3. Results
Chapter 3.1

3.1 Degradation of tyrosinase and role of proteasomes in the degradation of tyrosinase and tyrosinase related proteins in melanoma cells
Melanin biosynthesis, which takes place in the melanosomes in animals, requires participation of a number of melanocyte-specific proteins, such as tyrosinase (Tyr), tyrosinase related proteins 1 and 2 (TRP1 and TRP2/Dct) and gp100 (Kushimoto et al., 2003; Watabe et al., 2004). Among them, Tyr occupies a pivotal position in melanin synthesis. It initiates the process by the enzymatic oxidation of L-tyrosine to L-dihydroxyphenylalanine (L-dopa) in a reaction that uses L-dopa as a co-factor (Slominski et al., 1988). It is established that melanogenesis is a complex and highly regulated process.

The structure of Tyr protein is highly conserved among different species. It shows high homology with other tyrosinase related proteins including TRP1 and TRP2. Newly synthesized Tyr has a molecular weight of 55-58 kDa. Proteolytic cleavage of the transmembrane portion of the newly synthesized enzyme generates two soluble forms: a 53 kDa unmodified protein, or a 65 kDa glycosylated form, which may be active in the melanosome or secreted into the extracellular environment. After glycosylation in the trans-Golgi complex, Tyr increases in size to 65-75 kDa or even 80 kDa (Hearing and Tsukamoto, 1991; Del Marmol et al., 1993; Sanchez-Ferrer et al., 1995; Del Marmol and Beermann, 1996; Jimbow et al., 2000). Thus, although Tyr is crucial and rate-limiting enzyme for melanogenesis, pigmentation does not always correlate with the expression of Tyr (mRNA/protein); its function is also regulated at the posttranslational levels, namely, processing including folding and transport to Golgi (Slominski et al., 1991, 2004; Halaban et al., 2001).

As described above, in normal melanocytes, Tyr undergoes extensive glycosylation while it is being trafficked through endoplasmic reticulum, Golgi apparatus and vesicles to melanosomes. On the other hand, in
amelanotic melanoma cells, Tyr is retained within the endoplasmic reticulum, fails to reach the melanosomes and is eventually degraded by proteasomes, the multicatalytic proteases (Halaban et al., 1997; Pal, 2001; Kushimoto et al., 2003; Watabe et al., 2004). Further, expression of TRP1, which facilitates Tyr processing in the ER, has been shown to be down-regulated in the amelanotic cells (Watabe et al., 2004). Thus, there is more than one mechanism for amelanotic phenotype, particularly in Tyr positive melanoma cells. For example, there could be a defect in melanosomal synthesis or in Tyr translocation to melanosomes (Slominski et al., 1988, 1989a,b). Our interest has been to determine the role of proteasomes in the regulation of melanin biosynthesis in in vitro cultured mouse B16F10 melanoma cells.

Proteasomes are multicatalytic non-lysosomal proteases involved in intracellular protein degradation (Hilt et al., 1993; Scherrer and Bey, 1994; Coux et al., 1996; Voges et al., 1999; Ciechanover et al., 2000). Proteasomes exist in two forms as 26S and 20S proteasomes (Coux et al., 1996). The 20S proteasome is a core catalytic structure made up of 2 α and 2 β rings. The 26S proteasome is made up of 20S particle flanked by 19S regulatory subunits on both the sides, which activate the catalytic core. The 26S proteasomes are involved in ubiquitin-mediated protein degradation (Ciechanover et al., 2000; Brooks et al., 2000). Immunoproteasomes have 11S regulatory structures as REG, PA28 on both sides of 20S core and play a role in antigen presentation. Proteasomes have been shown to degrade a number of intracellular proteins (Scherrer and Bey, 1994). Besides this, they are proposed to be involved in many cellular functions such as cell cycle regulation by regulating the cyclin levels (Kawahara and Yokoawa, 1992; Amsterdam et al., 1993), and maintaining a constitutive stable expression of transcription factor c-myb during proliferation and differentiation of myeloid cells (Feikova et al., 2000). They have been shown to take part in neuronal apoptosis (Canu et al., 2000) and inhibition or regulation of apoptosis (Wojciak, 2002). Previous studies have
shown that there is differential synthesis of 20S proteasome subunits in chick embryos during development (Pal et al., 1994), and that the cytolocalization of 20S proteasomes changes as a function of development and differentiation (Pal et al., 1988; Grossi de Sa et al., 1988). It has also been shown that there is variation in 20S proteasome subunit composition in B-lymphoma cell lines and normal B cells (Frisan et al., 2000). A recent report suggests that localization to proteasomes is sufficient for degradation of any protein, and no polyubiquitination is necessary for proteolysis (Janse et al., 2004).

In the present study, we have used the mouse melanoma cell line B16F10 as a model system to determine the correlation between proteasomes and activity of Tyr, and thereby melanin biosynthesis in three different phenotypes of these cells that are spontaneously formed in in vitro cell culture. These three different phenotypes, namely amelanotic, partially melanotic and melanotic melanoma cells represent oscillations between pre-melanoblasts to committed non-terminally differentiated melanocytes (Sharma et al., 1998).
3.1.1 Reduced tyrosinase activity in amelanotic melanoma cells is due to its degradation

We determined the tyrosinase activity in the extracts of melanoma cells of different phenotypes, namely, melanotic, partially melanotic and amelanotic. Cell extracts were separated on SDS Polyacrylamide gels and the tyrosinase activity was determined in situ on the gel. Tyrosinase activity was seen in the form of a mature glycosylated tyrosinase with a molecular weight of 80-83 kDa in the melanotic melanoma cells. Whereas, in the amelanotic cells, there was significantly less amount of detectable activity (Fig. 9A).

On the other hand, as seen in the Western Blot (Fig. 9B), using mature 83 kDa form as well as the nascent form (60 kDa), whereas the amelanotic melanoma cells contained predominantly the immature form (60 kDa) of the protein. Thus, in amelanotic melanoma cells tyrosinase may be targeted for proteasomal degradation as shown by Halaban et al. (1997) and Watabe et al. (2003).
3.1.2 Role of proteasome in the generation of amelanotic phenotype

3.1.2a Increased expression of proteasome subunit p27 is inversely related to tyrosinase activity in the melanoma cells.

In order to determine if there is any correlation between proteasomes and tyrosinase degradation, we used the soluble protein extracts from the melanoma cells of 3 different phenotypes for determining the quantitative variations (if any) in proteasome subunits by Western Blot. As representative proteasome subunits, p27, p31 and α 1-7 (α-1,2,3,5,6,7) were chosen. As seen in figure 10, among the polypeptides (p27, p31, α1-7) analyzed, only p27 appeared to be down regulated in melanotic phenotype, in inverse correlation with melanogenesis. The proteasome subunit p27 was expressed 1.4-fold more (Statistically significant with p<0.02, S.D.± 0.0785) in amelanotic cells than in melanotic cells (Fig. 10B,C). However, the other two sets of subunits (p31 and α1-7) did not seem to change (Fig. 10B,C). These results together thus indicate that there is a negative correlation between expression of proteasome subunit p27 and tyrosinase activity in B16F10 melanoma cells.
Fig. 10. [A] Melanoma cell pellets of 3 different phenotypes used for experiments described in [B].
[B] Determination of proteasome subunit levels in melanotic (1), partially melanotic (2) and amelanotic (3) melanoma cells by Western Blot analysis using antibodies to p27, p31 and α 1-7 proteasome subunits, respectively. [C] The quantification profiles of the Western Blots in [B]. Significant level of increase in p27 expression was observed in amelanotic cells compared with melanotic cells as calculated from three independent experiments (⁎ p<.01, S.D. 0.076; ⁎⁎ p<.02, S.D. 0.0785). mel = melanotic, pmel = partially melanotic, amel = amelanotic.
3.1.2b Tyrosinase and tyrosinase related protein 1 co-localize with proteasomal subunits (p27 and p31) in amelanotic melanoma cells to a greater extent than in melanotic melanoma cells.

In order to further understand this differential expression of proteasomal subunits (p27 and p31), and also their co-localization with tyrosinase and tyrosinase related protein 1, we performed in situ localizations of these subunits by immunofluorescence using respective antibodies. As seen in Fig. 11, proteasomal subunit p27 co-localize with tyrosinase (Fig. 11A) or tyrosinase related protein 1 (Fig. 11B) to a greater extent in amelanotic melanoma cells (Fig. 11Ab, 11Bb) than in melanotic melanoma cells (Fig. 11Aa, 11Ba). Further, a relatively higher degree of co-localization of p27 with tyrosinase related protein 1 than with tyrosinase (Fig. 11B) was observed. Similar results were observed with reference to co-localization of p31 and tyrosinase (Fig. 11C). However, the degree of colocalization was significantly less than that between p27 and tyrosinase (compare Fig. 11C with Fig. 11A).

These data suggest that p27 and tyrosinase as well as tyrosinase related protein 1 co-localize more in the amelanotic melanoma cells and that might contribute to the degradation of tyrosinase leading to lack of melanin biosynthesis. Thus, a distinct role of p27 proteasome subunit in the generation of amelanotic phenotype in B16 mouse melanoma cells becomes a possibility. Our report is thus the first report on the possible role of p27 in controlling melanogenesis in B16 melanoma cells. Since this subunit is not a catalytic subunit, further work is necessary to establish the exact role of p27 in melanin biosynthesis.
Fig. 11. Immunofluorescence (confocal) localization of tyrosinase (Tyr) and tyrosinase related protein 1 (TRP1), proteasomal subunits, p27 and p31, in melanotic (a) and amelanotic (b) melanoma cells. [A], Tyr (1), p27 (2) and their co-localization (3); [B], TRP1 (1), p27 (2) and their co-localization (3); [C], tyrosinase (1), p31 (2) and their co-localization (3).
3.1.3 Enhanced co-localization of p27 and tyrosinase in amelanotic cells in absence of protein synthesis.

In order to determine the interactions (co-localization) of p27 with tyrosinase in cells subjected to inhibition of protein synthesis, cells were treated with 100 μM cycloheximide for 90 min and were processed for immunolocalization of p27 and tyrosinase. Concentration of cycloheximide and time of exposure were optimized through standardization. Interestingly, the co-localization of p27 and tyrosinase was significantly higher in the cycloheximide treated cells (Fig. 12Ab Bb) as compared to the control melanoma cells (Fig. 12Aa Ba). Further this co-localization was significantly higher in amelanotic cells (Fig. 12B) indicating that a higher rate of degradation of tyrosinase. Thus, these results further support the functional significance of co-localization of p27 and tyrosinase in terms of tyrosinase degradation. The higher level of co-localization due to inhibition of protein synthesis indicates that the half-life of tyrosinase being very short, in absence of protein synthesis, almost all the old protein molecules (tyrosinase) are targeted for degradation.
Fig. 12. Effect of cycloheximide (100 μM, 90 min) on co-localization (confocal immunofluorescence) of tyrosinase (Tyr) and proteasomal subunit p27 in melanotic [A] and in amelanotic [B] melanoma cells. In both [A] and [B], 1 and 2, localization of Tyr and p27; 3, co-localization of p27 and Tyr.
3.1.4 Proteasome inhibitor MG132 induces melanin synthesis in amelanotic melanoma cells.

Our data on the negative correlation between the level of the proteasome subunit p27 and tyrosinase activity in mouse melanoma cells, and an earlier report of degradation of tyrosinase by proteasomes (Halaban et al., 1997), raised the question of whether proteasomes have a direct role in regulating tyrosinase activity and melanin biosynthesis in the melanoma cells.

![Fig. 13](image_url)

**Fig. 13.** Effect of various concentrations of proteasome inhibitor MG132 on amelanotic B16 melanoma cells. [A] Cell pellets showing control amelanotic cells (1) and cells after being treated with 0.75 μM MG132 for 72 h (2). [B] Tyrosinase activity staining on gel (a) and Western Blot profile (b-d) of Tyr (b), TRP 1 (c) and p27 (d). In each profile (a-d), Lanes 1-6 are loaded with soluble proteins extracted from cells treated with 0 (control), 0.1 μM, 0.2 μM, 0.3 μM, 0.5μM and 0.75 μM MG132, respectively for 72 h.
To address this question further, another approach using a proteasome-specific inhibitor, MG132 was undertaken to determine its effect on tyrosinase activity and melanin synthesis. Amelanotic melanoma cells were treated with the inhibitor MG132 at various concentrations ranging from 0.1 μM-5 μM. However, the effective range was found to be 0.5 μM-0.75 μM and therefore results beyond 0.75 μM are not shown. Treatment of the cells with the inhibitor for 72 h led to induction of tyrosinase activity (Fig. 13Ba) and increased level of tyrosinase and tyrosinase related protein 1 (Fig. 13Bb,c) as a function of inhibitor concentration from 0.1 μM to 0.75 μM. Among the concentrations used, 0.75 μM inhibitor concentration gave highest tyrosinase activity in correlation with the melanogenesis as seen in the cell pellet color, which appeared distinctly darker as compared to the control cell pellet (Fig. 13A).

Fig. 14. Effect of high concentration of proteasome inhibitor MG132 on amelanotic B16 melanoma cells. Tyrosinase activity staining on gel after 8 h [A] and cells after being treated with 50 μM MG132 for 24 h [B]. (a) Control, (b) after treatment with 50 μM MG132.
Interestingly, higher concentrations (50 μM), inhibitor did not induce melanogenesis and the cells became apoptotic within 24 h of incubation with (Fig. 14Ba, 14Bb) although increase in tyrosinase activity could be seen within 8 hours (Fig. 14A).

3.1.5 Lawsone decreases melanin synthesis in melanotic melanoma cells

Lawsone, main component of Henna, is known to be used by leucoderma patients, possibly to conceal the white spots. However, there are some indications that this product may have some function in regulating melanin biosynthesis. In order to determine the molecular mechanism, we tested its effect on the melanoma cells in vitro, particularly on the tyrosinase activity and melanin biosynthesis. Lawsone did not affect the cell viability at a concentration as high as 1mM also. However, the melanin content in the melanotic cells reduced significantly within 48 h after treating the cells with 100μM lawsone (Fig. 15A), but the tyrosinase activity at 100 μM lawsone concentration showed a decrease in upper band of tyrosinase (Fig. 15B).
Fig. 15. Effect of lawsone on cell phenotype, melanin synthesis and tyrosinase activity. 
[A] Colour of the cell pellet. [B] Tyrosinase activity on SDS gel. (a) control, (b) 10μM lawsone and (c) 100 μM lawsone after 72 h.
3.2 Tyrosinase in Amelanotic and Melanotic Melanoma Cells: Study of Degradation Pattern
Several reports indicate that tyrosinase is sorted for degradation by the cytosolic proteasomes through the ER–associated degradation process (ERAD) (Halaban et al., 1997; Svedine et al., 2004) like other proteins that traverse the secretory pathway (Ahner and Brodsky, 2004). Only 50% wild-type tyrosinase reaches its mature form under optimal conditions (Halaban, 1997). Mutant tyrosinase from albino melanocytes can be completely retained in the ER and subsequently turned over through the ERAD pathway (Halaban et al., 2000; Svedine et al., 2004). This mutant tyrosinase is the immature monoglucosylated 70 kDa glycoform bound to both calnexin and calreticulin and is completely retained in the ER (Berson et al., 2000; Halaban et al., 2000; Toyofuku et al., 2001).

All these observations are made between cell types or under some experimental conditions or represent degradation of mutant tyrosinase. The degradation pattern of tyrosinase and loss of pigmentation under normal physiological conditions giving rise to amelanotic phenotype is unclear yet.

Study in the earlier chapter established that there is a negative correlation between proteasomes (particularly p27 subunit) and tyrosinase and at extremely low concentrations of proteasome inhibitor MG132, melanogenesis could be induced in amelanotic melanoma cells.

With these observations and available literature differential degradation pattern of tyrosinase in melanotic and amelanotic melanoma cells is studied, and the results are presented in this chapter.
3.2.1 Tyrosinase undergoes degradation at a rapid rate in amelanotic cells as compared to that in melanotic cells

To understand the reason for this differential expression of tyrosinase in amelanotic and melanotic melanoma cells we decided to determine the degradation rate of tyrosinase in both the cell phenotypes.

a) Biochemical analysis

As seen in figure 16, it was observed that after addition of cycloheximide tyrosinase level in amelanotic cells goes down in a time-dependent manner. The tyrosinase in melanotic cells gets degraded very slowly as compared to that in amelanotic cells where the half life of tyrosinase is approximately 3 h.
Fig. 16. Degradation rate of tyrosinase in melanotic (a) and amelanotic (b) cells. Cells were treated with 100μg/ml cycloheximide for 0-6h. Level of tyrosinase in each sample was determined by Western blot analysis. Arrow indicates GAPDH, as a loading control.
b) Immunofluorescence analysis

In order to verify the above observations similar experiment was carried out followed by immunofluorescence analysis. It was observed that after addition of cycloheximide tyrosinase level in amelanotic cells goes down (Fig. 17b) and is again increased after addition of proteasome inhibitor MG132 (Fig. 17c).

![Immunofluorescence analysis](image)

**Fig. 17. Tyrosinase level in melanotic and amelanotic cells following the treatment for 3h with 100μg/ml cycloheximide (b) or both 100μg/ml cycloheximide and 25μM MG132 (c) as compared to control untreated cells (a). The nuclei are stained with DAPI and appear blue, whereas tyrosinase is depicted by red colour.**
3.2.2 Degradation of tyrosinase in amelanotic cells is proteasome-dependent

To confirm that the degradation of tyrosinase is proteasome-dependent, the same experiment was repeated in presence or absence of proteasome inhibitor MG132. 25 μM MG132 was added 5 min before addition of cycloheximide.

\[
\begin{array}{c c c c c c c}
\text{25μM MG132} & - & + & - & + & - & + \\
\text{amelanotic} & \text{---} & \text{---} & \text{---} & \text{---} & \text{---} & \text{---} \\
\text{melanotic} & \text{---} & \text{---} & \text{---} & \text{---} & \text{---} & \text{---}
\end{array}
\]

Fig. 18. Degradation rate of tyrosinase in amelanotic cells is proteasome-dependent. Cells were treated with 100 μg/ml cycloheximide for 0-6 h with or without MG132 (25 μM). Level of tyrosinase in each sample was determined by Western blot analysis.

It was seen that tyrosinase was prevented from degradation as soon as MG132 was added (Fig. 18). Whereas, degradation of tyrosinase is seen in amelanotic cells in absence of MG132. Further, with increased duration of incubation with MG132, the quantity of Tyr increased. This reconfirms, that degradation of tyrosinase, at least at total cellular levels, is proteasome-dependent.
3.2.3 Tyrosinase in amelanotic melanoma cells appears to be more ubiquitylated

The degradation of a protein by ubiquitin-proteasome pathway can be regulated at the level of ubiquitylation or at the level of proteasome activity (Glickman and Ciechanover 2001). Normally the intracellular proteins targeted for proteasomal degradation are polyubiquitylated. Earlier results indicated that the accelerated degradation seen in amelanotic melanoma cells is proteasome-dependent. Hence the next obvious question was, whether tyrosinase is ubiquitylated more in amelanotic melanoma cells.

To determine the level of ubiquitylated tyrosinase from either of the phenotypes, immunoprecipitation-Western Blot analysis was done. Tyrosinase was immunoprecipitated using αPEP7 antibody. Ubiquitylation status of tyrosinase was determined by blotting the membrane with anti-ubiquitin antibody. The same membrane was then reblotted with anti-tyrosinase antibody.

![Image](image.png)

**Fig. 19.** Ubiquitylation of tyrosinase in amelanotic and melanotic melanoma cells. Tyrosinase was immunoprecipitated by anti-tyr (αPEP7) antibody and analysed by Western Blot using anti-ubiquitin antibody[A]. The same membrane was reblotted with anti-tyr antibody [B]. In order to equalize the amount of tyrosinase in both the phenotypes, 25μM MG132 was added 4h prior to harvesting the cells. As seen in [A] it appears that tyrosinase is ubiquitylated more in amelanotic melanoma cells.
Results of this experiment indicated that tyrosinase is ubiquitylated more in amelanotic melanoma cells than in melanotic melanoma cells (Fig. 19). Further the level of tyrosinase is substantially less in amelanotic melanoma cells. This may explain the higher degradation rate of tyrosinase in amelanotic melanoma cells.

3.2.4 Tyrosinase subjected to degradation represents fully mature Golgi form

In all the experiments done till now, it was observed that tyrosinase present in either of the cell types though in different proportion corresponds to the fully mature form i.e. the 80 kDa protein. Even after inhibitor treatment the tyrosinase that accumulates is not the immature 55 kDa form but the mature glycosylated Golgi protein (Fig 18). However, this was an assumption based on molecular weight and therefore it was necessary to determine whether the glycosylation status is indeed different in amelanotic and melanotic melanoma cells.

To determine the glycosylation status of tyrosinase in amelanotic and melanotic cells, total protein from both the cell types were treated with Endoglycosidase H (EndoH) and PNGase F, separately. 

PNGase F, is an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins, so essentially deglycosylates the protein completely. Whereas, Endoglycosidase H is a recombinant glycosidase, which cleaves within the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins i.e. Endo H cleaves only high mannose structures, and the proteins when reach Golgi turn EndoH resistant. So, cleavage with PNGase indicates that the protein is glycosylated and resistance to EndoH indicates that the protein is mature Golgi form.
Fig. 20. Glycosylation level of tyrosinase in amelanotic and melanotic cells. Total protein of either of the cell type was digested with PNGase (lane 2,5) or EndoH (lane 3,6) for 2h.

Treatment with PNGase F yielded a tyrosinase band of lower molecular weight (= 55 kDa) indicating complete deglycosylation in both phenotypes Fig. 20, lane 2 and 5, respectively). Whereas, digestion with EndoH did not yield a band with lower molecular weight as compared to the control (Fig. 20, lane 3 and 6, respectively). This indicates that tyrosinase in both the phenotypes is glycosylated (PNGase sensitive), fully mature Golgi form (EndoH resistant).
3.2.5 Subcellular fraction to detect the presence of tyrosinase in ER and Golgi

Considering this point the obvious question that comes to mind is, whether tyrosinase is subjected to degradation via some other post ER system along with/ other than ERAD. To determine this, experiment was designed to isolate ER as a source of immature form of tyrosinase and Golgi as a source of mature form and then study the degradation profile of tyrosinase. ER and Golgi were separated mainly on sucrose density gradients. Figure 21A gives a schematic representation of the gradient set. Golgi enriched fraction was isolated from 0.25 M-1.1 M sucrose interface and ER was isolated from 1.1 M-1.25 M sucrose interface.

Golgi and ER could be isolated in fairly pure form (as determined by blotting the fractions against respective organelle proteins). Presence of tyrosinase was detected in both the organelles, in both phenotypes (Fig. 21B). However, since these fractions were isolated in 1.1-2 M sucrose, using these as a source of tyrosinase for further experiments proved difficult. Therefore, it was planned to use purified melanosomes as source of purified form of tyrosinase.
Fig. 21. Isolation of ER and Golgi from melanotic and amelanotic cells and to determine the presence of tyrosinase in the same. [A] ER and Golgi were isolated on a sucrose step gradient (0.25-2 M). Golgi enriched fractions were collected from 0.25M-1.1M sucrose interface and ER enriched fractions were isolated from 1.1M-2M sucrose interface. [B] Tyrosinase is detected in Golgi (1,2) and ER (3,4) of melanotic (1,3) and amelanotic (2,4) phenotype at a variable proportion.
3.2.6 Purification of melanosomes and determination of presence of tyrosinase in the purified melanosome fractions

Melanosomes from both the phenotypes were isolated by sequential centrifugation at different speeds (Fig. 22A). Presence of tyrosinase in was determined at each step of purification (Fig. 22B).

![Diagram](image)
3.2.7 Determination of degradation rate of tyrosinase from melanosomes of melanotic cells as the source

As explained earlier, melanosomes were isolated from melanotic cells as source of tyrosinase and post melanosomal supernatant either from melanotic cells (self) or amelanotic cells (swap) as source of cytoplasmic supernatant. The reaction mixture was incubated for various time points namely 0h, 1h, and 5h. Though a slight decrease in tyrosinase is seen when incubated in presence of amelanotic supernatant (Fig. 23). This decrease is not significant enough to quantify and draw conclusions. However, the decrease seen is encouraging as it indicates a possibility that the membrane-bound mature tyrosinase is subjected to degradation and this degradation is accelerated in presence of certain cytoplasmic factors from amelanotic melanoma cells.

Fig. 23. Determination of degradation rate of tyrosinase with melanosomes from melanotic cells as source of tyrosinase. Black membrane + black sup (1-3), Black membrane + white sup (4-6) were incubated at 37°C for 0, 1 and 5 h respectively.
3.2.8 Cytoplasmic factors in amelanotic melanoma cells may induce accelerated degradation of tyrosinase

Results indicated that tyrosinase in amelanotic cells is degraded rapidly in amelanotic cells than in melanotic melanoma cells. It was therefore essential to find out the reason for this accelerated degradation.

The hypothesis put forth here was, that certain cytoplasmic factors in amelanotic melanoma cells induce accelerated tyrosinase degradation. To test this hypothesis tyrosinase, as well as cytoplasm from both the cell types was isolated. Briefly, cells of either phenotype were homogenized and lysed in hypotonic lysis buffer, nuclei and other cell debris was removed by centrifugation at 400rpm, 4°C, 15 min. total membrane and supernatant were isolated by further centrifugation at 100,000 X g, 1h, 4°C. The pellet was taken as total membrane fraction and the supernatant as cytoplasmic fraction.

The whole idea was to determine the role of cytoplasmic factors in amelanotic cells on degradation of tyrosinase. So it was essential to avoid cross contamination of tyrosinase through the cytoplasmic extracts.

Fig. 24. Majority of tyrosinase is membrane bound in either of the phenotypes. Total cell membrane and cytoplasm from melanotic and amelanotic melanoma cells. Presence of tyrosinase in each fraction was determined by Western blot analysis.
The level of tyrosinase in the cytoplasmic extracts and total membrane fractions was determined. If the hypotonic lysis was done carefully, it was seen that almost all tyrosinase was restricted to the membrane fraction (Fig. 24) and whatever tyrosinase present in the cytoplasmic fraction (either cytoplasmic or leaked from membranes due to mechanical shear) did not interfere with the experimental observations. This procedure was therefore used for setting up further experiments i.e. membranes (either total membrane or isolated melanosomes) to be used as a source of tyrosinase and cytoplasm as a source of cytoplasmic factors. The protocol of this experiment is detailed in figure 25.
Cells of both, amelanotic and melanotic phenotype were lysed by hypotonic lysis [A]. Total membrane and the cytoplasm isolated from both the phenotypes [B]. Different reactions were set by mixing the membranes and cytoplasm in different combinations [C] as detailed below,

- Black memb + black cyto = black self = original
- Black memb + white cyto = black swap
- White memb + white cyto = white self = original
- White memb + black cyto = white swap

Membranes (memb) were source of tyrosinase and Cytoplasm (cyto) was source of cytoplasmic factors.
- Black indicates the source was melanotic cells and white indicates the source was amelanotic cells.
- Black self indicates the reaction condition is similar as melanotic cells and white self indicates reaction condition is similar to amelanotic cells and therefore taken as reaction controls.
- Swap indicates membrane and cytoplasmic factors are from separate sources.
Melanotic membrane when incubated in presence of melanotic supernatant (Fig. 26A, lane 4-6) there is no significant change in tyrosinase level, whereas on incubation of melanotic membrane in presence of amelanotic supernatant, degradation of tyrosinase was observed (Fig. 26A, lane 7-9). Similarly when amelanotic membrane when incubated in presence of amelanotic supernatant (Fig. 26B, lane 4-6) degradation of tyrosinase was observed, whereas on incubation of amelanotic membrane in presence of melanotic supernatant, there is no significant change in tyrosinase level (Fig. 26B, lane 7-9).

These results indicate that certain factors in cytoplasm of amelanotic cells induce accelerated degradation of tyrosinase.
3.3 Phenotype-specific protein pattern of B16 melanoma cells, and purification of 20S and 26S proteasomes from B16 melanoma cells
Results reported in earlier chapters (3.1 and 3.2) indicated, 1) differential degradation pattern of tyrosinase between amelanotic and melanotic melanoma cells, 2) involvement of certain cytoplasmic factors in the preferential degradation of tyrosinase in amelanotic and melanotic melanoma cells, and 3) proteasome-dependent tyrosinase degradation. These observations, taken together, suggested the need to characterize proteasomes from both the cell phenotypes. This analysis may help in the characterization of cytoplasmic factors that may be responsible for accelerated degradation of tyrosinase.

Thus, to begin with, protein profiles of total soluble proteins from both amelanotic and melanotic melanoma cells were analysed by SDS PAGE as well as by two-dimensional electrophoresis. Subsequently the purification of proteasomes were attempted.

3.3.1a Differential protein profile in amelanotic and melanotic melanoma cells as determined by SDS PAGE

B16 melanoma cell cultures were established by raising tumors in C57BL/6J mice as described in materials and methods. The cells obtained were uniformly melanotic. The cells were then maintained in culture. After a few passages the cells started losing pigment and turned amelanotic. Uniformly melanotic cells and amelanotic cells were thus collected and frozen either for protein extraction or for revival as and when required in order to minimize the variation in pigmentation level of the cells between experiments. Total soluble protein was then extracted from both these phenotypes, as described in materials and methods.
To analyze protein pattern in amelanotic and melanotic B16 melanoma cells, equal amounts of total soluble protein from respective cell phenotypes were electrophoresed by 12% SDS PAGE (Fig. 27). Certain differences were seen between amelanotic and melanotic melanoma cells. Some polypeptides were over expressed or appeared unique in either of the phenotypes. Quantification of a few representative bands was done using BioRad Quantity one gel analysis software (Fig. 27B).

Fig. 27. Differential protein profile in amelanotic (a) and melanotic (b) melanoma cells as seen SDS PAGE [A]. Unique protein bands are depicted by •, and over expressed protein bands are expressed by T. [B] Quantification of the marked (*,T) protein bands from amelanotic and melanotic protein profile, as in A.
3.3.1b Differential protein profile in amelanotic and melanotic melanoma cells as seen by two-dimensional gel electrophoresis

SDS PAGE analysis did not give any clear idea about this differential expression pattern. Therefore, the differential protein expression pattern in amelanotic and melanotic melanoma cells was analyzed by two-dimensional gel electrophoresis. Unique protein bands are depicted by "x" and over expressed protein bands are expressed by "->". Isoelectric point (pI) and molecular weight of these protein spots showing are tabulated in [B]. Apparent unique spots (o) are made bold.

**Fig. 28.** (A) Differential protein profile in amelanotic and melanotic melanoma cells as seen by two-dimensional gel electrophoresis of B16 amelanotic melanoma cells [a] and B16 melanotic melanoma cells [b]. Proteins were separated in 1st dimension on immobilized pH gradient (3-10) and in 2nd dimension by SDS PAGE. Unique protein bands are depicted by "x", and over expressed protein bands are expressed by "->". Isoelectric point (pI) and molecular weight of these protein spots showing are tabulated in [B]. Apparent unique spots (o) are made bold.

**Table B**

<table>
<thead>
<tr>
<th>pI</th>
<th>mol. wt.</th>
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<tbody>
<tr>
<td>3.4</td>
<td>24.6</td>
<td>3.8</td>
<td>34.2</td>
</tr>
<tr>
<td>3.6</td>
<td>27.5</td>
<td>5.8</td>
<td>57.8</td>
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<tr>
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<td>36.2</td>
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<tr>
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<td>8.0</td>
<td>3.3</td>
<td>8.3</td>
<td>15.4</td>
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both the phenotypes was determined by two-dimensional gel electrophoresis. Similarly, some unique protein spots (indicated by \( \omega \)) and some over expressed protein spots (indicated by \( \rightarrow \)) were seen by two-dimensional gel analysis (Fig. 28A). Though the protein loading in both the gels is not equal, protein spots showing obvious difference in expression pattern were marked and a table showing pI and molecular weight of these differentially expressed protein spots is given (Fig. 28B).

As an attempt to characterize differentially expressed proteins further, proteasomes were purified from both the cell phenotypes, and the details are described later.
3.3.2 Purification of 20S proteasomes

For purification of proteasomes, approximately 4 g of the tumor was taken. Protein was extracted using lysis buffer. The protein was then dialyzed overnight against dialysis buffer, 140 mg of total protein was extracted, which was then subjected to purification according to Waxman et al., (1987) and Hough et al., (1987). A flow chart of steps involved in purification of 20S proteasome is given below.

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Flowchart of Purification of 20S proteasomes
DE-52 Anion Exchange Chromatography

The protein extract was passed through DE-52 anion exchange column (25 X 2.5cm) for purification. Prior to this, the packed column was equilibrated by passing 2 bed volumes of equilibration buffer. The proteins adhered to the column were eluted stepwise by 0.2 M NaCl elution buffer and 0.5 M NaCl elution buffer (Fig. 30). At each step of purification, the partially purified proteins were analyzed for the presence of proteasomes by SDS PAGE – CBB/silver staining (Fig. 31A) followed by Western blotting using antibody against proteasome subunit p27 (Fig. 31B). It is to be noted that for protein analysis, two consequent fractions were pooled onto one. As seen in the protein profile (Fig. 31B), most of the proteasomes were eluted in 0.2 M NaCl fractions (fraction 61-68) and in two 0.4 M NaCl fractions (fraction 165-166).

Fig. 30. DE-52 column (35cm X 2.5cm) chromatography profile of purification of proteasomes; Proteins were eluted by 0.2M NaCl elution buffer, fraction no. 55-155 and 0.5M NaCl elution buffer, fraction no.: 156-198. 148mg total protein was loaded.
Fig. 31. [A] SDS PAGE analysis of the DE-52 eluted fractions (CBB staining); (a) 0.2M NaCl elution fractions lane 1, molecular weight marker; lane 2, total protein; lane 3-10, fraction no. 55/56, 57/58, 59/60, 61/62, 63/64, 65/66, 67/68 respectively. (b) 0.5M NaCl elution fractions, lane 1, molecular weight marker; lane 3-6, fraction no. 165/166, 167/168, 169/170, 171/172 respectively. 30ug protein loaded in each lane.
[B] Western Blot analysis of the protein fractions (seen in gels in Fig. 31A) using antibodies against proteasome p27 subunit. Arrow indicates the presence of p27 in these fractions.
Ammonium sulfate precipitation

The 0.2 M NaCl elution fractions were then pooled and further purified by 0-40% and 40-80% ammonium sulfate precipitation in sequence (Fig. 32). The precipitated proteins were pelleted down by centrifugation at 10,000 rpm for 10 minutes at 4°C. The pellets were dissolved in minimum amount of dialysis buffer and dialyzed overnight. It was seen by Western blot analysis that proteasomes were precipitated mainly in 80% ammonium sulfate. This fraction is being purified further by Sephacryl S-500 HR gel exclusion chromatography.

Fig. 32. Western blot profile using antibody against proteasome subunit p27 of DE-52 fractions after ammonium sulfate precipitation. Lane 1, total protein; lane 2, 0-40% ammonium sulphate cut; lane 3, 40-80% ammonium sulphate cut. Arrow indicates the presence of proteasome subunit p27.
Gel filtration chromatography

The proteasome-containing fraction after ammonium sulfate precipitation was dialyzed and concentrated using a Centrikon P30 and were subjected to further purification by gel filtration chromatography (Fig. 33A). The matrix used was Sephacryl S-500 HR (2.5 X 30 cm). After equilibration blue dextran was loaded on the column to determine the void volume. The fractions were collected TCA precipitated and analyzed by SDS-PAGE and Western Blot (Fig. 33B). Fractions (fraction 63-79) showing proteasomes were pooled.

As seen in figure (Fig. 33C) though proteasome subunit could be detected in the fractions, after the fractions were pooled and concentrated, in SDS PAGE profile it was seen that the fractions were not completely purified and ideal 20S proteasome pattern as seen in literature was not obtained.
Fig. 33. Sephacryl S-500 HR (2.5 X 30 cm) gel filtration chromatography profile of purification of proteasomes. Proteins were eluted with elution buffer [A]. TCA precipitated fractions were analysed for presence of proteasomes by Western blot analysis using antibody against proteasome subunit p27 [B] Fractions 63/64, 65/66, 67/68, 69/70 (lane 1-4, respectively) showing presence of proteasomes were pooled concentrated and proteasome profile was determined by SDS PAGE [C] lane 1, total protein; lane 2 pooled concentrated fractions.
3.3.3a Purification of 26S proteasomes

The purification of 26S proteasomes was then attempted using BioLogic DuoFlow chromatography system (Bio-Rad). Initially the purification steps were standardized using HeLa cells as source of proteasomes and then similar procedure was followed for purification of 26S proteasomes from amelanotic and melanotic B16 melanoma cells. The flow chart of purification is given below:

- Extract protein by hypotonic lysis
- Centrifuge at 5500rpm, 15 min
- Collect supernatant
- Nuclei and cell debris
- Centrifuge at 36000rpm, 1 h, 4°C
- Centrifuge at 125,000 X g, 7 h, 4°C
- Load on UNO Q column
- Resuspend the pellet in buffer A
- Load on hydroxyapatite column
- Load on Superose 6 column
- Pool the fractions and concentrate
- Analyze for the purity and activity of 26S proteasomes

* Fractions showing proteolytic activity against SucLLVY (fluorogenic substrate) were pooled

Flowchart of Purification of 26S proteasomes
Cell lysis and 26S proteasome sedimentation

The cytoplasmic extract of \(5 \times 10^9\) HeLa cells was obtained by lysing the cells as described in materials and methods.

UNOQ Ion Exchange Column Protein Purification

The pellet was resuspended in Buffer A and insoluble material was separated by centrifugation. The supernatant (6.8 \(\mu\)l/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 (Fig.34 A). Fraction numbers 4-20 were analyzed for proteasome activity against SucLLVY (5\(\mu\)l/fraction) as the substrate. A peak was seen at fraction 13-15 (Fig.34 B).
Fig. 34. UNQ 12 elution profile for purification of 26S proteasomes. [A] Proteins were eluted by running a 0-0.5M NaCl gradient. [B] Proteasome activity profile as seen in fractions eluted from UnoQ column fraction no 4-20 were analysed for activity of proteasome against LLVY.
Hydroxyapatite elution profile

Fractions 12-15 of the UNO Q column elution 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 analysed for the proteasome activity against SucLLVY (Fig. 35).

Fig. 35. Hydroxyapatite elution profile for purification of 26S proteasomes; Proteasome activity profile as seen in fractions eluted from hydroxyapatite CHT II column. Proteins were eluted against a linear gradient of 20-200mM potassium phosphate buffer. Fraction no 1-24 were analysed for activity of proteasome against SucLLVY.

The fractions (No. 20-21) showing the highest activity were pooled and concentrated (ultrafree 1S, 50K MWCo) to a final volume including washing of the membrane to 250 µl before loading onto Superose column.
Superose 6 column elution profile

The concentrated pool was loaded onto a Superose 6 column 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 (Fig. 36). A peak of proteasome activity was seen at fraction 9-13.

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**Fig. 36.** Gel Filtration chromatography for purification of 26S proteasomes; Proteasome activity profile as seen in fractions eluted from Superose 6 column. Proteins were eluted against Tris-HCl buffer containing 100 mM NaCl. Fraction no 1-24 were analysed for activity of proteasome against SucLLVY.
The extent of purity and the activity of the 26S proteasome in the eluted fractions (5-17) by native gel electrophoresis followed by activity staining against SucLLVY in gel, in situ (Fig. 37A) and by staining the native gel by Coomassie blue (Fig. 37B). As seen the activity gel, as well as the Coomassie stained gel it was observed that fractions 8-11 contained mostly the 26S proteasome with least contamination of 20S.

These fractions were then pooled, concentrated as purified 26S proteasomes. After concentration the protein content was 0.8 µg/µl. To confirm the activity of purified proteasomes, activity staining in gel was
Further, protein profile was also checked by loading 4 μg of purified protein on SDS polyacrylamide gel (Fig. 38C). The 26S proteasomes were highly pure and contamination with other polypeptides was very less (Fig. 38B, C). The purified proteasomes were active, as seen by their activity against fluorogenic substrate SucLLVY (Fig. 38B,C).

![Proteasome activity on 4% native gel](image1)

![Colloidal blue staining 4% native gel](image2)

![12% SDS PAGE profile of purified 26S proteasome](image3)

**Fig. 38.** Activity and purity of 26S proteasome in the pooled Superose elution fractions showing proteasome activity; Proteasome activity on 4% native gel (A), Colloidal blue staining 4% native gel (B), 12% SDS PAGE profile of purified 26S proteasome (C).
3.3.3b Purification of 26S proteasomes from amelanotic and melanotic B16 melanoma cells

In order to compare the proteasome profile of amelanotic and melanotic melanoma cells. Cells of both phenotypes were harvested in large quantity (∼3 X 10⁸ cells/ phenotype). 26S proteasomes were purified from both the phenotypes precisely as described above. Figures 39 and 40 show the sequential purification steps and the activity of 26S proteasomes obtained after each purification step. The purified proteasomes from both the phenotypes were enzymatically active and showed typical 26S elution profile. These purified proteasomes need to be characterized further.
Purification of proteasomes from amelanotic melanoma cells

Fig. 39. Purification of proteasomes from amelanotic melanoma cells; 26S proteasomes from amelanotic cells were purified by sequential purification steps. Presence of active 26S proteasome was determined by activity assay against LLVY. 26S proteasome activity profile seen in fractions eluted from [A] UNOQ column [B] CHT II column, [C] Superose 6 column.
Purification of proteasomes from melanotic melanoma cell

Fig. 40. Purification of proteasomes from melanotic melanoma cells: 26S proteasomes from melanotic cells were purified by sequential purification steps. Presence of active 26S proteasome was determined by activity assay against LLVY. 26S proteasome activity profile seen in fractions eluted from [A] UNOQ column [B] CHT II column, [C] Superose 6 column.