Acta Derm Venereol (Stockh) 1990; 70: 291–294

# Isolation of Soluble Tyrosinase from Human Melanoma Cells

A. WITTBJER,[1] G. ODH,[1] A-M. ROSENGREN,[2] E. ROSENGREN[1] and H. RORSMAN[3]

*Departments of [1]Pharmacology, [2]Biochemistry, and [3]Dermatology, University of Lund, Sweden*

**In the human melanoma cell tyrosinase exists in a membraneous and a soluble form. The membraneous enzyme has an N-terminal amino acid sequence identical to that predicted from a human c-DNA clone by Kwon et al. (3, 13). The soluble form has now been isolated by a technique mainly based on the trypsin resistence of the enzyme and the use of hydrophobic interaction chromatography. The specific dopa oxidase activity of the soluble enzyme was 300 µmol/min x mg protein. On isoelectric focusing the enzyme was found in at least ten bands, *pI* between 3.8–4.6. The molecular weight was found to be 53,000 D. The N-terminal amino acid sequence was the same as that found in the membrane bound form of the enzyme, i.e. the protein maps at the c-albino locus (3). *Key words: Dopa; Cysteinyldopa; Melanin.***

(Accepted February 12, 1990.)

Acta Derm Venereol (Stockh) 1990; 70: 291–294.

H. Rorsman, Department of Dermatology, Lasarettet, S-221 85 Lund, Sweden.

Tyrosinase catalyzes the two first reactions in the melanin synthesis, tyrosine oxygenation and dopa oxidation. In recent reports the existence of two enzymes in the melanocyte with these catalytic activities has been proposed. In 1986, Shibahara et al. described a c-DNA clone encoding mouse tyrosinase (1) and in 1987, Kwon et al. (3), and Yamamoto et al. (2) cloned other c-DNA's from man and mouse, respectively, mapping another enzyme with tyrosinase activity. Both the proteins are expressed in pigmented tissues (1, 4, 5). The protein predicted by Shibahara and co-workers is encoded by the brown (b) locus gene but that of Kwon et al. and Yamamoto et al. by the albino (c) locus gene (3, 6). There are significant homologies between the two amino acid sequences in the proteins. Six histidines are found at such locations that they can be supposed to bind two copper atoms. Such positions of histidines are found to be present in some other proteins known to have tyrosinase activity (7). Both proteins show a short sequence of hydrophobic amino acid residues indicating a membraneous span. In most mammals, tyrosinase is mainly membrane bound, but a minor portion of the enzyme is soluble. Both the soluble and membraneous enzymes occur in multiple forms and the main fractions of the soluble tyrosinase have been named T1 and T2 (8, 9). This multiplicity is due to different degrees of glycosylation (10, 11, 12), as neuraminidase and glycosidases transform the different isozymes into only one form. The nature of soluble tyrosinase is not known.

In a previous study we have isolated the membrane bound tyrosinase from human malignant melanoma cells (13). The N-terminal amino acid sequence corresponded to a sequence predicted by Kwon et al. from a human tyrosinase c-DNA clone (3) and the membrane bound enzyme isolated is thus encoded by the albino (c) locus gene.

As two proteins with tyrosinase activity have been described we have now investigated if the soluble form of tyrosinase derived from the albino (c) gene or the brown (b) gene (3, 6). Isolation of tyrosinase is thwarted by many obstacles but we have developed a convenient method for its preparation. Our procedure is mainly based on tryptic digestion of other proteins and the use of hydrophobic interaction chromatography.

## MATERIAL AND METHODS

Cultures of a pigment producing human melanoma cell line (IGR-I) were obtained from Dr. Christian Aubert, Marseilles, and have been kept at the Tornblad Institute, University of Lund since March 1982 (14). In this investigation we have used material from subcultures number 150 to 165.

In order to test if the soluble tyrosinase did form during preparation a cocktail containing various protease inhibitors, (2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 15 µg/ml soybean trypsin inhibitor, 0.1 mM bezamidine, 5 µg/ml leukopeptin, 7 µg/ml pepstatin in 0.9% NaCl) was added to two culture flasks after the medium had been removed. The combination chosen contained several classes of protease inhibitors and was that used by Stiles et al. (15), to prevent degradation of the β-adrenergic receptor. The cells were loosened by a rubber policeman and homogenized in the presence of the additives with a Po-

lytron homogenizer as described below. The sample was centrifuged at 100,000 g for one hour. The cells in two other flasks were treated in the same way but without the addition of protease inhibitors. The tyrosinase activity was determined in the supernatant and in the pellet after the addition of 2% Triton X-100. The experiment was repeated twice.

In order to isolate soluble tyrosinase about 10 g of cells in 100 ml $H_2O$ were homogenized with a Polytron PT 10/35 homogenizer (Kinematica, Kriens Luzern, Switzerland) 3 × 1 sec (level 4). The sample was centrifuged at 100,000 g for one hour. The supernatant was passed through a Q-Sepharose column (17 mm × 150 mm) (Pharmacia, Sweden) equilibrated with 0.1 M TRIS-HCl buffer, pH 7.2. After sample application the column was washed with 50 ml of the buffer used for equilibration and then a gradient elution was carried out.

The mixing chamber contained 200 ml of equilibration buffer and the reservoir 0.1 M TRIS-HCl, 1.0 M NaCl, pH 7.2. Fractions of 10 ml were collected and the flow rate was 20 ml/h. Tyrosinase activity was measured as described below. The fractions containing tyrosinase activity were pooled and treated with 5 mg trypsin per mg protein (bovine pancreas, Type III, Sigma), and 30 µl 1 M $CaCl_2$, and kept at 37°C for 2 h. This step was performed in order to digest other proteins while tyrosinase stayed intact. The trypsinized sample was cleared by centrifugation at 30,000 g for 30 min, and then passed through a Concanavalin A-Sepharose column (9 mm × 35 mm) (Pharmacia, Sweden) equilibrated with 4 mM $KH_2PO_4$, 1M KCl, pH 7.2. The column was washed with 5 ml of 4 mM $KH_2PO_4$, 1 M KCl, pH 7.2, and 5 ml 4 mM $KH_2PO_4$, pH 7.2, and then eluted with 0.5 M methyl α-D-mannopyranoside, 4 mM $KH_2PO_4$, pH 7.2, in 4 ml fractions. Eluates containing tyrosinase activity were pooled and solid ammonium sulphate was added to give a final concentration of 1.0 M. The sample was then applied to a FPLC hydrophobic interaction column Phenyl-Superose HR 5/5 (Pharmacia, Sweden) equilibrated with 20 mM $KH_2PO_4$, 1.0 M ammonium sulphate, pH 7.2 (buffer A). The sample was applied with a superloop (50 ml) (Pharmacia, Sweden). After sample application the column was washed with 20 ml buffer A, and then eluted with a buffer gradient from 0 % buffer B: 20 mM $KH_2PO_4$, pH 7.2, to 100% buffer B in 40 min at a flow rate of 0.5 ml/min. The fraction containing tyrosinase was concentrated with a Centricon-30 microconcentrator (Amicon, Mass., USA), to a volume suitable for application at polyacrylamide gelelectrophoresis. The SDS-PAGE slab-gel 10% (140 mm × 129 mm × 1.5 mm) was prepared principally ad modum Laemmli (16), using a Bio-Rad electrophoresis apparatus.

*Sample preparation*

50 µl of the sample was mixed with 50 µl of a solubilization solution containing: 20% (w/v) glycerol, 4% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 0.125 M TRIS-HCl, pH 6.8, and a small amount of bromphenol blue. The mixture was boiled for 2 min. As molecular weight markers for the Coomassie Brilliant Blue R-250 staining we used Pharmacia Electrophoresis Calibration Kit for low molecular weight proteins (Pharmacia, Sweden).

*Running buffer (upper and lower):* 0.083 M TRIS (1/3 of the concentration used by Laemmli (16)), 0.192 M glycine, 0.1% (w/v) SDS, pH 8.3.

*Running conditions:* 7 mA for 30 min, 25 mA for 5 h and 30 min.

After SDS-PAGE the proteins were electroblotted to a PVDF (polyvinylidene difluoride) -membrane (Millipore, Bedford, Mass., USA) using a semi-dry electroblotting apparatus (JKA-Biotech, Denmark) according to the manufacturer's instructions. The membrane was stained in 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol for 5 min, and then destained in 50% (v/v) methanol, 10% (v/v) acetic acid for 5 min. After destaining the membrane was washed thoroughly with Millipore water (3x10 min) and then dried.

The main Coomassie Brilliant Blue stained protein band was cut out and sequenized on an Applied Biosystem model 470 sequenator (17). For the determination of the molecular weight of the membrane bound enzyme the Triton X-100 solubilized pellets (see above) were used. Polyacrylamide gelelectrophoresis was performed as described above, but β-mercaptoethanol was excluded from the solubilization solution and the sample was not boiled. The enzyme was stained with dopa (200 ml of 0.1 M BIS-TRIS, 0.08 g L-dopa (Merck), 0.05 g L-Tyrosine (Sigma), pH 6.5). Molecular weight markers were treated in the same way but were stained with Coomassie Brilliant Blue R-250.

*Tyrosinase determination*

The measurement of tyrosinase activity was based upon the determination of the stereospecific dopa oxidation by measurement of the quantity of 5-S-L-cysteinyl-L-dopa formed in the presence of D,L-dopa and L- cysteine. 0.1 ml of the eluate under investigation was added to 0.9 ml solution containing 1 mM L-dopa, 1 mM D-dopa, 3 mM L-cysteine, 10 µg catalase (bovine liver, Sigma) in 0.5 M $KH_2PO_4$, pH 7.2. The presence of catalase in the incubate limits non-specific oxidation. Incubation was performed at 37°C for 2 min under gentle air bubbling. The reaction was stopped by adding 0.1 ml of the incubate to 0.9 ml of 0.4 M PCA. The content of 5-S-L-cysteinyl-L-dopa was determined by means of HPLC and electrochemical detection (18).

## RESULTS AND DISCUSSION

In the experiments where the preparation was made in the presence of protease inhibitors we found that 20 per cent of the total tyrosinase activity in the sample was present in the supernatant after high speed centrifugation. The same ratio of tyrosinase activities of the supernatant and the pellet was found when the preparation was made without inhibitors. The findings indicate that the soluble form is not due to proteolysis during the preparation.

It is well-known that small membrane particles in

I. H F P R A C V S S K N L M E K E C C P P

II. H F P R A X V S S K N L M E K E X X P P

III. H F P R A X V S S K

*Fig. 1.* Amino acid sequence of the N-terminal region of human tyrosinase, *I*) predicted from cDNA by Kwon et al. (3), *II*) obtained from the isolated membrane bound form, *III*) obtained from the isolated soluble form.

some tissue samples cannot be plated by 100,000 g for one hour, because the presence of charged macromolecules keep them in suspension. That this is not the case in our preparation is evident from several findings. No particles were found in the supernatant when examined by electron microscopy after centrifugation for one hour. Addition of Triton X-100 to the supernatant did not give any increase in enzymatic activity. The protein also differed from the membrane bound form in our preparation schedule; in contrast to the membraneous tyrosinase the soluble enzyme can be eluted from the hydrophobic interaction column without the use of detergent. On polyacrylamide gelelectrophoresis the molecular weight of the native membrane bound tyrosinase was found to be 73,000 D. The soluble enzyme was found in a broad band with a molecular weight of about 53,000 D. The broadening of the protein band is probably due to the fact that tyrosinase is a glycoprotein with different degrees of glycosylation (10, 11, 12). The fact that tyrosinase has different charges due to the glycosylation makes the ion exchange and chromatofocusing chromatography almost useless. On isoelectricfocusing the soluble tyrosinase appears in at least 10 different bands with pI:s between 3.8 and 4.6. The specific dopa oxidase activity was found to be 300 μmol/min × mg protein.

The N-terminal amino acid sequence of the isolated soluble tyrosinase was found to be identical to that of the human membrane bound form previously described by us (13) (Fig. 1). The sequence is the same as that predicted from a cDNA clone mapping at the albino (c) locus (3). It should be noted that in the degradation procedure used by us, cysteine residues leave empty cycles, thus the Xs in Fig. 1 probably represent cysteine. The homology of the amino acid sequences determined proves that the soluble form of tyrosinase found also derives from the albino (c) locus. As in the membrane bound form of tyrosinase, histidine is the N-terminal amino acid.

Vertebrate tyrosinase is in contrast to most other proteins highly resistent to tryptic digestion, a fact which considerably simplified our isolation procedure (19, 20, 21). In order to check the trypsin effect on the protein purification was performed without protease digestion by using chromatofocusing after the Concanavalin step. With this procedure we got a pure enzyme fraction. When the enzyme obtained with this method was compared with that purified with the trypsin digestion step as described, we found no difference in molecular weight. The yield without tryptic digestion was, however, too low to allow sequenizing since the chromatography had to be carried out at a low pH-value, inactivating the enzyme.

Trypsin digestion of the membrane bound tyrosinase gave an enzyme with a molecular weight of 62,000 D. The cleavage by trypsin thus occurs close to the membrane span. The soluble tyrosinase now isolated has a molecular weight of 53,000 D indicating that the soluble form of the enzyme lacks a portion remaining in the trypsin digested enzyme. Another proteolytic enzyme must therefore be responsible for the formation of soluble tyrosinase in the melanoma cell.

Nishioka, who in contrast to us was using gelelectrophoresis without reducing agent, found that trypsination of the membraneous tyrosinase gave a product with the same molecular weight as the soluble form which suggested that the soluble form derived from the membrane bound form (20). The difference in molecular weight between the tryptic

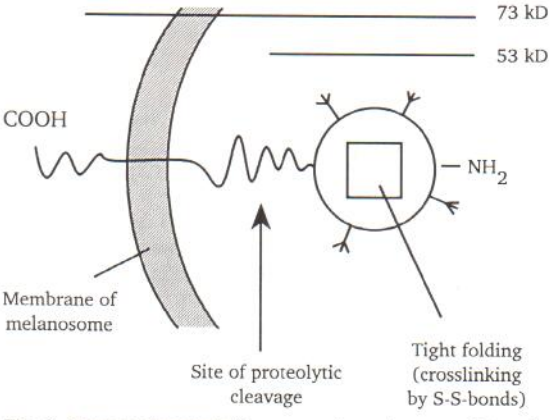


*Fig. 2.* Proposed model of monomeric melanosomal tyrosinase. The mature enzyme has a molecular weight of 73 kD. The protein has a single membrane span. The N-terminal portion is located inside the melanosome and carries the enzymatically active site. The aspargine linked glycoside residues are also present within the melanosome.

digested membraneous tyrosinase and the tyrosinase originally soluble found by us is probably real as we used β-mercaptoethanol to disrupt the polypeptide disulfide bonds. Our results from amino acid sequenizing demonstrate that soluble tyrosinase is a product of the membraneous form and that both are mapped by one gene. A schematic picture of how the enzyme may be located in the melanosome is shown in Fig. 2.

The results obtained did not give any support for the view that two tyrosinases occur in the melanocytes, as proposed from pigment cell specific cDNA clone studies. As the properties of c- and b-proteins (1, 3) should have great similarities it could be argued that the two proteins were not separated in our procedure and that both proteins were present in our final isolation step. When sequenizing the band from PVDF membrane the chromatograms, however, show peaks from just one protein. Still the b-locus protein may be blocked and if so, we would not be able to sequenize it.

## ACKNOWLEDGEMENTS

This investigation was supported by grants from the Swedish Cancer Society (Project 626-B86-14XB), The Swedish Medical Research Council, The Walter, Ellen, and Lennart Hesselman Foundation for Scientific Research, The Edvard Welander Foundation for Scientific Research, The Thure Carlsson Foundation for Scientific Research, The Crafoord Foundation, the donation funds of the Lund University Hospital and the donation funds of the Faculty of Medicine, University of Lund. We thank Ms I Dahlqvist at Malmö allmänna sjukhus for running the amino acid sequence.

## REFERENCES

1. Shibahara S, Tomita Y, Sakahura T, Nager C, Chaudhuri B, Müller R. Cloning and expression of cDNA encoding mouse tyrosinase. Nucleic Acids Res 1986; 14: 2413–2427.
2. Yamamoto H, Takeuchi S, Kudo T, Makino K, Nakata A, Shinoda T, Takeuchi T. Cloning and sequensing of mouse tyrosinase cDNA. Jpn J Genet 1987; 62: 271–274.
3. Kwon BS, Haq AK, Pomerantz SH, Halaban R. Isolation and sequence of a cDNA clone for human tyrosinase that maps at the mouse c-albino locus. Proc Natl Acad Sci USA 1987; 84: 7473–7477.
4. Jimenez M, Lee Maloy W, Hearing VJ. Specific identification of an authentic clone for mammalian tyrosinase. J Biol Chem 1989; 264: 3397–3403.
5. Yamamoto H, Takeuchi S, Kudo T, Sato C, Takeuchi T. Melanin production in cultured albino melanocytes transfected with mouse tyrosinase c-DNA. Jpn J Genet 1989; 64: 121–135.
6. Jackson IJ. A cDNA encoding tyrosinase related protein maps to the brown locus in mouse. Proc Natl Acad Sci USA 1988; 85: 4392–4396.
7. Lerch K, Germann UA. Evolutionary relationships among copper proteins containing coupled binuclear copper sites. Prog Clin Biol Res 1988; 274: 331–348.
8. Burnett JB. The tyrosinases of mouse melanoma. J Biol Chem 1971; 246: 3079–3091.
9. Burnett JB, Seiler H, Brown IV. Separation and characterization of multiple forms of tyrosinase from mouse melanoma. Cancer Res 1967; 27: 880–889.
10. Miyazaki K, Othaki N. Tyrosinase as a glycoprotein. In: Pigment Cell, vol. 3. Basel: Karger, 1976: 113–120.
11. Okhura T, Yamashita K, Mishima Y, Kobata A. Purification of hamster melanoma tyrosinases and structural studies of their aspargine linked sugar chains. Arch Biochem Biophys 1984; 235: 63–77.
12. Nishioka K. Conversion of particulate tyrosinase to soluble form and to desialylated tyrosinase in human malignant melanoma. FEBS Lett 1977; 80: 225–228.
13. Wittbjer A, Dahlbäck B, Odh G, Rosengren A-M, Rosengren E, Rorsman H. Isolation of human tyrosinase from cultured melanoma cells. Acta Derm Venereol (Stockh) 1989; 69: 125–131.
14. Aubert C, Rouge F, Galindo JR. Tumorigenicity of human malignant melanocytes in nude mice in relation to their differentiation. J Natl Cancer Inst 1980; 64: 1029–1040.
15. Stiles GH, Strasser RH, Lavin TN, Jones LR, Caron MG, Lefkowitz RJ. The cardiac β-adrenergic receptor. J Biol Chem 1983; 258: 8443–8449.
16. Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 1970; 227: 680–685.
17. Matsudaira P. Sequence from picomol quantities of proteins electroblotted onto polyvinylidene difluoride membrane. J Biol Chem 1987; 262: 10035–10038.
18. Agrup G, Edholm LE, Rorsman H, Rosengren E. Diastereomers of 5-S-cysteinyldopa. Acta Derm Venereol (Stockh) 1983; 63: 59–61.
19. Hearing VJ, Korner AM, Pawelek JM. New regulators of melanogenesis are associated with purified tyrosinase isozymes. J Invest Dermatol 1982; 79: 16–18.
20. Nishioka K. Particulate tyrosinase of human malignant melanoma. Eur J Biochem 1978; 85: 137–146.
21. Quevedo WC, Holstein TJ, Bienieki TC. Action of trypsin and detergents on tyrosinase of normal and malignant melanocytes. (39116) Proc Soc Exp Biol Med 1975; 150: 735–740.