Characterization of a Novel Yeast Synthesizing Melanin-like Pigment

By PRANAB ROY,* KALPANA K. NAYAK AND
NARENDRA K. PANDEY

Hindustan Lever Research Centre, Andheri (East), Bombay 400 099, India

(Received 27 June 1989; revised 22 August 1989; accepted 18 September 1989)

A laboratory yeast isolate identified as *Exophiala jeanselmei* synthesized melanin and showed polyphenol oxidase activity with dihydroxyphenylalanine (DOPA) and pyrogallol. Electrophoresis on native and denaturing gels, and activity staining with DOPA revealed two immunologically distinguishable bands of M_r about 100 000 and 120 000, which could be separated by $(\text{NH}_4)_2\text{SO}_4$ fractionation. The enzyme isolated from this yeast gave optimal activity at higher pH (8.5–10) and temperature (37–40 °C) than other known melanin-synthesizing activities.

INTRODUCTION

Melanin is synthesized by many bacteria, fungi, animals and plants (Lerch, 1981; Swan, 1974); however, to our knowledge, there is no report of its occurrence in yeast. In this paper, we report the isolation of a budding yeast which synthesizes melanin in different media, and describe the properties of the enzyme tyrosinase (polyphenol oxidase). Tyrosinase (EC 1.14.18.1), a key enzyme in the biosynthesis of melanin, catalyses at least three sequential reactions in the biosynthesis of dopaquinone (an intermediate in melanin synthesis) from tyrosine (Burnett, 1971; Korner & Pawalek, 1982). The rest of the pathway in melanogenesis is thought to be autocatalytic (Swan, 1974). Recently, dopachrome oxidoreductase has been implicated in blocking the biosynthesis of melanin at an intermediate stage (Barber *et al.*, 1984).

In the course of our experiments to transform *Saccharomyces cerevisiae* AH22 (*leu his*) with plasmid YEp13 (Broach *et al.*, 1979; Nayak *et al.*, 1988) carrying the tyrosinase gene, *mel*, from plasmid pIJ703 of *Streptomyces lividans* strain 3132 (Katz *et al.*, 1983), we observed a few black colonies on a selection plate. Light and scanning electron microscopy suggested that the pigmented colonies were composed of a budding yeast. Preliminary characterization of the isolate showed that it had no relationship to the host *S. cerevisiae* AH22 strain and did not contain a YEp13 derivative. A ^{32}P -labelled nick-translated *mel* gene probe from plasmid pIJ703 (Katz *et al.*, 1983) did not hybridize to the chromosomal DNA of the isolate. The isolated yeast strain has been identified as *Exophiala jeanselmei* (Langer) by the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

METHODS

Media. The yeast was grown aerobically on a gyratory shaker (200 r.p.m.) at 30 °C in minimal medium (containing, g l⁻¹: yeast nitrogen base, 1.7; ammonium sulphate, 5; glucose, 20; pH 6), Sabouraud's medium (containing, g l⁻¹: peptone, 10; glucose, 40; pH 6) or YEPD medium (containing, g l⁻¹: yeast extract, 10; peptone, 20; dextrose, 20; pH 6).

Cell extraction. Yeast cells were sonicated in a Branson sonicator at 80% duty cycle with three pulses of 1 min each at 0 °C in 10 mM-Tris/HCl, pH 7.5 and 1 mM-EDTA (TE buffer). The supernatant obtained by centrifugation at 10 000 r.p.m. for 15 min was used as the source of enzyme. Alternatively, the yeast cells were treated with the

Abbreviations: DOPA, DL-dihydroxyphenylalanine; NEM, N-ethylmaleimide.

enzymes driselase and β -glucuronidase as detailed previously (Nayak *et al.*, 1986). The protoplasts obtained were washed with citrate/phosphate buffer, pH 5.8, containing 1 M-sorbitol and then lysed with 1% SDS in TE buffer at 37 °C for 30 min. 1 mM-PMSF and 2.5 mM-*N*-ethylmaleimide (NEM) as protease inhibitors were added during extraction.

Extracts prepared by either method were fractionated by $(\text{NH}_4)_2\text{SO}_4$ as follows. Precipitation with $(\text{NH}_4)_2\text{SO}_4$ at 0–30% saturation was done in ice, maintaining the pH at 7.5, followed by centrifugation at 10000 r.p.m. for 10 min. The supernatant was saturated up to 60% with $(\text{NH}_4)_2\text{SO}_4$, following which, the remaining proteins were precipitated in the 60–90% $(\text{NH}_4)_2\text{SO}_4$ fraction. The fractions were dissolved in a minimum volume and dialysed against TE buffer.

Enzyme assay. Tyrosinase in the crude extract was assayed at pH 8.5 in 0.1 M-Tris/glycine buffer. For the $(\text{NH}_4)_2\text{SO}_4$ fractions, optimum activities were obtained at pH 10 in 0.1 M-bicarbonate buffer. The assay mixture contained 2 mg DOPA and 10 μg dialysed protein in 2.5 ml volume. Incubation was done at 37 °C for 1 h. Activities were calculated from ΔA_{475} obtained with correction for auto-oxidation of DOPA and the absorption due to inherent colour of the yeast extract. One unit of enzyme catalyses the transformation of 1 μmol of DOPA min^{-1} under the assay conditions. The molar absorption coefficient of oxidation products of DOPA at 475 nm is $3600 \text{ M}^{-1} \text{ cm}^{-1}$.

Gel electrophoresis and activity staining. SDS or native PAGE of the crude and $(\text{NH}_4)_2\text{SO}_4$ -fractionated cell extracts was done under standard conditions (Laemmli, 1970). The standard M_r markers were stained with Coomassie Brilliant Blue R250. The rest of the gel was immersed in DOPA solution (0.8 mg ml^{-1}) for 1–2 h. When distinct tyrosinase bands could be seen, the reaction was stopped by adding a few drops of β -mercaptoethanol.

RESULTS AND DISCUSSION

The doubling times of the yeast in minimal and Sabouraud's media were 4.5 and 3.5 h, respectively. The intracellular black pigment was produced in minimal and Sabouraud's media with no traces of production of ethanol (analysed by GLC on Porapak column at 85 °C). In YEPD medium the cells were cream-coloured rather than black and produced ethanol. Maximum growth and pigmentation occurred at 37–40 °C both on agar plates and in liquid media. Varying the glucose or $(\text{NH}_4)_2\text{SO}_4$ concentration from 5 to 50 g l^{-1} in minimal medium did not affect the growth and pigment production. There was no evidence of repression or the Crabtree effect (DeDeken, 1966). The pigment could be visualized after about 24 h incubation. In a typical shake culture experiment about 12 g glucose l^{-1} was utilized in 48 h in minimal medium. The addition of 20 g l^{-1} or more peptone to minimal medium drastically reduced melanogenesis without impairing growth. However, the addition of various amino acids, e.g. histidine, threonine, tyrosine, methionine, leucine, cysteine, proline or DOPA at 1 mM each did not affect pigmentation.

In order to characterize the pigment, the supernatant of sonicated cells was compared to the auto-oxidation product of DOPA, i.e. melanin. Both had identical UV and visible absorption spectra (190–700 nm). Most of the pigment remained in the cell pellet and, as expected of melanin, it could not be extracted with detergents such as SDS or Triton X-100 (used at 1%) or solvents such as chloroform, ethanol, methanol and acetone. Treatment with 2 M-NaOH at 50 °C for 16 h resulted in partial solubilization (40%, w/w) of the pigment. The neutralized alkali extract of the pigment and the *in vitro* product of the tyrosinase had the same absorption and fluorescence spectra as the auto-oxidation product of DOPA.

The supernatant (crude extract) was used as the source of tyrosinase and assayed with DOPA and pyrogallol as substrates. Electrophoresis of the crude extract or dialysates of $(\text{NH}_4)_2\text{SO}_4$ fractions on 7.5% or 10% polyacrylamide gel containing SDS showed two discrete bands of approximate M_r 100000 to 120000 when stained with DOPA (0.8 mg ml^{-1}) for tyrosinase activity (Fig. 1a, b). This is analogous to the pattern of tyrosinases from mouse or human melanoma, though they have different molecular sizes. *Saccharomyces cerevisiae* did not show any activity-stainable band either by native or denaturing gel electrophoresis. It has been reported that in mouse and human melanoma, the tyrosinase can be post-translationally modified to various isoenzymic forms (Burnett, 1971; Lerch, 1981). The enzyme activity was lost if the extracts were boiled in Laemmli buffer (Laemmli, 1970) but remained unaffected by 1% SDS.

Precipitation of tyrosinase activity from the crude extract showed it to be distributed in the 0–30%, 30–60% and 60–90% saturated $(\text{NH}_4)_2\text{SO}_4$ fractions (Table 1). This could be due to the

Fig. 1. (a). SDS-PAGE (7.5% acrylamide) of crude extracts of *Saccharomyces cerevisiae*, yeast isolate and *Streptomyces lividans*. 75 μ g protein was loaded in each lane, electrophoresed and stained with DOPA. The positions of standard M_r proteins (β -galactosidase, 116000; phosphorylase b, 97400) detected by Coomassie Blue R250 staining are shown by arrowheads. Lanes: 1, *Sacch. cerevisiae*; 2, yeast isolate grown in minimal medium; 3, as in 2 but grown in YEPD medium; 4, *Strept. lividans* strain 3132 expressing tyrosinase. (b) SDS-PAGE (conditions as in a) of various $(\text{NH}_4)_2\text{SO}_4$ fractions of the yeast isolate. Lanes: 1, crude extract; 2, 0–30% ammonium sulphate precipitate; 3, 30–60% ammonium sulphate precipitate; 4, 60–90% ammonium sulphate precipitate.

Table 1. *Fractionation of tyrosinase activity in crude extract*

Crude extract was assayed at pH 8.5 and the $(\text{NH}_4)_2\text{SO}_4$ fractions at pH 10.0, using 0.8 mg DOPA ml^{-1} as substrate.

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1})	Percentage of total activity	Activity staining
Crude extract	24.8	1550	62.5	—	Two bands of tyrosinase
0–30% $(\text{NH}_4)_2\text{SO}_4$	16.8	1170	69.6	59.3	Higher M_r band
30–60% $(\text{NH}_4)_2\text{SO}_4$	1.9	227	119.3	11.6	Lower M_r band
60–90% $(\text{NH}_4)_2\text{SO}_4$	4.9	573	117.0	29.1	Lower M_r band

Table 2. *K_m and maximum velocity (V) of different fractions*

K_m and V were calculated from Lineweaver–Burk plots of the respective substrates. Molar absorption coefficients for oxidation products of DOPA and pyrogallol were taken as 3600 and 2470 $\text{M}^{-1} \text{cm}^{-1}$, respectively, at 475 nm. Assays for pyrogallol and DOPA were done under identical conditions as mentioned in Methods.

Fraction	DOPA as substrate		Pyrogallol as substrate	
	$10^4 \times K_m$ (M)	$10^6 \times V$ (M min^{-1})	$10^4 \times K_m$ (M)	$10^6 \times V$ (M min^{-1})
Crude extract	7.6	4.6	7	5
0–30% Ammonium sulphate	8.3	7.6	25	19
60–90% Ammonium sulphate	5.6	5.9	25	19

multiple forms of tyrosinase, varying in charge, M_r or both, as found in mushroom and melanoma cells (Keevil & Mason, 1978; Lerch, 1981). The 0–30% ammonium sulphate fraction was enriched in the higher M_r tyrosinase band as seen by activity staining of electrophoresed gel (Fig. 1b). The 30–60% and 60–90% ammonium sulphate fractions were identical and enriched in the lower M_r band. The yeast enzyme was retained by DEAE-Sephacrose equilibrated with 50 mM-Tris/HCl, pH 7.5, and eluted with 0.5 M-NaCl in the same buffer, showing that the protein has a net negative charge at pH 7.5. Since the two tyrosinase bands could be separated by $(\text{NH}_4)_2\text{SO}_4$ fractionation, the dialysed fractions were used for further studies.

The K_m for DOPA was 7.6×10^{-4} M for the crude enzyme (Table 2), which is comparable to K_m values for tyrosinases from other sources. Apparent differences in K_m and V values of activities in different $(\text{NH}_4)_2\text{SO}_4$ fractions are suggestive of different isoenzymes. Surprisingly, the tyrosinase activity in the crude extract remained constant at a wide range of alkaline pHs (8.5 and above) as reported for choline esterase (Lehninger, 1982). The $(\text{NH}_4)_2\text{SO}_4$ fractions, however, exhibited a pH optimum of 10. Hence, tyrosinase in crude extracts was assayed at pH 8.5 and that in $(\text{NH}_4)_2\text{SO}_4$ fractions at pH 10. Preincubation of $(\text{NH}_4)_2\text{SO}_4$ fractions in 0.05 M-bicarbonate buffer at pH 10 for 2 h did not affect the activity, indicating the stability of the enzyme. At pH 8.5, all the $(\text{NH}_4)_2\text{SO}_4$ fractions showed a characteristic lag in tyrosinase activity which was overcome if the assay was performed at pH 10 (Fig. 2). This is analogous to the case of human melanoma and skin tyrosinase (Tripathi *et al.*, 1988) where there is a pH-dependent lag period in the cresolase activity of the tyrosinase. An important difference is that the pH range for activity of human tyrosinase is from 5.3 to 6.8 whereas in the yeast tyrosinase it is from 8.5 to 10. Under our assay conditions, enzyme activity was greatest at 37–40 °C, which is

Fig. 2. Comparison of tyrosinase activity (as a function of time) of the 0–30% and 60–90% $(\text{NH}_4)_2\text{SO}_4$ fractions at pH 8.5 and pH 10. The assays were carried out at a DOPA concentration of 2 mM, with 20 μg enzyme protein. For assay at pH 8.5, 0.05 M-Tris/glycine buffer was used and for assay at pH 10, 0.05 M-carbonate/bicarbonate buffer was used. The 30–60% ammonium sulphate fraction behaved identically to the 60–90% ammonium sulphate fraction. $(\text{NH}_4)_2\text{SO}_4$ saturation and assay pH: ●—●, 60–90%, pH 10.0; ●--●, 0–30%, pH 10.0; ○—○, 60–90%, pH 8.5; ○--○, 0–30%, pH 8.5.

similar to the optimum temperature for growth and pigmentation. The exclusion of protease inhibitors (PMSF, NEM) from the extraction buffer did not alter the tyrosinase activity. Additionally, the enzyme activity was also not affected by mild treatment of the crude extract with chymotrypsin, pronase or proteinase K [$12.5 \mu\text{g}$ protease (mg crude extract protein) $^{-1}$ at 37°C for 1 h]. However, incubation for 16 h with these proteases completely abolished the activity, as examined by activity staining on gels.

A polyclonal antibody raised against the higher M_r tyrosinase band obtained by electrophoretic elution from the SDS-PAGE was reacted with the extract. Various concentrations of crude extract proteins and antiserum were tested, and the antibody selectively neutralized the higher M_r protein. There was no cross-reaction with the lower M_r tyrosinase band (Fig. 3). The preimmune serum did not affect the activity of either band. Hence the antigenic determinants in the two polypeptides may be different. Native PAGE of the yeast extract also showed two distinct bands on activity staining.

Tyrosine with DOPA at 25:1 (w/w) gave enzyme activity in the crude extract that was 16% of that obtained with DOPA alone as substrate. Pyrogallol was a good substrate for the enzyme whereas tyrosine, catechol, 4-isopropylcatechol, orcinol and phenol were poor substrates (concentrations used in the assay varied between 0.32 mM to 4.0 mM in each case). Dopachrome oxidoreductase activity in the crude extract was found to be insignificant (Barber *et al.*, 1984). Table 3 shows the effects of various additions to the assay buffer on the tyrosinase activity. Tyrosinases are known to be copper containing enzymes, with possible involvement of cysteine

Fig. 3. 7.5% acrylamide gel electrophoresis of crude extract of yeast isolate (1) crude extract (2) supernatant after treatment with anti-tyrosinase antibody raised against higher M_r tyrosinase band. Activity staining of the gel was done with DOPA.

Table 3. *In vitro* assay for tyrosinase in the crude extract in presence of various compounds

The conditions of assay were as described in Methods. Dialysed crude extract (80 μ g) was used in each assay in 0.1 M-Tris/glycine buffer, pH 8.5. The control, with no addition, had an activity of 1.8 units with respect to DOPA.

Addition	Activity (%)
None	100
CuSO ₄ (1–10 μ M)	100
β -Mercaptoethanol or dithiothreitol (1 mM)	0
Dithiothreitol (1 mM)	0
Diethyldithiocarbamate (1 mM)	10
Bipyridyl (1 mM)	100
Sodium potassium tartrate (1 mM)	100
Cysteine. HCl (1–10 mM)	0
N-Ethylmaleimide (2.5 mM)	100
PMSF (1 mM)	100

and histidine at the active site (Lerch, 1978, 1981). The observation that NEM does not inhibit the tyrosinase activity indicates that thiol group(s) are not essential. Metal ion chelators like sodium potassium tartrate or bipyridyl may not be able to interact with the bound copper in the tyrosinase and hence do not inhibit the reaction as also reported for mouse melanoma tyrosinase (Barber *et al.*, 1984). The inhibition by dithiothreitol or 2-mercaptoethanol may be due to inhibition of oxidation of DOPA rather than to modification of the enzyme. Diethyldithiocarbamate, a known inhibitor of tyrosinase of mouse melanoma (Barber *et al.*, 1984) significantly inhibited the activity of yeast tyrosinase.

The properties of the tyrosinase reported here show that it is a polyphenol oxidase (Keevil & Mason, 1978). It is distinct from all reported tyrosinases in M_r , and pH and temperature characteristics. The two activity-stainable bands seem to be distinct enzymes rather than subunits of the same enzyme.

The authors thank Professor D. A. Hopwood for the *Streptomyces* plasmid pIJ703 and *Streptomyces lividans* strain 3132, also Mr R. M. Pradhan for electron microscopy.

REFERENCES

- BARBER, J. H., TOWNSEND, D., OLDS, D. P. & KING, R. A. (1984). Dopachrome oxidoreductase: a new enzyme in the pigment pathway. *Journal of Investigative Dermatology* **83**, 145–149.
- BROACH, J. R., STRATHERN, J. N. & HICKS, J. B. (1979). Transformation in yeast: development of a hybrid cloning vector and isolation of the *CAN1* gene. *Gene* **8**, 121–123.
- BURNETT, J. B. (1971). The tyrosinase of mouse melanoma. Isolation and molecular properties. *Journal of Biological Chemistry* **246**, 3079–3089.
- DEDEKEN, R. H. (1966). The Crabtree effect: a regulatory system in yeast. *Journal of General Microbiology* **44**, 149–156.
- KATZ, E., THOMPSON, J. C. & HOPWOOD, D. A. (1983). Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. *Journal of General Microbiology* **129**, 2703–2714.
- KEEVIL, T. & MASON, H. S. (1978). Molecular oxygen in biological oxidations — an overview. *Methods in Enzymology* **52**, 3–40.
- KORNER, A. & PAWALEK, J. M. (1982). Mammalian tyrosinase catalyses three reactions in the biosynthesis of melanin. *Science* **217**, 1163–1165.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LEHNINGER, A. L. (1982). Enzymes: kinetics and inhibition. In *Principles of Biochemistry*, pp. 147–168. New York: Worth Publishers.
- LERCH, K. (1978). Amino acid sequence of tyrosinase from *Neurospora crassa*. *Proceedings of the National Academy of Sciences of the United States of America* **75**, 3635–3639.
- LERCH, K. (1981). Copper monooxygenases: tyrosinases and dopamine beta monooxygenase. In *Metal Ions in Biological Systems*, pp. 143–186. Edited by H. Sigel. New York: Marcel Dekker.
- NAYAK, K. K., PADBIDRI, A., HIRLEKAR, M. G. & PANDEY, N. K. (1986). Rapid method for preparation of protoplasts of *Rhodotorula glutinis*. *Current Science* **55**, 926–927.
- NAYAK, K. K., PANDEY, N. K. & ROY, P. (1988). Cloning of tyrosinase gene from *Streptomyces lividans* in *Escherichia coli*. *Indian Journal of Biochemistry and Biophysics* **25**, 515–517.
- SWAN, G. A. (1974). Structure, chemistry and biosynthesis of the melanins. In *Progress in the Chemistry of Organic Natural Products*, vol. 31, pp. 521–582. Edited by W. Herz, H. Grisebach & G. W. Kirby. New York: Springer Verlag.
- TRIPATHI, R. K., CHAAYA DEVI, C. & RAMAIAH, A. (1988). pH dependent interconversion of two forms of tyrosinase in human skin. *Biochemical Journal* **252**, 481–487.