Chapter 2

Establishment of *in vitro* model system

2.1. INTRODUCTION

Due to its compartmentalized nature, multi subunit composition, a gated mechanism of substrate entry and requirement for substrate unfolding, the mechanism of protein degradation by the proteasomes and the various rate limiting steps involved are not well understood. Few well known factors that affect the half-life of proteins are ubiquitination, post-translational modification, misfolding of proteins (thyroglobulin, factor XII, antithrombin etc.) and loss of one of the binding partner (viral protein pM140 protects pM141 from degradation; free α and β globin subunit degrade fast but, hetero-tetramer is stable) (Bolin et al., 2010; Goldberg, 2003). Some substrate for which E2 and E3 ubiquitination enzymes are known can be ubiquitinated *in vitro* using E1, suitable E2 and E3 enzymes or a tetra ubiquitin tag can be fused to the substrate (Thrower et al., 2000). These modified substrates have been used to understand the role of ubiquitination and recruitment of substrate to the proteasome (Prakash et al., 2009). Interestingly, even ubiquitinated substrates were not amenable for degradation unless they also carry a long unstructured in trans (Prakash et al., 2009; Prakash et al., 2004). Nevertheless the numbers of examples are very few and there are fundamental questions that remain unanswered including the role of sequence, conformation and structure of the substrate in determining the half-life of the protein. Besides, signals other than ubiquitin that are likely to avoid premature release of the substrate are not well understood. The origin of intrinsic degradation signals is not known.

We decided to develop a new model system to address these questions and chose to test myoglobin (Mb) as a putative model substrate. Myoglobin (Mb) is a single-chain globular protein of 153 residues, exist in both heme (porphyrin ring with iron) bound holo and heme free apo form (Kendrew et al., 1958). Mb is oxygen binding protein found in muscle tissues. The additional characteristics that make myoglobin an

attractive model substrate are a) Both holo and apo forms have well defined tertiary structure, b) crystal structure of holo and NMR structure of apo form is known, allowing targeted protein manipulations and structural interpretations possible (Eliezer and Wright, 1996; Kendrew et al., 1958), c) protein is largely α-helical (expected to be less stable than the β-sheet), d) extensive studies have been done to understand the thermodynamics and kinetic properties of protein unfolding by chemical denaturants (Barrick and Baldwin, 1993; Eliezer et al., 2000; Eliezer et al., 1998).

To establish the model system we need pure, intact and active proteasome as well as Mb. Utilizing the basic nature (theoretical pI 9) of myoglobin we were able to purify it in one step. Proteasome was purified by affinity chromatography. We hypothesized that ligand free form of Mb will be susceptible for proteasomal degradation. We have standardized the *in vitro* proteasome degradation assay using purified components and established that a globular protein can be degraded *in vitro* without the assistance of trans-acting factors by purified proteasome.

2.2. MATERIALS and METHODS

2.2.1 Expression and affinity purification of 26S and 20S proteasome: Eukaryotic 26S proteasome has been purified from plants, yeast, rabbit muscle and red blood cells (Fischer et al., 1994; Otsuka et al., 1998; Yang et al., 2004). The homologous 20S proteolytic particles from archaea and mycobacteria as well as the ATPase homolog PAN (Protein Activated Nucleotidase) from archaebacteria have been purified by recombinant technology (Benaroudj and Goldberg, 2000; Wilson et al., 2000). Classical method of proteasome purification involves large amount of material from natural sources with protocols extending over a couple of days. The purification principle embodies both the acidic nature of the proteasome (using anion exchange

chromatography) and its huge size (using gel filtration chromatography or ultracentrifugation) (Fischer et al., 1994; Yang et al., 2004). Purification has become relatively easier due to advancement in genetic techniques and availability of several fusion tags. Commonly used tags are the Protein A and the FLAG (Leggett et al., 2002; Verma et al., 2000). Protein A is a 56 kDa protein found in the cell wall of the bacterium *S. aureus*. Due to very high affinity of protein A with IgG, proteasome from these strains are purified using IgG agarose. Unlike many common epitopes, the octapepide sequence DYKDDDDK or the FLAG tag, is position-insensitive and is the first example of a fully functional short epitope tag reported for the purification of proteasomes both from yeast and the mammalian cells. The anti-FLAG M2 antibody recognizes this epitope present in tandem and purification is normally done using this antibody coupled to agarose. These tags have been successfully fused to subunits of every sub-complex of the proteasome, for example RPN11 from lid, RPN1 from base and PRE1 from the 20S particle (Sone et al., 2004). These tags are generally incorporated by replacing the chromosomal copy and can precipitate/purify the entire 26S complex. Recently, such tags have been used creatively to either affinity purify or enrich the proteasomes from mammalian cells by co-immunoprecipitation (Bousquet-Dubouch et al., 2009; Wang et al., 2007). Not only were the resulting proteasomes characterized by mass spectrometry but, several interacting proteins were also identified providing a glimpse at the interaction network.

Two factors are indispensable for 26S proteasome purification - ATP and glycerol, both of which are included in all purification steps. Purification of different proteasomal sub-complexes like 20S core particle, base and lid has also been attempted. This strategy combines the power of sub-complex specific tag to capture the entire 26S and then the use of varying concentrations of salt for preferentially dissociating other

sub-complexes by differential elution. For example, to purify 20S proteasome, PRE1 subunit is used to capture 26S proteasome and incubation with high salt (and/or depleting ATP) to dissociate the 19S RP (Leggett et al., 2002).

We have used FLAG tagged subunits generated by Hideyoshi Yokosawa group (Hokkaido University, Japan) in *S. cerevisiae* for proteasome purification essentially following the methods described by them with some modifications (Sone et al., 2004). Liquid nitrogen was used for yeast cells lysis and purification steps were monitored by proteasomal activity assay using fluorogenic substrate.

***Material**: Anti-FLAG M2-agarose (Sigma), ATP (Sigma), 3XFLAG peptide (Sigma), Glycerol, $MgCl_2$, NaCl, liquid nitrogen and mortar-pestle.*

***Yeast strain:** YYS40 (for 26S proteasome) and YYS37 (for 20S proteasome) used for proteasome purification were kind gift from Hideyoshi Yokosawa (Hokkaido University, Japan). In these strains genomic copy of RPN11 (YYS40) or PRE1 (YYS37) was replaced by FLAG tagged version.*

***Instruments:** Sorval RC 5C (for centrifugation), Rotospin (Tarson).*

Essential buffers and regents:

100 mM ATP: 1 g ATP was dissolved in 20 ml sterile water. Wherever pH adjustment was required, it was done with 100 mM NaOH (care must be taken as pH increases suddenly), and then volume was adjusted to 20 ml. Small aliquots of stock was stored at -80°C freezer.

1 M $MgCl_2$: 2 g of $MgCl_2.6H_2O$ was dissolved in 8 ml sterile water and volume was adjusted to 10ml.

Note- $MgCl_2$ powder is hygroscopic, once opened must be stored in a desiccator.

Buffer A: 50 mM Tris pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$ and 10% glycerol

Buffer B: Buffer A + 4 mM ATP

Buffer C: Buffer A+ *2 mM ATP*

Buffer D: Buffer C+0.2% Triton X100

Buffer E: 50 mM Tris pH 7.5, 500 mM NaCl and 10% glycerol

Media: *Yeast nutrient-rich medium (YPD)- 2% glucose, 2% polypeptone, 1% yeast extract, 400 μg/ml adenine, and 20 μg/ml uracil.*

2.2.1A Yeast cell culture: Isolated colony of either YYS40 (for 26S proteasome) or YYS37 strain (for 20S proteasome) was inoculated in 10 ml YPD media and incubated at 25ºC at 200 rpm for 24 hr. 1 ml of starter culture was then inoculated in 500 ml of YPD media and incubated at 25ºC at 200 rpm. When A_{600} was ~2 (typically after 24 to 30 h of growth), cells were harvested by centrifugation at 6K for 5 min. Cell pellet was washed by resuspending the pellet in cold MQ water followed by cold buffer A and buffer B. After centrifugation cell pellet was stored at -80ºC freezer till further use.

Note: When proteasome was purified from cells harvested in late stationary phase, yield of 26S proteasome was relatively low. Unlike cultures grown from an inoculum, starting from a single colony ensures yield of consistently active, intact preparation of 26S proteasome.

2.2.1B Lysis: Yeast cell pellet was suspended in cold buffer B (1 ml buffer B/g of cell pellet) and added in drops to a pre-chilled mortar filled with liquid nitrogen to make what is typically called the noodle. Cells were ground in liquid nitrogen to a fine powder using pestle (1 h for 10 to 12 g pellet). Lysed cells in powder form were carefully collected in a centrifuge tube and stored on ice (20-30 min). Cell lysate was then supplemented with buffer B (in total for 2 ml buffer B/g of cell pellet) and centrifuged at 18K for 1 h to get rid of cell debris.

2.2.1C Affinity purification of proteasome: Anti-FLAG M2-Agarose beads (~100 to 150 μl for 500 ml culture) were equilibrated with buffer B in a 10 ml column. Cleared

cell lysate was filtered through cheese cloth to remove lipids and other debris. If needed pH was adjusted to 7.5. Lysate was incubated with M2-Agarose for 3 h (on rotospin at 15 rpm). Bound proteasomes were washed with 20 ml of buffer C followed by 20 ml of buffer D and 30 ml of buffer C. For 20S proteasome purification, all the purification steps were performed using the above buffer system without ATP. Subsequent to the washing step, M2-agarose beads were incubated with buffer E for 1 h to dissociate the base and lid components, beads were again washed with 10 ml buffer A. Either the bound 26S or the 20S proteasomes were eluted by incubating beads with 100 μg/ml 3X FLAG peptide (v/v typically 100 μl for 100 μl beads) in buffer C for 30 min. Purified proteasome may be dialyzed to remove FLAG peptide (dialysis buffer must contain at least 1 mM ATP and 10% glycerol) or snap frozen and directly stored as small aliquots at -80ºC freezer.

*Note: All the proteasome purification steps must be done in cold (4*ºC). *The elution step was repeated to get better yield.*

2.2.2 Characterization of purified proteasome: Proteasome activity assay, In-gel activity assay and degradation of a protein by proteasome are used to characterize proteasome purified from any source or method. Proteasome activity assay using fluorogenic substrate can be used not only to monitor the purification steps but also to characterize purified proteasome. In-gel activity combines the power of native PAGE in resolving intact complexes in their native state and ability of proteases to act on their substrates in situ (Elsasser et al., 2005). It provides unequivocal evidence of not only intact nature of huge multi-subunit complex but also its activity status. The ability of 26S and 20S proteasome to degrade unstructured protein casein and ATP hydrolysis by proteasomal ATPases can also be used to characterize purified proteasome.

2.2.2A Proteasomal activity assay: Purified proteasome was characterized by utilizing its protease (in this case chymotrypsin) activity. Most popular protease substrates are fluorogenic. Fluorophore such as 7-Amino-4-methylcoumarin (AMC) were attached after tetrapeptide protease recognition sequence. To monitor proteasome purification steps and to characterize purified proteasomes we used Suc-LLVY-AMC substrate.

Material: *Suc-LLVY-AMC (Calbiochem) and MG132 (Calbiochem), Black 96 well plate (Nunc).*

Reaction buffer: 50 mM Tris pH 7.5, 5 mM $MgCl_2$, 1 mM ATP and 50 μM Suc-LLVY-AMC.

In black 96 well ELISA plates 70µl reaction buffer was taken. 2µl of lysate, unbound or eluted proteasome (at least in duplicate) was added, and the amount of free AMC generation was measured by collecting the emission at 460 nm using Mithras (LB 940) plate reader (counting time: 0.1sec, lamp energy: 5000, total time: 5 min). Fluorescence intensity was corrected for 'substrate only' control and normalized for protein concentration (determined by Bradford reagent). To check the 20S proteasome activity, the assay was performed with or without 0.05% SDS.

2.2.2B In-gel activity assay: In-gel activity assay is a very informative technique as this single experiment can provide information whether proteasome is intact and active.

Essential buffers and regents:

5X gel buffer: 450 mM each Tris base and boric acid, final pH ~8.3 (no buffering required), can be stored at RT for few months.

5X non denaturing sample buffer: 250 mM Tris-HCl (pH 7.4), 50% glycerol and 60 ng/ml xylene cyanol (can be stored at RT for few weeks or -20°C freezer for long time).

Running buffer: 1X gel buffer supplemented with 1mM ATP, 5 mM $MgCl_2$ and 0.5 mM EDTA (for saving ATP, only cathode buffer was supplemented with ATP).

Developing buffer: 50 mM Tris pH 7.5, 1 mM ATP, 5 mM $MgCl_2$ and 50 μM Suc-LLVY-AMC.

Gel mixture: 90 mM Tris/borate, 4% acrylamide, 1 mM ATP, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 0.1% APS and before pouring the gel 0.1% TEMED.

4% Native PAGE: Gel mixture was prepared in 1X gel buffer with ATP and poured (~8ml) into a mini-gel apparatus set with 1.5 mm spacers (Bio-Rad) without stacking. 2 μg of proteasome was mixed with non-denaturing sample buffer and loaded carefully on the wells. Proteasome was resolved at 80 to 100V for 4 h (or until xylene cyanol run off) in cold room maintained at 4˚C.

Note: Glass plates used for casting native gel must be clean and dry otherwise gel might stick to the plate during removal after electrophoresis.

In-gel activity: After electrophoresis, gel was carefully dislodged in water. The plate may be dipped in ice cold water and the gel rolled off to keep it intact. Gel was incubated in developing buffer for 20 to 30 min at 37°C in an incubator. For 20S proteasome, developing buffer was supplemented with 0.05% SDS. Gel was carefully placed on clean UV transilluminator, visualized and photographed under UV. To monitor subpopulations of proteasome, gel was then stained with Coomassie brilliant blue (CBB).

Note – 4% gel is very fragile and care must be taken. Gel should not fold during developing as it may result in appearance of 'ghost bands'. It is better to use UV transparent dish or if placing gel directly on UV transilluminator, using small quantity of water will not only avoid distortion of gel but also it will make handling the gel essay.

2.2.2C SDS PAGE: Proteasome is a multi-subunit protein. Subunit composition and purity were assessed by resolving purified proteasome on 12% SDS PAGE followed by CBB staining.

2.2.2D Casein degradation: β-casein is a small unstructured protein, the degradation of which is used to check the activity of several proteases including the 26S proteasome.

Material: *ATP (Sigma), HEPES (Sigma), DTT (Sigma) and* $MgCl_2$

2X degradation buffer: 40 mM HEPES/NaOH pH 7.5, 6 mM ATP, 30 mM $MgCl_2$*, 2 mM DTT.*

25 μg β-casein was incubated with 2 μg of proteasome in degradation buffer (70 μl reaction volume). Casein without proteasome was taken as control. All reactions were carried out at 37ºC. 10 μl aliquots were withdrawn at 0, 0.5, 1 and 2 h in 3 X loading buffer (containing SDS), and stored at -20ºC freezer. These aliquots were resolved on a 15% SDS-PAGE. After CBB staining, substrate remaining was quantified by Image-J software.

2.2.3 Expression and purification Mb: Mb is all helical heme binding protein. Out of its eight alpha helices four are involved in heme binding. The protein is expressed in bacterial system and generally purified by combining two or more techniques like ammonium sulfate precipitation, ion exchange chromatography and gel-filtration chromatography (Springer and Sligar, 1987). Several mammalian proteins do not express and fold properly in bacterial system due to codon bias. Mammalian genes can be optimized for bacterial expression by using preferred codon. We utilized the basic nature of Mb (Calculated pI 9) and were able to optimize cation-exchange chromatography for one step purification. Due to the ability of Mb to bind heme that results in reddish brown color of the complex, it was relatively easy to monitor the purification steps.

***Material**: CM52 cellulose (Whatman).*

***Plasmid:** Synthetic sperm whale Mb in pUC19 vector, kind gift from S.G.Sligar (University of Illinois, llinois, USA)* (Springer and Sligar, 1987).

***Media:** LB broth, for selection 100 μg/ml ampicillin was used.*

***Instrument:** Ultrasonic homogenizer (300VT, BioLogics, Inc),* Sorval RC 5C (for centrifugation)

Essential buffers and regents:

Purification buffer: 10 mM sodium phosphate buffer pH 6.8

Elution buffer: 30 mM sodium phosphate buffer pH 6.8.

Synthetic sperm whale myoglobin cloned in pUC19 was transformed in DH5α. Single colony was inoculated in 10 ml LB broth and incubated for 5 h at 37ºC. The starter culture was then inoculated in 1 l LB broth and incubated for 20 h at 37ºC. Dark brown cells were harvested at 6K rpm for 7 min and either stored in -80ºC freezer or used directly for purification. Cell pellet was suspended in purification buffer (10 ml for 1 liter of pellet). Cells were lysed using 5 mm sonication probe for 10 cycles of 1 min each (1cycle=70% pulse). Cell debris was removed by centrifugation at 18K for 10 min. The pH of supernatant was adjusted to 6.4 using 1 M sodium dihydrogen phosphate with continuous stirring at 4ºC. Cell lysate was then incubated on ice for 1 h and centrifuged at 18K rpm for 1 h. CM 52 cellulose (5-7 ml) was equilibrated using five column volume of purification buffer, clear reddish brown lysate was then diluted $1/5^{th}$ with water and loaded on top of CM cellulose column. Mb binds on top of CM cellulose as a reddish brown ring. Bound Mb was washed with ten column volume of purification buffer. Eluted Mb was reddish brown in color. Purification steps and purity was checked by 15% SDS PAGE analysis.

Note: Accuracy of pH and salt concentration is essential for reproducible results. The pH meter should be calibrated before making buffers for purification.

2.2.3A ApoMb preparation: Classical technique to remove prosthetic group heme from the Mb combines denaturation of Mb by HCl (up to pH 2) and heme extraction using ethyl methyl ketone. We found this technique cumbersome and non-reproducible with ~50% loss of protein. To avoid such problems and to get high yield we have used acid-acetone method for apoMb preparation (Griko et al., 1988).

Material- *Acetone (SD fine), 3.5 kDa cutoff dialysis bag (Thermo scientific).*

Reagents*: Acidified acetone: 500 µl of 1 M HCl in 200 ml acetone. Store in -20ºC.*

For apoMb preparation, 25 ml chilled acidified acetone (-20ºC) was taken in a 100 ml beaker and holoMb was added in small drops. Contents were stirred for 5 min at -20ºC (color of the acetone turns light brown) and carefully transferred to precooled centrifuge tube and centrifuged at 18K for 30 min. Pellet was washed with 10 ml of chilled acetone and centrifuged at 18K for 20 min. Acetone was removed carefully and pellet was allowed to semi-dry. Pellet was dissolved in MQ water and dialyzed against MQ water at 4ºC. Protein was then centrifuged at 16K for 15 min to remove any insoluble aggregate. UV-visible spectrum was collected to monitor loss of soret band.

2.2.4 Characterization of purified Mb: Purified Mb is generally characterized by monitoring UV-visible spectrum for heme binding, SDS PAGE to access the purity and molecular mass.

2.2.4A UV-visible spectrometry: Heme binding proteins have characteristic absorbance at 410 nm referred as soret band. The classical method to characterize purified holoMb is to monitor the soret band, by collecting the UV-visible spectrum. UV-visible spectrum of holo or apoMb was collected (Jasco v-650) from 500 to 240 nm.

2.2.4B Gel-filtration chromatography: Size exclusion or gel-filtration chromatography has been used to purify and characterize the quaternary structure of the protein. In this technique molecule with larger molecular hydrodynamic volume migrate faster than the smaller one.

Material: *Supedex-75 column (GE Healthcare)-120 ml beads (in house packed)*

Instrument: *Biologic duo flow liquid chromatography system (Bio-Rad) equipped with on line UV-visible detector.*

To test if heme removal resulted in any dramatic change in the globular structure of apoMb, we performed gel-filtration chromatography using Superdex-75 matrix at 0.5 ml/min flow rate. Chromatogram was monitored by collecting absorbance at 280 nm.

2.2.5 Proteasomal degradation:

Material: ATP (Sigma), ATPγS (Sigma), PMSF (Sigma), MG132 (Calbiochem), Velcade (Janssen Pharmaceuticals) and epoxomicin (Calbiochem).

12 μM of holo or apoMb were incubated with 50 nM 26S proteasome in the degradation buffer (20 mM HEPES/NaOH pH 7.5, 3 mM ATP, 15 mM $MgCl_2$, 1 mM DTT, 2.5-3.5% glycerol). To test whether apoMb was degraded by 20S, proteasomal degradation reaction was performed using 50 nM 20S proteasome. Specificity of proteasomal degradation was checked by performing degradation reaction with or without active site inhibitors like MG132, Velcade, epoxomicin and nonspecific serine protease inhibitor PMSF. Degradation was also done in the presence of ATPγS a non-hydrolysable analogue of ATP. The nucleotides obtained from Sigma were used directly. In incubations with ATPγS (3mM), the final assay buffer contained 0.3 mM ATP from the elution buffer used to purify proteasomes.

All reactions were carried out at 37ºC. 10 μl aliquots were withdrawn at 0, 8, 12, 16 and 19h in 3 X samples loading buffer (containing SDS), and stored at -20ºC freezer. These

aliquots were resolved on a 15% SDS-PAGE. After CBB staining, substrate was quantified by Image-J software.

2.3. RESULTS and DISCUSSION

2.3.1 Purification and characterization of proteasome: Purification of unusually large multi-subunit protein complex with several enzymatic activities could be challenging. For 26S proteasome complex purification, it is essential to perform entire purification step in presence ATP and stabilizing agents like glycerol. We have adopted affinity method for the purification of 26S and 20S proteasome from *S. cerevisiae*. Due to the presence of a tough cell wall, lysis of yeast cell at the same time retaining active and intact proteasome is difficult. We used liquid nitrogen method for yeast cell lysis. Proteasomes were then purified using anti-FLAG M2-agarose. Average yield of 26S and 20S proteasome from a 7 g pellet grown from 500 ml culture was ~140 μg and ~80 μg respectively (26S proteasome= ~280 μg/lit and 20S proteasome=~160 μg/lit). Purification steps were monitored using fluorogenic substrate Suc-LLVY-AMC. Proteasomal chymotrypsin sites cleave this substrate after tyrosine to generate free AMC, fluorescent intensity of which can be measured by 340 nm excitation and recording emission at 460 nm. The chymotrypsin activity of proteasome in the lysate, unbound as well as the eluted fraction was measured and normalized for protein concentration (**Table 2.1**). To test the specificity of proteasome activity the assay was performed in the presence of 10 μM MG132 (**Fig. 2.1C**).

Table 2.1 Monitoring proteasome purification steps by fluorogenic substrate

Purification step	**Fluorescence intensity (AU/μg)**	
	Batch 1	Batch 2
Lysate	523	806
Unbound	335	445
Elution 1	127942	142328
Elution 2	50466	59014

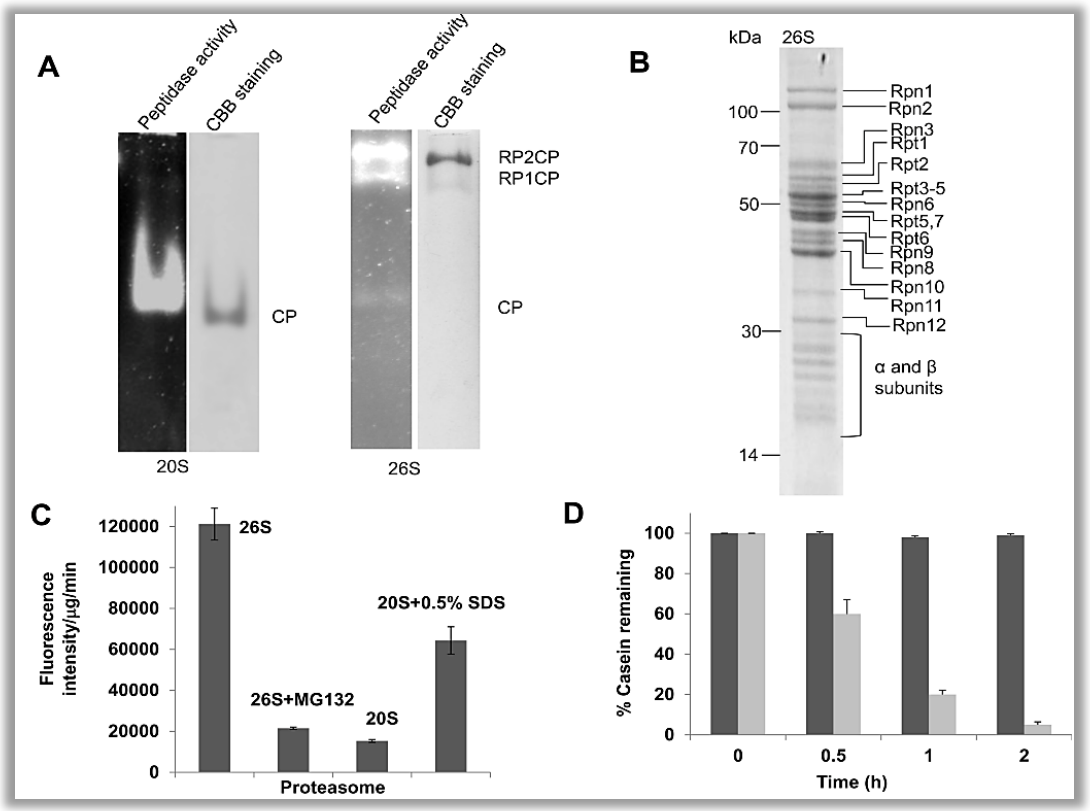

Figure 2.1 Purification and characterization of 26S and 20S proteasome: *Proteasome was affinity purified, in-gel activity assay showed both 26S and 20S proteasome were intact and active (A). 26S proteasome was resolved on SDS PASGE for verifying it composition (B). 26S and 20S protease activity was measured, 26S was active and its activity was inhibited in presence of MG132(C). Only in presence of SDS when the gate of catalytic core was opened, activity of 20S could be measured (C). In vitro purified proteasome was able to degrade casein (D) (black-casein only, gray casein with 26S proteasome). Data represent mean values of at least three independent ±S.D.*

In the absence of 19S regulatory particle, 20S proteasomes remain in 'gate closed' conformation. The gate of 20S proteasome can be stimulated to open even without regulatory particle using 0.05% SDS, salts or peptide derived from proteasomal

ATPases (Smith et al., 2007). To check the 20S proteasome activity, assay was performed with or without 0.05% SDS (**Fig. 2.1C**).

In order to avoid batch to batch variation and achieve data reproducibility, each batch of purified 26S and 20S proteasome was characterized by monitoring the protease activity of proteasome, In-gel activity assay, SDS PAGE and casein degradation. With the help of In-gel activity assay, the protease activity of intact proteasome was measured after resolving it on native PAGE. In-gel activity assay of purified 26S and 20S proteasome was done. In case of 26S proteasome, one or two bands are expected under UV. The top band corresponds to doubly capped (19S-20S-19S) and bottom to singly capped proteasome (19S-20S). 26S proteasome purified by above method results in >90% doubly capped population (**Fig 2.1A**). Similarly, in presence of SDS, single band corresponding to 20S was observed (**Fig. 2.1A**). Under UV we could only monitor the AMC fluorescence due the chymotrypsin activity of 20S (19S-20S, 19S-20S-19S or only 20S), the free 19S alone, free lid or base sub-complexes could be visualized by CBB staining of gel after In-gel activity assay. Subunit composition and purity of the proteasome was assessed by resolving purified proteasome on 12% SDS PAGE followed by CBB staining. All the component of 26S proteasome could be identified on the basis of their molecular weight (**Fig. 2.1B**). Purified proteasome was further characterized by testing its ability to degrade an unstructured protein *in vitro*. Purified 26S proteasome degraded casein in 2h (**Fig. 2.1D**).

From above results it was evident that eluted 26S and 20S proteasome was in holo form, active and retain all the essential functions of proteasome.

2.3.2 Purification and characterization of Mb: Due to codon bias several mammalian proteins may not express and fold properly in bacterial system. Mb cDNA was optimized for bacterial expression by using preferred codon (kind gift from S.G. Sligar,

University of Illinois, USA) (Springer and Sligar, 1987) and expressed in bacterial system. We utilized the basic nature of Mb (Calculated pI 9) and were able to optimize cation-exchange chromatography for one step purification. Due to the heme binding, it was relatively easy to monitor purification steps as holoMb was reddish brown in color (**Fig. 2.2A**). Purification steps were also monitored by SDS PAGE analysis (**Fig. 2.2B**). Complete binding of Mb to the column was achieved as negligible unbound Mb was seen by SDS PAGE analysis (**Fig. 2.2B**). Mb eluted from CM-cellulose column was found to be more than 95% pure on SDS PAGE (**Fig. 2.2B**). When UV-visible spectrum of Mb was collected from 500 nm to 240 nm two peaks were observed. Apart from the characteristic protein peak (280 nm) due to aromatic amino acids in protein, Soret peak at 410 nm due to heme binding was observed in case of holoMb. This band was absent when heme was removed (**Fig. 2.2C**). Heme binding also indicated that the purified Mb was well folded. We found acid extraction of heme cumbersome and non-reproducible with ~50% loss of protein. To avoid such problems and to obtain maximum yield, we have used acid-acetone method for apoMb preparation. Absence of heme was confirmed by loss of soret peak (**Fig. 2.2C**). Absorbance at 280 nm, 410 nm and 260 nm were analyzed to evaluate the extent of heme binding ($A_{410/280}$) and nucleotide contamination $A_{280/260}$. Proteins with $A_{410/280}$ to ~4 in the case of the holo form and apo proteins with $A_{280/260}$ ratio $\geq$1.5 were used for all assays.

Ligand removal might result in alteration in tertiary structure/quaternary structure of the protein or induce aggregation. Holo and apoMb was resolved by gel-filtration chromatography. Both holo and apoMb eluted at the same retention volume indicating that overall fold in apoMb has not been affected due to heme removal (**Fig. 2.2D**). The above observations suggest that purified Mb was pure, well folded and quaternary structure of apoMb was similar to that of holo form.

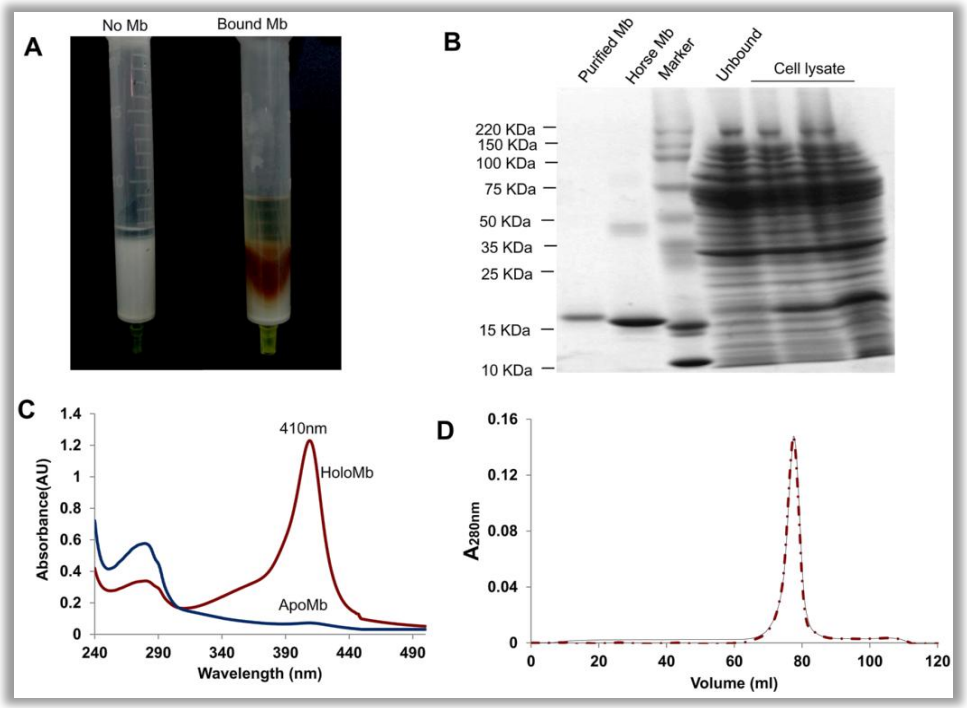

Figure 2.2 Purification and characterization of Mb: *Mb was purified by ion exchange chromatography, column bound protein was brownish red in color (A). Purification steps were monitored by SDS PAGE analysis, commercial horse Mb was used as control (B). HoloMb showed characteristic soret peak due to heme binding which was negligible in apoMb (C). Holo (solid line) and apoMb (dash line) elute at same retention volume indicating overall fold of apoMb is similar to holoMb (D).*

2.3.3 Degradation of apoMb by proteasome: To test the hypothesis whether *in vitro* proteasome will be able to degrade a well folded protein, apo or holoMb were incubated with 26S proteasome. Aliquots were withdrawn at different time points and degradation was monitored by decrease in band intensity of Mb on SDS PAGE. The holoMb was found to be resistant to proteasomal degradation, while ligand free apo form was degraded with the half-life of 12h (**Fig. 2.3A**). Degradation was inhibited by chymotrypsin active site inhibitor MG132 (200 μM) as well as by more specific inhibitors like Velcade (100 μM) and epoxomicin (200 μM) (**Fig. 2.3B**). Nonspecific serine protease PMSF (1 mM) had no effect on apoMb degradation (**Fig. 2.3B**). These data show that apoMb degradation was specific to purified 26S proteasome. The 20S

proteasome alone was unable to degrade apoMb indicating the necessity of 19S for upstream events like recognition and chain unraveling (**Fig. 2.3B**).

The process of unfolding and translocation requires active participation of proteasomal ATPases. Negligible degradation was observed when degradation reaction was performed in presence of non-hydrolysable analogue of ATP, ATPγS (**Fig. 2.3B**). This observation further proves that apoMb was degraded by purified 26S proteasome in an ATP dependent manner.

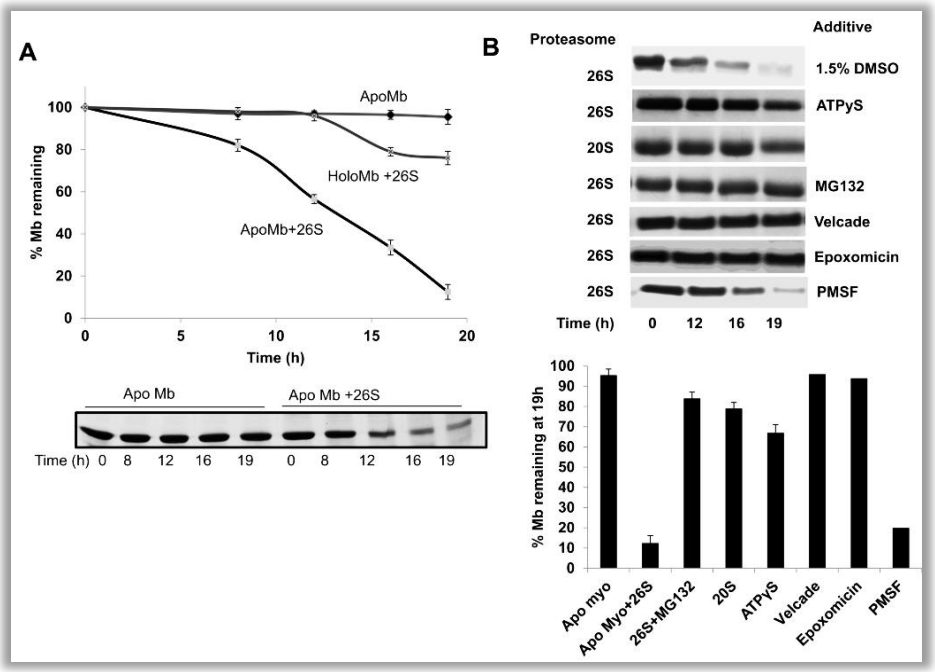

Figure 2.3 Purified 26S proteasome degrades apoMb in vitro: *Apo and holoMb when incubated with 26S proteasome only apoMb was amenable for proteasomal degradation (A). ApoMb degradation was inhibited in presence of proteasomal inhibitors (MG132, Velcade and epoxomicin) and non-hydrolysable ATP analogue, ATPγS (B). ApoMb was resistance for degradation by 20S proteasome indicating the importance of regulatory particles (B). Data represent mean values of at least three independent ±S.D.*

All the above observations provide unequivocal evidence that *in vitro* 26S proteasome can degrade a well folded apoMb without the help of 'trans-acting elements' in an ATP dependent manner.

2.4. SUMMARY

In order to develop an *in vitro* model system for in depth understanding of proteasomal degradation we chose to test Mb as a substrate. We successfully purified both the substrate and proteasome and found that proteasome is self-sufficient to degrade a globular substrate. The results obtain form the above experiments can be summarized as-

a) 26S and 20S proteasome purified by affinity chromatography were active and intact.
b) Purified 26S proteasome was able to degrade apoMb, a globular protein without the help of ubiquitin or other trans-acting elements *in vitro*.
c) Myoglobin purified by cation exchange chromatography was >95% pure.
d) Heme binding suggest that eluted myoglobin was in well folded holo form.
e) ApoMb prepared by acid acetone method was devoid of heme group.
f) Heme bound holoMb was resistance for degradation.
g) ApoMb was degraded specifically by proteasome as degradation was inhibited in presence of proteasome active site specific inhibitors.
h) 19S subunits were essential for apoMb degradation as 20S alone was unable to degrade apoMb.
i) Proteasomal ATPases were actively involved in apoMb degradation.

To the best of our knowledge this is the first demonstration of the inherent ability of 26S proteasome to degrade a globular protein in vitro in the absence of ubiquitin, adaptor or other trans-acting element.