Isolation of Soluble Tyrosinase from Human Melanoma Cells

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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 cDNA clone by Kwon et al. (3, 13). The soluble form has now been isolated by a technique mainly based on the trypsin resistance 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 membraneous bound form of the enzyme, i.e. the protein maps at the e-albino locus (3). Key words: Dopa; Cysteinyl-dopa; Melanin.

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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 cDNA clone encoding mouse tyrosinase (1) and in 1987, Kwon et al. (3), and Yamamoto et al. (2) cloned other c-DNAs 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 (e) 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 membraneous 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 cDNA clone (3) and the membraneous bound enzyme isolated is thus encoded by the albino (e) 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 (e) 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 trypitic 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, Marseille, 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 benzamidine, 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 p-adrenergic receptor. The cells were loosened by a rubber policeman and homogenized in the presence of the additivites 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 H2O 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, Swe-
den) 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
equilution 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 (bo-
vine pancreas, Type III, Sigma), and 30 μl 1 M CaCl2, 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, Swe-
den) equilibrated with 4 mM KH2PO4, 1 M KCl, pH 7.2.
The column was washed with 5 ml of 4 mM KH2PO4, 1 M
KCl, pH 7.2, and 5 ml 4 mM KH2PO4, pH 7.2, and then
equiluted with 0.5 M methyl α-D-mannopyranoside, 4 mM
KH2PO4, pH 7.2, in 4 ml fractions. Eluates containing
tyrosinase activity were pooled and solid ammonium sul-
phate was added to give a final concentration of 1.0 M. The
sample was then applied to a FPLC hydrophobic interac-
tion column Phenyln-Superose HR 5/5 (Pharmacia, Sweden)
equilibrated with 20 mM KH2PO4, 1.0 M ammonium sul-
phate, pH 7.2 (buffer A). The sample was applied with a
superloop (50 ml) (Pharmacia, Sweden). After sample ap-
lication the column was washed with 20 ml buffer A, and
then eluted with a buffer gradient from 0 % buffer B: 20
mM KH2PO4, pH 7.2, to 100% buffer B in 40 min at a flow
rate of 0.5 ml/min. The fraction containing tyrosinase was
centrifuged with a Centricon-30 microconcentrator (Am-
ticon, Mass., USA), to a volume suitable for application at
polyacrylamide gelelectrophoresis. The SDS-PAGE slab-
gel 10% (140 mm × 120 mm × 1.5 mm) was prepared prin-
cipally ad modum Laemmli (16), using a Bio-Rad elec-
trophoresis 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 Pharma-
cia 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 electrophoretically
transferred to a PVDF (polyvinylidene difluoride) - mem-
brane (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) meth-
anol, 10% (v/v) acetic acid for 5 min. After destaining
the membrane was washed thoroughly with Millipore water
(3×10 min) and then dried.

The main Coomassie Brilliant Blue stained protein band
was cut out and sequenced on an Applied Biosystem
model 470 sequenator (17). For the determination of the
molecular weight of the membrane bound enzyme the Tri-
ton X-100 solubilized pellets (see above) were used. Poly-
acrylamide gelelectrophoresis was performed as de-
scribed above, but β-mercaptoethanol was excluded from
the solubilization solution and the sample was not boiled.
The enzyme was stained with doph (200 μl 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 surfactant-soluble 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 solu-
tion containing 1 mM L-dopa, 1 mM D-dopa, β mM L-
cysteine, 10 μg catalase (bovine liver, Sigma) in 0.5 M
KH2PO4, 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
proteinolysis during the preparation.

It is well-known that small membrane particles in

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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 membranebound tyrosinase the soluble enzyme can be eluted from the hydrophobic interaction column without the use of detergent. On polyacrylamide gel electrophoresis 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 isoelectric focussing 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 resistant 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 trypsin digestion was, however, too low to allow sequencing 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 gel-electrophoresis without reducing agent, found that trypsinination 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 asparagine linked glycoside residues are also present within the melanosome.

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digested membranous 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 sequencing demonstrate that soluble tyrosinase is a product of the membranous 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 sequencing 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 sequence it.

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