BIOCHEMICAL CHARACTERIZATION OF RECOMBINANT 20S PROTEASOME FROM *THERMOPLASMA VOLCANIUM* AND CLONING OF IT'S REGULATORY SUBUNIT GENE

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Approval of the Graduate School of Natural and Applied Sciences

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ABSTRACT

BIOCHEMICAL CHARACTERIZATION OF RECOMBINANT 20S PROTEASOME FROM *THERMOPLASMA VOLCANIUM* AND CLONING OF ITS REGULATORY SUBUNIT GENE

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In this study, we have characterized some biochemical and electrophoretic features of recombinant 20S Proteasome from a thermoacidophilic archaeon *Thermoplasma volcanium*. As revealed by SDS-PAGE the 20S Proteasome was composed of two subunits, α- and β- subunits with estimated molecular masses of 24 kDa and 23 kDa, respectively. The highest chymotryptic activity was observed over an alkaline pH range (pH 8.0 – pH 9.0) and the optimum temperature for the activity was determined as 85^{o}C. The heat stability of proteasome was quite high after treatment at 98^{o}C for 30 minutes, 64 % of the activity has still been retained. The highest activity associated with the *Thermoplasma volcanium* proteasome was found to be peptidylglutamyl peptidase activity.

Within the scope of this project, also, we have cloned a 26S Proteasome related Regulatory Subunit gene of *Thermoplasma volcanium*. For cloning we have followed a PCR based approach. Amplification of 26S Proteasome Regulatory Subunit gene from chromosomal DNA of *Tp. volcanium* yielded a product of 1419 bp containing an open reading frame of 1128 bp comprising the structural gene. The PCR amplicon was cloned using pDrive vector in *E.coli* TG-1 cells. Out of ten putative recombinants, three plasmids, *E.coli* pD1-6, *E.coli* pD2-3, *E.coli* pD3-1, were proved to be true recombinants and selected for further characterization by restriction mapping and expression studies. ATPase activities of cell free extracts from both recombinant and non-recombinant *E.coli* strains were measured and found that ATPase activities in cell free extracts of recombinant strains were 10 times higher than non-recombinants. This result indicates sucessful expression of the cloned regulatory subunit gene with ATPase activity in *E.coli.*

Keywords: 20S Proteasome, 26S Proteasome Regulatory Subunit, Cloning, *Thermoplasma volcanium*.

ÖZ

THERMOLPASMA VOLCANIUM, REKOMBİNANT 20S PROTEAZOM'UNUN BİYOKİMYASAL KARAKTERİZASYONU VE DÜZENLEYİCİ ALT BİRİM GENİNİN KLONLANMASI.

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Bu çalışmada termoasidofilik bir arkea olan *Thermoplasma volcanium'un* rekombinant 20S Proteazom'unun bazı biyokimyasal ve elektroforetik özelliklerinin karakterizasyonu yapılmıştır. SDS-PAGE ile ortaya konulduğu üzere *Thermoplasma volcanium* 20S Proteazom'u moleküler ağırlıkları sırasıyla 24 kDa ve 23 kDa olan, α- ve β- olmak üzere iki altbirimden oluşmaktadır. En yüksek kimotriptik aktivite alkali pH aralığında (pH 8.0 – pH 9.0) gözlemlenmiştir ve aktivite için optimum sıcaklık 85^{o}C olarak belirlenmiştir. Proteazom'un ısı stabilitesi oldukça yüksek olup 98^{o}C'de 30 dakika ısıya tabi tutulduktan sonra aktivitenin % 64'ü korunmaktadır. *Thermoplasma volcanium* Proteazom'una ilişkin en yüksek aktivitenin peptidilglutamil peptidaz aktivitesi olduğu saptanmıştır.

Bu proje kapsamında ayrıca, *Thermoplasma volcanium*'un 26S proteazom'a ilişkin bir düzenleyici alt birim geni de klonlanmıştır. Klonlama için PCR temelli bir yaklaşım izlenmiştir. *Thermoplasma volcanium*'un kromozomal DNA`sından 26S Proteazom düzenleyici alt birim geninin amplifikasyonu sonucu 1128 bp büyüklüğünde yapısal geni içeren 1419 bp büyüklüğünde bir DNA fragmanı elde edilmiştir. PCR amplikon ile pDrive vektör, *E.coli* TG-1 hücrelerinde klonlanmıştır. On rekombinant arasından üç tanesi (*E.coli* pD1-6, *E.coli* pD2-3, *E.coli* pD3-1) gerçek rekombinant plazmit olarak belirlenmiş ve restriksiyon haritalaması yolu ile karekterize edilmek üzere seçilmiştir. Rekombinant ve rekombinant olmayan *E.coli* hücrelerinin, hücre özütünde ATPaz aktiviteleri ölçülmüş ve rekombinant suşların hücre özütündeki ATPaz aktiviteleri rekombinant olmayana göre on kat daha yüksek bulunmuştur. Bu sonuç ATPaz aktivitesini kodlayan klonlanmış düzenleyici alt ünite geninin *E.coli*'de başarılı bir şekilde anlatıldığını göstermiştir.

Anahtar Kelimeler: 20S Proteazom, 26S Proteazom düzenleyici alt birim, Klonlama, *Thermoplasma volcanium*.

Dedicated to My Family.

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CHAPTER I

INTRODUCTION

1.1. Cellular Proteolysis

Misfolded and malfunctioning proteins, prone to aggregation, must be scavenged and degraded. In addition to such "housekeeping" functions, protein degradation has a key regulatory role in many cellular pathways and must therefore be subject to spatial and temporal control. Regulatory proteins of cell cycle, apoptosis, differentiation, growth, signal transduction, antigen processing, transcription, DNA repair and metabolic pathways display high turnover rates (*1, 2, 3, 4, 5, 6*). When these high turnover rates are coupled with transcriptional control, cells are able to respond effectively to changes in the neutral environment by rapidly altering the concentrations of enzymes in the cells. After cells are exposured to stresses such as heat shock or oxidative damage, some abnormal proteins accumulate and form protein aggregates, same as those produced by errors in transcription, translation, genetic mutation, RNA damage or misfolding of proteins in the presence of molecular chaperones. These abnormal proteins are degraded by proteasome in a processive mechanism (*7*), so that the protein is completely degraded into peptides without releasing any intermediates.

Little more than a decade ago, the lysosomes, with their substantial complement of broad-specificity proteases, were considered the locus of cellular protein degradation by relatively non-selective bulk processes (*8*).

In contrast to the lysosome the "nonlysosomal system" is responsible for the remarkably selective turnover of intracellular proteins that occurs under basal metabolic and stress conditions. The most important nonlysosomal proteolytic system is the energy-dependent proteolytic system, of which the proteasome pathway is the major mechanism through which soluble nuclear and cytoplasmic proteins are specifically recognized and finally removed by the proteasome.

1.2. Energy-dependent Proteases

Energy-dependent proteolysis is not only vital to the elimination of defective proteins but also central to the specific regulation of essential functions in both procaryotic and eukaryotic cells.

There is only a small group of energy-dependent proteases known (*9*) and they are classified into four families based on the primary sequence of their proteolytic active sites.

1.2.1. Clp Family

The Clp family includes the ClpAP and ClpXP proteases, in which ClpP, a serine protease, associates with either ClpA or ClpX ATPases of differing substrate specificities.

1.2.2. Lon Family

The Lon family members have ATPase and serine protease domains on the same polypeptide.

1.2.3. FtsH Family

The FtsH family consists of membrane-associated, Zn^{+2}-dependent metalloproteases with ATPase domains on the same polypeptide.

1.2.4. Proteasome Family

Proteasome family proteases appear to have a single threonine residue active site at the amino terminus. They include the 20S proteasomes found in eukarya, archaea, and Gram-positive actinomycetes, all of which are formed by four stacked monomeric or heptameric rings and presumed to associate with energy-dependent regulatory components. The simpler eubacterial HslUV protease, composed of the HslU ATPase and HslV protease, is formed by two rings and also included in the proteasome family.

Based on genome sequences, however, FtsH and Clp proteases are not found in the archaea. Instead, the proteasome may be responsible for degradation of the majority of proteins in the archaeal cytosol (*10, 11*).

Although these four families of energy-dependent proteases share limited primary sequence identity, they appear to have converged into a common self-compartmentalized quaternary structure or nanocompartment which sequester rather non-specific proteolytic active-sites away from other cellular components and prevent the unregulated hydrolysis of proteins in the cell (*12, 13, 14*).

It has been shown that the ATP consumption of ClpXP is more than PAN-20S proteasome complex for degradation of proteins. ATP hydrolysis by the Lon protease is proportional to the number of cleavages made in the substrate, but with this type of enzyme significantly less ATP is consumed than by PAN-20S complex (*15, 16)*.

1.3. The 20S Proteasomes

Proteasomes are large, multimeric, self-compartmentalizing proteases, which play a crucial role in the protein quality control, clearance of misfolded proteins, and processing by specific partial proteolysis as well as preparation of peptides for immune presentation (virus defence) (*17*).

1.3.1. General Structure of 20S Proteasome

X-ray diffraction studies of 20S proteasomes from the archaeon *Thermoplasma acidophilum* (*18*) and yeast *Saccharomyces cerevisiae* (*19*) revealed three inner cavities joined by a central channel (Figure 1.1). These cavities include two antechambers (59 nm^3) formed by the α and β rings and a central chamber (84 nm^3) formed by the two β rings. It is the central chamber which harbors the 6-14 active sites required for peptide bond hydrolysis (Figure1.1). The role of the antechambers, is not as clear. In fact, many self-compartmentalized proteases (e.g. ClpP and HslV) do not have antechambers (*14*, *20*, *21*, *22*).

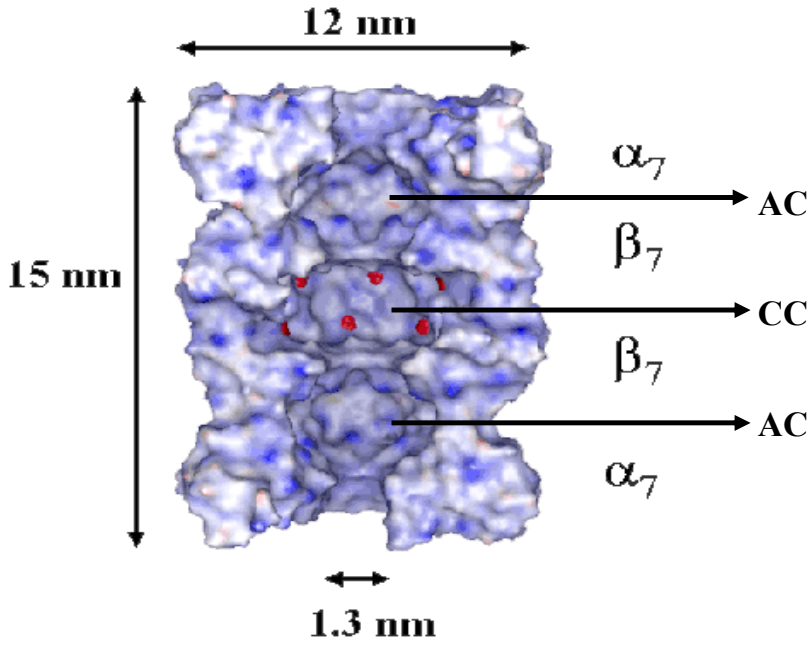

Figure 1.1 Predicted structure of the 20S proteasome from the methanoarchaeon *Methanosarcina thermophila*. The 20S cylinder is composed of four stacked heptameric rings arranged as $\alpha_7\beta_7\beta_7\alpha_7$. A central channel joins three inner chambers, two anthechambers (AC) and one central chamber (CC). The central chamber harbors the proteolytic active sites required for peptide bond hydrolysis. The active sites are indicated in red (*23*).

The substrate entry to the central chamber is restricted by 1.3 nm axial pores on each end of the cylinder. The substrates enter the lumen of the barrel through openings at both ends (*24, 25)*.

The overall architecture of 20S proteasomes is highly conserved from bacteria to man (*26*). It is a 650-700 kDa cylindrical or barrel-shaped particle (~12nm x 15nm) of four stacked heptameric rings (*15, 21*) (Figure 1.1). 20S proteasome core particle is composed of two outer α rings of α subunits and two central β rings of β subunits, which contains the proteolytic active sites sequestered within a central chamber (*18, 19, 27*).

Each ring consists of seven identical (in archea) or similar (in eucarya) subunits. The substrates enter the lumen of the barrel through openings at both ends. This notion is supported by the observation that regulators located at the openings increase the proteolytic activity of the proteasome (*24, 25)*.

1.4. The 20S Proteasomes Found in Three Domains of Life

Proteasomes are large, energy-dependent proteases found in all three domains of life. The 20S proteasome has been found in archaea, eukarya and surprisingly, in *Actinomycetales* that belong to the bacterial subdivision of high G+C Gram-positives (*24*) (Table 1.1).

The simplest form of the proteasome is the HslUV protease which has been identified in *Eubacteria* (*28, 29*). It consists of two stacked rings of proteolytic subunits (HslV) capped on each end by ATPase rings (HslU) of the AAA^+ superfamily (*30, 31*). More elaborate proteasomes are found in *Eucarya*. These consist of a proteolytic 20S core which associates with regulatory components including ATPases of the AAA subfamily (*24, 32, 33*).

Searching entire microbial genomes it became apparent that not all organisms contained proteasomal sequences (*24, 33*).

Table 1.1 Six organisms exemplifying proteasomes of bacteria, archaea and eukarya (*24*).

Organism	Kingdom	Monomers	Genes	Active sites	Proteasome type	Reference
Archaeoglobus fulgidus	A	28	3	14	20S	106
Thermoplasma acidophilum	A	28	2	14	20S	122
Escherichia coli	B	12	1	12	HslV	137
Saccharomyces cereviciae	E	28	14	6	20S	74
Homo sapiens	E	28	17	6	20S	86

In archaea, the ancestral proteasome gene has diverged into two different genes, coding for subunits termed α and β, which differentiated into seven subtypes in *Eucaryotic* proteasomes by further gene duplications (*24, 26*, *33)*. The amino acid sequences of the α and β subunits are similar and this supports that both were derived from a single ancestral gene (*34*). The archaeal proteasome is believed to be the precursor of the ubiquitin-proteasome protein degradation pathway in eukaryotes (*35*).

In the archaea *Thermoplasma acidophilum* the proteasome is essential under stress conditions, but not during normal growth, whereas in the actinomycete *Mycobacterium smegmatis* its knock-out does not have a phenotype under any of the conditions tested (*36, 37*).

Primitive eucaryotes (e.g. Yeast, *Caenorhabditis elegans)* synthesize only one type of 20S proteasome. In contrast, higher eucaryotes have a constitutive housekeeping 20S proteasome and other proteasomes which are induced. These ancillary proteasomes include the immunoproteasome of vertebrates which is induced by IFN-γ and spermatogenesis-specific proteasome of insects (*38*). Multiple 20S proteasomes may also be common in plants where up to 23

different α or β type genes have been identified (*39*). Although, the common form of proteasome is 20S proteasome in archaeon surprisingly, at least two 20S proteasomes with different α subunit composition ($\alpha_1\beta$ and $\alpha_1\alpha_2\beta$) are found in the archaeon *Haloferax volcanii* (*40*).

1.4.1. The Eucaryal 20S Proteasomes

The proteasome holoenzyme was first identified in mammalian cells in 1986 (*41, 42*). The eukaryotic proteasome as a central player in controlling protein degradation; is responsible for rapid degradation and processing of regulatory proteins tagged with ubiquitin (*43*) such as cell-cycle regulators (*3, 7, 44*) oncogens, tumor suppressors (*45*) and transcription factors (*46*). Proteasomes are found free in the cytosol, attached to the endoplasmic reticulum (ER) and in the nucleus and functional in bulk turnover of cytosolic proteins (80–90%) (*47,48*), co-translational degradation (*49*), processing of antigens (*50*) and ER-associated degradation.

The Eukaryotic 26S proteasome is the most complicated form, where a 20S proteasome core complex capped by two 19S regulatory complexes containing ATPases of the AAA subfamily (*18, 46, 51, 52*). The Eucaryal 20S proteasome i.e., that of yeast has maximal subunit complexity with seven different α type subunits, (α_1, α_2, α_3, α_4, α_5, α_6, α_7) and seven different β type subunits, (β_1, β_2, β_3, β_4, β_5, β_6, β_7) (*53, 54, 55*). In yeast, the archaeal α and β subunits have diverged into seven different subunits each, which are present in unique locations in two copies per 20S proteasome so that the D7 symmetry of the archaebacterial particle is reduced to C2 symmetry (*56*). Of the seven different β subunits, only three (β_1, PGPH activity, β_2, Tryptic activity, β_5, Chymotryptic activity) are 'catalytically active' resulting in six proteolytic active sites per Eucaryal 20S proteasome (*19, 57*).

Eukaryotic 26S proteasome is a very large complex with a mass of around 2000 kDa, consisting of at least 62 proteins encoded by 31 genes (*51*) (Figure 1.2).

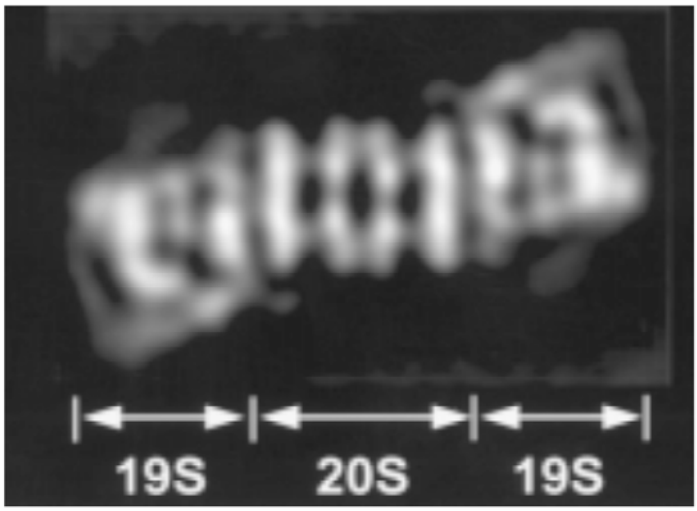

Figure 1.2 Structure of the *Droshophila* 26S proteasome derived from electron micrographs of a negatively stained sample. The 20S core structure and the regulatory 19S "caps" are marked. The total length of the complex is 45 nm (*58*).

The peptide products generated around 7–9 amino acids by the 20S core particle may be incorporated finally into MHC (Major Histocompatibility Complex)-class I molecules (*59*, *5*) or alternatively further degraded to amino acids by other endopeptidases and aminopeptidases like tricorn proteases (*60*, *61*).

1.4.1.1 The ubiquitin-proteasome pathway

In eukaryotes, the regulated proteolysis of intracellular proteins occurs through specialized enzymatic machinery, the ubiquitin-proteasome pathway. The ubiquitin-proteasome pathway has been well characterized in the recent decade (*62, 63*). Here proteins are specifically recognized and marked for degradation by addition of poly-ubiquitin chains before being degraded by the 26S proteasome. Ubiquitin is a 76 amino-acid protein that is highly conserved among eukaryotes, and covalently bound to proteins through a series of reactions.

The reactions of the pathway can be summarized in seven steps as given in Figure 1.3. First, the activation of ubiquitin, by an ubiquitin activating enzyme (E1) and ATP. Second, transfer of activated ubiquitin from E1 to a member of the ubiquitin carrier or conjugating enzymes (E2). Third, formation of a E2-E3 complex by binding of E2 either to a RING Finger E3 (yellow) or a HECT domain type E3 (blue), which might not or might have substrate already bound via a defined recognition motif. Fourth, direct transfer of activated ubiquitin from E2 to a lysine of the E3-bound substrate via an isopeptide bond occurs in substrates targeted by RING finger E3s. Multiple (n) cycles of ubiquitin conjugation to previously bound ubiquitin moieties on the target substrate leads to synthesis of a poly-ubiquitin chain. Fifth, as in step fourth, but the activated ubiquitin moiety is first transferred from the E2 to the E3, yielding a high-energy thiol ester intermediate, before its conjugation to the E3-bound substrate or to the previously conjugated ubiquitin moiety of the substrate. Sixth, binding of the polyubiquitinated substrate to the ubiquitin receptor subunit in the 19S complex of the 26S proteasome and degradation of the substrate to short peptides by the 20S complex with release of short peptides. Seventh, recycling of ubiquitin via the activity of the deubiquitinating enzymes (DUBs) or ubiquitin hydrolases and ubiquitinspecific processing proteases (UBPs).

In Eucaryotes, ubiquitin chains of four or more ubiquitin molecules serve as a proteasome targeting signal (*64*). The ubiquitin molecules in these ubiquitin chains are linked via the C-terminal glycine of one ubiquitin molecule to lysine 48 of a second ubiquitin (*65*). The polyubiquitinated substrate protein is then recognized and degraded by the 26S proteasome complex, whereby free and re-utilizable ubiquitin is released (*66, 67*).

The ubiquitin-proteasome system is also involved in generation of peptides destined for presentation by class I molecules of the major histocompatibility (MHC) complex (*48*). In this capacity, the system is involved in the regulation of many cellular processes besides playing a key role in cellular quality control and defense mechanisms.

Southern blotting experiments suggested the presence of ubiquitin-like sequences in a Methanogenic Archaeon, *Methanosarcina thermophila* (*23*).

This ATP-dependent modification of substrates facilitates recognition by the 26S proteasome and is, for many substrates, an essential step for degradation. Ubiquitin conjugation allows tight regulation of degradation, and ensures that this process is highly selective. A few examples of ubiquitin-independent degradation have been reported such as ornithine decarboxylase (ODC) (*68*). However, that ubiquitin-independent degradation may be more common than presumed. Ornithine decarboxylase is the rate-limiting enzyme in polyamine biosynthesis. Accumulation of polyamines stimulates synthesis of antizyme protein. Antizyme negatively regulates ODC by binding to ubiquitin and enabling its destruction by the 26S proteasome in the absence of ubiquitination.

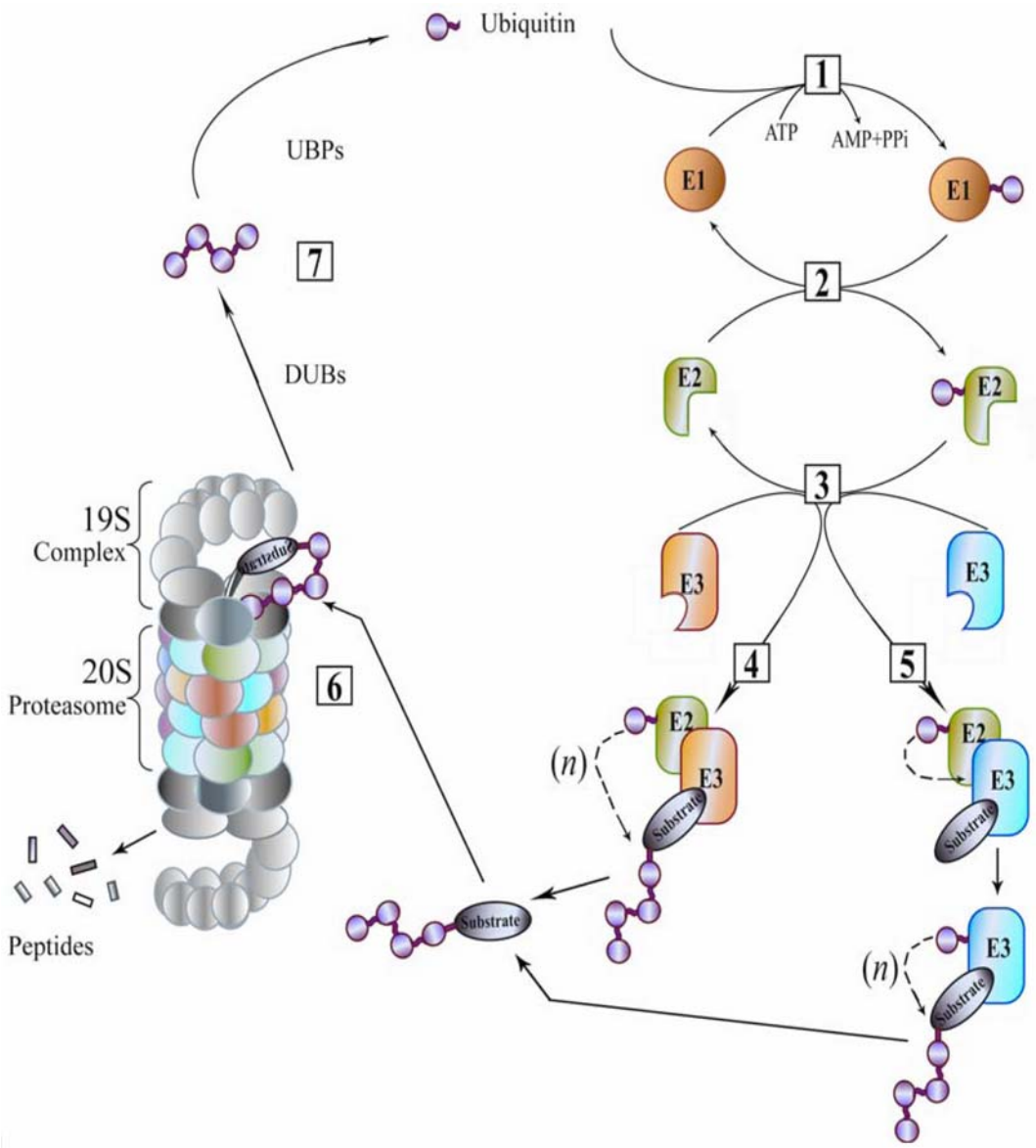

Figure 1.3 The Ubiquitin-proteasome proteolytic pathway (*55*, *69*).

1.4.2. The Bacterial 20S Proteasomes

Although other self-compartmentalizing proteases (such as ClpP and HslV) occur in *Bacteria*, remarkably, genuine 20S proteasomes are only present in one phylum of *Bacteria*, the actinomycetes (subdivision with high G+C, *Rhodococcus erythropolis*) (*61, 70*). It is possible that these organisms have acquired the protease complex by horizontal gene transfer (*71*). Most eubacteria contain a proteasome homologue called HslV that does not form 20S particles (Figure 1.4). Most actinomycetes appear to have additional proteases that are predicted to compensate for loss of the proteasome (*72*). Consistent with this, mutations in the genes encoding the 20S proteasome and Lon protease *(prcab lon)* of *Mycobacterium smegmatis* do not influence growth rates or stress tolerance (*37*).

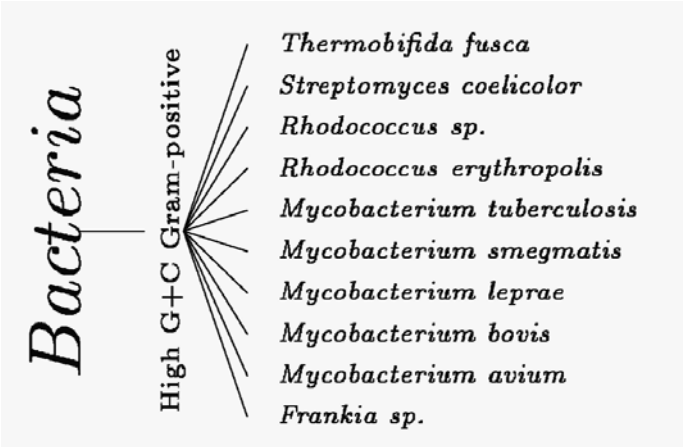

Figure 1.4 Bacteria that have a 20S proteasome instead of HslV (*24*).

The 20S proteasomes isolated from *Rhodococcus*, are composed of two different α and two different β type subunits (*71, 73*) while, the other actinomycetes like *Mycobacterium smegmatis* (*36*) and *Streptomyces coelicolor* (*74*) are built of multiple copies of only one α and one β type subunit. The *in vitro* assembly of chimeric proteasomes composed of *Rhodococcus erythropolis* and *Frankia* subunits are correctly assembled and proteolytically active.

1.4.3. The Archaeal 20S Proteasomes

Since the 1989 discovery of the proteasome complex from the moderate thermophile *Thermoplasma acidophilum* (*26*) wild-type proteasomes have been isolated and characterized from the moderate thermophile *Methanosarcina thermophila* (*23*) the hyperthermophile *Pyrococcus furiosus* (*75*) and the extreme thermophile *Methanococcus jannaschii* (*76*). The distribution of genes coding for subunits of the proteasome is apparently common in archaea, including *Thermoplasma acidophilium* (*26*), *Methanosarcina thermophila* (*23*), *Methanococcus jannaschii* (*76*), *Methanobacterium thermoautotrophicum DH* (*77*), *Archaeoglobus fulgidus* (*78*), and *Pyrococcus horikoshii* (*79*). A 20S proteasome, comprising two subunits α and β, was purified from the extreme halophilic archaeon *Haloarcula marismortui*, which grows only in saturated salt conditions. (*80, 81*). Genomic sequencing revealed single α and β type genes in other archaea but *Pyrococcus horikoshii (79), Pyrococcus furiosus* (*75*) *(http://www.genome.utah.edu) and Pyrobaculum aerophilum* (*82*) contain two distinct β type genes and a single α type gene. A notable exception is the recent finding of two distinct α proteasome genes in the halophilic archaeon *Haloferax volcanii* (*83*). Surprisingly, at least two 20S proteasomes with different α subunit compositions ($\alpha_1\beta$ and $\alpha_1\alpha_2\beta$) are found in the archaeon *Haloferax volcanii* (*83*). Its widespread occurrence in archaea is further inferred from the identification of homologs for α and β subunit genes in the genomes of *Archaeoglobus fulgidus*, *Methanococcus jannaschii*, *Methanobacterium thermoautotrophicum*, and *Pyrococcus horikoshii* (http://www.tigr.org/tigr_home/tdb/mdb/mdb.html). The 20S proteasome from *Thermoplasma acidophilum* was the first proteasome cloned and over-expressed in *E. coli* (*84*), and several papers have since appeared describing various aspects of that archaeal proteasome's structure, activity, and assembly (*18, 85, 86*).

Electron microscopy images showed that the archaebacterial 20S proteasome has an overall architecture closely resembling that of the eukaryotic 20S complex, but it contains homo-oligomeric rings composed of either the single α type or β type subunits, respectively (*87*). The β subunits form two rings, that are sandwiched in between two rings of α subunits, as first demonstrated by immunoelectron microscopy. The X-ray crystal structure of the 20S proteasome from *Thermoplasma acidophilum* (*18*) showed for the first time the architecture of the core particle at atomic resolution (Figure 1.5).

Much like eucaryal proteasome, archaeal proteasomes appear to be central players in regulating the degradation of cytosolic proteins and several AAA^+ proteins are predicted to serve as molecular chaperones and/or as unfoldases that assist the proteasome (*24, 88*).

The proteasomes occur in physiologically and phylogenetically diverse in Archaea. Based on genomics, the archaeal proteasome appears central to protein degradation and linked to an exosome involved in RNA processing and modification.

It is not yet known whether the proteasome is essential for growth of archaea. An *Haloferax volcanii psm C* (α_2) null mutation does not influence growth under normal conditions, but limits production of 20S proteasomes to those containing α_1 and β subunits (*40*). Inhibition of 80% of the *Thermoplasma acidophilum* 20S proteasomes does not modify growth but reduces survival after heat shock (*89*). Thus, the physiological role of proteasomes in the archaea is not known but the proposed function is survival under stress conditions.

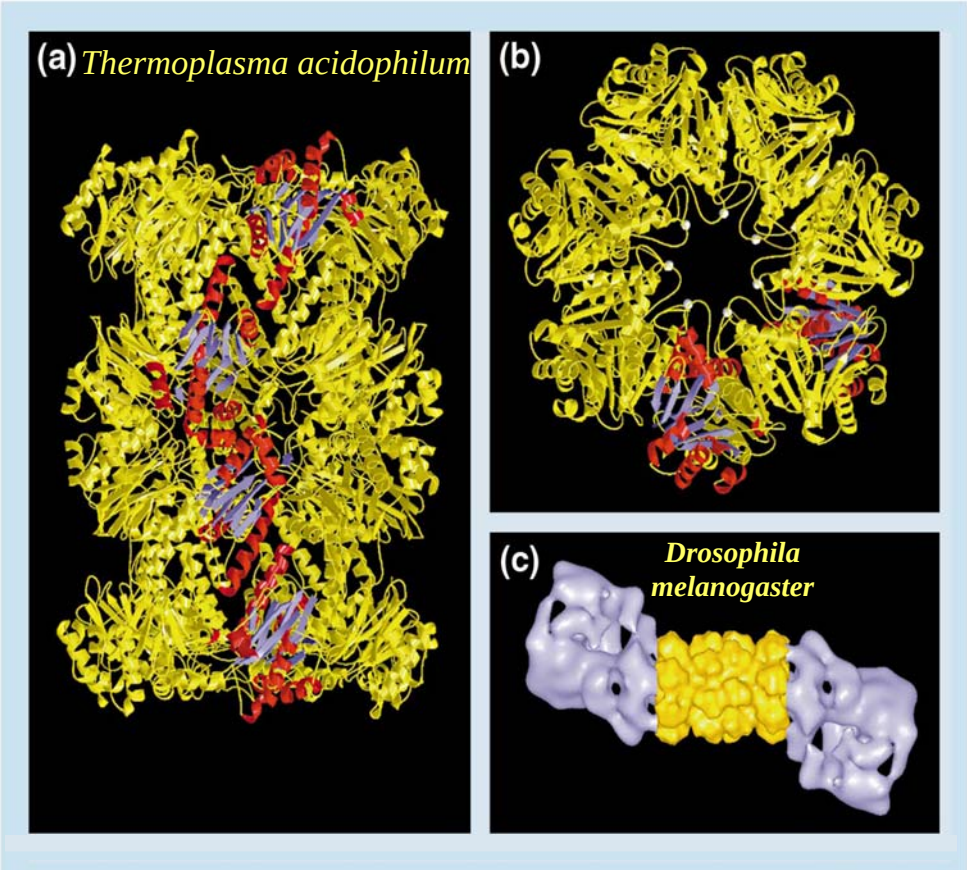

Figure 1.5 Structure of the *Thermoplasma acidophilum* 20S proteasome. (a) Ribbon drawing indicating one subunit in each of the four rings by colour coding. (b) A half-proteasome (one α and one β ring) viewed down the sevenfold axis. (c) A composite model of the electron microscopic structure of the 19S caps combined with the 20S proteasome of *Drosophila melanogaster* (*12*).

1.5. The Assembly and Processing of Proteasome Complex

The assembly pathways of 20S proteasomes from *Thermoplasma*, *Rhodococcus*, *S. cerevisiae* and mammals are shown in Figure 1.6. In *Tp. acidophilum,* formation of seven-membered α rings is the first step in the proteasome assembly and subsequently, these α rings function as docking platform and serve as a site-specific template for proper folding and assembly of β-subunit precursors (*12, 86*). Monomeric β-subunit precursors may also be stabilized as they stack on α ring scaffolds by simple mass action. Possibly, conformational changes in the β subunits, induced upon binding to the α ring, are the prerequisite for the dimerization of the two half-proteasome precursors, which again is needed to trigger processing of the β-subunits (Figure 1.6) (*90, 86*). Upon dimerization of half-proteasomes autocatalytic removal of the N-terminal β propeptide occurs to generate the active site Thr1, concomitantly yielding fully assembled proteolytically active particles both *in vivo* and *in vitro* (*17*).

The β propeptides are not essential for assembly, but their absence retards proteasome formation (*32*). The rate-limiting step in assembly appears to be the processing of the β propeptide after dimerization of the two half proteasomes.

The *Thermoplasma acidophilum* β subunit is synthesized as a precursor with an N-terminal propeptide of eight amino acid residues (*17*), which is autocatalytically cleaved off during proteasome assembly (*90*). When expressed alone, *Thermoplasma acidophilum* α subunits assemble into seven-member rings, whereas β precursors and processed β subunits, lacking propeptides, do not form an ordered structure (*86*). Co-expression of α and β genes yields fully assembled and proteolytically active proteasomes, independent of the presence or absence of the propeptide of the β precursors (*84*). *In vitro*, *Thermoplasma acidophilum* proteasomes can also be reassembled after complete dissociation, reemphasizing that the propeptideis not essential for assembly (*27*).

Some additional factors that probably act as chaperones and are only transiently associated with the nascent complex are also involved in proteasome assembly. Eucaryal proteasome assembly and maturation factors have been identified including Ump1, Nob1 and Pno1. Interestingly, Nob1 and Pno1 homologues are found in archaea. It is possible that these are accessory proteins that stabilize interaction of 20S proteasomes with PAN and/or other AAA ATPases in the archaea (*22*).

Unlike other recombinant archaeal protein complexes studied to date, assembly of the 28-mer complex from *Methanococcus janaschii* was highly temperature dependent. The lowest temperature compatible with active proteasome production was found to be 50°C, with an optimal assembly temperature of 70°C (*91*).

1.6. The Proteolytic Activity of Proteasome Complex

The precursors of the proteasomal β-subunits are synthesized as proproteins and become active by autolytic processing between Gly-1 and Thr-1 in a process that requires the presence of Thr-1, Gly-1 and Lys33 (*92, 93*). A critical and common function of the propeptides in precursors of active subunits is to protect the N-terminal catalytic threonine residue against N-α-acetylation until the catalytic chamber has been sealed off by formation of the 20S proteasome from two half-complexes.

The major peptidase activities catalyzed by 20S proteasomes include hydrolysis of peptide bonds after large hydrophobic or aromatic (chymotrypsin-like, CL), basic (trypsin-like, TL) and acidic amino acid residues (caspase-like or postglutamyl peptide hydrolyzing, PGPH) (*4, 20, 92, 94, 95*). The minor peptidase activities include cleavage between small neutral amino acids (SNAAP) and after branched chain residues (BrAAP) (*61, 96*).

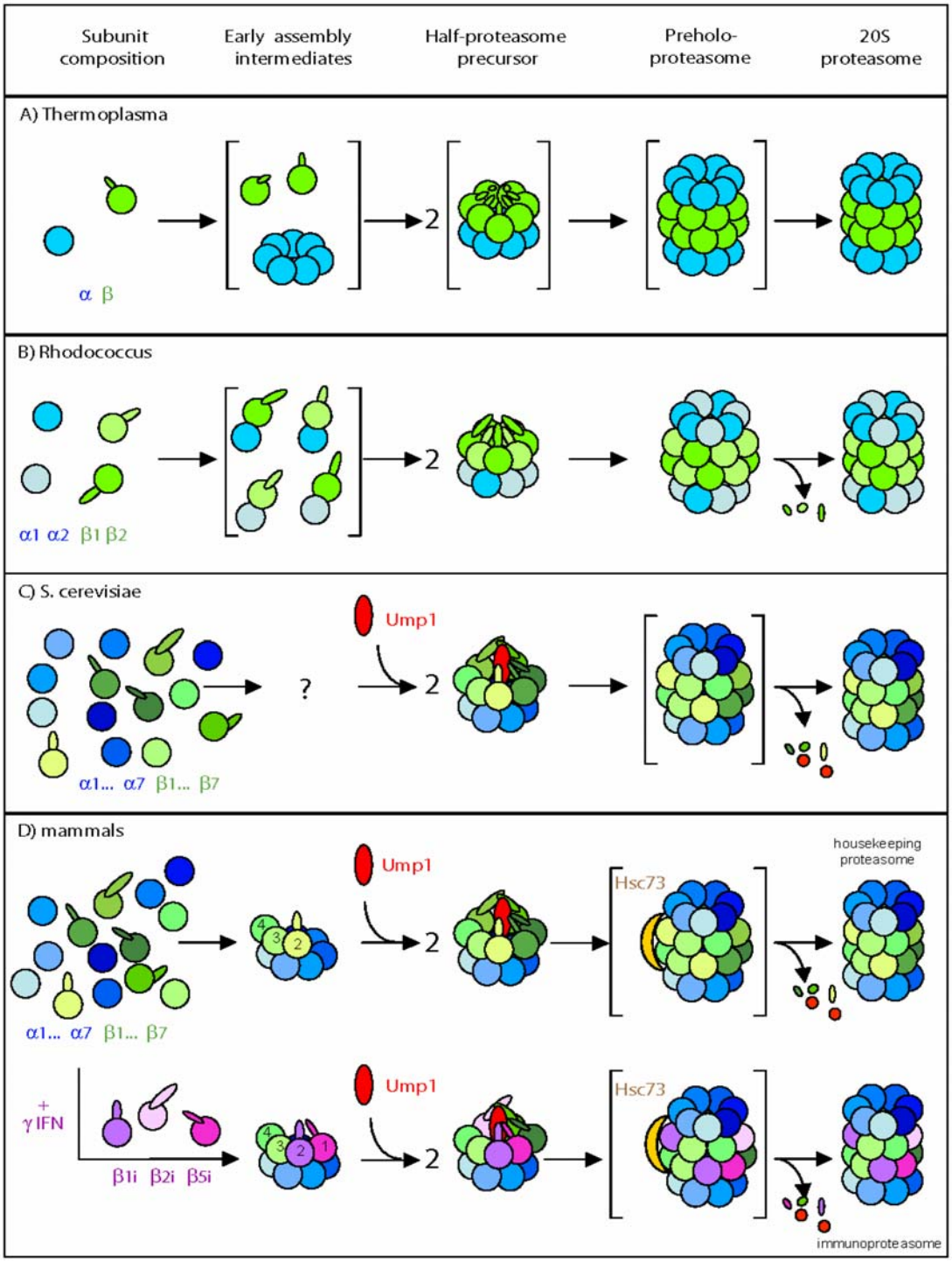

Figure 1.6 Models of the 20S proteasome biogenesis in different organisms (*97*).

Archaeal and eubacterial 20S proteasomes purify in a relatively active state but several are stimulated by chaotrophic agents (*28, 60, 85, 98, 99*). Most are able to hydrolyze unfolded proteins and chymotrypsin-like peptides (*75, 85*) while only the 20S proteasomes of methanogens appear to have intrinsically high-level PGPH peptidase activity (*98, 99*) the proteasomes of *Thermoplasma* and of the bacterium *Rhodococcus* have only chymotrypsin-like activity, consistent with the fact that they have only one type of active site (*85, 71*). *Thermoplasma acidophilum* proteasomes degrades partially unfolded and ubiquitin-associated proteins (*100*).

Eucaryal 20S proteasomes, in contrast, purify as latent enzymes which are activated by heat, chaotrophic agents and hydrostatic pressure (*101*). The β_1; β_2 and β_5 subunits of eucaryotic enzymes are responsible for PGPH, TL and CL activities, respectively (*19, 102, 103*). The β_1 subunit also cleaves BrAAP substrates (*104*). In mammals, the housekeeping β_1, β_2 and β_5 subunits are duplicated and replaced by β_{1i}, β_{2i} and β_{5i} alternative subunits after IFN-γ induction to generate an immunoproteasome with altered peptide cleavage specificity to degrade a broader variety of proteins (*105*) and facilitate the generation of antigenic peptides (*52, 106, 107*).

Experimental evidence supporting that the peptidase activity of eukaryotic 20S proteasomes can be stimulated by a variety of treatments; (*108, 109, 110, 111, 112, 113*) (i) activation occurs when the core particle interacts with the Regulatory Particle and forms the 26S holoenzyme; (ii) there exist other endogenous regulators of the core particle, which include the γ-interferon induced PA28 complex (also known as the 11S regulator) (*114, 115*); and (iii) mild chemical treatments, such as exposure to low levels of sodium dodecylsulfate, are also effective in activating the core particle (*116*). SDS activation of the wild type complex is mediated by channel opening (*61, 110*).

The number of active sites in the central cavity is six in eucaryotes, instead of fourteen as in *Thermoplasma* proteasome (*61*, *92*).

20S proteasomes belong to the amino-terminal (Ntn) hydrolase family, where the N-terminal threonine residue functions as the nucleophile (*117*). Another feature of this family is the exposure of an N-terminal active site after autocatalytic removal of a propeptide (*118*). The Thr1 γ-oxygen is believed to act as the nucleophile and the α-amino group as the proton acceptor in the hydrolysis of peptide bonds. In support of this, peptide bond hydrolysis is inhibited when Thr1 is modified by site-directed mutagenesis (*98*) chemical inhibition (*18, 19, 105*) or N-α-acetylation. Additional conserved residues essential for proteolytic activity are Glu-17, Lys-33, and Asp-166 (*102, 94, 117*). Other conserved residues, e.g. Ser-129 and Ser-169, seem to be required for structural integrity (*19*), and/or β precursor processing (*119*).

The central cavity, which is less hydrophobic than the antechambers, has a volume of ~84 nm in *Thermoplasma acidophilum*. The central cavity do not usually accommodate more than one polypeptide at a time (*60*). The proteasome degrades proteins in a highly processive manner, i.e. the enzyme makes multiple cuts in a protein and converts it to oligopeptides before attacking the next substrate molecule (*60*).

With the longer substrates more time is probably necessary for a cleavable bond on the substrate to reach the active site(s). Thus, the sequence and length of a protein substrate can influence the fraction of large peptides generated by the proteasome. To reach the active sites in the proteasome's central chamber, substrates must first traverse the narrow opening in the α ring, the outer chamber, and finally the opening in the β ring. These multiple chambers probably help prevent the premature release of substrates until the products are small enough to exit the particle. The proteasome may take a longer time to degrade a protein than the ribosome takes to synthesize it (*120, 121*)**.**

Proteasomes often degrade proteins from free N- or C-termini into oligopeptides of 3-30 amino acids in length by processive hydrolysis (*60, 122*). In *Thermoplasma acidophilum* it was proposed that the main factor determining product size (the molecular ruler) was the distance between adjacent active sites, which corresponds to a 7- or 8-residue peptides in an extended conformation (*18, 123*). Huber and co-workers (*18, 25*) proposed that the substrate remains covalently attached to one active site threonine residue, while it is attacked by an active site on an adjacent subunit. Such a mechanism would generate predominantly 7–8-residue peptides. However, if cleavages are made by nonadjacent active sites, longer products would be generated. Peptides smaller than 7 residues are probably produced if the cleaved fragments diffuse to and are cut by other active sites (*120*).

Furthermore, reducing the number of catalytic sites does not influence the average product length (*124*). Instead, it appears more likely that the dimensions of the axial gates formed by the α ring play a role in determining the size of products released than the spacing of the catalytic sites. Consistent with this, regulatory components that associate with and modify the axial gate influence product size (*103*).

Processive degradation is an intrinsic feature of 20S proteasomes (*60*). Interestingly, 20S proteasomes degrade some proteins by non-processive hydrolysis (*125*) and do not require substrates to have free N- or C-termini (*126*). In fact, some substrates (e.g., NF- κB p105, NF- κB p100) are predicted to have disordered, internal loops that enter the axial channel of 20S proteasomes which result in substrate processing and activation (*110, 112, 127*). Substrates hydrolysed by the 20S proteasome in the cell can be grouped as;

Transcription Factors; such as NF-κB p105 (p50 precursor) and IκBα (inhibitor of NF-κB) with the two-step activation roles in the immune and inflammatory responses, Rpn4 with transcriptional activator role on the proteasomal genes and

E2F which is a family of transcription factors, cell-cycle regulation (*7*) and proliferative responce.

Cell-Cycle Regulators; S-phase CDK inhibitors ($p27^{Kip1}$, $p21^{Cip1}$) which is resulted with G1 arrest, G1 cyclin (Cyclin D1) transition to S, Mitotic cyclins (Cyclin A, Cyclin B) completion of mitosis and Cyclin E for maintenance of the karyotype and normal cell homeostasis at late G1 and early S.

Oncoproteins and tumor suppressors; Protooncogene products (c-Jun, c-Fos, c-Myc) with physiologic role of transcriptional activation, cell growth, differentiation, transformation and proliferation.

Enzymes; DNA Polymerase II which has a role in DNA. Nuclear enzymes DNA topoisomerase I and II which modify DNA topology, and Fructose-1,6-biphosphatase which is a key enzyme of gluconeogenesis in glucogen metabolism.

Membrane proteins; such as CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) with a physiological role of transmembrane transport of chlorine ions in Cl^- secreting and reabsorbing epithelial cells.

1.7. Substrate Entry to the Proteasome Complex

In the *Thermoplasma* proteasome, two entry ports of 1.3 nm diameters, made by turn-forming segments of the seven identical α subunits, can be visualized at the ends of the cylindrical particle (*18*). In Archaeal proteasomes, because of the tight packing of subunits, substrates enter through a narrow channel in α ring which only permits entrance of unfolded polypeptide chains or short peptides (*15, 18*). By contrast, the hydrolytic chamber of the yeast 20S proteasome is tightly sealed (*19*). The N-termini of α subunits project into the ports seen in *Thermoplasma*, and fill them completely in several layers with tightly interdigitating side chains. Thus access to the interior of the particle requires substantial structural rearrangement (*61*). The deletion of the nine residues from the N-terminal tail of

α_3 resulted in stimulation of proteasomal peptidase activity and disordering of the remaining pore residues and the crystal structure analysis of this mutant has an axial channel through the molecule whose dimensions are comparable to those of the *Thermoplasma* core particle channels (*61, 110*).

All sequenced archaebacterial core particles to date contain the characteristic Tyr8-Asp9-Arg10 (YDR-motif) in their α subunits, which emphasises the importance of this structural element in proteasomes. Remarkably the YDR motif appears to be present in all organisms but is different in tertiary structure and function between eukaryotes and prokaryotes (*61*) (Figure 1.7).

In eukaryotes, YDR element in the α_3 and α_4 subunits maintain the regulatory gate. In contrast, YDR motif in prokaryotic core particles interact specifically with the YDR motif of the adjacent α subunits and mainly contribute to the open axial channel (*128*).

1.8. Regulation of 20S Proteasome Complex

1.8.1. Regulatory AAA ATPases of 20S Proteasome

The proteasomal ATPases belong to the Triple-A family of ATPases (AAA ATPases; ATPases Associated with a variety of cellular Activities) (*129, 130, 131*). AAA^+ proteins all possess a conserved ATPase domain (CAD or AAA cassette) made of approximately 250 amino acid residues, which contains the Walker A and B motifs typical for P-loop ATPases as well as a the second region of homology (SRH) motif (*31, 33, 132*). The AAA family members are characterized by the presence of either one or two CADs in their protein structure. CAD encompasses the consensus Walker A (G*xx*G*x*GKT, *x*; any amino acid) and Walker B (*hhhh*DE*xx*, *h*; hydrophobic residue) motifs which are critical for ATP binding and hydrolysis functions, respectively. SRH motif [(T/S)-(N/S)-X5-D-X-A-X2-R-X2-R-X-(D/E)] is also involved in ATP hydrolysis.

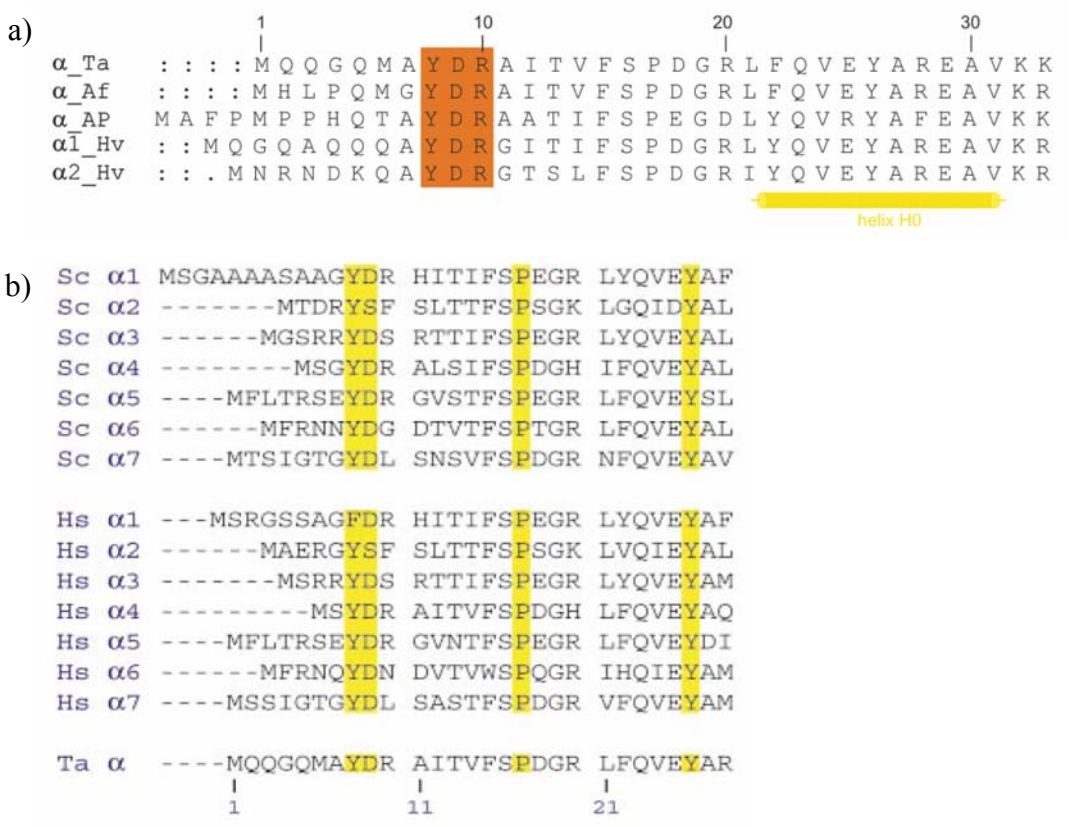

Figure 1.7 (a) Alignment of the N-terminal regions of α subunits from *Thermoplasma acidophilum* (Ta), *Archaeoglobus fulgidus* (Af), *Aeropyrum pernix* (Ap) and *Haloferax volcanii* (Hv). Residue numbers for the α subunits are assigned on the basis of the alignment to Ta. To the left of the vertical box lie the tail sequences with the conserved YDR motif. The H0 helices are indicated in yellow in all panels (*128*). (b) Sequence alignment of 20S proteasome α-subunit N-terminal residues. The seven (α_1-α_7) subunits of yeast (Sc) and human (Hs) with Ta (*133*). Proteasomes are shown with the single α subunit of the Ta proteasome. The four conserved residues that stabilize the open gate conformation are shown on a yellow background. Other conserved residues generally perform obvious structural roles that do not change between open and closed proteasome conformations.

The activities carried out by these well-conserved AAA proteins in the cell are numerous and include controlling protein folding and unfolding by manipulation of protein structure with nucleotide-dependent conformation changes, assembly and disassembly of protein complexes, protein transport through membrane fusion and programmed protein degradation. Such operations are the result of chaperonin-like abilities and include unfolding of proteins prior to degradation; chaperonin foldase/unfoldase activities, DNA replication, transcription, recombination, cell cycle control and various ‘‘activase’’ operations (*31, 131, 134, 135, 136*). Although the exact role of ATP hydrolysis remains to be established in the protein degradation by a 26S proteasome, it is likely that the energy-dependent steps are unfolding and translocation (*32*).

The Rpt (regulatory particle triple-A) subunits of the 26S proteasome, archaeal PAN, ARC of the actinomycetes, ClpX, ClpA, HslU, Lon, and FtsH which are all likely involved in proteolysis and are included in the AAA^+ superfamily (*31*).

The AAA^+ ring, which is often hexameric, appears to have an internal cavity and a central pore forming a passage that is continuous with the central channel of proteases such as the 20S proteasome and ClpP (Figure 1.8). Thus, as substrate protein becomes unfolded it may first pass through the central cavity of the AAA^+ complex and then through a passage formed by a combination of the pore of the AAA^+ ring and the narrow openings located at either end of the protease cylinder. Perhaps, this ATP-dependent passage through AAA^+ ring eliminates residual secondary structural elements of substrate protein that prevents its diffusion into the open gate 20S particles (*22*).

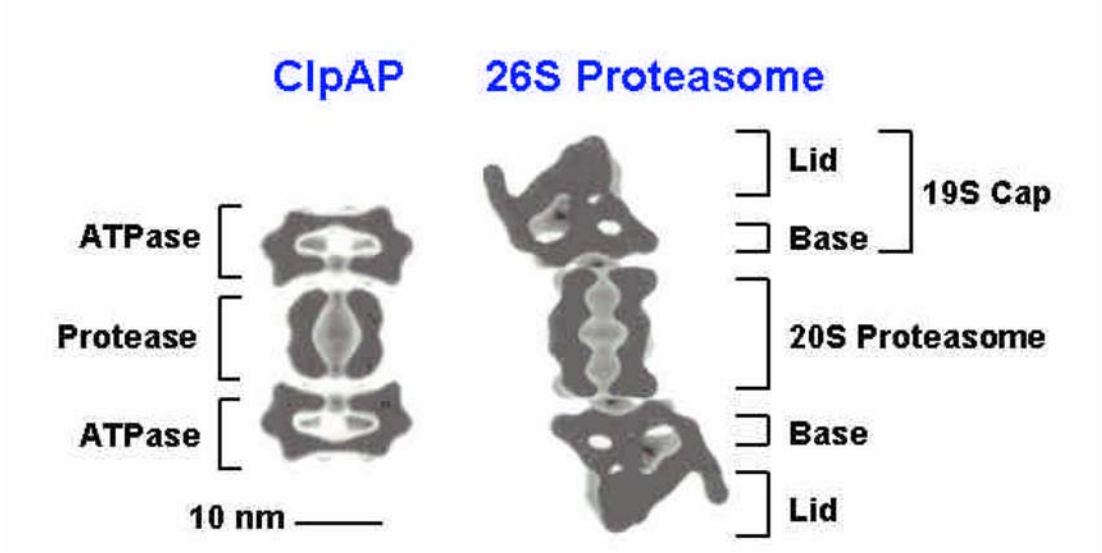

Figure 1.8 The selfcompartmentalized proteolytic chambers (20S proteasome and ClpP protease) and energy-dependent AAA^+-regulatory complexes (19S cap and ClpA ATPase) are indicated. The Base domain is composed of eight subunits which include six AAA^+ proteins which are proposed to form a hexmeric ring similar to ClpA (*10*).

1.8.1.1. Bacterial ARC Complex

In actinomycetes, a gene encoding an AAA ATPase that is distantly related to proteasomal ATPases was identified in the vicinity of the operon for the proteasome subunit genes. This protein, denoted as ARC for AAA ATPase ring-forming complex, forms single and double-stacked homo-hexameric rings which hydrolyzes nucleotides including ATP, CTP, and ADP. The ARC protein of the actinomycetes is predicted to activate proteolysis by 20S proteasomes similar to the archaeal PAN protein, but an interaction or activation of the proteasome with ARC could not be substantiated experimentally (*72, 137*).

Until now, *arc* gene homologs have only been found in Actinobacteria. The *Rhodococcus erythropolis* ARC forms 600 kDa hexameric rings with sixfold symmetry and is encoded by a separate operon but linked to genes encoding the

α_2 and β_2 subunits of the 20S proteasome (*32*). The diameter of the complex is approximately 8 nm and the pore has a diameter of approximately 1.5 nm. The N-terminal coiled coil of ARC is neither necessary for complex formation, nor for ATPase activity.

1.8.1.2. Eucaryal 11S Regulatory Subunit

Degradation of small peptides (not ubiquitinated or denatured proteins) by the proteasome is greatly stimulated by the 11S regulatory subunit (PA28 activator) in an ATP-independent manner. PA28 is a cytoplasmic complex formed by equal amounts of two different, but related, 28 kDa subunits, PA28α and PA28β, which are suggested to form a 200 kDa heteroheptamer (*114, 138, 139, 140*).

The formation of the 11S-20S complex through ATP-independent mechanisms is induced by γ-interferon, 11S regulators bind to one or both ends of 20S proteasomes triggers the exchange of three particular subunits (β_{1i}, β_{2i}, β_{5i}) in the 20S core particle, and dramatically enhances its proteolytic capacity (*113*). The modified 20S core then associates with 19S caps to constitute the so-called 'immunoproteasomes' which are responsible for antigen processing. The main function of 11S complex is to enhance the production of antigenic peptides for presentation by MHC class I molecules (*141, 107*). The substitution of three subunits leads to the generation of oligopeptides that have an higher affinity to bind to MHC class I molecules by their C-terminal anchor residues.

Endogenous antigens are degraded by the proteasomes or the immunoproteasomes into short peptides. These peptides are transported into the endoplasmic reticulum (ER) lumen by the TAP1/TAP2 heterodimer. Polypeptides of 8–11 residues bind with the TAP (transporter associated with antigen presentation) in the cytosol, are transferred into the endoplasmic reticulum, form complexes with MHC class I molecules, and are eventually delivered to the cell surface. Such antigen presentation allows cytotoxic T cells of the immune system to detect and destroy cells expressing viral or other unusual polypeptides (*120, 5*).

This central role of the proteasome in antigen presentation by MHC class I molecules was demonstrated with proteasome inhibitors.

The existence of hybrid proteasomes, in which one 11S regulator and one 19S complex bind simultaneously to a 20S proteasome has been demonstrated. Such complexes are induced by γ-interferon and play a role both in antigen presentation and in the breakdown of some proteins.

The heptameric barrel-shaped 11S Regulatory Particle contains a central channel that has an opening of 20 A° diameter at one end and another of 30A° diameter at the presumed proteasome-binding surface. The binding of 11S Regulatory Particle probably causes conformational changes that open a pore in the proteasome α-subunits through which substrates and products can pass (*142*) (Figure 1.9).

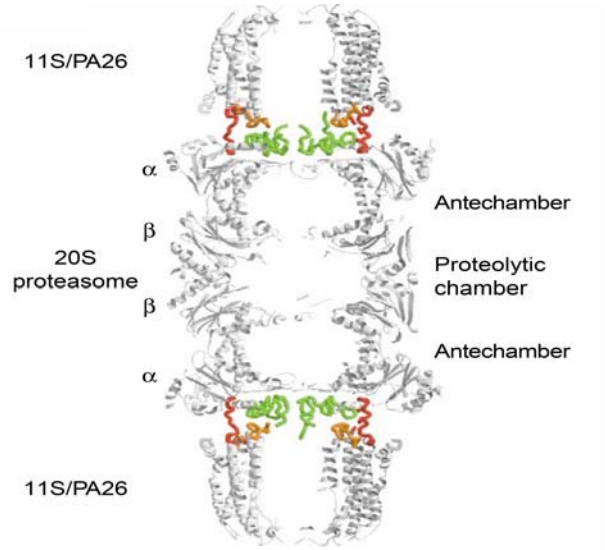

Figure 1.9 Ribbon representation of the 20S-11S proteasome complex structure. Residues of the N-terminal tails of α-subunits are depicted in green, residues of the 11S C-terminus are red and residues of the 11S activation loop are orange (*143*).

1.8.1.3. Eucaryal 19S Regulatory Subunit

The eucaryal 20S proteasome is fully functional in degrading proteins only when associated with an energy-dependent 19S cap regulatory complex or an eight-subunit derivative of this complex. The 19S cap regulatory complex has been separated into two domains; Lid and Base (*144*) (Figure 1.10). Base domain is composed of eight subunits. Six of these subunits are ATPases which are members of the AAA family (*145*) and have recently been named as Rpt proteins (Regulatory Particle Triple-A, Rpt1-Rpt6) (*13*). Each of the six ATPases contains an 230-aminoacid-long CAD (conserved ATPase domains, including Walker A and B sequences of P-loop ATPases, and the second region of homology region; SRH region) (*132, 146, 33*) which is a characteristic of the AAA ATPases family (*147, 129*). These energy-dependent subunits are responsible for the recognition, unfolding and/or translocation of substrate proteins into the central proteolytic chamber of the 20S proteasome (*144*). The six Rpt proteins of the 26S proteasome base domain may form a hexameric ring which interfaces the heptameric α-ring of the 20S core with symmetry mismatch similar to ClpAP protease (*33*). Lam *et al.* (2002) recently provided evidence that in the base domain, Rpt5 interacts with the polyubiquitin chains, functioning as a degradation signal by eukaryotic 26S proteasomes, and that this interaction is modulated by ATP hydrolysis. It has also been shown that a non-ATPase subunit, Rpn10, binds polyubiquitin chains. The base and the lid domains are jointly required for degradation of ubiquitin-protein conjugates.

The non-ATPase subunits of the base complex Rpn's (Rpn1 and Rpn2) (*148*) are the largest among which eight subunits, Rpn3, Rpn5, Rpn6, Rpn7, Rpn8, Rpn9, Rpn11, and Rpn12 composes the lid. The lid is needed for recognition of ubiquitinated proteins and does not directly interact with the 20S core (*60*). Six related ATPases of the base and two additional proteins (Rpn1 and Rpn2) which can independently bind to 20S core (Figure 1.11).

In the eucaryal 26S proteasome, the base of the 19S cap (PA700) which contains six Rpt (AAA^+) subunits may also participate in maintaining the quality control of proteins by functioning as an unfoldase, a molecular chaperone, inhibits the aggregation of incompletely folded, nonubiquitinated proteins and promotes the refolding of denatured proteins (*e.g.* citrate synthase) to their native state similar (*9, 136, 149*) to molecular chaperones.

Several functions of 19S Regulatory particle ATPases have been proposed as follws: First, the hydrolysis of ATP by the 19S ATPases promote the assembly of 26S proteasomes (*111, 150, 151*). Second, the ATPases have a role in the gating of the translocation channels, which are closed in the yeast proteasome (*19*), substrate proteins are possibly bound and unfolded by the ATPases (*21*), a function reminiscent of the activity of molecular chaperones during refolding of damaged or aberrant proteins (*152*). Finally, the ATPases might assist in the translocation of unfolded substrate proteins into the central chamber of the 20S proteasome (*153*).

The base contains the six related ATPases (Rpt1 – Rpt6) and two additional proteins (Rpn1 and Rpn2) which can bind independently to the 20S core. The other subcomplex (the lid) contains Rpn3, Rpn4–Rpn9, as well as Rpn11 and Rpn12 (Figure 1.11). Rpn10 is thought to provide a ‘‘bridge’’ between the base and the lid. The lid is positioned distal to the 20S core (*52*).

Deletion of the 19S regulator subunit Rpn10 yields 26S proteasomes from which a complex of eight subunits, called the lid, can be dissociated at high salt concentrations (*32*). The ability of a 50 kDa subunit Rpn10 of the 26S complex from human erythrocytes to bind Lys-48–linked ubiquitin chains was first discovered by Deveraux *et al* (*154*). Rpn10 is the only subunit in the 19S complex yet identified which binds Lys-linked ubiquitin chains *in vitro*.

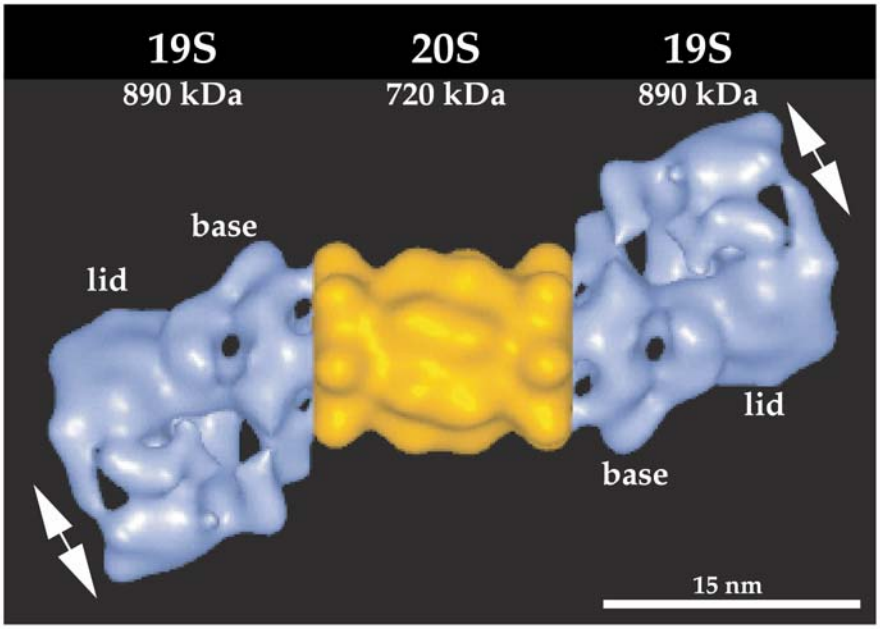

Figure 1.10 Model of the three-dimensional (3D) structure of the 26S proteasome from *Drosophila* based on electron microscopy and using the crystal structure of the 20S proteasome from *Thermoplasma* (*64*). The 19S cap, which may attach to one or both ends of the 20S core (shown in yellow), is colored in blue. The indicated masses of the 19S and 20S complexes from *Drosophila* were determined by scanning transmission electron microscopy (*46*).

It has been reported that Rpt2, one of the six ATPases in the base of the 19S complex, gates the proteasome channel and controls both substrate entry and product release (*112*).

The base is sufficient to activate the 20S core particle for degradation of peptides, but the lid is required ATP-dependent degradation of ubiquitinated proteins. In conjunction with the base complex, the 20S proteasome is able to degrade peptides and non-ubiquitinated proteins in an ATP-dependent manner. The lid subunits share sequence motifs with components of the COP9/signalosome complex, suggesting that these functionally diverse particles have a common evolutionary ancestry.

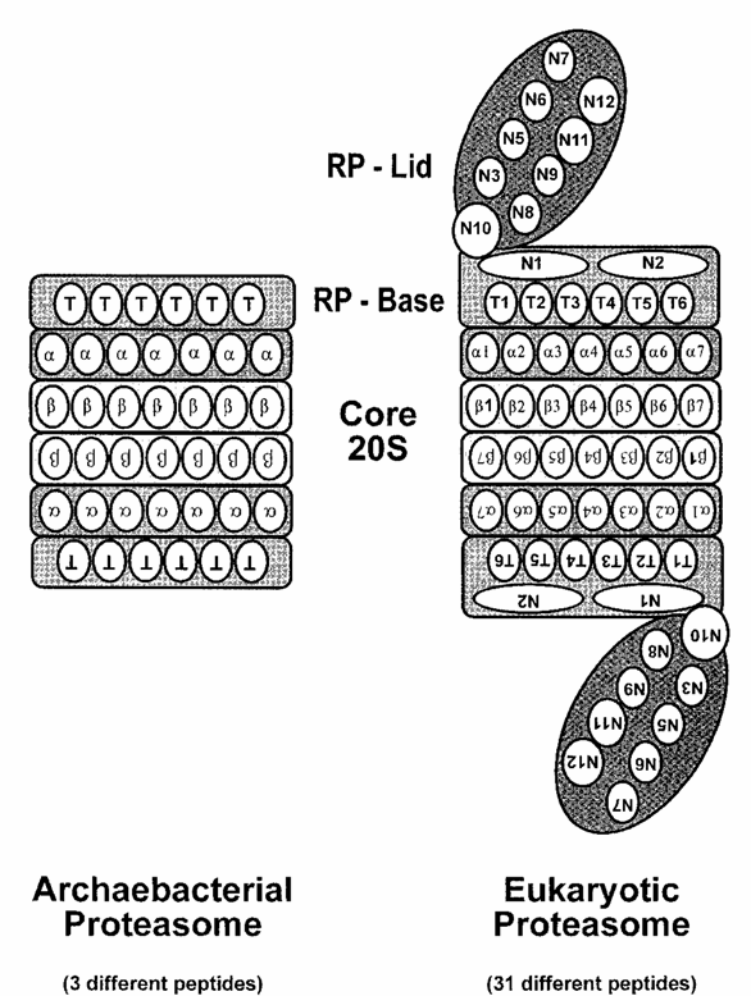

Figure 1.11 Structure and subunit distribution of archaebacterial and eucaryotic proteasomes (T: Rpt, Regulatory particle ATPase; N:Rpn, Regulatory particle non-ATPase) (*52*).

In vitro studies with citrate synthase has implied that refolding of enzyme is mediated by the Rpt1- Rpt6 family of ATPases, although the participation of the non-ATPase components of the base, Rpn1 and Rpn2 cannot be excluded (*149*).

A hallmark of the 19S cap ATPases is a predicted coiled coil segment near the N terminus (*21*). The N-terminal regions of the 19S ATPases, which are predicted to form coiled coils (*155*), were suggested to promote binding of substrate proteins by the 26S proteasome or interactions between individual ATPases. In archaea, close relatives of the 19S cap ATPases have been discovered in the genomes of *Methanococcus jannaschii* and *Archeoglobus fulgidus* (*12*).

For exspression of Rpt genes it was found (*116*) that the promoter regions of the Rpt genes share a unique nonamer sequence motif (5‘-GGTGGCAAA-3‘ or its complement). Promoter analysis showed that, in fact, it serves as a control element in the expression of Rpt genes. Interestingly, the element is found in the promoters of 28 of the 32 proteasomal genes implying that these subunits are subject to coordinate regulation at the transcriptional level. Therefore, we named the sequence element PACE (for proteasome associated control element). Further, PACE is found in the promoter regions of ~40 additional genes that are supposed to be involved in the ubiquitin-proteasome pathway, thus forming a regulatory network.

1.8.1.4. Archaeal PAN Complex

Closely related homologs of the eucaryal proteasome ATPase subunits are predicted from the genome sequences of the archaea (*76, 79, 77*). One homolog from *Methanococcus jannaschii* (MJ1176) has recently been purified from recombinant *Escherichia coli* and named PAN for proteasome-activating nucleotidase (*35*). PAN is identified as a 650 kDa ATPase complex from this archaebacterium and probably the evolutionary precursor of the eukaryotic 19S Regulatory Particle (*131*). The purified PAN complex had ATPase activity and activated the energy-dependent degradation of proteins by 20S proteasomes from

Methanosarcina thermophila and *Thermoplasma acidophilum* by four to ninefold (*99*).

PAN is composed of identical subunits closely homologous to the six ATPases of the 19S Regulatory Particle (Rpt1-Rpt6) with a (*S4*) gene and stimulates markedly the degradation of proteins, but not of tetrapeptides, by archaeal 20S proteasomes. This process requires hydrolysis of ATP (*99, 131*). PAN exhibits certain activities characteristic of molecular chaperones, e.g., it prevents aggregation and promotes the refolding of denatured proteins (*156*). In addition, in the presence of ATP, PAN catalyzes the unfolding of the globular green fluorescent protein when its C terminus is fused to the 11 residue ssra peptide (GFPssrA) (*156*). This ATP-dependent "unfoldase" activity is essential for the degradation of GFPssrA by archaeal 20S proteasomes, which otherwise do not degrade GFPssrA (*15*).

The axial channel of archaeal 20S proteasomes in its native state is still permeable to tetrapeptides, but is not large enough to accommodate poly-peptides, which require the presence of PAN and ATP for efficient degradation. Once the channel of the 20S proteasome is opened by deleting the N-terminal extremities of the α subunits, the dependence on PAN and ATP is abrogated for unfolded proteins.

PAN shows disaggregating and unfolding activities against heterologous substrates (*156*). When mixed with the *Thermoplasma* 20S proteasome, PAN from *Methanococcus jannaschii* stimulates the degradation of several unfolded proteins (*35*) suggesting that the although *Thermoplasma sp.* does not have PAN, it's 20S proteasome has not lost the ability to interact with an activating ATPase and that one or several of the other AAA^+ ATPases substitute for the missing PAN function (*157*). Two genes were identified as PAN homologs in the genome from *Halobacterium sp.*, an extreme halophile (*81*).

The ATPases catalyze unfolding of protein substrates and promote their translocation into the central chamber of 20S proteasomes where proteolysis occurs (*21*). More recently, it has been shown that bacterial ATP-dependent proteases (ClpAP and ClpXP) and the PAN (proteasome-activating nucleotidase) complex (the archaeal 19S homolog) unfold globular proteins (*9, 156, 158, 159, 160, 161*) (Figure 1.12).

ATP consumption was identical whether or not proteasomes were present. Large number of ATP molecules (at least 300) are hydrolyzed during degradation of a protein substrate, regardless of its folding status. The folded and unfolded protein substrates stimulate PAN's ATPase activity to a similar extent (Figure 1.12).

The protein substrate that binds to PAN triggers a change in its conformation leading to an increased capacity to hydrolyze ATP. Substrate binding to PAN can occur on the isolated PAN or on the PAN-20S complex (*15*) This substrate-activated ATPase (i) facilitates unfolding of the substrate, probably at the surface of the ATPase ring (*160*), (ii) favors an open form of the 20S axial channel, and (iii) promotes substrate translocation into the internal chamber of the 20S particles where peptide bond cleavage occurs (*15*) (Figure 1.12).

Therefore, we conclude that PAN, in the presence of ATP, facilitates the opening of the gate in the 20S proteasome, enabling the translocation of unfolded polypeptides. By interacting with 20S particles, PAN probably induces an ATP-dependent movement of the N termini of α subunits that displaces them from the surface of the axial pore. A similar mechanism has been suggested for the activation of 20S proteasomes by PA28 (*15, 35, 113*). Indeed, deletion of amino-terminal residues of 20S α subunits increases rate of degradation of unfolded proteins and eliminates situmulation by PAN.

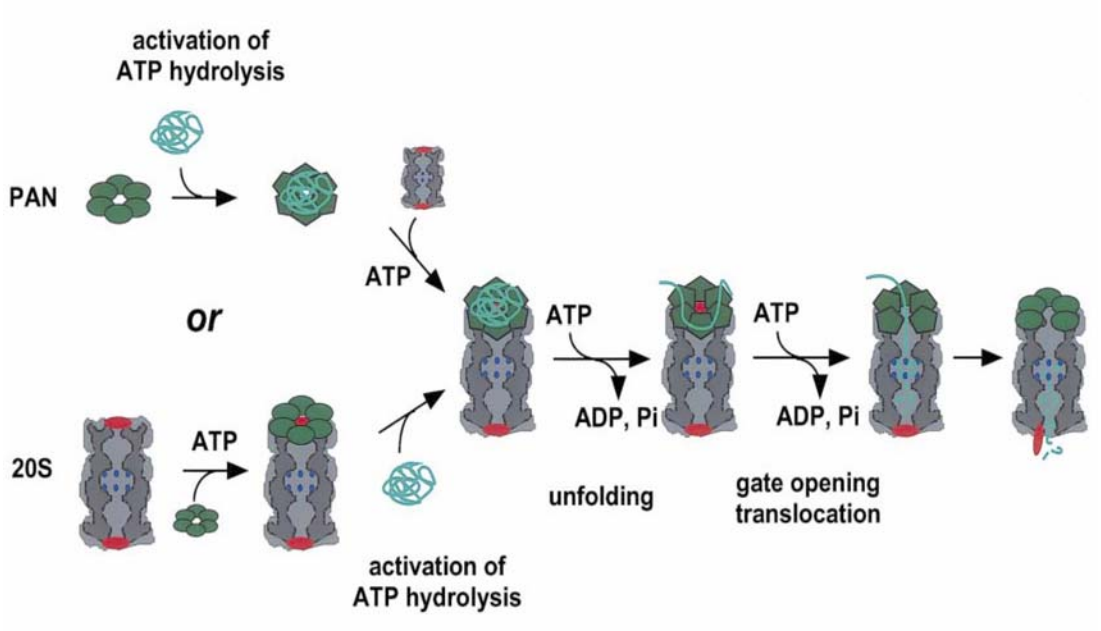

Figure 1.12 Model for the energy-dependent steps of PAN and proteasome-mediated protein degradation Cut side views of 20S particles are schematized with the active sites represented as blue dots in the internal chamber. PAN is schematized as a hexameric ring. The circle and pentagonal shape of the subunits represent the substrate-free and substrate-bound forms of PAN, respectively. The gate which precludes entry of protein into 20S particles is represented in red (*15*).

1.8.1.5. Archaeal VAT Complex

VAT (valosine containing protein-like ATPase from *Thermoplasma acidophilum*), an archaeal member of the AAA-family that possesses foldase as well as unfoldase-activity. These chaperone or chaperone-like activities are carried out by the conserved ATPase domains, which provide the needed energy through ATP hydrolysis. Electron cryo-microscopic studies revealed that *Thermoplasma acidophilum* VAT exhibits the tripartite domain architecture typical for type II AAA-ATPases: N-D1-D2, whereby N is the substrate binding N-terminal domain preceding domains D1 and D2, both containing conserved ATPase domains. The hexameric core of the VAT-complex (diameter 13.2 nm,

height 8.4 nm) encloses a central cavity and the substrate-binding N-domains are clearly arranged in the upper periphery (*162*) (Figure 1.13). The deduced product of the VAT gene is 745 residues long (Mr 83 000) and 500-kDa. Biochemical characterization studies showed that VAT from *Thermoplasma acidophilum* possesses Mg^{+2} dependent ATPase activity with a specific activity of 5.43 μmol/mg/h at an optimum temperature of ~70°C (*163*). Electron microscopy shows the purified protein to form single and double homo-hexameric rings (*163*) (Figure 1.13).

One attractive hypothesis how AAA-ATPases like VAT can act as unfoldases, is the enforcement of conformational changes by pushing or pulling the substrate through the central opening. Subsequently, such an unfolded substrate can be passed on to a proteolytical complex like the 20S proteasome. The type II AAA-ATPases has a driving force for these movements, protein unfolding and further translocation to an acceptor protein, could be provided by a molecular ratchet mechanism of the type proposed by Zhang *et al*. D1 and D2 form two interacting but negatively cooperating rings, ratcheting back and forth between ATP and ADP-bound states (*163*).

The VAT is a homologue of SAV from *Sulfolobus acidocaldarius* and CdcH of *Halobacterium salinarium*, and belongs to the CDC48/VCP/p97 subfamily due to being an archaeal homologue of the Cdc48 and p97 proteins of eukaryotes, which mediate homotypic membrane fusion and are involved in ubiquitin dependent protein degradation (*164*) (Figure 1.14). In Eucarya (human), in addition to the substrate-binding N domain, the ATPase domains of VCP (valosine containing protein) are also required in mediating the ubiquitin-proteasome degradation. The ATPase activity of VCP provide the first evidence that the two domains are not catalytically equal: D2 is responsible for the major enzyme activity at physiological temperature, whereas D1 is involved in the regulation of heat-induced ATPase activity (*164*).

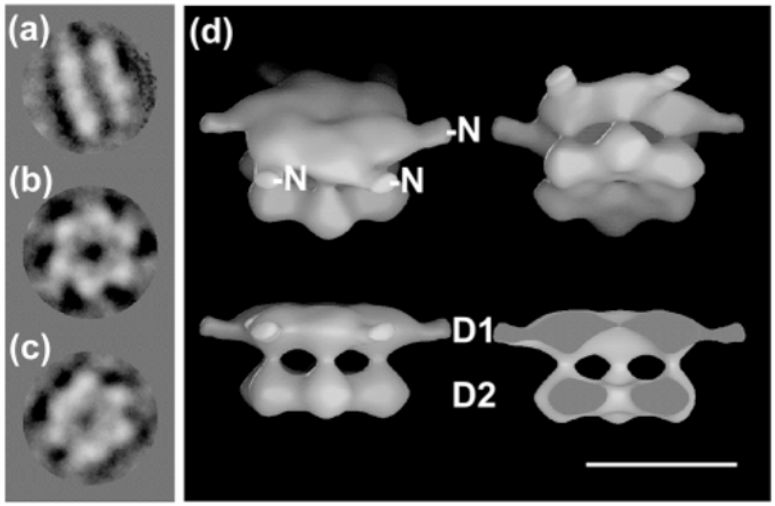

Figure 1.13 Calculation of a reference structure from three characteristic views. (a)-(c) Three characteristic views obtained from reference-free alignment were used to calculate the reference model in (d). Scale bar represents 15 nm. Domains N, D1 and D2 are labelled (*163*).

Thermoplasma contains several representatives of the AAA^+ family that are likely to have chaperone-like roles in the disassembly and unfolding of proteins and protein complexes; e.g., the Cdc48 homologue VAT (Valosine containing protein-like ATPase, a truncated form of VAT (VAT-2) lacking one of the AAA domains present in VAT, an archaeal- type Lon protease, and a related ATPase lacking the protease domain (*163*).

Depending on the Mg ion concentration, VAT can act in either an unfolding or a refolding mode (*32*). Both reactions were ATP-dependent. Simultaneously it was shown that refolding of substrate, cyclophilin is not obligatorily dependent on ATP hydrolysis (*134*). The N-terminal domain of VAT, which lacks ATPase activity, also accelerated the refolding of cyclophilin but showed no effect on penicillinase (*134*).

Thermoplasma does not contain a proteasomal ATPase PAN, which is found in all archaeal genomes and stimulates the degradation of several proteins by the *Thermoplasma* 20S proteasome (*165, 166*). Since folding and unfolding activities of VAT have been demonstrated *in vitro*, it seems plausible that VAT could substitute for the missing PAN function.

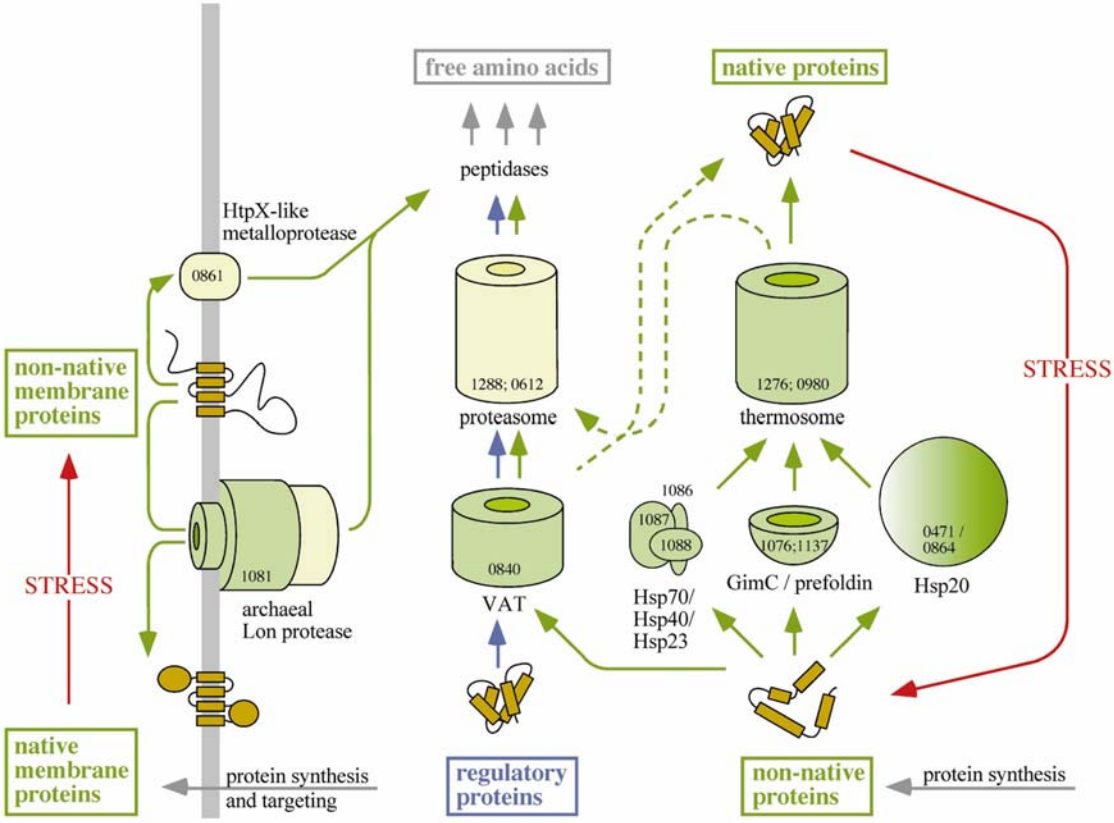

Figure. 1.14 Schematic overview of the putative pathways for protein quality control (green) and regulatory protein degradation (blue) in *Thermoplasma acidophilum.* Only a limited number of arrows in this chematic have been substantiated experimentally thus far. Chaperones are shown in green and proteases in yellow. Numbers denote the open reading frame number in the *Thermoplasma* genome (*157*).

Unlike any other Cdc48 protein, the second cdc48 homologue in *Thermoplasma,* VAT-2, consists of only the second ATPase domain (truncated form of VAT), which is preceded by a unique N-domain. So far, the function of this homologue remains unknown (*157*).

1.9. *Thermoplasma volcanium* GSS1 strain

Domain:	*Archaea*
Kingdom:	*Euryarchaeota*
Order :	*Thermoplasmales; Thermoplasmaceae*
Genus :	*Thermoplasma*
Species :	*Thermoplasma volcanium*
Strain :	*Thermoplasma volcanium* GSS1 (DSM 4299, JCM 9571)

Thermoplasma volcanium GSS1 strain is the source organism in this study, which is gram-negative thermoacidophilic archaeon lacking a cell wall, isolated from coal refuse piles and solfatara fields. The optimum growth conditions are pH 2-3 and 60°C.

Thermoplasma volcanium is (next to *T. acidophilum*) the second *Thermoplasma* species whose genomic sequence was completed and published. The genus *Thermoplasma* is unique among archaea, as it is a candidate for the origin of eukaryotic nuclei in the endosymbiosis hypothesis and it is adaptable to aerobic as well as anaerobic environments. Although genetically distinct, both *Thermoplasma* species share many phenotypic characteristics, including high motility with multiple flagella, quite irregular shape due to the absence of cell wall and a unique cell membrane chemistry.

1.10. Scope and Aim of the study

Previously; in our laboratory, *Thermoplasma volcanium* 20S proteasome α and β subunit genes have been separately cloned and then combined in pUC18 vector (pUC-SK-αβ). The heterologous expression of *in-vivo* assembled 20S proteasome was achieved in the recombinant *E.coli* SK-αβ cells. Also the recombinant 20S proteasome has been purified to homogenity by two colon chromatographies.

In this study, we have aimed at biochemical characterization of the purified recombinant 20S proteasome of *Tp. volcanium*, and cloning and expression of a putative proteasome regulatory gene (TVN0382) of *Tp. volcanium* in *E. coli* which belongs to AAA^+ ATPase family.

In addition, a through database search regarding the proteasomal regulatory subunits belonging to AAA^+ ATPase family, and sequence analyses such as multiple sequence alignment and domain search were also undertaken.

The data would provide more insights into our understanding of the structure and function of archaeal proteasomes as well as regulatory circuits that modulate their functions.

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial Strains and Culture Growth

Thermoplasma volcanium GSS1 type strain 4299 was purchased from DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and its genomic DNA was used as template DNA for cloning of *Thermoplasma volcanium* 26S proteasome regulatory subunit gene. *Thermoplasma volcanium* cells were grown in liquid Volcanium Medium (pH 2.7) (Robb and Place, 1990), which was supplemented with glucose (0.5%, w/v) and yeast-extract (0.1%, w/v) at 60°C. The cultures were refreshed by subculturing once a week.

E. coli strain TG1 (*supE hsd*Δ5 *thi*Δ(*lac-proAB*) F[*traD*36 *proAB*$^{+}$ *lacl*^{q}*lac Z*ΔM15]) from our laboratory collection was used as the recipient strain in the transformation studies. *E. coli* cells were grown on Luria-Bertani (LB) agar (*Bertani, G.,* 1951) (*167*) plates supplemented with ampicillin (final concentration 50 µg/ml) at 37°C. The cultures were renewed by subculturing to LB agar plates, monthly.

2.1.2. Chemicals, Enzymes, Kits

All chemicals used were analytical grade. The chromogenic substrates; Alanine-Alanine-Phenylalanine-pNA (Ala-Ala-Phe-pNA), L-Leucine-pNA (L-Leu-pNA), N-Suc-Alanine-Alanine-Proline-Phenylalanine-pNA (N-Suc-Ala-Ala-Pro-Phe-pNA), Alanine-Alanine-Alanine-pNA (Ala-Ala-Ala-pNa), N-Suc-Phenylalanine-pNA (N-Suc-Phe-pNA), (N-Carbobenzoxyl-Arginine-pNA (N-CBZ-Arg-pNA), Alanine-Alanine-Valine-Alanine-pNA (Ala-Ala-Val-Ala-pNA), N-CBZ-Leucine-Leucine-Glutamine-βNA (N-CBZ-Leu-Leu-Glu-βNA) and Cysteine(Cys) were purchased from Sigma Chemical Company, Saint Louis, Missouri, U.S.A.

Acetic acid-glacial, ammonium sulfate [$(NH_4)_2SO_4$], Bisacrylamide, Bromophenol Blue ($C_{19}H_{10}BrO_5S$), Chloroform, Citric acid, Dimethyl sulfoxide (DMSO), Dodecyl Sulfate Sodium Salt (SDS), Ethylenediaminetetraacetic acid (EDTA), Glucose, Guanidine HCl, Hydrochloric acid (HCl), Isoamylalcohol, Magnesium Chloride ($MgCl_2$), Magnesium Sulfate ($MgSO_4$), Manganase Chloride ($MnCl_2$), β-Mercaptoetanol, Methanol (CH_3OH), Potassium dihydrogen phosphate (KH_2PO_4), Sodium Chloride (NaCl), Sucrose, Tris-base, Tryptone, Yeast extract were purchased from Merck, Darmstadt, Germany.

Acrylamide, Ammonium per-sulfate (APS), Ampicillin, Adenosine triphosphate (ATP), Calcium Chloride ($CaCl_2$), Sodium per-chlorate ($NaClO_4$), Coomassie brilliant blue R-250, Ethidiumbromide (EtBr), Glycerol, Isopropyl β-D-1-thiogalactopyranoside (IPTG), Potassium Chloride (KCl), Sodium Molybdate, N,N,N',N'-tetramethylenediamine (TEMED), Trisma Hydrochloride, Triton X-100, 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), were purchased from Sigma Chemical Company, Saint Louis, Missouri, U.S.A.

Ethanol (C_2H_5OH) was purchased from Riedel-de-Haën, Seelze, Germany. Agarose was from Applichem, Darmstadt, Germany. Agar was purchased from Difco, Detroit, Michigan, USA. N-2-Hydroxyethylpiperezine-N'-2 Ethanesulfonic Acid (HEPES) was purchased from Calbiochem, Los angeles, USA. Yeast

Extract was puchased from Difco, Detroit, USA and OXOID Basingstoke, England.

Taq DNA polymerase, restriction endonucleases (EcoRI, HindIII, PstI, AvaII) and their buffers were from MBI Fermentas AB, Vilnius, Lithuania. All of the primers were synthesized by Applichem, Darmstadt, Germany.

Wizard® Plus SV Miniprep DNA Purification System Kit was purchased from Promega Co, WI, USA. QIAGEN PCR Cloning Kit were obtained from Qiagen Inc., Valencia, USA.

2.1.2.1. Plasmids, Molecular Size Markers

The cloning vector p-Drive, used throughout the study, was purchased from Qiagen Inc., Valencia, USA and diagramaticaly shown in Appendix A. DNA and protein molecular size markers are supplied from MBI Fermentas AB, Vilnius, Lithuania and Sigma Chemical Co., St. Louis, Missouri, USA, respectively and are shown in Appendix B.

2.1.2.2. Recombinant *Tp. volcanium* 20S Proteasome Enzyme

Thermoplasma volcanium (*Tp. volcanium*) 20S proteasome has recently been cloned, expressed and purified in our laboratory. The recombinant *E.coli* pUC-sk-α/β strain that carries the pUC-18 vector containing proteasome α- and β- subunit genes was the source of recombinant 20S Proteasome. The recombinant 20S proteasome was previously purified in our laboratory by using two sequential colon chromatographies (gel filtration and ion-exchange chromatography) and kept at -20°C until use. This enzyme preperation was used throughout the study, for electrophoretic and biochemical analyses.

2.1.2.3. Buffers and Solutions

The buffers and the solutions used and recipes for their preparations are given in the Appendix C.

2.2. Methods

2.2.1. Electrophoretic Analyses

2.2.1.1. Native Gel Electrophoresis

For detection of 20S proteasome in the native state, purified protein samples were electrophoresed in a non-denaturing polyacrylamide slab gel composed of a stacking gel and a resolving gel containing 4.95% and 5,1% acrylamide, respectively.

The following molecular size standards were used to estimate the apparent molecular weight of the 20S proteasome complex.

α_2 macroglobulin	Mr 340 000	(non reduced)
β-galactosidase	Mr 116 400	(non reduced)
Fructose-6-phosphate	Mr 85 200	(non reduced)

The samples were prepared by mixing 3 µl of protease or size marker with 2 µl of sample buffer (Glycerol 20% in 50 mM Tris HCl, pH of 7,5) and 1 µl dye (Bromo Phenol Blue, 0,5%).

Electrophoresis was performed by using Biometra Vertical Minigel System (Biometra Biomedzinische Analytic Gmbh, Göttingen, Germany) at 90 V (volt) and 20m Amp (current), at 4^{o}C for 6 hours.

After electrophoresis, the polyacrylamide gel was stained with the Coomassie Brilliant Blue R250 for 1 hour on a plate shaker and then, destained within the Destaining Solution until bands become visible against a clear background.

2.2.1.2. SDS Polyacrylamide Gel Electrophoresis of Purified *Tp.volcanium* 20S Proteasome

The SDS polyacrylamide slab gel electrophoresis was performed to determine the molecular weights of recombinant alpha and beta subunits of *Tp. volcanium* 20S proteasome in the presence of the anionic detergent SDS (sodium dodecyl

sulphate, 0.1%). Since the charge on proteins is roughly proportional to size, it is possible to estimate the molecular weight of proteins by SDS-PAGE.

Five proteins given below were used as molecular weight standards, in molecular weight determination.

Urase (monomer)	Mr 90 000
Bovine Albumin (monomer)	Mr 66 000
Egg Albumin	Mr 45 000
Carbonic Anhydrase	Mr 29 000
α-Lactoalbumin	Mr 14 200

Vertical polyacrylamide gel electrophoresis was performed with 5 % stacking and 12.5% separating gel concentration as described by Laemmli (1970) using the Biometra Vertical Minigel System (Biometra Biomedzinische Analytic Gmbh, Göttingen, Germany).

The aliquots (about 30 μg protein) of purified proteasome enzyme sample or size standard were mixed with 2x sample buffer, containing 1% SDS. After denaturation in a boiling water-bath for 7 minutes and incubation on ice for 4 minutes, samples were loaded onto the gel and run at 90 V and 20 mAmp, at 4^{o}C for 6 hours. After the electrophoresis, the gel was stained with the Coomassie Brilliant Blue R250 and then, destained with the Destaining Solution until a clear background appears and bands become visible.

A standard curve graph of relative mobility (Rm) vs log molecular weight of known protein standards were plotted. The relative mobility of each protein was measured from the migration distance between the center of wells and center of the protein bands. The molecular weight of an unknown protein band can then be estimated by comparing its Rm value to that of the standards in the calibration curve.

2.2.2. Biochemical Analyses

2.2.2.1. Measuring the Chymotrypsin-like Activity of Recombinant *Tp. volcanium* 20S Proteasome Enzyme

The proteolytic activities associated with the recombinant *Tp. volcanium* 20S proteasome were measured with synthetic chromogenic peptide substrates, which are N-terminally blocked by succinyl (Suc) or carbobenzoxyl (Cbz) groups and the C-termini contain aromatic amines such as para-nitroanilide (pNA) or β-naphthylamide (βNA). The reaction mixture contained an aliquot of purified proteasome sample in a 820 µl proteasome assay buffer (pH 7,5). After preheating at 55°C, the enzymatic reaction was started by adding 0,6 mM oligopeptide substrate. The chymotrypsin-like protease activity of proteasome was measured by using Ala-Ala-Phe-*p*-nitroanilide as substrate. The proteolytic activity was determined based on the measurement of *p*-nitro aniline liberation by recording the absorbance at 410 nm contionously using Shimadzu UV-1601A double beam spectrophotometer (Shimadzu Analytical Co., Scientific Instruments Division, Kyoto, Japan) against a blank (proteasome assay buffer). The rate of βNA formation was measured at 340 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 nmol of p-NA or β-NA, per minute (Σ_{410}=8800 $M^{-1}cm^{-1}$, Σ_{340}=1780 $M^{-1}cm^{-1}$).

2.2.2.2. Effect of Temperature on Recombinant *Tp. volcanium* Proteasome Activity

The optimum temperature of proteasome enzyme activity was determined by measuring the peptidolytic activity at different temperatures over the range from 35°C to 98°C. The assays were performed using Ala-Ala-Phe-*p*-nitroanilide as substrate.

The residual activity was expressed as the percentage of the highest enzymatic activity measured. The background measurements due to non-enzymatic degradation of the peptide substrate were taken, to correct the activity results.

2.2.2.3. Determination of the Substrate Profile of the Recombinant *Tp. volcanium* Proteasome Enzyme

In order to find out the substrate profile of recombinant 20S proteasome, peptide hydrolysing activities were measured using various synthetic chromogenic polypeptides such as Ala-ala-phe-pNA, L-leu-pNA, N-Suc-Ala-ala-pro-phe-pNA, Ala-ala-ala-pNA, N-Suc-phe-pNA, N-CBZ-Arg-pNA and Ala-ala-val-ala-pNA as described before. The activity with the oligopeptide, N-CBZ-leu-leu-glu-βNA was assayed at 340 nm and 55°C using the same spectrophotometer (Shimadzu Analytical Co., Scientific Instruments Division, Kyoto, Japan). The activities were expressed as the percentage of the highest enzymatic activity measured.

2.2.2.4. Effect of pH on the Recombinant *Tp. volcanium* Proteasome Enzyme Activity

To determine the effect of pH in purified protease, peptidolytic activity was measured at different pH values, using Ala-Ala-Phe-p-NA as substrate under standard assay conditions. The buffers used were Tris acetate (pH 4,0 - 5,5), Tris phosphate (pH 5,7 - 8,0) and Tris glycine (pH 8,3 - 9,5). The residual activity was expressed as the percentage of the highest enzyme activity.

2.2.2.5. Effect of Denaturating Agent on the Recombinant *Tp. volcanium* Proteasome Activity

The chymotrypsin-like activity of 20S proteasome was measured in the presence of activating agents such as SDS, at various concentrations. The enzyme samples were incubated with SDS (over range between $2x10^{-4}$ mM and $35x10^{-4}$ mM) at room temperature for 10 min, and then residual activity was measured at 55°C as described before, using Ala-ala-ala-pNA as substrate. Controls with no substrate were also included into each assay, as control.

2.2.2.6. Effect of Various Activators and Inhibitors on the Recombinant *Tp. volcanium* Proteasome Activity

The effects of various metal ions (Mg^{+2}, Mn^{+2}, Ca^{+2}, Na^{+}, K^{+}), Cysteine ATP and EDTA on peptide hydrolyzing activity of the purified proteasome were assesed. The enzyme sample (5µg) was incubated with the activators and inhibitors at room temperature for 10 min. The residual activity was determined as described before, by measuring the Ala-Ala-Phe-p-NA hydrolysing activity and expressed as the percentage of the activity in the absence of inhibitor and activator.

2.2.3. Gene Manipulation Analyses

2.2.3.1. Genomic DNA Isolation of *Thermoplasma volcanium*

The genomic DNA was isolated using a modified method of Marmur *et al.*1962. *Tp. volcanium* cells cultivated in 200 ml Darland's broth medium until they reach late log phase. Then, cells were harvested by centrifugation at 8000 rpm (Sigma Sorvall Laboratory Centrifuge, Sigma Chemical Co., St Louis, Missouri, USA) for 10 minute at 4°C. The supernatant was discarded. The pellet was resuspended and washed twice in Saline-EDTA. The pellet was resuspended in Saline-EDTA-lysosyme solution (for 2 g cell pellet, 10 mg lysosyme in 25 ml Saline-EDTA, pH of 8.0) and incubated at 37˚C waterbath for 1 hour. After cells were lysed, 25% SDS solution was added and incubated at 60°C waterbath until it gets clear. After addition of 0,2 volume (v/v) $NaClO_4$ (5M) and 1 volume chloroform:isoamylalcohol (24:1, v/v) solution, the tubes were stirred in watery-ice bath for 30 minutes until homogenous suspension was obtained. Two phases were appeared after the centrifugation at 12000 rpm, 4˚C, for 10 minutes. The DNA in the aqueous upper phase was transferred into a new tube and incubated within the 2 volume (v/v) of cold absolute ethanol at -20°C and washed twice in 5 ml 70% ethanol by centrifugation at 11000 rpm, 4˚C, for 45 minutes. After ethanol was totally vaporized by aeration the pellet was dissolved in 500 µl sterile double distilled water. The samples were kept in eppendorph tubes at

-20°C freezer. DNA samples were further purified by RNAse treatment and phenol/chloroform extraction as described by Southerland *et al*., (1990). To check purity, genomic DNA was cut by the restriction enzyme EcoRI (Fermentas) following the manufacturer's instructions and analyzed by agarose gel electrophoresis.

2.2.3.2. Agarose Gel Electrophoresis

The DNA fragments after restriction endonuclease digestion, PCR amplification and all other manipulations were analyzed by gel electrophoresis using 1% (w/v) agarose gel (Agarose low EEO, AppliChem GmbH, Ottoweg, Darmstadt, Germany). DNA samples were mixed with 1/10 volume (v/v) of tracking dye and loaded onto the ethidium bromide (0.5 µg/ml) supplemented gel, and electrophoresis was performed in a submarine agarose gel apparatus (Mini SubTM DNA Cell, Bio Rad, Richmond, CA, U.S.A.) containing 1xTAE buffer.

Electrophoresis was carried out at 80 V, and 6,4 watts. After electrophoresis was completed the bands were visualized on a UV transilluminator (Vilber Lourmat, Marne La Vallée Cedex 1, France) and gel photographs were taken using a gel imaging and analysis system (Vilber Lourmat Gel Imaging and Analysis System, Marne La Vallée Cedex 1, France).

The molecular sizes of DNA fragments were determined by reffering to calibration curves, which were obtained by plotting the logarithms of molecular weights (bp) of size marker DNA molecules against their migration distances (cm) on the gel. The calibration curve shows *Eco*RI+*Hind*III cut Lambda DNA (MBI Fermentas AB, Vilnius, Lithuania).molecular size marker.

2.2.3.3. Design of PCR Amplification Primers

The primers were designed by consulting the nucleotide sequence of *Thermoplasma volcanium* 26S proteasome regulatory subunit gene in National Center for Biotechnology Information (NCBI) - Nucleic Acid Sequence Database

with the abbreviation of TVN0382 and in Archaic Database with the abbreviation of TVG0373521 (Kawashima *et al.*, 2000).

The length of 26S Proteasome Regulatory Subunit gene was 1128 bp. The forward (FPg, 22 bp) and reverse (RPg, 21 bp) primers were so designed that the up-stream regulatory sequences and some down-stream sequences to be included in the amplified gene. The list of PCR primers and their sequences are given in Table 2.1.

Both forward and reverse primers were synthesized by Applichem, Darmstadt, Germany. These primers were used to amplify the DNA fragment of the 26S Proteasome Regulatory Subunit gene from the isolated genomic DNA.

Table 2.1 Forward and reverse primer sequences used in amplifications of *Thermoplasma volcanium* 26S proteasome regulatory subunit gene.

PCR Primers	**Sequences**
FPg	5’ ctg ccg ggc agt caa acc caa c 3’
RPg	5’ cga tgc agg gga tct att gcc 3’

2.2.3.4. PCR Amplification of 26S Proteasome Regulatory Subunit Gene

The isolated and purified genomic DNA of *Tp. volcanium* was used as template in Polymerase Chain Reaction (PCR). The PCR reaction was performed by using Techne Thermal Cycler (Techgene, Techne Inc., NJ. USA). The reaction mixture (100 µl) was composed of 72 µl double distilled sterile water, 10 µl 1xPCR buffer (750 mM Tris-HCl pH 8.8 at 25°C, 200 mM $(NH_4)_2SO_4$, 0.1 % Tween20), 6 µl $MgCI_2$ (1.5 mM), 3 µl template DNA, 2 µl from each deoxyribonucleoside triphosphates (dNTPs) and 100 pmoles of each primers (FPg and RPg). The PCR mixture within the 0,2 ml eppendorph tubes was preincubated at 94^{o}C for 5

minutes and then, 2.5 U of *Taq* DNA polymerase (MBI Fermentas AB, Vilnius, Lithuania) was added and the PCR was carried out for 30 cycles.

The steps of thermal cycling per cycle were as follows:

Denaturation :at 94^{o}C for 1 minute,
Primer Annealing :at 40-60^{o}C for 2 minutes,
Primer Extension :at 72^{o}C for 3 minutes,
Final Extension :at 72^{o}C for 10 minutes,
Storage temperature was 4 ^{o}C.

The PCR was performed at various annealing temperatures, between 40^{o}C and 60^{o}C to findout the optimum hybridization temperature that yields PCR amplicons with the highest purity.

The amplified PCR products were analyzed by agarose gel electrophoresis (1% agarose gel). A molecular size marker, *Eco*RI+*Hind*III cut Lambda DNA (MBI Fermentas AB, Vilnius, Lithuania) was used for molecular size determination.

2.2.3.5. Cloning of PCR Amplified *Tp.volcanium* 26S Proteasome Regulatory Subunit Gene

2.2.3.5.1. Preperation of Competent *E. coli* Cells

Competent *E. coli* (TG1 strain) cells were prepared according to the modified method of Chung (1989). The *E. coli* cells, in 20 ml LB broth medium through were grown vigorous shaking at 37°C until early log. phase. (Heidolph UNIMAX 1010 Incubator, Heidolph Instruments GmbH, Kelheim, Germany). The growth was monitored by measuring the optical density of cell culture at 600 nm on the spectrophotometer (Shimadzu Analytical Co. Kyoto, Japan). When it reached to the logarithmic phase of growth (the number of cells were 10^{8}), the cells were harvested by centrifugation at 4000 rpm for 10 minutes (Sigma 3K30 Centrifuge, Sigma Chemical Co., St. Louis, MO, USA). The supernatant was discarded and the pellet was dissolved in TSS solution. The competent cell suspension was distributed as 100 µl aliquots into eppendorph tubes and stored at -80°C.

2.2.3.5.2. Ligation Protocol

The PCR amplified 26S proteasome regulatory subunit gene was ligated into the pDrive Cloning Vector by the QIAGEN PCR Cloning Kit (Qiagen Inc., Valencia, USA) according to the manufacturer's instructions. QIAGEN PCR Cloning Kit uses the single A overhang at each end of PCR products generated using *Taq* and other non-proofreading DNA polymerases and supplies the vector in a linear form with a U overhang at each end, to hybridize them with high specificity.

The vector contains several unique restriction endonuclease recognition sites, T7 and SP6 promoters on either side of the cloning site, allowing easy restriction analysis of recombinant plasmids and *in vitro* transcription of cloned PCR products as well as sequence analysis using standard sequencing primers. This vector also allows ampicillin and kanamycin selection, as well as blue/white colony screening by the expression of the amicillin, kanamycin resistance and *Lac-Z* genes. A map of the pDrive Cloning Vector and the sequence of the region surrounding the cloning site are given in Figure 2.1.

2.2.3.5.3. Transformation of Competent Cells

Ligation mixture (10 µl), containing 1 µl pDrive Cloning Vector (provided by the Kit), 5 µl Ligation Master Mix (2x) (provided by the Kit), 2 µl PCR product fragments, and 2 µl distilled water (provided by the Kit), was prepared by incubation at 11°C waterbath (Huber Ministat Waterbath) for 30 min - 1,5 hrs.

The control lacking the transforming DNA, was included into each assay. The competent *E. coli* (TG1 strain) cells, taken from -80°C deep freezer, were thawed on ice. An aliquot of 10 µl ligation mixture was added into thawed competent cells, mixed by gentle flippings and incubated on ice for 30 minutes. Cells were

transferred into glass tubes containing 600 µl LB broth supplemented with glucose and incubated at 37°C with vigorous shaking for 1 hr (Heidolph UNIMAX 1010, Heidolph Instruments GmbH, Kelheim, Germany). Then, a series of ten-fold competent cell dilutions were spread onto selective LB agar

plates supplemented with Ampicillin, IPTG and X-Gal. The agar plates were overnight incubated at 37°C.

Cells, which received the recombinant plasmids, formed white colonies on IPTG and X-Gal supplemented medium, due to the insertional inactivation of *Lac-Z* gene. Putative recombinant colonies were picked up and their pure cultures were prepared on the selective LB agar plates supplemented with Ampicillin, IPTG and X-Gal.

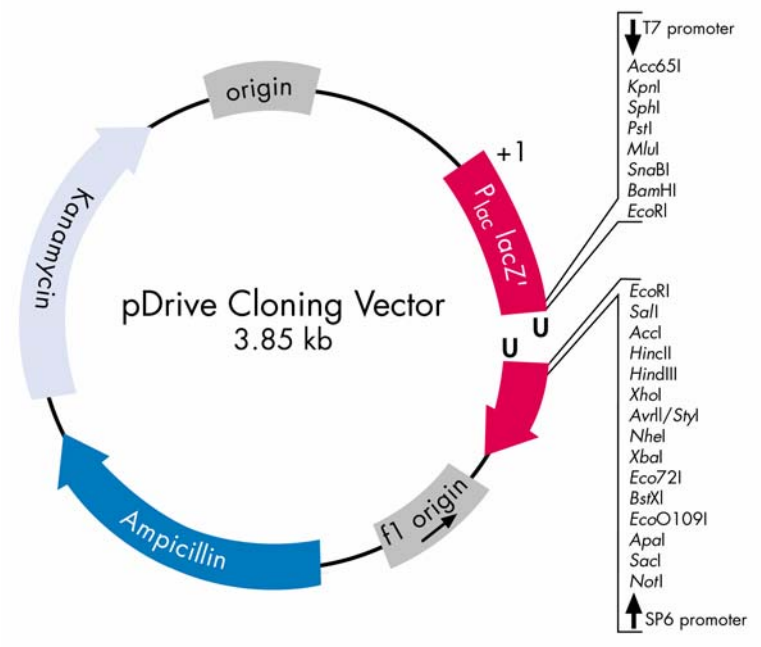

Figure 2.1 Diagram of pDrive cloning vector and restriction enzyme cut sites in the Multiple Cloning Site (MCS).

2.2.3.5.4. Plasmid Isolation from Recombinant Colonies

The recombinant plasmid, containing the complete *Tp. volcanium* 26S proteasome regulatory subunit gene in the pDrive cloning vector were isolated using Wizard® Plus SV Miniprep DNA Purification System (Promega Co., Madison, WI, USA). *E. coli* cells from the white colonies, harboring the recombinant plasmids, were innoculated into 10 ml ampicillin containing LB broth medium, and incubated at 37°C, in a shaking incubator (Heidolph UNIMAX 1010, Heidolph Instruments GmbH, Kelheim, Germany) overnight.

The cells were harvested by centrifugation at 4000 rpm for 15 minutes (IE Clinical Centrifuge). The pelleted bacterial cells were resuspended in 250 µl Cell Resuspension Solution (provided by the kit) and vortexed. The cells were transferred to sterile 1,5 ml eppendorph tubes and lysed by the addition of 250 µl Cell Lysis Solution (provided by the kit). The endonucleases, which degrades double-stranded DNA, were inactivated and other proteins were nonspecifically degraded by the addition of 10 µl Alkaline Protease Solution (provided by the kit) to reduce the overall level of protein contaminants in the cleared bacterial lysate. After the addition of 350 µl Neutralization Solution (provided by the kit), the tubes were immediately mixed by gently invertions, incubated for 5 minutes and the cell debris was pelleted by centrifugation at 13000 rpm for 10 minutes in Biofuge 15 Heraeus Spatech GmbH, Osterode am Harz, Germany. The supernatant containing the plasmid was transferred into Spin Column (provided with the kit), and centrifuged at 13000 rpm for 1 minute in order to bind the DNA to the column. The bound DNA was washed twice, with Wash Solution (provided by the kit), and then, DNA was eluted with Nuclease-Free Water (provided by the kit).

Finally 100 µl of plasmid sample, at a concentration of 1.5 mg/ml, was obtained from the starting material, and was stored at -20ºC until further use.

2.2.3.5.5. Restriction map of pDrive Cloning Vector

The pDrive Cloning Vector was supplied in a linear form with an U overhang at each end. The first base of the *Lac-Z* 5' upstream region is designated as nucleotide number one. Figure 2.1 shows restriction enzymes that cut the pDrive Cloning Vector in the Multiple Cloning Site (MCS).

2.2.3.5.6. Restriction Endonuclease Digestion of the Isolated Recombinant Plasmid DNA

Structural analysis of the cloned DNA was achieved through digestion with various restriction enzymes individually and in combinations. Thus existance of the cloned gene in the vector, and restriction map derived from nucleic acid sequence data were confirmed. Also, the orientation of the clone in the pDrive vector was determined.

Single or double digestions with the restriction enzymes, *Ava*II, *Eco*RI, *Hin*dIII and *Pst*I were performed following the instructions of the manufacturer. *Ava*II and *Eco*471 were isochisomers, which have the same recognition sequence but may or may not share the same cut site.

2.2.4. ATPase Activity Analyses of Recombinant Cells

2.2.4.1. Preperation of Cell Free Extracts of Both the Recombinant and Non-recombinant Cells

Recombinant *E.coli* cells (*E.coli* pDrive1-6 and *E.coli*, pDrive2-3), harboring the pDrive-26S Proteasome Regulatory Subunit and non-recombinant *E. coli* cells (TG1-15 and TG1-13), lacking the recombinant plasmid, were grown in 10 ml LB broth medium supplemented with Ampicillin (50 µg/ml) through incubation at 37°C overnight. Aliquotes of the cultures (2 ml) were transferred into 300 ml of fresh LB broth, supplemented with ampicillin (50 µg/ml), and incubated in a shaking incubator (Heidolph UNIMAX1010, Heidolph Instruments GmbH, Kelheim, Germany) at 160 rpm and 37°C, overnight. The cells were harvested by

centrifugation (Sigma 3K30 Centrifuge, Sigma Chemical Co., St. Louis, MO, USA) at 8000 rpm, for 15 minutes, at 4°C and washed with 50 ml of Tris buffer (50 mM Tris, 100 mM NaCl). The pellet was dissolved in 15 ml Tris buffer supplemented with ATP and β-mercaptoetanol (50 mM Tris, 100 mM NaCl, pH8.0, 5 mM β-mercaptoetanol, 5 mM $MgCl_2$, 1 mM ATP). The cells were disrupted by ultrasonication (Sonicator VC100, Sonics and Materials, CT, USA). The sonicate was centrifuged at 12000 rpm for 2 hours at 4°C (Sigma 3K30 Centrifuge, Sigma Chemical Co., St. Louis, MO, USA) and the half of the supernatant was stored at -20°C until use. The second half of the supernatant was incubated at 60°C water-bath for 15 minutes and cenrtifuged at 12000 rpm, 4°C for 1,5 hours. The supernatant (heat treated cell free extracts) was kept at -20°C until use.

2.2.4.2. ATPase Activity Assay

ATPase activity was assayed by measuring the release of P_i (inorganic phosphate) from the hydrolysis of ATP, by the modified method of Lanzetta (*168*). The Cell Free Extract (20 µl) and ATPase Assay Buffer (30 µl) were incubated at 40°C for 40 minutes. Then, as the color reagent 800 µl Sodium Molybdate Malachite Green Reagent was added into the tubes containing enzyme and buffer mixtures. Subsequently, 100 µl Citric Acid (34 %, v/v) was added and mixed by inverting the tubes a few times. The citric acid was used as a quencher and the reaction stopped immediately upon its addition. So, the colour of the solution was stabilized. The tubes were incubated at room temperature for 40 minutes and the absorbance was read by using the Shimadzu UV-1601A double beam spectrophotometer at 660 nm (Shimadzu Analytical Co., Scientific Instruments Division, Kyoto, Japan). Duplicate reactions were performed in parallel.

The amount of P_i released was calculated from the standard curve and the equation $y = 0{,}0832\ x + 0{,}1055$.

2.2.4.3. Time Dependent Measurement of ATPase Activity

Five different reaction mixtures were prepared, using heat-treated cell-free extract of *E. coli* p-Drive1-6, which were incubated at 40°C for 0, 5, 10, 15, 20, 25 and 30 minutes. The assays were run as described before and time course of absorbance readings were recorded at 660nm.

2.2.4.4. Standard Curve for Inorganic Phosphate Determination

The reactions containing a series of different amounts of inorganic phosphate, P_i (1/50-20/50 nmloes) were prepared using 1mM KH_2PO_4 stock solution and treated as described in the ATPase assay procedure. Standard curve for the inorganic phosphate determination was prepared by using the same ATPase assay buffer (with no ATP). The absorbance of each reaction was measured at 660 nm (Shimadzu Analytical Co., Scientific Instruments Division, Kyoto, Japan) after incubation at room temperature for 40 min.

2.2.5. Sequence Data Search and Analyses

The putative nucleotide and amino acid sequences of *Thermoplasma volcanium* GSS1, "26S Proteasome Regulatory Subunit" were obtained from the NCBI (National Center for Biotechnology Information) Database. (BAB59528 database with TVG0373521 locus tag and NP_110901 database with TVN0382 locus tag.)

The multiple sequence alignment of "26S proteasome regulatory subunit" based on amino acid sequences were achieved by using CLUSTAL W (1.82) multiple sequence alignment program in EMBL-EBI (European Molecular Biology Laboratories-European Bioinformatics Institute) web site database.

CHAPTER III

RESULTS

3.1. Biochemical Analyses

3.1.1. Effects of Different Temperatures on Enzyme Activity and Stability

In order to find out the optimum temperature for the chymotripsin-like activity of the recombinant *Tp. volcanium* 20S proteasome, the activities were measured at various temperatures as described before, between 35°C and 98°C. The remaining enzyme activities were expressed as the percentage of the maximum activity (relative activity) (Figure 3.1). Enzyme activity was increased as the temperature of preincubation increased, up to 85°C. The optimum temperature for the maximum proteolytic activity was determined as 85°C. Enzyme activity was increased about ten-fold, when the assay temperature was increased from 35°C to 85°C.

For the stability assays, enzyme samples were incubated between 35°C and 98°C for 30 minutes, and then assays were run at 55°C as desribed before. Full activity was retained after incubation at 80°C and 85°C for 30 minutes (Figure 3.2). The 64% of the activity was retained after heat treatment at 98°C for 30 mins.

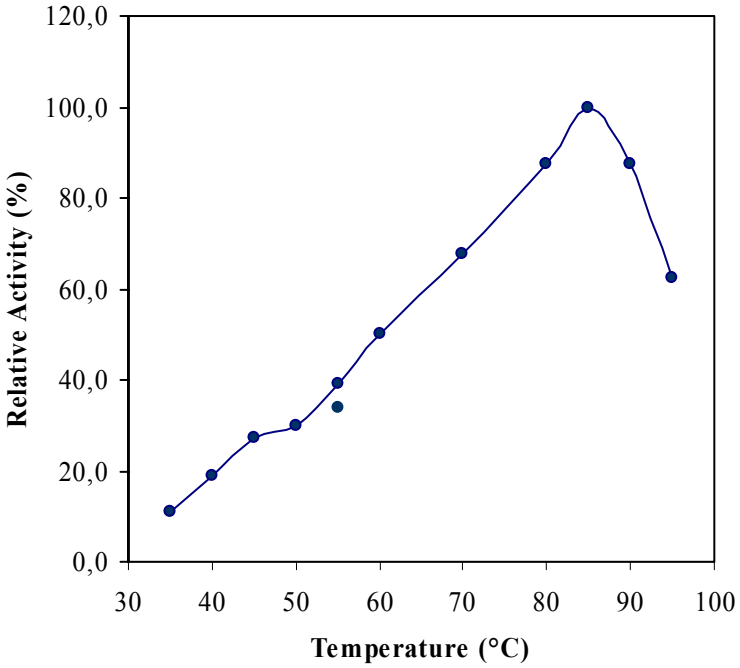

Figure 3.1 Effect of temperature on activity of recombinant *Tp. volcanium* 20S Proteasome.

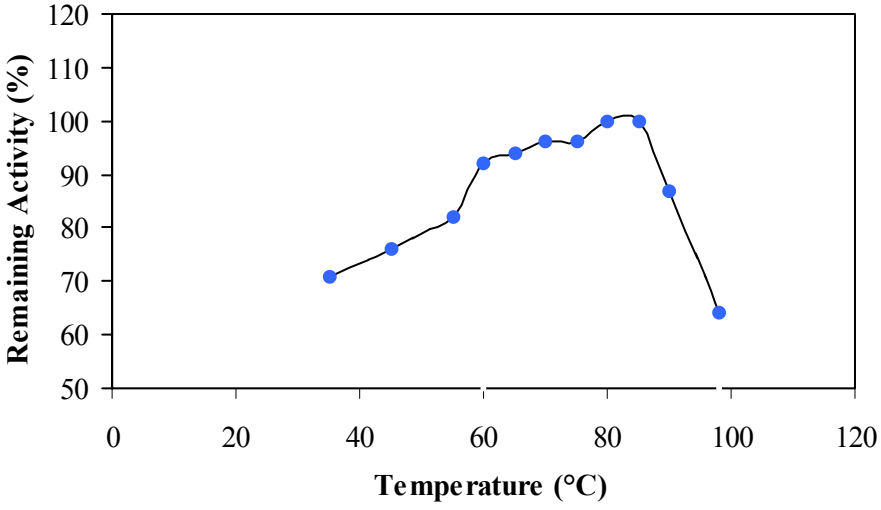

Figure 3.2 Effect of temperature on stability of recombinant *Tp. volcanium* 20S Proteasome.

3.1.2. Substrate Profile of *Tp. volcanium* 20S Proteasome Enzyme

The substrate profile of 20S proteasome was assayed in order to determine peptide substrate specificity of the enzyme complex. The assays were as described in Materials and Methods. Our results showed that the proteasome has the highest activity on the oligopeptide substrate, N-CBZ-leu-leu-glu-βNA, which is a typical ubstrate for peptidylglutamyl peptidase activity. The 20S proteasome of *Tp. volcanium* displayed second highest enzymatic activity against Ala-ala-phe-pNA, which is a specific substrate for chymotrypsin-like activity. But relatively low activity against N-CBZ-Arg-pNA, which is a typical substrate for trypsin activity was observed. The activities were expressed relative to that of N-CBZ-leu-leu-glu-βNA, which displayed the highest activity (Table 3.1).

Table 3.1 Substrate profile of *Tp. volcanium* 20S Proteasome.

Substrates	**Relative Activity (%)**
N-CBZ-leu-leu-glu-βNA	100
Ala-ala-phe-pNA	88
L-leu-pNA	52
N-Suc-Ala-ala-pro-phe-pNA	39
Ala-ala-ala-pNA	35
N-Suc-phe-pNA	28
N-CBZ-Arg-pNA	27
Ala-ala-val-ala-pNA	21

3.1.3. Effects of pH on 20S Proteasome Enzyme Activity

The enzymatic activity was measured to determine the optimum pH of the enzyme using Ala-ala-phe-pNA as substrate, at different pH values under the same assay conditions. The buffer systems used were Tris-acetate buffer (pH 4.0-5.5), Tris-phosphate buffer (pH 5.7-8.0) and Tris-glycine buffer (pH 8.3-9.5). The optimum pH for the activity was found to be pH 8.7 (Figure 3.3). The highest chymotrypsin-like activity was observed over an alkaline pH range and between pH 7.0 and pH 9.0 enzyme displayed 50% to 100% of the maximum activity. On the other hand, more than 50% of the activity was retained at pH 7.0.

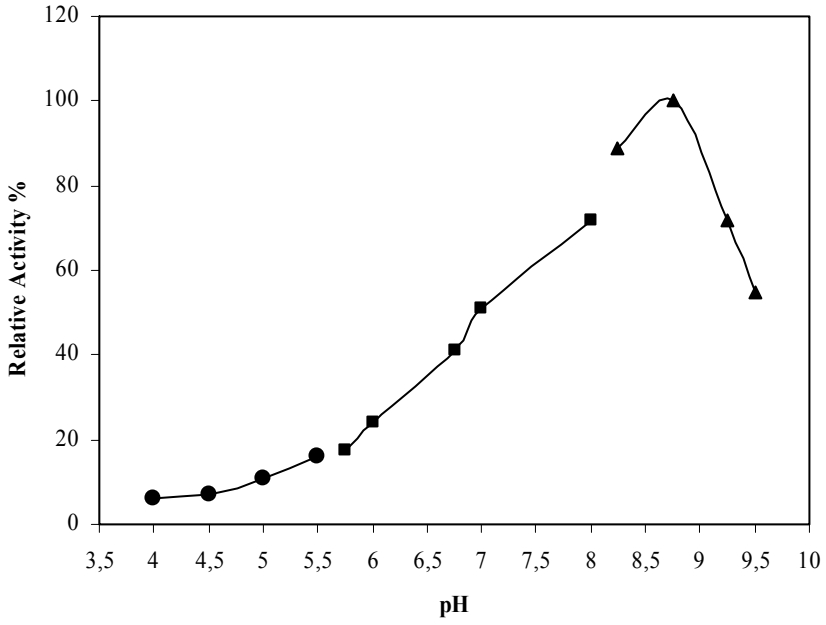

Figure 3.3 Effect of pH on activity of 20S Proteasome from *Tp. volcanium*. The protease was assayed over a range of pH 4,0 - pH 9,5.

Acetate buffer (● , pH 4,0 – 5,5) , Phosphate buffer (■ , pH 5.7 – 8,0), Glycine buffer (▲, pH 8,3 – 9,5).

3.1.4. Effects of Various Metal Ions and Other Effectors on 20S Proteasome Activity

An anionic detergent, SDS at different concentrations was tested as described in the Materials and Methods. In the presence of 3 μM SDS 20S proteasome activity has increased 300 fold while there was no detectable change up to 10 μM concentration (Figure 3.4).

Effects of various molecules such as $MgCl_2$, $MnCl_2$, Cysteine, ATP, $CaCl_2$, NaCl, KCl, EDTA, Guanidine HCl and SDS on chymotrypsin-like enzyme activity of recombinant 20S proteasome has been shown on the Figure 3.5. The enzyme activity was not significantly effected by Mn^{+2}, Ca^{+2}, K^{+} and EDTA, while Mg^{+2}, Cysteine, ATP, Na^{+2}, SDS and GdmCl reduced activity by ≥55% as compared to control.

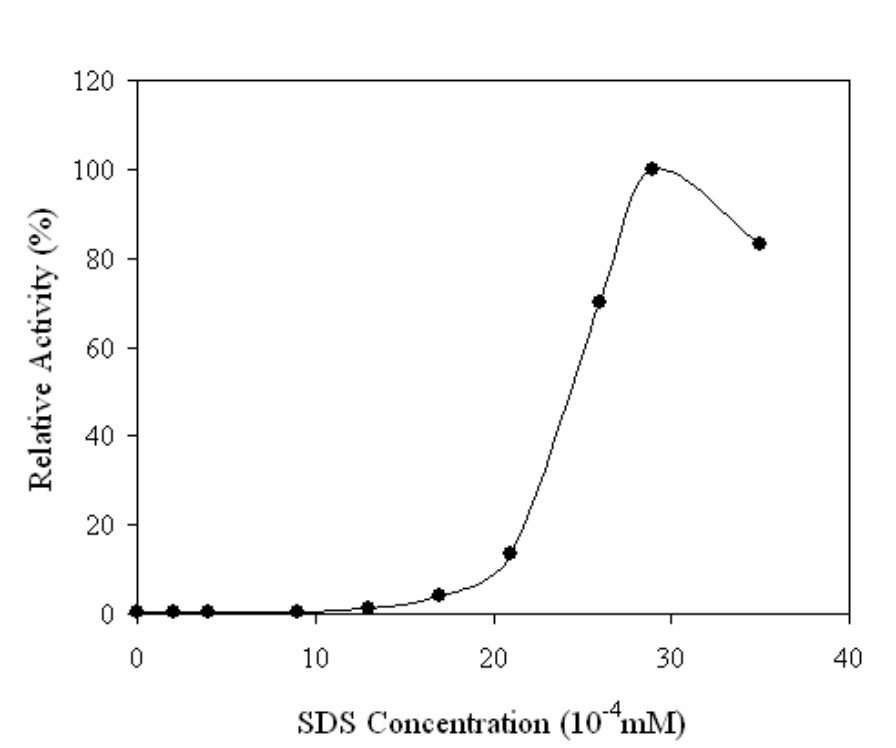

Figure 3.4 Effect of SDS on the activity of *Tp. volcanium* 20S proteasome.

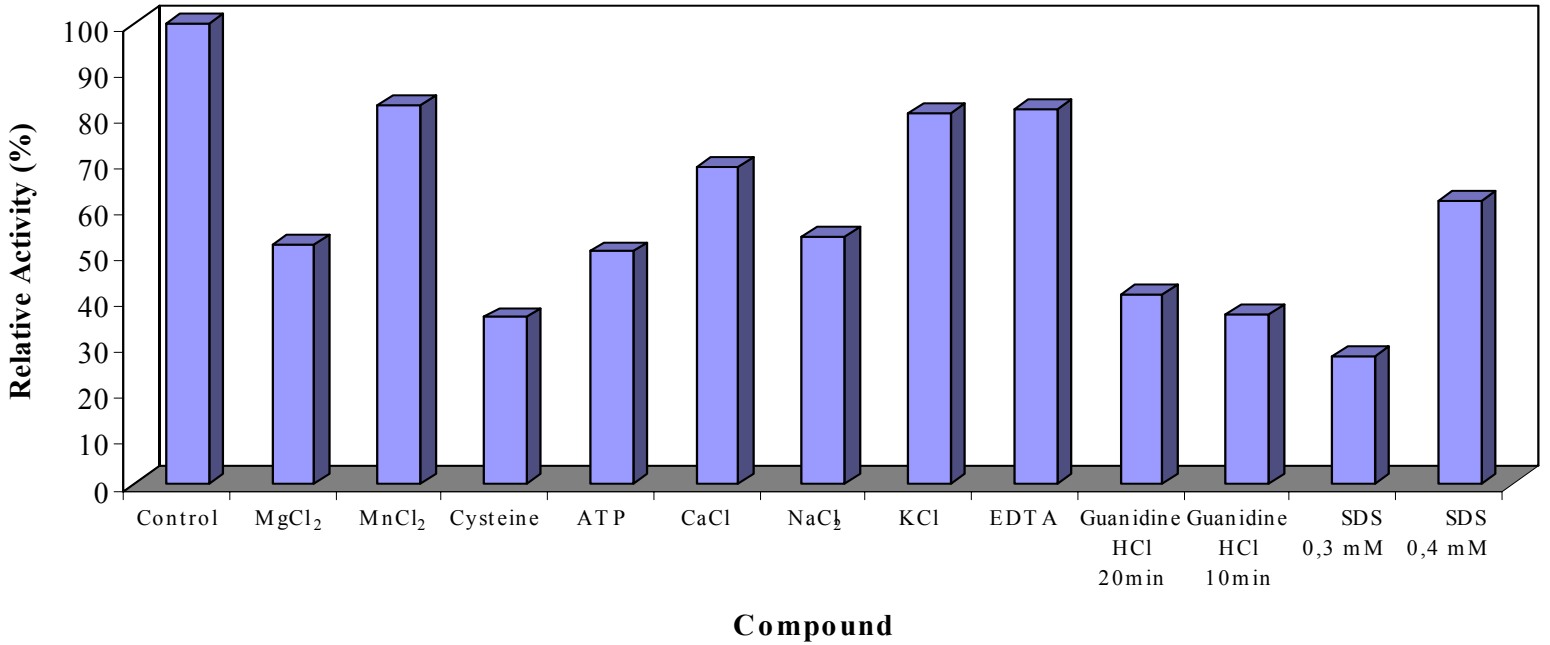

Figure 3.5 The effects of various compounds on activity has been shown on the graph. Control, $MgCl_2$, $MnCl_2$, Cysteine, ATP, $CaCl_2$, NaCl, KCl, EDTA, GdmCl (20 min), GdmCl (10 min), SDS (0,3 mM), SDS (0,4 mM)

3.2. Electrophoretic Analyses

3.2.1. Native Polyacrylamide Gel Electrophoresis

Native gel electrophoresis was performed in order to visualize the purified 20S proteasome complex using ion-exchange chromatography in a non-denatured form on the polyacrylamide gel. Single unique band observed on 5% polyacrylamide gel after staining indicating the homogenity of the proteasome sample.

Single protein band possibly representing 20S Proteasome complex was observed at the same position as α-macroglobulin (standard size marker) (340 kDa). This result shows that *Tp. volcanium* 20S proteasome was purified to homogenity by ion-exchange chromatography (Figure 3.6)

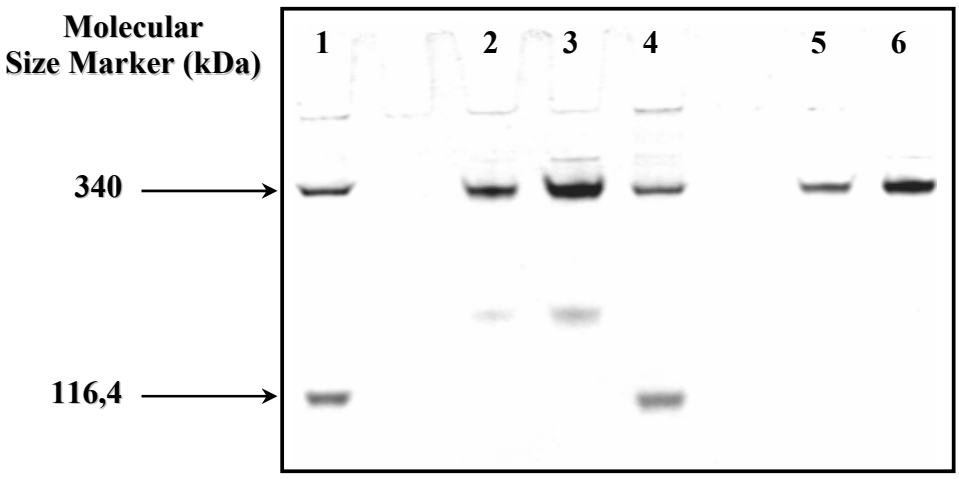

Figure 3.6 The native gel photograph of *Thermoplasma volcanium* recombinant 20S proteasome. Lane 1 and 4, protein molecular size marker (α_2 macroglobulin: 340 kDa; β-galactosidase: 116,4 kDa), lane 2 and 3, gel filtration samples, lane 5, ion exchange chromatography, lane 6, protein molecular size marker (α_2 macroglobulin, 340 kDa).

3.2.2. SDS Polyacrylamide Gel Electrophoresis

In order to determine the subunit composition and molecular weight, purified samples of *Tp. volcanium* 20S proteasome were run on SDS-polyacrylamide gel (12,5 %). The electrophoresis revelaed that 20S proteasome is composed of two subunits, *Tp.v*-α subunit and *Tp.v*-β subunit, with estimated molecular sizes of 24 kDa and 23 kDa, respectively (Figure 3.7). Figure 3.8 shows the standard curve used in determination of molecular sizes of *Tp.v*-α and *Tp.v*-β subunits.

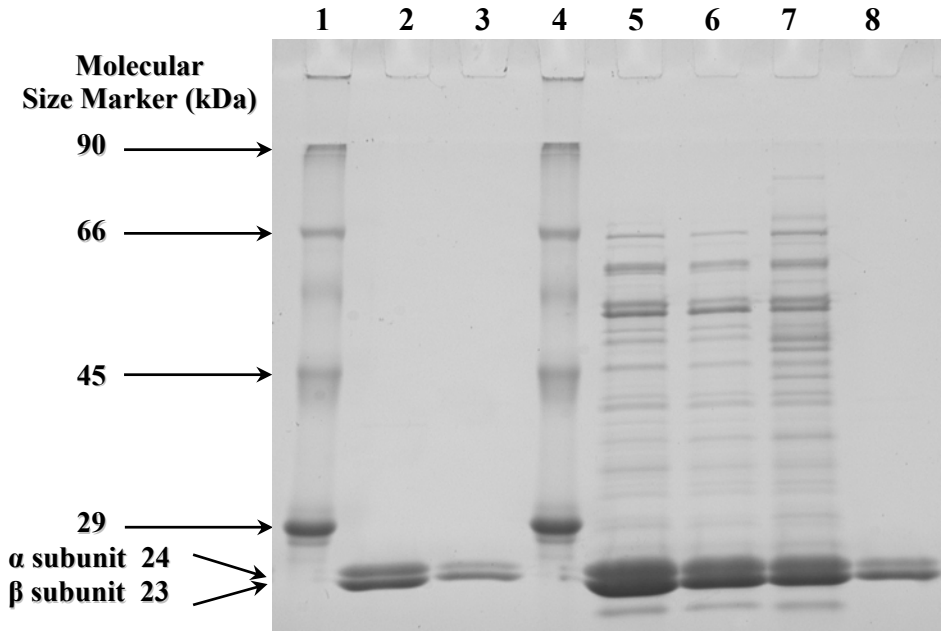

Figure 3.7 SDS-PAGE of the *Tp. volcanium* 20S Proteasome. Lane 1, Protein molecular size marker (Carbonic Anhydrase: 29 kDa, Egg Albumin: 45 kDa, Bovine Albumin: 66 kDa and Urease: 90 kDa); lanes 2, 3 and 8, samples of 20S proteasome after ion-exchange chromatography, lanes 5, 6 and 7, 20S proteasome samples after gel filtration chramatography.

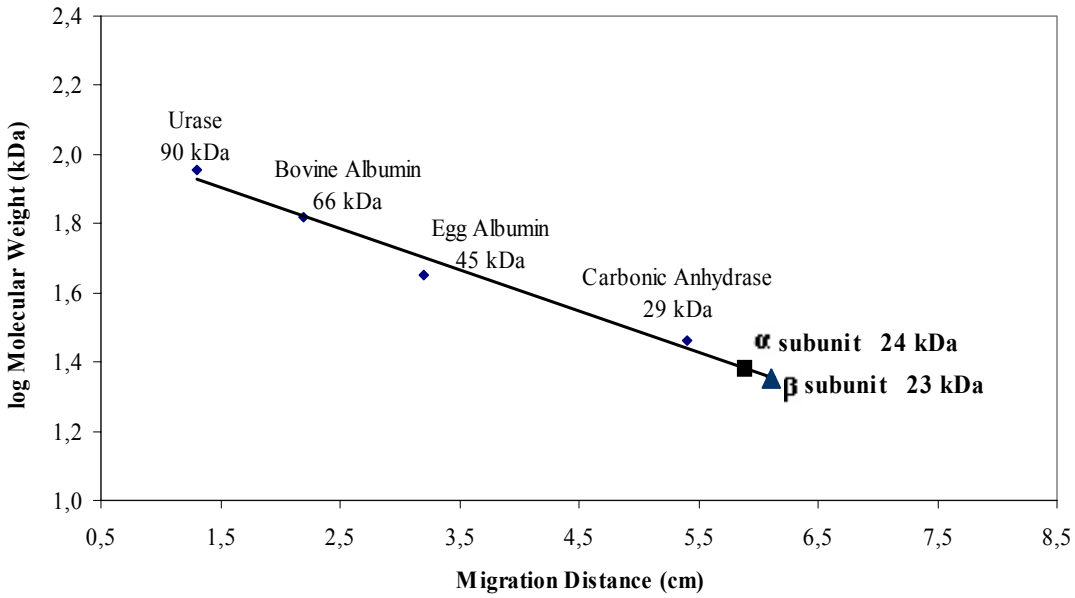

Figure 3.8 Standard Curve for determination of apparent molecular sizes of α and β subunits of *Tp. volcanium* 20S Proteasome .
(■ ; 23,87 kDa α subunit, ▲; 22,02 kDa β subunit)

3.3. Gene Manipulation Analyses

3.3.1. Isolation, Digestion and Visualisation of *Thermoplasma volcanium* Genomic DNA

The total genomic DNA of *Tp. volcanium* was isolated and digested with *Eco*RI restriction endonuclease and visualized by agarose gel electrophoresis, as described in the Materials and Methods. The spectrophotometric analysis (OD_{260}/OD_{280} = 1,8) and restriction digestion results (Figure 3.9) indicated that the purified *Tp. volcanium* genomic DNA was pure enough to be used in genetic manipulations.

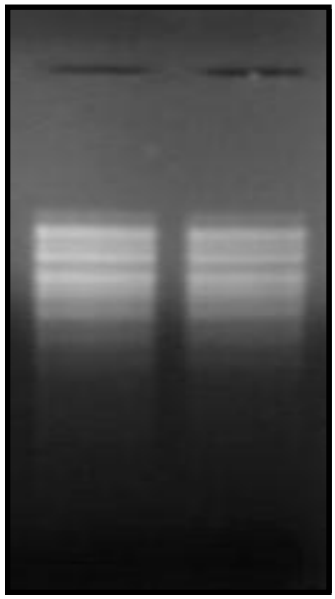

Figure 3.9 Digestion of purified Genomic DNA of *Tp. volcanium* with *Eco*RI.

3.3.2. Amplification by PCR and Cloning of PCR Fragment

3.3.2.1. Amplification of 26S Proteasome Regulatory Gene by PCR

Purified *Tp. volcanium* genomic DNA was used as template for amplification of 26S Proteasome Regulatory Subunit gene (TVN0382) using FPg and RPg amplification primer set by PCR. PCR reactions were carried out at different hybridization temperatures between 40°C and 60°C. The amplications at ≥ 50°C hybridization temperatures all yielded a single unique band on agarose gel (Figure 3.10). The PCR product obtained at 60°C hybridization temperature was cloned.

The size of the amplified PCR fragment was estimated as 1419bp (Figure 3.10). The calibration curve used for calculation of the molecular size of the PCR amplicon is shown in Figure 3.11. This fragment is composed of a 1128 bp open reading frame corresponding to 26S Proteasome Regulatory Subunit structural gene sequence and, a 213 bp upstream (including the promoter) and a 78 bp downstream, untranslated regions.

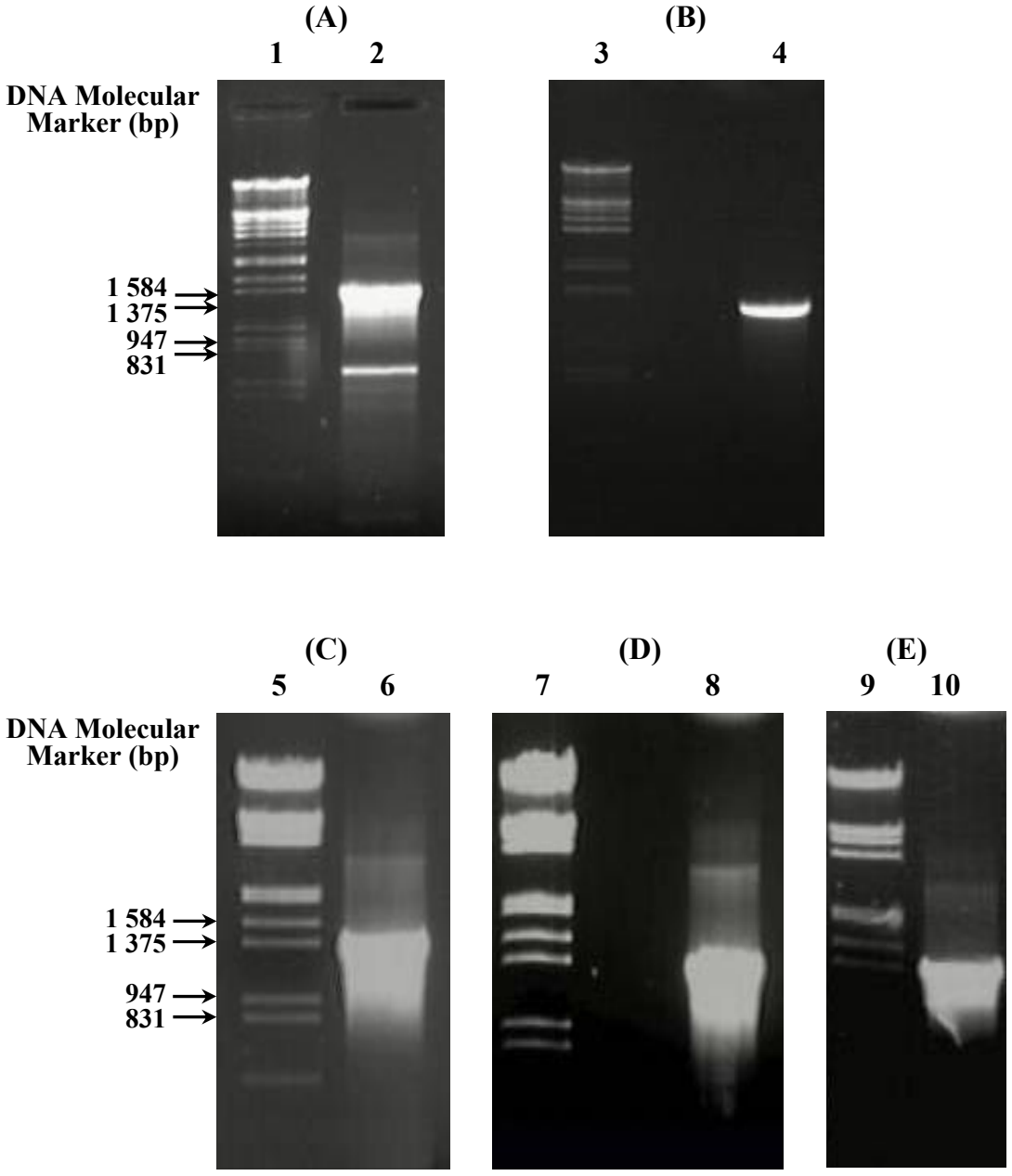

Figure 3.10 The PCR amplification of 26S Proteasome Regulatory Subunit Gene of *Tp. volcanium* at various annealing temperatures. PCR performed at 40 ^{o}C (A), at 50 ^{o}C (B), at 55 ^{o}C (C), at 57 ^{o}C (D) and at 60 ^{o}C (E) yielded single unique band of 1419 bp length. Lanes 1,3,5,7,9; molecular size markers, *Eco*RI+*Hind*III cut Lambda DNA (MBI Fermentas AB, Vilnius, Lithuania)

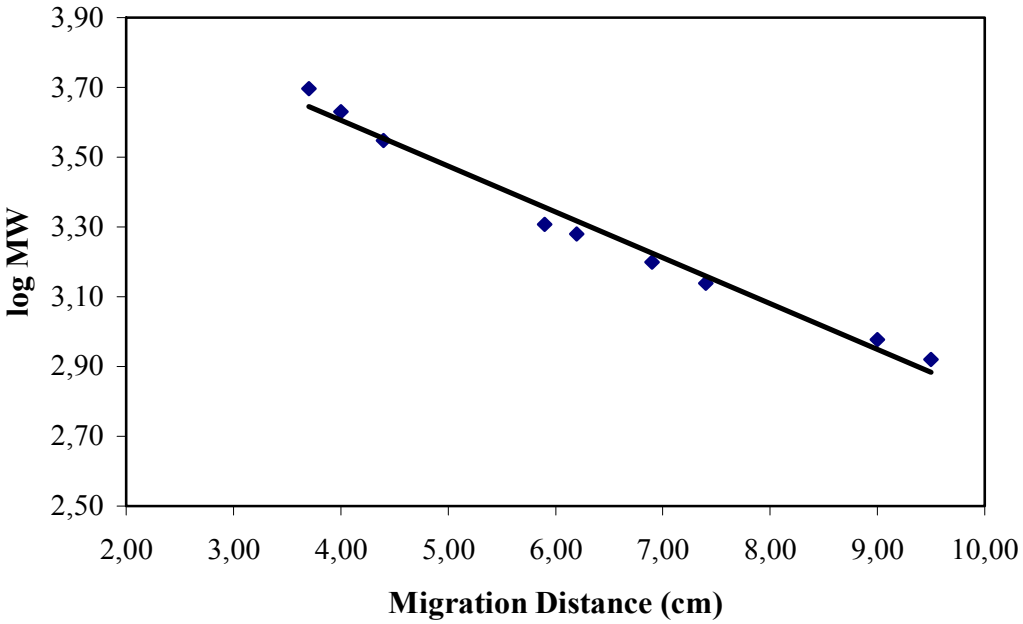

Figure 3.11 Calibration curve for DNA molecular size marker, *Eco*RI and *Hind*III cut Lambda DNA (MBI Fermentas AB, Vilnus, Lithuania)

3.3.2.2. Cloning of PCR Amplified Fragment

The 1419 bp, PCR amplified gene fragment was ligated into the pDrive cloning vector as illustrated in the Figure 3.12 and cloned by using Qiagen PCR Cloning Kit (Qiagen Inc., Valencia, USA). Qiagen PCR Cloning Kit is highly efficient in cloning of PCR products via UA (uracil-adenin) hybridization based ligation. The PCR amplified fragments have A overhangs at each 3'-ends which are generated by *Taq* Polymerase and other non-proofreading DNA polymerases. These enzymes add a non template specified adenine (A) residues to 3'-termini of both strands of amplicons, which can be made use of in cloning. The pDrive cloning vector was available in linear form with a U overhang at each 3'-end in the Kit and hybridized with the single A overhang at each end of PCR products in the ligation mixture with high specificity. The competent cells were treated with

the ligation mixture containing the recombinant vectors as described in Materials and Methods for transformation.

The pDrive cloning vector allows ampicillin and kanamycin selection as well as blue/white colony screening. Thus, the transformed colonies were distinguished through blue/white screening. The transformation efficiency (based on blue+white colonies) and recombination efficiency (based on white colonies) were calculated as $2x10^3$ transformants/µg DNA and $1x10^3$ recombinants/µg DNA, respectively. Total 21 white colonies (putative recombinant clones) were obtained. The plasmids were isolated from 10 white colonies (Figure 3.13) and plasmids from 3 of which were proved to be recombinants (*E.coli* pD1-6, *E.coli* pD2-3, *E.coli* pD3-1).

*Eco*RI digestion of these recombinant plasmids *E.coli* pD1-6, *E.coli* pD2-3, *E.coli* pD3-1, and *E.coli* pD3-4 released 1419 bp cloned gene fragment from the vectors (Figure 3.14).

3.3.2.3. Restriction Map of *Tp. volcanium* 26S Proteasome Regulatory Subunit Gene (TVN038)

The restriction maps of the cloned 26S proteasome regulatory subunit structural gene (1128 bp) itself and together with 3' and 5' untranslated sequences (1419 bp) were prepared using "Restriction Mapper" and "New England Biolabs NEBcutter" programs (Figure 3.15). Table 3.2 and Table 3.3 also lists the major restriction enzymes that cut and do not cut the cloned 26S proteasome regulatory subunit fragment.

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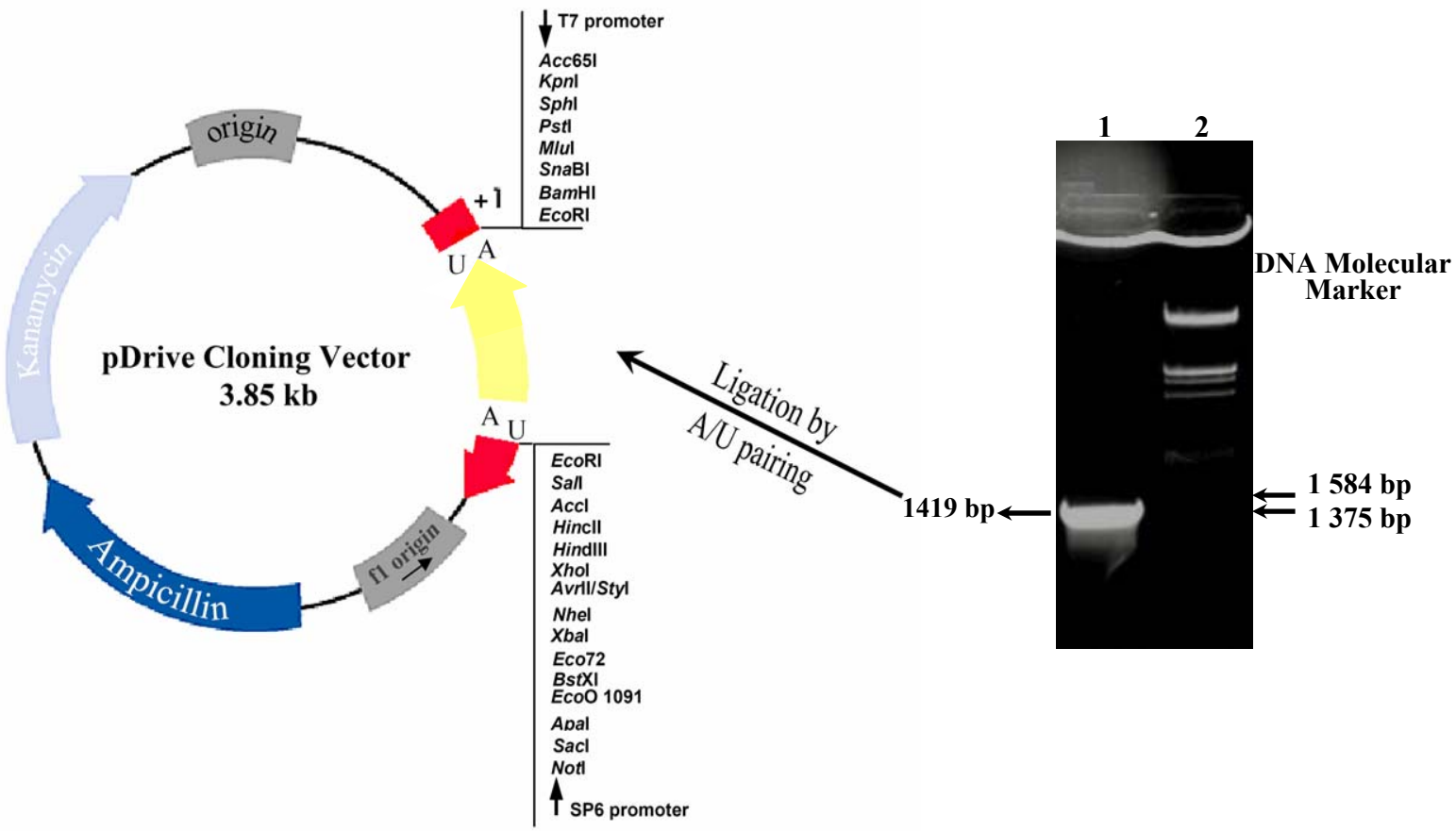


Figure 3.12 The schematic representation of cloning of 26S Proteasome Regulatory Subunit into pDrive Cloning Vector. Lane 1, 1419 bp PCR amplified 26S Proteasome Regulatory Subunit gene fragment, and lane 2, Lambda DNA/*Eco*RI+*Hind*III cut, molecular size marker.

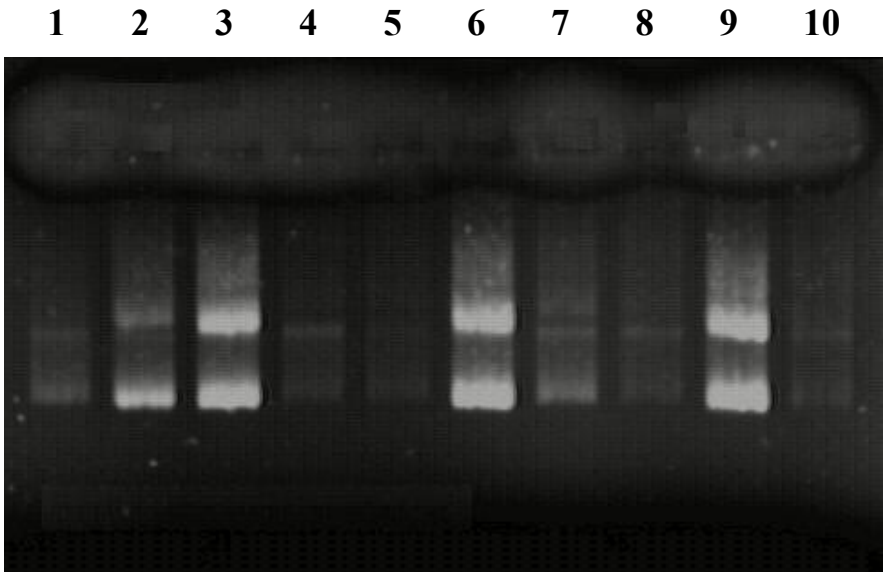


Figure 3. 13 Plasmids isolated from 10 putative transformed colonies containing recombinant plasmid vector. Lane 1: *E.coli* pD3-5, lane 2: *E.coli* pD3-4, lane 3: *E.coli* pD3-1, lane 4: *E.coli* pD2-7, lane 5: *E.coli* pD2-5, lane 6: *E.coli* pD2-3, lane 7: *E.coli* pD2-1, lane 8: *E.coli* pD1-7, lane 9: *E.coli* pD1-6, lane 10: *E.coli* pD1-2.

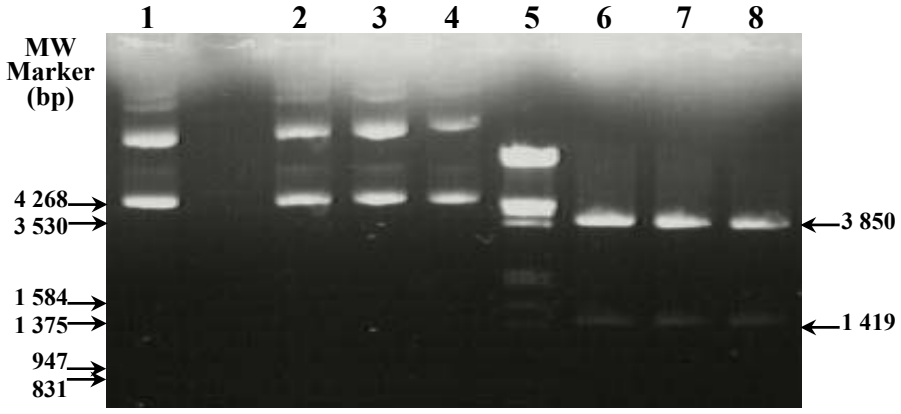


Figure 3.14 *Eco*RI digestion released 1419 bp cloned gene fragment from the recombinant vectors. Lane1; *E.coli* pD1-6-U, lane 2: *E.coli* pD2-3-U, lane 3: *E.coli* pD3-1-U, lane 4: *E.coli* pD3-4-U, lane 5: Marker, lane 6: *E.coli* pD1-6-D, lane 7: *E.coli* pD2-3-D, lane 8: *E.coli* pD3-1-D. U: undigested, D: digested.

Table 3.2 List of Restriction Endonucleases which cut cloned sequence containing 26S Proteasome Regulatory Subunit gene.

Cutter Restriction Endonucleases	Cut Positions
*Ava*II	667
*Hind*III	150, 796
*Hph*I	496, 1230
*Xba*I	558
*Acc*I	1121
*Bc*II	387

Table 3.3 List of Restriction Endonucleases which do not cut cloned sequence containing 26S Proteasome Regulatory Subunit gene.

Non- Cutter Restriction Endonucleases
*Bam*HI
*Bg*lII
*Eco*RI
*Eco*RV
*Hind*II
*Hpa*I
*Pst*I
*Pvu*II
*Sal*I
*Smu*I
*Sph*I

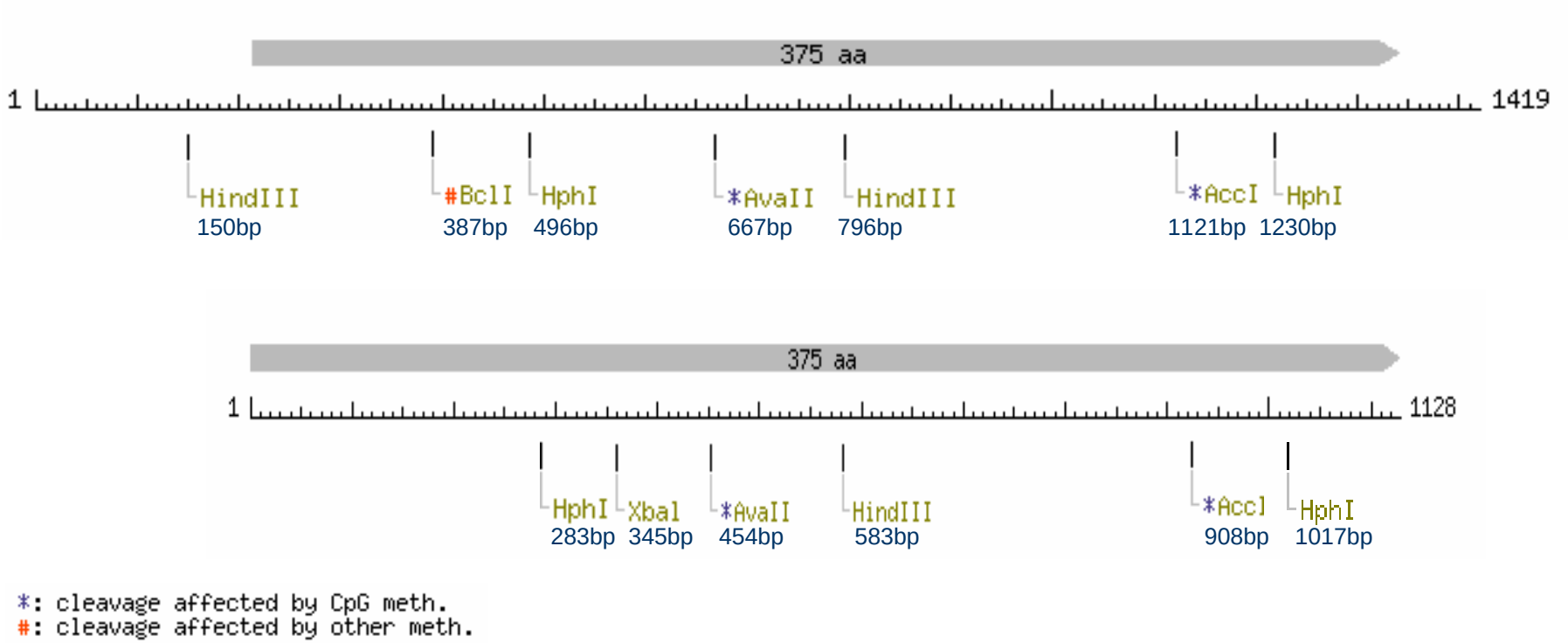


Figure 3.15 Restriction map of 26S Proteasome Regulatory Subunit; Restriction sites and their cut positions in *Tp. volcanium* 26S proteasome regulatory subunit gene which were determined by New England Biolabs NEBcutter program (http://tools.neb.com/NEBcutter2/index.php).

3.3.2.4. Characterization of Recombinant Plasmid Containing 26S Proteasome Regulatory Subunit Gene

Restriction enzyme digestion was performed to control whether the cloned 26S proteasome regulatory subunit fragment is inserted into the plasmid or not and to determine the direction of the inserted fragment on the plasmid. Also, single and double restriction digestions were performed to find out the restriction map of the cloned fragment experimentally.

In order to monitor the presence of insert in the 10 putative recombinant plasmids, which were isolated form white colonies, restriction digestion with *Eco*RI was performed. Closer to the open ends of pDrive vector there are two *Eco*RI sites (1 site per end).

Two HindIII sites are located at positions 150 bp and 796 bp on the cloned PCR fragment. There is also one *Hind*III site on the vector's MCS. *Hind*III digestion of the two recombinant plasmids produced DNA fragments of 177 bp, 646 bp and 4447 bp sizes (Figure 3.16). On the other hand, cloned fragment does have not any *Pst*I site, while there is one on the MCS. *Hind*III/*Pst*I double digestion yielded 177 bp, 646 bp, 66 bp and 3801 bp (vector DNA) digestion fragments (Figure 3.16).

There is one site for *Ava*II restriction enzyme on the cloned fragment at position 667 bp and two sites on the vector's MCS. Digestion with *Ava*II yields 3 DNA fragments: 2307 bp, 1944 bp and 222 bp (Figure 3.16) Thus, the restriction map derived from the sequence of the cloned 26S Proteasome Regulatory Subunit gene fragment using Restriction Mapper Program (Figure 3.17) was confirmed by experimental data. Restriction map analysis revealed that in both recombinant plasmids (*E.coli,* pDrive1-6, *E.coli,* pDrive2-3 and *E.coli,* pDrive3-1) the cloned gene fragment was inserted into cloning vector in opposite direction with respect to the *Lac Z*' gene.

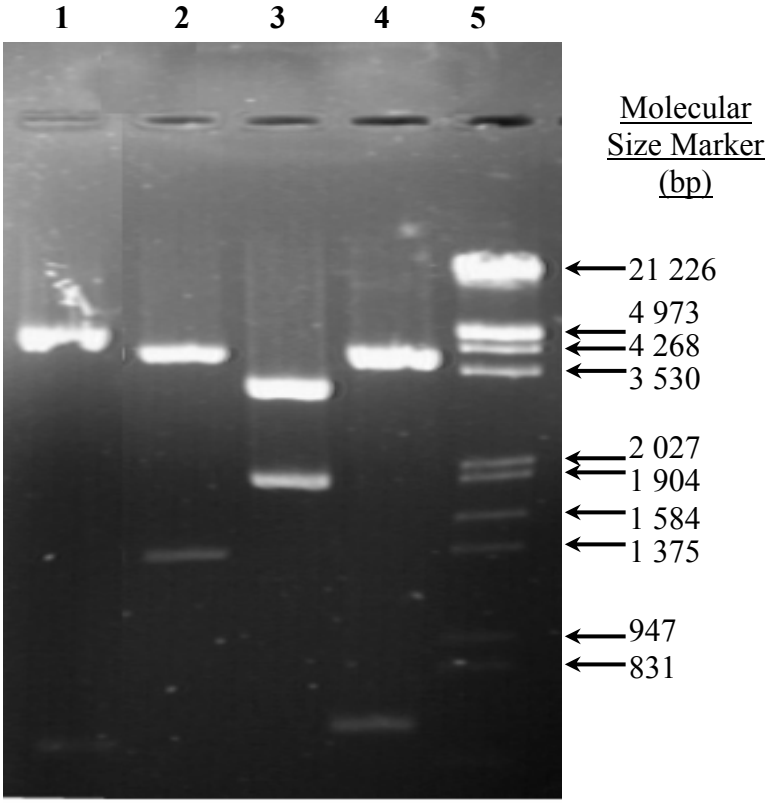


Figure 3.16 Restriction analysis of the pDrive Cloning Vector. pDrive Cloning Vector was subjected to single digestions with *Hin*dIII (lane1), *Eco*RI (lane 2) and AvaII (lane3). pDrive Cloning Vector was subjected to double digestion with *Hin*dIII-*Pst*I (lane4). Marker (lane5) Lambda DNA/*Eco*RI+*Hin*dIII, molecular size marker.

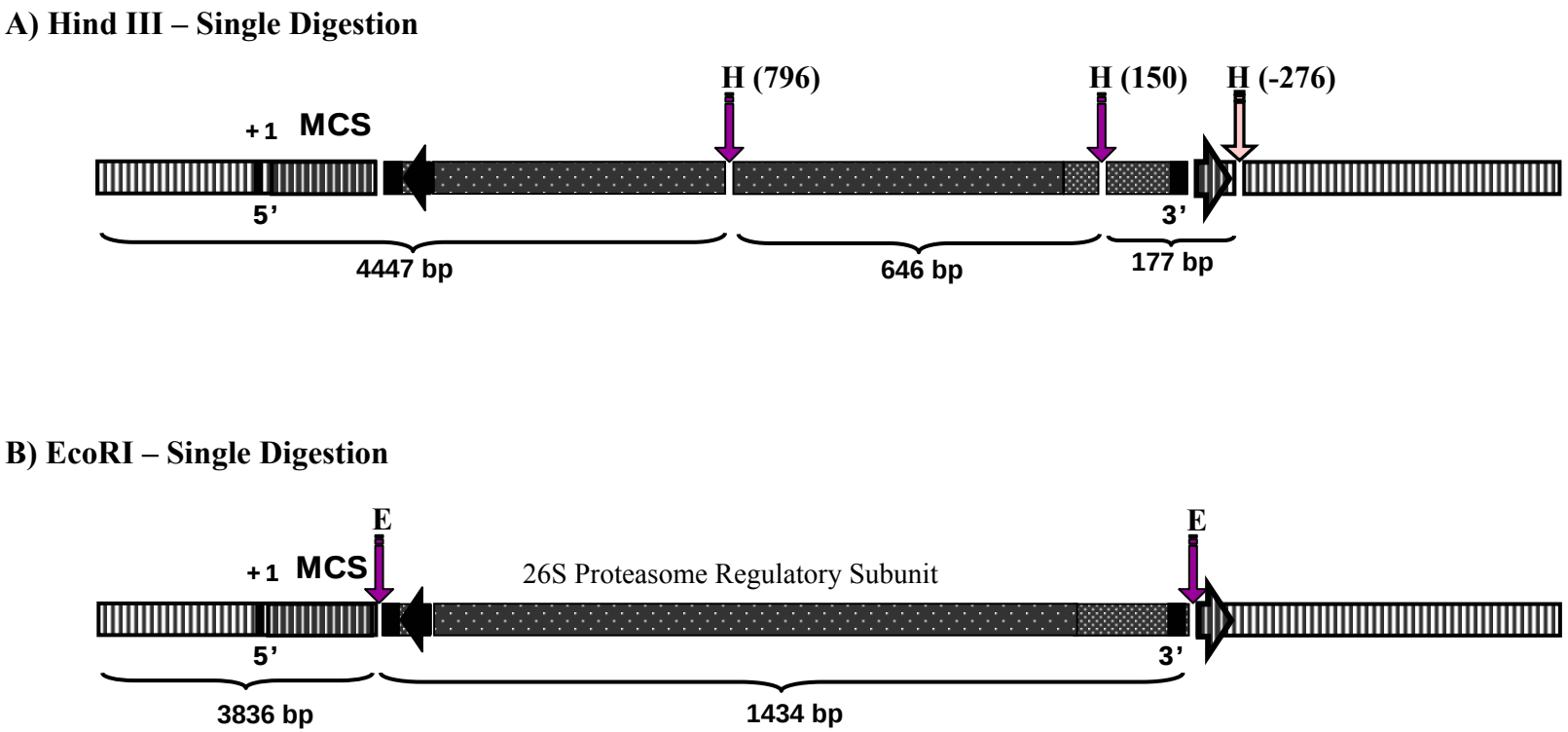
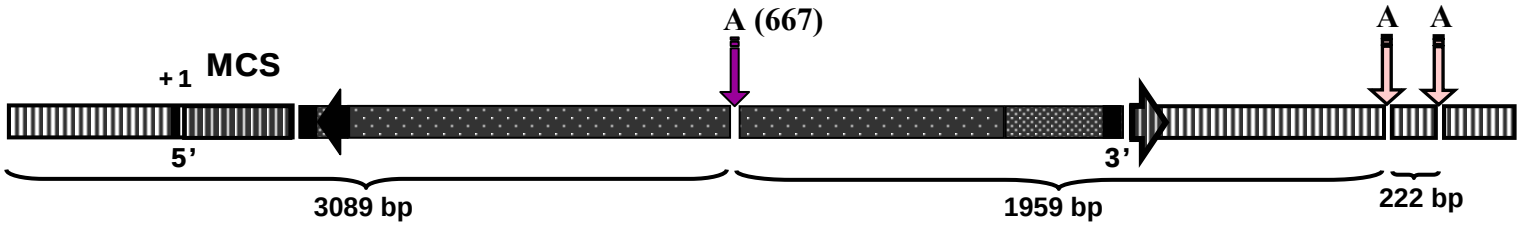


Figure 3.17 Restriction map of the recombinant pDrive vector containing the 26S Proteasome Regulatory Subunit gene. : pDrive Cloning Vector, : Multiple Cloning site (MCS), : 26S Proteasome Regulatory Subunit, : Amplified Region for Primers. A:*Ava*II, E:*Eco*RI, H:*Hin*dIII, P: *Pst*I

D) Ava II – Single Digestion



C) Hind III / PstI – Double Digestion

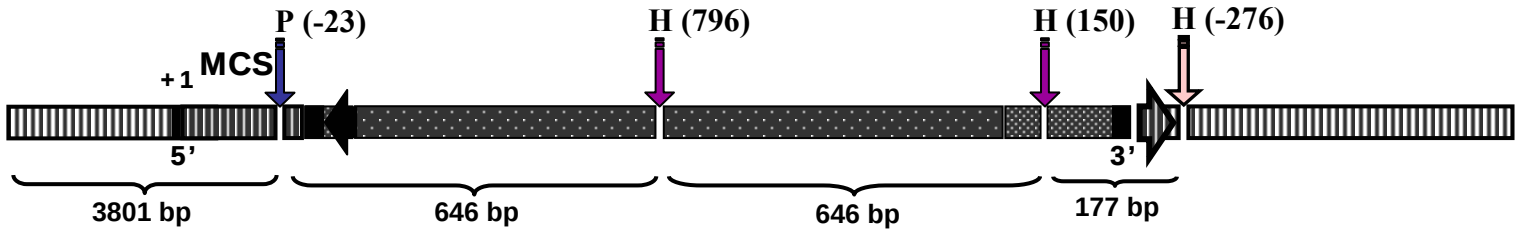


Figure 3.17 Continued.

3.4. ATPase Activity Analyses

3.4.1. ATPase Activity Assay

ATPase activities of two recombinant cells (*E.coli* pDrive 1-6 and *E.coli* pDrive 2-3) in cells free extracts were determined and the results were given in Table 3.4. The activities in cell free extracts of the recombinant strains were 10 times higher than non-recombinant *E.coli* strain TG-1. After heat-treatment the activities were higher as compared to activities measured before-heat treatment of the cell free extracts.

Table 3.4 ATPase activities of the cell free extracts from recombinant and wild-type strains.

Cell Free Extracts	**A_{660}**	**P_i released (pmoles/µl)**
BH *E.coli* pDrive 1-6	1,068	11,6
BH *E.coli* pDrive 2-3	0,728	7,5
AH *E.coli* pDrive 1-6	1,385	15,4
AH *E.coli* pDrive 2-3	1,345	14,9
AH *E.coli* TG-1 (Control)	0,138	0,4

* Results are average of two readings.

3.4.2. Time Dependent Measurement of ATPase Activity

For time-dependent assay of the ATPase activity the cell free extract of *E.coli* pDrive 1-6 cells was used as enzyme source (Figure 3.18). The activity of the enzyme sample was 0,1097 Abs_{660}/min or 0,1 P_i/min.

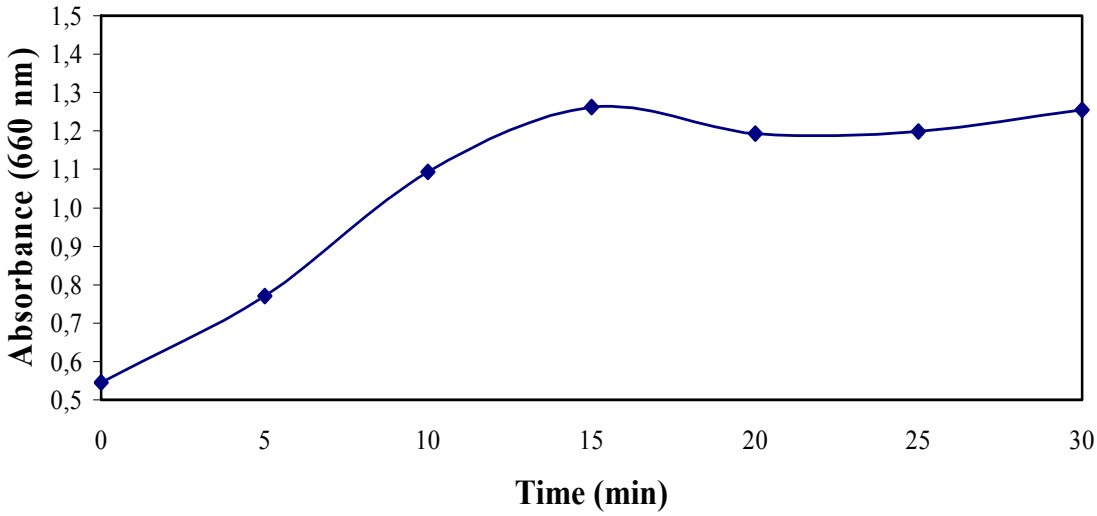


Figure 3.18 Measurement of time dependent ATPase activity in the cell free extract of *E.coli* pDrive 1-6 cells.

3.4.3. Standard Curve for Inorganic Phosphate Determination

Standard curve graph was plotted with the relased inorganic phosphate (P_i) measurements at 660 nm absorbance in the spectrophotometer (U2800 Spectrophotometer, DigiLab, Hitachi) (Figure 3.19).

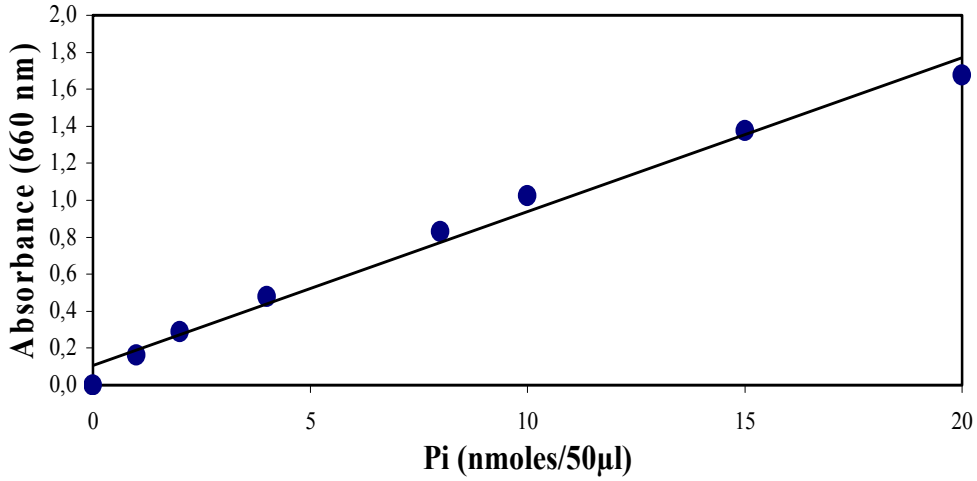


Figure 3.19 Standard Curve of ATPase Assay, prepared using KH_2PO_4 stock solution.

3.5. Sequence Data Search and Analyses

3.5.1. Sequence Analysis of *Tp. volcanium* 26S Proteasome Regulatory Subunit Gene

Sequence analysis of 26S Proteasome Regulatory Subunit Gene (TVG0373521 also known as TVN0382) of *Thermoplasma volcanium* (strain DSM 4299 / GSS1 / JCM 9571) revealed a 1128 bp open reading frame (Figure 3.20). The coding region has a G+C content of 39%. This is higher than the G+C content of genomic DNA which is about 38% (Segerer e*t al*., 1988). The coding region encodes a protein of 375 amino acids with estimated molecular size of Mr=42005 and isoelectric point of pI 5,2729 (Figure 3.21 and Table 3.5).

A putative promoter sequence of 5'-ATGT-3' (Box B) was observed at -6 to -2 upstream region of 26S Proteasome Regulatory Subunit Gene (Kawashima *et al*., 2002).

Figure 3.20 The location of 26S Proteasome Regulatory Subunit, TVN0382 Gene in the *Tp. volcanium* genome is indicated. Gene locates in between the sequences of 372394 – 373521.

FPg

ctgccgggcagtcaaacccaacggccgaccgcgattatatatttttttcatc

taaattcagccacatccttacggaatatgcaatatttgcagaggaatatgaa

gttaaagtggagtttgtaagcgcagcctataaattgaagaaggtaaagcttt

atacaaagggcactgtgatggatagttactccgaggagaaaagcgagggag

atgtaa

1

atgagtgatgatgttcagaataagattgaaaaagccacaaaactactgcacgca
M S D D V Q N K I E K A T K L L H A

gggataaaagaagaggaaaatggacataaagataaggcaaaggagtactatctt
G I K E E E N G H K D K A K E Y Y L

gtggcttacagggtaatgttagaagctgcaaacgactctccatcggatcttaag
V A Y R V M L E A A N D S P S D L K

aagaaaagacttgatcaatgtgctttaatattaaatgcctacaaacgcgttagt
K K R L D Q C A L I L N A Y K R V S

ggagaaaggacagacttcacagtgaaaaaacaaaatgatgatgaaatagtagaa
G E R T D F T V K K Q N D D E I V E

ggtgaggctctactagaagagataggaatagaaaaaccagagataccaaaagta
G E A L L E E I G I E K P E I P K V

acctttgaagatgttgcaggtctagatgacgttaaaaatgagatattaggaaaa
T F E D V A G L D D V K N E I L G K

attatttacccaatgaaatttaaggaattatcgcaggaatataatatacaattc
I I Y P M K F K E L S Q E Y N I Q F

ggaggcggaatgttattgtacggtccgccaggcactggaaaaacctttattgtt
G G G M L L Y G P P G T G K T F I V

aaggctatagccaacgaggtaaaggccagatttatcaacgtaaatccttccaca
K A I A N E V K A R F I N V N P S T

Figure 3.21 Nucleotide sequence and deduced amino acid sequence of the gene encoding 26S Proteasome Regulatory Subunit from *Tp. volcanium.* The open reading frame is shown with corresponding amino acid sequences.

ctctacagtgaatggtttggtgttttgaaaagaatatatccaagctttcagg
L F R L Y S E W F G V F E K N I S K

gcagcgtcattgctatctccagcaataatattctttgatgagatagatgctttg
A A S L L S P A I I F F D E I D A L

gtgccaaagagagacacctccaattcagatgctgcaaagagaggagttgcacag
V P K R D T S N S D A A K R G V A Q

cttttgaatgaggttggtggtataaattctcagaagaacaaaaatatattcatc
L L N E V G G I N S Q K N K N I F I

atcgcagcaacaaacaatccctgggaagtagatgaggccatgcttaggccaggt
I A A T N N P W E V D E A M L R P G

cgcttcgatataaagatatacgtgccacctccagacatagtcgctagaaagaaa
R F D I K I Y V P P P D I V A R K K

atatttcagcttaatctagcgaaaattaaacaggtaggaaacgtagactacgat
I F Q L N L A K I K Q V G N V D Y D

ctgctggcagaggagacagaaggatacagtggtgcagatatagagttcatatgc
L L A E E T E G Y S G A D I E F I C

aaaaaggcagctcagaatgtattcatggaagcggtgaaaacagggaagagcaga
K K A A Q N V F M E A V K T G K S R

ccagttgaaacgagggacgtcatagacgttataggaagcatcaaaccttccatt
P V E T R D V I D V I G S I K P S I

gattatgaactgctggaaaaatattcaaagtatggttccgcttttag**373521**
D Y E L L E K Y S K Y G S A F *

gcaatagcaattcaataaaaaggcataaaagtttatatgtttatttcgttta

ttatt**ggcaatagatcccctgcatcg**

RPg

Figure 3.21 Continued.

Table 3. 5 Amino acid composition of *Tp. volcanium* 26S Proteasome Regulatory Subunit. The data is obtained from the sequence given in Figure 3.21. Values are given as residues/molecules. Total number of amino acids: 375.

Amino acids			**Content**
Alanine	Ala	A	33
Arginine	Arg	R	13
Asparagine	Asn	N	21
Aspartik acid	Asp	D	25
Cysteine	Cys	C	2
Glutamine	Gln	Q	10
Glutamic acid	Glu	E	33
Glycine	Gly	G	24
Histidine	His	H	2
Isoleucine	Ile	I	33
Leucine	Leu	L	29
Lysine	Lys	K	42
Methionine	Met	M	6
Phenylalanine	Phe	F	17
Proline	Pro	P	16
Serine	Ser	S	19
Threonine	Thr	T	12
Tryptophan	Trp	W	2
Tyrosine	Tyr	Y	14
Valine	Val	V	26

3.5.2. BLAST Search

The 375 letters length amino acid sequences of *Tp. volcanium GSS1,* "26S Proteasome Regulatory Subunit" protein (also called as "ATPase of the AAA^+ class involved in cell division") was BLAST (Basic Local Alignment Search Tool) searched in the complete *Tp. volcanium GSS1* amino acid sequence database, which is composed of totaly 450,115 letters, to find out the paralogies within the aligned protein sequences. (Table 3.6)

Table 3.6 BLAST search of 26S Proteasome Regulatory Subunit gene within the completely sequenced *Tp. volcanium GSS1* genome; 1499 sequences, 450,115 total letters.

Sequences producing significant alignments:	**Score (bits)**	**E Value**
ATPase of the AAA^+ class involved in cell division	692	0.0
ATPase of the AAA^+ class involved in cell division	193	$5x10^{-51}$
ABC-type multidrug transport system, ATPase component	31	0.053
ATPase (AAA superfamily)	28	0.26
Predicted GTPase	28	0.26
Predicted ATP-dependent protease	27	0.58
Predicted ATPase (PilT family)	27	0.76
Mg-chelatase, I and D subunits	27	1.00
Predicted ATPase	27	1.00
Malate oxidoreductase (malic enzyme)	26	1.7
Glutamyl-tRNA reductase	26	1.7
ABC-type multidrug transport system, ATPase component	26	1.7
ATPase (AAA superfamily)	25	2.2
ABC-type multidrug transport system, ATPase component	25	2.2

NCBI data search revealed that based on 200 BLAST hits the most related protein sequences to *Tp. volcanium* 26S Proteasome Regulatory Subunit protein were a number of archaeal AAA^+ ATPases (such as VAT and cell division control protein 48, cdc48).

3.5.3. Multiple Sequence Alignment

3.5.3.1. Multiple Sequence Alignment Among Archaea

Multiple Sequence Alignment using CLUSTAL W (1.82) program was performed to find out the best matches for the *Tp. volcanium* 26S Proteasome Regulatory protein and to see the identities, similarities and differences between the aligned protein sequences (Figure 3.22). The alignment data revealed the highest homology (score 96) between *Tp. acidophilum* VCP-like ATPase (Ta0840) and *Tp. volcanium* ATPase of the AAA^+ class involved in cell division (TVN0947) which were grouping together in the phylogram. The second high homology (score 81) is found between *Tp. volcanium* 26S Proteasome Regulatory Subunit (TVN0382, reported in this work) and *Tp. acidophilum* VAT-2 protein and two were group altogether in the phylogram. Although homology between two proteasome activating nucleotidase, Pan A and (Hv Pan A) and Pan B (Hv Pan B) of *Haloferax volcanii* was quite high (score 60), similarity between 2 *Methanocaldococcus jannaschii* regulatory proteins (MT 1176 and MJ 1156) was too low (score 30) for these proteins to be grouped together (Table 3.7). In addition, evolutionary relationships between the proteins can be seen via viewing cladograms or phylograms (Figure 3.23).

```
CLUSTAL W (1.82) multiple sequence alignment

TVN0947         MDNSSGIILRVAEANSTDPGMSRVRLDESSRRLLDAEIGDVVEIEKAR-KTVGRVYRARP 59
Ta0840          MESNNGIILRVAEANSTDPGMSRVRLDESSRRLLDAEIGDVVEIEKVR-KTVGRVYRARP 59
MJ1156          ----MVKELKVAEAYQGDVGRGIARIDPYTMEELGLKPGDVIEIEGPKGKAYAIVYRGFL 56
HvPanA          ------------------------------------------------------------
HvPanB          ------------------------------------------------------------
MJ1176          ------------------------------------------------------------
TVG0373521      ------------------------------------------------------------
Ta1175          ------------------------------------------------------------

TVN0947         EDENKGIVRIDSVMRNNCGASIGDKVRVRKVRTEIAKKVTLAPIIRKDQRLKFGEGIEEY 119
Ta0840          EDENKGIVRIDSVMRNNCGASIGDKVKVRKVRTEIAKKVTLAPIIRKDQRLKFGEGIEEY 119
MJ1156          EDAGKGIIRIDGYLRQNAGVAIGDRVKVKRVEIKEAKKVVLAP----TQPIRFGPGFEDF 112
HvPanA          ------------------------------------------------------------
HvPanB          ------------------------------------------------------------
MJ1176          ------------------------------------------------------------
TVG0373521      ------------------------------------------------------------
Ta1175          ------------------------------------------------------------

TVN0947         VQRALIRRPMLEQDNISVPGLTLAGQTGLLFKVVKTMPGKVPVEIGEETKIEIREEPASE 179
Ta0840          VQRALIRRPMLEQDNISVPGLTLAGQTGLLFKVVKTLPSKVPVEIGEETKIEIREEPASE 179
MJ1156          VKRKILGQVLSKGSKVTIG--VLG--TALTFVVVSTTP-AGPVRVTDFTHVELKEEPVSE 167
HvPanA          ------------------------------------------------------------
HvPanB          ------------------------------------------------------------
MJ1176          ------------------------------------------------------------
TVG0373521      ------------------------------------------------------------
Ta1175          ------------------------------------------------------------

TVN0947         VLE-EVSRVSYEDIGGLSEQLGKIREMIELPLKHPELFERLGITPPKGVILYGPPGTGKT 238
Ta0840          VLE-EVSRISYEDIGGLSEQLGKIREMIELPLKHPELFERLGITPPKGVILYGPPGTGKT 238
MJ1156          IKETKVPDVTYEDIGGLKEEVKKVREMIELPMRHPELFEKLGIEPPKGVLLVGPPGTGKT 227
HvPanA          ------------------------------------------------------------
HvPanB          ------------------------------------------------------------
MJ1176          ------------------------------------------------------------
TVG0373521      ------------------------------------------------------------
Ta1175          ------------------------------------------------------------

TVN0947         LIARAVANESGANFLSINGPEIMSKYYGQSEQKLREIFSKAEETAPSIIFIDEIDSIAPK 298
Ta0840          LIARAVANESGANFLSINGPEIMSKYYGQSEQKLREIFSKAEETAPSIIFIDEIDSIAPK 298
MJ1156          LLAKAVANEAGANFYVINGPEIMSKYVGETEENLRKIFEEAEENAPSIIFIDEIDAIAPK 287
HvPanA          ----------------------MMTDTVDDVDLPYDKD-SASQQEKITALQERLEVLETQ 37
HvPanB          ----------------------MSRSPSLPERPHLDLDPEMSDAERLSALRQHFERMVDV 38
MJ1176          ---------------MVFEEFISTELKKEKKAFTEEFKEEKEINDNSNLKNDLLKEELQ 44
TVG0373521      ----------------------------MSDDVQNKIEKATKLLHAGIKEEENGHKDKA 31
Ta1175          ----------------------------MRVLDDIDEKIDKATKLLQGGIKEESLGHKDKA 33
                                           .          .  .  .        :

TVN0947         REEVQGEVERRVVAQLLTLMDGMKERGHVIVIGATNRIDAVDPALRRPGRFDREIEIGVP 358
Ta0840          REEVQGEVERRVVAQLLTLMDGMKERGHVIVIGATNRIDAIDPALRRPGRFDREIEIGVP 358
MJ1156          RDEATGEVERRLVAQLLTLMDGLKGRGQVVVIGATNRPNALDPALRRPGRFDREIVIGVP 347
HvPanA          NE----EMRDKLLDTNAENNKYQQKLERLTHENKKLK------------QSPLFVATVQ 80
HvPanB          NR----ELDQRLQNADDRHAELVDEVDQMKARNEALK------------TASYYIATVE 81
MJ1176          EKARIAELESRILKLELEKKELERENLQLMKENEILRRELDR------MRVPPLIVGTVV 98
TVG0373521      KEYYLVAYRVMLEAANDSPSDLKKKR---------------------------------- 57
Ta1175          KEYYFAAYRLMLEAANASPPDLKKKR---------------------------------- 59
                .          :        .           .     .
```

Figure 3.22 Multiple Sequence Alignment Results of the putative 26S Proteasome Regulatory Subunit among other Archeal ATPases.

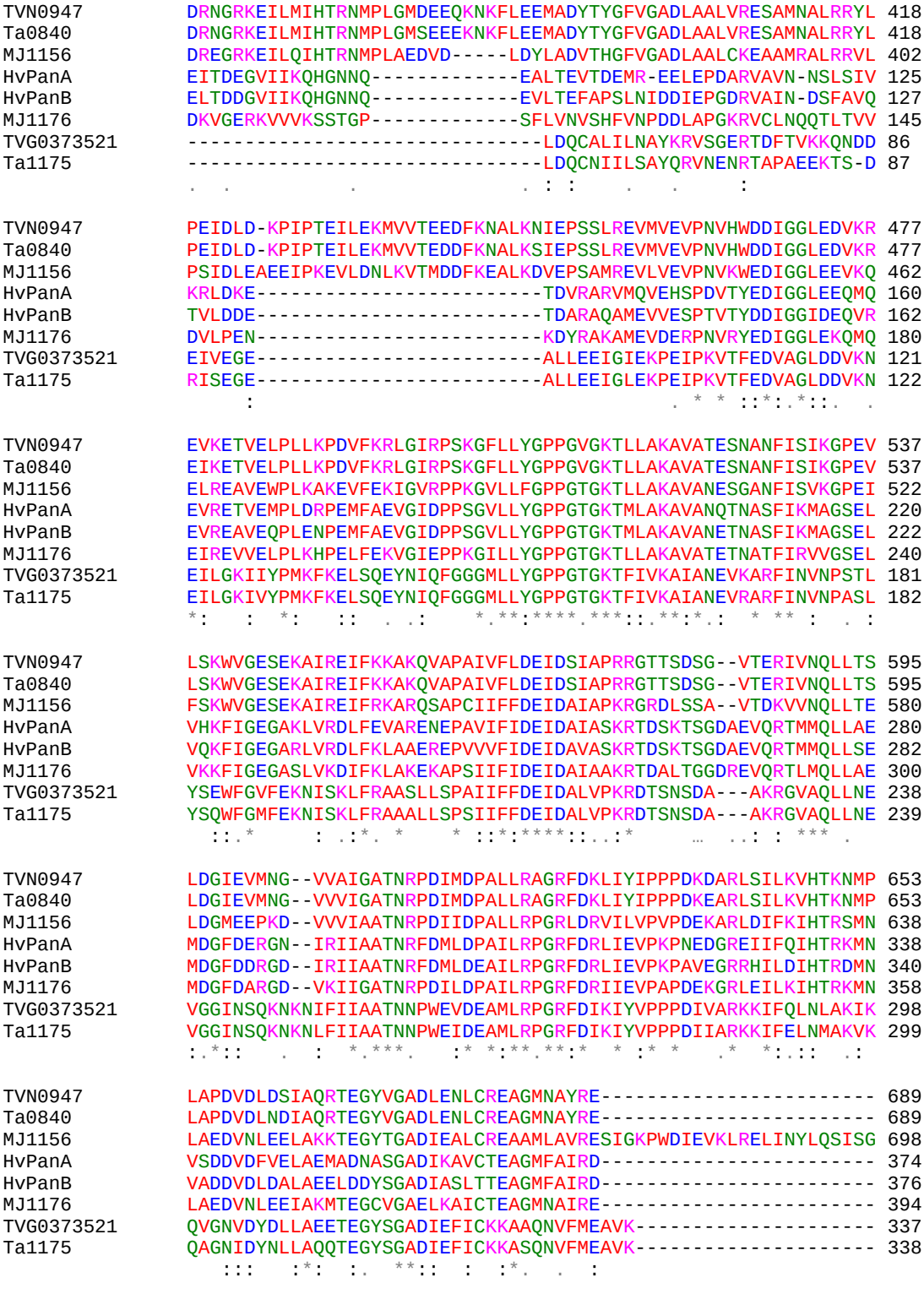


Figure 3.22 Continued.

```
TVN0947         ------------------------------------------------------------
Ta0840          ------------------------------------------------------------
MJ1156          TFRAAAVELNSVIKATKERESAEAGEFSELKNAIGKIISVLSPAKEKIEAVEKEIDKFLE 758
HvPanA          ------------------------------------------------------------
HvPanB          ------------------------------------------------------------
MJ1176          ------------------------------------------------------------
TVG0373521      ------------------------------------------------------------
Ta1175          ------------------------------------------------------------

TVN0947         --NPDATQVSQKN------------------------------------------------ 700
Ta0840          --NPDATSVSQKN------------------------------------------------ 700
MJ1156          VINKEELKPSEKDEAQKLAKYLKDILGKLKEMIDNIYELENKLNTLKEQVSAEEIDEIIK 818
HvPanA          ------------------------------------------------------------
HvPanB          ------------------------------------------------------------
MJ1176          ------------------------------------------------------------
TVG0373521      ------------------------------------------------------------
Ta1175          ------------------------------------------------------------

TVN0947         ----------------------------------------FIDALKTIRPSIDEEVIKF 719
Ta0840          ----------------------------------------FLDALKTIRPSVDEEVIKF 719
MJ1156          TTQNIIQRFTTSLDELKNILKDIESIRLKVSTKDVKIKKEHFMKALEKIKPSVSKEDMRV 878
HvPanA          ------------------------------------------------------DRTEIFMQD 383
HvPanB          ------------------------------------------------------GRTEVTGAD 385
MJ1176          ------------------------------------------------------LRDYVTMDD 403
TVG0373521      ---------------------------------------------TGKSRPVETRDV 349
Ta1175          ---------------------------------------------SGKQRQVETRDL 350

TVN0947         YKSISETMGKSVSEKRKELQDQGLYL- 745
Ta0840          YRTLSETMSKSVSERRKQLQDQGLYL- 745
MJ1156          YEKLAQEYGRATSVEKKKEEGKEVI---903
HvPanA          FVDAWEKIQQEASDETEVSRAFA---- 406
HvPanB          FDAAHEKLSNVDESGTGPISGFTDYQY 412
MJ1176          FRKAVEKIMEKKKVKVKEPAHLDVLYR 430
TVG0373521      IDVIGSIKPSIDYELLEKYSKYGSAF- 375
Ta1175          LDVIGSIKPSIDADLLSKYREFAGF---375
```

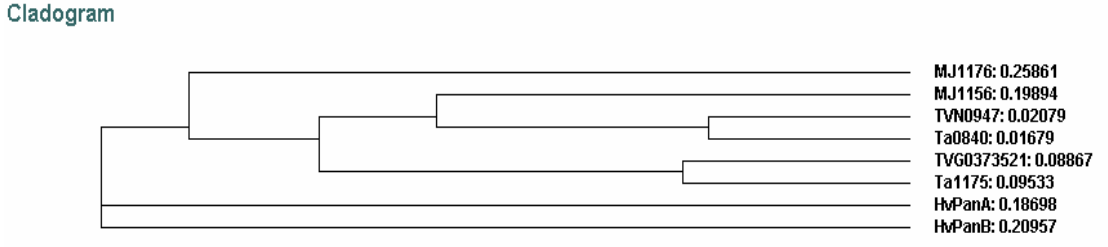
Figure 3.22 Continued.

Table 3.7 Homology scores between *Tp. volcanium* 26S Proteasome Regulatory Subunit and that of other archaea are tabulated in the following table and obtained by multiple sequence alignment.

26S Proteasome Regulatory Subunit Source	Percent Identity with 26S Proteasome Regulatory Subunit from							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
(1) TVG0373521	**100**	**27**	**26**	**34**	**25**	**32**	**30**	**81**
(2) HvPanA		**100**	**60**	**35**	**49**	**30**	**29**	**25**
(3) HvPanB			**100**	**33**	**45**	**30**	**30**	**24**
(4) MJ1156				**100**	**34**	**57**	**57**	**31**
(5) MJ1176					**100**	**31**	**34**	**26**
(6) TVN0947						**100**	**96**	**31**
(7) Ta0840							**100**	**31**
(8) Ta1175								**100**

(1) TVG0373521 (TVN0382) : *Thermoplasma volcanium,* Proteasome regulatory subunit
(2) HvPanA **:** *Haloferax volcanii,* proteasome-activating nucleotidase A (panA) gene
(3) HvPanB **:** *Haloferax volcanii,* proteasome-activating nucleotidase B (panB) gene
(4) MJ1156 **:** *Methanocaldococcus jannaschii,* cell division control protein 48 (cdc48)
(5) MJ1176 **:** *Methanocaldococcus jannaschii,* Proteasome Reg AAA ATPase, PAN
(6) TVN0947 **:** *Thermoplasma volcanium,* ATPase of the AAA^+ class involved in cell division
(7) Ta0840 **:** *Thermoplasma acidophilum,* VCP-like ATPase
(8) Ta1175 **:** *Thermoplasma acidophilum,* VAT-2 protein

a)



b)

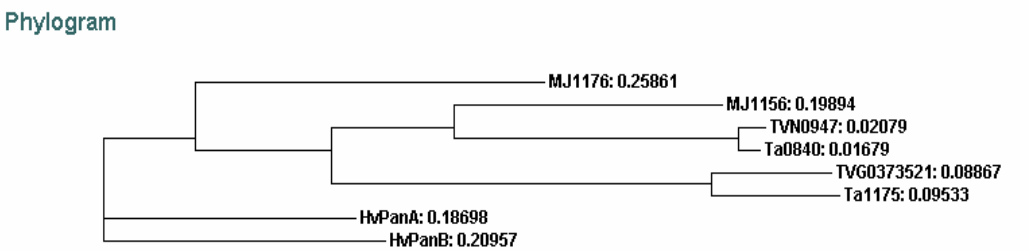


Figure 3.23 (a) Cladogram and (b) phylogram of the cloned putative 26S Proteasome Regulatory Subunit.

3.5.3.2. Multiple Sequence Alignment Among Diverse Organisms

Multiple sequence alignment of 26S proteasome regulatory proteins (of AAA family ATPase) from diverse sources (from archaea to human) shows that *Tp. volcanium* and *Tp. acidophilum* proteins, (score 81), *Aspergillus*, *Dictiyostelium*, *Saccharomyces* and *Oryzae* proteins (score 49-60) have fairly similar sequences. (Figure 3.24, Table 3.8 and Figure 3.25).

Three hyperthermophilic archaeal ATPases (*Pyrococcus, Sulfolobus* and *Methanocaldococcus*), five eucaryotic ATPases (*Plasmodium*, *Oryza*, *Aspergillus*, *Dictyostelium* and *Saccharomyces)* and seven human ATPases are grouped separately. Suprisingly, *Tp.* has diverged as a distinct lineage from hyperthermophilic Archaeal lineage.

```
CLUSTAL W (1.82) multiple sequence alignment

PH1840                  ------------------------------------------------------------
MJ1156                  ------------------------------------------------------------
AN7254.2                ------------------------------------------------------------
DDB0191154              ------------------------------------------------------------
ScCdc48                 ------------------------------------------------------------
OSJNBa0007M04.24        ------------------------------------------------------------
PF07_0047               MKVRKMHYPLFSFTEYLSCYIILYFFVFLPNYSYAININNYHNKNNLWNINNKWNNQQSN 60
SSO2420                 ------------------------------------------------------------
TVG0373521              ------------------------------------------------------------
Ta1175                  ------------------------------------------------------------
GI_4506209              ------------------------------------------------------------
GI_24430160             ------------------------------------------------------------
GI_24497435             ------------------------------------------------------------
GI_24430151             ------------------------------------------------------------
GI_5729991              ------------------------------------------------------------
GI_21361144             ------------------------------------------------------------
GI_78070571             GGTCAAGAGAAGGCTTTAAGAAAGACGGTATTAATCTCCCGTTGCGGCTCCCGCCTGGTC 60

PH1840                  ------------------------------------------------------------
MJ1156                  ------------------------------------------------------------
AN7254.2                ------------------------------------------------------------
DDB0191154              ------------------------------------------------------------
ScCdc48                 ------------------------------------------------------------
OSJNBa0007M04.24        ------------------------------------------------------------
PF07_0047               HFSNNRIGHFLWGSKIRKNPLASISTSITTKKLENQKKKTNKNESYSNVNDKYEHNNNTA 120
SSO2420                 ------------------------------------------------------------
TVG0373521              ------------------------------------------------------------
Ta1175                  ------------------------------------------------------------
GI_4506209              ------------------------------------------------------------
GI_24430160             ------------------------------------------------------------
GI_24497435             ------------------------------------------------------------
GI_24430151             ------------------------------------------------------------
GI_5729991              ------------------------------------------------------------
GI_21361144             ------------------------------------------------------------
GI_78070571             CCATCTTCTGCCCGCTCCTCCAGGAAATGAATCTGCTGCCGAATATTGAGAGTCCAGTGA 120

PH1840                  ------------------------------------------------------------
MJ1156                  ------------------------------------------------------------
AN7254.2                ------------------------------------------------------------
DDB0191154              ------------------------------------------------------------
ScCdc48                 ------------------------------------------------------------
OSJNBa0007M04.24        ------------------------------------------------------------
PF07_0047               HFVQINYKNDQSNDTLSTYKNNNEMTVEGKPNNLASGEGKKEIHNTTQKILEKVDYFKER 180
SSO2420                 ------------------------------------------------------------
TVG0373521              ------------------------------------------------------------
Ta1175                  ------------------------------------------------------------
GI_4506209              ------------------------------------------------------------
GI_24430160             ------------------------------------------------------------
GI_24497435             ------------------------------------------------------------
GI_24430151             ------------------------------------------------------------
GI_5729991              ------------------------------------------------------------
GI_21361144             ------------------------------------------------------------
GI_78070571             CTCGGCAGGAGAAGATGGCGACCGTGTGGGATGAGGCCGAGCAAGATGGAATTGGGGAGG 180
```

Figure 3.24 Multiple Sequence Alignment Results of the putative 26S Proteasome Regulatory Subunit among diverse organisms

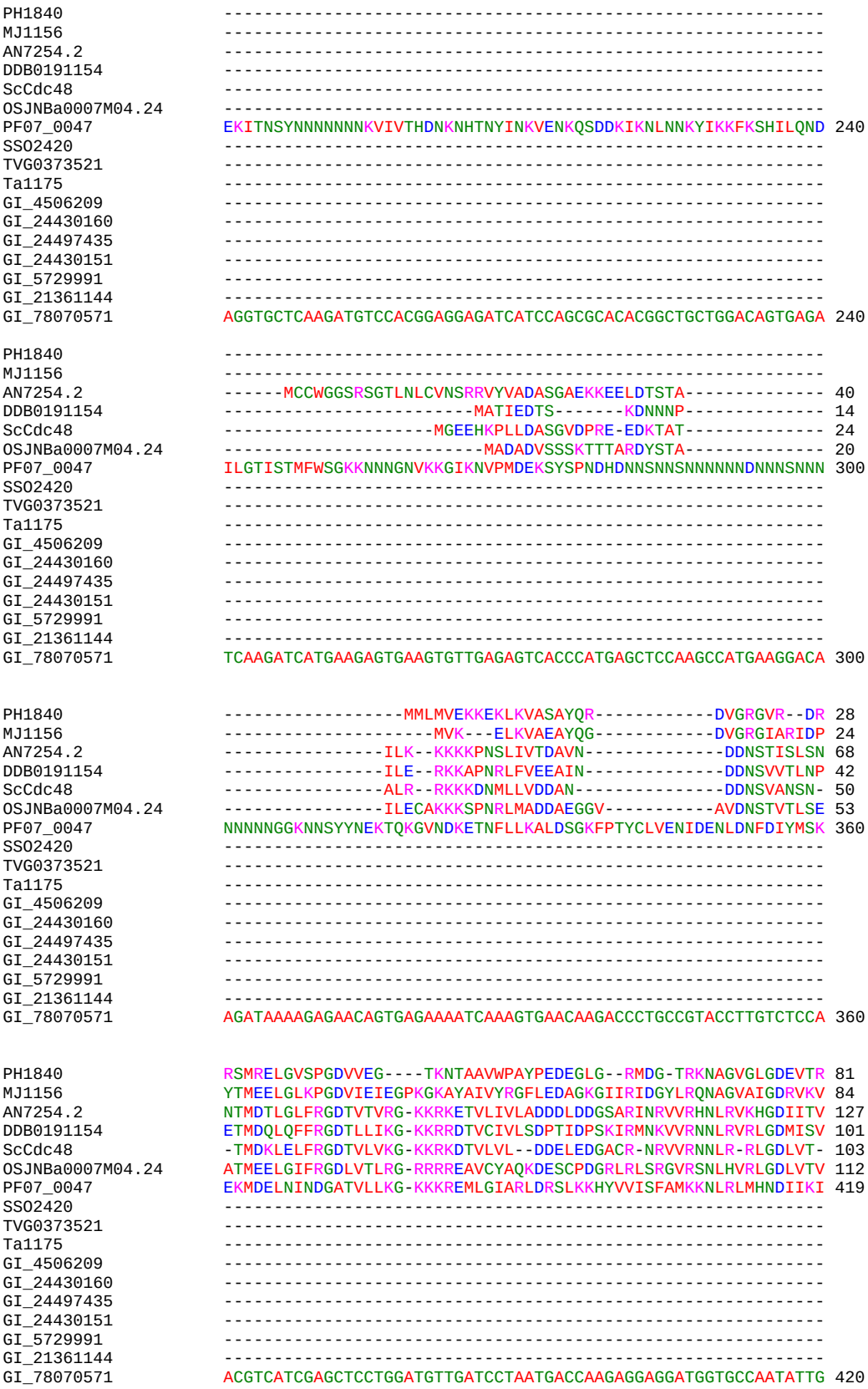


Figure 3.24 Continued.

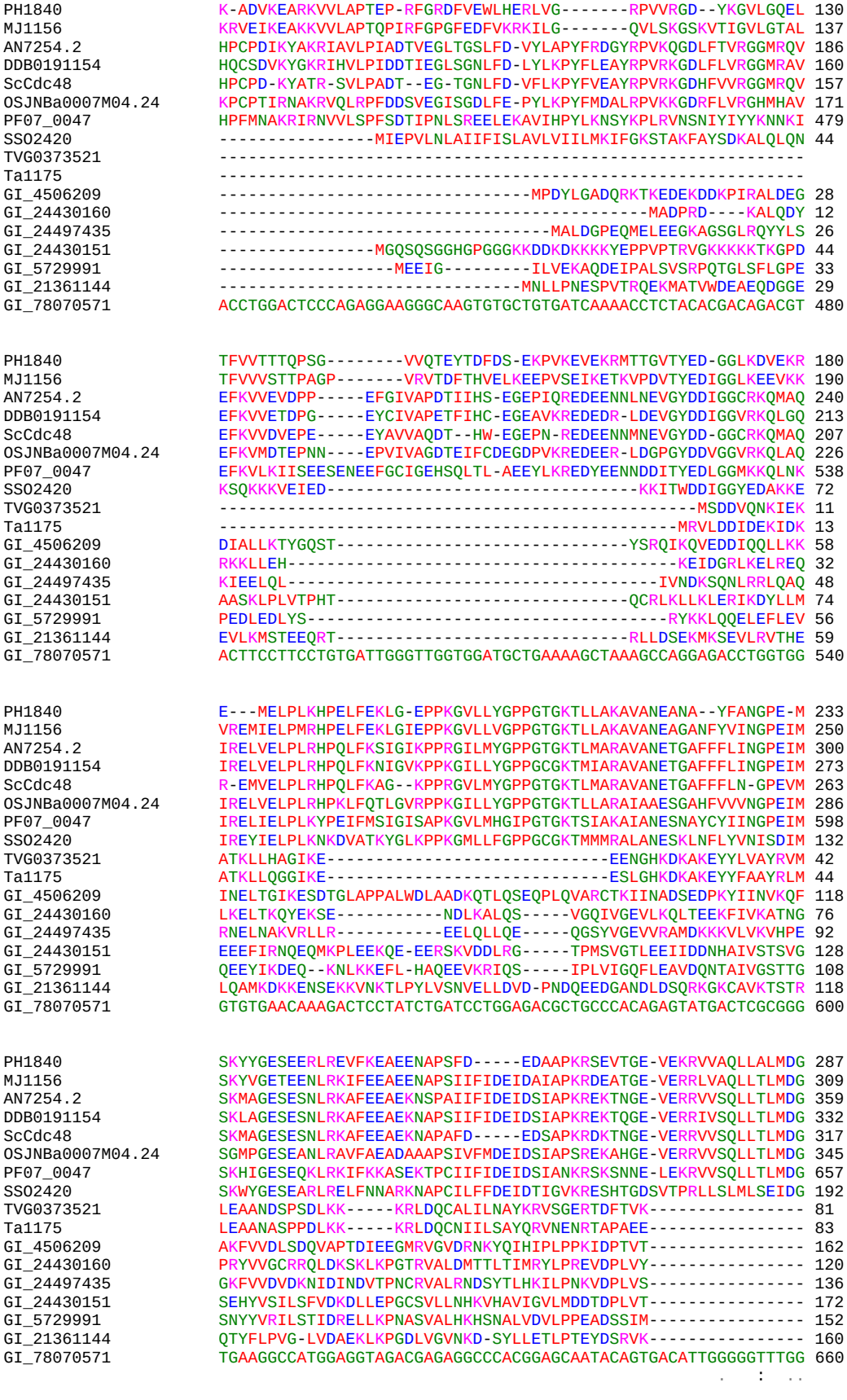


Figure 3.24 Continued.

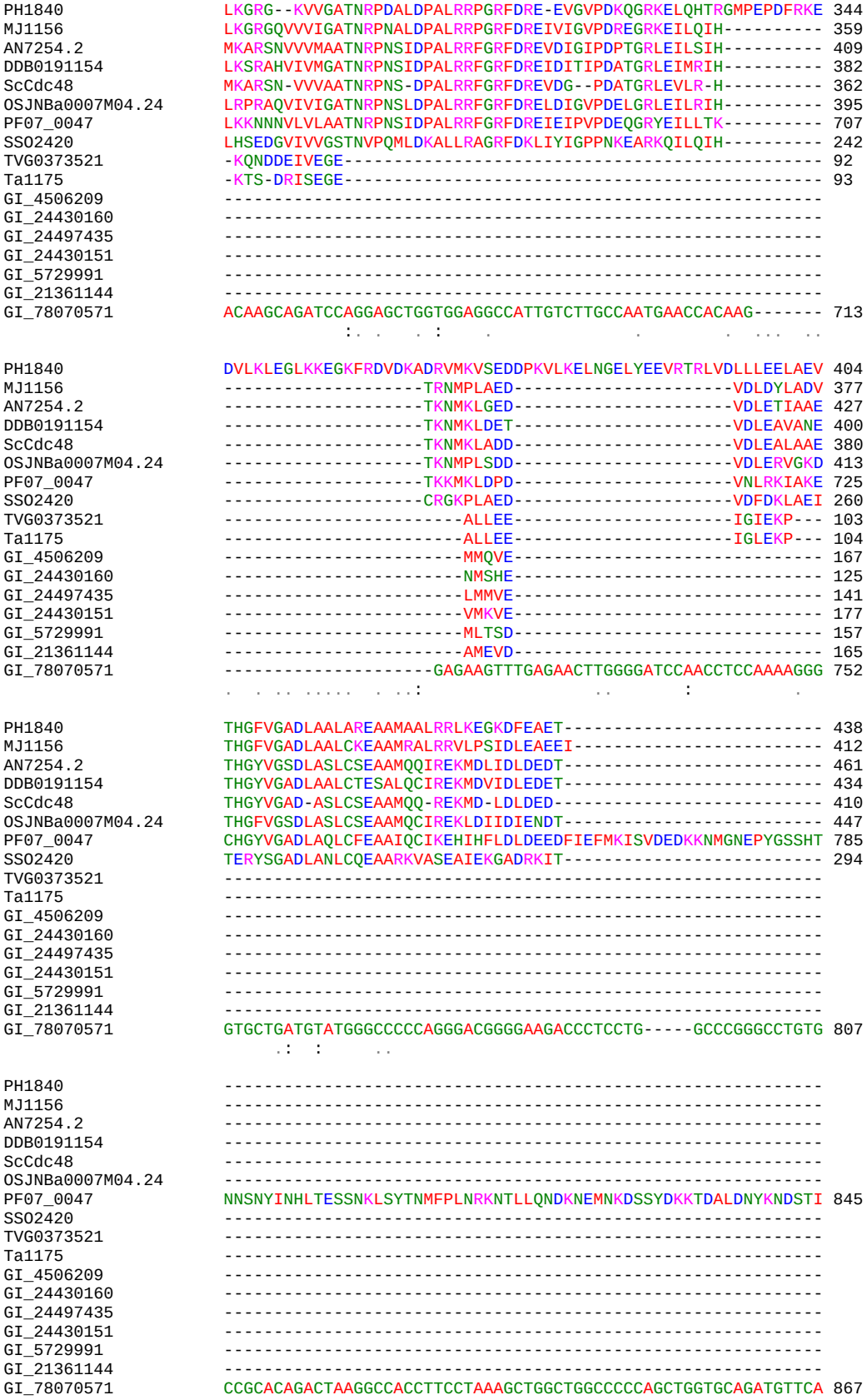


Figure 3.24 Continued.

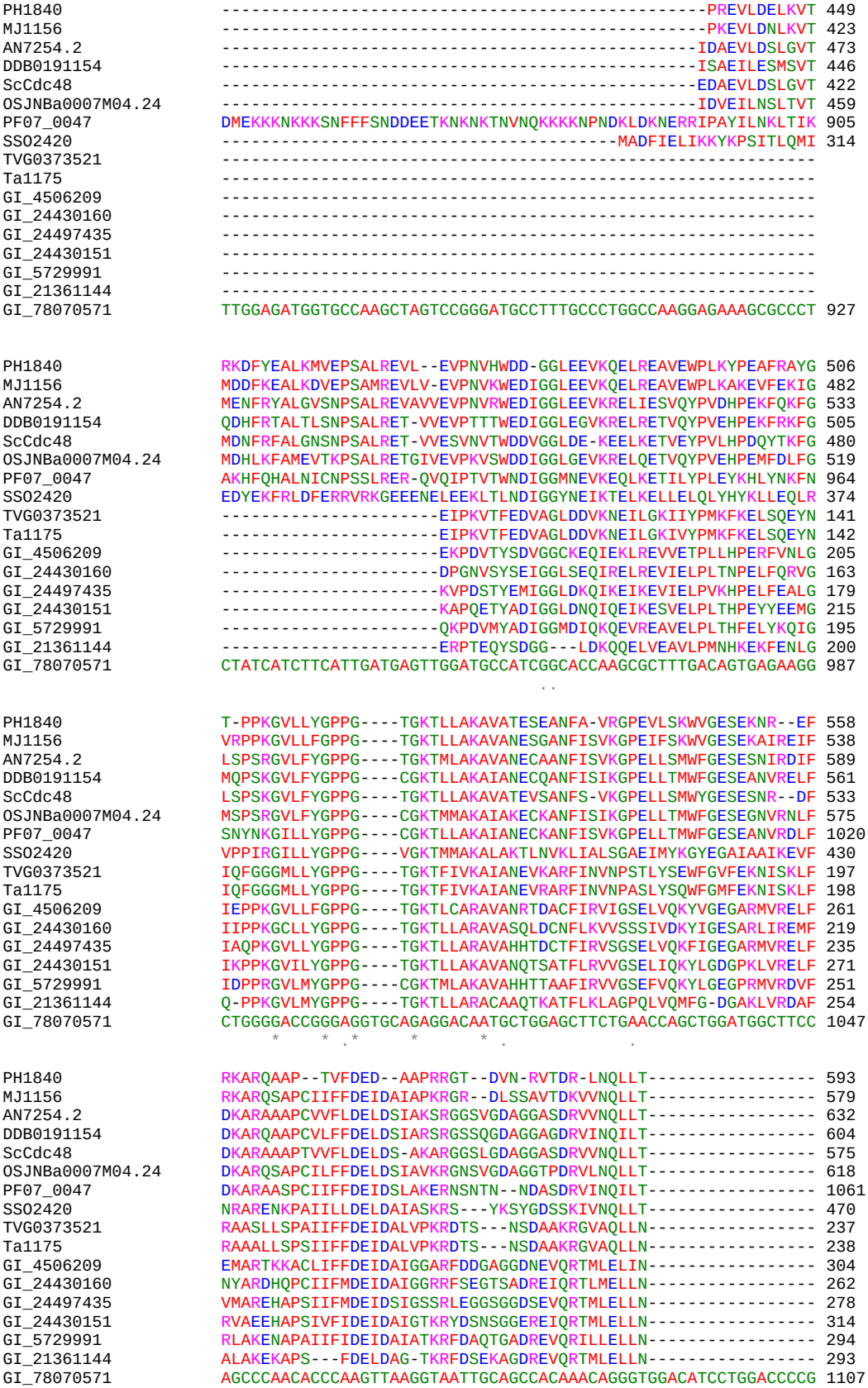


Figure 3.24 Continued.

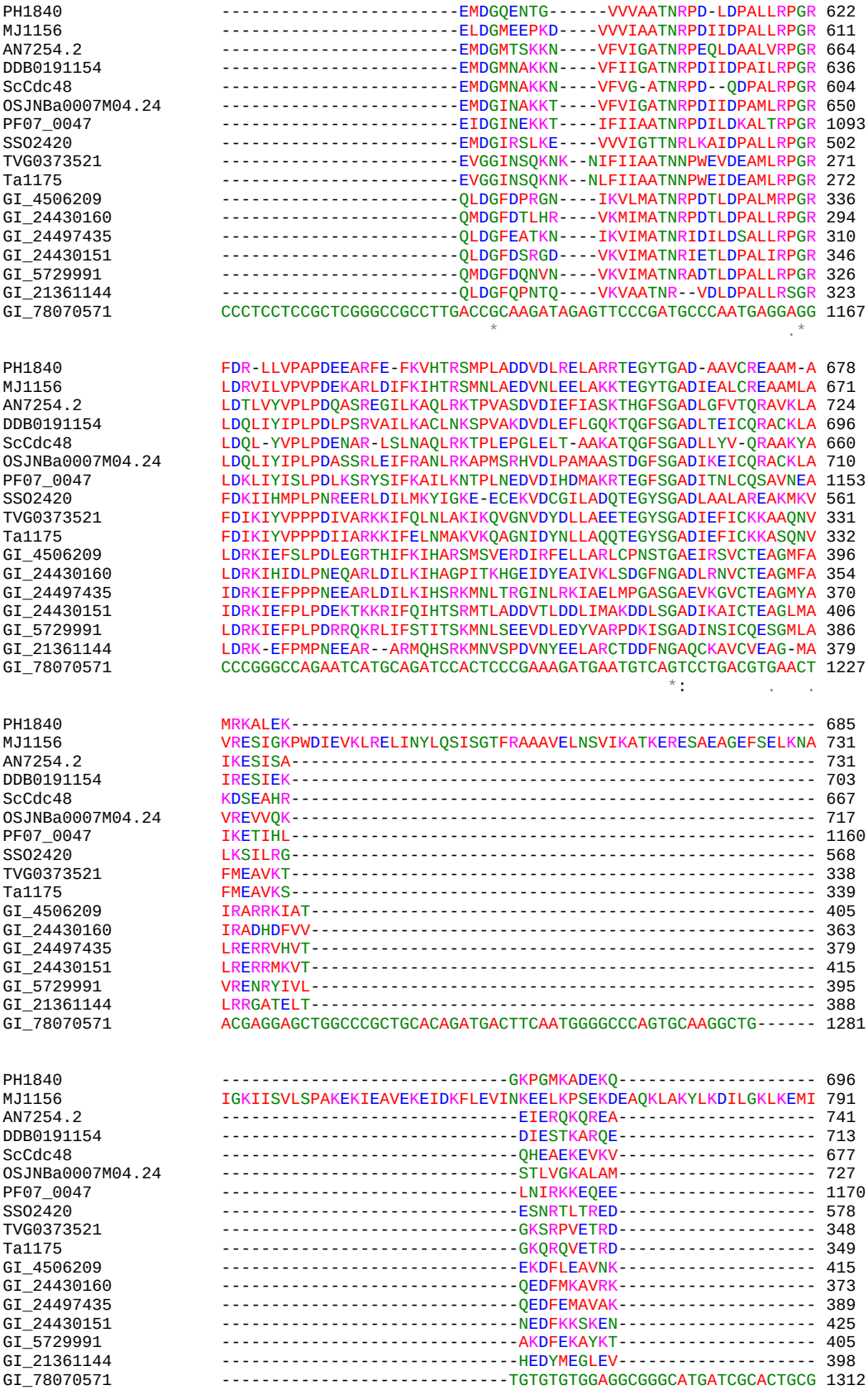


Figure 3.24 Continued.

```
PH1840                ------------KAKVTMKDFEEALKK------------------------------- 711
MJ1156                DNIYELENKLNTLKEQVSAEEIDEIIKTTQNIIQRFTTSLDELKNILKDIESIRLKVSTK 851
AN7254.2              ------------AGEDVKMEDEEEGEDPV------PELT-----RAHFEEAMKTAR--- 774
DDB0191154            ------------SGD-TKMED--DSVDPV------PEIT-----RDHFQEAMRSAR--- 743
ScCdc48               ------------EGEDVEMTDEGAKAEQE------PEVDPVPYTKEHFAEAMKTAK--- 715
OSJNBa0007M04.24      ------------AGAELTVDHFKSAMKHARKSVSELDVIKYEYFKHKFSGGIPDEEEAP 774
PF07_0047             ------------QRKKNKNSFKIDDTDTYDP---VPTLS-----KKHFDLAFKNAR--- 1206
SSO2420               ------------LIDALNKIHPSVKKRLS----------------------KGSS--- 599
TVG0373521            ------------VIDVIGSIKP------------------------------------ 358
Ta1175                ------------LLDVIGSIKP------------------------------------ 359
GI_4506209            ------------------------------------------------------------
GI_24430160           ------------------------------------------------------------
GI_24497435           ------------------------------------------------------------
GI_24430151           ------------------------------------------------------------
GI_5729991            ------------------------------------------------------------
GI_21361144           ------------------------------------------------------------
GI_78070571           CAGGGGTGCCACGGAGCTCACCCACGAGGACTACATGGAAGGCATCCTGGAGGTGCAGGC 1372

PH1840                ----------------GPSVSKETMEYYRKQEQ---------FKQARG------------ 734
MJ1156                DVKIKKEHFMKALEKIKPSVSKEDMRVYEKLAQEYGRATSVEKKKEEGKEVI-------- 903
AN7254.2              -----------------RSVSDVEIRRYEAFAQSLKNSGGS-SFFRFPSANE-------- 808
DDB0191154            -----------------RSVSDNDIRKYESFAQTLVQSRGLGNNFKFPDQ---------- 776
ScCdc48               -----------------RSVSDAELRRYEAYSQQMKASRGQFSNFNFNDAPLGTTATDNA 758
OSJNBa0007M04.24      ATEPELPVGQLRLVAKPKTKTKAELEAEAEAAAKAKAEAEAKAKAKLKGKAVAVD----- 829
PF07_0047             -----------------ISIQPEDVLKYEKFKEKLSLQDF-------------------- 1229
SSO2420               -----------------SNSDHETR-------------------------------- 607
TVG0373521            -----------------SIDYELLEKYSKYGSAF------------------------- 375
Ta1175                -----------------SIDADLLSKYREFAGF------------------------- 375
GI_4506209            -----------------VIKSYAKFSATPRYMTYN----------------------- 433
GI_24430160           -----------------VADS-KKLESKLDYKPV------------------------ 389
GI_24497435           -----------------VMQKDSEKNMSIKKLWK------------------------ 406
GI_24430151           -----------------VLYKKQEGTPEGLYL-------------------------- 440
GI_5729991            -----------------VIKKDEQ--EHEFYK-------------------------- 418
GI_21361144           -----------------QAKKKANLQYYA----------------------------- 410
GI_78070571           CAAGAAGAAAGCCAACCTACAATACTACGCCTAGGGCACACAGGCCAGCCCCAGTCTCAC 1432

PH1840                ----------------------------------
MJ1156                ----------------------------------
AN7254.2              ------AADSGNTFGE-AGNDDSLYD--------- 827
DDB0191154            --------ESSGQFNQ-DQSDDLFSN--------- 793
ScCdc48               NSNNSAPSGAGAAFGSNAEEDDDLYS--------- 784
OSJNBa0007M04.24      ------DDDDDSTYSMDSMDEDLLY---------- 848
PF07_0047             ----------------------------------
SSO2420               ----------------------------------
TVG0373521            ----------------------------------
Ta1175                ----------------------------------
GI_4506209            ----------------------------------
GI_24430160           ----------------------------------
GI_24497435           ----------------------------------
GI_24430151           ----------------------------------
GI_5729991            ----------------------------------
GI_21361144           ----------------------------------
GI_78070571           GGCTGAAGTGCGCAATAAAAGATGGTTTTAAAGTG 1467
```

Figure 3.24 Continued.

Table 3.8 Homology scores between *Tp. volcanium* 26S Proteasome Regulatory Subunit and that of other organisms from archaea to human are tabulated in the following table and obtained by multiple sequence alignment.

26S Proteasome Regulatory Subunit Source	Percent Identity with 26S Proteasome Regulatory Subunit from																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
(1) TVG0373521	100	27	1	20	21	23	26	22	22	30	23	32	30	28	81	34	30
(2) ScCdc48		100	60	23	20	21	23	25	23	49	38	40	55	26	23	38	63
(3) GI_78070571			100	1	1	1	2	24	1	1	1	0	0	1	1	0	0
(4) GI_21361144				100	34	36	37	31	31	24	30	23	21	17	18	26	19
(5) GI_4506209					100	39	42	36	43	24	26	26	24	19	20	31	26
(6) GI_24430151						100	44	44	39	26	26	26	25	20	25	30	25
(7) GI_24497435							100	40	46	27	28	27	28	23	22	35	29
(8) GI_5729991								100	39	30	25	28	27	21	20	33	28
(9) GI_24430160									100	28	27	27	28	27	20	33	30
(10) OSJNBa0007MO4.24										100	37	40	60	33	29	41	55
(11) PH1840											100	28	33	29	28	55	35
(12) PF07_0047												100	47	33	32	32	45
(13) DDB0191154													100	33	32	44	67
(14) SSO2420														100	26	39	34
(15) Ta1175															100	31	30
(16) MJ1156																100	42
(17) AN7254.2																	100

(1) TVG0373521 (TVN0382) : *Thermoplasma volcanium* 26S proteasome regulatory subunit

(2) Ta1175 : *Thermoplasma acidophilum* VAT-2 protein

(3) PH1840 : *Pyrococcus horikoshii* OT3, transitional ER ATPase

(4) SSO2420 : *Sulfolobus solfataricus* P2, AAA fam. ATPase, Cellular processes

(5) MJ1156 : *Methanocaldococcus jannaschii* (cdc48), AAA family

(6) PF07_0047 : *Plasmodium falciparum* 3D7, cell division cycle ATPase,

(7) OSJNBa0007M04.24 : *Oryza sativa*, putative cell division cycle protein

(8) AN7254.2 : *Aspergillus nidulans* FGSC A4, hypothetical protein, Cdc48p

(9) DDB0191154 : *Dictyostelium discoideum* AX4, cell division cycle protein 48

(10) GI:78070571 : *Homo sapiens*, proteasome 26S ATPase subunit 3

(11) ScCdc48 : *Saccharomyces cerevisiae*, CDC48 ATPase in ER, nuclear membrane and cytosol with homology to mammalian p97; in a complex with Npl4p and Ufd1p participates in retrotranslocation of ubiquitinated proteins from the ER into the cytosol for degradation by the proteasome

(12) GI:21361144 : *Homo sapiens* proteasome 26S subunit, ATPase, 3 (PSMC3),

(13) GI:4506209 : *Homo sapiens* proteasome 26S subunit, ATPase, 2 (PSMC2),

(14) GI:24430151 : *Homo sapiens* proteasome 26S subunit, ATPase, 1 (PSMC1),

(15) GI:24497435 : *Homo sapiens* proteasome 26S subunit, ATPase, 5 (PSMC5),

(16) GI:5729991 : *Homo sapiens* proteasome 26S subunit, ATPase, 4 (PSMC4),

(17) GI:24430160 : *Homo sapiens* proteasome 26S subunit, ATPase, 6 (PSMC6),

a)

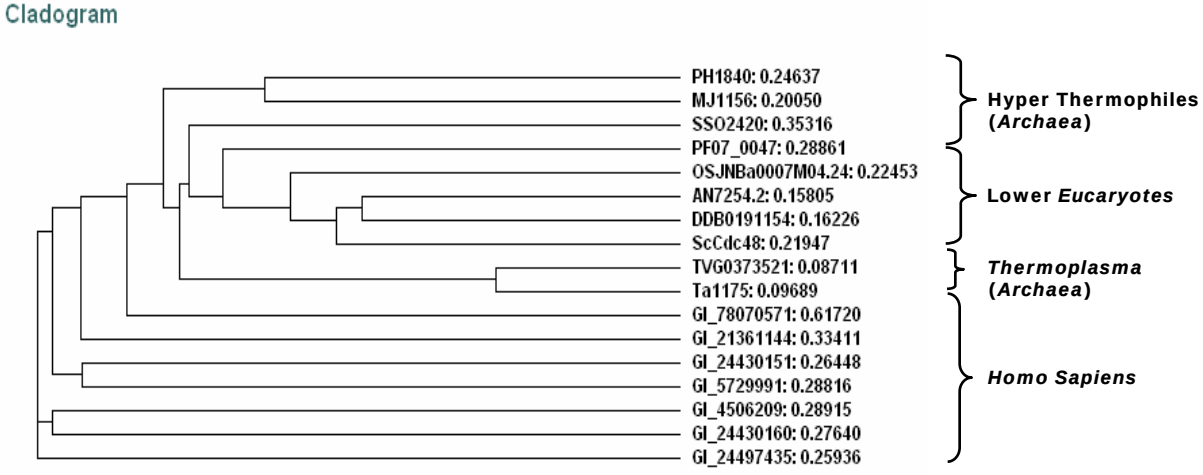


Figure 3.25 (a) Cladogram and (b) phylogram of the cloned putative 26S Proteasome Regulatory Subunit.

b)

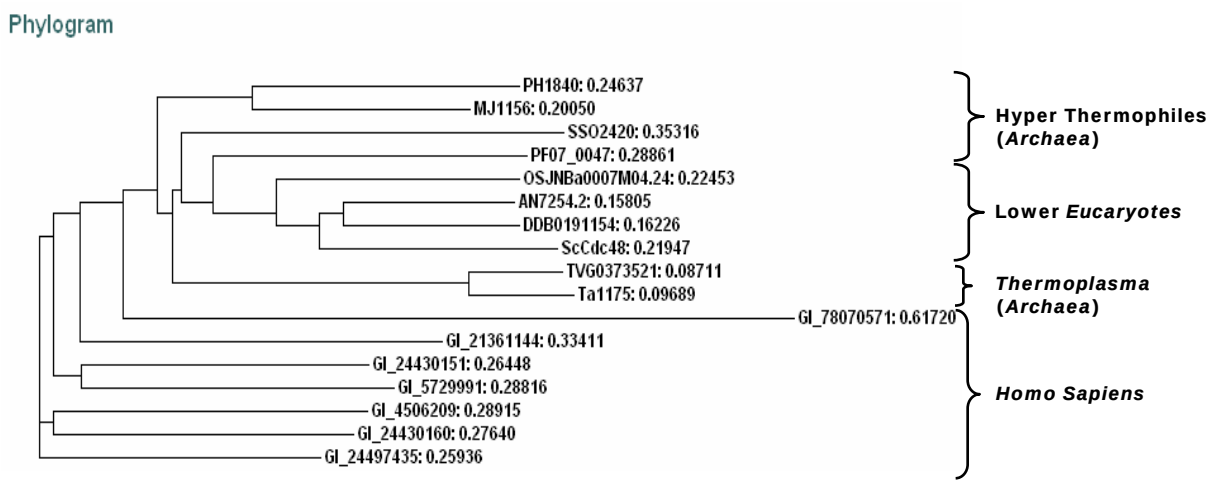


Figure 3.25 Continued.

3.5.4. Conserved Domain Search

NCBI Conserved Domain Search showed that there are two conserved domains, AAA and Lon domains associated with the amino acid sequences of *Tp. volcanium* 26S Proteasome Regulatory Subunit (Figure 3.26).

Tp. volcanium 26S Proteasome Regulatory Subunit gene has an ATPase domain which designates AAA ATPase family associated with various cellular activities. This family proteins often perform chaperone-like functions that asist in the assembly, operation, or disassembly of protein complexes. It was found that the *Tp. volcanium* 26S Proteasome Regulatory Subunit gene is the member of ATPases of the AAA^{+} class (COG0464) of Clusters of Orthologous Groups of Proteins, which has Posttranslational modification, protein turnover and chaperone activities.

Conserved AAA domain covers the sequences between 147 and 275. Three motifs, Walker A, Walker B and SRH are shown in the alignment presented in the Figure 3.27.

Conserved Lon sequrnces lie between the position 1 and 175, and typical alignments for this motif is given in the Figure 3.28.

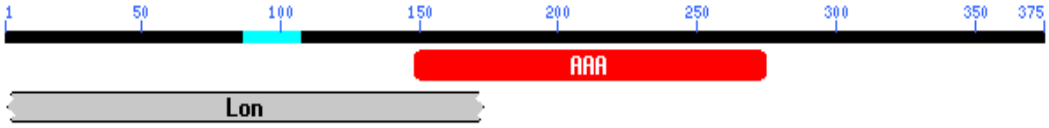


Figure 3.26 Conserved domain sequences in the putative 26S Proteasome Regulatory Subunit.

3.5.4.1. Conserved AAA Domains

AAA-superfamily of ATPases associated with a wide variety of cellular activities, including membrane fusion, proteolysis, and DNA replication.

ATP binding site also called Walker A and Walker B motifs:

Walker A motifs (G*xxGx*GKT, *x* _ any amino acid) for ATP binding,

Walker B motifs (*hhhh*DE*xx*, *h* _ hydrophobic residue) for ATP hydrolysis,

SRH (Second Region Homology) motif [(T/S)-(N/S)-X5-D-X-A-X2-R-X2-R-X-(D/E)] also involved in ATP hydrolysis.

```
                      10        20        30        40        50        60        70        80
                 ....*....|....*....|....*....|....*....|....*....|....*....|....*....|....*....|
Feature 1                 #####
1DO2_A            51 NILMIGPTGVGKTEIARRLAKla----naPFIKVEAtkfte---------------------------------vgyvG 92
query            147 gMLLYGPPGTGKTFIVKAIANev----kaRFINVNPstlyse-------------------------------wfgvfE 190
1E32_A           240 GILLYGPPGTGKTLIARAVANet----gaFFFLINGpeimsk-------------------------------lagesE 283
1NSF              65 SVLLEGPPHSGKTALAAKIAEesn---fpFIKICSPdkmigf-------------------------------setakC 109
1MAB_B           151 KIGLFGGAGVGKTVLIMELINnvakahggYSVFAGVgertregndlyhemiesgvinlkdatskvalvygqmneppgarA 230
gi 1729896       246 GILLFGPPGTGKTLIARAVANet----gsFFFLINGpevmsk-------------------------------msgesE 289
gi 2492526       241 GVLLHGPPGCGKTSIANALAGel----qvPFISISApsvvsg-------------------------------msgesE 284
gi 2492504       517 GVLFYGPPGCGKTLLAKAIANec----qaNFISVKGpelltm-------------------------------wfgesE 560
gi 3122632       177 GVLLYGPPGTGKTLLAKAVANqt----raTFIRVVGsefvqk-------------------------------yigegA 220
gi 548458        733 GILLYGYPGCGKTLLASAVAQqc----glNFISVKGpeilnk-------------------------------figasE 776
```

Figure 3.27 Conserved AAA domains alignment of the putative 26S Proteasome Regulatory Subunit

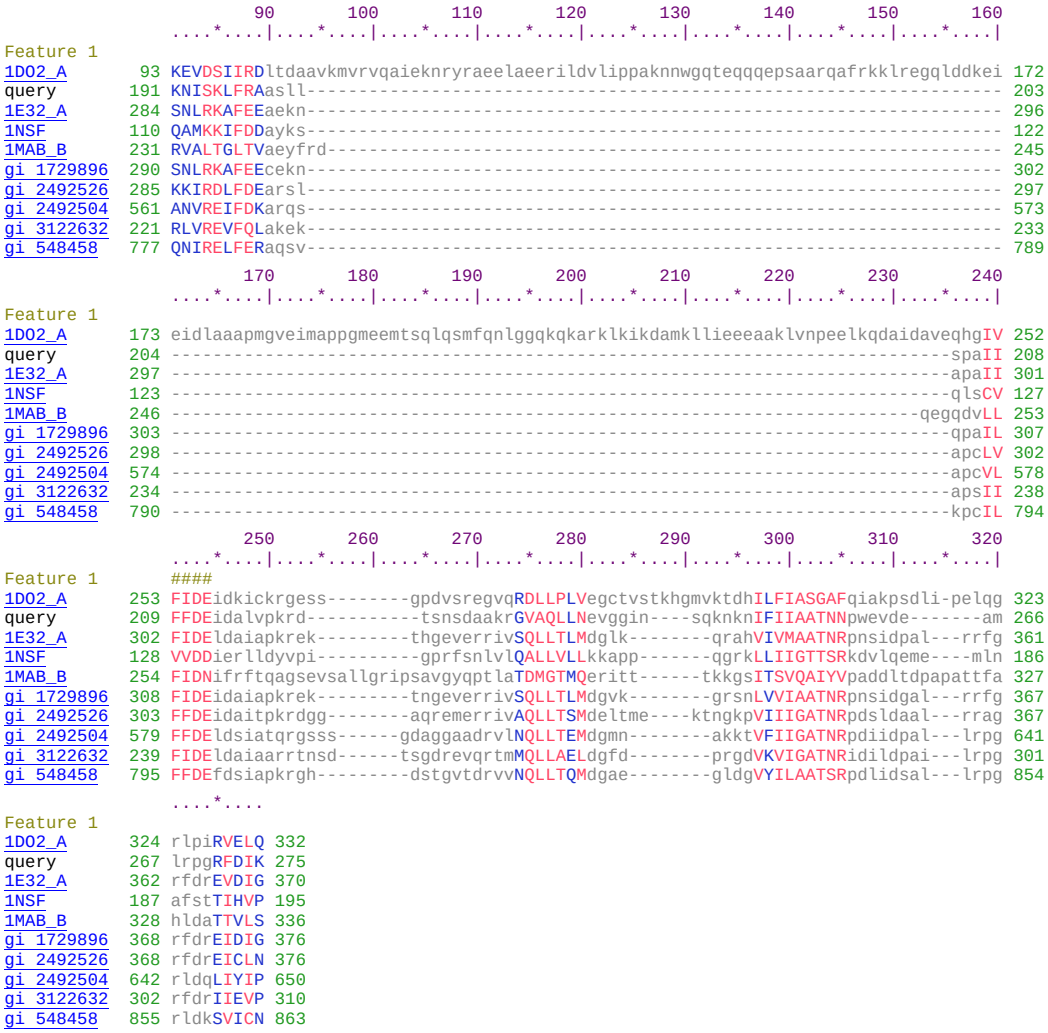


Figure 3.27 Continued.

1DO2_A : *Escherichia coli*, HslU

1E32_A : *Mus musculus*, Membrane Fusion Atpase P97.

1NSF : *Cricetulus griseus*, N-Ethylmaleimide Sensitive Factor (Nsf).

1MAB_B : *Rattus norvegicus*, Rat Liver F1-Atpase.

gi 1729896 : *Caenorhabditis elegans*, p97/CDC48 homolog 1

gi 2492526 : *Saccharomyces cerevisiae*, Ribosome biogenesis ATPase RIX7.

gi 2492504 : *Capsicum annuum*, Cell division cycle protein 48 homolog.

gi 3122632 : *Archaeoglobus fulgidus*, (PAN)

gi 548458 : *Saccharomyces cerevisiae*, Peroxisome biosynthesis protein PAS1

3.5.4.2. Conserved Lon Sequences

ATP-dependent bacterial type Lon protease sequences, functional in posttranslational modification, protein turnover and chaperone activities.

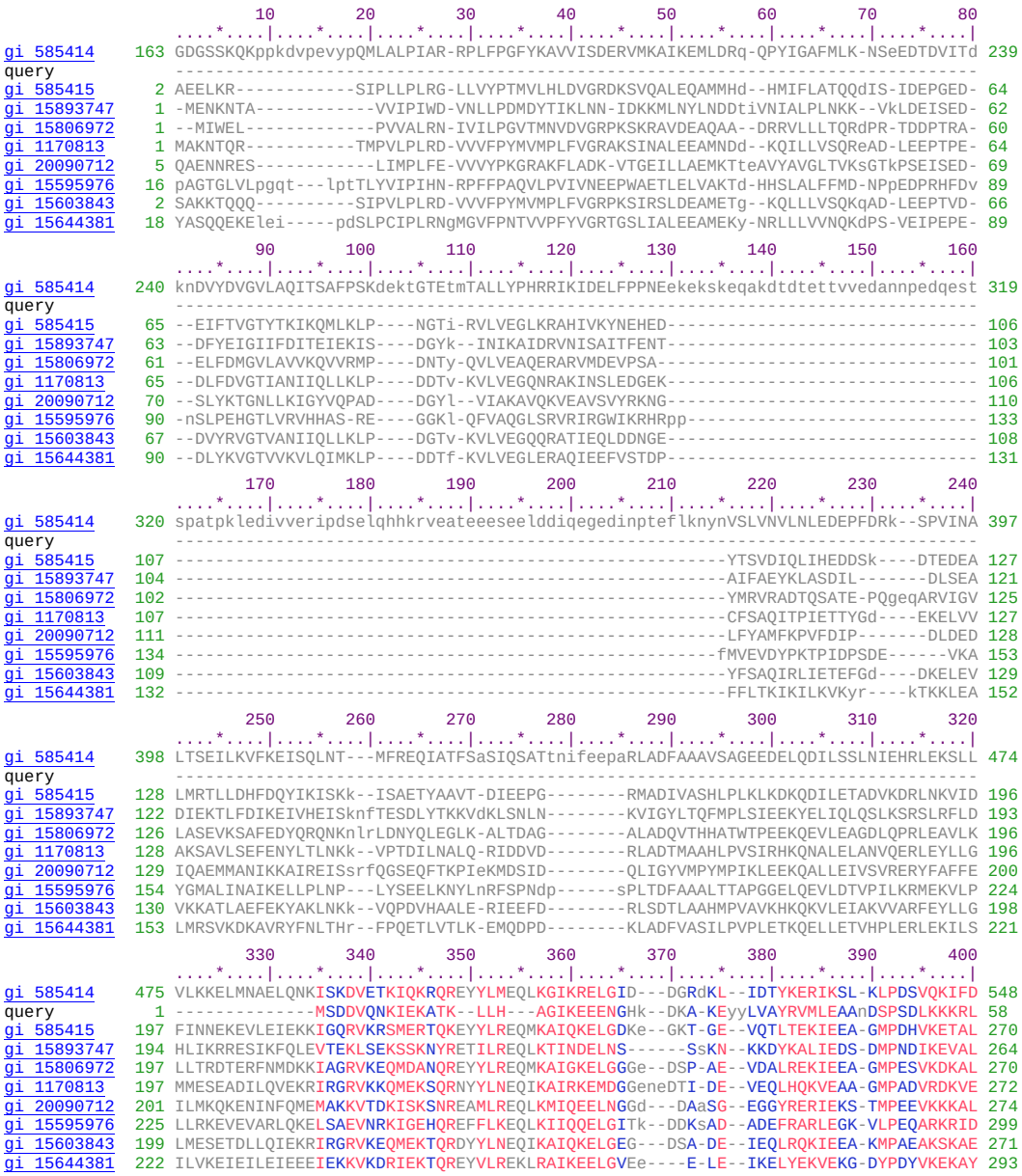


Figure 3.28 Conserved Lon domain alignment of the putative 26S Proteasome Regulatory Subunit.

Figure 3.28 Continued.

```
                    970       980       990      1000
                ....*....|....*....|....*....|....*....|....*.
gi 585414   1077 LNDWEELPDNVKEGLEPLAADWYNDIFQKLFKDvntkegNSVWKAE 1122
query             ---------------------------------------------
gi 585415    739 EKDIEDIPESVREGLTFILASHLDEVLEHALVGe----kK------ 774
gi 15893747  731 IDDLKDIPDEVKESLQIIPVKTIEDVLKEGSDIsv--pkAECLIST 774
gi 15806972  760 EPSLQEVPESIRGDLKVHAVEHVSEVLELLLLP------KPEQAEQ 799
gi 1170813   741 VKDLEEIPENVKQNLAIHAVETIDEVLGFALEN------PPEGIEF 780
gi 20090712  743 ERDLEDVPEEVRNELKFVPVETIEEVLKEALGLdl---pGHVLSSS 785
gi 15595976  767 RGHFEELPDYLREGLTVHFAKRYGDVAKVLFPA------------- 799
gi 15603843  740 VKDLEEIPDNAKSNLTIHAVETIDEVLTIALEN------APTGVEF 779
gi 15644381  755 RPDVEKIPKEYLNGMEIVYCSEIQEVLKEAIVR------------- 787
```

Figure 3.28 Continued.

gi 585414 : *Saccharomyces cerevisiae*, Lon protease homolog,
gi 585415 : *Bacillus subtilis*, ATP-dependent protease La 1.
gi 15893747 : *Clostridium acetobutylicum*, ATP-dependent protease (lonA).
gi 15806972 : *Deinococcus radiodurans* R1, ATP-dependent protease LA.
gi 1170813 : *Haemophilus influenzae*, ATP-dependent protease La.
gi 20090712 : *Methanosarcina acetivorans* C2A, endopeptidase La.
gi 15595976 : *Pseudomonas aeruginosa*, probable ATP-dependent protease.
gi 15603843 : *Pasteurella multocida*. Pm70, Lon.
gi 15644381 : *Thermotoga maritima* MSB8, ATP-dependent protease LA.

CHAPTER IV

DISCUSSION

In eurkaryotic cells, controlled protein degradation is important for the removal of 'abnormal' proteins and thus for cell viability. The selective degradation of many short-lived proteins is carried out by the proteasome with ubiquitin system. Although prokaryotes lack ubiquitin and 26S proteasomes, archaebacteria and certain eubacteria contain 20S proteasomes whose structural organization and catalytic mechanism are very similar to those of the eukaryotic 20S particles (*12,* 32, *72*). The subunits are arranged in four rings; α-subunits form the outer rings of the stack, while the two rings composed of β-subunits which carry active sites (*87*). Archaea have a reduced proteasome complex equivalent to the 20S subunit which was reported first for *Thermoplasma acidophilum* (*85*) and later for *Methanosarcina thermophila* (*23*) and *Methanococcus jannaschii* (*76*). Subsequently 20S proteasome is also found in *Pyrococcus furiosus* (*75*), *Methanobacterium thermoautothrophicum* (*77*), *Archaeoglobus fulgidus* (*78*), *Pyrococcus horikoshii* (*79*), *Haloferax volcanii* (*83*) and *Thermoplasma volcanium*.

In archaea, proteasomes are central players in energy-dependent proteolysis and form elaborate nanocompartments that degrade proteins into oligopeptides by processive hydrolysis. The exact physiological role of proteasomes in archaea is not clear, and to our knowledge, no strains that are deficient in 20S proteasomes have been created. Inhibitor studies suggest that 20S proteasomes in these organisms are not essential and the other pathways for protein degradation exist

may compensate by performing many of the critical metabolic functions of the proteasome. This could allow *Thermoplasma* to survive without functional proteasomes under non-stress conditions. Only under stress conditions, that is when the level of misfolded proteins will become very high, blockage of the proteasome-dependent degradation pathway results in growth arrest.

In this study, firstly we have focused on previously cloned and purified *Thermoplasma volcanium* 20S proteasome for biochemical characterization assays and a putative proteasome regulatory subunit gene (TVN0382) belonging to AAA^+ ATPase family of *Thermoplasma volcanium* was cloned and expressed in *E. coli.* Subsequently the expression of cloned proteasome regulatory subunit was measured by an ATPase assay.

ATPase activity of VAT(VCP-like ATPase of *Thermoplasma*), a protein of the CDC48/p97 subfamily from the archaeon *Thermoplasma acidophilum* (like CDC48 and p97) has Mg^2-dependent ATPase activity with a specific activity of 5,43 μmol/mg/h at an optimum temperature of approximately 70°C. The optimum temperature of VAT was approximately 10°C above the growth temperature of *Tp. acidophilum* (~58-60°C) (*89*).

The whole archaeal 20S proteasome structure is build up from only two types of subunits designated as α and β. The 19S Regulatory Subunit is not found in Archaea. Instead, in all archaea, except *Thermoplasma* has PAN, which is proteasome activating nucleotidase. PAN is identified as a 650 kDa ATPase complex from the archaebacterium *Methanococcus jannaschii* and probably the evolutionary precursor of the eukaryotic 19S. It is composed of identical subunits closely homologous to the six ATPases of the 19S and stimulates markedly the degradation of proteins, but not of tetrapeptides, by archaeal 20S proteasomes. This process requires hydrolysis of ATP (*35*). PAN exhibits certain activities characteristic of molecular chaperones, e.g., it prevents aggregation and promotes the refolding of denatured proteins (*156*). In *Thermoplasma*, genome analysis revealed that two ATPases of AAA^+ class (eg. VAT) putative regulatory might substitute

for PAN. This thesis project is secondly focused on one of these proteasomal regulatory ATPases.

Purity of recombinant 20S proteasome samples of *Thermoplasma volcanium,* used in biochemical characterization assays was confirmed by gel electrophoresis. In the native gel proteasome complex migrated as a distinct band, with an estimated molecular mass of 340 kDa. SDS-PAGE result showed that 20S proteasome from the *Tp. volcanium* was composed of two subunits, α and β subunits, with relative molecular masses of 24 kDa and 23 kDa, respectively. This was in good agreement with the reports on 20S proteasomes of other archaeabacteria, *Tp. acidophilum* (25.8 kDa α- and 22.3 kDa β-subunits), *Methanosarcina thermophila* (24 kDa α- and 22 kDa β-subunits) and *Pyrococcus furious* (25 kDa α- and 22-kDa β subunits) (*11, 98, 169*). Exceptionally, the actinomycete, *Frankia sp.* revealed two types of subunits, α and β with a molecular mass of 31 kDa and 28 kDa, respectively (*170*).

Thermoplasma proteasomes, which contain one type of active site, exhibit not only "chymotrypsin-like" activity, but also some "post-glutamyl" and "trypsin-like" activities. Like eukaryotic proteasomes, its activity can be stimulated by SDS, Mg^{+2}, and also guanidine HCl (*102*). Unlike conventional proteases, proteasome degrades proteins processively without release of polypeptide intermediates. Upon activation by SDS, guanidine, heat (55 °C), proteasomes still functioned processively, but generated a different pattern of peptides under each condition. The Mg^{+2} and Ca^{+2} at 25-500 mM also enhance peptidase activity of *Tp. acidophilum* proteasome several fold.

In *Methanosarcina thermophila,* SDS and divalent or monovalent ions stimulate chymotrypsin-like (CL) activity and inhibit postglutamyl peptide hydrolyzing (PGPH) activity (*98*).

The activiy of *Tp. volcanium* 20S proteasome samples treated with 3 μM SDS has increased 300 fold. We have also detected the effects of various agents ($MgCl_2$, $MnCl_2$, Cysteine, ATP, $CaCl_2$, NaCl, KCl, EDTA, Guanidine HCl and SDS) on the

enzymatic activity of *Tp. volcanium* 20S proteasome and found that Mg^{+2}, Cysteine, ATP, NaCl, Guanidine HCl and SDS reduced the activity 50% as compared to control.

The purified *Methanosarcina thermophila* 20S proteasome display chymotrypsin-like (CL) activity with the hydrolysis of Suc-Ala-Ala-Phe-AMC (7-amido-4-methyl-coumarin) and also has postglutamyl peptide hydrolyzing (PGPH) activity with the CBZ-Leu-Leu-Glu-β-NA.

The highest activity measured with Suc-Ala-Ala-Phe-AMC (7-amido-4-methyl-coumarin) which is a typical substrate for chymotrypsin-like (CL) activity of 20S proteasome from *Haloferax volcanii*.

The recombinant 20S proteasome of *Methanococcus jannaschii* has high postglutamyl peptide hydrolyzing (PGPH) activity with CBZ-Leu-Leu-Glu-βNA substrate and also has significant activity with Suc-Leu-Leu-Val-Try-AMC and Suc-Ala-Ala-Phe-AMC substrates (*99*).

The substrate profile of the *Tp. volcanium* recombinant 20S proteasome has been determined by using various synthetic chromogenic substrates at 55°C. The highest activity has been obtained with N-CBZ-Leu-Leu-Glu-βNA, by measuring the postglutamyl peptide hydrolyzing (PGPH) activity. The chymotrypsin-like (CL) activity has been measured with Ala-Ala-Phe-pNA and found as 88 % of the PGPH activity. The trypsin-like activity (TL) has been detected with peptide substrate N-CBZ-Arg-pNA and found as 27% of the PGPH activity.

The chymotryptic activity of *Tp. acidophilum 20S* proteasome when assayed with Ala-Ala-Phe-pNA substrate the optimum temperature has been determined as 85°C. The heat stability of *Tp. volcanium* proteasome has been found quite high. After the heat treatment at 98°C for 30 minutes, 64% of the activity has been retained. In a large, multimeric enzyme like the poteasome, where stability may ultimately be governed by intersubunit interactions, activity and stability need not

be mechanistically linked. The optimum temperature and pH are 75°C and 7,7 for the activity of *Haloferax volcanii* 20S proteasome, respectively.

The enzymatic activity was measured to determine the optimum pH of the enzyme using Ala-ala-phe-pNitro-Anilide as substrate, at different pH values ranging between 4 to 9,5 in *Tp. volcanium*. There was considerable activity at alkaline pH.

The second focus of interest was cloning of a putative proteasome regulatory subunit gene (*Tv*PRS) which is belong to AAA^+ ATPase family of *Tp. volcanium* and over-expression in *E.coli*. For cloning of *Tv*PRS, a PCR based strategy was used. The nucleotide sequences for putative proteasome regulatory subunit gene (TVN0382) were taken from GenBank DNA sequence database of NCBI (National Center for Biotechnology Information). The putative sequence has been amplified by PCR from *Tp. volcanium* genome. PCR was performed under highly stringent annealing conditions with the amplification primers complementary to the 5' and 3' non-coding regions and yielded unique 1419 bp regulatory subunit gene. These PCR amplified gene fragments were cloned into the cloning site of pDrive cloning vector via A/U complementation. The prescence of PCR amplified cloned gene was confirmed by restriction analysis of recombinant plasmids. The restriction endonuclease digestion results also have revailed the reverse orientation of the insert with respect to the *lac Z* promoter. Thus, for the first time, a 20S proteasome regulatory gene was cloned and expressed in *E.coli*.

ATPase activities in cell free extracts of the recombinant *E.coli* strains were determined as 10 times higher than the non-recombinants. In the assay conditions, some samples were incubated at 40°C, some of them were treated with heat at 65°C, the ATPase activity of heat treated ones were found higher when measured.

Domain search revealed that the cloned proteasome regulatory subunit has conserved domains typical of the AAA^+ ATPase family; Walker A motifs (GxxGxGKT, x-any amino acid) for ATP binding, Walker B motifs (hhhhDExx, h-hydrophobic residue) for ATP hydrolysis and Second Region Homology (SRH)

motif [(T/S)-(N/S)-X5-D-X-A-X2-R-X2-R-X-(D/E)] also involved in ATP hydrolysis.

When we aligned the predicted amino acid sequences of the proteasome regulatory subunit (TVN0382, TVG0373521) with various archaeal species proteins, including VAT-2 protein (VCP-like ATPase of *Tp. acidophilum*) of *Tp. acidophilum* (Ta1175), using CLUSTAL W (1,82) program in European Bioinformatics Institute (EBI)'s website, a remarkable similarity (81 %) was found. On the other hand, proteasome regulatory AAA ATPase, (PAN, Proteasome Activating Nucleotidase) of *Methanocaldococcus jannaschii* (MJ1176) has a relatively low homology with an identity score of 25%.

Detailed functional analyses of recombinant *Tp. volcanium* proteasome regulatory subunit protein including purification is still underway, in our laboratory.

CHAPTER V

CONCLUSION

SDS-PAGE analysis revealed that 20S proteasome from *Tp. volcanium* was composed of two subunits, α and β with relative molecular masses of 24 kDa and 23 kDa, respectively.

The highest chymotryptic activity was observed over an alkaline pH range (pH 8.0 – pH 9.0) and the optimum temperature for the activity was determined as 85^{o}C. The heat stability of proteasome was quite high after treatment at 98^{o}C for 30 minutes, 64% of the activity has still been retained. The highest activity associated with the *Tp. volcanium* proteasome was found to be peptidylglutamyl peptidase activity.

The PCR amplified putative 26S Proteasome related Regulatory Subunit gene (1128 bp) of *Tp. volcanium* was cloned in *E. coli*. Cloning was comfirmed by restriction map analyses of isolated plasmids from recombinant colonies. This is the first report on the cloning of a 26S Proteasome related Regulatory Subunit gene from *Tp. volcanium* and the first one from the *Thermoplasma.*

The successful expression of the cloned gene was proved from high rates of increase in the ATPase activity in *E.coli.* because of the ATPase activity of the recombinant protein.

Future research in the field of archaeal proteasomes is focused on understanding their physiological role and the mechanism of how substrate proteins are recognized for

destruction. The thermophilic archaeon *Thermoplasma volcanium* is amendable to genetic studies, which allows the proteasome to be examined in a cellular context. Integrating genetics with the detailed biochemical and biophysical information already available will assist in understanding how these elaborate energy-dependent proteases work in the cell.

The variety of human diseases that can be traced to malfunctioning ubiquitin/proteasome process is significant and diverse. Cancer arising from defective proto-oncogene expression patterns can, in many cases, be traced to a faulty proteasome degradation profile of members belonging to this group. Numerous neurodegenerative diseases including Alzheimer's, Parkinson's, Huntington's, amyolateral lateral sclerosis and spongiform encephalopathies (Mad Cow) also can be linked to faulty proteasomal degradation of specific proteins. Some inflammatory and auto-immune diseases also arise due to inappropriate proteasome pathway activity.

Although prokaryotes lack ubiquitin and 26S proteasomes, archaebacteria and certain eubacteria contain 20S proteasomes whose structural organization and catalytic mechanism are very similar to those of the eukaryotic 20S particles. In the view of medical sciences and human health; the studies of archaeal proteasomes have provided important insights into the structure and enzymatic mechanisms of their eukaryotic homologs**.**

REFERENCE LIST

1. Deshaies, R.J., Make it or break it: the role of ubiquitin-dependent proteolysis in cellular regulation Trends Cell Biol., vol. 5, pp. 428– 434 (1995).

2. Gottesman, S., and M. R. Maurizi.. Regulation by proteolysis: energy dependent proteases and their targets. Microbiol. Rev. 56:592–621 (1992).

3. King, R. W., R. J. Deshaies, J. M. Peters, and M. W. Kirshner. How proteolysis drives the cell cycle. Science 274:1652–1659 (1996).

4. Kirscher M., Intracellular proteolysis. TIBS. 24:12, M42-45 (1999).

5. Rock K.L., Goldberg A.L., Degradation of cell proteins and generation of MHC class I-presented peptides, Annu. Rev. Immunol. 17 739-779 (1999).

6. Schauber C., Chen L., Tongaonkar P., Vega I., Lambertson D., Potts W., Madura K., Rad23 links DNA repair to the ubiquitin/proteasome pathway, Nature 391 715-718 (1998).

7. Pagano M. Cell cycle regulation by the ubiquitin pathway.FASEBJ. 11:1067–75 (1997).

8. Bohley, P., and Seglen, P. O. Proteases and proteolysis in the lysosome. Experientia 48, 151-157 (1992).

9. Schmidt, M., Lupas, A.N., Finley, D., Structure and mechanism of ATP-dependent proteases. Curr. Opin. Chem. Biol. 3, 584–591 (1999).

10. Maupin-Furlow, J.A., Wilson, H.L., Kaczowka, S.J., Ou, M.S.,. Proteasomes in the archaea: from structure to function. Front. Biosci. 5, d837–d865 (2000).

11. Maupin-Furlow, J.A., Kaczowka, S.J., Ou, M.S., Wilson, H.L.,. Archaeal proteasomes: proteolytic nanocompartments of the cell. Adv. Appl. Microbiol. 50, 279–338 (2001).

12. Baumeister,W., J. Walz, F. Zühl & E. Seemüller: The proteasome: paradigm of a self-compartmentalizing protease. Cell 92, 367-380 (1998).

13. Baumeister,W., Z. Cejka, M. Kania & E. Seemüller: The proteasome: a macromolecular assembly designed to confine proteolysis to a nanocompartment. Biol Chem 378, 121-130 (1997).

14. Lupas, A., J.M. Flanagan, T. Tamura & W. Baumeister: Self-compartmentalizing proteases. Trends Biochem Sci 22, 399-404 (1997).

15. Benaroudj, N., Zwickl, P., Seemuller, E., Baumeister, W., Goldberg, A.L., ATP hydrolysis by the proteasome regulatory complex PAN serves multiple functions in protein degradation. Mol. Cell 11, 69–78.) (2003).

16. Menon, A.S., Waxman, L., and Goldberg, A.L. The energy utilized in protein breakdown by the ATP-dependent protease (La) from *Escherichia coli.* J. Biol. Chem. 262, 722–726 (1987).

17. Zwickl, P., A. Grizwa, G. Puhler, B. Dahlmann, F. Lottspeich, and W. Baumeister. Primary structure of the *Thermoplasma* proteasome and its implications for the structure, function, and evolution of the multicatalytic proteinase. Biochemistry 31:964–972 (1992).

18. Lowe, J., Slock, D., Jap, B., Zwickl, P., Baumeister, W. Crystal structure of the 20S proteasome from the archaeon *T. acidophilum* at 3.4 A° resolution. Science, 268, 533–539 (1995).

19. Groll, M., Ditzel, L., Lowe, J., Stock, D., Bochtler, M., Bartunik, H. D. & Huber, R. Structure of 20S proteasome from yeast at 2.4 A° resolution. Nature, 386, 463–471. (1997).

20. Bochtler, M., Ditzel, L., Groll, M., Huber, R., Crystal structure of heat shock locus V (HslV) from *Escherichia coli.* Proc. Natl. Acad. Sci. USA 94, 6070–6074 (1997).

21. Lupas, A., Koster, A.J., and Baumeister, W. Structural features of 26S and 20S proteasomes. Enzyme Protein 47, 252–273 (1993).

22. Maupin-Furlow, J. A., Kaczowka, S. J., Reuter, C. J., Archaeal proteasomes: potential in metabolic engineering. Metab. Eng. 5, 151–163 (2003).

23. Maupin-Furlow, J. A., and J. G. Ferry.. A proteasome for the methanogenic archaeon *Methanosarcina thermophila*. J. Biol. Chem. 270:28617– 28622 (1995).

24. Gille C., Goede A., Preißner C. S. R., Kloetzel P. M., Göbel U. B. A Comprehensive View on Proteasomal Sequences: Implications for the Evolution of the Proteasome J. Mol. Biol. 326, 1437–1448 (2003).

25. Stock, D. et al. Catalytic mechanism of the 20S proteasome of *Thermoplasma acidophilum* revealed by X-ray crystallography. Cold Spring Harb. Symp. Quant. Biol. 60, 525–532 (1995).

26. Dahlmann B, Kopp F, Kuehn L, Niedel B, Pfeifer G, Hegerl R & Baumeister W The multicatalytic proteinase (prosome) is ubiquitous from eukaryotes to archaebacteria. FEBS Lett. 251: 125–131 (1989).

27. Grziwa, A., Baumeister, W., Dahlmann, B., Kopp, F., Localization of subunits in proteasomes from *Thermoplasma acidophilum* by immunoelectron microscopy. FEBS Lett. 290, 186–190 (1991).

28. Lupas, A., Zuhl, F., Tamura, T., Wolf, S., Nagy, I., De Mot, R. & Baumeister, W. Eubacterial proteasomes. Mol. Biol. Rep. 24, 125–131 (1997).

29. Missiakas, D., Schwager, F., Betton, J. M., Georgopoulos, C. & Raina, S. Identification and characterization of HslV HslU (ClpQ ClpY) proteins involved in overall proteolysis of misfolded proteins in *Escherichia coli.* EMBO J. 15, 6899–6909 (1996).

30. Couvreur, B., Wattiez, R., Bollen, A., Falmagne, P., Le Ray, D., Dujardin, J.C., Eubacterial HslV and HslU subunits homologs in primordial eukaryotes. Mol. Biol. Evol. 19, 2110–2117 (2002).

31. Neuwald, A. F., L. Aravind, J. L. Spouge, and E. V. Koonin. AAA:a class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. Genome Res. 9:27–43 (1999).

32. Zwickl, P., Baumeister, W. and Steven, A. Dis-assembly lines: the proteasome and related ATPase-assisted proteases. Curr. Opin. Struct. Biol., 10, 242-250 (2000).

33. Swaffield JC & Purugganan MD The evolution of the conserved ATPase domain (CAD): reconstructing the history of an ancient protein module. J. Mol. Evol. 45: 549–563 (1997).

34. Zwickl, P., Lottspeich, F., Dahlmann, B., and Baumeister, W. Cloning and sequencing of the gene encoding the large subunit of the proteasome from *Thermoplasma acidophilum*. FEBS Lett. 278: 217–221 (1991).

35. Zwickl, P., D. Ng, K. M. Woo, H.-P. Klenk, and A. L. Goldberg. An archaebacterial ATPase, homologous to ATPases in the eukaryotic 26S proteasome, activates protein breakdown by 20S proteasomes. J. Biol. Chem. 274:26008–26014 (1999).

36. Knipfer N, Shrader TE: Inactivation of the 20S proteasome in *Mycobacterium smegmatis*. Mol Microbiol, 25:375-383 (1997).

37. Knipfer, N., Seth, A., Roudiak, S. G. & Shrader, T. E. Species variation in ATP-dependent protein degradation: protease profiles differ between mycobacteria and protease functions differ between *Mycobacterium smegmatis* and *Escherichia coli*. Gene, 231, 95–104 (1999).

38. Ma, J., Katz, E., Belote, J.M., Expression of proteasome subunit isoforms during spermatogenesis in *Drosophila melanogaster*. Insect Mol. Biol. 11, 627–639 (2001).

39. Fu, H., J. H. Doellıng, C. S. Arendt, M. Hochstrasser, And R. A. Vıerstra. Molecular organization of the 20S proteasome gene family from *Arabidopsis thaliana.* Genetics 149:677–692 (1998).

40. Kaczowka, S.J., Maupin-Furlow, J.A., Subunit topology of two 20S proteasomes from *Haloferax volcanii*. J. Bacteriol. 185, 165–174 (2003).

41. Glickman M.H., Rubin D.M., Fu H., Larsen C., Coux O., Wefes I., Pfeifer G., Cjeka Z., Vierstra R., Baumeister W., Functional analysis of the proteasome regulatory particle Molecular Biological Report, 26, 21–28 (1999).

42. Hough R, Pratt G, Rechsteiner M. Ubiquitin-lysozyme conjugates. J. Biol. Chem. 261:2400–8 (1986).

43. Brodsky, ER-associated and proteasome-mediated protein degradation: how two topologically restricted events came together. Trends in Cell Biology 7, 151-155 (1997).

44. Townsley, F.M. and Ruderman, J.V. Proteolytic ratchets that control progression through mitosis. Trends Cell Biol. 8, 238-244 (1998).

45. Huibregtse JM, Maki CG, Howley PM. In Ubiquitin and the Biology of the Cell, ed. JM Peters, JR Harris, D Finley, pp. 324–43 New York: Plenum (1998).

46. Voges D, Zwickl P, Baumeister W: The 26S proteasome: a molecular machine designed for controlled proteolysis. Annu Rev Biochem 68:1015-1068 (1999).

47. Hirsch, C., Ploegh, H.L., Intracellular targeting of the proteasome. Trends Cell Biol. 10, 268–272 (2000).

48. Rock K.L., Gramm C., Rothstein L., Clark K., Stein R., Dick L., Hwang D. and Goldberg A.L. Inhibitors of proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell, 78, 761–771 (1994).

49. Turner, G.C., Varshavsky, A., Detecting and measuring cotranslational protein degradation in vivo. Science 289, 2117–2120 (2000).

50. Kloetzel P: Antigen processing by the proteasome. Nat Rev Mol Cell Biol 2:179-187 (2001).

51. Glickman M.H., Rubin D.M., Fried V.A. and Finley D. The regulatory particle of the *S. cerevisiae* proteasome. Mol. Cell. Biol., 18, 3149–3162 (1998).

52. Wollenberg K. And Swaffield J.C. Evolution of Proteasomal ATPases, Molecular Biology and Evolution, 18 (6): 962-974 (2001).

53. Arendt, C.S. and Hochstrasser, M. Identification of the yeast 20S proteasome catalytic centers and subunit interactions required for active-site formation. Proc. Natl Acad. Sci. USA, 94, 7156–7161(1997).

54. Heinemeyer, W., Trondle, N., Albrecht, G. & Wolf, D. H. PRE5 and PRE6, the last missing genes encoding 20S proteasome subunits from yeast Indication for a set of 14 different subunits in the eukaryotic proteasome core. Biochemistry, 33, 12229–12237 (1994).

55. Peters, J. M., Cejka, Z., Harris, J. R., Kleinschmidt, J. A., and Baumeister, W. Structural features of the 26 S proteasome complex. J Mol Biol 234, 932-937. (1993).

56. Dahlmann, B., Kopp, F., Kristensen, P., Hendil, K.B., Identical subunit topographies of human and yeast 20S proteasomes. Arch. Biochem. Biophys. 363, 296–300 (1999).

57. Groll M, Bochtler M, Brandstetter H, Clausen T and Huber R, Molecular Machines for Protein Degradation, ChemBioChem, 6, 222-256 (2005).

58. De Mot, R., Nagy I. and Baumeister W., A self-compartmentalizing protease in *Rhodococcus*: the 20S proteasome. Antonie van Leeuwenhoek 74: 83–87, (1998).

59. Pamer, E. & Creswell, P. Mechanisms of MHC class Irestricted antigen processing. Annu. Rev. Immunol. 16, 323–358 (1998).

60. Akopian, T.N., Kisselev, A.F., and Goldberg, A.L. Processive degradation of proteins and other catalytic properties of the proteasome from *Thermoplasma acidophilum.* J Biol Chem 272: 1791±1798 (1997).

61. Groll M, Huber R Substrate access and processing by the 20S proteasome core particle, The International Journal of Biochemistry & Cell Biology 35: 606–616 (2003).

62. Hershko, A., and A. Ciechanover. The ubiquitin system. Annu. Rev. Biochem. 67:425–479 (1998).

63. Ciechanover, A. The ubiquitin-proteasome pathway: on protein death and cell life. EMBO J 17, 7151-7160 (1998).

64. Thrower, J. S., L. Hoffman, M. Rechsteiner, and C. M. Pickart. Recognition of the polyubiquitin proteolytic signal. EMBO J. 19:94-102 (2000).

65. Chau V, Tobias JW, Bachmair A, Marriot D, Ecker DJ, et al. A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. Science 243:1576–831989.

66. Cıechanover, A., A. Orıan, And A. L. Schwartz. Ubiquitin-mediated proteolysis: biological regulation via destruction. BioEssays 22:442–451.

67. Ciechanover, A. And Schwartz, A. The ubiquitin-proteasome pathway: the complexity and myriad functions of proteins death. Proc Natl Acad Sci U S A 95, 2727-2730 (1998).

68. Murakami, Y., Matsufuji, S., Kameji, T., et al. Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination. Nature 360, 597–599 (1992).

69. Yoshimura, T., Kameyama, K., Takagi, T., Ikai, A., Tokunaga, F., Koide, T., Tanahashi, N., Tamura, T., Cejka, Z., Baumeister, W., and et al. Molecular characterization of the "26S" proteasome complex from rat liver. J Struct Biol 111, 200-211. (1993).

70. Kunst F, Ogasawara N, Moszer I, Albertini AM, Alloni G, et al. The complete genome sequence of the grampositive bacterium *Bacillus subtilis*. Nature 390:249–56 (1997).

71. Tamura, T., Nagy, I., Lupas, A., Lottspeich, F., Cejka, Z., Schoofs, G. et al. The first characterization of a eubacterial proteasome: the 20S complex of *Rhodococcus*. Curr. Biol. 5, 766–774 (1995).

72. De Mot, R., Nagy, I., Walz, J., Baumeister, W., Proteasomes and other self-compartmentalizing proteases in prokaryotes. Trends Microbiol. 7, 88–92 (1999).

73. Zühl F, Tamura T, Dolenc I, Cejka Z, Nagy I, DeMot R, Subunit topology of the *Rhodococcus* proteasome. FEBS Lett. 400: 83–90 (1997b).

74. Nagy I, Tamura T, Vanderleyden J, Baumeister W & De Mot R The 20S proteasome of *Streptomyces coelicolor*. J Bacteriol 180: 5448–5453 (1998).

75. Bauer, M. W., S. H. Bauer, and R. M. Kelly. Purification and characterization of a proteasome from the hyperthermophilic archaeon *Pyrococcus furiosus*. Appl. Environ. Microbiol. 63:1160–1164 (1997).

76. Bult, C. J., O. White, G. J. Olsen, L. Zhou, D. Fleischmann, G. G. Sutton, J. A. Blake, Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. Science 273:1058–1073. (1996).

77. Smith, D. R., L. A. Doucette-Stamm, C. Deloughery, H. Lee, J. Dubois, T. Aldredge, R. Bashirzadeh, D. Blakely, R. Cook, K. Gilbert, D. Harrison, L. Hoang, P. Keagle, W. Lumm, B. Pothier, D. Qiu, R. Complete genome sequence of *Methanobacterium thermoautotrophicum* DH: functional analysis and comparative genomics. J. Bacteriol. 179:7135–7155 (1997).

78. Klenk, H. P., R. A. Clayton, J. F. Tomb, O. White, K. E. Nelson, K. A. Ketchum, R. J. Dodson, M. Gwinn, E. K. Hickey, J. D. Peterson, D. L. The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon *Archaeoglobus fulgidus*. Nature 390:364–370 (1997).

79. Kawarabayasi, Y., Sawada M., Horikawa H., Haidawa Y., Hino Y., Yamamoto S., Sekine M., Baba S., Kosugi H., Hosoyama A., Complete sequence and gene organization of the genome of a hyper-thermophilic archaebacterium, *Pyrococcus horikoshii* OT3. DNA Res. 5:147–155 (1998).

80. Franzetti, B., G. Schoehn, D. Garcia, R. W. H. Ruigrok, and G. Zaccai. Characterization of the proteasome from the extremely halophilic archaeon *Haloarcula marismortui*. Archaea 1:53–61 (2002).

81. Ng, W.V., S.P. Kennedy, G.G Mahairas, et al. From the cover: genome sequence of *Halobacterium* species NRC-1. Proc. Natl. Acad. Sci. 97:12,176–12,181 (2000).

82. Gibbon, Fitz ST, Ladner H, Kim UJ, Stetter KO, Simon MI, Miller JH: Genome sequence of the hyperthermophilic crenarchaeon *Pyrobaculum aerophilum.* Proc Natl Acad Sci USA, 99:984- 989 (2002).

83. Wilson, H.L., Aldrich, H.C., Maupin-Furlow, J.A., Halophilic 20S proteasomes of the archaeon *Haloferax volcanii*: purification, characterization, and gene sequence analysis. J. Bacteriol. 181, 5814–5824 (1999).

84. Zwickl, P., Lottspeich, F., and Baumeister, W. Expression of functional *Thermoplasma acidophilum* proteasomes in Escherichia coli. FEBS Lett. 312: 157–160 (1992).

85. Dahlmann, B., Kuehn, L., Grziwa, A., Zwickl, P., and Baumeister, W. Biochemical properties of the proteasome from *Thermoplasma acidophilum*. Eur. J. Biochem. 208: 789–797 (1992).

86. Zwickl, P., Kleinz, J., and Baumeister, W. Critical elements in proteasome assembly. Struct. Biol. 1: 765–769 (1994).

87. Pűhler, G., Weinkauf, S., Bachmann, L., Műller, S., Engel, A., Hegerl, R., Subunit stoichiometry and three-dimensional arrangement in proteasomes from *Thermoplasma acidophilum*. EMBO J 11: 1607-1616 (1992).

88. Leonhard, K., Stiegler, A., Neupert, W., and Langer, T. Chaperone-like activity of the AAA domain of the yeast Yme1 AAA protease. Nature 398, 348 – 351 (1999).

89. Ruepp, A., Eckerskorn, C., Bogyo, M., Baumeister, W., Proteasome function is dispensable under normal but not under heat shock conditions in *Thermoplasma acidophilum*. FEBS Lett. 425, 87–90 (1998).

90. Seemüller E, Lupas A & Baumeister W Autocatalytic processing of the 20S proteasome. Nature 382: 468–470 (1996).

91. Rob J.Frankenberg, Tına S.Hsu, Hısao Yakota, Rosalınd Kım, And Douglas S.Clark Chemical denaturation and elevated folding temperatures are required for wild-type activity and stability of recombinant *Methanococcus jannaschii* 20S proteasome, Protein Science, 10:1887–1896 (2001).

92. Chen, P., Hochstrasser, M., Autocatalytic subunit processing couples active site formation in the 20S proteasome to completion of assembly. Cell 86, 961–972 (1996).

93. Schmidtke, G., Kraft, R., Kostka, S., Henklein, P., Frommel, C., Lo¨ we, J., Huber, R., Kloetzel, P.M., Schmidt, M., Analysis of mammalian 20S proteasome biogenesis: the maturation of bsubunits is an ordered two-step mechanism involving autocatalysis. EMBO J. 15, 6887–6898 (1996).

94. Heinemeyer, W., Fischer, M., Krimmer, T., Stachon, U. & Wolf, D. H. The active sites of the eukaryotic 20S proteasome and their involvement in subunit precursor processing. J. Biol. Chem. 272, 25200–25209 (1997).

95. Rivett, A.J., Proteasomes: multicatalytic proteinase complexes. Biochem. J. 291, 1–10 (1993).

96. Orlowski, M., Cardozo, C., Michaud, C., Evidence for the presence of five distinct proteolytic components in the pituitary multicatalytic proteinase complex. Properties of two component cleaving bonds on the carboxyl side of branched chain and small neutral amino acids. Biochemistry 32, 1563–1572.

97. Heinemeyer, W., Ramos, P. C., and Dohmen, R. J. The ultimate nanoscale mincer: assembly, structure and active sites of the 20S proteasome core. Cell Mol Life Sci 61, 1562-1578 (2004).

98. Maupin-Furlow, J. A., H. C. Aldrich, and J. G. Ferry.. Biochemical characterization of the 20S proteasome from the methanoarchaeon *Methanosarcina thermophila*. J. Bacteriol. 180:1480–1487 (1998).

99. Wilson, H.L., Ou, M.S., Aldrich, H.C., Maupin-Furlow, J.A., Biochemical and physical properties of the *Methanococcus jannaschii* 20S proteasome and PAN, a homolog of the ATPase (Rpt) subunits of the eucaryal 26S proteasome. J. Bacteriol. 182, 1680–1692 (2000).

100. Wenzel, T., and W. Baumeister. *Thermoplasma acidophilum* proteasomes degrades partially unfolded and ubiquitin-associated proteins. FEBS Lett. 326:215–218 (1993).

101. Gardrat, F., Fraigneau, B., Montel, V., Raymond, J., Azanza, J.L., Effect of high hydrostatic pressures on 20S proteasome activity. Eur. J. Biochem. 262, 900–906 (1999).

102. Arendt, C.S., Hochstrasser, M., Eukaryotic 20S proteasome catalytic subunit propeptides prevent active site inactivation by N-terminal acetylation and promote particle assembly. EMBO J. 18, 3575–3585 (1999).

103. Emmerich, N.P., Nussbaum, A.K., Stevanovic, S., Priemer, M., Toes, R.E., Rammensee, H.G., Schild, H., The human 26S and 20S proteasomes generate overlapping but different sets of peptide fragments from a model protein substrate. J. Biol. Chem. 275, 21140–21148 (2000).

104. Dick, T.P., Nussbaum, A.K., Deeg, M., Heinemeyer, W., Groll, M., Schirle, M., Keilholz, W., Stevanovic, S., Wolf, D.H., Huber, R., Rammensee, H.G., Contribution of proteasomal b-subunits to the cleavage of peptide substrates analyzed with yeast mutants. J. Biol. Chem. 273, 25637–25646 (1998).

105. Fenteany, G., Standaert, R.F., Lane, W.S., Choi, S., Corey, E.J., Schreiber, S.L., Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. Science 268, 726–731 (1995).

106. Groetrupp, M., A. Soza, M. Eggers, L. Kuehn, T. P. Dıck, H. Schıld, H. G. Rammensee, U. H. Koszınowskı, And P. M. Kloetzel A role for the proteasome regulator PA28 in antigen presentation. Nature 381:166-168 (1996).

107. Groettrup, M., A. Soza, U. Kuckelkorn, And P. M. Kloetzel. Peptide antigen production by the proteasome: complexity provides efficiency. Immunol. Today 17:429– 435 (1996).

108. Chu-Ping, M., Vu, J. H., Proske, R. J., Slaughter, C. A., and DeMartino, G. N. Identification, purification, and characterization of a high molecular

weight, ATP-dependent activator (PA700) of the 20S proteasome. J. Biol. Chem. 269, 3539-3547 (1994).

109. DeMartino, G.N., The proteasome, a novel protease regulated by multiple mechanisms. J. Biol. Chem. 274, 22123–22126 (1999).

110. Groll, M., Bajorek, M., Kohler, A., Moroder, L., Rubin, D. M., Huber, R. et al. A gated channel into the proteasome core particle. Nature Struct. Biol. 7, 1062–1067 (2000).

111. Hoffman, L. and Rechsteiner, M. Activation of the multicatalytic protease. J. Biol. Chem., 269, 16890–16895 (1994).

112. Kohler, A., Cascio, P., Leggett, D.S., Woo, K.M., Goldberg, A.L., Finley, D., The axial channel of the proteasome core particle is gated by the Rpt2 ATPase and controls both substrate entry and product release. Mol. Cell 7, 1143–1152 (2001).

113. Whitby, F.G., Masters, E.I., Kramer, L., Knowlton, J.R., Yao, Y.,Wang, C.C., Hill, C.P., Structural basis for the activation of 20S proteasomes by 11S regulators. Nature 408, 115–120 (2000).

114. Ma, C.P., Slaughter, C.A., and DeMartino, G.N. Identification, purification, and characterization of a protein activator (PA28) of the 20S proteasome. J. Biol. Chem. 267, 10515–10523 (1992).

115. Rechsteiner, M., Realini, C., Ustrell, V., The proteasome activator 11 S REG (PA28) and class I antigen presentation. Biochem. J. 345, 1–15 (2000).

116. Coux O, Tanaka K & Goldberg AL Structure and functions of the 20S and 26S proteasomes. Annu. Rev. Biochem. 65: 801– 847 (1996).

117. Seemüller E, Lupas A, Stock D, Löwe J, Huber R & Baumeister W Proteasome from *Thermoplasma acidophilum:* a threonine protease. Science 268: 579–582 (1995).

118. Brannigan, J.A., Dodson, G., Duggleby, H.J., Moody, P.C.E., Smith, J.L., Tomchick, D.R., A protein catalytic framework with an N-terminal nucleophile is capable of selfactivation. Nature 378, 416–419 (1995).

119. Seemüller, E., Lupas, A., Zuhl, F., Zwickl, P., and Baumeister, W. The proteasome from *Thermoplasma acidophilum* is neither a cysteine nor a serine protease. FEBS Lett 359, 173-178.

120. Kisselev, A.F., Akopian, T.N., and Goldberg, A.L. Range of sizes of peptide products generated during degradation of different proteins by Archaeal proteasomes. J. Biol. Chem. 273, 1982–1989 (1998).

121. Kisselev, A.F., Akopian, T.N., Woo, K.M., Goldberg, A.L., The sizes of peptides generated from protein by mammalian 26 and 20S proteasomes. Implications for understanding the degradative mechanism and antigen presentation. J. Biol. Chem. 274, 3363–3371 (1999).

122. Lee, C., Schwartz, M.P., Prakash, S., Iwakura, M., Matouschek, A., ATP-dependent proteases degrade their substrates by processively unraveling them from the degradation signal. Mol. Cell 7, 627–637 (2001).

123. Wenzel, T., Eckerskorn, C., Lottspeich, F., and Baumeister, W. Existence of a molecular ruler in proteasomes suggested by analysis of degradation products. FEBS Lell. 349, 205-209 (1994).

124. Nussbaum, A.K., Dick, T.P., Keilholz, W., Schirle, M., Stevanovic, S., Dietz, K., Heinemeyer, W., Groll, M., Wolf, D.H., Huber, R., Rammensee, H.G., Cleavage motifs of the yeast 20S proteasome β subunits deduced from digests of enolase 1. Proc. Natl. Acad. Sci. USA 95, 12504–12509 (1998).

125. Cardozo, C., Michaud, C., Proteasome-mediated degradation of tauproteins occurs independently of the chymotrypsin-like activity by a nonprocessive pathway. Arch. Biochem. Biophys. 408, 103–110 (2002).

126. Liu, C.W., Corboy, M.J., DeMartino, G.N., Thomas, P.J., Endoproteolytic activity of the proteasome. Science 299, 408–411 (2003).

127. Lee, C., Prakash, S., Matouschek, A., Concurrent translocation of multiple polypeptide chains through the proteasomal degradation channel. J. Biol. Chem. 277, 34760–34765 (2002).

128. Groll,M., Brandstetter,H., Bartunik,H., Bourenkow,G. and Huber,R. Investigations on the maturation and regulation of archaebacterial proteasomes. J. Mol. Biol., 327, 75-83 (2003).

129. Patel, S., and Latterich, M. The AAA team: related ATPases with diverse functions. Trends Cell. Biol. 8, 65 –71 (1998).

130. Vale, R. D. AAA proteins: lords of the ring. J. Cell Biol. 150:F13–F19 (2000).

131. Zwickl, P., Baumeister, W., AAA-ATPases at the crossroads of protein life and death. Nat. Cell Biol. 1, E97–E98 (1999).

132. Beyer A. Sequence analysis of the AAA protein family. Protein Sci. 6: 2043–2058 (1997).

133. Förster A, Whitby F. G.and Hill C. P. The pore of activated 20S proteasomes has an ordered 7-fold symmetric conformation The EMBO Journal Vol. 22 No. 17 pp. 4356-4364, (2003).

134. Golbik R., Lupas A.N., Koretke K.K., Baumeister W., Peters J., The Janus face of the archaeal Cdc48/p97 homologue VAT: protein folding versus unfolding. Biol. Chem. 380, 1049–1062 (1999).

135. Schtılerman, M., G. H. Lorımer, And S. W. Englander. Chaperonin function: folding by forced unfolding. Science 284:822–825 (1999).

136. Strickland, E., Hakala, K., Thomas, P.J., DeMartino, G.N., Recognition of misfolding proteins by PA700, the regulatory subcomplex of the 26 S proteasome. J. Biol. Chem. 275, 5565–5572 (2000).

137. Wolf S, Nagy I, Lupas A, Pfeifer G, Cejka Z, Müller S, Engel A, De Mot R, Characterization of ARC, a divergent member of the AAA ATPase family from *Rhodococcus erythropolis*. J. Mol. Biol. 277: 13–25 (1998).

138. Ahn, K. et al. In vivo characterization of the proteasome regulator PA28. J. Biol. Chem. 271, 18237– 18242 (1996).

139. Hill, C.P., Masters, E.I., Whitby, F.G., The 11S regulators of 20S proteasome activity. Curr. Top. Microbiol. Immunol. 268, 73–89 (2002).

140. Kuehn L, Dahlmann B. Reconstitution of proteasome activator PA28 from isolated subunits: optimal activity is associated with an alpha,beta-heteromultimer. PG. FEBS Lett 394: 183–186 (1996).

141. Dick, L.R., T. Ruppert, M. Groettrup, P. M. Kloetzel, L. Kuehn, U. H. Koszınowskı, Coordinated dual-cleavage induced by the proteasome regulator PA28 leads to dominant MHC ligands. Cell 86:253–262 (1996).

142. Knowlton,J.R., Johnston,S.C., Whitby,F.G., Realini,C.R., Zhang,Z., Rechsteiner,M.C. and Hill,C.P. Structure of the proteasome activator REGa (PA28a). Nature, 390, 639-643 (1997).

143. Groll M., Clauseny T., Molecular shredders: how proteasomes fulfill their role, Current Opinion in Structural Biology, 13:665–673 (2003).

144. Aravind, L., and Ponting, C.P. Homologues of 26S proteasome subunits are regulators of transcription and translation. Protein Sci. 7, 1250–1254 (1998).

145. Coles, M., Diercks, T., Liermann, J., GroÈ ger, A., Rockel, B., Baumeister, W. et al. The solution structure of VAT-N reveals a “missing link” in the evolution of complex enzymes from a simple βαββ element. Curr. Biol. 9, 1158-1168 (1999).

146. Confalonieri F & Duguet M A 200-amino acid ATPase module in search of a basic function. BioEssays 17: 639–650 (1995).

147. Dubiel,W., Ferrell,K., Pratt,G. and Rechsteiner,M. Subunit 4 of the 26S protease is a member of a novel eukaryotic ATPase family. J. Biol. Chem., 267, 22669–22702 (1992).

148. Finley, D., Tanaka, K., Mann, C., Feldmann, H., Hochstrasser, M., Vierstra, Unified nomenclature for subunits of the *Saccharomyces cerevisiae* proteasome regulatory particle. Trends Biochem. Sci. 23, 244–245 (1998).

149. Braun, B.C., Glickman, M., Kraft, R., Dahlmann, B., Kloetzel, P.M., Finley, D., Schmidt, M., The base of the proteasome regulatory particle exhibits chaperone-like activity. Nat. Cell Biol. 1, 221–226 (1999).

150. Armon,T., Ganoth,D. and Hershko, A. Assembly of the 26S complex that degrades proteins ligated to ubiquitin is accompanied by the formation of ATPase activity. J. Biol. Chem., 265, 20723–20726 (1990).

151. Peters, J.M. Proteasomes: protein degradation machines of the cell. Trends Biochem. Sci. 19, 377–382 (1994).

152. Hartl, F.U.: Molecular chaperones in cellular protein folding. Nature 381, 571-579 (1996).

153. Larsen, C. N., and Finley, D. Protein translocation channels in the proteasome and other proteases, Cell 91, 431–434 (1997).

154. Deveraux, Q., V. Ustrell, C. Pickart, A 26S protease subunit that binds ubiquitin conjugates. J. Biol. Chem. 269:7059-7061 (1994).

155. Rechsteiner, M., L. Hoffman and W. Dubiel. The multicatalytic and 26S proteases. J. Biol. Chem. 268:6065–6068 (1993).

156. Benaroudj, N., Goldberg, A.L., PAN, the proteasome-activating nucleotidase from archaebacteria, is a protein-unfolding molecular chaperone. Nat. Cell Biol. 2, 833–839 (2000).

157. Ruepp, A., Rockel, B., Gutsche, I., Baumeister, W. & Lupas, A. N. The chaperones of the archaeon *Thermoplasma acidophilum*. J. Struct. Biol. 135, 126-138 (2001).

158. Hoskins, J. R., S. K. Singh, M. R. Maurizi, and S. Wickner. Protein binding and unfolding by the chaperone ClpA and degradation by the protease ClpAP. Proc. Natl. Acad. Sci. USA 97:8892–8897 (2000).

159. Kim, Y.I., Burton, R.E., Burton, B.M., Sauer, R.T., and Baker, T.A. Dynamics of substrate denaturation and translocation by the ClpXP degradation machine. Mol. Cell 5, 639–648 (2000).

160. Navon, A. and Goldberg, A.L. Proteins are unfolded on the surface of the ATPase ring before transport into the proteasome. Mol. Cell, 8, 1339±1349 (2001).

161. Weber-Ban, E. U., Reid, B. G., Miranker, A. D. & Horwich, A. L. Global unfolding of a substrate protein by the Hsp100 chaperone ClpA. Nature, 401, 90-93. (1999).

162. Rockel, B., Jakana, J., Chiu, W., Baumeister, W., Electron cryomicroscopy of VAT, the archaeal p97/CDC48 homologue from *Thermoplasma acidophilum*. J. Mol. Biol. 317, 673–681. (2002).

163. Pamnani, V., Tamura, T., Lupas, A., Peters, J., Cejka, Z., Ashraf, W., and Baumeister, W. Cloning, sequencing and expression of VAT, a Cdc48/P97 ATPase homologue from the Archaeon *Thermoplasma acidophilum.* FEBS Letters 404, 263-268 (1997).

164. Song, C., Wang, Q., Liu, C.C., ATPase activity of p97-VCP: D2 mediates the major enzyme activity and D1 contributes to the heatinduced activity. J. Biol. Chem. 278, 3648–3655 (2003).

165. Ruepp A, Graml W, Santos-Martinez ML, Koretke KK, Volker C, Mewes HW, Frishman D, Stocker S, Lupas AN, Baumeister W: The genome sequence of the thermoacidophilic scavenger *Thermoplasma acidophilum*. Nature, 407:508-513 (2000).

166. Kawashima T, Amano N, Koike H, Makino S, Higuchi S, Kawashima-Ohya Y, Watanabe K, Yamazaki M, Kanehori K, Kawamoto T, et al. Archaeal adaptation to higher temperatures revealed by genomic sequence of *Thermoplasma volcanium*. Proc Natl Acad Sci USA 97:14257-14262 (2000).

167. Bertani G Studies in lysogenesis I. The mode of phage liberation by lysogenic *Escherichia coli*. J Bacteriol 62:293-300 (1951).

168. Lanzetta, P. A., Alvarez, L. J., Reinach, P. S. and Candia, O. A. An improved assay for nanomole amounts of inorganic phosphate. Anal. Biochem. 100, 95-97 (1979).

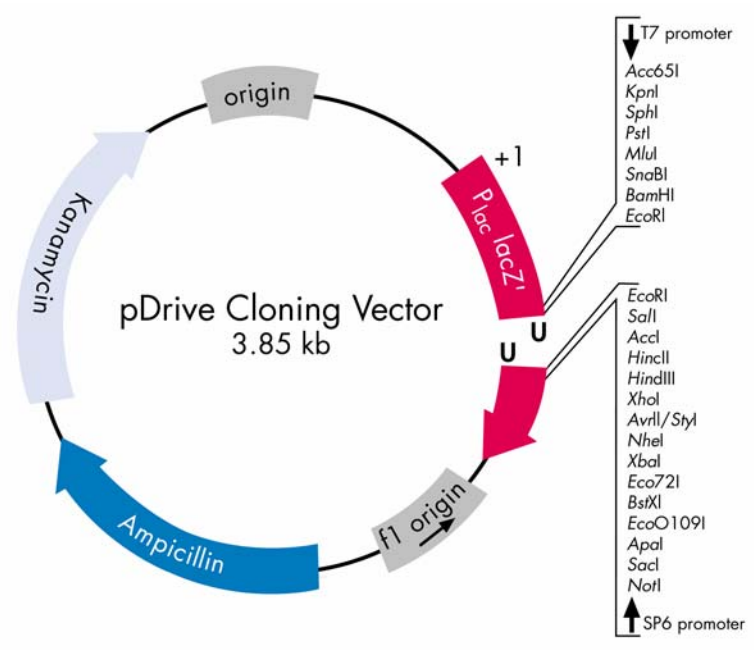
169. Zwickl, P., Grziwa, A., Puhler, G., Dahlmann, B., Lottspeich, F., and Baumeister, W., Structure of the *Thermoplasma* proteasome and its implications for the function and evolution of the multicatalytic proteinase. Biochemistry 31: 964–972 (1992).

170. Pouch, M.N., Cournoyer, B., Characterization of the 20S proteasome from the actinomycete *Frankia*. Mol. Microbiol. 35, 368–377 (2000).

APPENDICES

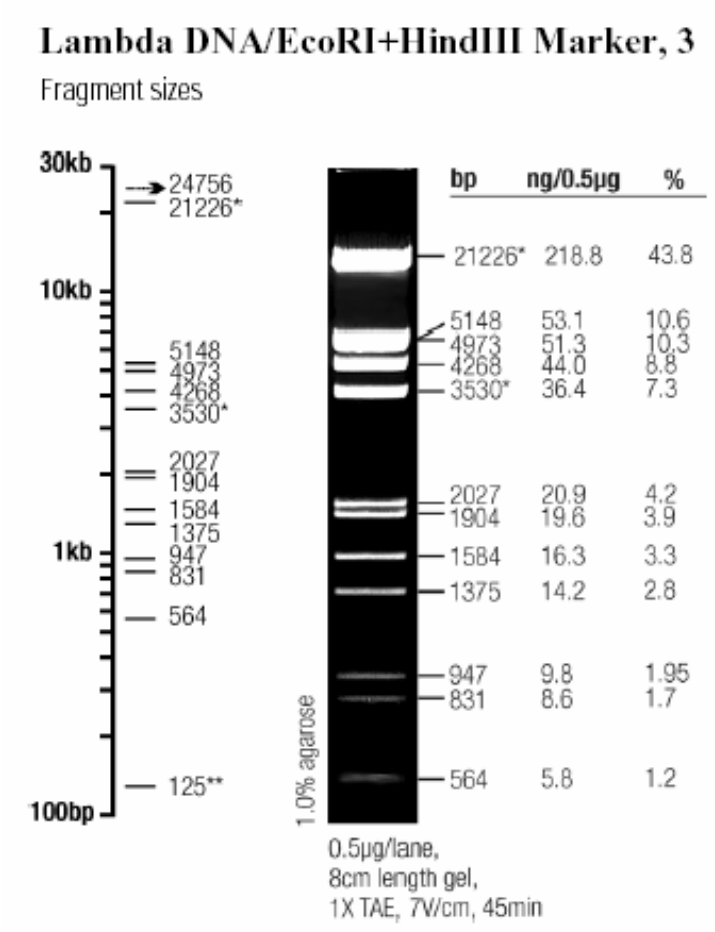
APPENDIX A

pDRIVE CLONING VECTOR



APPENDIX B

EcoRI+HindIII cut Lambda DNA (MBI Fermentas AB, Vilnius, Lithuania) molecular size marker.



APPENDIX C

BUFFERS AND SOLUTIONS

LB Medium

10 g tryptone

5 g yeast extract

10 g NaCl

Proteasome Assay Buffer

20 mM Tris-HCl

1 mM EDTA

1mM NaN_3

Adjust pH to 7.5 with 10M HCl.

TE Buffer

10 mM Tris-HCl, pH 7.4

1 mM EDTA, pH 7.0

Autoclave the solution at 121^{o}C for 20 min. And store at 4^{o}C.

Tris HCl with Sucrose pH 8.0

50 mM Tris

25% Sucrose

50X TAE Buffer

242 g Tris-HCl

57.1 ml Glacial acetic-acid

100 ml 0.5 M EDTA, pH 8.5

Complete the solution to 1 liter with distilled water, autoclave at 121°C for 20 minutes and store at 4°C.

Tracking Dye Type IV

0.25% Bromophenol Blue

40% (w/v) Sucrose in ddH_2O

Autoclave at 121°C for 20 minutes and store at 4°C.

TSS solution

0.5 M $MgCl_2$ (or $MgSO_4$) 10 ml

50% PEG-800 20.0 ml

DMSO 5.0 ml

LB-Broth 65 ml

Store the solution inside of a dark bottle, at 4^oC.

Malachite Green Reagent (in 100 mL 0,7 M HCl)

0,2 gr Sodium Molybdate

30 mg Malachite Green oxalate salt

50 µL Triton X-100

Tris Buffer supplemented with ATP and β-mercaptoetanol

50 mM Tris

100 mM NaCl, pH 8.0

5 mM β-mercaptoetanol

5 mM $MgCl_2$

1 mM ATP

Gel Loading Buffer

0.25% Bromophenol blue

40% Sucrose

2X Sample Buffer for SDS-PAGE (1 Lt)

50 mM Tris-HCl, pH 6.8

10% (v/v) Glycerol

Dissolve in 35 ml distilled water and adjust pH to 6.75 with concentrated HCl; then add,

1% (w/v) SDS

0.01% (m/v) Bromophenol Blue

Dilute with distilled water to a final volume of 100 ml, and store the solution at -20^{o}C.

ATPase Assay Buffer (in 10 mL 18 mM HEPES)

10 mM β-mercaptoetanol

5 mM $MgCl_2$

2 mM ATP

Running Buffer for SDS-PAGE (500 ml)

1.51 g Tris-HCl

7.2 g Glycine

0.5 g SDS

Complete the solution to 1 liter with distilled water, read pH about 8.3, autoclave at 121°C for 20 minutes and store at 4°C.

Coomassie Blue Staining Solution (1 Lt)

1 g Coomassie Brilliant Blue R-250

450 ml Methanol

450 ml dH_2O

100 ml Glacial acetic acid

Destaining Solution (1 Lt)

100 ml Methanol

100 ml Glacial acetic acid

800 ml dH_2O