Immunological quantitation of tyrosinase from wild-type and albino mutant mice

Hideotoshi B. Tamate, Takejiro Kuzumaki, Jun-ichi Suzuki and Kiichi Ishikawa*

Department of Biochemistry, Yamagata University School of Medicine, Zaoh-Iida, Yamagata 990-23, Japan

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The relationship between gene dosage, enzyme activity, and level of immunologically cross-reacting material (CRM) was examined in mammalian tyrosinase (EC 1.14.18.1) by rocket immunoelectrophoresis. Skin extracts from mice heterozygous (*C/c*) and homozygous (*c/c*) for the albino locus contain 46% and 0% of CRM, respectively, as compared with wild-type (*C/C*) animals. Enzyme activity and CRM level were directly proportional in these genotypes, suggesting that the albino locus controls the quantity of tyrosinase produced in melanocytes.

Tyrosinase *Albino locus* *Rocket immunoelectrophoresis* *Skin homogenate*

1. INTRODUCTION

Mammalian tyrosinase, a copper-containing glycoprotein, is a key enzyme in melanin synthesis. In the mouse, the albino (*c*–) locus at chromosome 7 has been assumed to be the structural gene for tyrosinase, since mice homozygous for albino (*c*) alleles at this locus entirely lack melanin pigment. Some reports have shown that there is no detectable tyrosinase activity in skin extracts of albino mice [1,2]. In contrast, other reports have shown minimal but significant activity in skins [3] and eyes [4] of albino mice, and in the skins of albino hamsters [3]. Moreover, the presence of tyrosinase inhibitors has been suggested in albino axolotls [5], human albinos [6], and mice carrying a mutation at the *c*-locus [7]. Therefore, the real reason for the absence of tyrosinase activity in albino mutants remains unclear. Here, the level of tyrosinase CRM was measured directly by rocket immunoelectrophoresis (RIE), to determine whether the albino mutants produce inactive tyrosinase.

Abbreviations: DOC, sodium deoxycholate; TRX, Triton X-100; Dopa, L-3,4-dihydroxyphenylalanine; CB, Coomassie brilliant blue; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; RIE, rocket immunoelectrophoresis

* To whom correspondence should be addressed

2. MATERIALS AND METHODS

2.1. *Preparation of antiserum*

Melanosomal tyrosinase used as an antigen was purified from Harding-Passey (HP) mouse melanoma as described [8]. Briefly, melanosomal tyrosinase was solubilized from large granule fraction [9] with trypsin and DOC, and subjected to chromatography on Ultrogel AcA 34. Eluate was further subjected to DE32 in batch, chromatography on Ultrogel AcA 44, DEAE-Sephadex A-25 at pH 7.4 and pH 6.0. At the final purification step, tyrosinase was separated by PAGE followed by electroelution [10]. Purified tyrosinase was also subjected to analytical SDS-PAGE according to Laemmli [11]. Antiserum against the purified tyrosinase was raised in a rabbit. Specificity of the antiserum was determined by Ouchterlony double immunodiffusion in agarose (medium EEO, Sigma) containing 1% TRX [12].

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2.2. *Immunoelectrophoresis*

Immunoelectrophoresis was carried out as in [13] except that 1% TRX was added to agarose gels. For RIE, 5 μl samples were placed in 2-mm wells in agarose plate (12 × 10 cm), and run at 20 V for 18 h. To detect tyrosinase activity, gel plates were incubated in 0.1% Dopa, 0.1 M sodium phosphate buffer (pH 7.2) for 1 h at 37°C. Gels were also stained with CB to visualize immunoprecipitin lines.

2.3. *Preparation of tissue extracts*

Albino (DDY) and black (C57BL/6) mice were obtained from Funabashi Farm Co., Japan. Mice heterozygous (*C/c*) for the *c*-locus were the offspring of a cross between DDY (*c/c*) and C57BL/6 (*C/C*). Dorsal skins from 5-day-old newborn mice were excised, washed 3 times with phosphate-buffered saline to remove blood contaminant, and homogenized with 20 vols of 0.5% DOC, 50 mM Tris-HCl (pH 7.4). After being left for 1 h at 4°C, the homogenate was centrifuged at 100000 × *g* (max) for 90 min. The resulting supernatant was mixed with 1/9 vol. of 10% trypsin (Difco 1:250) and incubated for 1 h at 37°C. Tryptic digestion was stopped by adding 4 mM PMSF. The supernatant was then subjected to ammonium sulfate fractionation. The precipitate at 60% saturation was recovered, dissolved with a minimum amount of 10 mM Tris-HCl buffer, pH 7.4, dialysed against distilled water for 18 h at 4°C, and lyophilized to concentrate the sample. The activity of tyrosinase was measured colorimetrically at 475 nm with Dopa as substrate [14]. Protein content was measured according to Lowry et al. [15].

3. RESULTS AND DISCUSSION

The M_r of the purified tyrosinase was estimated to be about 68000 by SDS-PAGE (fig.1). This value is similar to those reported for melanosomal tyrosinase [16–18].

Monospecificity of the antiserum was confirmed by double immunodiffusion. A single precipitin line was formed against the purified tyrosinase and crude homogenate of HP melanoma (fig.2). Dopa oxidase activity was detected on the precipitin line (not shown).

The purified tyrosinase used as an antigen was supposed to be the particulate form (T3) according to Nishioka [16]. However, the antiserum also cross-reacted with soluble tyrosinase which corresponds to T1 and T2 forms (fig.3). The precipitin lines against soluble and particulate tyrosinase fused completely, showing the presence of identical determinants in these isoforms as reported in [19].

Since most of the tyrosinase exists in the particulate form, solubilization of tyrosinase from the

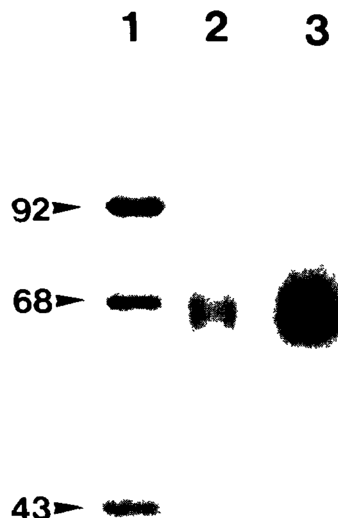

Fig.1. SDS-PAGE pattern of purified tyrosinase. Lane 1, marker proteins: phosphorylase *a* (92 kDa), bovine serum albumin (68 kDa), and ovalbumin (43 kDa). Lanes 2 and 3, purified tyrosinase (5 μg). Gels were stained with CB (lane 2), or incubated with Dopa to detect Dopa-oxidase activity (lane 3).

Fig.2. Ouchterlony double immunodiffusion analysis. Well 1, 5 μl anti-tyrosinase antiserum; well 2, serum from DDY mouse; well 3, liver cell sap of DDY mouse; well 4, homogenate of HP mouse melanoma; well 5, purified tyrosinase. Gel was stained with CB.

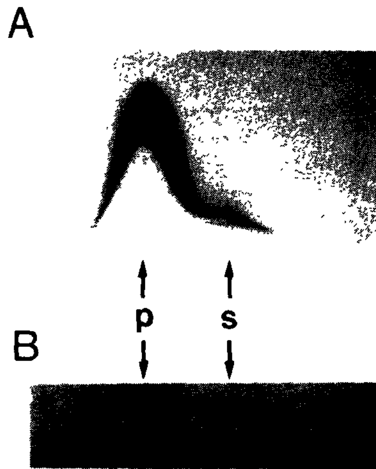

Fig.3. Immunological cross-reaction of anti-tyrosinase antiserum with soluble and particulate forms of tyrosinase. Tyrosinases were solubilized from HP mouse melanoma and subjected to crossed immunoelectrophoresis (A) or immunoelectrophoresis (B), without further purification. Precipitin lines against particulate (p) and soluble (s) tyrosinase fused completely on crossed immunoelectrophoresis (A). It was also confirmed by immunoelectrophoresis (B). Lane 1, solubilized fraction of HP melanoma, which contains both soluble and particulate tyrosinases. Lane 2, particulate tyrosinase purified from large granule fraction. Gels were stained with Dopa.

organelles in a prerequisite for the accurate quantitation of the total amount of the enzyme in tissues. Here, approx. 70% of the tyrosinase activity was recovered in the solubilized samples at the final preparation step as compared with the crude homogenates. For immunodiffusion and immunoelectrophoresis, TRX was added to agarose gels to prevent non-specific aggregation of proteins.

Although there are several reports on the immunological identification of tyrosinase [12,19–21], a quantitative study on the amount of the enzyme in skin has not been reported. Halaban et al. [22] determined the abundance of tyrosinase in cultured melanocytes by using immunoprecipitation of ^{35}S-labeled proteins [22]. Their method, however, is rather difficult to apply for measurement of the amount of tyrosinase in skin. Alternative techniques, such as ELISA and radioimmunoassay, may offer higher sensitivity than our method, but may be less reliable for the quantitation since non-specific aggregation of detergent-solubilized proteins often interferes with the specific binding of an antibody. In our method, the specificity of the immunoprecipitation of tyrosinase was further confirmed by staining precipitin lines with Dopa. Usually, gels containing a 1:200 dilution of the antiserum were used. The area under the rocket is proportional to the amount of antigen in the range 0.2–3.5 μg (fig.4). By using different concentrations of antiserum, the linearity of the quantitation of antigen was well preserved up to 20 μg antigen concentration.

Previously, Coleman [1] determined the amount of tyrosine incorporation into melanin pigment in skins from various *c*-locus genotypes, and found that the value obtained in heterozygotes (*C/c*) was intermediate between those in wild (*C/C*) and albino (*c/c*) homozygotes. Recently, Murray et al. [2] reported the absence of tyrosinase and Dopa-oxidase activities in extracts of albino skin. However, these workers did not measure the amount of tyrosinase in mice of different genotypes. In this study, we found that the tyrosinase activity in skin extract was directly proportional to the amount of tyrosinase determined by RIE, in both *C/C* and *C/c* genotypes (table 1). Both the

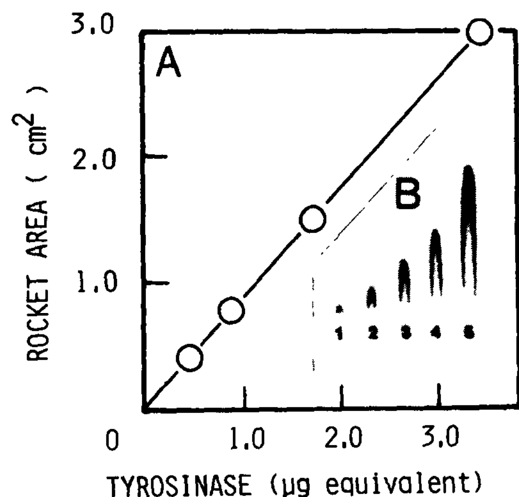

Fig.4. Standard curve for quantitation of the amount of tyrosinase CRM. (A) Purified tyrosinase was subjected to RIE as standard. Area under the rocket was measured and plotted against the dose of standard tyrosinase. (B) Gel plate for RIE after staining it with Dopa. Each well contained 0.22 μg (1), 0.44 μg (2), 0.88 μg (3), 1.76 μg (4), and 3.52 μg (5) of purified tyrosinase.

Table 1

The relationship between tyrosinase activity and its content estimated from RIE in skin extracts

Genotype	Activity[a] (units × 10^{-3}/mg total protein)	Content (μg tyrosinase/mg total protein)	Specific activity (units/mg tyrosinase)
C/C	7.84 ± 0.87	0.72 ± 0.13	10.9
C/c	3.48 ± 1.23	0.33 ± 0.10	10.5
c/c	0.09 ± 0.02	ND	–

[a] Specific activity of tyrosinase was calculated as a/b. ND, no precipitin line was detected. Each value is the mean ± SD of 3 experiments

total enzyme activity and the amount of tyrosinase in *C/c* skin were almost half of those in *C/C* skin, although the specific activity of tyrosinase did not differ between the two. Precipitin line was not formed against the extract of albino (*c/c*) skin, showing the absence of tyrosinase CRM in detectable amounts in albino mice. This result clearly suggests that the dose effect of the albino gene plays a definite role in tyrosinase production.

It must be mentioned here that the c^{p} and c^{h} genes, the other mutations at the *c*-locus, have been suggested to influence the structure of tyrosinase [1,7,23]. The polyclonal antibody used in our study is supposed to recognize various immunological determinants on tyrosinase. Therefore, it is difficult to distinguish minor changes in the immunogenicity of tyrosinase such as expected in these mutations. The use of monoclonal antibodies in our immunological method will provide further information on the relationship between gene doses and its expression as well as the structural specificity of the albino tyrosinase.

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