CONVERSION OF PARTICULATE TYROSINASE TO SOLUBLE FORM AND TO DESIALYLATED TYROSINASE IN HUMAN MALIGNANT MELANOMA

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1. Introduction

Studies of mammalian tyrosinase (*o*-diphenol: O_2 oxidoreductase, EC 1.10.3.1), a key enzyme in melanogenesis, have been conducted principally with soluble forms (T^1 faster, T^2 slower migrating tyrosinases by gel electrophoresis) obtained from mouse and hamster melanoma [1–5] and these T^1 and T^2 tyrosinases are considered as isozymes [5,6]. However, mammalian melanocytes are known to contain active tyrosinase in both soluble and insoluble melanosome (melanin-producing organelle)-bound (T^3) forms [2,3]. The majority of tyrosinase activity from mouse and human melanoma tissue is in particulate form [3,6,7]. Recently it has been shown by serological studies that human soluble tyrosinase is a glycoprotein [8]. This communication describes the partial purification of tyrosinase with concanavalin A (Con A) affinity chromatography and demonstrates the interconversion of the different enzymes forms (T^1, T^2, and T^3).

2. Materials and methods

Human malignant melanoma tissue was obtained from metastatic deposits in the liver of a patient with disseminated melanoma and kept in a Revco at −75°C. It was thawed, minced, and homogenized by a Polytron (Brinkman Instruments) for a total period of 1 min at 4°C in 10 volumes of medium A (50 mM sodium phosphate pH 7.0, 0.25 M sucrose, 25 mM KCl, 4 mM $MgCl_2$ and 4 mM phenylmethylsulfonyl fluoride (Sigma)). This homogenate was centrifuged at 700 × *g* for 10 min. The supernatant obtained was further centrifuged at 100 000 × *g* for 1 h. This supernatant was used as soluble tyrosinase fraction. The pellet containing melanosomes was washed by suspending it in medium A and centrifuging twice to eliminate any contaminating soluble tyrosinase. It was then subjected to a tyrosinase solubilization step, in which melanosomes were suspended at 5 mg protein/ml in 0.05 M Tris/HCl pH 7.4 with 0.5% (w/v) sodium cholate by sonication. After stirring for 30 min, this suspension was centrifuged at 100 000 × *g* for 1 h. The supernatant was then subjected to $(NH_4)_2SO_4$ fractionation after dialysis against 10 mM Tris/HCl (pH 7.4), containing 0.1% sodium cholate. No activity was detected in the pellet. The precipitate obtained from the 40 to 60% $(NH_4)_2SO_4$ saturation range was dissolved into 4 mM sodium phosphate (pH 7.0), with 0.1% (v/v) Triton X-100, and dialyzed against the same buffer for Con A-Sepharose (Pharmacia) affinity chromatography.

Tyrosinase activity was measured by oxidation of L-3,4-dihydroxyphenylalanine (DOPA) as described by Pomerantz and Li [9], but carried out at 37°C utilizing a Gilford Stasar III equipped with a data lister. In this study, 1 unit of tyrosinase is defined as the amount that will catalyze the transformation of 1 μmole of DOPA per min at 37°C. To confirm the identity of tyrosinase, each prepared tyrosinase fraction was also examined for tyrosine hydroxylation activity [9]. Protein was measured by the method of Lowry et al. [10]. When Triton X-100 was present, each tube was centrifuged at 1000 × *g* for 10 min before reading the absorbance as described by ChandraRajan and Klein [11].

Utilizing Davis' method [12], polyacrylamide gel electrophoresis (7%) was carried out in Canalco system at 4°C with 3 mA per column (6.5 X 140 mm) using a standard Tris-glycine buffer system and 2.5% stacking gel. When the presence of Triton X-100 was required, 0.1% was included in all gels and tank buffers. For tyrosinase activity staining, the gel was rinsed with 0.2 M sodium phosphate buffer (pH 6.8), then incubated in 0.3% (w/v) DOPA solution in 0.1 M sodium phosphate buffer (pH 6.8), for 45 min at 37°C. For preparation purposes, tyrosinase was extracted from gels containing 200 μg protein in the following manner: the gel, after electrophoresis, was kept in 0.2 M sodium phosphate buffer pH 6.8 for 10 min. The reference gel was stained with DOPA. The portion corresponding to the DOPA positive area on the reference gel was cut off. The gel fragments were homogenized in 5 ml of 0.2 M sodium phosphate buffer (pH 6.8), and centrifuged at 3000 X *g* for 10 min. The supernatant containing tyrosinase was concentrated by an Amicon standard UF cell using PM-10 membrane. Neuraminidase *(Vibrio cholerae)* was obtained from Behring Diagnostics and the activity unit defined by Schultze et al. [13] was employed.

3. Results and discussion

Soluble tyrosinase and sodium cholate-solubilized tyrosinase were partially purified by Con A-Sepharose column as shown in fig.1. The affinity chromatography for solubilized tyrosinase was carried out in the presence of 0.1% (v/v) Triton X-100 to avoid aggregation (fig.1b). Tyrosinase was never eluted with even the highest possible concentration of α-methyl-D-mannoside until the column temperature was raised. This suggests a high mannose content in this enzyme.

To examine the possibility of proteolytic conversion of particulate tyrosinase to soluble form, solubilized tyrosinase was treated with trypsin and the result of this time-course-experiment is shown in fig.2. Upon trypsin digestion, the majority of solubilized tyrosinase was converted to a fast-migrating active tyrosinase in 6 h as shown by gel electrophoresis. The detergent-solubilized T^3 tyrosinase seems to be an intact melanosomal tyrosinase giving a definite band in gel electrophoresis in the presence of Triton X-100. The stability of the trypsin-cleaved tyrosinase against further tryptic action appears to indicate that solubilized tyrosinase has a specific

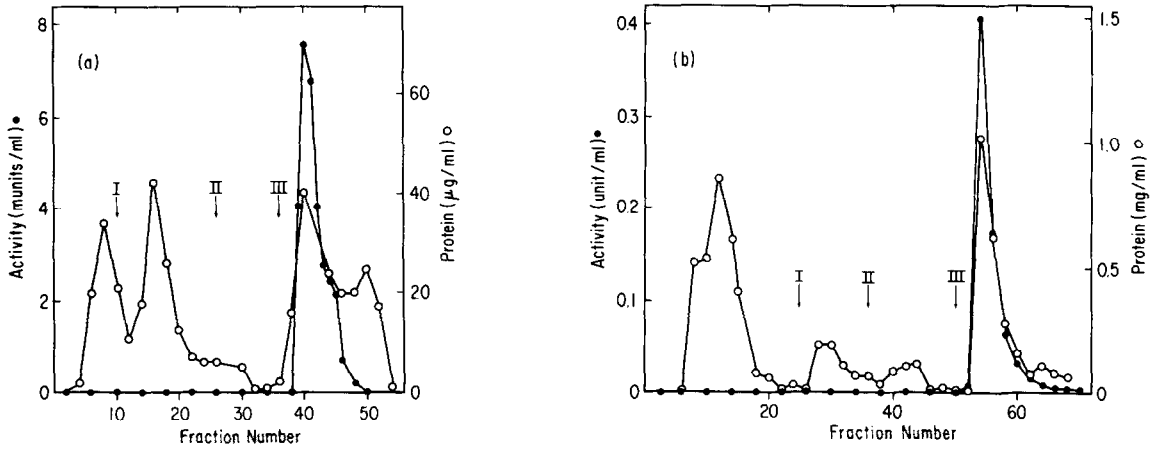

Fig.1. (a) Con A affinity column chromatographies of soluble tyrosinase and (b) sodium cholate-solubilized tyrosinase. Tyrosinase fractions were prepared as described in Materials and methods. Contaminating proteins were eluted by buffer I (4 mM potassium phosphate pH 7.0, 1 M KCl), KCl was then eliminated by buffer II (4 mM potassium phosphate pH 7.0) washing, and the flow was stopped. Column temperature was raised from 4°C to 28°C and tyrosinase was eluted by buffer III (4 mM potassium phosphate (pH 7.0), 1 M α-methyl-D-mannoside). Flow rate was 15 ml/h and 2 ml was collected in each fraction. Aliquots were taken for measurements of tyrosinase activity and protein concentration. Column sizes were 1.4 X 2 cm (a) and 1.2 X 7 cm (b). Proteins applied to each column were 1.6 mg (a) and 28.2 mg (b). Column b was run throughout with 0.1% (v/v) Triton X-100 in the solutions.

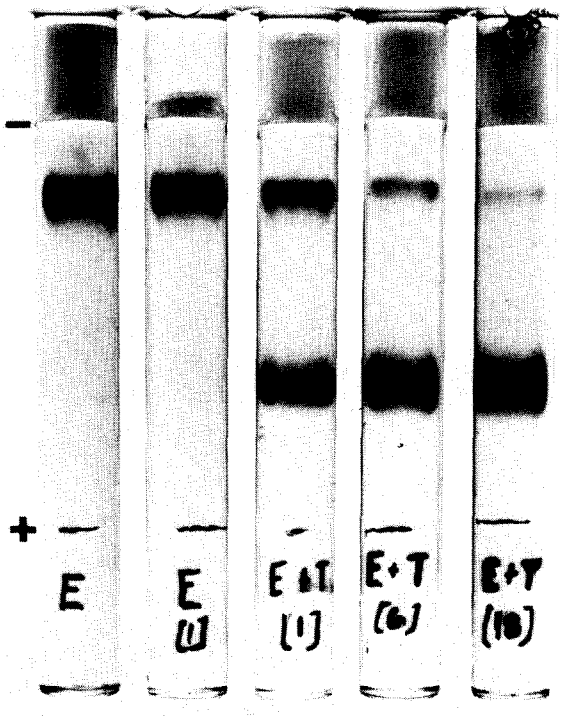

Fig.2. Time course of tryptic cleavage of solubilized tyrosinase examined by 7% polyacrylamide gel electrophoresis in the presence of 0.1% Triton X-100. Partially purified solubilized tyrosinase (E) was incubated with trypsin (T) at 37°C in 10 mM Tris/HCl (pH 8.1), with 10 mM $CaCl_2$ for the period of hours as indicated in [ ]. The final composition of the reaction mixture was as follows: tyrosinase, 0.2 mg/ml; TPCK-trypsin, 0 or 0.2 (for up to 6 h incubation) or 0.4 mg/ml (for a longer than 6 h incubation, trypsin was increased to 0.4 mg/ml after 9 h incubation). Two μg of tyrosinase sample was applied to each gel. After electrophoresis, gels were stained by DOPA as described in Materials and methods. Tyrosinase migrated from top (cathode) to bottom (anode). India ink indicates the position of the front marker dye, bromophenol blue (BPB). Note that tyrosinase incubated alone released no fast-migrating tyrosinase.

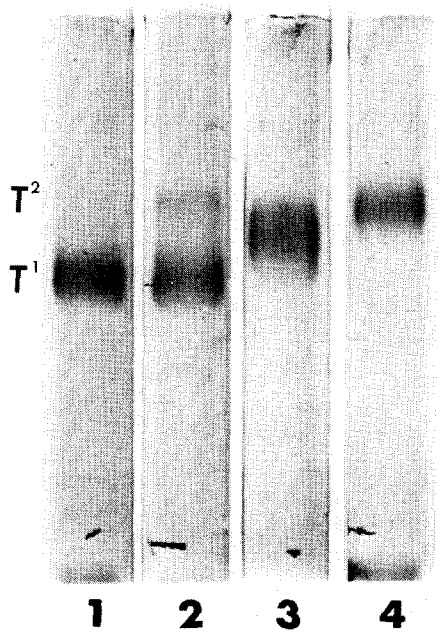

Fig.3. Polyacrylamide gel electrophoresis pattern of neuraminidase-treated tyrosinase. Trypsin-cleaved tyrosinase was prepared as described in Materials and methods, and treated with neuraminidase at 37°C in 5 mM potassium phosphate (pH 7.0), 50 mM KCl and 1 mM $CaCl_2$. Final composition of the reaction mixture was as follows: tyrosinase, 0.5 mg/ml neuraminidase, 0 or 1 unit/ml. Ten μg equivalents of tyrosinase were applied to each gel. After electrophoresis, gels were stained by DOPA. (1) Trypsin-cleaved tyrosinase, (2) soluble tyrosinase containing T^1 and T^2 tyrosinases, (3) trypsin-cleaved tyrosinase treated with neuraminidase for 15 min, (4) trypsin-cleaved tyrosinase treated with neuraminidase for 30 min.

site susceptible to proteolytic action. This tryptic conversion is also in agreement with the results obtained by Miyazaki and Seiji [14] and Quevedo et al. [15], in which mouse melanosomes were treated with trypsin, causing release of soluble tyrosinase. This fast-migrating trypsincleaved tyrosinase was prepared utilizing the gel electrophoresis system as described in Methods, then treated with neuraminidase, and the effect of neuraminidase treatment examined by gel electrophoresis, as shown in fig.3. The result indicated that trypsin-cleaved tyrosinase (R_F: 0.51) corresponded to T^1 (R_F: 0.51), and upon neuraminidase treatment shifted to R_F 0.37 which corresponds to the T^2 tyrosinase position (R_F: 0.36), indicating overall conversion of $T^3 \rightarrow T^1 \rightarrow T^2$. To further confirm this $T^1 \rightarrow T^2$ conversion, T^1 and T^2 tyrosinases were prepared by gel electrophoresis, and treated with neuraminidase. As shown in fig.4, while T^1 tyrosinase shifted to T^2 position upon neuraminidase treatment, T^2 retained its position.

These data strongly support the dynamic conversion pathway of $T^3 \rightarrow T^1 \rightarrow T^2$ rather than their existence as separate enzymes. The presence of carbohydrate

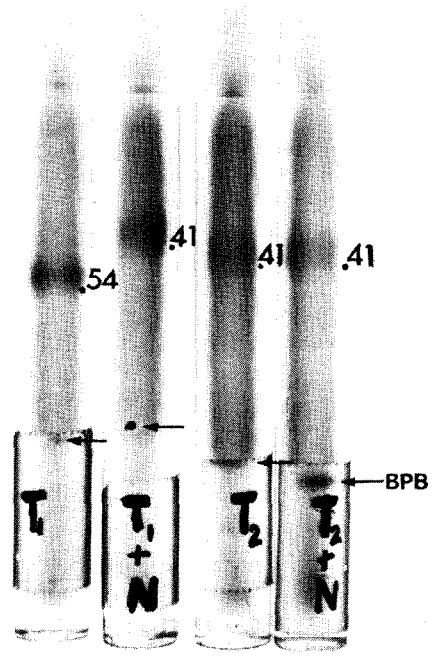

Fig.4. Effect of neuraminidase treatments of T^1 and T^2 tyrosinases examined by 7% polyacrylamide gel electrophoresis. T^1 and T^2 tyrosinases were prepared from partially purified soluble tyrosinase (fig.1a) as described in Materials and methods and were treated with neuraminidase for 16 h otherwise as described in fig.3 legend. Fifty-nine μg equivalent tyrosinase were applied to each gel. Gels were stained by DOPA. T_1 : T^1 tyrosinase, T_2 : T^2 tyrosinase, N: neuraminidase.

moiety in soluble tyrosinase itself also suggests that soluble tyrosinase might stem from melanosome-bound tyrosinase.

It has been reported that melanoma-bearing mice possess circulating serum tyrosinase which stems from the melanoma tissue and a similar report has appeared in conjunction with human melanoma patients [16]. On the basis of the above conversion scheme, circulating tyrosinase should be T^1, since desialylated tyrosinase containing a galactose end-group should be trapped by the liver, as shown by Ashwell and Morell [17]. Whether this conversion is a physiological process is not yet clear. Since there is much necrotic tissue inside the tumor mass, the release of lysosomal enzyme might play a role in this conversion. Yet, T^1 and T^2 tyrosinases can be observed in cytosol fractions of human melanoma tissue culture lines, although inclusion of phenylmethylsulfonyl fluoride during the homogenization step tends to lessen the ratio of soluble tyrosinase relative to particulate form. This aspect requires further studies.

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