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BIOGENESIS OF BETALAINS: PURIFICATION AND PARTIAL CHARACTERIZATION OF DOPA 4,5-DIOXYGENASE FROM *AMANITA MUSCARIA*

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Key Word Index—*Amanita muscaria;* mushroom; DOPA; ring-cleavage; betalamic acid; biosynthesis; betaxanthins; betalains.

Abstract—3,4-Dihydroxyphenylalanine 4,5-dioxygenase, a central enzyme in the biogenesis of betalains, has been purified from the mushroom *Amanita muscaria.* Like other extradiol-cleaving dioxygenases, this enzyme is an oligomer; however, DOPA 4,5-dioxygenase is composed of varying number of an identical subunit of M_r 22 000. It is inhibited by cyanide, diethylpyrocarbonate and various nitrogen-containing ion chelating agents. The enzyme does not exhibit a strict specificity for DOPA. Other p-dihydric aromatic compounds such as dopamine and catechol are also converted to α-hydroxymuconic ε-semialdehyde derivatives. This is the first report of an enzyme involved in the metabolism of betalain pigments.

INTRODUCTION

Betalains, a class of yellow and violet nitrogenous water-soluble pigments, are restricted to plants belonging to the order Centrospermae (see [1,2] for review) and they have also been shown to be the pigments of the cap (pileus) of mushrooms of the genera Amanita and Hygrocybe [3,4].

On the basis of radiotracer feeding experiments (Fig. 1), it has been postulated that the pigment chromophore, betalamic acid (4), originates from 3,4-dihydroxyphenylalanine (DOPA; 1) by an extradiol fission (A) of the aromatic ring and subsequent closure of the resultant intermediate to a heterocyclic system [5]. These findings are in agreement with proposals based on the structural investigations [6].

This oxidative disruption of the ring system appears to be analogous to the aerobic cleavage of the aromatic ring of catechols in microbes, which is catalysed by specific dioxygenases, a class of enzymes that contain ferrous ion as an essential metal cofactor [7-10]. A similar reaction can be seen in stizolobic acid (5) and stizolobinic acid (6) biosynthesis in *Stizolobium hassjo* [11, 12]. The former amino acid arises from the same extradiol cleavage of DOPA (1) as 4. Stizolobic acid synthase has been shown to attack at the carbon-carbon double bond adjacent to the p-hydroxyl group on the aromatic nucleus of DOPA. In this extradiol type of ring fission, α-hydroxyl-γ-alanine muconic-ε-semialdehyde (2) is formed before further dehydrogenation and recyclization [13]. Although the proposed mechanism for biosynthesis of 4 requires an enzyme of the extradiol dioxygenase type, there is no information on the enzymes involved in typical reactions of betalain biosynthesis. The present paper describes the isolation of DOPA 4,5-dioxygenase prepared from coloured tissues of *Amanita muscaria* based on the combination of two affinity chromatographies and its characterization.

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RESULTS

Purification of DOPA 4,5-dioxygenase

The purification procedure for DOPA 4,5-dioxygenase from *A. muscaria* is described in detail in the Experimental. In order to protect the enzyme from partial digestion by proteases released during cell breakage, it was necessary to maintain a concentration of 1 mM PMSF in the buffer. DTT was also added to inhibit polyphenol oxidase activity. The yellow-brown acetone precipitate gave a yellow-orange solution after treatment with calcium phosphate gel. In the presence of 17% glycerol, this enzyme preparation appeared to be very stable to storage at -20° and to several freeze/thaw cycles.

The affinity chromatography steps proved to be an effective method for separation of DOPA 4,5-dioxygenase from contaminating proteins which were not removed by the other procedures. Two affinity columns were prepared by coupling L-DOPA (1) and isonicotinic acid with its carboxyl group to EAH-Sepharose 4B (Pharmacia). The elution profile on the DOPA-column is shown in Fig. 2. No metabolism of DOPA linked to Sepharose has been observed, although the ligand was readily oxidized. One interesting feature is that the enzyme was not eluted by DOPA (10 mM) when it was added to the buffer. We did notice however, that the column turned yellow with a maximum absorbancy at 424 nm as the DOPA solution was passing through the column, indicating that catalytic sites were free to interact with the substrate. Although the DOPA 4,5-dioxygenase-containing peak is rather broad and seems to be composed of different species, the activity

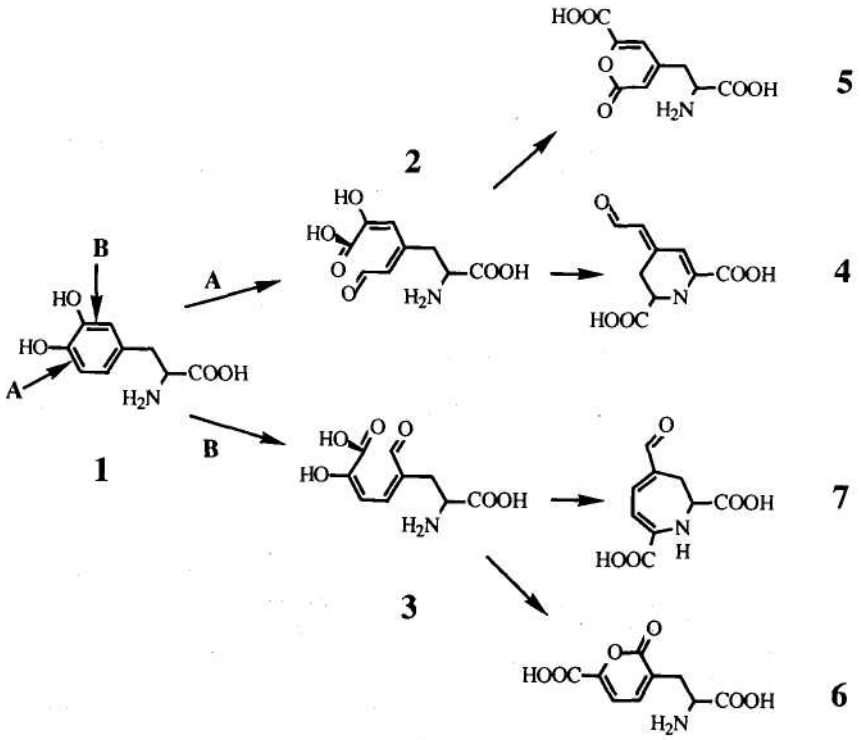


Fig. 1. Biosynthetic route to betalamic acid [1, 2].

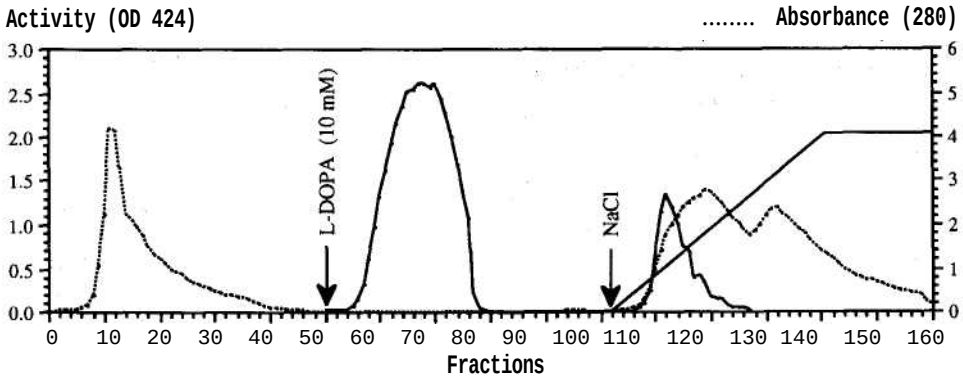


Fig. 2. Elution profile of DOPA 4,5-dioxygenase on affinity column to which L-DOPA is linked. The column effluent was monitored at 280 nm. The precipitate obtained by $(NH_4)_2SO_4$ precipitation was applied to the column equilibrated in 50 mM Tris-HCl buffer containing 5 mM DTT. After washing, L-DOPA (l0 mM) was added to the buffer. Elution of the column was achieved by a linear gradient (0-0.4 M NaCl) in the same buffer. Fractions (400 *µl)* were collected and analysed for their activity according to the following assay mixture: 5 *µl* of each fraction was diluted in 20 *µ1* of buffer containing 10 mM L-DOPA and incubated at 20° for 30 min. The mixture was made 200 *µl* with water and the absorbance measured at 424 nm.

decreased in the trailing fractions and they were more coloured (orange) than those of the peak fractions. However, gel electrophoresis showed no additional protein contaminants. The DOPA 4,5-dioxygenase pool was concentrated and applied to the isonicotinic acid-column. Analysis of the active fractions from this column by SDS-PAGE showed that a single product of relative M_r of 22000 was retained while a significant amount of the samples were not adsorbed (compare lanes 14 and 15 in Fig. 3). Thus, the affinity column appears to be somewhat selective by separating the inactive from the active forms of enzyme molecules. The purified enzyme exhibited characteristic absorption spectrum of ferroprotein in both its ferric (λ = 635 nm) and ferrous (λ = 432, 553 nm) states [14, 15], a property not shared by other extradiol dioxygenases which lack the visible spectrum or exhibit a yellow colouration (λ = 414 nm). It is interesting to notice that protocatechuate 4,5-dioxygenase (EC 1.13.11.7) contains equal quantities of ferrous and ferric iron, this latter not being involved in the enzyme activity [10].

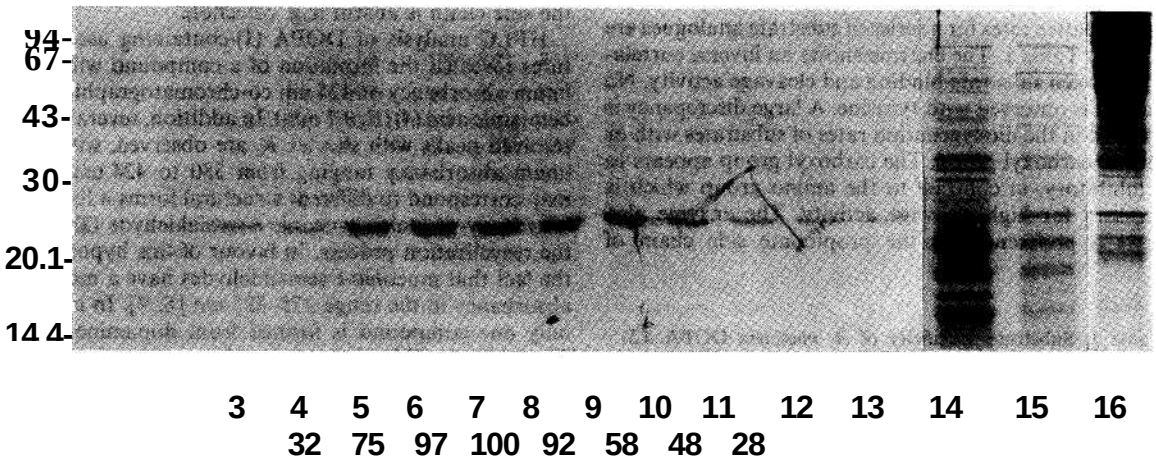


Fig. 3. SDS-PAGE analysis of the active fractions collected after affinity chromatography on both matrices. The different aliquots of protein were precipitated with acetone and the pellet resuspended in 25 µl of sample buffer and incubated 5 min at 100°. The separating gel contained 14% acrylamide. Protein was visualized by Coomassie Blue staining. Lanes 1-13, fractions from the peak of DOPA 4,5-dioxygenase activity (the relative activity of each fraction is indicated below); lane 14, proteins not retained by the first affinity column; lane 15, $(NH_4)_2SO_4$ precipitate (step 3); lane 16, crude extract.

M_r *and subunit structure*

The purified enzyme moved as a broad diffuse band when gel electrophoresis was conducted under non-denaturing condition. When enzyme activity was directly tested in the gel, the specific stain showed that DOPA 4,5-dioxygenase was present throughout this band. From calibrated gels with proteins of known M_r, the M_r of functional DOPA 4,5-dioxygenase was estimated to be 75000-140000. When the gel containing the broad protein band was placed at the top of a slab gel containing SDS and electrophoresis conducted in a second dimension, only the 22000 protein was observed (Fig. 4) and several spots could be distinguished. Isoelectricfocusing conducted under native conditions (an essential factor seems to be removed during IEF) and subsequent blotting of the protein onto nitrocellulose membrane showed an activity within the pI range 4.6-5.2 whereas IEF under denaturing conditions showed only one band having a pI value of 4.55. This subunit is not glycosylated as judged by the absence of any electrophoretic mobility shift after treatment with trifluoromethanesulphonic acid [16]. Together, these data suggest that the enzyme can exist in several different forms in solution, all of which are composed of the same subunit.

Effect of pH

The DOPA 4,5-dioxygenase exhibits a pH activity profile with a maximum near pH 8.5 (assaying the appearance of yellow products at 424 nm). At pH 7.0, the activity represents only 14% of the maximum value, 50% at pH 7.6, whereas it is still 80% at pH 10.

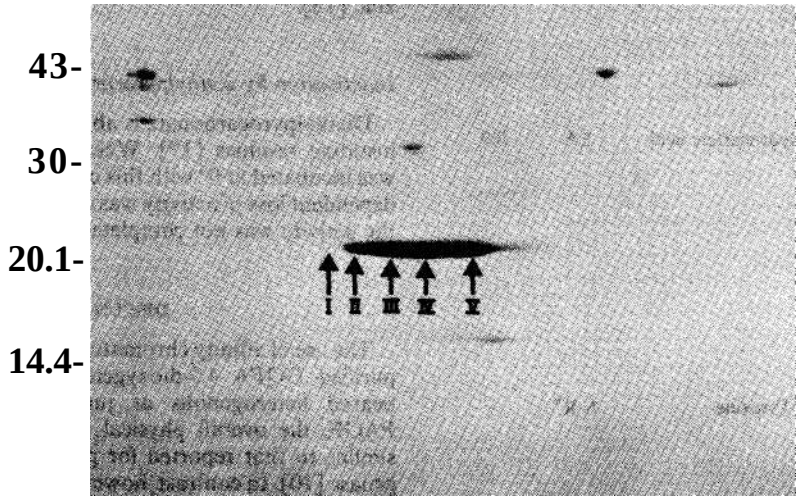
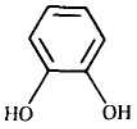
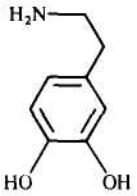
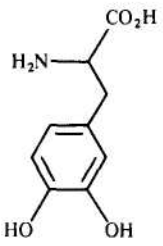
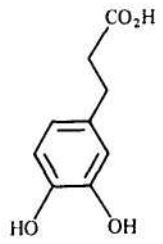
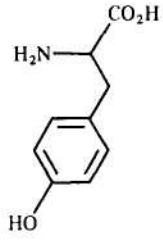


Fig. 4. Two-dimensional electrophoretic analysis of DOPA 4,5-dioxygenase. Panel is shown with the non-denaturing dimension horizontally with high molecular weight on the left and the SDS-PAGE dimension vertically. I-V are spots of the different oligomers.

Kinetic parameters

The relative rates for a series of substrate analogues are shown in Table 1. The enzyme shows an inverse correlation between substrate binding and cleavage activity. No reaction is observed with tyrosine. A large discrepancy is observed in the dioxygenation rates of substrates with or without carboxyl group. The carboxyl group appears to be inhibitory, in contrast to the amino group which is necessary for high cleavage activity. The enzyme also shows a preference for the propionate side chain of hydrocaffeic acid with substrate affinity decreasing when the side chain is absent (e.g. catechol).

Table 1. Substrate specificity of *A. muscaria* DOPA 4,5-dioxygenase. Relative rate of 100 corresponds to a molecular specific activity of 235 + 42 per monomer.

Substrate		V_{max}	K_M (mM)
	Catechol	375	75
	Dopamine	274	6.3
	L - DOPA	100	3.9
	Hydrocaffeic acid	5.6	0.9
	L-Tyrosine	N.R.*	–

* N.R.,no reaction

HPLC analysis of DOPA (l)-containing assay mixtures revealed the formation of a compound with maximum absorbancy at 424 nm co-chromatographing with betalamic acid (4) *(R,* 9.7 min). In addition, several poorly resolved peaks with shorter R_t are observed, with maximum absorbancy ranging from 380 to 424 nm. These may correspond to different structural forms adopted by α-hydroxy-γ-alanine muconic-ε-semialdehyde (2) during the recyclization process. In favour of this hypothesis is the fact that muconic-ε-semialdehydes have a maximum absorbancy in the range 375-385 nm [8, 9]. In contrast, only one compound is formed from dopamine by the dioxygenase, with a spectrum identical to that of betalamic acid, but with a longer R_t (13.2 min). These properties suggest that this product is the monodecarboxylated form of betalamic acid. The presence of free 2 in the DOPA assay mixture could be the consequence of repulsion effect between the negatively charged carboxyl groups of the alanyl side chain and of the α-hydroxy muconic-£-semialdehyde at the optimum pH of the enzyme. Such effect did not occur with dopamine, so that the recyclisation of the cleavage reaction product to the α-pyronyl aminoacid occurred spontaneously in basic condition. Beside these compounds, dopaxanthine and dopaminexanthine (miraxanthine V) respectively are also present in the extracts, as well as a new xanthine resulting from the condensation of Tris with the α-pyronyl amino acid. Neither stizolobic acid (5) nor stizolobinic acid (6) have been detected as reaction products.

Studies of various potential inhibitors have shown that the enzyme was inhibited by iron ligands such as pyridine, o-phenanthroline and CN^- at 1 mM concentration. In contrast, EDTA had no effect on enzyme activity, and the inhibitory effect of p-chloromercuribenzoate was ascribed to its binding to the active site of the enzyme. The initial velocities measured did not depend on whether enzyme or substrate was added last to the assay mixture. The anaerobic addition of DOPA to the native enzyme also effected a marked decrease in the cleavage activity. Moreover, DOPA 4,5-dioxygenase showed no enhancement of activity with added ferrous ion, manganese [9] or zinc [13].

Inactivation by diethylpyrocarbonate

Diethylpyrocarbonate is able to react specifically with histidine residues [17]. When DOPA 4,5-dioxygenase was incubated at 0° with this compound, a concentration dependent loss of activity was observed (Fig. 5). However, the activity was not completely inhibited.

DISCUSSION

The use of affinity chromatography resulted in a highly purified DOPA 4,5-dioxygenase. The preparation appeared heterogenous as judged by non-denaturing PAGE, the overall physical size of our enzyme being similar to that reported for protocatechuate 4,5-dioxygenase [10]. In contrast, however, the investigation of the subunit structure revealed one major band on SDS-PAGE. Therefore, it is possible to ascribe a composition of 4 to 7 (8?) identical subunits per native enzyme, but the exact stoichiometry of binding is not known.

The specific molecular activity of the enzyme for DOPA is usually in the range 235 + 42 molecules per subunit, although activity of the preparations varied considerably. This is ascribed to the removal of an essential cofactor which is assumed to be iron, or oxidation of ferrous ion to ferric form during the handling procedures, purification or freeze/thaw cycles. The inability of ascorbate and DTT to effect reactivation may indicate that the inactivation is progressive and is only initiated by the oxidation. The prevention of DOPA ring-cleavage by diethylpyrocarbonate suggests that histidyl residues are essential for enzyme activity. As for other reported dioxygenases [10, 18], iron is assumed to be coordinated to the imidazole group of histidine, but the fact that a residual activity remains may indicate that histidine residues have different reactivities or that the iron is also bound by ligands other than histidine.

The enzyme underwent a rapid loss of activity during catalysis with all substrates tested, the extent of this inactivation being apparently related to the amount of product formed. The product does not seem to inhibit the reaction however, because different V_{max} values were observed. Kinetic analysis suggests that the side chain moiety is important for binding of substrate and analogues to DOPA 4,5-dioxygenase, and is required for rapid substrate turnover. Thus, it is likely that the primary role of the side chain is to correctly position the substrate in the active site although it probably also modulates the reaction via inductive effects through the aromatic ring.

The reaction product appears to be α-hydroxy-γ-alanylmuconic ε-semialdehyde (2). In this respect, the enzyme cleaves specifically at the double bond adjacent to the p-hydroxyl group, although extradiol-type cleavage adjacent to the *meta* position might occur occasionally as a side reaction. The latter is not observed at the pH conditions used for the enzyme assay (only one peak is detected from catechol or dopamine containing assay mixture by HPLC). However, it can not be ruled out that, at neutral pH, the net charge of the ionized form of the carboxyl and the amino group may allow the substrate to bind in two orientations with 3-hydroxyl and 4-hydroxyl competing for the same ligand site. In such conditions, muscaflavine (7) would be generated by analogous ring closure of α-hydroxy-δ-alanine muconic-e-semialdehyde (3) as in the case of betalamic acid. It is interesting to notice that, if 7 is detected in *Amanita muscaria*, it exists as a minor pigment [19].

The isolation of the DOPA 4,5-dioxygenase from *A. muscaria* represents an important step in resolving the biogenesis of betalains. These results may aid in the purification and characterisation of the corresponding plant enzyme and hence in the study of the regulation of the biosynthesis of betaxanthins and betacyanins and their metabolic relationship [20].

EXPERIMENTAL

Biological material. Young carpophore of the mushroom *Amanita muscaria* were collected from the field in September and October 1987. The orange coloured tissue was removed, frozen in liquid N_2 and stored at —20°.

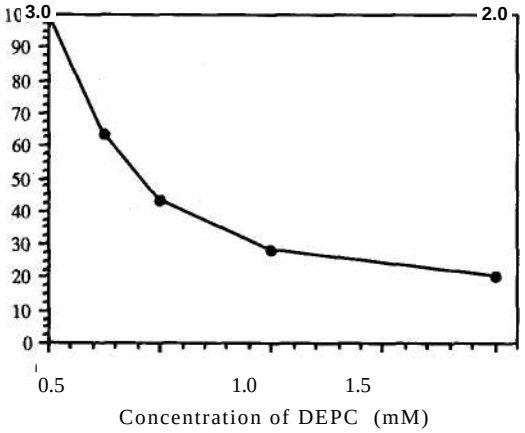


Fig. 5. Effect of diethylpyrocarbonate (DEPC) on enzyme activity. DOPA 4,5-dioxygenase in 10 mM Tris-HCl pH 8.5 buffer, was incubated at 0° with 0.25, 0.5, 1 and 2 mM of diethylpyrocarbonate. After 15 min, 10 *μl* aliquots of the mixture were added to 40 *μ1* of assay mixture. The extent of inhibition was calculated by comparing the modified enzyme to reference treated in a similar manner but without DEPC.

Purification of DOPA 4,5-dioxygenase. Step 1: crude extract. All purification steps were carried out at 4° unless otherwise specified. The purification buffer contained 50 mM Tris adjusted to pH 7.5 with HC1, 1 mM PMSF and 5 mM DTT. Frozen coloured tissue of the cap (pileus) of *A. muscaria* (10 g) was ground to a fine powder with liquid N_2 and resuspended in 50 ml of buffer. The suspension was sonicated with a Branson Sonifier at maximum power for 5 min. The supernatant soln was separated from the residue by centrifugation at 20 000 *g* for 30 min. The pellet was washed once with 25 ml buffer.

Step 2: acetone fractionation. Cold Me_2CO (1 vol., -20°) was added slowly to the combined supernatants and, after 60 min of incubation at —20°, the resulting ppt. was removed by centrifugation. Another 0.5 vol of cold Me_2CO was added to the supernatant to give a final concn of 66% Me_2CO. After 60 min incubation at —20° the resulting ppt. was collected by centrifugation and resuspended in *ca* 25 ml of 100 mM Tris-HCl, pH 7.5.

Step 3: calcium phosphate gel treatment. To this soln was added 2.5 ml of calcium phosphate gel suspension [21] with stirring. After 10 min, the gel was collected by centrifugation and washed once with 25 ml H_2O. The enzyme was then eluted twice from the gel, each time with 12.5 ml 100 mM Na_2HPO_4 adjusted to pH 7.0 with H_3PO_4. The supernatant was brought to 44% saturation in $(NH_4)_2SO_4$. After overnight precipitation at 4°, proteins were collected by centrifugation. The pellet was dissolved in 2.5 ml glycerol-H_2O (5:1). The preparation was stable indefinitely when stored at —20°.

Step 4: affinity chromatography. The enzyme preparation was applied to the affinity columns (approximately 2 ml resin) prepared by coupling L-DOPA or isonicotinic acid to EAH-Sepharose 4B (Pharmacia) with a water-soluble carbodiimide (EDC; Sigma) according to established procedures and the manufacturer's instructions. The affinity matrix was equilibrated with 50 mM Tris-HCl, pH 7.5 buffer containing 5 mM DTT. The flow rate was set at 24 ml hr^{-1} and the fraction volume was 400 μl. The enzyme activity was eluted with a linear gradient (20 ml) of buffer and buffer containing 0.4 M NaCl. The active

Dr F. Terradas has shown that α-hydroxyl-γ-alanine muconic-ε-semialdehyde (2) and α-hydroxy-δ-alanine muconic-ε-semialdehyde (3) recyclize spontaneously to betalamic acid (4) and muscaflavine (7) respectively, in acidic condition (Ph.D. thesis. University of Lausanne, 1989).

fractions were pptd by addition of 2 vol cold Me_2CO and resuspended in 100 μ\ glycerol soln.

Enzyme activity determination. DOPA 4,5-dioxygenase was routinely assayed by measuring the appearance of the absorbance due to product from 190 to 600 nm. A typical assay was carried out at 23° for 3 min in a total vol. of 50 µl in buffer containing 50 mM Tris adjusted to pH 8.5 with 50 mM PIPES and 1 mM DTT and 10 mM L-DOPA. Inhibitors (dissolved in water with the pH adjusted to 8.5) were used at a 1 mM concn.

Identification of the reaction products. Analytical HPLC (Spectra Physics system) was performed on a RP column (250 x 4.6 i.d. mm) packed with Hypersil ODS RP18 (3 μm particle size, Shandon), fitted with a guard column (20 x 4.6 i.d. mm) containing LiChroprep RP18 (Merck). An HP ChemStation (Hewlett Packard) was used for column eluent analysis. Detection was undertaken from 190 to 600 nm. Buffer A contained 50 mM NaH_2PO_4 and 2.5 mM Et_3N and was adjusted to pH 4.2 with H_3PO_4 acid. Buffer B consisted of a 40% MeCN. The column was equilibrated in 100% A at a flow rate of 1.0 ml min^{-1}. The analytical gradient was 0-15 min: 0-30% B; 15-20 min: 30-60% B; 20-25 min: 60-80% B. The injection vol. was 20 μl. Peaks were identified by their UV/visible spectrum, by comparison with lit. data [8, 9, 22] and by co-chromatography with authentic references prepared following the procedure described in [22] from crystallized betanin extracted from red beet.

Treatment with diethylpyrocarbonate. The enzyme was treated with diethylpyrocarbonate following the procedure described in ref. [23].

Electrophoresis. SDS-PAGE was performed according to ref. [24] and gels were stained with Coomassie Brilliant Blue R-250. Electrophoresis conducted under non-denaturing conditions was carried out in discontinuous acrylamide gel: the separating gel (1.5 mm thick) consisted of 7.6% acrylamide and 0.4% Bis in 0.24 M Tris-HCl buffer, pH 8.5, 0.04% TEMED; polymerization was induced by the addition of 0.04% ammonium persulphate. The stacking gel contained 4.85% acrylamide, 0.15% Bis in 0.04 M Tris-Pi buffer pH 6.9, 0.08% TEMED. Electrophoresis buffers contained 63 mM Tris-HCl pH 7.5 at the anode and 37.6 mM Tris, 40 mM glycine pH 8.9 at the cathode. The enzyme was loaded in upper gel buffer. The electrophoresis conditions were: 100 V until the sample had run into the separating gel and then 200 V. The DOPA 4,5-dioxygenase activity was specifically stained by incubating the gel in a 10 mM soln of dopamine at pH 8.0 in 50 mM Tris-HCl. The activity was indicated by formation of the yellow colour of the reaction product. The gel was then stained in Coomassie Brilliant Blue R-250. The band was cut out from the gel, weighed and equilibrated in an equivalent vol. of sample buffer [24] for 10 min. The band was then placed at the top of a SDS containing slab gel (3 mm thick), sealed with a 1 x concd sample buffer solution containing 1% agarose LMT and electrophorased in a second dimension.

Isoelectric focusing, pi determination of native enzyme was performed in thin layer gels (4.85% acrylamide, 0.15% Bis) containing 7% ampholines pH 4.0-6.5 (LKB) and 20% glycerol at 800 V constant voltage for 1 hr at 4°. Proteins were blotted onto nitrocellulose and enzyme activity assayed as described previously. Proteins were stained with Amidoblack.

Denaturing IEF was performed in polyacrylamide gel with Immobiline pH 4.2-4.9 gradient (LKB) containing 8 M urea, 0.5% ampholines pH 4.0-6.5 **(LKB),** 50 mM DTT and 20% glycerol. 1500 V (1 mA, 5 W) was applied overnight at 12°. Gel was soaked in 10% TCA overnight and then stained with Coomassie Brilliant Blue R-250.

Acknowledgements—We thank Dr A. Komamine for the gift of the samples of stizolobic acid and stizolobinic acid. We are grateful to Prof. H. Wyler for having focused our attention on the interest of *Amanita muscaria* as a source of enzyme for the synthesis of betalains.

REFERENCES

1. Mabry, T. J. (1980) in *Encyclopedia of Plant Physiology* **New** Series, Vol. 8, *Secondary Plant Products* (Bell, E. A. and Charlwood, B. V., eds), pp 513-533. Springer, Berlin.
2. Reznik, H. (1980) in *Pigments in Plants* 2nd Edn (Czygan, F. C, ed.), pp. 370-392. Gustav Fischer, Stuttgart.
3. Döpp, H. and Musso, H. (1973) *Chem. Ber.* **106,** 3473.
4. Von Ardenne, R., Döpp, H., Musso, H. and Steglich, W. (1974) Z. *Naturforsch.* 29, 637.
5. Impellizzeri, G. and Piattelli, M. (1972) *Phytochemistry* **11,**2499.
6. Wyler, H., Mabry, T. J. and Dreiding, A. S. (1963) *Helv. Chim. Acta* 46, 1745.
7. Nozaki, M. (1970) *Meth. Enzymol.* **17A,** 522.
8. Nakai, C, Hori, K., Kagamiyama, H., Nakazawa, T. and Nozaki, M. (1983) *J. Biol. Chem.* **258,** 2916.
9. Que, L., Widom, J. and Crawford, R. L. (1981) *J. Biol. Chem.* **256,** 10941.
10. Arciero, D. M., Lipscomb, J. D., Hanh Huynh, B., Kent, T. A. and Münck, E. (1983) *J. Biol. Chem.* **258,** 14981.
11. Ellis, B. E. (1976) *Phytochemistry* **15,** 489.
12. Saito, K. and Komamine, A. (1976) *Eur. J. Biochem.* **68,**237.
13. Saito, K. and Komamine, A. (1978) *Eur. J. Biochem.* **82,**385.
14. Fujiwara, M. and Nozaki, M. (1973) *Biochim. Biophys. Acta* **327,** 306.
15. Ishimura, Y. (1970) *Meth. Enzymol.* **17A,** 429.
16. Edge, A. S. B., Faltynek, C. R., Hof, L., Reichert, L. E. and Weber, P. (1981) *Analyt. Biochem.* **118,** 131.
17. Miles, E. W. (1977) *Meth. Enzymol.* 47, 431.
18. Ohlendorf, D. H., Lipscomb, J. D. and Weber, P. C. (1988) *Nature* 336, 403.
19. Barth, H., Burger, G., Döpp, H., Kobayashi, M. and Musso, H. (1981) *Liebigs Ann. Chem.* 2164.
20. Girod, P. A. and Zryd, J. P. (1990) *Plant Cell Tiss. Organ Cult,* (in press).
21. Colowick, S. P. (1965) *Meth. Enzymol.* 1, 90.
22. Döpp, H., Maurer, S., Sasaki, A. N. and Musso, H. (1982) *Liebigs Ann. Chem.* 254.
23. Aunis, D., Miras-Portugal, M. T., Mandel, P. (1973) *Biochim. Biophys. Acta* **327,** 313.
24. Laemmli, U. K. (1970) *Nature* **227,** 680.