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# A Sensitive Tyrosinase Method for Human Skin

A. WITTBJER[1], G. ODH[1], E. ROSENGREN[1] and H. RORSMAN[2]

[1]*Department of Pharmacology and* [2]*Department of Dermatology, University Hospital, Lund, Sweden*

**A sensitive competitive radioimmunoassay for quantitative analysis of tyrosinase in biological tissues has been developed. The binding sites of antityrosinase antibodies were competed for by $^{125}$I-labelled human tyrosinase and a known (standard) or unknown (sample) amount of tyrosinase in solution. A sensitivity range of about 5–500 pmol/l was obtained when a solubilized melanoma tyrosinase with a molecular weight of 66,000 D was used as a standard. The method was used on 0.5 cm$^2$ skin samples from which tyrosinase was solubilized with Nonidet P-40. The method is useful for measuring the content of tyrosinase in crude samples where the enzyme activity may be affected by inhibitors or by unspecific oxidation.**
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H. Rorsman, Department of Dermatology, Lasarettet, S-221 85 Lund.

Tyrosinase (EC.1.14.18.1.) catalyzes the formation of the polymeric pigment, melanin. The enzyme is localized exclusively in melanocytes which are above all found in the skin but are also present in the eye. The total volume of human skin melanocytes is 1.5 cm$^3$ (1). As tyrosinase represents only 0.01–0.1% of the total protein content of the melanocytes (2, 3) not more than 15 – 150 μg tyrosinase could be expected to be found in the skin of the body. This fact makes the enzyme difficult to detect and determine and there are only a few reports on tyrosinase in skin. In 1975 Pomerantz & Ances (4) determined the tyrosinase activity in human skin by measuring the amount of $^3H_2O$ formed from $^3$H-tyrosine by tyrosine hydroxylation (5). They examined the influences of race and age to the activity, and found that the tyrosinase activities in the foreskins of black babies were more than double that of white babies (4). Iwata et al. (6) determined the tyrosinase activity in human skin by the same method and also determined the amounts of $^{14}$C-melanin formed from $^{14}$C-tyrosine (7). They found that the activity in foreskin of blacks were about three times higher than that in whites (6). They also found a correlation between the tyrosinase activity and the melanin formation in skin.

Besides determination of tyrosinase by measurement of the enzymatic activity there are also immunological methods for quantification of the enzyme. Halaban et al. (8) used a preparation of polyclonal antibodies to determine the tyrosinase content in cultured melanocytes. Vachtenheim et al. (9) have developed a RIA, Ishiguro et al. (10) and Fuller et al. (11) have described an enzyme-linked immunosorbent assay (ELIZA) for determination of tyrosinase.

We have developed a method for measuring the amount of tyrosinase by radioimmunoassay, a method which is specific and sensitive in crude samples as well. In this study we describe our RIA for tyrosinase quantification applied on seven pieces (0.5 cm$^2$) of human skin. Tyrosinase was solubilized in Nonidet P-40.

## MATERIAL AND METHODS

Tyrosinase was purified as previously described (12). Antiserum to tyrosinase was prepared by injecting the preparation from bovine eye into white rabbits as follows. The antigen had been electroblotted onto a nitrocellulosa membrane. The membrane was cut into small pieces and sonicated in 0.9% saline solution. The sample was thoroughly emulsified in an equal volume of Freund's complete adjuvant and injected into two sites of the neck of the rabbits (12). After four months of biweekly injections high titers of antibodies were obtained.

### *Preparation of $^{125}$I-labelled tracer*

Purified tyrosinase from cultured melanoma cells (13), which was labelled with $^{125}$I was used as the tracer for characterizing the antibody. Chloramine-T was used as an oxidant in the labelling process. A calculated quantity of 5 μg of tyrosinase in 25 μl 20 mM PBS (1.44 g $Na_2HPO_4$, 7.1 g NaCl, 0.2 g $KH_2PO_4$, 0.2 g KCl/l $H_2O$), pH 7.4, 0.4 mCi $^{125}$I, and 2 μl chloramine T (1mg/ml in 50 mM PBS, pH 7.4) was incubated for 10 min on ice. The reaction was stopped by adding 50 μl $NaHSO_3$ (1 mg/ml in 50 mM PBS, pH 7.4). For preservation of the sample 50 μl 2% BSA, 0.02% $NaN_3$, was added. The sample was then passed through a PD-10 Sephadex column (Pharmacia, Sweden), and the column was washed with PBS, pH 7.4. The fractions containing the material not adsorbed were measured for radioactivity for two min/sample in a gamma counter (LKB Wallac 1260 Multigamma, 80% counting efficiency for $^{125}$I), and the fractions with high activity were pooled and stored at +4°C.

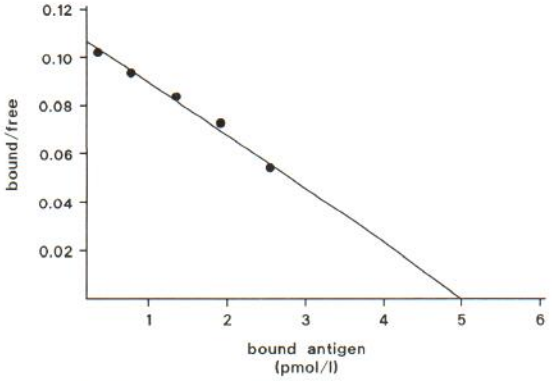


*Fig. 1.* Scatchard plot of rabbit anti-tyrosinase antibody, with fixed amount of antigen and varying concentrations of rabbit anti- tyrosinase antibody. Each value is a mean of triplicate samples.

*Scatchard plot*

The affinity constant of the antibody was determined by performing a Scatchard plot. Anti-tyrosinase antiserum (100 µl) diluted 1:1500 in 50 mM PBS, pH 7.4 containing 0.5% BSA and 100 µl of the $^{125}$I-labelled tyrosinase tracer at different dilutions in the same buffer was incubated for 24 h at +4°C. The antibody-bound tracer was separated from the free tracer by precipitation of the complex with a combination of 500 µl of goat anti-rabbit globulin (GAR) (Milab, Malmö, Sweden) diluted 1:40 and 7.5% PEG in PBS, pH 7.4. The radioactivities in the supernatant and the pellet were measured for two min/sample in a gamma counter (Fig.1).

*Specificity determination of the antibody*

For determination of the specificity of the antibody, anti-tyrosinase antiserum was diluted 1:250 and 1:2000 in PBS, pH 7.4, containing 0.5% BSA. Serum from rabbits which had not received antigen was diluted in the same way. The anti-tyrosinase antiserum and the serum from normal rabbit was incubated with $^{125}$I-labelled human tyrosinase for 24 h at +4°C. The antibody-antigen complex was precipitated with GAR 1:40, 7.5% PEG in PBS, pH 7.4 as described above. The radioactivity was measured in a gamma counter (Table I). The specificity of the antibody was also tested by competing the binding of antigen to the antibody with other proteins. Antibody diluted 1:200 in PBS, 0.5% BSA, pH 7.4, $^{125}$I-labelled tyrosinase about 20,000 CPM, and either of: tyrosinase 20 ng, ovalbumine 2000 ng, hemoglobin 2000 ng, carbonic anhydras 2000 ng was incubated for 24 h at +4°C. The complexes were then precipitated with GAR 1:40, 7.5% PEG in PBS, pH 7.4, as described above and the radioactivity was measured in a gamma counter.

*Class determination of the antibody*

A gelfiltration column FPLC, Superose 12 (Pharmacia, Sweden) was used for determination of class of the antibody. The running buffer was 50 mM PBS, pH 7.4. The antiserum (50 µl) was diluted to 200 µl in the running buffer and applied to the column. The sample was eluted with 50 ml of the buffer, at a flow rate of 0.25 ml/min. As a standard we used IgG and BSA ( 1 mg of each per ml in running buffer), which was filtrated in the same way. The fractions (50 µl) were incubated with $^{125}$I-labelled tyrosinase (about 10,000 CPM) diluted in 100 µl of 0.5% BSA in PBS, pH 7.4 for 20 h at +4°C and then precipitated with GAR 1:40 in 7.5% PEG, PBS, pH 7.4. The radioactivity was measured in a gamma counter as previously described. The protein peak containing radioactivity was compared with the known sample of IgG from the standard (Fig.2).

*Preparation of skin samples*

Pieces of skin were obtained from patients undergoing plastic surgery. Samples of 0.5 cm$^2$ were excised from the skin and cut into small pieces. They were then washed three times in 5 ml of Krebs buffer (equal volumes of A and B, A: 0.59 g $CaCl_2$, 0.56 g $MgSO_4$/l $H_2O$, B: 13.9 g NaCl, 0.72 g KCl, 3.2 g $Na_2HSO_4$, 0.36 g $NaH_2SO_4$/l $H_2O$). After washing they were homogenized in a Polytron homogenizer (Kinematica, Luzern, Switzerland) 3 × 2 sec at level 4 in 0.5 ml of PBS, pH 7.4. Nonidet P-40 was added to a final concentration of 1% and the samples were kept at +4°C over night. The homogenate was then centrifuged at 100,000 × g for 1 h. The supernatant was used for RIA determination.

*Radioimmunoassay*

To decide the most suitable dilution of the antiserum to work at, we incubated our tyrosinase tracer with different amounts of antibody. Antiserum was diluted 1:100 – 1:100,000 in 100 µl 50 mM PBS, 0.5% BSA, pH 7.4. The antisera of different dilutions were incubated with 100 µl of the $^{125}$I-labelled tyrosinase tracer (about 20,000 CPM) in PBS, 0.5% BSA, pH 7.4, for 20 h at +4°C. The antibody-antigen complexes were precipitated with GAR 1:40, 7.5%

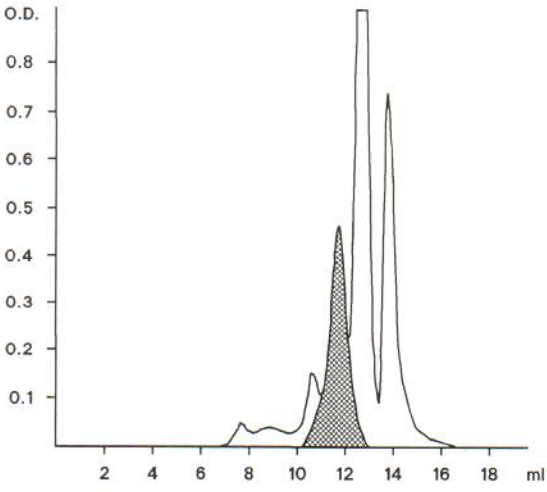


*Fig. 2.* Gelfiltration chromatography on Superose$^R$ column of rabbit anti- tyrosinase antiserum. The shadowed peak indicates radioactivity of $^{125}$I-labelled tyrosinase. IgM is expected to eluate together with the front and IgG is known to have an elution volume of 11.5 ml. An absorbance of 1.0 corresponds to 500 mg protein.

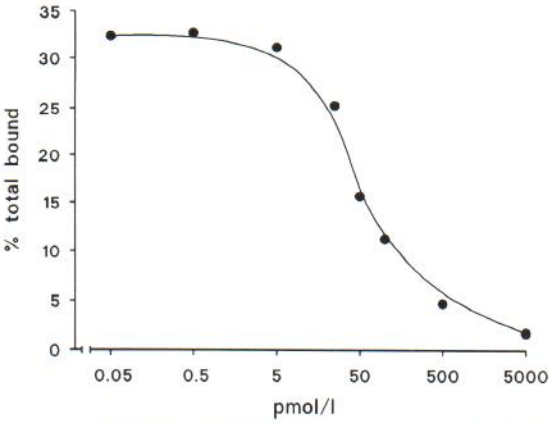


*Fig. 3.* Competitive displacement curve with 40,000 CPM of $^{125}$I-labelled tyrosinase and varying amounts of purified unlabelled tyrosinase at a dilution of 1:3000 of the anti-tyrosinase antiserum. Each value is a mean of duplicate samples.

PEG in PBS, pH 7.4. The radioactivity was measured in a gamma counter as described above. For the RIA we used the antiserum diluted 1:3000. For the RIA standard curve we used 500 µl of PBS, pH 7.4, 0.5% BSA with purified tyrosinase (12).

To the supernatant from the skin BSA was added to reach a concentration of 0.5%. The standard curve and the samples with antibody were incubated for 24 h at +4°C. After 24 h the $^{125}$I-labelled tyrosinase tracer was added (40,000 CPM), and the samples were incubated for 48 h at +4°C. The standard samples and the skin samples were then precipitated with GAR 1:40, 7.5% PEG, in PBS, pH 7.4 as described above. The radioactivity was measured in a gamma counter.

*Precision of the RIA*

For determination of the intraassay variation coefficient, 13 identical samples of medium from cultured melanoma cells (IGR 1) were measured by our radioimmunoassay as described above.

## RESULTS

The affinity constant of the antibody to the antigen was found to be $2.2 \times 10^{10}$ as calculated from the Scatchard curve (Fig. 1). There was no binding of tyrosinase to serum from the nonimmunized rabbits whereas the binding of rabbit anti-tyrosinase antibody to tyrosinase was high (Table I). The specificity of the assay was also ascertained using competition of tyrosinase to hemoglobin, ovalbumine and carbonic anhydras. None of the three proteins even at 100 times excess concentration competed for the binding sites of antityrosinase antibodies to the antigen. The antibody class was IgG (Fig. 2). The range of sensitivity of the RIA was about 5–500 pmol tyrosinase/L (based on a molecular weight of 66,000 D). An example of a RIA standard curve with known tyrosinase amounts is seen in Fig. 3. The precision of the RIA was measured by determination of the intraassay variation coefficient. It was found to be 8.3%. The range of the amount of tyrosinase in skin was 0.2 to 1.1 ng/cm$^2$. Four different samples from the breast showed values between 0.2 to 0.4 ng tyrosinase/cm$^2$. In a skin sample from the face we found 0.6 ng tyrosinase/cm$^2$ and in a sample from the abdomen 0.7 ng tyrosinase/cm$^2$ skin. The highest value, 1.1 ng tyrosinase/cm$^2$ was found in a scrotal skin.

## DISCUSSION

Tyrosinase is a bifunctional enzyme with monooxygenase and oxidase activity. Both functions have been used for quantifying the enzyme. Pomerantz (5) measured the oxygenase activity by determination of the amount of $^3H_2O$ formed from L-(3,5$^3$H) tyrosine. The method is sensitive and specific. The oxidase function has been used by several workers. Graubard & Nelson (14,15) measured the consumption of $O_2$ to estimate the enzyme. Another widely used method is based on measurement of the red color of an oxidation product of dopa, dopachrome (16). Measurement of the amount of $^{14}$C-melanin formed from $^{14}$C-dopa by tyrosinase (6) involves many late reactions not related to tyrosinase. Finally an enzymatic method for measurement of tyrosinase developed at this laboratory determines the dopaoxidase function as the amount of L-5-S-L-cysteinyl-dopa formed from L-dopa in the presence of L-cysteine. This method is highly specific and takes in account the nonspecific dopa oxidation by the presence of D-dopa in the incubate (17,18). Ishiguro et al. (10) and Fuller et al. (11) developed an ELIZA method whereas Vachtenheim et al. (9) used a RIA for determination of tyrosinase in crude samples.

We have developed a RIA for tyrosinase quantification. Our antibody had a high affinity for tyrosi-

Table I. *Specificity of anti-tyrosinase antiserum*

| Antiserum | Titer | CPM values from $^{125}$I tyrosinase |
|---|---|---|
| Anti-tyrosinase | 1:250 | 9389 |
| | 1:2000 | 2409 |
| Normal rabbit | 1:250 | 250 |
| | 1:2000 | 0 |

nase as can be seen in the Scatchard plot (Fig.1). It also shows a high specificity for the antigen as there is no binding of the antigen to serum from non-immunized rabbits whereas the binding to anti-tyrosinase antiserum is high (Table I). The specificity is also shown in competition experiments, as the binding sites of the antibody are not occupied by other proteins even when in great excess. The sensitivity is good, our RIA is more than one order of magnitude higher than earlier described methods. Neither 3% Triton X-100 nor 1% Nonidet P-40 interferes with the antigen-antibody binding. Thus the method can be used for determination of the enzyme in the presence of detergents as well and hence allows analysis of the membrane bound form of the enzyme.

The values obtained from human skin by our RIA show agreement with those reported by Pomerantz & Ances (4) and Iwata et al. (6) who used the $^3H_2O$ formation from $^3H$-tyrosine as indicator of tyrosinase function. They found 0.126 μmol $^3H_2O$/h and g, and 10 – 124 pmol $^3H_2O$/h and mg skin, measured as tyrosinhydroxylase activity. The specific activity of human tyrosinase is 20 μmol/mg protein × min with tyrosine as substrate and 300 μmol/mg protein × min with L-dopa as substrate according to our determinations. Calculated on these figures the tyrosinase content in skin determined by Pomerantz & Ances (4) is somewhat higher and the figures of Iwata et al. (6) are somewhat lower than our values. The skin samples could not be compared as they come from different populations and different regions of the body.

Our values correspond well to the expected quantities of tyrosinase in skin considering that the melanocyte volume of 1.5 $cm^3$ and tyrosinase constitute about 0.01% of the protein in the melanocytes, i.e. about 0.15 mg in the total skin (1,2,3). The method offers good qualities for the determination of minute amounts of tyrosinase even in crude tissue samples.

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