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TYROSINASE ACTIVITY IN THE MEDIUM OF HUMAN MELANOMA CELL CULTURES

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Abstract. The medium of cultured melanoma cells was studied for tyrosine hydroxylation and dopa-oxidizing activity. The supernatant obtained after centrifugation at 100000 *g* for 2 hours was treated with ammonium sulphate, and the precipitate obtained between 35 and 50% saturation was used. Dopa was determined as the product of tyrosine hydroxylation and 5-S-cysteinyldopa as the product of dopa oxidase activity. Determinations were performed with HPLC and electrochemical detection. Our preparation of culture medium of cells showed the following. 1) No hydroxylation of tyrosine in the absence of co-factor. 2) Hydroxylation of L-tyrosine in the presence of dopamine. No hydroxylation with boiled medium. Minimal effect of catalase on hydroxylation. 3) Hydroxylation of tyrosine in the presence of ascorbic acid. Hydroxylation was catalysed also with boiled medium. Catalase strikingly diminished hydroxylation. 4) Oxidation of L-dopa to dopaquinone determined as its main reaction product with cysteine, 5-S-cysteinyl-dopa. There was negligible oxidation with boiled medium. 5) With dopamine as co-factor the catalysis of tyrosine hydroxylation was stereospecific for L-tyrosine. Dopa oxidase activity was also stereospecific for L-dopa.

Tyrosinase is the enzyme responsible for production of melanin. It has two functions, as *oxygenase* and as *oxidase*. Tyrosine is the natural major substrate for the enzyme in its *oxygenase function*, with dopa as the product. Under certain circumstances dopa may also be hydroxylated with 5-OH-dopa as the product (3, 8). In its *oxidase* function, tyrosinase has dopa as natural substrate, and the product dopaquinone is transformed into indolines and indoles or becomes combined with cysteine to form cysteinyldopas. All these compounds are precursors of melanins, and dopaquinone also acts as an oxidant of the reduced forms of these precursors (7, 9, 11).

Several methods have been used for measuring tyrosinase activity. Pomerantz and Li (15) recommend the initial rate of formation of dopachrome as indicated by a change in absorbancy at 475 nm as the most convenient method of assay during purification of tyrosinase. They advocate measurement of ^{3}HOH formed during the hydroxylation of 3,5-^{3}H-L-tyrosine as the method of choice for the assay of low activity samples. Other methods involving manometric measurement of oxygen uptake or measurement of the non-enzymatic oxidation of ascorbate by dopaquinone to give dehydroascorbate and dopa have been extensively used in classical studies on tyrosinase. In a fifth method, the incorporation of L-tyrosine-^{14}C into melanin is measured. This method has been carefully studied and compared with the tritiated water method (10).

We have for several years been developing methods for the investigation of melanin precursors in melanin-forming tissues, serum or plasma, and urine. These methods have been adapted for studies on tyrosinase activity (3). The present preliminary report describes the measurement of tyrosinase activity in a crude extract of the medium of cultivated melanoma cells, and demonstrates the possibility of distinguishing between tyrosinase-induced specific oxidation and non-specific oxidation by using the L- and D-forms of tyrosine and dopa in the incubate. In contrast to mushroom tyrosinase, human tyrosinase, like other mammalian tyrosinases (10), seems to be highly specific for L-tyrosine and L-dopa.

MATERIAL AND METHODS

Chemicals

L-Tyrosine (Sigma), D-tyrosine (Sigma), L-dopa (Merck), D-dopa (Sigma), Dopamine (Sigma), Ascorbic acid (Analar), L-cysteine (Merck), Mushroom tyrosinase (Sigma), Catalase (Sigma), MEM (Flow). 5-S-L-Cysteinyl-L-dopa was prepared enzymatically, and purified as recently described (1).

Cultures of human pigment-producing melanoma cell line (IGR 1)

IGR 1 were obtained from Dr Christian Aubert, Marseille, and kept since March 1982 in culture at the Tornblad

Table I. *The effect of dopamine and the stereospecificity of tyrosine hydroxylation*

The source of enzyme was an ammonium sulphate precipitate (35–50% saturation) of culture medium. All incubates contained the precipitate, and incubations were performed for 9 min at 37°C

Incubate	Dopa (μg/ml medium)
L-Tyrosine	None
L-Tyrosine[a] Dopamine	0.29
L-Tyrosine[a] Dopamine Catalase	0.24
D-Tyrosine[b] Dopamine	0.04
D-Tyrosine Dopamine Catalase	0.03

[a] No dopa was detected after incubation with boiled precipitate.
[b] 0.02 μg dopa was found after incubation with boiled precipitate.

Institute, University of Lund, by methods previously described (4). The medium used was minimal essential medium MEM + 15% fetal calf serum (Flow). 75 ml of medium was collected from cultures on the 5th day. Cells were sedimented by centrifugation at $1\,000 \times g$, and the medium was then centrifuged at $100000 \times g$ for 2 hours to separate any particulate from soluble tyrosinase. The supernatant was treated with ammonium sulphate, and the precipitate obtained at 35–50% saturation was dissolved in 20 ml of 0.5 M phosphate buffer (pH 7.4) and used for experiments.

Liquid chromatographic (HPLC) analysis of dopa and 5-S-cysteinyldopa

A Varian model 5000 (Varian, Palo Alto, Calif., USA) solvent delivery system was used.

Samples were injected with a valve injector Rheodyne model 7120 (Rheodyne, Berkeley, Calif., USA) equipped with a 100-μl loop. A model LC-10 amperometric detector (Bioanalytical Systems Inc., West Lafayette, Ind., USA) was used. The detector potential was +0.75 V vs. the Ag/AgCl (3 M NaCl) reference electrode.

The working electrode was prepared from carbon paste (CPO) material. Columns were packed in 250×4.6 mm stainless steel tubes with a chemically bonded 5 μm C_{18} material (Nucleosil C_{18}, Machery, Nagel and Co., Düren, GFR). The mobile phase contained 6.0 g of methane sulphonic acid and 3.0 g of orthophosphoric acid per litre of MilliQ purified water. pH was adjusted to 3.00.

EXPERIMENTS

Oxygenase function of tyrosinase

Incubations were performed in 1 ml of 0.5 M phosphate buffer (pH 7.4) at 37°C under constant air bubbling using the ammonium sulphate precipitate as the source of enzyme. L-Tyrosine and D-tyrosine were used as substrates (10^{-3}M concentration), and dopamine and ascorbic acid, both 10^{-3} M, were examined as co-factors. The influence of hydrogen peroxide, possibly formed during the incubation, on tyrosine hydroxylation, was investigated by adding 20 μg of catalase per ml incubate. Incubations were interrupted after 9 min by the addition of perchloric acid to a concentration of 0.4 M, and the quantities of dopa present, which reflected oxygenase activity, were determined. The results are given in Tables I and II. All activities are calculated per ml of the original culture medium.

Table I shows that hydroxylation of L-tyrosine was not catalysed by the precipitate of the culture medium in the absence of added reducing compound. On adding *dopamine*, a pronounced hydroxylation occurred. Addition of catalase did not markedly affect the rate of hydroxylation. Boiling of the precipitate destroyed the oxygenase activity. The hydroxylation of D-tyrosine was slow and not entirely inhibited by boiling the precipitate.

Table II shows that the hydroxylation of L-tyrosine in the presence of the precipitate and *ascorbic acid* differed in nature from that catalysed in the presence of dopamine. Thus catalase prevented the hydroxylation of tyrosine which was promoted by ascorbic acid, and boiling of the medium instead increased the reaction rate.

Oxidase function of tyrosinase

Oxidation of dopa was studied by incubations of L- or D-dopa (10^{-3} M) with cysteine (3×10^{-3} M) in the presence of ammonium sulphate precipitate (35–50% saturation) for 9 min at 37°C and air bubbling. Perchloric acid was added to a concentration of 0.4 M, and the quantities of 5-S-cysteinyldopa present were determined.

Table III shows that the precipitate catalysed the oxidation of L-dopa efficiently. Boiling of the precipitate destroyed the catalysing effect.

Table II. *The effect of ascorbic acid on tyrosine hydroxylation*

Source of enzyme and incubation conditions as in Table I

Incubate	Dopa (μg/ml medium)
Precipitate L-Tyrosine Ascorbic acid	0.19
Precipitate L-Tyrosine Ascorbic acid Catalase	0.05
Boiled precipitate L-Tyrosine Ascorbic acid	0.27
Boiled precipitate L-Tyrosine Ascorbic acid Catalase	0.03

COMMENTS

The results show that the medium of cultured human melanoma cells possesses a pronounced tyrosine hydroxylation activity that is dependent on reducing agents such as dopamine or ascorbic acid. Dopamine was chosen instead of dopa as co-factor for the reaction, because the hydroxylation product, dopa, could then be readily distinguished in the analysis.

The hydroxylation of tyrosine was stereospecific for L-tyrosine when dopamine was the co-factor and the catalytic effect was then abolished by boiling. Catalase had only a slight effect on the hydroxylation, indicating that any H_2O_2 formed during incubation was not responsible for the hydroxylation.

Ascorbic acid promoted hydroxylation differently from dopamine. Thus boiling of the precipitate enhanced the formation of dopa in the presence of ascorbic acid. Further, catalase inhibited most of the hydroxylation, indicating that the reaction was due to H_2O_2 formed during the incubation. Hydroxylation of tyrosine by the culture medium in the presence of ascorbic acid is similar to the catalysis of tyrosine hydroxylation by methaemoglobin and ascorbic acid as recently described (2). It seems possible that haem-containing compounds are responsible for the oxidation of tyrosine by the precipitate in the presence of ascorbic acid also in the present study.

Our results show that a tyrosine-hydroxylating and dopa-oxidizing enzyme is present in the melanoma culture medium. The enzyme seems to be stereospecific for L-tyrosine and L-dopa. However, we have not yet performed closer kinetic studies on the enzyme, and some caution concerning the stereospecificity is still necessary, as is illustrated in Fig. 1, which shows a Lineweaver-Burke plot for mushroom tyrosinase. It can be seen that K_M for D-dopa is five times greater than K_M for L-dopa (5×10^{-4} and 1×10^{-4} respectively), and that V_{max} of D-dopa is more than twice V_{max} of L-dopa. In another experiment, equal amounts of L- and D-dopa were added to the incubate containing cysteine and tyrosine, and the formation of the two 5-S-cysteinyldopa diasteromers was measured. The ratio of the formation of the two isomers tallied closely with that calculated from the constants obtained from the reciprocal plots in Fig. 1. A closer kinetic investigation of human melanoma tyrosinase is planned and under preparation.

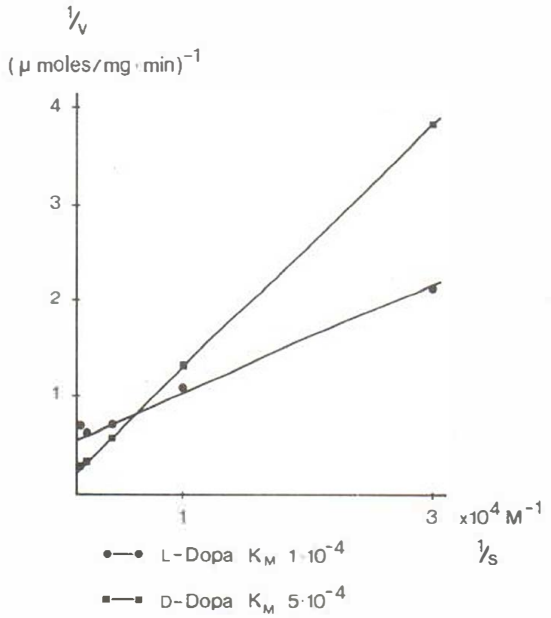

Fig. 1. Lineweaver-Burke plot for mushroom tyrosinase. Incubation mixtures contained mushroom tyrosinase (10 μg), L-dopa, or D-dopa as substrate, and cysteine (10^{-3}M). 5-S-Cysteinyldopa was taken as a measure of dopa oxidation. Incubation time was 45 sec at 0°C.

Table III. *Stereospecificity of dopa oxidation*

Source of enzyme and incubation conditions as in Table I

Incubate	5-S-Cysteinyldopa (μg/ml medium)
Precipitate L-Dopa L-Cysteine	5.4
Precipitate D-Dopa L-Cysteine	0.4
Boiled precipitate L-Dopa L-Cysteine	0.07

Difficulty in obtaining enough human material has hampered studies on tyrosinase in man. It has been demonstrated however that a large proportion of the tyrosinase in human melanoma tissue is associated with particulate material, and that soluble tyrosinase occurs in two forms, T_1 and T_2, which can be separated by gel electrophoresis (5, 12, 13). On treatment with neuraminidase, T_1 is shifted to the T_2 position (13). Nishioka examined

soluble tyrosinase for tyrosine hydroxylase and dopa oxidase activity; similar activities for the two functions were found.

The sensitivity of our method will permit kinetic studies on human tyrosinase, examination of the co-factor requirement, and establishment of substrate stereospecificity of the enzyme, investigations which will facilitate studies on the possible occurrence of tyrosinase in serum of patients with metastasizing melanoma (6, 14).

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