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POLYPEPTIDE COMPOSITION OF TWO FUNGAL TYROSINASES

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Key Word Index—*Neurospora crassa*; *Agaricus bisporus*; fungi; mushroom; tyrosinase; MW; active enzyme centrifugation.

Abstract—The presence of two distinct molecular structures for tyrosinase in fungi is confirmed. The enzyme from *Agaricus bisporus* is acidic and comprises two dissimilar subunits which aggregate to form a tetramer. This tetramer constitutes the majority both in the resting and functional states. In *Neurospora crassa*, tyrosinase is slightly basic and contains only one subunit, similar in size to the larger subunit of the *Agaricus* enzyme. In the resting state *Neurospora* tyrosinase is distributed among a number of forms, from the monomer to the tetramer. In this case it was possible to show that a species smaller than the tetramer, probably the monomer, was fully active.

INTRODUCTION

For some years the minimal MW of tyrosinases (monophenol oxygenase EC 1.14.18.1) has been considered to be *ca* 32000 [1, 2] since purest preparations contained 0.2% copper and enzymes of this size have been isolated from various organisms, including bacteria [3] higher plants [4, 5] and one fungus, *Neurospora crassa* [6–8]. However this assumption has been challenged recently following an investigation of the quaternary structure of *Agaricus bisporus* tyrosinase [9] which indicated that the enzyme contained two dissimilar subunits, and with the determination of the complete amino acid sequence of the *Neurospora* enzyme [10]. Both these studies were conducted on the denatured state of the enzyme and it is now important to re-examine the properties of the native form in the knowledge of these findings. Thus we wish to report an ultracentrifugal study of both enzymes, performed in the presence and absence of phenolic substrates, and to compare the results obtained with the polypeptide composition of each enzyme.

RESULTS

Properties of isolated enzymes

Tyrosinase was purified from *N. crassa* and *A. bisporus* as previously described [7, 11] except that an additional step, column chromatography on hydroxyapatite, was added to the preparation of the latter. This procedure resolved the mushroom enzyme into four forms [12, 13] and the second in order of elution was used in this study. The properties of both preparations are summarised in Table 1. Specific activities compare favourably with previous reports in which the same assay was used [6, 7, 14], indicating that a high degree of purity had been obtained.

Table 1. Physico-chemical properties of *Agaricus* and *Neurospora* tyrosinases

	Agaricus	*Neurospora*
Specific activity (μmol L-DOPA oxidised/min per mg)	720	750
$s_{20,w}$ (sedimentation velocity)	6.48 S	4.50 S
MW (sedimentation equilibrium)	110000	47000–170000
Isoelectric point (5°)	4.70, 4.80, 4.85, 4.95	7.8
MW after denaturation		
major component	48000	46000
minor component	13000	34000
$s_{20,w}$ (active enzyme centrifugation)	6.53 S	4.30 S

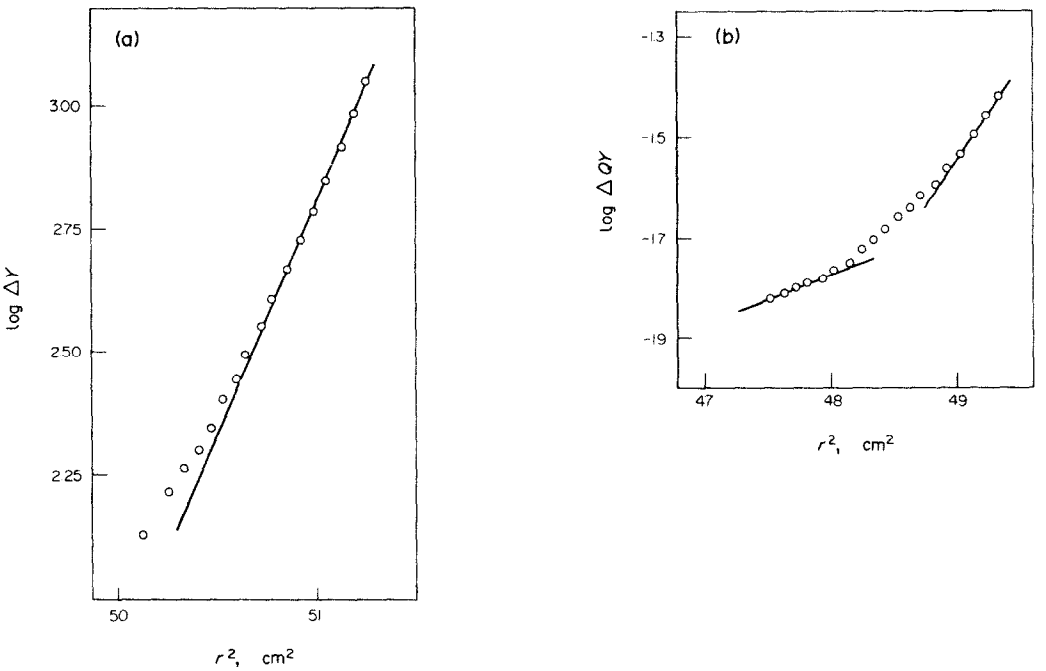

Fig. 1. Sedimentation equilibrium of (a) *Agaricus* tyrosinase 2.5 mg/ml in 0.05 M phosphate buffer pH 7, rotor speed 18 000 rpm, temperature 10° (b) *Neurospora* tyrosinase, 2 mg/ml in 0.1 M phosphate buffer pH 6, rotor speed 9943 rpm, temperature 20°. Logarithms of the fringe displacements are plotted against r^2 where r is the distance from the cell position to the axis of rotation; the ordinate in (b) is modified according to ref. [35]. The straight lines represent the theoretical slopes for MW of 47 000 and 170 000 in (b) and for 110 000 in (a).

Ultracentrifuge studies by the sedimentation velocity method showed that each sample sedimented in a single boundary but with quite different sedimentation coefficients. Sedimentation equilibrium studies showed that the *Agaricus* preparation consisted of a component with a MW of 110 000 somewhat contaminated with a low MW species, (Fig. 1a) while the *Neurospora* enzyme displayed a more complex behaviour, typical of an aggregating system (Fig. 1b). Several distinct populations appeared to be present, the limits apparently representing monomer and tetramer with a MW for the monomer of 47 000.

Polyacrylamide disc gel electrophoresis of the *Agaricus* preparation showed a single band (R_f, mobility relative to the dye front, 0.57) when stained for activity, and one major band of protein (R_f 0.57), although a further component (R_f 0.35) was also detected in trace amounts. The *Neurospora* preparation was poorly resolved containing a diffuse band of low mobility. Isoelectric focusing resolved the *Agaricus* enzyme into four closely spaced components with isoelectric points in the range 4.7–4.9, quite distinct from the isoelectric point determined for the *Neurospora* enzyme of 7.8. The difference in isoelectric points helps explain why the *Agaricus* enzyme is better resolved under the conditions used for gel electrophoresis, for which the running pH is 9.5.

In an attempt to assess the effect of substrate on the state of aggregation of the enzymes, active enzyme centrifugation was employed [15]. This method requires a trace amount of enzyme and normally measures the sedimentation coefficient of the heaviest active species, but Arnaud [16] has described a modification applicable to tyrosinase which is theoretically capable of yielding information on more slowly sedimenting species as well. This modification was employed with the *Agaricus* enzyme (Fig. 2a) but yielded only one boundary characterized by a sedimentation coefficient very similar to that determined in the absence of substrate (Table 1). No satisfactory boundary was obtained with the *Neurospora* enzyme until a different detection system with *p*-cresol as substrate and benzene sulphinate as indicator was substituted (Fig. 2b). The quinone produced in the oxidation of *p*-cresol was trapped as the sulphone [7, 17] and this was monitored at 325 nm. With *Neurospora* also the sedimentation coefficient obtained by this method was not significantly different from that obtained by the sedimentation velocity method (Table 1).

Polypeptide composition

Electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS) [18], showed that each preparation was heterogenous, each containing one strongly staining band and one more weakly staining component (Fig. 3). Using proteins of known MW as standards, allowed the MW of these components to be determined (Table 1) but it should be noted that for the band of highest mobility, (lowest MW) the assigned value is tentative for it is at the limit of the calibration range. For *Neurospora* tyrosinase the value determined for the major component, 46 000, agrees well with the minimum value obtained from the ultracentrifuge study. The discrepancies between the values obtained for the *Agaricus* enzyme by sedimentation equilibrium and electrophoresis in the

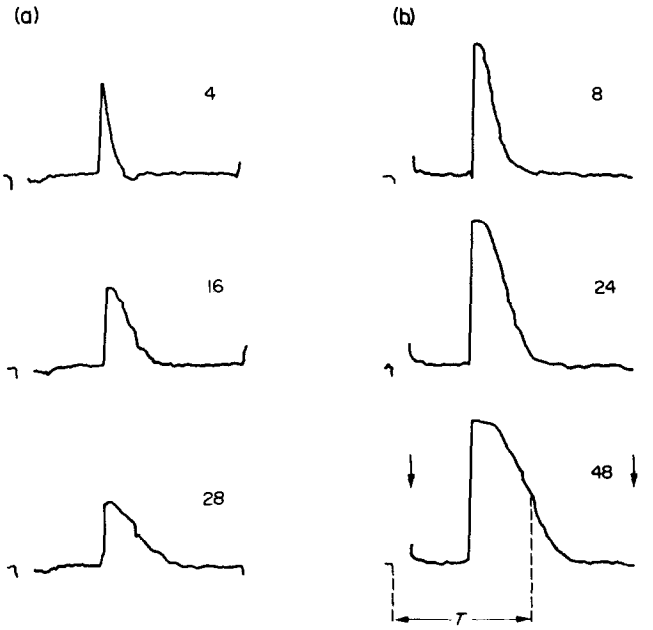

Fig. 2. Active enzyme centrifugation of (a) *Agaricus* and (b) *Neurospora* tyrosinases. Representative scans of the reaction products are shown, taken at 340 nm and 325 nm respectively, at times indicated in min above each curve; the arrows indicate the top and bottom of the cell with the top on the left. The distance (T) from the midpoint of the boundary to the inside edge of the reference line was measured for each scan and used to calculate the sedimentation coefficient as described in [36].

presence of SDS indicated the existence of quaternary structure and prompted a further experiment; the native enzyme was treated with a diimido-ester, as described by Davies and Stark [19] in an attempt to introduce intramolecular cross-links between subunits. Since the trace impurity, separated by disc gel electrophoresis with an R_f of 0.35, has a very similar mobility to the lowest MW band under the conditions used, it was removed by gel filtration [13] before the modification was attempted. The treatment caused no loss in enzyme activity but SDS gel electrophoresis showed that an additional species (of MW 92 000) was produced (Fig. 3c).

DISCUSSION

This study was made with tyrosinases which had been extensively purified. The molecular properties of the *Agaricus* enzyme are comparable with previous reports which cite $s_{20,w}$ values in the range 6.3–6.7 S [12, 20–22] and MWs of 116 000–119 000 [12, 23], but distinct from the aggregating system reported in [14]. For the *Neurospora* enzyme our data are superficially similar to the report of Fling *et al.* [6] who determined an $s_{20,w}$ value of 4.1–4.4 S and also obtained a distribution characteristic of an aggregating system by the sedimentation equilibrium method, but in their report a lower limiting MW of 33 000 was observed.

That both enzymes are composed of polypeptides with a MW of 33 000 has been advocated and confirmed repeatedly. Thus the proposal of Fling *et al.* [6] for the *Neurospora* enzyme was confirmed by us [7] and others [8]. Also the proposal that *Agaricus* tyrosinase was a tetramer of identical subunits was based on end group determination [23] and studies on the multiple forms and their interconversions [22]. It has proved impossible to

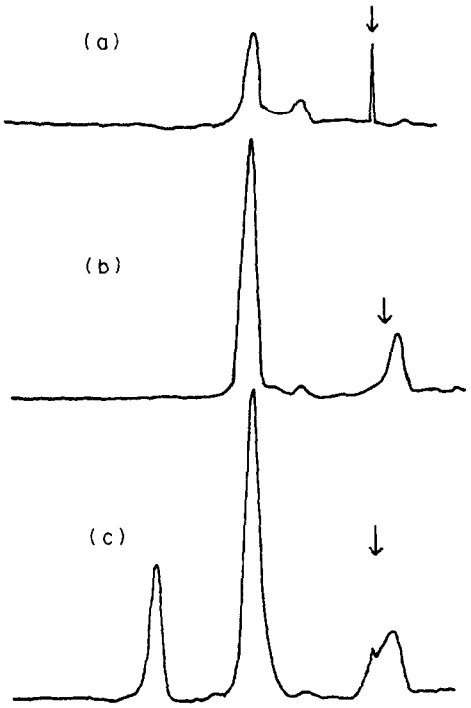

Fig. 3. Densitometric scans of tyrosinase from *Neurospora* (a) and *Agaricus*, before (b) and after (c) cross-linking with dimethyl suberimidate. Separations were made on 5% polyacrylamide gel rods 11 cm in length, in Na Pi containing SDS [18]. The tops of the gels are on the left and the ordinate refers to absorbance; the arrows denote the position of the tracking dye.

obtain in a reproducible manner, reasonable amounts of an active *Agaricus* enzyme corresponding to the proposed monomer. However its existence was favoured by the following observations: (a) gel filtration on a calibrated column in the presence or absence of substrate [24]; (b) $s_{20,w}$ value of 2.30 S determined by active enzyme centrifugation [24]; (c) occasional isolation of an enzyme of the expected molecular size [37, 22].

In contrast, this study shows that such an entity is present only in minor amounts and provides additional data to support the revisions proposed recently that the *Neurospora* enzyme has a MW of 46 000 [10, 25] and the *Agaricus* enzyme is a tetramer of the form H_2L_2 comprising two subunits of MW 43 000 and two with a MW of 13 000 [9]. Thus the major polypeptide present in our *Neurospora* preparation has a MW of *ca* 46 000 as determined by sedimentation equilibrium of the native enzyme and by electrophoresis on polyacrylamide gels in the presence of SDS. For the *Agaricus* enzyme our results are in agreement with the model of Strothkamp *et al.*, although we determine a slightly larger MW for the H subunit [9]. Since the H_2 complex is easily obtained on cross-linking, the failure to observe H_3 or H_4 complexes is significant and suggests that the *Agaricus* enzyme is not a homopolymer of three or four similar subunits. Recently this work has been extended and cross-linked complexes of the H and L subunits have now been identified [26].

To reconcile these results with earlier work, it is suggested that limited proteolysis may be a problem in the preparation of tyrosinase from these two sources. The *Neurospora* enzyme characterized in earlier reports with a MW of 33 000 may have arisen by proteolysis of the 46 000 polypeptide, and it is noteworthy that such an entity is commonly present in *Neurospora* preparations, and its extent is variable. Estimation of the amount of the 33 000 component present in this study is difficult since it was not detected in the ultracentrifugal studies and since it is known that different proteins do not necessarily take up stain to the same extent [27]. A very minor contaminant with a similar MW was also evident in the *Agaricus* preparation (Fig. 3b) and it too may have been formed by proteolysis from the H subunit. However, while proteolysis in extracts of *Neurospora crassa* has been observed, and has caused problems in the isolation of several enzymes [28], no such literature exists for *Agaricus*.

Active enzyme centrifugation is carried out with very low enzyme concentrations, when dissociation is favoured, and is a useful technique for searching for the smallest functional unit. Thus for *Neurospora* the results show that a slowly sedimenting species, probably the monomer, is functional. (The MW of the equivalent anhydrous sphere calculated from the sedimentation coefficient is 41 000). In contrast, the complex observed for the *Agaricus* enzyme was the tetramer, i.e. the same as that found in the absence of substrate. A similar value was obtained by Arnaud [16], who determined an $s_{20,w}$ value of 6.8–7.2 S by this method; the smaller species reported by Jolley *et al.* [24] were not observed.

Thus, compared to the *Neurospora* enzyme, *Agaricus* tyrosinase dissociates less readily. Whether this is due to the presence of the lighter subunit has yet to be explored. The similarity in size between the heavier subunit and the *Neurospora* polypeptide is also shared by the enzyme from spinach beet [29], but further work is required to establish if such a species is commonly present in plant tyrosinases.

EXPERIMENTAL

Preparation of enzymes. Conditions of growth of *N. crassa* strain number 1757 and isolation of the enzyme have been detailed previously [7]. The *Agaricus* enzyme was purified according to a published method [11] extended by fractionation on hydroxylapatite [13] and for the SDS gel electrophoresis experiments, filtration through Sephacryl S200 [13].

Assays. The dopachrome assay of Fling *et al.* [6] was employed to determine enzyme activity and protein was estimated spectrophotometrically at 280 nm assuming an $E^{1\%}_{1cm}$ of 26.5 and 22 for the *Agaricus* and *Neurospora* enzymes respectively [12, 25].

Ultracentrifuge studies. Sedimentation velocity studies were conducted in 0.2 M NaPi pH 6.8 using an ultracentrifuge equipped with a photoelectric scanner operating at 280 nm. The enzyme concns used were in the range 0.1–0.3 mg/ml and the partial sp. vol. was assumed to be 0.73 ml/g [6, 23]. The density and relative viscosity of the solvent were estimated to be 1.0203 g/ml and 1.099 respectively. In the sedimentation equilibrium studies, measurements were made with interference optics at 546 nm, 4–12 hr after equilibrium was attained. Short columns were used, requiring 24 hr centrifugation before equilibrium was reached. Active enzyme centrifugation was conducted following the recommendations of ref. [15]. For *Agaricus* tyrosinase, 15 ng in 10 μl was layered onto an oxygenated soln of *p*-cresol (2.6 mM) and NAD^+ (145 μM) in 0.2 M Pi buffer and the enzyme was sedimented through the substrate at a rotor speed of 56 000 rpm. The progress of the enzyme front was monitored at 340 nm. For *Neurospora* tyrosinase the same technique was used with 114 ng protein, but benzene sulphinate (12.2 mM) was substituted for NAD^+ and the progress of the enzyme was estimated by monitoring the product of homoquinone and benzene sulphinate at 325 nm.

Electrophoresis. Analytical disc gel electrophoresis was conducted in 6.6% polyacrylamide rods according to the method of Davis [30]. Gels were stained for activity with DL-DOPA in H_2O (1 mg/ml) and for protein with 0.04% Coomassie Brilliant Blue G-250 in 3.5% $HClO_4$ [31]. Denatured samples were analysed by electrophoresis in the presence of SDS in 5% and 10% gels [18] using rabbit-muscle phosphorylase, BSA, hen ovalbumen, bovine carbonic anhydrase, staphylococcal penicillinase, equine myoglobin and equine cytochrome *c* as MW markers. Samples were denatured by boiling in SDS for 5 min. Cross-linking studies using Me suberimidate were conducted at pH 8.0 in triethanolamine buffer [28]. After an incubation of 1 hr, SDS was added to the samples prior to denaturation by boiling, and electrophoresis was conducted in the presence of SDS [18]. After staining and destaining the gels were scanned with a densitometer.

Isoelectric focusing. Agaricus tyrosinase was spotted onto a commercially prepared slab gel (LKB Instruments) containing ampholytes. After electrofocusing for 90 min at 30 W according to ref. [32] a stable pH gradient between 3.5 and 9.0 was developed across the gel. The enzyme was located by staining for protein and activity after the pH gradient had been determined by the method of Beeley *et al.* [33]. For *Neurospora* tyrosinase a sucrose column containing ampholytes was used [34]. After elution the fraction containing activity was located and its pH determined.

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REFERENCES

1. Vanneste, W. H. and Zuberbuhler, A. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O. ed.) p. 371. Academic Press, New York.
2. Mayer, A. M. and Harel, E. (1979) *Phytochemistry* **18,** 193.
3. Lerch, K. and Ettlinger, L. (1972) *Eur. J. Biochem.* **31,** 427.
4. Harel, E. and Mayer, A. M. (1968) *Phytochemistry* **7,** 199.
5. Combs, J., Baldry, C., Bucke, C. and Long, S. P. (1974) *Phytochemistry* **13,** 2703.
6. Fling, M., Horowitz, N. H. and Heinemann, S. F. (1963) *J. Biol. Chem.* **238,** 2045.
7. Gutteridge, S. and Robb, D. (1975) *Eur. J. Biochem.* **54,** 107.
8. Lerch, K. (1975) Abstract 582, Tenth Meeting of the Federation of European Biochemical Societies, Paris 1975.
9. Strothkamp, K., Jolley, R. L. and Mason, H. S. (1976) *Biochem. Biophys. Res. Commun.* **70,** 519.
10. Lerch, K. (1978) *Proc. Nat. Acad. Sci. U.S.A.* **75,** 3635.
11. Gutteridge, S., Dickson, G. and Robb, D. A. (1977) *Phytochemistry* **16,** 517.
12. Bouchilloux, S., McMahill, P. and Mason, H. S. (1963) *J. Biol. Chem.* **238,** 1699.
13. Robb, D. (1978) *Biochem. Soc. Trans.* **7,** 131.
14. Duckworth, H. W. and Coleman, J. E. (1970) *J. Biol. Chem.* **245,** 1613.
15. Cohen, R. and Mire, M. (1971) *Eur. J. Biochem.* **23,** 267.
16. Arnaud, Y. (1968) *J. Polymer Sci.* **16c,** 4103.
17. Hinsberg, O. and Himmelschein, A. (1896) *Bericht* **29,** 2023.
18. Weber, K., Pringle, J. R. and Osborn, M. (1972) in *Methods in Enzymology* (Hirs, C. and Timasheff, S. N., eds) Vol. 26, p. 3. Academic Press, New York.
19. Davies, G. and Stark, G. R. (1970) *Proc. Nat. Acad. Sci. U.S.A.* **66,** 651.
20. Mallette, M. F. and Dawson, C. R. (1949) *Arch. Biochem. Biophys.* **23,** 29.
21. Kertesz, D. and Zito, R. (1965) *Biochim. Biophys. Acta* **96,** 447.
22. Jolley, R. L., Robb, D. A. and Mason, H. S. (1969) *J. Biol. Chem.* **244,** 1593.
23. Jolley, R. L., Nelson, R. M. and Robb, D. A. (1969) *J. Biol. Chem.* **244,** 3251.
24. Jolley, R. L., Nelson, R. M., Mason, H. S. and Ganapathy, K. (1972) in *Pigmentation: Its Genesis and Biologic Control* (Riley V., ed.) p. 607. Appleton Century Crofts, New York.
25. Lerch, K. (1976) *FEBS Letters* **69,** 157.
26. Gutteridge, S. and Mason, H. S. (1980) in *Biochemical and Clinical Aspects of Oxygen* (Caughey, W. S., ed.) p. 589. Academic Press, New York.
27. Bickle, T. A. and Traut, R. R. (1971) *J. Biol. Chem.* **246,** 6828.
28. Lumsden, J. and Coggins, J. R. (1977) *Biochem. J.* **161,** 599.
29. Vaughan, P. F. T., Eason, R., Paton, J. Y. and Ritchie, G. (1975) *Phytochemistry* **14,** 2383.
30. Davies, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121,** 404.
31. Reisner, A. H., Nemes, P. and Buckoltz, C. (1975) *Analyt. Biochem.* **64,** 509.
32. Vesterberg, O. (1973) *Science Tools* **20,** 22.
33. Beeley, J. A., Stevenson, S. M., Beeley, J. G. (1972) *Biochim. Biophys. Acta* **285,** 293.
34. Vesterberg, O. (1971) in *Methods in Enzymology* (Jakoby, W. B. ed.) Vol. 22, p. 389. Academic Press, New York.
35. DiCamelli, R. F., Holohan, P. D., Basinger, S. F. and Lebowitz, J. (1970) *Analyt. Biochem.* **36,** 470.
36. Kemper, D. L. and Everse, J. (1973), in *Methods in Enzymology* (Hirs, C. and Timasheff, S. N., eds.) Vol. 27, p. 67. Academic Press, New York.
37. Kertesz, D. and Zito, R. (1957) *Nature* **179,** 1017.