity (lane 3).

Enzyme purification and subunit composition

Purification of the tyrosinase hydroxylating activity from the coloured parts of the mushroom resulted in the accumulation of two proteins of M_r 30 000 and 27 000, but the extract still contained minor impurities (Fig. 3A, lane 4). The purified preparation was subjected to native PAGE, which was either stained for activity or run on a second dimension denaturing PAGE (Fig. 3B). The activity coincided with the double band. The tyrosinase activity eluted from the gel filtration column at a M_r of about 50 000, indicating that the enzyme is active as a heterodimer. In comparison, the tyrosinase from *Agaricus bisporus* consists of two subunits that form a tetrameric structure with an apparent M_r of 120 000 [14] and that from *Neurospora crassa* is a monomeric protein **[15],** but tetrameric forms have also been reported [16]. Plant polyphenol oxidases were previously considered to be proteins with M_r*s* of *ca* 45 000, but recent work on PPO from broad bean leaves [17] and tomato [18] has shown that the native M_r is in the order of 60 000. In terms of quaternary structure, the *A. muscaria* tyrosinase resembles the *A. bisporus* enzyme, which also consists of two different subunits. However, unlike the *A. bisporus* enzyme, the tyrosinase

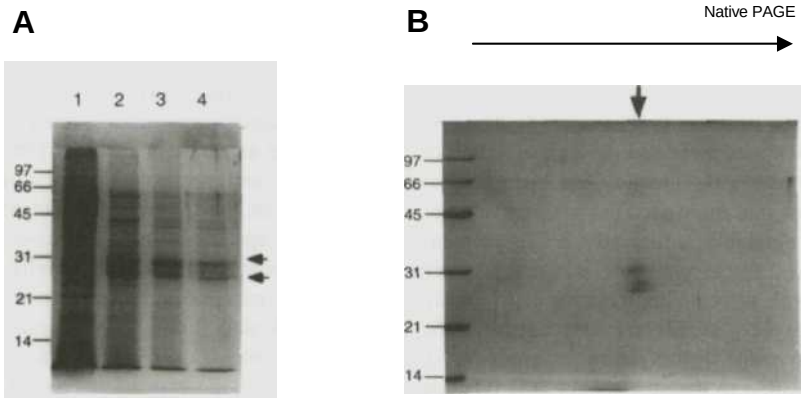

Fig. 3. (A) Purification of *Amanita muscaria* tyrosinase: lane 1 shows a crude extract of *A. muscaria* proteins; lane 2 is after step 3, lane 3 after step 4, and lane 4 after step 7, as described in the methods. (B) Lane 4 (Fig. 3(A)) was subjected to 2D PAGE. The first dimension was run under non-denaturing conditions and the second in the presence of SDS. Activity stain of the first dimension gel revealed the presence of tyrosinase, and is indicated by the arrow. The activity coincides with the two bands with M_rs of 27 000 and 30 000.

subunits from *A. muscaria* apparently form a heterodimer and not a heterotetramer. Structural differences to other tyrosinases were also reflected in the fact that the *A. muscaria* tyrosinase did not cross-react with antibodies against *A. bisporus* or *N. crassa* tyrosinases (results not shown).

Native polyacrylamide gels that were run with crude extracts and stained for activity showed a pattern of tyrosinase isoforms, which changed during purification. The purified extracts yielded only one band, although in some preparations a faint second band was also detected. Isoenzyme forms of tyrosinases have been described from other sources and have been suggested to arise from conformational changes [19], partial digestion and post-translational modifications [20], covalent attachment of phenolic material [21] or association and dissociation equilibria, which may occur either between tyrosinase subunits or arise from interactions with other proteins [22]. Which of the effects are involved in the case of the *A. muscaria* tyrosinase has not been investigated.

Substrate specificity and effect of pH

The tyrosinase was not specific for tyrosine. It hydroxylated other monophenols, like phenol, tyramine and 3-(4-hydroxyphenyl)propionic acid, and oxidized L-DOPA, D-DOPA, 3-hydroxytyramine and 3-hydroxycaffeic acid to their corresponding *o*-quinones (Table 1). Monophenols that contained their hydroxyl group in the *meta*-position and aromatic rings without hydroxyl groups were not hydroxylated. L-Tyrosine had the lowest K_m of all substrates tested, and had the lowest V_{max}. The amino-group seems to be important for strong binding to the enzyme, since L-tyrosine and tyramine have relatively low K_m values. The diphenolase reaction inactivated the enzyme, which is typical for tyrosinases (data not shown) [21]. The pH optimum for the hydroxylation reaction was very large, with a maximum of *ca* pH 6. No activity was observed below pH 3, but at pH 8 the enzyme still retained 75% of the maximal activity. These kinetic data are similar to those observed for other tyrosinases [23–25].

Table 1. Kinetic properties of the tyrosinase from *Amanita muscaria*. Values are given for monophenol and diphenol substrates. The V_{max} values are given relative to L-tyrosine

Substrate	K_m (mM)	V_{max} (rel.)
Monophenols		
L-Tyrosine	0.3	1.0
Tyramine	0.71	6.3
p-Hydroxyphenylpropionic acid	2.31	14.1
Phenol	2.3	2
m-Tyrosine	*	*
Diphenols		
L-DOPA	1.2	8.3
D-DOPA	5.3	22
Dopamine	0.65	11.9
Hydrocaffeic acid	21.4	7.9
Catechol	83	42.6

*No reaction.

Effect of inhibitors

The *A. muscaria* tyrosinase was strongly inhibited by typical tyrosinase inhibitors such as 2-mercaptobenzothiazole (2-MBT) and tropolone [26]. Tropolone is a structural analogue of L-mimosine, an often used, but weaker inhibitor than tropolone. The inhibitor concentrations for half maximal activity (i_{50}) of the monophenolase activity were of the order of 1 μM for tropolone and 3 μM for 2-MBT, when assayed with the substrate tyramine. The addition of 10 μM tropolone inhibited tryosinase activity completely, while a concentration of 50 μM 2-MBT was necessary for complete inhibition. Inhibition by benzoic acid showed the characteristic pH dependence [27]. Benzoic acid at 1 mM inhibited tyrosinase activity at pH 5 by more than 95%, but no inhibition was observed at pH 7.

Stability

The purified enzyme was stable for about three months at −20° and sustained several freeze/thaw cycles when 10% glycerol was added to the freezing buffer. When stored at 4°, the addition of $CuSO_4$ stabilized the enzyme, in the absence of $CuSO_4$ half of the activity was lost in three days.

Co-factor requirements

A general feature of tyrosinases is the presence of Cu^{2+} at their active site. The inhibition of tyrosinase by tropolone has been attributed to the Cu^{2+}-chelating properties of this compound [26]. In mushroom tyrosinase, addition of $CuSO_4$ to enzyme inhibited by tropolone restored enzymic activity [26]. The addition of $CuSO_4$ to a reaction mixture containing 10 mM tyramine and 5 μM tropolone restored activity of *A. muscaria* tyrosinase, although a large excess of $CuSO_4$ (0.5 mM) was needed. Addition of 1 mM K_2SO_4 failed to restore activity, whereas addition of the sulphate salts of manganese, magnesium or iron restored only *ca* 10% of the activity when compared to $CuSO_4$ (100%). This indicates that the *A. muscaria* enzyme contains Cu^{2+} at the active site.

Tyrosinase function

There has long been speculation of the involvement of a tyrosinase-type enzyme in betalain biosynthesis [5, 28]. Progress has been made recently by using PCR techniques to amplify PPO fragments from a *Phytolacca americana* cDNA library [29], but this method is limited to a characterization of the enzyme at the molecular biology level. The results have to be inter-

preted with caution, because PPOs are almost ubiquitous enzymes. They are often found in fruits, and are present in a latent form [17, 30]. There is still much speculation on the function of PPOs, and their role in plant defence, phenolic metabolism and photosynthesis [31] has been discussed, but they have never been associated with a defined biosynthetic pathway.

The tyrosinase from *A. muscaria*, by comparison, is located exclusively in the coloured parts of the mushroom, and readily hydroxylates tyrosine to DOPA, which corresponds to the first step in betalain biosynthesis. Other mushroom tyrosinases have only been linked to the synthesis of melanin pigments, but our results strongly suggest that a tyrosinase is involved in the biosynthesis of betalains in *A. muscaria*.

EXPERIMENTAL

Reagents. All reagents were purchased from Fluka, except DEAE Sepharose and Sephadex G-100 (Pharmacia).

Biological material. Young *A. muscaria* mushrooms were collected in September and October 1992 and 1993. The orange coloured tissue was removed, frozen in liquid N_2 and stored at −70°.

Purification. All purification steps were carried out at 4°.

Step 1: crude extract. Frozen coloured tissue of the pileus of *A. muscaria* (*ca* 100 g fr. wt, containing *ca* 300 mg protein) was ground to a fine powder in liquid N_2. Extraction buffer (100 mM K-Pi buffer (pH 7.5), 1 mM phenylmethanesulphonyl fluoride, 10% glycerol and 10 mM ascorbic acid) was added and the suspension sonicated on ice 5 times during 30 sec. The extract was centrifuged at 5000 *g* to remove large debris.

Step 2: $(NH_4)_2SO_4$ pptn. The supernatant was brought to 45% satn by slowly adding 0.258 mg ml^{-1} solid $(NH_4)_2SO_4$. The pptd proteins were pelleted at 20 000 *g*. The supernatant was then brought to 65% satn, by adding 0.123 mg ml^{-1} $(NH_4)_2SO_4$ and again centrifuged. The pellet was resuspended in 0.01 M K-Pi (pH 7), containing 10% glycerol and 5 mM ascorbic acid, and dialysed against the same buffer for 4 hr with several changes of buffer.

Step 3: ion exchange chromatography. The soln (50 ml, *ca* 3 mg ml^{-1} protein) was applied to a DEAE Sepharose column (50 ml bed vol.) and the active frs were collected after elution with 0.5 M NaCl. The pooled frs (30 ml) contained *ca* 0.7 mg ml^{-1} protein.

Step 4: gel filtration. The pooled active frs were pptd with 75% $(NH_4)_2SO_4$ and loaded on a Sephadex G-100 gel-filtration column (100 ml bed vol.). The elution buffer was 0.01 M K-Pi (pH 7), 5 mM ascorbic acid and 0.5 M NaCl. The resulting prepn (18 ml) contained 0.2 mg ml^{-1} protein.

Step 5: DEAE-Sephadex chromatography. The active frs were pooled and dialysed and again applied to a DEAE-Sephadex column (5 ml bed vol.) equilibrated with 0.01 M K-Pi buffer (pH 6), 10% glycerol and 5 mM ascorbic acid. A 50 ml gradient from 0 to 0.5 M NaCl was applied.

Steps 6 and 7: steps 4 and 5 were repeated, but the DEAE column was run at pH 7. The final prepn contained 0.1 mg ml^{-1} protein in a total vol. of 10 ml.

Tyrosinase assays. Monophenolase activity was routinely assayed using a method adapted from ref. [32]. The enzyme soln (5 μl) was added to 50 μl 0.1 M K-Pi buffer (pH 6.8), containing 5 mM substrate (tyramine), 50 μM $CuSO_4$ and 5 mM ascorbic acid. The addition of ascorbic acid prevented formation of quinone products, and tyramine was preferred to tyrosine as a substrate because of its higher V_{max} values (Table 1). The incubation was stopped after 30 min by acidifying the reaction mixt. with 50 μl 0.5 M HCl. After addition of 50 μl of a soln of 100 mg ml^{-1} $NaNH_2$ and 100 mg ml^{-1} $MbNO_3$, samples were allowed to stand for 5 min. Alkalization with 50 μl of 2 μ NaOH generated a red colour with an A_{max} of *ca* 490 nm, which was immediately measured using an ELISA-Reader.

Effect of inhibitors. The effect of tropolone and 2-MBT was measured using the assay for monophenolase activity as described above, except that inhibitors were added from 25× stock solns in the desired concns (0.1, 0.5, 1, 2, 5 and 10 μM). The pH dependence of benzoic acid inhibition was measured by assaying enzyme activity between pH 3.5 and 7.0, with steps of 0.5 pH unit, in the presence of 1 mM inhibitor and in its absence.

Diphenolase assay. For determination of diphenolase activity, 5 μl of each fr. was assayed in 50 μl 0.1 M K-Pi buffer (pH 6.8), 50 μM $CuSO_4$ and 5 mM tyramine. The assays were carried out in multi-well plates and the *A* of the quinone measured at 490 nm using an ELISA-Reader.

Measurement of kinetic parameters. The consumption of O_2 during the reaction was measured using a Clark-type oxygen electrode (Radiometer Copenhagen) [23]. Substrate concns were varied from 0.1 to 10 mM. Values for K_m and V_{max} were determined on Lineweaver–Burk plots. Diphenolase activity was measured using a Shimadzu photometer, by directly monitoring *A* of the respective quinone formed during the reaction. A_{max} and specific absorbances were taken from ref. [33].

Electrophoresis. SDS–PAGE was performed according to ref. [34]. A polyacrylamide concn of 15% was used in the separating gel.

Native PAGE. This was carried out according to the method for denaturing PAGE, but SDS was omitted, as was addition of DTT and boiling of the sample. The polyacrylamide concn was 10%. Electrophoresis was performed at 4° if the gels were to be stained for activity.

Activity staining after native PAGE. Tyrosinase activity was stained by incubating the native gels after electrophoresis in 5 mM tyramine. After 15–20 min, dark bands appeared, indicating formation of melanin-like products and the presence of tyrosinase.

2D PAGE. The first dimension was run as a native PAGE, as described above. The lanes were excised from the gel and soaked in sample buffer containing

SDS before being placed on top of a conventional SDS-PAGE second-dimension gel.

Protein concn. This was measured according to the method of ref. [35].

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