2. Materials and Methods
2.1 Chemicals and reagents:

Various chemicals and reagents were purchased from various companies. The details are as stated below.

Sigma Chemical Co., USA.

DMEM; Fetal Bovine Serum (FBS); Antibiotic-antimycotic solution; BSA (fraction IV and V); Dimethyl sulfoxide (DMSO); Coomassie Brilliant Blue R-250; Coomassie Brilliant Blue G-250; Gel chromatography matrices viz., Sephacryl S-500-HR, DE-52; Gel filtration markers; Blue Dextran; 3-Methyl-2-Benzothiazolinone Hydrazone (MBTH); L-3, 4-Dihydroxyphenyl-Alanine (L-DOPA); N, N, N', N'-Tetramethylethylenediamine (TEMED); Ammonium Persulfate (APS); TRIZMA base; Triton X-100; Polyoxyethylene sorbitan monolaurate (Tween 20); 1,2-Diazobicyclo[2.2.2] octane (DABCO); Cycloheximide (from microbial source); Dialysis tubing; Trichloroacetic acid (TCA); Silver nitrate; D(+)-Glucose; Sucrose; HEPES.

GIBCO Life Technologies/ Invitrogen™ Life Technologies

Sodium dodecyl sulfate (SDS); Ethylenediaminetetraacetic acid (EDTA); Trypsin (1:250); Glycine; Urea; HEPES; Dithiothreitol (DTT); Phenylmethylsulfonyl fluoride (PMSF); Hank’s Balanced Salt Solution (HBSS); NBT/BCIP.

MERCK India
Methanol, Acetic acid, Isopropanol, ortho-Phosphoric acid, Disodium Hydrogen Phosphate 2-hydrate, Sodium Dihydrogen Phosphate-2-Hydrate, Calcium Chloride Fused, Sodium Chloride, Sodium thiosulphate, Absolute Ethanol, Glycerol.

**Roche**

Protease Inhibitor Cocktail tablets; BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit).

**BioRad**

UnoQ, Superose, Hydroxyapatite columns

**2D Gel Electrophoresis**; ReadyStrip™ IPIG Strip, all accessories for IEF and second dimension SDS PAGE.

**Antibodies**

**ICN Biomedicals Inc.**

**Primary antibodies**

- p27K, mouse anti-duck monoclonal antibody
- p31K, mouse anti-duck monoclonal antibody
- p30-33K, mouse anti-duck monoclonal antibody

**BIOMOL International LP**

- Anti-Subunit α1,2,3,5,6&7, Monoclonal (clone MCP231)
Secondary Antibodies

Banglore Genei

For Western Blotting
Goat anti-mouse IgG HRP and ALP conjugates
Goat anti-rabbit IgG ALP conjugates

For Immunofluorescence Staining
Goat anti-mouse IgG FITC conjugates
Goat anti-rabbit IgG TRITC conjugates

αPEP1, αPEP7 and αPEP8 antibodies
The peptide antibodies used in this study, αPEP1, αPEP7 and αPEP8 were a generous gift from Prof. VJ Hearing, National Institute of Health, Bethesda. The antibodies generated in rabbits against synthetic peptides correspond to the carboxyl termini of murine melanogenic proteins TRP1, Tyr, Dct, respectively, and specifically recognize each melanogenic protein.

New England Biolabs
Materials and Methods

Prestained Molecular weight Marker, EndoH, PNGase F

BIOMOL International LP
Proteasome inhibitor MG-132 [Z-Leu-Leu-Leu-H]

2.2 Experimental Procedures

1. Cell Culture
B16F10 mouse melanoma cells were originally obtained from NCCS repository and then were maintained in DMEM containing 10% FCS, supplemented with antibiotic-antimycotic in 5% CO₂ in humidified atmosphere. The melanotic cells from the initial cultures were passaged through a few generations till they turned amelanotic. Cells of all three phenotypes (melanotic, partially melanotic and amelanotic) were harvested and used for subsequent experiments.

2. Tumor induction in C57BL/6J mice
To obtain a uniformly melanotic population of B16F10 mouse melanoma cells, the cells grown in culture were injected subcutaneously in C57BL/6J mice as described by Chaubal et al. (2002). The protocols used have been duly approved by the National Centre for Cell Sciences Biosafety and Bioethics Committee. Two weeks later the tumors were dissected out, freed from the connective tissue, washed in PBS and were used to set primary culture of B16 melanoma cells.

3. Protein Extraction
Protein Extraction Buffer (PEB):
Tris-HCl (pH 8.0)- 50 mM
EDTA - 5 mM
MateriaCs and Methods

Triton-X 100 - 0.1%
PMSF - 1 mM

Protease Inhibitor Cocktail (PI):
1 tablet of P.I. cocktail (mini, EDTA free, B.M.) was dissolved in 1.5 ml of deionized water. Aliquots of 150μl were stored at −20°C.

PMSF - 100 mM

Working solution of lysis buffer was prepared by mixing 847 μl PEB+ 143 μl Pl + 10μl PMSF.

Total soluble proteins from the cells were extracted by homogenizing the cell pellet in 2-3 volumes of lysis buffer on ice. The homogenates were then centrifuged 10,000 rpm for 20 min at 4°C. The supernatant was then stored at −70°C till further use.

4. Protein Estimation

Protein content was estimated by Bradford’s method (Bradford, 1976).

**Bradford’s reagent (5X)**

To prepare Bradford’s reagent 50mg of Coomassie Brilliant Blue-G 250 was dissolved in 25ml of ethanol with constant stirring. 50ml of ortho-phosphoric acid was added with constant stirring and the volume was made up to 100ml by adding deionized water dropwise with continuous and vigorous shaking on magnetic stirrer. The solution was stored in dark at 4°C.

**Microassay procedure**

Protein content of cell extracts was determined by adding 0.2 ml of Bradford’s reagent to 0.8ml of appropriately diluted sample followed by vortexing. Absorbance of the mixture was recorded at 595nm. Bovine Serum Albumin (BSA) was used as the standard.

5. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

**Gel solution 30: 1 Acrylamide: Bisacrylamide**
Materials and Methods

10% Ammonium persulfate solution
10% Sodium dodecyl sulfate (SDS)
0.5 M Tris-HCl (pH 6.8)
1.5 M Tris-HCl (pH 8.8)

Tris-glycine electrophoresis buffer for SDS-PAGE
   Tris base - 25 mM
   Glycine - 190 mM
   SDS - 0.1%

10% running gel (SDS-PAGE)
   1.5 M Tris (pH 8.8) - 2.5 ml
   30% gel solution - 3.35 ml
   10% SDS - 100 μl
   10% APS - 50 μl
   TEMED - 5 μl
   Distilled water - 4 ml

4% Stacking gel (SDS-PAGE)
   0.5 M Tris (pH 6.8) - 1.25 ml
   30% gel solution - 650 μl
   10% SDS - 50 μl
   10% APS - 37.5 μl
   TEMED - 5 μl
   Distilled water - 3.05 ml

Sample buffer (Laemmli, 1970)
   2X         5X
Materials and Methods

- 0.5M Tris (6.8) - 2.5 ml 6.25 ml
- 10% SDS - 4 ml -
- SDS - - 1.0 g
- Glycerol - 2 ml -
- Sucrose - - 2 g
- 2-mercaptoethanol - 1.0 ml 2.5 ml
- Bromophenol Blue (BPB) - 0.05% w/v 0.125% w/v
- Distilled water - 0.5 ml 1.25 ml

SDS PAGE was carried out as described in Laemmli (1970). Appropriate amount of protein sample containing equal amounts of protein, as determined by Bradford’s method, from cell extracts were mixed with equal volume of 2X sample buffer and denatured by heating for 3-5 min at 100 °C. The samples were then electrophoresed on 10% or 12% SDS polyacrylamide gel at a constant current (25 mA) at room temperature. After the electrophoresis, the gels were processed either for CBB staining, Silver staining or for Western Blotting.

6. Gel Staining

Coomassie Brilliant Blue- R 250 staining

Coomassie Brilliant Blue -R 250
0.125% in destainer 1

Destainer I
Methanol - 50%
Glacial acetic acid - 10%
Distilled water - 40%
Destainer II
Methanol - 10%
Glacial acetic acid - 10%
Distilled water - 80%

Gel storage solution -
7% glacial acetic acid in distilled water
The gels were stained overnight with 0.125% Coomassie Brilliant Blue-R 250, followed by destaining to clear the background in destainer I and destainer II.

Silver Staining (Blun et al., 1987)
Following electrophoresis the gels were fixed in fixative (50% methanol, 12% acetic acid in deionized water) overnight. The gel was washed with 50% methanol three times for 15 min each. The gel was then washed with 0.02% Sodium thiosulfate for 2 min, followed by two washes with distilled water 10 min each. The gel was then transferred to the freshly prepared silver staining solution (0.2g AgNO3 + 190µl 40% formaldehyde in 100ml distilled water) for 20 min in dark. The gel was then rapidly rinsed with distilled water. The gel was transferred to the developer (6gm Na2CO3 + 25µl 40% formaldehyde + 20µl hypo in 100ml distilled water) till bands of sufficient intensity without background developed. The gel was then immediately washed with distilled water and transferred to the fixative to stop the developing.

7. Western Blotting (Towbin et al., 1979)
Transfer buffer
Tris - 25 mM
Glycine - 190 mM
Methanol - 20%
Materials and Methods

**Phosphate buffered saline (PBS) (pH 7.4 1X)**

- NaCl - 8 g
- KCl - 0.2 g
- Na₂HPO₄ - 1.44 g
- KH₂PO₄ - 0.24 g

All the above components were dissolved in 800 ml distilled water and pH was adjusted to 7.4 with 1N HCl. The final volume was made up to 1 litre.

**Tris buffered saline (TBS) (pH 7.5)**

- Tris - 6.05 g
- NaCl - 8.76 g

All the above components were dissolved in 400 ml distilled water and pH was adjusted to 7.5 with 1N HCl. The final volume was made up to 1 litre.

**Blocking reagents**

- 3% BSA in PBS (pH 7.4) for colour detection
- 1% Blocking reagent (Roche Biochemicals) in TBS (pH 7.5) for chemiluminescence detection

**Colour developing solution** (for peroxidase conjugated secondary antibody)

- 0.3% 4-Chloro-l-Naphthol in Methanol - 1.2 ml
- PBS (pH 7.5) - 4.8 ml
- 30% H₂O₂ - 6 μl

Following the electrophoresis, the proteins were transferred to nitrocellulose membrane (Millipore 45μM), at a constant current of 70 mA for 12-14 h at 4 °C by wet transfer method using the Hoeffer scientific instruments apparatus. After transfer, blot was stained with Ponceau red S to check the efficiency of
transfer. The blots were immediately processed for immunoreaction or stored dry at 4 °C for a few weeks, until further use.

**Colour detection method (for Western blot)**
Blot was destained and equilibrated by washing thrice, 10 min each with PBS, followed by saturation/blocking with 3% BSA in PBS for 4 h at room temperature with gentle shaking. Blot was incubated overnight in primary antibody (diluted in PBS) at 4 °C, followed by incubation in secondary antibody, anti-mouse IgG peroxidase (diluted in PBS containing 0.1% BSA) for 2-3 h at room temperature with gentle shaking. After blocking and antibody incubations blot was washed thrice, 10 min each, with PBS. Blot was reacted with colour developing solution containing, PBS (pH 7.4), 4-chloro-lnaphthol and H₂O₂ in dark. Reaction was stopped by washing the blot with distilled water. Blot was stored dry at 4 °C and then photographed / scanned.

**Chemiluminescence detection**
Blot was washed twice, 15 min each, with TBS (7.5) for to equilibrate the membrane and remove the Ponceau S stain, followed by saturation/blocking with 1% blocking reagent overnight at 4°C or 4 h at room temperature on a shaker. Blot was incubated overnight in primary antibody (diluted in 0.5% blocking reagent) at 4°C, followed by washing twice, 15 min each, with TBS containing 0.1% Tween-20 (TBST) and then with 0.5% blocking reagent. Blot was incubated in secondary antibody, anti-mouse IgG peroxidase (diluted in 0.5% blocking reagent) for 4 h at room temperature with shaking, followed by washing three times, 10 min each, with 1X TBS containing 0.1% Tween-20. Then the blot was reacted with chemiluminescent substrate (Luminol and H₂O₂) in dark. Signal was trapped on a X-ray film, followed by developing in
Materials and Methods

Kodak DA-163 developer (Kodak India Ltd., India) and fixing with sodium thiosulfate. Signals were scanned using BioRad gel documentation system.

8. Tyrosinase Activity Staining in situ on SDS polyacrylamide gel.

Requirements
- 50mM Sodium Phosphate buffer
  The phosphate buffer was prepared just before use by mixing 600μl Na₂HPO₄ (1M) and 4.4ml NaH₂PO₄ (1M) to make total 50ml of 50mM Phosphate buffer (pH 6).
- L-DOPA
- MBTH

The tyrosinase activity assay on polyacrylamide gel in situ was done as described in Jimenez-Cervantes (1993). The melanoma protein samples were mixed with sample buffer without β-mercaptoethanol (0.18M Tris-HCl pH 6.8, 15% glycerol, 0.075% Bromophenol blue, 9% SDS) in a ratio of 2:1. Heat treatment was not given to the samples. The samples were then resolved by SDS-PAGE (12%) at 30 mA. After electrophoresis the gel was equilibrated in 50 mM Na-Phosphate buffer for 15 min at 37°C. Tyrosinase activity was detected by immersing the equilibrated gels in prewarmed 50 mM Na-phosphate buffer (pH 6.0) containing 1.5 mM L-dopa and 4 mM MBTH at 37°C with gentle shaking till dark pink-brown coloured bands developed.

9. Immunofluorescence

In situ localization of various proteins was done by indirect single/double-label confocal immunofluorescence detection. The B16 melanoma cells used were grown on glass coverslips precut in size of 5 mM X 5 mM. The coverslips were thoroughly cleaned with detergent, and then immersed in chromic acid for few h. To remove the traces of acid the coverslips were
MateriaCs and Methods

thoroughly washed in running tap water and then kept immersed in water overnight. The coverslips were then washed in deionized water. The coverslips were then oven dried individually wrapped in aluminum foil, sterilized and stored under sterile conditions till further use.

Indirect Immunofluorescence staining

A general protocol (with minor variations and standardizations as per the antibody and antigen) was followed for immunofluorescence studies.

The cells grown on coverslips were placed in 24 well plates and rinsed in PBS (x 2) for 10 min each without disturbing the monolayer. The cells were then fixed in methanol: acetic acid (3:1) at −20°C. The cells were then rinsed with PBS (x 3) 10 min each, permeabilized with 0.1% PBST 10 min at 4°C, rinsed in PBS for 5 min (x 2). After the permeabilization step the cells were incubated in 5% normal goat serum (NGS) to avoid the nonspecific binding of IgG. After the blocking the cells were rinsed in PBS (x 3). They were further incubated in appropriately diluted primary antibody/antibodies (in case of double immunostaining) overnight. The cells were then rinsed in PBS (x 3) blocked with 5% NGS for 30 min and a mixture of appropriately diluted secondary antibodies (either goat anti mouse IgG-FITC or goat anti rabbit IgG-TRITC or both) was added to the cells. After incubation for 1h the cells were washed in PBS for 10 min (X 3). All the excess PBS from the coverslips was removed action by gently tapping the coverslips on a Whatmann filter paper taking care that the adhered cells do not dry out. The coverslips were then mounted in a drop of mounting medium taking care that no bubble is formed. Excess mounting medium was removed and edges were sealed with a nail polish. The slides were allowed to dry.

An alternative immunofluorescence procedure
Materials and Methods

The cells were washed in PBS and fixed in 3.7% formaldehyde (diluted in PBS) for 30 min, on ice. If the cells were not treated for immunofluorescence immediately, cells were fixed for longer time (40 min) and conserved in PBS + 1% serum at 4°C. The cells were permeabilized with 0.25% Triton X-100, 5 min (room temperature), washed 3 min X 3 in PBS and then the cells were treated with 100% methanol at -20°C, 10 min. followed by a wash in PBS 3 min X 3. The coverslips containing cells were incubated in 1% serum (10 min) at room temperature, then in primary antibody (diluted in 1% serum) for 1h, washed in PBS 3 min X 3. This was followed by incubation in secondary antibody (diluted 1:1000 in 1% serum) for 30-40 min at room temperature, washed in PBS +S (3 min X 2), followed by one wash in PBS. Incubated in 0.1 μg/ml DAPI, 10 min (room temperature). Washed in PBS +S 3 min X 2 followed by one wash in PBS. The coverslips were rapidly wash in distilled water and mounted in Mowiol.

10. Effect of cycloheximide on melanotic and amelanotic B16 melanoma cells

Equal amounts of melanotic and amelanotic melanoma cells were seeded and cultured in DMEM supplemented with 10% FCS and 5% CO2 for 60 h and then exposed to different concentrations of cycloheximide (100-200 mM) in the medium for 90 min; concentrations above 200 mM became toxic to the cells. The cells were harvested, cell count was done and the effect of cycloheximide on co-localization of Tyr (αPEP7) and proteasomal p27 subunit was observed by double label confocal immunofluorescence microscopy.
11. Inhibitor studies

The proteasome-specific inhibitor MG132 (BIOMOL International LP) was dissolved in DMSO (52.5 mM stock). MG132 is a peptide aldehyde inhibitor (benzyloxy carbonyl-Leu-Leu-Leucinal), which inhibits the chymotrypsin-like activity. Amelanotic melanoma cells were exposed to different concentrations of MG132 (0.1-5 mM) in the medium for different periods of time. The cells were then harvested and the effect of the inhibitor was analyzed.

12. PNGase and EndoH glycosidase assay

20μg protein from amelanotic and melanotic cells was denatured in 10X glycoprotein denaturation buffer at 100°C for 10 min. A reaction mixture was made by addition of 1/10th volume of G7 reaction buffer, 10% NP40 and 2 μl PNGase F to one aliquot of the sample and other equal aliquot was kept as control. The samples were incubated at 37°C for 2h.

For EndoH assay 20μg protein from amelanotic and melanotic cells was denatured in 10X glycoprotein denaturation buffer at 100°C for 10 min. A reaction mixture was made by addition of 1/10th volume of G5 reaction buffer 2 μl Endo H to one aliquot of the sample and the other aliquot was kept as control. The samples were incubated at 37°C for 2h.
13. Purification of 20S Proteasomes

Stock Solutions

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
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<tr>
<td>Tris-HCl (pH 7.6)</td>
<td>1 M</td>
</tr>
<tr>
<td>KCl</td>
<td>2 M</td>
</tr>
<tr>
<td>[(CH₃COO)₂Mg.4H₂O</td>
<td>1 M</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 M</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.5 M</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>1.0 M</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
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</tr>
<tr>
<td>HCl</td>
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<tr>
<td>NaOH</td>
<td>0.5 N</td>
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<tr>
<td>(NH₄)₂SO₄</td>
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DE 52 Anion Exchange Chromatography

Equilibration Buffer (Buffer A)

<table>
<thead>
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<tbody>
<tr>
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<tr>
<td>Tris-HCl (pH 7.6)</td>
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</tr>
<tr>
<td>EDTA</td>
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</tr>
<tr>
<td>DTT</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Glycerol</td>
<td>20 % v/v</td>
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0.2M NaCl Elution Buffer (Buffer B₁)

<table>
<thead>
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<th>Component</th>
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<tbody>
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<tr>
<td>Tris-HCl (pH 7.6)</td>
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<tr>
<td>EDTA</td>
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</tr>
<tr>
<td>DTT</td>
<td>0.5 mM</td>
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<tr>
<td>Glycerol</td>
<td>20 % v/v</td>
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</tbody>
</table>
0.5M NaCl Elution Buffer (Buffer B2)
NaCl                      - 500 mM
Tris-HCl (pH 7.6)         - 20 mM
EDTA                     - 0.1 mM
DTT                      - 0.5 mM
Glycerol                 - 20% v/v

Ammonium Sulfate Precipitation
Saturated Ammonium Sulfate Solution.

Gel Filtration Chromatography
Sephacryl S-500-HR matrix
Blue Dextran 5mg/ml
Elution Buffer
50 mM Tris-HCl pH 7.6
5 mM β-Mercaptoethanol

Gel Filtration Markers
Cytochrome C, Carbonic Anhydrase, Bovine Serum Albumin, Alcohol Dehydrogenase, β-Amylase, Dextran.

14. Purification of 26S Proteasomes

Hypotonic Buffer
HEPES                     - 10 mM
MgCl2                     - 1.5 mM
KCl                       - 10 mM
PMSF*                     - 0.2 mM
DTT*                      - 0.5 mM
### Materials and Methods

* to be added just before use

#### Equilibration Buffer (Buffer A)
<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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<tr>
<td>Tris-HCl (pH 7.6)</td>
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<tr>
<td>EDTA</td>
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</tr>
<tr>
<td>DTT*</td>
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<tr>
<td>ATP*</td>
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<td>Glycerol</td>
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* to be added just before use

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<td>EDTA</td>
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<tr>
<td>DTT*</td>
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</tr>
<tr>
<td>ATP*</td>
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<td>Glycerol</td>
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* to be added just before use

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<td>MgCl₂</td>
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<tr>
<td>ATP*</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>DTT*</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10% v/v</td>
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</tbody>
</table>

* to be added just before use

#### Buffer D
<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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</thead>
<tbody>
<tr>
<td>K phosphate pH 7.8</td>
<td>200 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>5 mM</td>
</tr>
<tr>
<td>ATP*</td>
<td>0.5 mM</td>
</tr>
</tbody>
</table>
Materials and Methods

DTT* - 0.5 mM
Glycerol - 10%

* to be added just before use

Cell lysis and 26S proteasome sedimentation
The cytoplasmic extract was obtained by lysing the cells (5 X 10^9, PCV 20ml) in 100 ml hypotonic buffer. The cell debris and nuclei were pelleted by low speed centrifugation (5500 rpm, 15min, SS34 rotor) and the post-nuclear supernatant was collected. The supernatant was collected, the isotonicity was restored by adding 1/7 vol. of 2M (15.5ml) sucrose. The extract was then centrifuged at 36K, 1h, 4°C in Beckman 5Ti rotor. The supernatant was filtered through a cheesecloth. The supernatant was collected and again centrifuged at 125,000 X g, 7h, 4°C in Beckman 45Ti rotor.

UNOQ Ion Exchange Column Protein Purification
The pellet was resupended in Buffer A and insoluble material was separated by centrifugation. The supernatant (6.8 ml/15.5 mg/ml protein) was loaded on an UNOQ 12 column. 5ml fractions were collected by running a gradient of 0-0.5M NaCl. Fractions were analyzed for proteasome activity against SucLLVY (5ml/fraction).

Hydroxyapatite elution profile
Fractions showing peak activity were pooled and loaded onto a CHT II 5ml column (BioRad). 2.5ml fractions were eluted against a linear gradient of 20 mM (Buffer C) -200mM (Buffer D) potassium phosphate. Again the fractions were analyzed for the proteasome activity against SucLLVY. The fractions showing the highest activity were pooled and concentrated (ultrafree IS, 50K
Materials and Methods

MWCo) to a final volume including washing of the membrane to 250 ml before loading onto Superose column.

Superose 6 elution profile

The concentrated pool was loaded onto a Superose 6 pre-equilibrated with Buffer A + 100 mM NaCl. The first 20 fractions collected were of 0.5 ml volume and the next ones 1 ml. Proteasome activity was determined by an activity assay against SucLLVY. The extent of purity of the 26S proteasome in the eluted fractions was checked by SDS PAGE (Fig. 37B) of various fractions (5-17) and also by native gel electrophoresis followed by in gel activity against SucLLVY. As seen the activity gel and the same gel after staining it was observed that fraction 8-11 contained mostly the 26 S proteasome with least contamination of 20S. These fractions were then pooled, concentrated as purified 26S proteasomes. After concentration the protein content was 0.8 mg/ml. To confirm the purified proteasomes are active activity staining in gel was done. And protein profile was checked by loading 4 mg of purified protein on SDS polyacrylamide gel.
15. Preparation of cytoplasmic extract by hypotonic lysis
The medium was removed from B16 monolayer cultures. Cells were washed with PBS, scrapped in PBS and spun at 3000 X g, 10min, 4 °C. The supernatant was decanted and pcv was measured. The cells were rapidly resuspended in 5 volumes hypotonic buffer just to remove residual salts and spun at 3,000 X g, 5min, 4 °C. The pellet was resuspended in 3 volumes (original pcv) hypotonic buffer and allowed to swell on ice for 10min. The cells were homogenized in Dounce’s homogenizer using type B pestle with 10 up and down strokes to achieve approximately 80-90% lysis. The cells were transferred to centrifuge tubes. The nuclei were collected by centrifuging for 15min, 4000 rpm. The volume of cytoplasmic extract was measured and 0.11 volume of 10X cytoplasmic extract buffer was added, mixed and spun at 100,000 X g, 1 h, 4°C in. The supernatant was taken as total cytoplasmic extract and the pellet as total membranes.

16. Subcellular fractionation
Isolation of Endoplasmic reticulum and Golgi apparatus
The cells were lysed by hypotonic lysis, sucrose was added to final concentration of 1.25M. Sucrose density gradient was set up as: 2M sucrose, clarified sample with 1.25M sucrose, 1M sucrose, and 0.25M sucrose. The organelles were separated by density by centrifugation at 100,000Xg (3h, 40°C). The off-white ER enriched fraction was collected at the 1.25M-1.1M sucrose interface and Golgi enriched fraction at the 1.1M-0.25M sucrose interface. The organelle enriched fraction was used for further experimentation.
Materials and Methods

An alternative procedure (Zhang & Herscovitz, 2003)
The cells were harvested and spun at 1000 X g (10 min, 4°C) in 0.25M Sucrose buffer. They were homogenized on ice giving 20 strokes and spun at 1000 X g (10 min, 4°C). The supernatant was spun at 2000 X g (30 min, 4°C) then at 105,000 X g (60 min, 4°C). The pellet was resuspended in 1.35 M sucrose and layered in the middle of 0.8-2.1 M sucrose (0.8, 1, 1.2, 1.35, 2.1 M sucrose) step gradient, and spun at 100,000 X g (6 h, 4°C). 0.8 M and 1 M layer was carefully collected as Golgi rich fraction and 1.35 M and 2.1 M layers as ER rich fraction.

Purification of melanosomes
The cells were harvested and washed once in 0.25 M sucrose, spun at 1000 X g (5 min, 4°C). The supernatant was further centrifuged at 19,000 X g (30 min, 4°C). The pellet was resuspended in 2M sucrose and layered at the bottom of a 1-2 M sucrose (1.0, 1.2, 1.4, 1.5, 1.6, 1.8, 2.0 M sucrose) step gradient, spun at 100,000 X g (1 h, 4°C). Various layers of the gradient were carefully removed. The 1 M fraction was further layered in between 0.8-1.0M sucrose (0.8, 1.0, 1.2 and 1.4 M sucrose) step gradient, spun at 100,000 X g (1 h, 4°C). The 0.8 M and 1 M samples were recovered carefully, and the various fractions were analyzed by Western blot.

17. Proteasome activity assay against LLVY
Proteasome Activity Assay Buffer
Tris-HCl (pH 7.6) - 20 mM
MgCl₂ - 5 mM
DTT - 0.5 mM
ATP* - 1 mM
LLVY* - 1 μM
- added just before use
Proteasome Activity Assay in solution
5 μl of the proteasome containing fraction was mixed with 50 μl assay buffer. The reaction mixture was incubated at 37°C for 30 min. The reaction was stopped by addition of 1ml 1% SDS. The samples were read on a fluorimeter at 380nm excitation wavelength and 440 nm emission wavelength.

4% native polyacrylamide gel
Electrophoresis Buffer
- Tris-Borate: 90 mM
- MgCl₂: 5 mM
- EDTA: 0.1 mM
- DTT*: 0.5 mM
- ATP*: 0.5 mM
*to be added just before use
A 4% polyacrylamide gel was prepared in 1X electrophoresis buffer. Samples were loaded and electrophoresed at 80-100 V in a cold room till the xylene cyanol dye front came out of the gel completely (4 h approximately).

Proteasome Activity Assay in gel
The gel was incubated in 10-15 ml assay buffer for 20 min. at 37°C and it was observed under UV (BioRad gel documentation system).

18. Tyrosinase Immunoprecipitation
(Protocol initially referred from Millipore standard protocols)
Modified RIPA (Radio-Immunoprecipitation Assay) Buffer
- Tris-HCl: 50 mM, pH 7.4
- NP-40: 1%
- NaCl: 150 mM
- EDTA: 1 mM
Materials and Methods

PMSF: 1 mM

Protease inhibitor cocktail

The adherent cells were washed twice in the dish or flask with ice-cold PBS, the PBS was drained ice-cold RIPA buffer was added to the cells (1 ml per 10^7 cells/100 mM dish). The cells were scrapped with a plastic cell scraper that had been cooled in ice-cold distilled water. The cell suspension was transferred into a centrifuge tube. The suspension was gently rocked on an orbital shaker at 4°C for 5 min to lyse cells. The lysate was centrifuged at 14,000 x g in a precooled centrifuge rotor for 15 min. The supernatant was immediately transferred to a fresh centrifuge tube and the pellet was discarded. To prepare protein A agarose beads, they were washed twice with PBS and restored to a 50% slurry with PBS. The cell lysate was pre-cleared by adding 100 μl of either protein A agarose bead slurry (50%) per 1 ml of cell lysate and incubating at 4°C for 10 min on an orbital shaker. Pre-clearing the lysate will reduce non-specific binding of proteins to the agarose when it is used later on in the assay. The protein A beads were removed by centrifugation at 14,000 X g at 4°C for 10 min. The protein concentration of the cell lysate was determined. The cell lysate was diluted to approximately 1 μg/μl total cell protein with PBS to reduce the concentration of the detergents in the buffer. The immunoprecipitating antibody (αPEP7) was added to 500 μl of cell lysate. The optimal amount of antibody that will quantitatively immunoprecipitate the protein of interest was empirically determined. The cell lysate/antibody mixture for either was gently rocked for 2h or overnight at 4°C on a rocker The immunocomplex was captured by adding
100 μl protein A agarose bead slurry (50 μl packed beads) and gently rocking on either a rocker or orbital shaker for either 1 hour or overnight at 4°C. The agarose beads were collected by pulse centrifugation (i.e. 5 seconds in a microcentrifuge at 14,000 rpm). The supernatant was discarded and the beads were washed 3 times with 800 μl ice-cold modified RIPA buffer. The agarose beads were resuspended in 60 μl 2x sample buffer and mixed gently. The agarose/sepharose beads were boiled for 5 min to dissociate the immunocomplexes from the beads. The beads were collected by centrifugation and SDS-PAGE was performed with the supernatant, followed by Western Blot analysis.
19. 2D Gel Electrophoresis

Rehydration Buffer
Standard Method
8 M urea
2% CHAPS
50 mM dithiothreitol (DTT)
0.2% Bio-Lyte ampholytes

SDS-PAGE Equilibration Buffer I (With DTT):
6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2% (w/v) DTT

SDS-PAGE Equilibration Buffer II (With Iodoacetamide):
6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2.5% (w/v) iodoacetamide

Sample preparation and isoelectric focusing
Samples were prepared by lysing equal amount of cells in 2-3 volumes of rehydration buffer. To ensure complete protein extraction samples were homogenized, centrifuged at 10000 rpm and the supernatant was collected. The ampholytes were added to the samples by adding 100X ReadyStrip buffer (final 1X). 300 µL/18 mM IPG strip of sample was loaded with a pipette in IEF focusing tray along the whole length of the channel except for 1 cm at each end. Care was taken not to introduce any bubbles. Cover sheet from IPG strips was peeled off and the strips were placed gel side down in the IEF focusing tray such that the acidic (marked with ‘+’) is at anode of IEF cell taking care that gels made contact with the electrodes. The strips were then overlayed with mineral oil. Rehydration was allowed overnight. After rehydration was complete, two paper wicks were wetted with deionized water (10 µl per paper
wick) and inserted between the IPG strip and the electrodes. The PROTEAN IEF cell was programmed as follows:

<table>
<thead>
<tr>
<th>Step</th>
<th>Start Voltage</th>
<th>End Voltage</th>
<th>Set Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>0 V</td>
<td>250 V</td>
<td>15 min</td>
<td>20°C</td>
</tr>
<tr>
<td>Step 2</td>
<td>50 V</td>
<td>10,000 V</td>
<td>2 h</td>
<td>20°C</td>
</tr>
<tr>
<td>Step 3</td>
<td>10,000 V</td>
<td>10,000 V</td>
<td>30-45,000 V-h</td>
<td>20°C</td>
</tr>
</tbody>
</table>

The default cell temperature was set at 20°C, with a maximum current of 50 μA/IPG strip. When the electrophoresis run was complete, the IPG strips were removed, mineral oil was drained off and care was taken not to lose the orientation. The strips were stored in -70°C.

**Equilibration and SDS-PAGE**

The frozen IPG strips were thawed for 15-20 min and equilibrated in equilibration buffer for 15-20 min for 10 min. on orbital shaker. The buffer was discarded gently and 8 ml/strip equilibration buffer was added in the equilibration tray and incubated for another 10 min on orbital shaker. The strips were then rinsed in 1X TGS and gently kept on the back plate of 7.5-20% Gradient SDS polyacrylamide gel. With a pipette tip the strip was gently slid onto the surface. Molten agarose was overlaid. A well was casted for loading molecular weight marker. The gels were mounted in electrophoresis assembly and were elecrophoresed at a constant current (50 mA) till the dye front reached the end of the gel. The gels were then silver stained, observed and the results were documented.