PURIFICATION AND CHARACTERIZATION OF CYTOPLASMIC AND PROTEASOME ASSOCIATED CHYMOTRYPSIN-LIKE PROTEASES FROM *THERMOPLASMA VOLCANIUM*

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Approval of the Graduate School of Natural and Applied Sciences.

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ABSTRACT

PURIFICATION AND CHARACTERIZATION OF CYTOPLASMIC AND PROTEASOME ASSOCIATED CHYMOTRYPSIN-LIKE PROTEASES FROM *THERMOPLASMA VOLCANIUM*

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In this study, two novel cytoplasmic serine proteases were isolated and characterized from thermophilic archaea *Thermoplasma volcanium*. The first protease was purified by ion exchange and affinity chromatographies and identified as a chymotrypsin-like serine protease mainly based on its substrate profile and inhibition pattern. The presence of protease activity was analyzed by gelatin zymography which was detected as a single band (35 kDa). Optimum temperature was found to be 60^{o}C for azocasein hydrolysis and 50^{o}C for N-Suc-Phe-pNA hydrolysis. Optimum activity was observed in the pH range of 6.0-8.0 with a maximum value at pH 7.0. The K_m

and V_{max} values for the purified protease were calculated to be 2.2 mM and 40 μmoles of p-nitroanilide released $min^{-1}.ml^{-1}$, respectively, for N-Suc-Phe-PNA as substrate. Ca^{2+} and Mg^{2+} at 4 mM concentrations were the most effective divalent cations in activating the enzyme.

In the second stage of this study, 20S proteasome of *Tp. volcanium* with substantial chymotrypsin-like activity was purified and characterized. This enzyme complex was purified with 19.1 U/mg specific activities from cell free extract by a four-step procedure. SDS-PAGE analysis revealed two strong bands with relative molecular masses of 26 kDa (α-subunit) and 21.9 kDa (β-subunit). *Tp. volcanium* 20S proteasome predominantly catalyzed cleavage of peptide bonds carboxyl to the acidic residue Glu (postglutamyl activity) and the hydrophobic residue Phe (chymotrypsin-like activity) in short chromogenic peptides. Low-level hydrolyzing activity was also detected carboxyl to basic residue Arg (trypsin-like activity). Chymotrypsin-like activity of *Tp. volcanium* 20S proteasome was significantly inhibited by chymotrypsin specific serine protease inhibitor chymostatin. When N-CBZ-Arg was used which is a substrate for trypsin, 20S proteasome was strongly inhibited by TLCK. The optimum temperature for Ala-Ala-Phe-pNA hydrolysis by the *Tp. volcanium* 20S proteasome was $55^{o}C$ and the optimum pH was 7.5. The chymotryptic activity was significantly enhanced by divalent cations such as Ca^{+2} and Mg^{2+} at high concentrations, i.e. 125-250 mM.

Keywords:Serine protease, 20S proteasome, archaea, thermophilic protease, *Thermoplasma volcanium*, chymotrypsin-like serine protease.

ÖZ

THERMOPLASMA VOLCANIUM'DAN SİTOPLAZMİK VE PROTEAZOMAL KİMOTRİPSİN BENZERİ PROTEAZLARIN SAFLAŞTIRILMASI VE KARAKTERİZASYONU

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Eylül, 147 sayfa

Bu çalışmada thermophilik bir arkea olan *Thermoplasma volcanium*'dan sitoplazmik iki yeni serine proteaz enzimi saflaştırılmış ve karakterize edilmiştir. Birinci proteaz enzimi iyon değiştirici ve afinite kolon kromatografisi ile saflaştırılmış ve esas olarak substrat profili ve inhibasyon özelliklerine dayanılarak bir kimotripsin benzeri serin proteaz olarak tanımlanmıştır. Proteaz aktivitesinin varlığı tek bir bant olarak (35 kDa) jelatin-zimografisi ile analiz edilmiştir. Optimum sıcaklık, azocazein hidrolizi için 60^{o}C ve N-Suc-Phe-pNA hidrolizi için 50^{o}C bulunmuştur. Optimum enzim aktivitesi pH 6.0-8.0 aralığında gözlenmiş olup maksimum aktivite pH 7.0'de saptanmıştır. Saflaştırılan proteaz N-Suc-Phe-pNA substratı için K_m ve V_{max}

değerleri sırası ile 2.2 mM ve 40 μmoles.min^{-1}.ml^{-1} dir. Enzim aktivasyonu için en etkin iki değerlikli katyonlar 4 mM konsantrasyonunda Ca^{2+} ve Mg^{2+}'dur.

Çalışmanın ikinci kısmında, önemli düzeyde *Tp. volcanium* 20S proteazomu saflaştırılmış ve karakterize edilmiştir. Bu enzim kompleksi hücre içi özütünden 4 aşamadan sonra spesifik aktivitesi 19.1 U/mg olarak saflaştırılmıştır. SDS-PAGE analizi yaklaşık moleküler ağırlıkları 26 kDa (α-alt ünitesi) ve 21.9 kDa (β-alt ünitesi) olan iki güçlü bandın varlığını göstermiştir. *Tp. volcanium* 20S proteazom, başlıca küçük chromogenic peptitlerin karboksil bölgesinde yer alan asidik Glu (postglutamil aktivite) ve hidrofobik Phe (kimotripsin benzeri aktivite) rezidülerinin peptit bağlarının kesilmesini katalize etmektedir. *Tp. volcanium* 20S proteazomunun kimotripsin benzeri aktivitesi bir kimotripsin spesifik serine proteaz inhibitörü olan kimostatin ile önemli ölçüde inaktive edilmiştir. Tripsin için substrat olan N-CBZ-Arg kullanıldığında ise 20S proteasom aktivitesi TLCK tarafından güçlü bir şekilde inhibe edilmiştir. *Tp. volcanium* 20S proteazomuun Ala-Ala-Phe-pNa hidrolizi için optimum sıcaklık 55^{o} derecesi ve optimum pH derecesi 7.5'dur. Kimotriptik aktivite yüksek konsantrasyonlardaki (125-250 mM) Ca^{+2} and Mg^{2+} gibi metal iyonların varlığında belirgin olarak artmıştır.

Anahtar kelimeler: Serin proteaz, 20S proteasom, archaea, termofilik proteaz, *Thermoplasma volcanium*, kimotripsin benzeri serin proteaz.

Dedicated to my Parents

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APS	Ammonium Persulfate
Bis	N, N'-methylene-bisacrylamide
BSA	Bovine serume albumin
CBZ	carbobenzoxyl
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediamineteraacetic acid
EGTA	Ethyleneglycol-bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid
EtBr	Ethidiumbromide
MOPS	3-[N-morphalino] propanesulfanic acid
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethylsulfonyl flouride
pNA	Para nitroanilide
SDS	Dodecylsulphate sodium salt
Suc	Succinyl
TBS	buffer for color development.
TBS	Tris buffered saline
TEMED	N, N, N, N'-tetramethylenediamine
TPCK	Tosyl-L-phenylalanine-chloromethyl ketone
T_M	Melting temperature

CHAPTER 1

INTRODUCTION

1.1. Proteases

Proteases are highly complex group of enzymes which occupy a central position with respect to their applications in both physiological and commercial fields. Proteolytic enzymes, which are produced intracellularly an extracellularly, play an important role in the metabolic and regulatory processes of animal and plant cells, as well as in those of prokaryotic and eukaryotic microorganisms. Extracellullar proteases are involved mainly in the hydrolysis of large polypeptide substrates, such as proteins into smaller molecular entities which can subsequently be absorbed by the cell. Intracellular enzymes play a key role in the regulation of metabolic processes. They also play a vital role in protein turnover, maintaining a balance between protein synthesis and degradation. Proteinases are also involved in control of many other physiological functions, such as digestion, maturation of hormones, viral assembly, immune response, inflammation, fertilization, blood coagulation, fibrinolysis, control of blood pressure, sporulation, germination, and pathogenesis. Proteinases have also been implicated in the regulation of gene expression, DNA repair and DNA synthesis (reviewed in references Gottesman and Maurizi, 1992, Kalisz, 1988, Rao *et al*., 1998).

Besides their large variety of complex physiological functions, proteases also play an important role in industrial processes, and account for about 60% of the totalworldwide sale of enzymes. They have several applications, mainly in detergent and food industries. In view of the recent trend of developing environmentally friendly technologies; proteases have extensive applications in leather treatment and in several bioremediation processes. Proteases are used extensively in the pharmaceutical industry for preparation of medicines. Proteases that are used in the food and detergent industries are prepared in bulk quantities and used as crude preparations; whereas those that are used in medicine are produced in small amounts but require extensive purification before they can be used (Garcia-Carreno and Del Toro, 1997).

Since protease enzymes are physiologically necessary for living organisms, they are ubiquitous, being found in a wide diversity of sources such as plants, animals, and microorganisms. The use of plants as a source of proteases is executed by several factors such as the availability of land for cultivation and the suitability of climatic conditions for growth. Moreover, production of proteases from plants is a time-consuming process. Papain, bromelain and keratinases are the well known proteases of plant origin. The most familiar proteases of animal origin are trypsin, chymotrypsin, pepsin and rennins (Rao *et al*., 1998). These are prepared in pure form in bulk quantities. However, their production depends on availability of livestock for slaughter, which in turn is governed by political and agricultural policies. Thus, the inability of the plant and animal proteases to meet current world demands has led to increase interest in microbial proteases. Microbial protease account for approximately 40% of the total worldwide enzyme sales (Kalisz, 1988).

Microorganisms (bacteria, fungi, viruses) represent an excellent source of enzymes because of their rapid growth, the limited space required for their cultivation, broad biochemical diversity and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications. Proteases from microbial sources are preferred to enzymes from plant

and animal sources since they posses almost all the characteristics desired for their biotechnological applications.

1.2. Classification of Proteases

The classification system for microbial proteinases, recommended by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB), divides the proteolytic enzymes into two major groups on the basis of their nature of attack: endopeptidases and exopeptidases (Barrett and McDonald, 1985).

Exopeptidases cleave the peptide bond proximal to the amino or carboxyl termini of the substrate, whereas endopeptidases cleave peptide bonds distant from the termini of the substrate. The exopeptidase acting at a free amino-terminus may liberate a single amino acid residue (aminopeptidases), and those acting at a free carboxy-terminus liberate a single residue (carboxypeptidase) (Barret, 1994).

Both exopeptidases and endopeptidases exhibit “sequence specificity”, i.e. they show varying preferences for particular amino acids near the peptide bonds to be cleaved. The active site of a protease is commonly located in a groove on the surface of the molecule, and substrate specificity is dictated by the properties of binding sites arranged along the groove on one or both sides of the catalytic site that is responsible for the hydrolysis of the peptide bond. Accordingly, the specificity of a peptidase is described by use of a model in which each specificity subside is able to accommodate the side chain of a single amino acid residue (Barret, 1994). The sides are numbered from catalytic site, S1, S2 and so on towards the amino-terminus of the substrate, and S1’, S2’ and so on towards the carboxy-terminus. The substrate amino acids they accommodate are numbered P1, P2, etc., and P1’, P2’. etc., respectively (Figure 1.1).

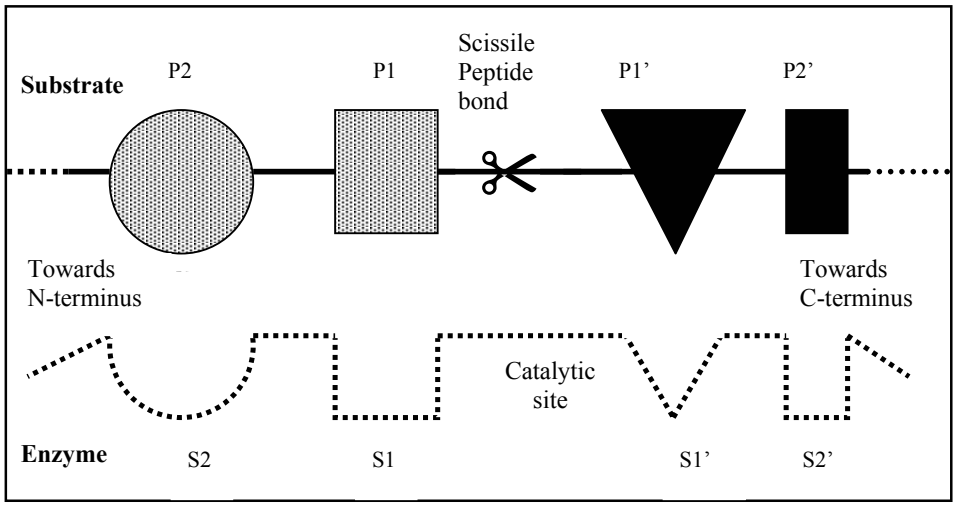


Figure 1.1. Scheme for the specificity subsites of proteases.

The endopeptidases are also called proteinases classified according to the chemical nature of the amino acid residues that are responsible for the catalytic activity of the enzyme (Table 1.1).

Table1.1. Five catalytic types of proteases (Barret, 2001).

Catalytic Type	**Primary Catalytic Group**	**Secondary Catalytic Group**
Serine	Hydroxyl group of serine	Imidazole nitrogen histidine
Threonine	Hydroxyl group of threonine	Amino-terminal α-amino group
Cysteine	Thiol group of cysteine	Imidazole nitrogen of histidine
Aspartic	Carboxyl groups of two aspartic acid residues	-
Metallo	Zinc atom (sometimes another metal)	Carboxyl group of glutamate

The enzymes of sub-subclass serine endopeptidases have an active center serine involved in the catalytic process, those of cysteine endopeptidases have a cysteine in the active center, those of aspartic endopeptidases depend on aspartic acid residues (or typically two) for their catalytic activity and those of metalloendopeptidases use a metal ion (often, but not always Zn^{2+}) in the catalytic mechanism. Recently, sub-subclass has been added for threonine peptidases, which have threonine in their active site and the major example is the prosteasome. The initial assignment of endopeptidase to a catalytic type commonly depends upon the results of tests with inhibitors (Barret, 2001).

Unlike the IUBMB system, the structure-based approach can be applied to a protease that is known only from its gene, and so can allow predictions, about properties and functions at a very early stage in the study of the enzyme, which is particularly important in the genomic area. The present form of the classification can be found in the MEROPS database on the World Wide Web (http://.merops.sanger.ac.uk). In the MEROPS system, individual proteases are first grouped into families on the basis of statistically significant similarities between the amino acid sequences of the parts that are most directly responsible for catalytic activity. The families are then grouped into clans when it is apparent from similar 3-D structures or amino acid sequence motifs that they have evolved ultimately from a single peptidase.

1.3. Serine Proteases

Serine proteases are a large class of proteolytic enzymes found in viruses, bacteria, and eukaryotes and they are distinguished by the reactivity of a serine residue in the active site. Serine proteases have been classified into evolutionary unrelated clans, which have been subdivided into families of proteases whose homology can be established statistically (Rawlings and Barret, 1993). The 'family' describes a group of enzymes in which each member resembles another, either throughout the whole sequence or at least in the part of the sequence responsible for the proteolytic activity. Serine proteases are divided into 22 families (S1 to S2, where S indicates

serine) and into six clans (Nduwimana *et al*., 1995). The cysteine types are arranged in 14 families (C1 to C14, where C indicates cysteine) and two clans. Two families (A1 and A2, where A indicates aspartate) group the aspartate proteases in a single clan. The metalloproteases consist of 25 families (M1 to M25, where M indicates metalloprotease) and five clans. Finally, there are 21 other families (U1 to U21, where U indicates unknown).

The primary structures of the members of four clans of serine proteases, chymotrypsin (SA), subtilisin (SB), carboxypeptidase C (SC), and *Escherichia* D-Ala-D-Ala peptidase A (SE) are totally unrelated, suggesting that there are at least 4 separate evolutionary origins for serine proteases. The clans SA and SC maintain strictly conserved active site geometry among their common catalytic triad of the three amino acids Ser (nucleophile), His (base) and Asp (electrophile) (Rawlings and Barret, 1994).

They are recognized by their irreversible inhibition by diisopropylphosphofluoridate (DFP) which is historically one of the most widely used protease inhibitors (Walsh and Wilcox, 1970). 3, 4-dichloroisocoumarin (3, 4-DCI), L-3-carboxytrans 2, 3-epoxypropyl-leucylamido (4-guanidine) butane (E.64), Phenylmethylsulphonyl fluoride (PMSF), tosyl-L-lysine chloromethyl ketone (TLCK) are other well-known irreversible inhibitors of serine proteases. Some of the serine proteases are inhibited by thiol reagents such as p-chloromercuribenzoate (PCMB) due to presence of a cysteine residue near the active site. Serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7.0 and 11.0. They show broad substrate specificities including esterolytic and amidase activity. The molecular masses of serine proteases with a few exceptions range between 18 and 35 kDa and their isoelectric points are between pH 4.4 and 6.2 (Kalizs, 1988, Barret, 1994, Rao *et al*., 1998).

1.3.1. Serine Alkaline Proteases

Serine alkaline proteases represent the largest subgroups of serine proteases that are produced by several bacteria, molds, yeasts and fungi. The optimum pH of alkaline proteases is generally between pH 9.0 and 11.0. They also have high isoelectric point which is around pH 6.0-12.0. The optimum temperatures of alkaline proteases range from 50 to 70^{o}C. Serine alkaline proteases are completely inhibited by PMSF and DFP. In this regard, PMSF sulfonates the essential serine residue in the active site and results in the complete loss of activity. Serine alkaline proteases are active against aromatic or hydrophobic amino acid residues such as tyrosine, phenylalanine, or leucine at the carboxyl side of the splitting bond, having specificity similar to, but less stringent than, α-chymotrypsin. They require a divalent cation like Ca^{2+}, Mg^{2+}, Mn^{2+} or a combination of these cations, for maximum activity. Their molecular masses are in the range of 15 to 30 kDa (Rao *et al*., 1998, Kumar and Takagi, 1999).

1.3.2. Subtilisins

Subtilins of *Bacillus* origin represent the second largest family of serine proteases. Two different types of alkaline proteases, subtilisin Carlsberg and subtilisin Novo or bacterial protease Nagase (BPN'), have been identified (Phadtare *et al*., 1997). Both subtilisin have a molecular mass of 27.5 kDa but differ from each other by 58 amino acids. They have similar properties such as an optimal temperature of 60^{o}C and an optimal pH of 10.0. Both enzymes exhibit broad substrate specificity and have an active-site triad made up of Ser221, His64, and Asp32. The Carlsberg enzyme has broader substrate specificity and does not depend on Ca^{2+} for its stability. The active site conformation of subtilisin is similar to that of trypsin and chymotrypsin despite the dissimilarity in their overall molecular arrangements (Rao *et al*., 1998).

1.3.3. Applications of Alkaline Proteases

Alkaline proteases or subtilisins are physiologically and commercially important group of the enzymes. In recent years there has been growing interest in use of alkaline proteases as industrial catalysts. These enzymes offer various advantages, for example they exhibit high catalytic activity, and a high degree of substrate specificity can be produced in large amounts and are economically viable. All these reasons provide wide applications for microbial alkaline proteases.

The main industrial application of alkaliphilic proteases is in the detergent industry, as detergent enzymes. Today, detergent enzymes account for 89% of the total protease sales in the world (Gupta *et al*., 2002). Alkaline proteases added to laundry detergents enable the release of proteinaoceous material from stains. Detergents available in the international market such as Dynumo©, Era plus© (Procter and Gavable), Tide© (Colgate and Palmolive) contains proteolytic enzymes, the majority of which are produced by member of the genus *Bacillus*. The ideal detergent protease should possess broad substrate specificity to facilitate the removal of a large variety of stains due to food, blood, and other body secretions. Activity and stability at high pH and temperature and compatibility with other chelating and oxidizing agents added to the detergents are among the major prerequisites for the use of proteases in detergents. A protease is most suitable for this application if its pI coincides with the pH of the detergent solution (Kumar and Takagi, 1999). Ideally, proteases used in detergent formulation should have good activity at relevant washing pH and temperature. The current trend in laundering processes is the use of proteases that are active at lower temperature, due to present energy crisis, however the use of the thermophilics in laundry detergents for institutional (e.g., hospital) use may well be a future application because in this process especially high temperatures are required (Buriens *et al*., 2001). A combination of lipase, amylase, and cellulase is expected to enhance the performance of protease in laundry detergents.

Alkaline proteases with elastolytic and keratinolytic activity offer an effective biotreatment of leather, especially dehairing and bating of skins and hides. The alkaline conditions enable the swelling of hair roots and subsequent attack of proteases on the hair follicle protein allows easy removal of the hair. The traditional method in leather processing involve the use of the hydrogen sulfide and other chemicals which contributes to 100 % of sulfide and over 80% of the suspended solids in tannery effluents, creating environmental pollution and safety hazards. At present, alkaline proteases with hydrated lime and sodium chloride are used for dehairing, resulted in a significant reduction in the amount of waste water generated. This enzyme-assisted de-hairing provides several advantages, e.g. easy control, speed and waste reduction, thus being ecofriendly (Anwar and Saleemuddin, 1998, Gupta *et al*., 2002). Currently, trypsin is used in combination with other *Bacillus* and *Aspergillus* proteases for bating. The selection of the enzyme depends on its specificity for matrix proteins such as elastin and keratin. Novo Nordisk manufactures three different proteases, Aquaderm, NUE, and Pyrase for use in soaking, dehairing and bating, respectively.

Alkaline proteases play a crucial role in the bioprocessing of used X-ray or photographic films for silver recovery. Used X-ray film contains 1.5-2.0% silver (by weight) in its gelatin layers. The conventional practice of silver recovery by burning film causes a major environmental pollution problem. Enzymatic hydrolysis of gelatin layers on the X-ray film enables not only the silver, but also polyester film base can be recycled. There are several reports for the use of alkaline proteases from *Bacillus* sp, which were efficient in decomposing the gelatinous coating on used X-ray films and releasing silver particles (Masui *et al*., 1999).

Alkaline proteases are also used for developing products of medical importance. Collagenases with alkaline protease activity are increasingly used for therapeutic applications in the preparation of slow-release dosage forms (Kumar and Takagi, 1999, Gupta *et al*., 2002). A semi-alkaline protease with high collagenolytic activity from *Asperillus niger*, hydrolyzed various collagen types without amino acid release

and liberated low molecular weight peptidases of potential therapeutic use (Barthomeuf *et al*., 1992). Kudrya and Simonenko (1994) exploited the elastolytic activity of *B. subtilis* for preparation of elastoterase which was applied for the treatment of burns, and wounds. Kim *et al.* (1996) reported the use of alkaline protease from *Bacillus* sp. as a thrombolytic agent having fibrinolytic activity.

The basic function of proteases is to hydrolyze proteins; and this property has been exploited for the preparation of protein hydrolysates of high nutritional value. The alkaline proteases can hydrolyze proteins from plants, fish, or animals to produce hydrosylates of well-defined peptide profile. Protein hydrolysates have several applications, e.g. as constituents of dietic and health product, in infant formulae and clinical nutrition supplements, and as flavoring agents. The commercial protein hydrolysates commonly generated from casein, whey protein and soyaprotein find major application in hypo allergic infant food formulation. They can also be used for the fortification of fruit juices or soft drinks and in manufacturing protein-reach therapeutic diets. Further, proteases play a prominent role in the meat tenderization, especially of beef. The tenderization process can be achieved by sprinkling the powdered enzyme preparation or by immersion in an enzyme solution and/or by injecting the concentrated protease preparation into the blood stream or meat (Kumar and Takagi, 1999, Gupta *et al*., 2002).

Alkaline proteases provide potential application for the management of waste from various food processing industries and household activities. Proteases solublize proteinaceous waste and thus help lower the biological oxygen demand of aquatic systems. An enzymatic process using a *B. subtilis* alkaline protease in the processing of waste feathers from poultry slaughterhouse has been reported (Dalev, 1994). The end product was a heavy, grayish powder with a very high protein content which could be used as a feed additive. The use of keratinolytic protease in feed industry for production of amino acids or peptides, for degrading waste keratinous material from poultry refuse and as depilatory agent to remove hair in bath tub from drains has been reported (Kumar and Takagi, 1999, Gupta *et al*., 2002).

1.3.4. Alkaline Serine Proteases from Microorganisms

The inability of the plant and animal proteases to meet current world demands has led to an increase interest in microbial proteases. Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Microbial proteases account for approximately 40% of the world wide enzyme sales (Kalisz, 1988). Most commercial alkaline proteases are produced by organisms belonging to the genes *Bacillus* and *Aspergillus* genera. The majority of the *Bacillus* species are harmless, non-toxin producing saprophytes, which are easy to grow in high density without a requirement for extensive growth factor. The asporagenic mutants and proteinase-hyperproducing strains of *Bacilli* have also been developed (Jolliffe *et al*., 1980). The *Aspergillus* species, like *Bacillus*, are highly variable and widespread and the majority of the species are non-pathogenic and non-toxin forming. The most frequently used species for enzyme production are the *A. niger* and *A. oryzea*.

Bacterial alkaline proteases are characterized by their high activity at alkaline pH, e. g., pH 10, and their broad substrate specificity. Their optimal temperature is around 60^{o}C. These properties of bacterial alkaline proteases make them suitable for use in detergent industry. For example, subtilisins are a prototype group of bacterial alkaline serine proteases including Subtilisin BPN' from *B. amyloliquefaciens* (Wasantha *et al.,* 1984), subtilisins E from *B. subtilis* (Stalh and Ferrari, 1984), subtilisin Carlsberg from *B. licheniformis* (Jacobs *et al*., 1985), subtilisin amylosacchariticus from *B. subtilis*, (Yoshimato *et al*., 1988), subtilisin NAT from *B. subtilis* (natto) (Nakamura, 1992, Yamagato, 1995) which all an of industrial importance.

There are several studies published, from various bacteria, mainly *Bacillus sp*. concerning purification, and cloning of alkaline serine proteases (Babé and Schmidt, 1998, Han and Damodaran, 1998, Sahoo *et al*., 1999, Kumar *et al*., 1999, Singh *et al*., 2001, Joo *et al*., 2002, De Toni *et al*., 2002, Lee *et al*., 2000, Kitadokoro *et al*.,

1993. Fungi, especially *Aspergillus* sp. also elaborate a wide variety of alkaline proteases, with similar properties to the serine proteases of the *Bacillus* sp. (Chakrabarti *et al*., 2000).

1.4. Thermophilic Proteases

Most of proteases are stable in normal (mesophilic) temperature; however, mesophilic enzymes are often not optimally adapted to conditions where they are to be applied. For this reason several strategies are being used to improve the characteristics of biocatalysts, such as stability, activity, specificity, and pH optimum. In this respect, isolation of enzymes from organisms that are able to survive under extreme conditions eventually in combination with directed evolution approaches has been shown be important source for new biocatalysts (Fujiwara *et al*., 1993). Because of that, in recent years much attention has been given to proteases from extromophilic microorganisms. During their evolution these microorganisms adapted themselves to grow over a wide range temperature, pH and pressure. Much of interest has been especially focused on those living at high temperatures particularly on their enzymes (Niehaus *et al*., 1999). Thermozymes are optimally active between 60^{o}C and 125^{o}C evolved in thermophiles (organisms thriving at 60-80^{o}C) and hyperthermophiles (organisms thriving at >80^{o}C). Whereas thermophiles are classed as bacteria or archaea, most hyperthermophiles are archaea (Vieille and Zeikus, 1996). Species found at higher temperatures (105-113^{o}C) are exclusively archaea (Stetter, 1996).

Thermostable proteases from thermophilic bacteria are given much attention due to their stability at higher temperatures. Because thermophilic enzymes are optimally active under more severely denaturing conditions than mesophilic enzymes, they need to be more rigid than mesophilic enzymes. Basic mechanisms of stability are sequence modifications such as replacement of conformationally strained residues by glycines, addition of salt bridges, increases hydrophobic interactions, high packing density, additional ion pairings and hydrogen bonding, minimization of accessible

hydrophobic surface area, helix stabilization, shortening of loops and subunits assembly. Oligomer formation and other environmental factors can further stabilize the enzyme (Vieille and Zeikus, 1996). These strategies, used to differing extents by different thermophilic proteins, confer not higher thermal stability to proteins but also enhanced rigidity and resistance to chemical denaturation and proteolytic attack. Their high stability makes them attractive for several industrial processes. A further advantage is the performing biological processes at high temperature reduce risk of contamination. Another advantage of applying thermostable enzymes for production purposes is lower viscosity of process fluids. At higher temperatures viscosity is usually reduced, which lowers shear and, consequently, the cost of pumping, filtration, and centrifugation, or allow the use of lower water levels during processing. Heat and mass transfer rates are also improved. At higher temperatures diffusion rates will be higher and mass transfer is less limiting. Moreover, at high temperature, more substrates will dissolve and this can shift the equilibrium to higher product yields (Bruins *et al*., 2001). It is often speculated that thermozymes should have higher maximal catalytic rates. However, these higher maximal catalytic rates are not found experimentally. Thermophilic enzymes catalyze reactions at high temperatures with K_m and V_{max} values similar to those of their mesophilic counterparts at their respective optimal temperature.

1.4.1. Thermophilic Alkaline Serine Protease from *Archaea*

Serine proteases from alkalophiles are generally stable in highly alkaline environments while being not very heat-resistant. On the other hand, proteases from thermophiles retain their catalytic activity at high temperatures and also exhibit a high degree of resistance to denaturing reagents, organic solvents and other deleterious influences, however unstable at high pH. Because of these reasons, proteases with high activity and stability in high alkaline range and temperature have attracted a great deal interest for bioengineering and biotechnological applications as well as for protein chemistry (Lee *et al*., 2000). Several studies have been reported about serine alkaline proteases which are isolated and purified from thermophilic microorganisms

The quantity of proteases produced on a commercial scale worldwide is grater than any other enzymatic group of biotechnological relevance (Niehaus *et al*., 1999). As such, the isolation and characterization of heat-stable microbial proteases are the focus of much interest. There are several reports of thermophilic serine alkaline proteases isolated and purified from thermophilic bacteria (Manachini *et al*., 1988, Fujiwara *et al*., 1993, Ferrero *et al*., 1996, Hutalidok-Towatana *et al*, 1999, Johnvesly and Naik, 2001, Zvauya and Zvidzai, 2001, Kim *et al*., 1996, Kristjansson and Kinsella, 1990, Lee *et al*., 2000, Matsuzawa *et al*., 1983, Tsuchiya *et al*., 1997). Besides, various proteolytic enzymes from thermophilic and hyperthermophilic archaea, mostly of the serine protease type, have been isolated. Pryrolysin is a serine protease-type protease which has been identified and characterized in heterotrophic anaerobe *Pyrococcus furiosus*. The enzyme is associated with the cell envelope, has a temperature optimum of 110^{o}C and a half-life of 4 h at 100^{o}C. The gene encoding the pro-form of the enzyme has been cloned and sequenced (Voorhorst *et al*., 1996). Bluemental *et al*. (1990) showed that *P. furiosus* produces several intracellular proteolytic species, as determined by clearing zones on gelatin overlays by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell extracts. One of these serine protease, now designated PfpI, with an electrophoretic mobility of 66 kDa by SDS-PAGE was shown to be highly thermostable (a half-life of over 30 H at 98^{o}C) and resistant to denaturant (urea, dithiothreitol and β-mercaptoethanol). The enzyme inhibited by the serine protease inhibitors, phenylmethylsulfonyl fluoride and diisopropyl fluorophosphates but not ethylene glycol-bis (β-aminoethyl ether)-N-N-N-N-teraacetic acid. Halio *et al* (1997) isolated the PfpI gene and they showed that it coded for a protein 18.8 kDa. The pfpI gene was expressed in *Escherichia coli* to yield an active protease, but no protease activity was detected. A serine protease was isolated and purified from the supernatant of a culture of another *Pyrococcus* species which is *Pryroccus abyssi*, strain st 549. The protease showed high affinity for aromatic (Phe and Tyr) and hydrophobic amino acids (mainly Leu) in P1 position of synthetic substrates. The optimum temperature and pH of the enzyme was 95^{o}C and at pH 9.0, respectively (Dib *et al*., 1998). The properties of extracellular serine proteases from a number of *Thermoccoccus* species (*T. celer, T.*

stetteri, *T. litoralis* and *Thermoccus* strain AN 1) have been analyzed (Klingeberg *et al*., 1991). The extracellular serine protease from Archaeon *Thermococcus stetteri,* was purified and characterized. The purified enzyme has a molecular mass of 68 kDa, shows maximal activity at 85^{o}C and is active over a broad pH range (between 5.0 and 11.0). By phenylmethylsulfonyl fluoride and di-isopropyl fluorophosphates inhibited *T. stetteri* protease enzyme display a relative narrow substrate specificity, catalyzing the hydrolysis of N-protected pNA of basic (Arg or Lys) or hydrophobic amino acids (Phe or Tyr). The purified enzyme displays a half-life of 2.5 h at 100^{o}C and is able to retain 70% of its catalytic activity even in the presence of 1% SDS (Klingeberg *et al*., 1995). Archaelysin, is another heat-stable serine protease was isolated and purified from the cell-free supernatant of *Desulforococcus* strain $Tok_{12}D_1$. Purified serine protease exhibits a molecular mass of 52 kDa and is optimally active at 100^{o}C. Archaelysin was inhibited by phenylmethylsulfonyl fluoride, di-isopropyl phoshorofluoridate and chymostatin. Substrate specificity studies revealed that the preference for hydrolytic residues on the C-terminal side of the splitting point (Cowan *et al*., 1987). A proteinase was purified from thermoacidophilic archeabacterium *Sulfolobus solfataricus*. The enzyme behaves as a chymotrypsin-like serine proteinase, as shown by the inhibitory effects exerted by phenylmethylsulfonyl fluoride (PMSF), 3, 4-dichloroisocoumarin, tosyl-L-phenylalanine-chloromethyl ketone (TPCK) and chymostatin, but displaying a specificity much narrower than and clearly different from that of chymotrypsin. The enzyme also displayed a broad pH optimum in the range 6.5-8.0. At 50^{o}C, it could withstand 6 M urea and, to some extent, different organic solvents. None of the chemical physical properties of the enzyme, including amino-acid analysis, provided evidence of a possible relation to other well-known microbial serine proteinases (Burlini *et al*., 1992). Recently, several thermoacidophilic archeal strains from hot-spring and hot-soils were screened for their ability to produce intracellular alkaline proteases in our laboratory. Especially three archeal strains (898-BS1, 898-BS17 and 898-BS24) which displayed higher enzyme activities then others were further examined for their protease activities. According to gelatin zymography studies, two of the archeal strains (898-BS1, 898-BS17) produced multiple active SDS-resistance

proteases. And also, amplification of multiple, distinct fragments of 650, 450, 400 and 300 bp by PCR with the template DNAs from these strains support the possibility of existence of several protease activities in one strain. The maximum protease activities in the cell free extract of these three strains were at 60-70^{o}C and pH optimum over a range of neutral and/or alkaline pH (pH 7.0-8.5) for hydrolysis of azocasein. Inhibitor studies showed that serine protease inhibitors such as PMSF (2 mM), TPCK (1 mM) and Chymostatin (400 µg/ml) inhibited more than 90% enzyme activities (Kocabıyık and Erdem, 2002). Serine proteases have also been characterized from the halophilic archeabacteria. An extracellular serine protease was isolated from the culture filtrate of extremely halophilic *Halobacterium halobium*. The protease consists of one polypeptide chain with a molecular weight of about 41,000. The optimum pH of the enzyme activity against L-pyroglutamyl-Lyro-Alanyl-L-Alanyl-Leucine-p-nitroanilide was between 8.0 and 9.0. The protease was inhibited by DFP and PMSF at a concentration of 1 mM, indicating that it is a serine protease (Izotova *et al.*, 1983). A serine protease was isolated and purified from the haloalkaliphilic archaeon *Natronomonas pharaonis*. Its molecular mass was 30 kDa and its isoelectric point was 4.55. The enzyme hydrolyzed synthetic peptides, preferentially at carboxyl terminus of phenylalanine or leucine, as well as large proteins. The optimum temperature and pH was 61^{o}C and pH 10, respectively (Stan-Lotter *et al*., 1999). An extracellular serine protease was purified and characterized from another *Natronoccus* species which was *N. occultus*. The enzyme has a native molecular mass of 130 kDa. *N. occultus* extracellular protease has chymotryptic-like activity which was inhibited by serine protease inhibitors PMSF (1 mM) and chymostatin (8 µM). The enzyme active in a broad pH rage (5.5-12) and it is rather thermophilic, showing maximal activity at 60^{o}C in 2 M NaCl (Studdert *et al.,* 2001). Another serine protease was isolated and characterized from the haloalkaliphilic archaeon *Natrialba magadii*. The protease was inhibited by PMSF, DFP, and chymostatin, indicating that it is a serine protease. It hydrolyzed large proteins such as casein and gelatin and synthetic peptides, preferentially at the carboxyl terminus of phenylalanine, tyrosine, or leucine. These results show that *N. magadii* protease is a chymotrypsin-like serine protease. The native molecular mass

of the enzyme determined by gel filtration was 45 kDa. The optimum temperature for N.magadii extracellular protease was 60^{o}C and the enzyme was active and stable broad range (6.0-12.0) and showed the highest activity for azocasein hydrolysis at a pH of 8.0-10.0 (Giménez *et al*., 2000).

1.5. Catalytic Activity of Serine Proteases

Serine proteases follow a two step reaction for hydrolysis in which a covalently linked enzyme-peptide intermediate is formed with the loss of the amino acid or peptide fragment. This acylation step is followed by a deacylation process which occurs by nucleophilic attack on the intermediate by water, resulting in hydrolysis of the peptide.

Serine endopeptidases can be classified into three groups based on their primary substrate preference: (i) trypsin-like, which cleaves a chain just after carbonyl group on a basic amino acid either lysine or arginine, (ii) chymotrypsin-like, which cleaves the peptide bonds on the carboxyl side chains tyrosine, tryptophan, and phenylalanine and of large hydrophobic residues such as methionine, (iii) elastase-like, which cleave after small hydrophobic residue (Walker and Lynes, 2001). The P1 residue exclusively dictates the site of peptide bond cleavage. The catalytic mechanism of serine endopeptidases is illustrated in the Figure 1.2 by using trypsin as an example.

In serine proteases from chymotrypsin and subtilisin families, the active site architecture, S1-specificity determinants and reaction mechanism have been studied intensively based on recently determined 3-D structures of free enzymes and their inhibitor complexes. Serine proteases with a chymotrypsin fold is one of the largest and most comprehensively studied of all enzyme families. Over 20 unique structures have been determined to date, and the number of available sequences exceeds 500 (Perona and Craik, 1997). All of the enzymes process an identical fold consisting of 2 perpendicular β-barrel domains with catalytic Ser195, His57 and Asp102 amino

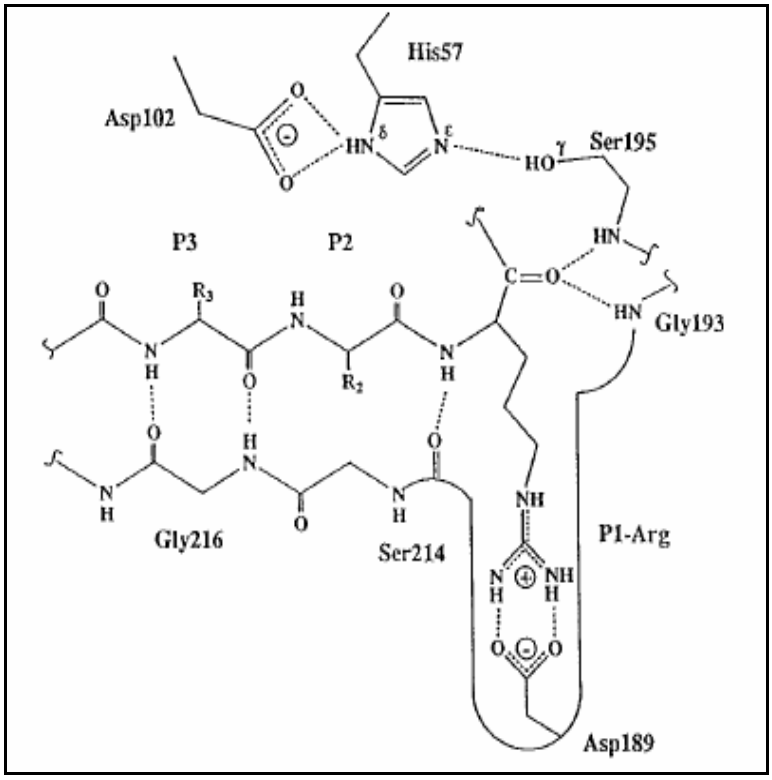


Figure 1.2. Chemical mechanism of catalysis for serine proteases (Perona and Craik, 1997). The catalytic groups of chymotrypsin-fold enzymes are shown interacting with a peptide substrate binding to the P1 site (Nomenclature for the substrate amino acid residues is Pn...,P2,P1,P1',P2',...Pn', where P1-P1' donates the hydrolyzed bond, Sn,...,S2,S1,S1', S2',...,Sn') donates the corresponding enzyme binding sites. Five enzyme-substrate hydrogen bonds at positions P1 and P3 are shown in addition to hydrogen bonds the members of the catalytic amino acids.

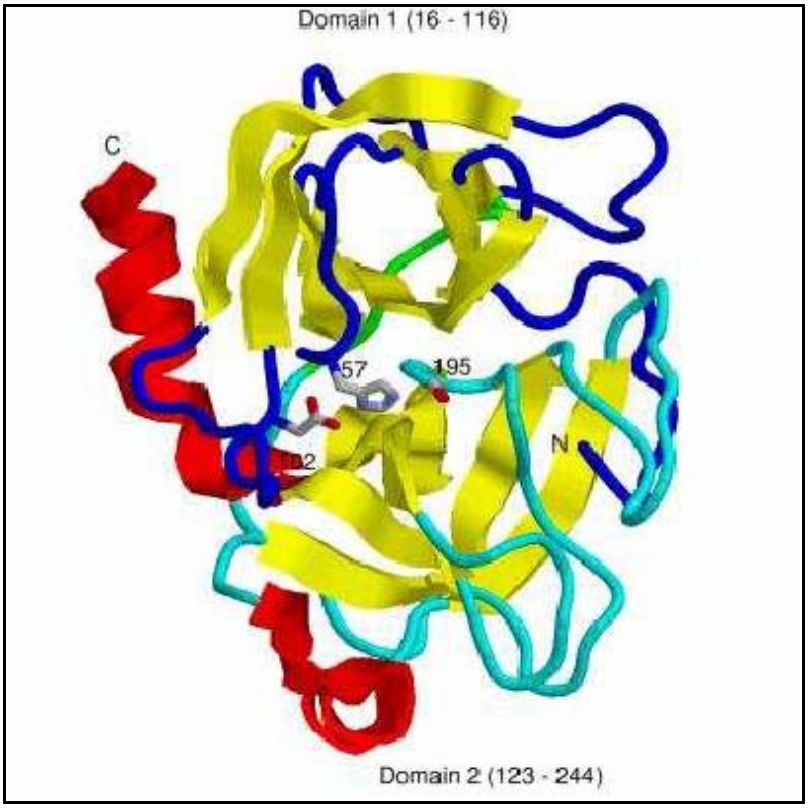


Figure 1.3. The structural fold of alpha-chymotrypsin (bovine). Helices are shown in red and sheets in yellow. The two domains are indicated with loops from domain 1 colored blue and loops from domain 2 colored cyan. The strap connecting the domains is shown in green. The catalytic triad (active site) is shown as a stick drawing. Note that the catalytic triad is comprised of residues from both domains and is located at the domain interface.

acids found at the interface of the 2 domains (Figure 1.3). Common features present in all structures, including five enzyme-substrate hydrogen bonds at position P1 and P3, serve to properly juxtapose the scissile peptide bond adjacent to the Ser-His catalytic couple, such that the nucleophilic Ser 195 O-γ is accurately positioned for attacked.

Subtilisins are arranged in a fold radically different from enzymes of the chymotrypsin family but with similar spatial organization of the catalytic triad, oxyanion-binding whole and hydrophobic S1 pocket. The core of subtilisins contains ~ 190 superimposable residues, including the central part of an enzyme composed of 5 parallel β-strands surrounded by four helices (Figure 1.4). The S1-site of subtilisins is more open than in chymotrypsin-like enzymes and resembles a hydrophobic cleft, which is typical in subtilisin BPN'.

Chymotrypsin is one of the best known and best characterized protein-hydrolyzing enzymes. It is synthesized in the acinar cells of the pancreas as an inactive precursor (zymogen) known as chymotrypsinogen that is activated in small intestine by action of proteases. This 25 kDa enzyme, consist of three polypeptide chains connected by two interchain disulfide bonds and contains several antiparalel β-pleated sheet regions and α-helix (Appel, 1986).

Physiologically, chymotrypsin hydrolyses peptide bonds in proteins and polypeptides. They are highly selective for peptide bonds with aromatic or large hydrophobic side chains (tyrosine, tryptophan, phenylalanine, methionine) on the carboxyl side of the bond. Non-physiologically, the enzyme also cleaves low molecular weight substrates such as ester, amides, acrylamides or substituted aromatic amides like β-naphtylamide or p-nitroanilide (Appel, 1986).

The mechanism of chymotrypsin has been extensively studied and probably better understood than any other enzyme mechanism. Chymotrypsin is serine proteases, which contain catalytic triad of Asp-His-Ser. In the serine proteases, the catalytic

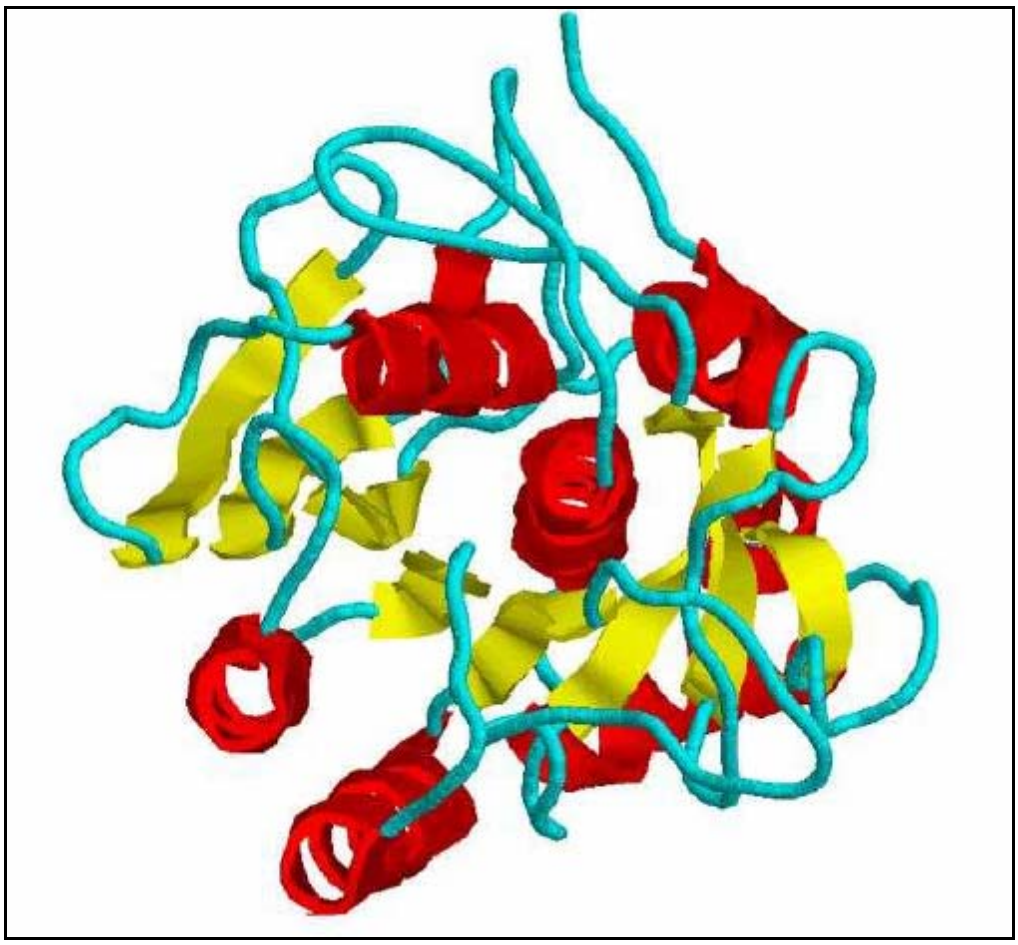


Figure1.4. The structural fold of alpha-subtilisin.

triad acts as a "charge relay" system. Histidine removes a proton from the serinecharged histidine intermediate. Deacylation of the enzyme involves an activated water molecule acting as the nucleophile. Crucial to this catalytic scheme is the formation of unusually short catalytic hydrogen bond between histidine and aspartic acid. This makes the histidine a more potent base, facilitating its deprotonation of serine (Paetzel and Dalbey, 1997, Shaw *et al*., 2002). Proton transfer, in general has charged histidine intermediate. Deacylation of the enzyme involves an activated water molecule acting as the nucleophile. Crucial to this catalytic scheme is the formation of unusually short catalytic hydrogen bond between histidine and aspartic acid. This makes the histidine a more potent base, facilitating its deprotonation of serine (Paetzel and Dalbey, 1997, Shaw *et al*., 2002). Proton transfer, in general has a high-energy barrier, but for a number of hydrogen-bonded system it has only a very low barrier, leading to proton delocalization in the hydrogen bond that strongly depends on the environments. Such hydrogen bonds are called "sort-strong" or "low-barrier" hydrogen bonds (SSHBs or LBHBs). Frey and coworkers suggested that the LBHB in the protonated dyad (Asp-His) should increase the basicity of His 57 strongly enough to abstract a proton from Ser 195. The basicity of His 57 should be decreased again later to give a proton from its conjugate acid form to leaving group (Kim and Ahn, 2001). In the chymotrypsin-like serine proteases, the hydrophobic side chain of the substrate makes a number of favorable contacts with the non-polar side-chains lining the pocket.

The catalytic mechanism of serine endopeptidases is illustrated in Figure 1.5 by using chymotrypsin as an example (Nduwimana *et al*., 1995). Catalytic mechanism for chymotrypsin has been deduced from extensive X-ray crystallographic and chemical data. Hydrolysis of the substrate occurs in two successive stages: acylation and deacylation of the enzyme. In the first step, enzyme-substrate complex formed; the substrate being held by H bonding between the substrate and two amino acid carbon to form a tetrahedral intermediate. Three amino acid residue at the catalytic site (catalytic triad) participate in the reaction. His 57 removes a proton from Ser 195 generating a powerful nucleophile, thus His becomes the conjugate acid (positively

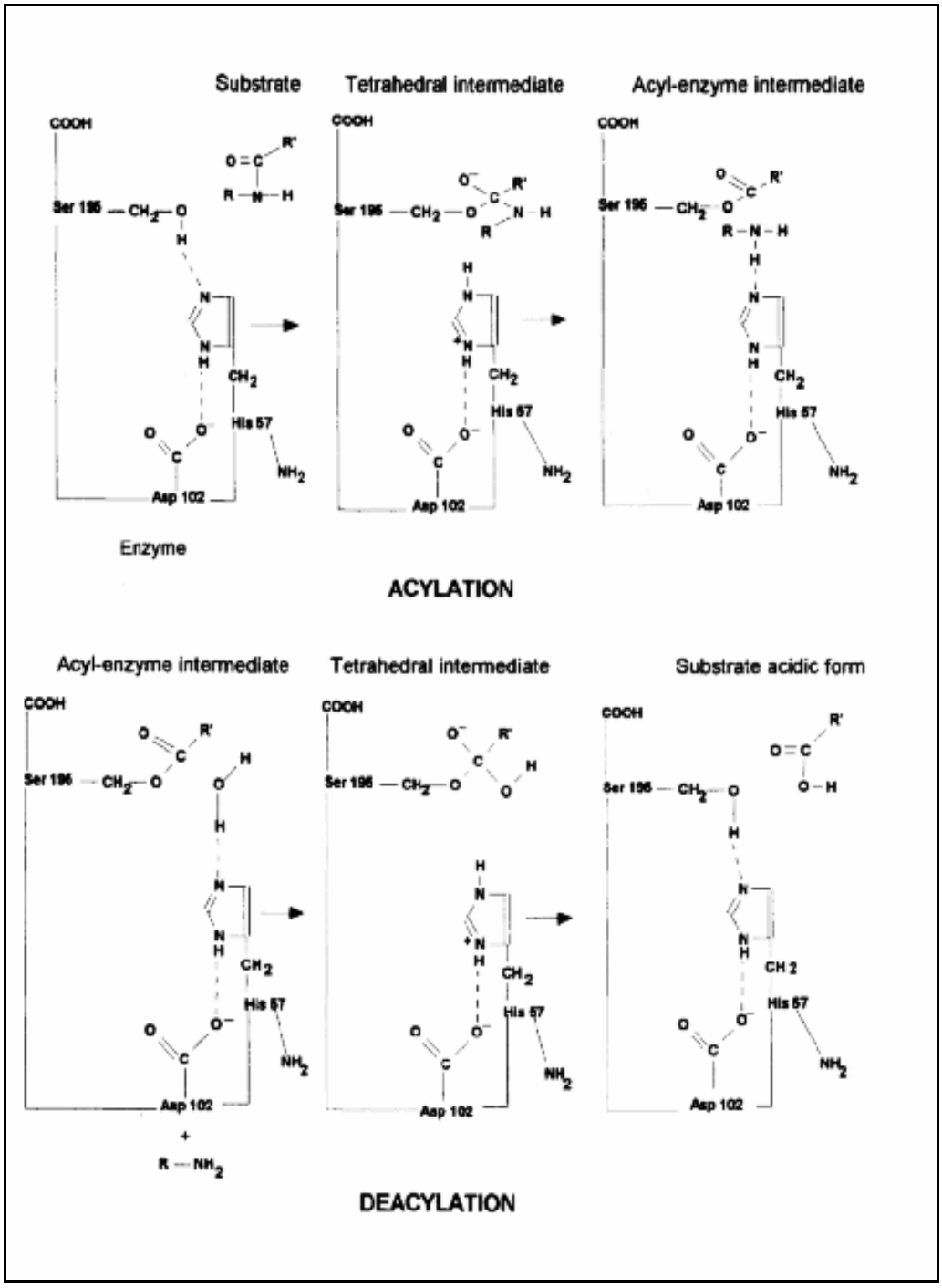


Figure 1.5. Catalytic mechanisms of serine endopeptidases (Nduwinmana *et al.*, 1995).

charged). Asp 102 is H bonded to His, stabilizing this transition state. H-bonding to His 57 also stabilizes the amide linkage on the backbone. The tetrahedral intermediate is stabilized by H bonds between the oxyanion (O^-) of the peptide and Ser 195 and Gly (193). The net effect of these interactions is to lower the activation energy due to the negative H effects. The peptide bond is broken; the leaving group (the amino side of the original peptide bond) picks up a proton from the imidazolium ion of His 57. The carboxyl side of the original peptide bond remains attached to the enzyme by H bonds and covalent bonds to Ser 195. Water takes place the leaving part of the substrate and protonates His 57 (acting as an acid) and the resulting OH- makes a nucleophilic attack on the carbonyl carbon of the remaining polypeptide. A second tetrahedral intermediate is formed, representing a second transition intermediated. This collapses, with hydrolysis of the acyl-enzyme intermediate, and release of the remaining part of the original polypeptide. Ser 195 recovers a proton from His 57, His remains associated with this H by H binding. This proton comes from the incoming water molecule. The catalytic cycle is complete and new round of substrate binding begins (Dodson and Wlodawer, 1998).

Chymotrypsin family serine proteases play essential roles in key biological and pathological processes and are frequently targets of drug discovery efforts. The three-dimensional structures of numerous complexes between a chymotrypsin family enzyme and peptide or proteinoceosus inhibitors have been solved, and these structures provided many important insights into the mechanism and structure/function relationship of serine proteases. The majority of these protein-protein complexes contain standard mechanism of action. Serine proteases can inhibited either by small compounds (organophosphates, sulfonyl fluorides, coumarins, heterocyclic derivatives, boronic acid derivatives, chloromethyl ketones) or by protein protease inhibitors (serpins, kunitz type inhibitors), or peptide inhibitors (fairly specific microbial inhibitors) (Walker and Lynas, 2001).

Inhibitors behave as limited proteolysis substrates; residues interacting with proteinase at the reactive site of the inhibitor determine specificity and typically

reflect the substrate specificity of the target proteinase. The identity of the P1 residue is considered to be the main determinant of specificity (Coombs *et al*., 1999). Diisopropylphosphofluoridate (DIPF—potent nerve poison), one of the most widely used protease inhibitors, is a member of the orgonophosphorus compounds. Using DIFP, through chemical labeling, the presence of the active Ser of serine proteases is identified. This binds irreversibly to active serine and inactivates the enzyme. Other Ser residues, including those on the same protein, do not react with DIPF. DIPF reacts only with Ser 195 of chymotrypsin, thereby demonstrating that this residue is the enzyme's active Ser. His 57, a second catalytically important residue, was discovered through affinity labeling. Chymotrypsin specifically binds tosyl-L-phenylalanine chloromethyl ketone (TPCK), because of its resemblance to a Phe residue (one of chymotrypsin's preferred residues). Active site-bound TPCK's chloromethyl ketone group is a strong alkylating agent; it reacts only with His 57 thereby inactivating the enzyme.

1.6. Proteasome

The proteosomes are giant, multisubunits proteases that are found in the cytosol, both free and attached to the endoplasmic reticulum, and in the nucleolus of eukaryotic cells. The energy dependent 26S proteasomes in eukaryotes are involved in ubiquitin-mediated protein degradation. Ubiquitin is very abundant and highly conserved 76-residue protein.

Ubiquitination of protein substrates proceeds through a tightly regulated, multistep pathway (Figure 1.6). The first stage, activation of ubiquitin, is catalyzed by E1, the ubiquitin-activating enzyme, and one of several E2 or ubiquitin-conjugating (or carrier) enzymes. In the first reaction, ubiquitin molecules are linked through their C-terminal glycine residues to cysteine residue on E1 via thioester bond. Activated ubiquitin is then transferred to cysteine residue on the E2 enzymes. The second stage, substrate recognition, occurs independently and involves one of several E3 enzymes or ubiquitin ligases. E3 enzymes constitute a large family of proteins and

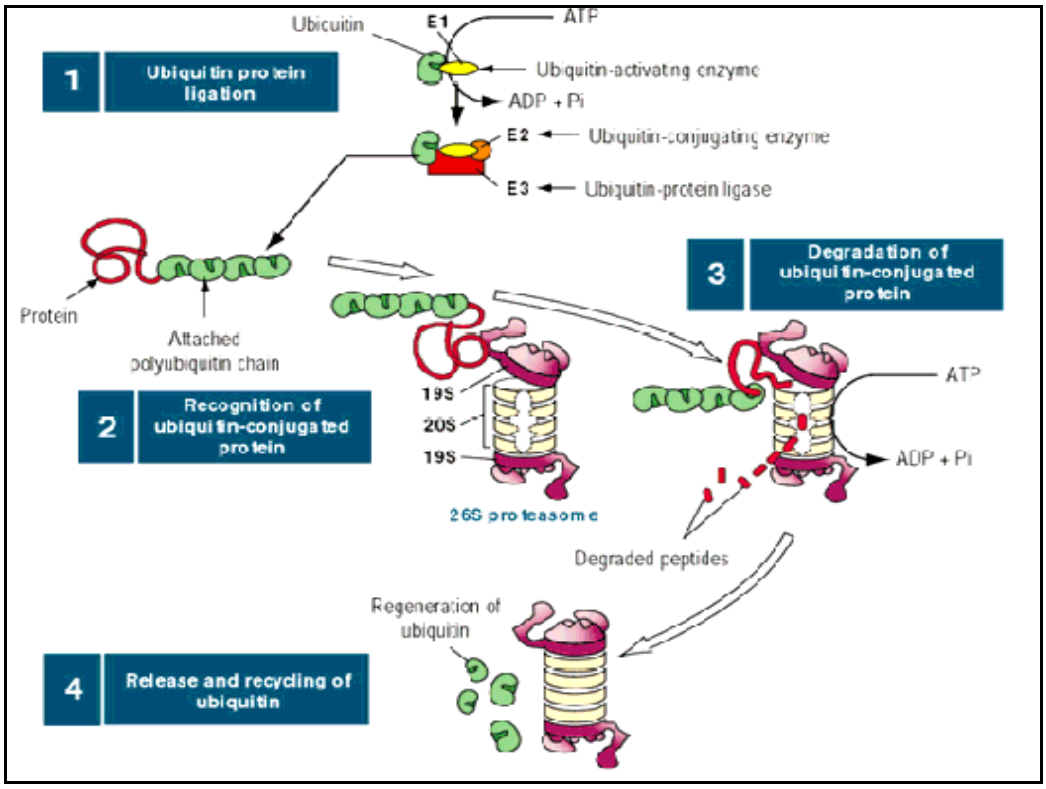


Figure 1.6. Proteasome-ubiquitin pathways (Kierszenbaum, 2000).

protein complexes that confer specificity to the ubiquitin reaction. The E3 enzyme catalyzes the transfer of ubiquitin to the protein substrate from E2-ubiquitin intermediate either directly or in two steps through an E3-ubiquitin intermediate. This process is repeated several times to generate a long polyubiquitin chain attached to the substrate protein destinated for degradation by the 26S proteasome (Tanaka and Tsurumi, 1997, Kierszenbaum, 2000). There are many signals which are recognized by the ubiquitin system for degradation by the 26S proteasome including: proline-glutamate/aspartate-serine-threonine (PEST) sequences, destabilizing N-terminal residues, hydrophobic regions, phosphorylation states, glycine-rich regions (GRRR), the destruction box of mitotic cyclins, and glycocylation state of proteins (Maupin-Furlow *et al*., 2000, Ulric, 2002).

The 26S proteasome in eukaryotes in general mediate the ATP-dependent degradation of ubiquitin bound proteins. Their ubiquitous presence and high abundance (comprise up to 1% of the cytosolic proteins) reflects their central role in cellular protein turnover (Bochtler *et al*., 1999). These multi-subunit protease complexes are involved in remarkable broad range of functions such as metabolic adaptation, cell differentiation, cell-cycle control, control of gene expression, turnover of normal proteins, apoptosis, signal transduction, stress response, antigen presentation. Besides that, the proteasome is responsible for the elimination of abnormal proteins include those which accumulate after exposure to stresses such as heat shock or oxidative damage as well as those which are produced by errors in transcription or translation, genetic mutations, or RNA damage and also subunits of multi-protein complexes are often targets for degradation when stoichiometry becomes disturbed (DeMartino *et al.,* 1996, Maupin-Furlow *et al*., 2000, Stolzing and Grune, 2001).

The proteasome holoenzyme (also known as the 26S proteasome) can be divided into two major subcomplexes: the 20S core particle, which forms the proteolytically active core and regulatory particle (RP) (also known as PA700, 19S cap particle, or μ), which confers polyubiquitin binding and energy dependence (Bloemendal *et al*., 1998, Glickman, 2000, Wollenberg and Swaffield, 2001). The holoproteasome is a very large complex with a mass of approximately 2,600 kDa containing at least 62 protein subunits encoded by 31 genes. The 20S core proteasome has a hollow barrel structure consisting of four stacked rings in the order αββα, with each ring consisting of seven subunits each (Löwe *et al*., 1995, Groll *et al*., 1997, Gerrards *et al*., 1998). The particle is self-compartmentalized, with the catalytic sites, formed by β-subunits, sequestered within the central cavity of the "barrel", rendering the isolated particle inactive (Lupas *et al*., 1997).

Approximately 700 kDa 19S or PA700 regulator binds to both end of the central catalytic portion in opposite orientations in an ATP-dependent manner to form the 2000 kDa, 26S dumbbell-shaped particle. 19S composed of about 20 subunits of 25-

110 kDa and can be dissociated *in vitro* defined subcomplexes, called the base and lid. The base contains six ATPases of the AAA family and they play role in the dissociation of target protein oligomers, protein unfolding. The 19S regulatory complex has about 14 non ATPase subunits which are involved in recognition of ubiquitinated proteins and in debiquitination, a process required to recyle ubiquitin from proteins destined for degradation (Tanaka, 1998, Tanahashi *et al*., 1999).

20S proteasome also serves as catalytic core of another larger proteasomal assemble, the "Activated" proteasome which has a ring-shaped multimeric regulator complexes, the 11S cap or PA28 or REG, attached to two ends of the 20S core and dramatically stimulate its capacity to hydrolyze small peptides. Association of PA28 with 20S proteasome does not require ATP-hydrolysis, differing from assembly of the 26S proteasome that occurs in an ATP-dependent fashion (Figure 1.7).

In mammalian cells, the proteasome population consists of a mix of [RP-20S-RP], [RP-20S-PA28], and [PA28-20S-PA28] (Tanahashi *et al*., 2000). PA28 consist of a single hexameric ring structure composed of two related proteins (PA28α and PA28β); with a total mass about 200 kDa (Wollenberg and Swaffield, 2001). The 20S/PA28 form of the proteasome is involved in the production and presentation of immunogenic peptides in vertebrates with an adaptive immune system (immunosomes) (Groetrupp *et al*., 1996a, 1996b). Each subunit is included by δ-interferon which is a potent stimulator of major histocompatibility complex (MHC) class I antigen presentation (Rock and Goldberg, 1999). This complex has been reported by one group to be hexameric (3α3β), and others to be a heptamer (3α4β) (Zhang *et al*, 1999).

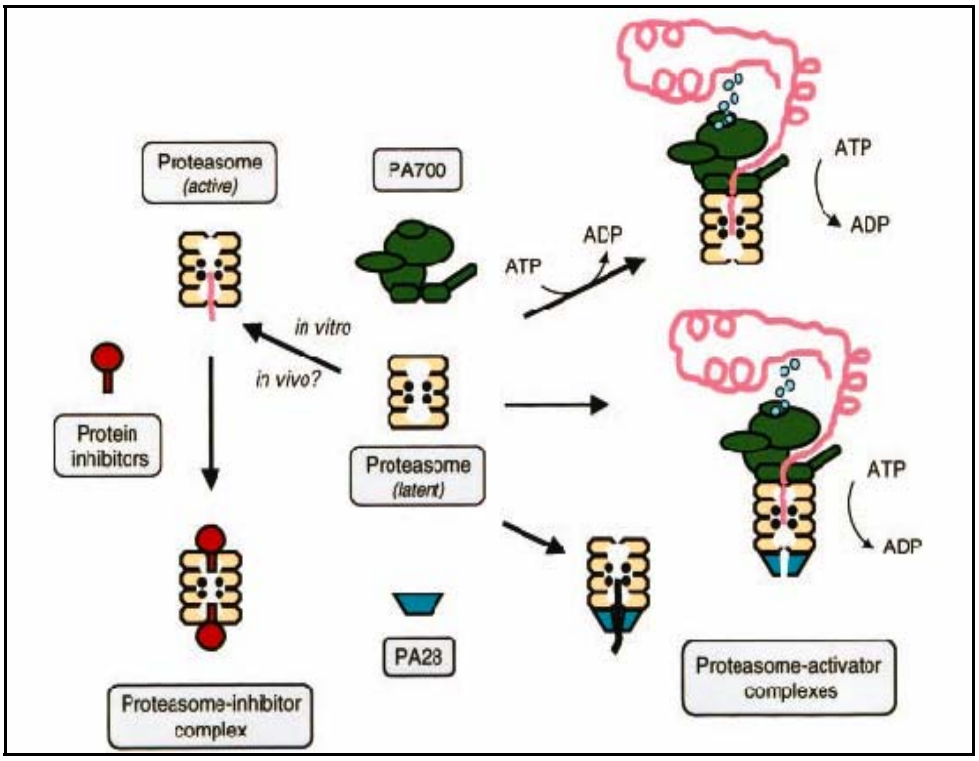


Figure 1.7. Regulation of proteasome activity by regulatory proteins (DeMartino and Slaughter, 1999).

1.6.1. 20S Proteasome Structure

The proteasome 20S core structure is conserved and found in the *Archaea, Eukarya* and gram-positive *actinomycetes* (Ward *et al*., 2002). The eukaryotic 20S core particle (about 700kDa) composed of 28 subunits with molecular weight ranging from 20 to 30 kDa. The 20S proteasome built from four stacked, seven membered rings. The rings are formed by 14 different but related subunits, which fall into two families, with the α-type subunits forming outer ring and the β-type subunit forming the inner rings of the complex (DeMartino and Slaughter, 1999, Gaczynska *et al*., 2001). The overall subunit composition can be therefore presented an organization of α7β7β7α7.

The simplest form of 20S proteasome has been found in many archeal species and bacteria i.e. gram-positive *actinomycetes* (Barber and Furry, 2001). Much of the early information about the structure of the proteasome come from studies of the enzyme from *Thermoplasma acidophilum* (Dahlmann *et al*., 1989*)* and it becomes prototype for the quaternary structure and topology of the enzyme. Unlike eukaryotic proteasome, the *T. acidophilum* proteasome is made of two subunits only, α and β, with molecular weights of 25.8 and 22.3 kDa, respectively (Zwickle *et al*., 1992 a). The two subunits have significant sequence similarity (26%) suggesting that their genes arose from common ancestral gene (Zwickle *et al*., 1992b, Schmidt *et al*., 1997). The archaebacterial and eukaryotic proteasomes are almost identical in size and shape; the subunits are arranged in four rings; α-subunits form the two outer rings of the stack, while the two rings composed of β-subunits which carry active site (Figure 1.8) (Pühler *et al*., 1992).

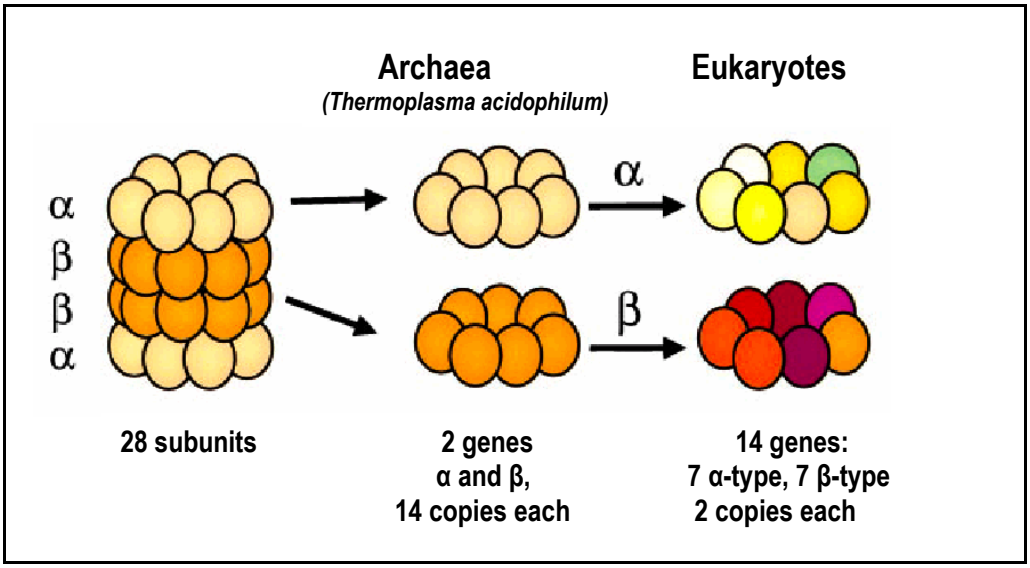


Figure 1.8. Structural organization of the 20S proteasome (DeMartino and Slaughter, 1999).

The 20S proteasome resembles a hollow cylinder, or barrel, with diameter of 11 nm and a length of 15 nm contains three inner cavities separated by narrow constructions (Figure 1.9). The two cavities between the α-subunits and β-subunit rings (the 'antechambers'), which have unknown function. The third cavity at the centre of the complex, where the proteolysis occurs has a height of 4 nm and a maximum diameter of 5.5 nm (Baumeister *et al*., 1997).

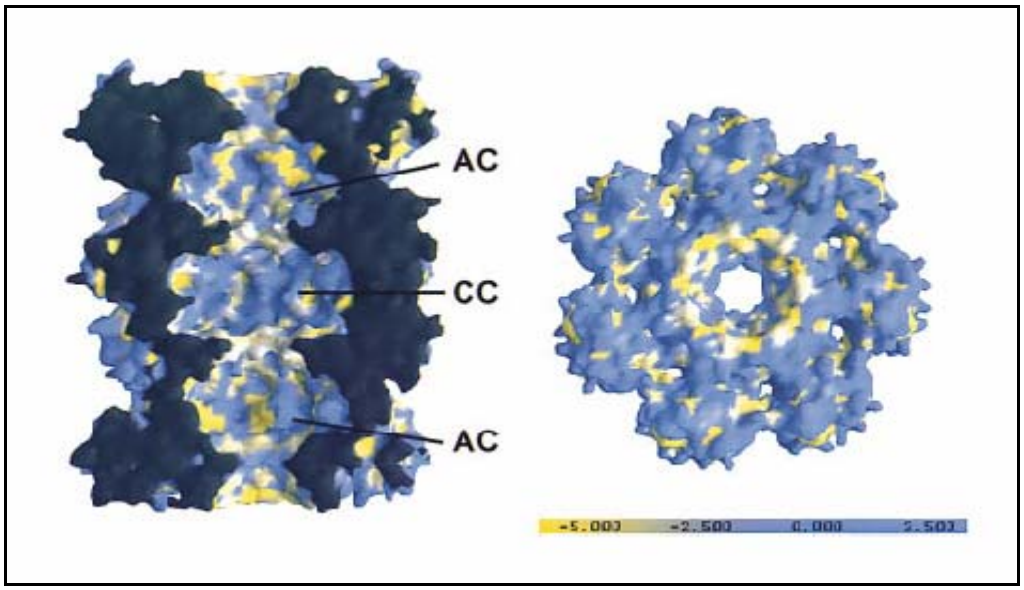


Figure 1.9 Inner and outer surface region of *Thermoplasma acidophilum* 20S Proteasome (Baumeister *et al.,* 1998). The left side shows a cut-open model of the complex with the antechambers (AC) formed jointly by α and β ring, and the central cavity (CC) formed by two β rings. On the right side, the outer surface of one α ring is shown. The scale bar shows the color coding of the hydrophobicity in kcal/mol.

It has been demonstrated that the 20S proteasome degrades proteins in a highly possessive manner chopping protein substrates at multiple places to yield peptide fragments of 3 to 30 amino acids in length (Akopian *et al*., 1997, Kisselev *et al*.,

1998). It means this particle digest proteins to small peptides before attacking another protein molecule. The central cavity, which less hydrophobic than the antechambers are narrow (1.3 nm in diameter), allowing it, in principle, to accommodate a single folded protein of about 70 kDa; a loosely packed unfolded polypeptide requires much more space. Since polypeptides can only enter the cavity one after the other, the central cavity will not usually accommodate more than one polypeptide at a time (Akopian *et al*., 1997). This self compartmentalized structure also act as safeguards against unwanted protein degradation (Lupas *et al*., 1997). According to current model, proteasomal α-subunits spontaneously self-assemble into seven-membered rings that serve as templates into which the β-subunits are incorporated (Baumeister *et al*., 1998).

1.6.2. Proteolytic Activity of the Proteasome

The eukaryotic proteasome characterized at least five types of proteolytic activity against short synthetic peptides and they have been classified with regard to residue at P1 position: chymotrypsin-like (hydrophobic), trypsin-like (basic), and post-glutamic or post acidic (acid) (PGPH), branched chain amino acid preferring (BrAAP), and small neutral amino acid preferring (SNAAP) activities (Orlowski and Wilk, 2000). Mutational analysis of yeast and mammalian proteasomes has been reported indicating only three active sites resides in this complex, β1 subunit is necessary for expression of PGPH activity, β2 for the trypsin-like activity and for β5 for chymotrypsin-like activity.

A hierarchy has been found among the different catalytic sites in terms of contribution to protein degradation with the chymotrypsin-like activity normally determining the rate of protein breakdown (Jäger *et al*., 1999, Krüger *et al*., 2001). Goldberg and colleagues demonstrated the existence of mutual allosteric regulation between the active centers for chymotrypsin-like and post-acidic activities (Goldberg et al., 2002). According to this “bite and chew” mechanism, the chymotrypsin-like site initially cleaves (bites) the protein substrate, which stimulates post-acidic sites.

These sites perform subsequent cleavages of the substrate previously cleaved (chew) while the chymotrypsin-like activity is inhibited as long as the post acidic sites are occupied. When there is no more substrates for post acidic sites, the allosteric inhibition of the chymotrypsin-like site stops and cycle is repeated (Papapostolou *et al*, 2002). In this model, protein degradation continues until the substrate is hydrolyzed to small peptides that vary in length from 4 to 25 residues.

The chymotryptic-like activity is possibly the main and one of the most active catalytic components of the proteasome. Therefore, it is the best characterized peptidase activity of 20S proteasome. The chymotryptic-like activity measured with chromogenic substrates is the most sensitive to inactivation by synthetic covalent inhibitors that bind covalently to the hydroxyl group of the N-terminal Thr. The general serine protease inhibitor 3, 4 dichloroisocoumarin (3, 4-DCI) is one of the first found proteasome irreversible inhibitor. 3, 4-DCI functions as a masked acid chloride that binds in the active site near the base-activated hydroxyl side chain of threonine to form a covalent ester linkage (Orlowski and Michaud, 1989). The α',β' epoxyketone is another irreversible proteasome inhibitor that was initially designed to target serine proteases then was successfully converted into proteasome inhibitors. Like the aldehydes,αβ' epoxyketones have two electron deficient carbon atoms that are susceptible to attack by the proteasomes threonine hydroxyl (Spaltenstein *et al*., 1996). Peptide vinyl sulfones which are electrophiles are found to be another type of proteasome reversible synthetic inhibitors. They inhibit the proteasome through covalent bond formation with the active site threonine hydroxyl (Bogyo *et al*., 1998).

The chymotryptic activity is also inhibited by peptidyl aldehydes having either a phenylalaninal or a leucinal residue as the aldehyde moiety. Peptide aldehydes such as leupeptin and calpain inhibitors I and II, Cbz-Leu-Leu-leucinal are frequently used to block proteasome activity both *in vitro* and *in vivo*. These agents are substrate analogues and potent transition-state inhibitors primarily of the chymotrypsin-like activity of the proteasome (Lee and Goldberg, 1998).

Lactacystin and its derivative clastro-lactacystin β-lactone, which are natural products structurally different from the peptide aldehydes, are much more specific proteasome inhibitors. Lactacystin was isolated originally from actinomycetes by its ability to inhibit cell division and induce neurite outgrowth in neural cell lines. The studies showed that lactacystin acts by inhibiting proteasome function as a presudosubstrate that becomes link covalently to the hydroxyl groups on the active site threonine of the β subunits (Dick *et al*., 1997, Bogyo and Wang, 2002).

Archaeal 20S proteasomes, which contain only one type of active site, exhibit various peptidase activities; most have only chymotrypsin-like activity, although some of them have high trypsin-like activity or post glutamyl activity (Akopian *et al*., 1997, Ward *et al*., 2002). The proteolytic mechanism of the 20S proteasome has been a matter of debate for a long time. Proteasomal subunits showed no sequence homology to member of four classical proteases: the serine, cysteine, aspartic acid or metallopeptidases. Studies with standard inhibitors revealed that the proteasome might be an unusual type of serine endopeptidase. However, unlike typical serine endopeptidases, it is insensitive to diisopropyl fluorophoshate and peptide chloromethyl ketones (Seemüller *et al*., 1995a). Mutagenenesis studies showed that neither serine nor histidine residues of the β-subunits are essential. Deletion of the amino-terminal threonine or its replacement by alanine residue abolished the enzyme activity, suggesting that threonine residue is near or in the catalytic center. Replacement, however, of the N-terminal threonine by serine generated fully active enzyme which was over ten times more sensitive to the serine protease inhibitor 3,4,-diisochloroisocoumarine (DCI). In combination with the crystal structure of a proteasome-inhibitor complex, the data indicated that nucleophilic attack is mediated by the amino-terminal threonine of processed β-subunits. Significantly, 20S proteasome from *T. acidophilum* has seven identical β-subunits, and thus seven active sites, and 20S proteasome from mammals has only four subunits with N-terminal Thr residues (Seemüller *et al*., 1995b).

Although the physiological role of the archaeal proteasome is unclear, inhibitor-based studies show *T. acidophilum* cells cannot grow without proteasome activity under heat shock conditions (Ward *et al*., 2002). In eukaryotes, it is known that 20S proteasome interplay in various cellular function that modulate the proteolytic activity of this enzyme. In eukaryotes, 20S proteasomes assemble with 19S cap complexes to form larger ATP-dependent 26S proteasome required for degradation of proteins often covalently modified ubiquitin. The 19S cap complexes appear to be responsible for recognition, unfolding, and translocation of substrate proteins into catalytic chamber of the 20S proteasome. Although the ubiquitin tagging mechanism appears to be restricted to eukarya, homologues, of the ATPase subunits of the 19S cap are highly conserved in the Archaea. One of these homologous, the archaeal PAN or proteasome-activating nucleotidase, has been shown to assemble into 550 kDa complexes and stimulate the hydrolysis of substrate proteins in presence of ATP that has been characterized from *Methanococcus jannaschii* (Wilson *et al*., 2000). When this ATPase mixed with the well characterized 20S proteasome from *Thermoplasma acidophilum* and ATP, this complex stimulated degradation of several unfolded proteins 8-25 fold. By Zwickle and his colleagues, this homohexameric ATPase ring complex named as PAN first time and they added that PAN did not promote the degradation of small peptides, which, unlike proteins should readily diffuse into the proteasome (Zwickle *et al*., 1999).

Benaroudj and his colleague's studies have indicated that PAN is a molecular chaperone that catalyzes the ATP-dependent unfolding of globular proteins. Presence of 20S proteasome, this unfoldase activity, is linked to degradation. Thus PAN, and presumably the 26 ATPase, unfold substrates and facilitate their entry into 20S particle, 26 proteasomes preferentially degrade ubiquitinated proteins (Benaroudj *et al*., 2001).

1.6.3. Features of Archaeal Proteasomes

Proteasomes have been purified from several thermophilic archaea. One of the archeal proteasomes isolated from well-studied *Thermoplasma acidophilum*. It is purified to apparent homogeneity with molecular mass of about 650 kDa and isoelectric point of 5.6. *Thermoplasma acidophilum* proteasome hydrolyses peptide substrates containing an aromatic residue adjacent to the reporter group, as well as [^{14}C] methylated casein optimally at pH 8.5 and 90^{o}C. The enzyme activity is enhanced several fold by Mg^{2+} and Ca^{2+} at 25-500 mM. The purified proteasome was irreversibly inhibited serine protease inhibitor diisopropyflourophosphate and 3, 4-dichloroisocoumarin (Dahlmann *et al*., 1992). *T. acidophilum* proteasome is made of multiple copies of two subunits, only α and β, with molecular weights of 25.8 and 22.3 kDa, respectively. Analysis of the derived amino acid sequence of the β subunits (211) and comparison with α subunit (233) show that two amino acid sequences are showing 24 % identity (Zwickle *et al.,* 1992a). The two genes encoding the constituent subunits of *T. acidophilum* proteasome were co-expressed in *E.coli* yielding correctly folded and assembled proteolytically active proteasomes, which do not influence the viability of host cells (Zwickle *et al*., 1992b). The catalytic mechanism of the 20S proteasome from the archeabacterium *T. acidophilum* has been analyzed by site-directed mutagenesis of the β-subunit and by inhibitor studies (Baumeister *et al*., 1997).

A 645 kDa proteasome was purified from *Methanosarcina thermophila*. Purified proteasome gave two bands on 12% SDS-polyacrylamide gel, 24 kDa (α-subunits), 22 kDa (β-subunits). The purified *M. thermophila* enzyme displayed chymotrypsin-like activity catalyzing hydrolysis of Suc-Ala-Ala-Phe-AMC (7-amido-4-methyl-coumarin). The enzyme also had peptidylglutamyl-peptide hydrolyze activity catalyzing the hydrolysis of Cbz-Leu-Leu-Glu-β-NA (Maupin-Furlow and Ferry, 1995). α-subunit and β-subunit encoding genes of the *M. termophila* 20S proteasome were cloned, expressed and purified independently in *Escherichia coli*. Characterization studies showed that heterogously produced *M. thermophila* 20S

proteasome predominantly catalyzed cleavage of peptide bonds carboxyl to the acidic residue Glu (postglutamyl activity) and the hydrophobic residues Phe and Tyr (chymotrypsin-like activity) in short chromogenic and fluorogenic peptides. Low level hydrolyzing activities were also detected carboxyl to the acidic residue Asp and basic residue Arg (trypsin-like activity). SDS and divalent or monovalent ions stimulated chymotrypsin-like activity and inhibited postglutamyl activity (Maupin-Furlow *et al*., 1998).

One of the 20S proteasome purified from a hyperthermophilic archaeon, *Pyrococcus furious* using following column chromatography methods*;* DEAE Sepharose, Hydroxyapatite, Phenyl Sepharose 650, Sepharacyl S300 gel filtration column chromatographies. It was found that 640 kDa purified proteasome comprised of 25 kDa α-subunit and 22 kDa β-subunit. The pH optimum of the proteasome was slightly acidic at pH 6.5 and optimum temperature was 95^{o}C. The *P. furious* proteasome cleaved peptidyl linkages on the carboxyl side of some hyrophobic residues, including methionine, tyrosine, and phenylalanine (Bauer *et al*., 1997).

Another 20S proteasome purified from a halophilic archaeon *Haloferax volcanii*. A 600-kDa purified proteasome was found to be composed of α_1- and β-subunit with relative molecular masses of 37,5 and 30 kDa, respectively. The optimum temperature and pH were obtained 75^{o}C and 7.7 in 2 mM NaCl-Tris buffer, respectively. The highest measured of the peptide with synthetic peptides was cleavage carboxyl to the hydrophobic residues phenylalanine, tyrosine, and tryptophan (Chymostrypsin like activity) as determined by using Suc-Ala-Ala-Phe-AMC, Suc-Leu-Leu-Val-Tyr-Amc, Suc-Ile-Ile-Trp-Amc (Wilson *et al*., 1999).

One of the 20S proteasome was purified from *Methanococcus jannaschi* as a 700 kDa complex by *in vitro* assembly of the α and β subunits and it was determined that *M. jannaschi* has high rate hydrolyzing activity against peptide and unfolded-polypeptide at 100^{o}C. The enzyme also had high rate peptidylglutamyl-peptide hydrolyzing activity catalyzing CBZ-Leu-Leu-Glu-βNA and also it had significant

chymotrypsin-like activity and catalyzing Suc-Leu-Leu-Val-Try.AMC and Suc-Ala-Ala-Phe-AMC. Low levels of trypsin-like activity were also observed for the *M. jannaschi* (Wilson *et al*., 2000).

Recently, one of the 20S proteasome, comprising two subunits α (32 kDa) and β (25 kDa), was purified and characterized from the extremely halophilic archaeon *Holoarcula marismortui*. The optimum temperature for *H. marismortui* proteasome activity was found 60^{o}C in assay buffer containing 2 M KCl. Frantzetti and colleagues were investigated that *H. marismortui 20S* proteasome eliminates a misfolded protein (Frantzetti *et al*., 2002).

1.7. Biology of *Thermoplasma volcanium*

The source of the enzymes for the experiments in this thesis, the archaebacteria *Thermoplasma* (*Tp*) *volcanium*, is an interesting organism. As a member of the thermophilic eubacteria within the archae kingdom, it thrives best acidic and warm conditions (Darland *et al*., 1970). *Tp. volcanium* originally was isolated from submarine and continental solfataras at Vulcano Island, Italy. The genus *Thermoplasma* is unique among archae as it is adaptable to aerobic environment as well as anaerobic environment. *Tp. volcanium* has optimal growth conditions at approximately pH 2-3 and temperatures between 55-60^{o}C (Segerer *et al*., 1988). Although the organism thrives in an acidic environment, a nearly neutral cytosolic pH is maintained without the use of a rigid cell wall (Langworthy *et al*., 1972). The complete genomic sequence of the archaeon *Tp. volcanium* was determined as 1,584,799 bases (Kawashima *et al*., 2000). GC content of DNA is about 46 mol% (Tm, Bd, and direct analysis).

1.8. Scope and Aim

In this study, an intracellular serine protease and 20S proteasome from thermoacidophilic archaeon *Thermoplasma volcanium*, have been purified and

characterized first time. Purification of the novel proteases were achieved by gel permeation, ion-exchange, hydroxylapatite and affinity colon chromatographies. Biochemical characterizations involved a series of optimization experiments, such as determination of effect of metal ions, optimum temperature, optimum pH, substrate specificity and Michaelis constants. The result of these studies would be useful in better understanding of the molecular biology of the thermophilic proteases. Since thermophilic proteases have several biotechnological applications, this new thermophilic proteases also could be an alternative as biocatalysts for future use.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1 Chemicals

Acrylamide, agarose, ammonium sulfate [$(NH_4)_2SO_4$], ammonium persulfate (APS), azocasein, calcium chloride ($CaCl_2$), chromogenic substrates (N-Suc-Phenilalanine-pNA (N-Suc-Phe-pNA), Alanine-Alanine-Phenilalanine-pNA (Ala-Ala-Phe-pNA), N-Carbobenzoxyl-Arginine-pNA (N-CBZ-Arg-pNA), N-Suc-Alanine-Alanine-Alanine-pNA (N-Suc-Ala-Ala-Ala-pNA), N-Suc-Alanine-Alanine-Valine-Alanine-pNA (N-Suc-Ala-Ala-Val-Ala-pNA), N-Suc- Alanine-Alanine-Valine-pNA (N-Suc-Ala-Ala-Val-pNA), L-Leucine-pNA (L-Leu-pNA), N-Suc-Alanine-Alanine-Proline-Penilalanine-pNA (N-Suc-Ala-Ala-Pro-Phe-pNA), N-CBZ-Leucine-Leucine-Glutamine-βNA (N-CBZ-Leu-Leu-Glu-βNA)),chymostatin, coomassie brillient blue R-250, glucose, glycerol, ethidiumbromide (EtBr), dithiothreitol (DTT), ethyleneglycol-bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), iodoacetic acid (IAA), 3-[N-morphalino] propanesulfanic acid (MOPS), pepstatin, phenol, phenylmethylsulfonyl flouride (PMSF) sodium perchlorate ($NaClO_4$), N,N,N',N'-tetramethylenediamine (TEMED), N-[N-(l-3-trans-carboxyloxirane-2-carbonyl)-L-leucyl]-agmatine L-3-carboxytrans 2,3-epoxypropyl-leucylamido (4-guanidine) butane (E.64), trichloroacetic acid (TCA), tris [hydoxymethl] aminoethane ($C_4H_{11}NO_3$), tosyl-L-phenylalanine-chloromethyl ketone (TPCK), triton

X-100 were purchased from Sigma Chemical Company, Saint Louis, Missouri, U.S.A.

Acetic acid-glacial, bromophenol blue ($C_{19}H_{10}Br_4O_5S$), chloroform, dimethyl sulfoxide (DMSO), dodecylsulfate sodium salt (SDS), ethylediamineteraacetic acid (EDTA), glucose, glycine, isoamyl alcohol, magnesium chloride ($MgCl_2$), manganase chloride ($MnCl_2$) N,N'-methylene-bisacrylamide (Bis), hydrochloric acid (HCl), cobalt (II) chloride ($CoCl_2$), mercaptoethanol, sodium chloride (NaCl), sodium per chlorate, sodium hydroxide (NaOH), were purchased from Merck, Darmstadt, Germany.

Yeast extract was purchased from Difco, Detroit, U.S.A. Ethanol was from Reidel de Höen. N, N, dimethyl formamide from Fluka Garatie.

Tag DNA Polymerase was purchased from MBI Fermantas AB, Vilniues, Lithuania and RNAase A were purchased from Sigma Chemical Company. Saint Louis, Missouri, U.S.A.

2.1.2. Buffers and Solutions

Compositions of buffers and solutions used in the experiment are given in Appendix A .

2.1.3. Molecular Size Markers

DNA molecular size markers are given in Appendix B.

Protein molecular size markers are given in Appendix C.

2.2. Methods

2.2.1 Strains and Media

Thermoplasma volcanium culture was purchased from DSMZ-Deutsche Sammlung von Mikroordanismen und Zellkultured GmbH (Braunschweig, Germany). *Thermoplasma volcanium* cells were grown in Volcanium Medium, pH 2.7 which was supplemented with glucose (0.5%, w/v) and yeast-extract (0.1%, w/v) (Robb and Place, 1990). The cultures were renewed by subculturing at 60^{o}C once a week.

2.2.2. Purification of *Thermoplasma volcanium* Chromosomal DNA

The genomic DNA was isolated essentially according to protocol described by Sutherland *et al*. (1990). *Thermoplasma* cells were grown in 200 ml Volcanium Medium in 1 lt flask until the late log phase was reached and then the cells were harvested by centrifugation at 7 000 rpm (Herause Sepatech Centrifuge 17RS) for 15 minute at 4^{o}C. The supernatant was discarded and, the pellet was resuspended in 3 ml of 50 mM Tris-HCl, pH 8.0, containing 25% glucose. After cell lyses, sodium perchlorate solution was added to give final concentration of 1 M. An extraction with chloroform: isoamylalcohol (24:1 v/v) was performed. After centrifugation at 7 000 rpm for 10 min, the aqueous phase was transferred to a fresh tube and 2 volumes of absolute ethanol was added for DNA precipitation at –20^{o}C followed by centrifugation at 12 000 rpm for 30 min. The pellet was washed with 70% ethanol and dissolved in minimal volume of TE buffer. The RNase treatment and phenol extraction was carried out as described by Sambrook *et al*. (2001). After ethanol precipitation, the pellet was dissolved in TE buffer and stored at -20^{o}C. Purified chromosomal DNA was analyzed by gel electrophoresis with 1% agarose gel.

2.2.3. Amplification of 16S rRNA of *Thermoplasma volcanium*

The purity of *T. volcanium* cells were checked by specific amplification of 16S rDNA sequences by Polymerase Chain Reaction (PCR). We have used PCR primers which were designed for amplification of conserved regions of *archaea* and *bacteria* 16S rDNA sequences (Robb and Place, 1990). The oligonucleotides are synthesized by Integrated DNA Technologies, Coralville, IA, U.S.A. Each 100 µl PCR amplification reaction mixture contained 10 µl of reaction buffer (750 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.1% Tween-20), 500 ng of template DNA, 1.5 mM $MgCl_2$, 0.2 mM of each deoxyribonucleoside triphosphates (dNTPs) and 50 pmole of each primer, in a 0.5 ml volume test tube, which was filled up to a volume of 100 µl with sterile distilled water. The mixtures were overlaid with 70 µl mineral oil. The samples were preincubated at 95°C for 5 minute and then, 2.5 U of Taq DNA polymerase (MBI Fermentas AB, Vilnius, Lithuania) was added. The PCR was performed with a Hybaid Thermal Cycler (Hybaid, Hook and Tucker Inst., UK). The thermal cycling was as follows: denaturation at 94°C for 1 minute, annealing at 37°C for 2 minute, and amplification at 72°C for 3 minute, for a total of 30 cycles. Primer extension was carried out at 72°C for 10 minute. Amplified PCR products were analyzed by gel electrophoresis with 1% agarose gel and then by ethidium bromide staining. A 1 kb ladder standard (Gene Ruler, 1 kb DNA Ladder, MBI Fermentas) was used for length calibration.

2.2.4. Agarose Gel Electrophoresis

Submarine agarose gel apparatus (Mini Sub™ DNA Cell, Bio Rad, Richmond, CA,U.S.A) (40 ml) was used in the gel electrophoresis experiments using 1X TAE (Tris-acetate-EDTA) as the running buffer. Ethidium bromide at final concentration of 1.25 µg/ml was added to cooled agarose gel before solidification. A 1/10 volume of tracking dye was mixed with each DNA sample before loading. The size standard used was Gene Ruler 1 kb DNA Ladder (MBI Fermentas AB, Vilnius, Lithvania). Electrophoresis was carried out at 60 volts.

2.2.5. Preparation of Cell Free Extract.

Thermoplasma volcanium cell in 250 ml cultures were harvested by centrifugation at 8 000 rpm (Sigma 3K30 Centrifuge, Sigma Chemical Co., St. Louis, Missouri, U.S.A) for 15 minute at 4^{o}C. The cells were washed in unsupplemented growth medium. Washed cells were centrifuged (Sigma 3K30 Centrifuge, Sigma Chemical Co., St. Louis, Missouri, U.S.A) at 8 000 rpm for 15 minute at 4^{o}C. The pellet was resuspended in 1/5 volume of protease assay buffer. The cells were disrupted by sonication (Sonicator VC 100, Sonics and Materials, CT, U.S.A) 1 minute with 30 second intervals and the cell free extract (CFE) was obtained by centrifugation (Sorwall Combi Plus Ultracentrifuge, Wilmington, Delaware, U.S.A) at 12 000 rpm for 1.5 hour at 4^{o}C. This extract was stored -20^{o}C until use.

2.2.6. Determination of Enzyme Activity

2.2.6.1. Determination of Protease Activity by Using Azocasein

Alkaline protease activity of *T. volcanium* was measured using azocasein as substrates according to the method of Cowan *et al*., (1987). The assay mixture contained, 600 µl of 0.5% (w/v) azocasein (Sigma St. Louis, Missouri, U.S.A) in 50 mM Tris HCl buffer (pH 7.0) containing 5 mM $CaCl_2$ and 40 µl cell free extract. The assay was carried out at 60^{o}C in a time dependent manner, and the reaction terminated by addition of 480 µl of 15% trichloroacetic acid (TCA). The samples were incubated on ice and the precipitate was removed by centrifugation (Heraus Sepatech, Biofuge15, GmbH, Osterode, Germany). After addition of 10 µl of 10 M NaOH into 900 µl of the supernatant, the absorbance of the supernatant was monitored at 440 nm using UV/Visible 160A spectrophotometer (Schimadzu, Kyoto). One unit of proteinase activity was defined as the amount of enzyme required to produce an increase in absorbance at 440 nm of 0.1 under the above-mentioned assay conditions.

2.2.6.2. Determination of Protease Activities by Using Chromogenic Substrate

The alkaline protease activity was also determined by measuring p-nitroanilide liberation from N-Succinyl-Phenylalanine-para-Nitroanilide (N-Suc-Phe-pNA) which is a chromogenic synthetic peptide substrate according to the method of Kirchke and Wiederonsders (1999). The continuous assay was carried out at 55^{o}C. The absorbance was measured at 410 nm using UV/visible160A spectrophotometer (Shimadzu, Kyoto). One unit of p-nitroanilide-hydrolyzing activity was defined as the amount of enzyme that liberated 1 nmol of p-nitroanilide per minute (Σ_{410}=8800 $M^{-1}cm^{-1}$).

2.2.7. Purification of Serine Protease from *Tp. volcanium* by Column Chromatography

2.2.7.1. Ammonium Sulfate Precipitation

The cell free extract was subjected to ammonium sulfate precipitation by dissolving solid ammonium sulfate to give about 80% saturation at 4^{o}C. The precipitate formed was collected by centrifugation (Sigma 3K30 Centrifuge, Sigma Chemical Co., St. Louis, Missouri, U.S.A) at 4^{o}C and the pellet was dissolved in 1/20 volume (v/v) protease assay buffer pH 7.5.

The 80% $(NH_4)_2SO_4$ fraction was filtered using 0.45 µl millipore millex HV. Then, to remove residual ammonium sulfate it was washed several times using 5 000 cut-off ultrafiltration device (Vivascience, Sartorious Group, Stonehouse, Gloucestershire, U.K.) with protease assay buffer pH 7.5. The volume increased to 10 ml with protease assay buffer pH 7.5, and then concentrated.

2.2.7.2. Ion Exchange Chromatography

Ion-exchange chromatography was conducted by using Econo-Pac Q ion exchange cartridge (Bio Rad) which was equilibrated with protease assay buffer. One ml of desalted and concentrated enzyme sample was loaded and then, the column was washed with protease assay buffer until the absorbance at 280 nm of the eluate had returned to the baseline. About 900 µl fractions were collected by the aid of a fraction collector (BioRad Model 2110 Richmond, CA, U.S.A). The bound proteins were eluted with linear gradient of 0-1 M NaCl using a gradient maker. Protein contents of the fractions were monitored by absorbance measurement at OD_{280}. The proteolytic activities of the fractions associated with the protein peaks were measured based on N-Suc-Phe-pNA hydrolysis as described before. The fractions with detectable proteolytic activity were pooled, then desalted and concentrated by ultrafiltration using 5 000 cut-off ultrafiltration device (Vivascience, Sartorious Group, Stonehouse, Gloucestershire, U.K.). During ultrafiltration, then enzyme was washed with protease affinity chromatography buffer solution.

Protease ion exchange column was cleaned following a series of washing steps. The column was first washed with 1 M NaOH, and then equilibrated with protease ion exchange buffer pH 7.5. After washing the column with high salt buffer, it was equilibrated with protease ion exchange buffer pH 7.5 again.

2.2.7.3. Affinity Chromatography

Concentrated fractions from the ion exchange column were applied to α-casein agarose column (Sigma, chemical Co. St Louis, Missouri, U.S.A), which was preequilibrated with Buffer A. Contaminating proteins were removed from the column by washing with the same buffer, then by additional washing with buffer A containing 1 M NaCl (buffer B). The protease fraction was eluted with 50 ml buffer B containing 25% isopropanol (Manachini *et al*., 1988). Protein amount of the fractions were determined by measuring absorbance at 280 nm.

Fractions with protease activity against N-Suc-Phe-pNA were pooled, then desalted and concentrated using 5 000 cut-off ultrafiltration device (Vivascience, Sartorious Group, Stonehouse, Gloucestershire, U.K.). The α-casein agarose column was washed with 50 ml buffer A, followed washing with 100 ml buffer A containing 1 M NaCl. The column then equilibrated by washing with 100 ml buffer A.

2.7.3.4. Determination of Enzyme Activity by Gelatin Zymography

The protease activity was also detected by Gelatin zymography as described by Kocabıyık and Erdem (2002). In this process, samples were electrophoresed in zymogram resolving gel containing 5% polyacrylamide, 0.1% SDS and 0.2% (w/v) copolymerized gelatin (Connaris *et al*., 1991). Enzyme samples were mixed with 2X sample buffer containing 1% SDS but not reducing agent. The electrophoresis was carried out at 100 V, 4^{o}C per gel using a Biometra Vertical Minigel System (Biometra Biomedzinische Analytic Gmbh, Göttingen, Germany). After the electrophoresis was completed, the gel was washed twice in 2.5% Triton X-100 solution for 30 minute to remove SDS. The gel was transferred to zymogram development solution and incubated for 18 hours in the zymogen development solution to allow the enzyme to digest gelatin. The gel was stained in the Coomassie Blue Staining solution and clear zones were observed after several washings in the Destaining solution.

2.2.8. Biochemical Characterization of *Thermoplasma volcanium* Serine Protease

2.2.8.1. Substrate Specificity of Purified Serine Protease

The purified enzyme was tested for its ability to hydrolyze synthetic peptide substrates which are N-terminally blocked by succinyl (Suc) or carbobenzoxyl (CBZ) and the C-terminus contain aromatic amines such as para-nitroanilide (pNA). The continuous spectrophotometric assay was in a total volume of 800 µl, as

described before. Reaction was started by adding substrate solution to give final concentration of 0,625 mM. One unit of p-nitroanilide-hydrolyzing activity was defined as the amount of enzyme that liberated 1 nmol of p-nitroanilide per minute (Σ_{410}=8800 $M^{-1}cm^{-1}$) (Erlanger, 1961).

2.2.8.2. Kinetic Measurement of Purified Serine Protease

The K_m and V_{max} values of the purified protease for N-Suc-Phe-pNA were determined from the Lineweaver-Burk plot generated from the initial reaction velocities obtained with substrate concentrations in the range 0.12-1.2 mM. Each assay was carried out at 55°C in protease assay buffer pH 7.5. The change in absorbance at 410 nm was monitored continuously and initial velocity was used for calculation of the kinetic constants.

2.2.8.3. Proteolytic Activity of *Tp. volcanium* Protease Against Natural Proteins

2.2.8.3.1. Determination of Enzyme Activity Against Casein

Serine protease activity of *T. volcanium* was measured using casein as protein substrates according to the method of Anson (1938). 100 µl of enzyme was incubated with 700 µl of 1% (w/v) casein solution (prepared in Protease assay buffer, pH 7.5) for 0 to 20 min at 55°C. The reaction was stopped by addition of 900 µl 15 % tricholoroacetic acid (TCA) and kept for 15 minute at room temperature. After the contents were centrifuged (Herause Sepatech, Biofuge15, GmbH, Osterode, Germany) at 13 000 rpm for 10 minute at 55°C, 1.5 ml of supernatant was filtered through Whatmann no.1 filter paper. The optical density of samples was measured at 280 nm on a Shimadzu spectrophotometer (model UV 160 A). One unit of enzyme activity was defined as an increase in absorbance reading ($A_{280\ nm}$) of 0.01/60 min, at 55°C.

2.2.8.3.2. Determination of Enzyme Activity Against Bovine Serum Albumin

The proteolytic activity assay was determined using other protein substrates. Stock solution of bovine serum albumin was prepared in Protease assay buffer pH 7.5. The activity was determined by the method described Foltman *et.al.* (1985). 1 ml of 1% (w/v) bovine serum albumin preparation was added to 100 µl enzyme solution incubated for 0 to 60 minute at 55^{o}C. The reactions were stopped with addition of 3 ml 15 % tricholoroacetic acid (TCA). After centrifugation for 20 minute at 5 000 rpm (Herause Sepatech, GmbH, Osterode, Germany), the absorbance of the supernatant was read at 280 nm using UV/Visible 160A spectrophotometer (Schimadzu, Kyoto) against a blank. One unit of enzyme activity was defined as an increase in absorbance reading ($A_{280\ nm}$) of 0.01/60 min, at 55^{o}C.

2.2.8.4. Effect of Temperature on Caseinolytic Activity of *Tp. volcanium* Protease

The optimum temperature for the protease activity was determined by caseinolytic activities at various temperatures ranging between 40 and 80°C. Otherwise, the reaction conditions were same as the one described before. The residual activity was expressed as the percentage of the highest enzymatic activity measured at one of the incubation temperatures.

2.2.8.5. Effect of Temperature on N-Suc-Phe-pNA Hydrolysis by *Tp. volcanium* Protease

The optimum temperature of the enzyme was also determined by using N-Suc-Phe-pNA as the substrate and incubating the enzyme-substrate mixture at various temperatures ranging between 35 and 58^{o}C under the standard assay conditions as described before. The residual enzyme activity was then measured as described before.

2.2.8.6. Effect of pH on Protease Activity

The pH optimum of the serine protease activity was measured at different pH values using N-Suc-Phe-pNA under the standard assay conditions as the one described before. The buffer used were 50 mM Sodium acetate in the pH range of 4.0- 6.0, 50 mM MOPS in the pH range of 6.0- 7.0, 50 mM Tris-HCl in the pH range of 7.5-9.0, 50 mM Glycine in the pH range of 9.5-12.0 each contains 2 mM $CaCl_2$. The residual activity was expressed as the percentage of the highest enzymatic activity measured at one of the pH systems. To measure pH stability of the enzyme, residual protease activities were assayed after incubation for 1 hour in these buffer solutions at different pH values.

2.2.8.7. Effects of Inhibitors on Enzyme Activity

The inhibitor sensitivity of the purified enzyme at 1 to 2 mM concentrations of phenylmethylsulfonyl flouride (PMSF) as described by Levy and Chou (1974), at 200 to 400 μg/ml concentration of chymostatin as described by Feinstain *et al*. (1976) and 0.5 to 1 mM of tosyl-L-phenylalanine-chloromethyl ketone (TPCK) as described by Grammer and Blenis (1996). After treatment of aliquots of enzyme solution with inhibitors the remaining enzyme activity was determined using azocazein as substrate, as described before. The activity was expressed as the percentage of the enzyme activity measured under identical conditions but in the absence of inhibitors. Effects of ethyleneglycol-bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) (10 mM), ethylenediamine teraaceticacid (EDTA) (10 mM) and 1,4 dithiothreitol (DTT) (20 mM) on caseinolytic activity were also measured by azocasein assay, as described before.

2.2.8.8. Effect of Metal Ions on Enzyme Activity

The effect of various metal ions on the protease were assayed by measuring the N-Suc-Phe-pNA hydrolysis activity of the purified enzyme in the presence of the

various ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+} in chloride salts) at a concentration of 4 mM under the standard assay conditions. The activity was determined as described before and expressed as the percentage of the activity of the enzyme under the identical conditions in the absence of inhibitors.

2.2.8.9. Effect of $CaCl_2$ and $MgCl_2$ on Enzyme Activity

For determining the influence of Ca^{2+} and Mg^{2+} on enzyme activity, calcium and magnesium salts ($CaCl_2$ and $MgCl_2$) at different concentrations (0.5- 12.5 mM) was added into assay mixture. The enzyme activity was determined by measuring the activity at 55°C under the standard assay conditions as described before using N-Suc-Phe-pNA as substrate.

2.2.9. Purification of 20S Proteasome by Colon Chromatography

2.2.9.1. Preparation of Cell Free Extract

Thermoplasma cells are grown in 250 ml Volcanium Medium in 1 lt flask and the precipitated by centrifugation at 8 000 rpm (Sigma 3K30 Centrifuge, Sigma Chemical Co., St. Louis, Missouri, U.S.A) for 15 minute at 4°C. After washing in the unsupplemented Volcanium Medium, the pellet was dissolved in 1/5 volume of proteasome assay buffer solution. The cell were disrupted by sonication (Sonicator VC 100, Sonics and Materials, CT, U.S.A) 1 minute with 30 second intervals, and cell free extract (CFE) was obtained by centrifugation at 12 000 rpm, for 1 hour and 15 minute at 4°C (Sorwall Combi Plus Ultracentrifuge, Wilmington, Delaware, U.S.A). The extract was frozen at -20°C, and thawed whenever required.

2.2.9.2. Ammonium Sulfate Precipitation

The proteins in the cell free extract were precipitated in two steps by ammonium sulfate solutions at 40 % (w/v) and 80 % (w/v) concentrations at 4°C. The last

precipitate collected by centrifugation 10 000 rpm (Sigma 3K30 Centrifuge, Sigma Chemical Co., St. Louis, Missouri, U.S.A) for 30 minute, was resuspended in a 1/10 volume of proteasome assay buffer and desalted by ultrafiltration using 10 000 cut-off membrane filter (Vivascience, Sartorious Group, Stonehouse, Gloucestershire, U.K.).

2.2.9.3. Gel Permeation Chromatography

Desalted and concentrated 80% ammonium sulfate fraction was applied onto a gel filtration column (DEAE-Sepharose fast flow column, Amersham Pharmacia Biotech, Sweden). The proteasome associated chymotryptic like activity was eluted with the chromatography buffer and 900 µl fractions were collected with 0.8 µl/ml flow rate using peristaltic pump (Whatson Marlow 302S). Proteolytic activity of the fractions were measured using Ala-Ala-Phe-pNA as substrates, and those showing chymotryptic activity were pooled and desalted using 10 000 cut-off (Vivascience, Sartorious Group, Stonehouse, Gloucestershire, U.K.) ultrafiltration membrane filter.

2.2.9.4. Ion Exchange Chromatography

Ion exchange chromatography was performed on Sepharacyl S-300 column (Amersham Pharmacia, Biotech, Sweden). After loading the pooled active enzyme fractions, the column was washed with the same buffer; the bound proteins were eluted with a linear NaCl gradient from 0 to 0.5 M in 50 ml of the chromatography buffer. 900 µl eluents collected by help of peristaltic pump (Whatson Marlow 302S). The fractions of the first peak which showed chymotriptic-like activity were pooled and concentrated using a 10 000 cut-off ultrafiltration membrane filter (Vivascience, Sartorious Group, Stonehouse, Gloucestershire, U.K.).

2.2.9.5. Hydroxylapatite Column Chromatography

Hydroxyapatite column chromatography was performed on a Econo-Pac HTP Cartridge (Bio Rad, Richmond, CA, U.S.A), which had been equilibrated with 0.1 M

potassium phosphate-buffer pH 7.5 (PPB). The concentrated active fractions from previous chromatography were directly loaded onto column. A 0.1 M to 0.6 M potassium phosphate linear gradient (25 ml) was used for elution, and fractions with chymotryptic activity were pooled. The pooled fractions was desalted using 5 000 cut-off filter (Centrisart, Sartorious Group, Stonehouse, Gloucestershire, U.K.) using 3 mM MOPS buffer pH 7.5 and then concentrated using the same filtration device.

2.2.10. Biochemical Characterization of the Purified 20S Proteasome

2.2.10.1. Substrate Specificity of Purified 20S Proteasome

The purified protease enzyme was tested for it is ability to hydrolyze synthetic peptide substrates which are N-terminally blocked peptides by succinyl (Suc) and carbobenzoxyl (Cbz) group and the C-therminus contain aromatic amines such as para-nitroanilide (pNA) and β-naphthylamide (βNA). An aliquot of purified enzyme was preincubated at 55^{o}C in proteasome assay buffer, pH 7.5. Reaction was started by adding of each substrate, to give a final concentration of 0,625 mM. The rate of pNA formation was measured at 55^{o}C spectrophotometrically at 410 nm and the rate of βNA formation was measured 55^{o}C at 340 nm using UV/Visible 160A spectrophotometer. One unit of p-nitroanilide-hydrolyzing activity was defined as the amount of enzyme that liberated 1 nmol of p-NA or β-NA per minute. (Schimadzu, Kyoto) (Σ_{410}=8800 $M^{-1}cm^{-1}$, Σ_{3}=1780 $M^{-1}cm^{-1}$) (Erlanger, 1961, Kirschke and Wiederonders, 1999).

2.2.10.2. Kinetic Measurement of Purified 20S Proteasome

The K_m and V_{max} values of the purified protease for Ala-Ala-Phe-pNA were determined from the Line weaver-Burk plot generated from the initial reaction velocities obtained with substrate concentrations in the range of 0.03-0.73 mM. Each assay was carried out at 55^{o}C in proteasome assay buffer pH 7.5. The change in absorbance at 410 nm was monitored continuously and initial velocity was used for calculation of the kinetic constants.

2.2.10.3. Effect of Temperature on Enzyme Activity

The optimum temperature for the enzyme activity was determined by measuring the activity at various temperatures ranging between 35-58^{o}C under the standard assay conditions as described before using Ala-Ala-Phe-pNA as substrate.

2.2.10.4. Effect of $CaCl_2$ and $MgCl_2$ on Enzyme Activity

To determine if the activity of the enzyme was affected by Ca^{2+} or Mg^{2+} concentration, the enzyme was pre-incubated at room temperature in the presence of different concentrations of $CaCl_2$ and $MgCl_2$. The activity was measured as described using the Ala-Ala-Phe-pNA as substrate.

2.2.10.5. Effect of Inhibitors on Enzyme Activity

Enzyme samples were preincubated at room temperature for 15 minute in the presence of following proteinase inhibitors: phenylmethanesulphonyl fluoride (PMSF) (serine protease inhibitor), chymostatin, tosyl-L-phenylalanine-chloromethyl ketone (TPCK) (chymotrypsin-like serine protease inhibitors), Leupeptin, tosyl-L-lysine-chloromethyl ketone (TLCK) (trypsin-like serine protease inhibitors), iodoacetic acid (IAA), L-3-carboxytrans 2, 3-epoxypropyl-leucylamido (4-guanidine) butane (E-64) (Cysteine protease inhibitors), pepstatin (aspartic acid protease inhibitor) (Beynon and Salvasen, 2001) Remaining activity were determined by Ala-Ala-Phe-pNA hydrolysis under the standard assay conditions and expressed as the percentage of the enzyme activity of under identical conditions in the absence of inhibitors.

2.2.11. Determination of Protein

The amount of protein in the samples was calculated by spectrophotometrically according to Whitaker and Granum (1980) method. The difference in absorbance

between 235 and 280 nm, due primarily to peptide bond, used to calculate protein concentration by the equation

$$\text{Protein concentrations (mg/ml)} = (A_{235}-A_{280})/2.51$$

where the factor 2.51 is the difference between the average measured extinction coefficient ($E^{0.1\%}$) at 235 and 280 nm. After each purification step, the protein content of the purified enzyme was determined by this method.

2.2.12. SDS-Polyacrylamide Gel Electrophoresis

Polyacrylamide slab gel electrophoresis, in the presence of the 0.1% anionic detergent SDS, was performed on the 5 % stacking gel and 12.5% separating gel as described by Laemmli (1970). The five proteins given below were used as molecular weight standards.

Urase (monomer)	M_r90 000
Bovine Albumin (monomer)	M_r66 000
Egg Albumin	M_r45 000
Carbonic Anhydrase	M_r29 000
α-Lactoalbumin	M_r14 200

Vertical slab gel electrophoresis was carried out using the Biometra Vertical Minigel System (Biometra Biomedzinische Analytic Gmbh, Göttingen, Germany). The assembly of the glass plate cassettes and the process of the gel casting were done according to instruction manual provided with the apparatus.

10 µl aliquots (about 30 µg protein) of enzyme samples were mixed with 2X sample buffer containing 1% SDS and placed in a boiling water bath for 5 minutes. Afterwards, protein samples and molecular weight standards were loaded onto gel. The gel was run at 100 V at 4^{o}C. After the electrophoresis was completed, the

polyacrylamide gel was then stained in Coomassie Brilliant Blue R250 and then, destained by placing in a destaining solution that was changed several times.

The relative mobility (R_f) of each protein was determined by measuring its migration distance from the top of the separating gel to the center of the protein band. The Rf values were plotted against the known molecular weights (logarithmic scale ordinate) and standard line was drawn and its slope was used in the calculation of the molecular weight of proteins.

CHAPTER 3

RESULTS

3.1. Amplification of 16S rDNA Sequences from Genomic DNA of *Tp. volcanium* by PCR

Throughout this study the archaeon *Tp. volcanium* was used as the source organism for production of protease and proteasome enzymes. The purity of the *Tp. volcanium* culture which was maintained through continuous subculturing into fresh medium was checked periodically, by specific amplification of its 16S rDNA sequences, besides microscopic examination. By using a combination of the *archaea* specific (8-27) forward primer and universal primer (1510-1492) in PCR, we have obtained unique 1.2 kb 16S rDNA fragments with the template DNA isolated from *Tp. volcanium*. However, there was no amplification, when the same templates were used in the PCR together with bacteria specific (333-348) forward primer and universal primer, to the exclusion of archael 16S rDNA genes (Figure 3.1.). This result indicated the purity of the culture in all the tests. In every test, a control PCR with DNA from the bacterium *Bacillus* sp. was also run.

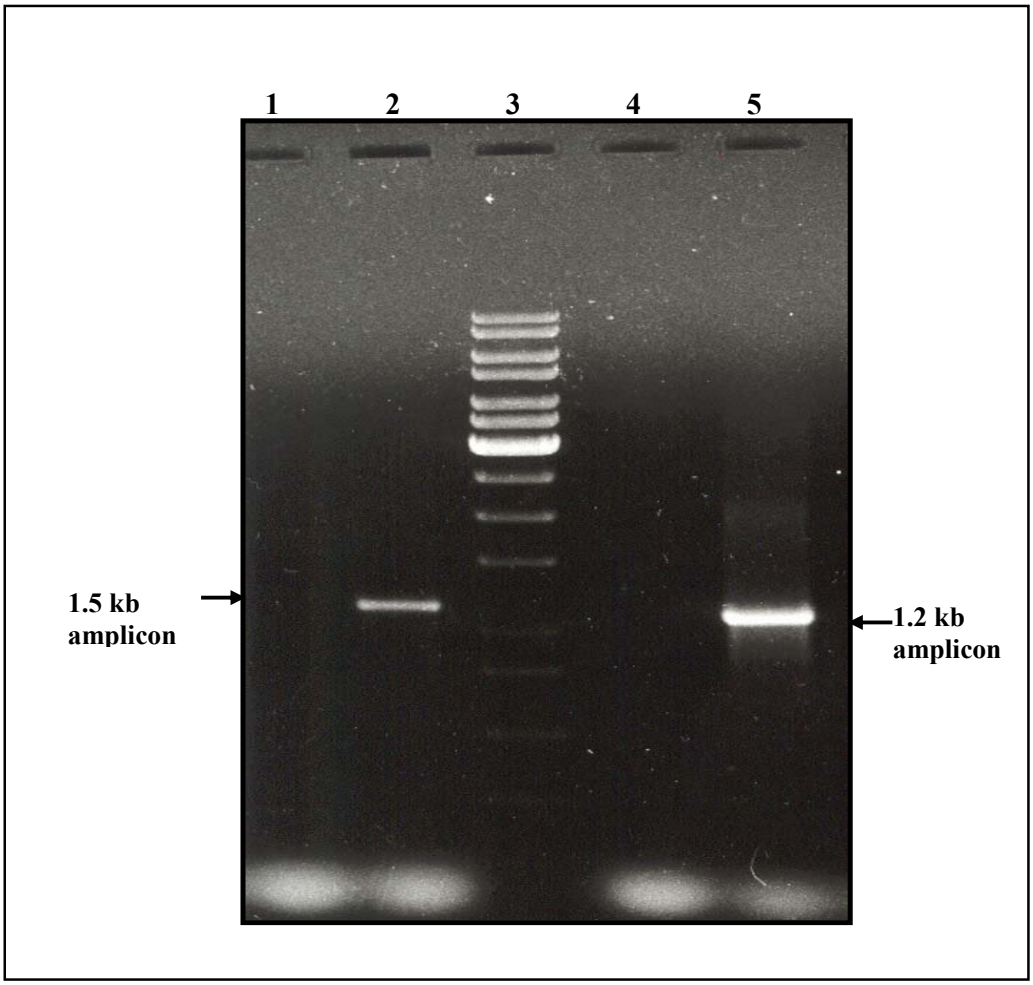


Figure 3.1. PCR amplification of 16S rDNA gene sequences. Total genomic DNAs from the bacterium *Bacillus* sp. (lanes 1 and 2) and *Tp. volcanium* (lanes 4 and 5) were used as templates. Odd numbered lanes show the results of PCR with bacterial specific, even numbered lanes show the results of PCR with Archaea specific primer sets. Lane 3, DNA size marker.

3.2. Purification of *Tp. volcanium* Serine Protease by Colon Chromatography

The protease was purified from the cell free extract of *Tp. volcanium* by the procedure described in Materials and Methods. The purification procedure was consisted of cell disruption by sonic treatment (step 1), fractionation with solid ammonium sulfate (step 2), anion exchange chromatography on Econopac Q ion exchange cartridge (step 3) and affinity chromatography on α-casein agarose column (step 4) and the results are summarized in Table 3.1.

Proteins in the cell-free extract were precipitated by ammonium sulfate (up to 80% saturation). The precipitate was washed and concentrated by ultrafiltration. Desalted enzyme was applied to a column of anion exchanger (Econo Pac Q Ion Exchanger Cartridge). The bound proteins were eluted with a linear gradient of NaCl in the range of 0-1.0 M NaCl in the equilibrating buffer, as two distinct peaks (minor and major peaks). Elution profile is shown in Figure 3.2. The protease activity was eluted at 0.75 M NaCl, as a fraction of the second (major) protein peak, in the form of a single peak of N-Suc-Phe-pNA hydrolysis activity. This step resulted in 3.56 fold purification with a specific activity of 3.86 U/mg.

In the final step of the purification, a substrate affinity chromatography (on α-casein agarose column) was used and a single peak of protease activity was eluted from column in the presence of 25% isopropanol (Figure 3.3). Thus, the enzyme was purified by approximately 5.5 fold with a specific activity of 6.0 U/mg.

The protease activity after purification by α-casein affinity chromatography was visualized by using gelatin zymography. The enzyme activity was detected as a single clear band against dark background of SDS-PAGE copolymerized with gelatin (Figure 3.4).

Table 3.1. Purification table for intracellular thermophilic serine protease from *Tp. volcanium*.

	Protein			Enzyme				
Purification Step	**Volume (ml)**	**Concentration (mg/ml)**	**Total (mg)**	**Concentration (nmole/mg/ml)**	**Specific Activity (units/mg)**	**Total (units)**	**Yield (%)**	**Purification (fold)**
Crude extract	27.0	4.67 (± 0.112)	133.55 (± 1.345)	5.06 (± 0.0)	1.084	1.084	100	1
$(NH_4)_2SO_4$ Precipitation	3.3	5.66 (± 0.810)	18.96 (± 1.605)	11.4 (± 0.184)	2.014	2.014	27.4	1.85
Ion Exchange Chromatography	2.8	3.47 (± 0.135)	9.72 (± 1.13)	13.411 (± 0.651)	3.862	3.862	27.0	3.56
Affinity Chromatography	2.5	2.2 (± 0.120)	5.5 (± 1.30)	18.0 (± 0.52)	6.000	33.00	23.7	5.5

(S.D values for the measurements from five purification experiments)
Unite (U): The amount of enzyme that liberated 1 nmol of p-nitroanilide per minute.

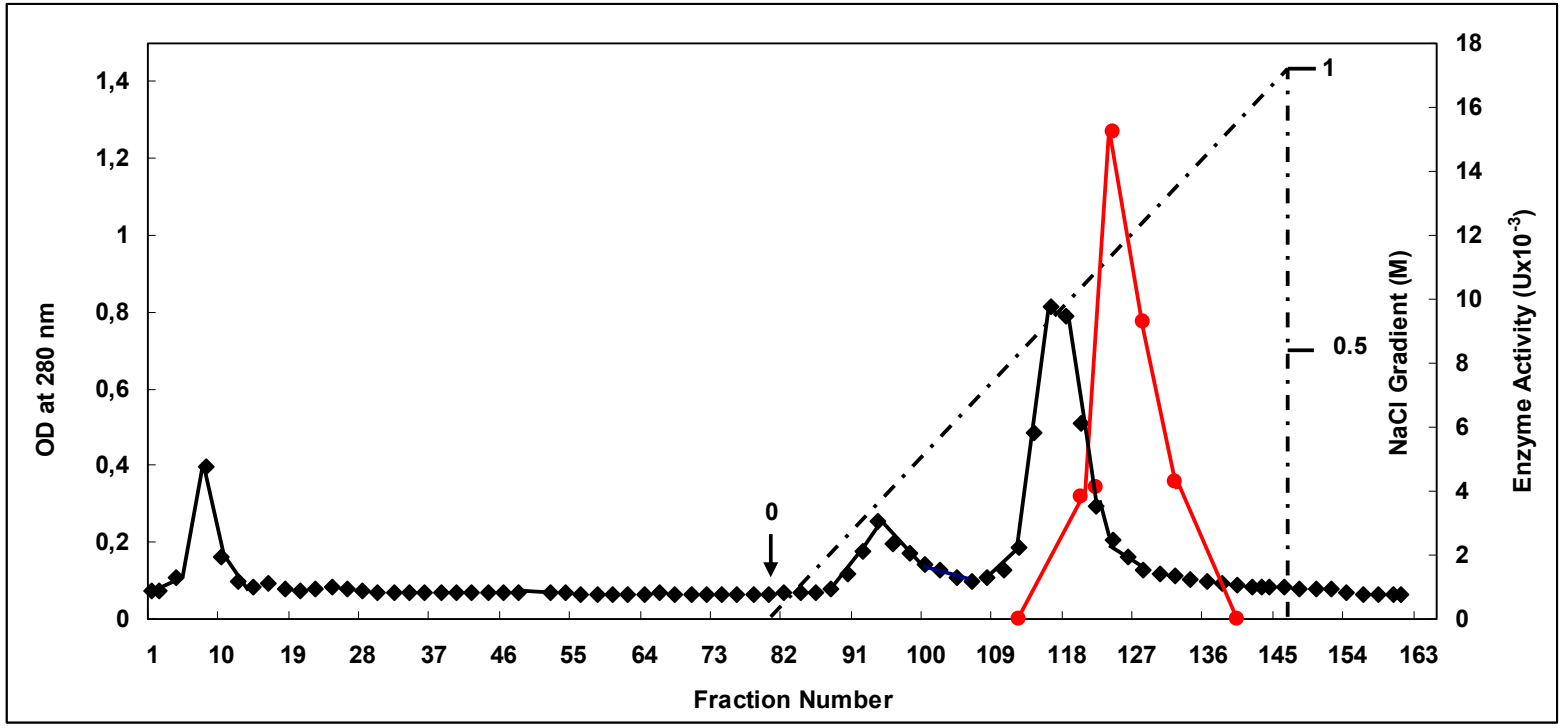


Figure 3.2. Chromatogram of *Tp. volcanium* serine protease on Econo Pac Q Ion Exchanger Cartridge. The protease was eluted with a linear NaCl gradient (0-1 M). The arrow indicates the start of the salt gradient. Symbol and lines: –♦– absorbance at 280 nm;

–●– proteolytic activity on N-Suc-Phe-pNA; –·–·– NaCl.

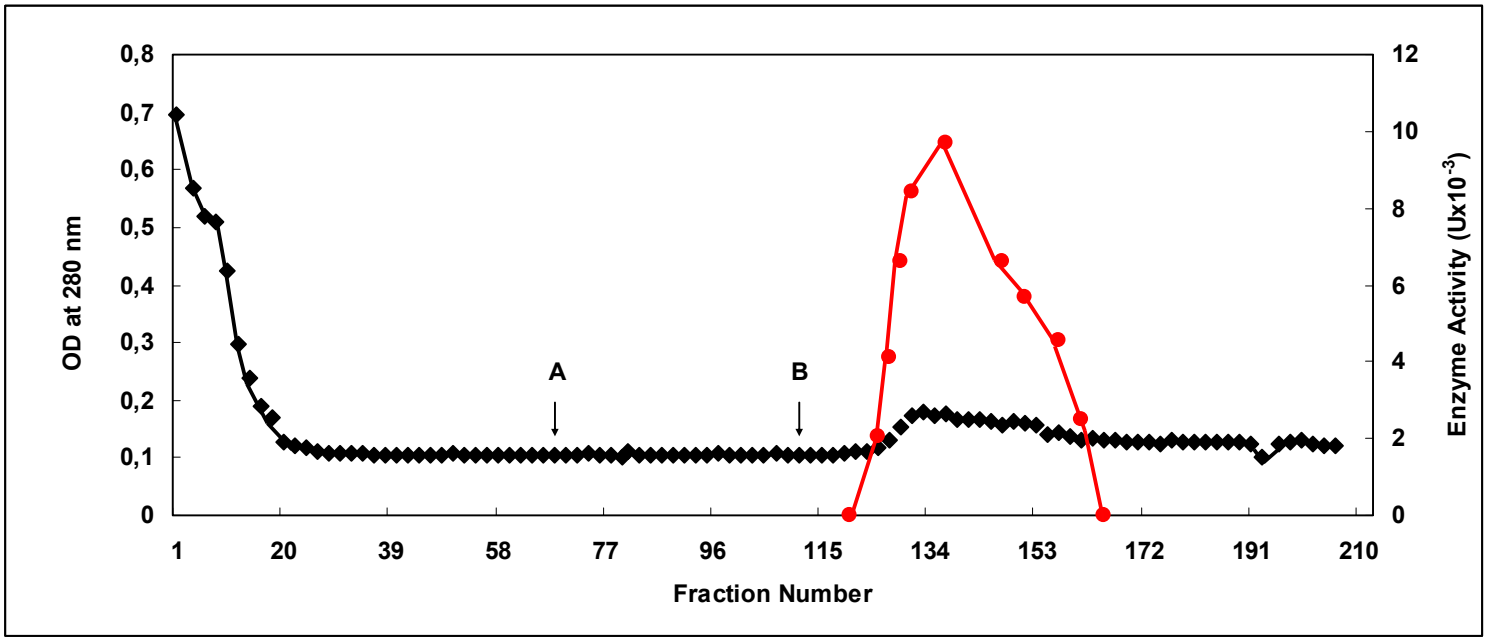


Figure3.3. Elution profile of *Tp. volcanium* chymotryptic-like serine protease on α-casein agarose. First and second arrows *indicate* the application of (A) elution buffer containing 1 M NaCl and (B) the same buffer with the addition of 25% isopropanol, respectively. Symbol and lines –♦–, absorbance at 280 nm; –●– proteolytic activity on N-Suc-Phe-pNA.

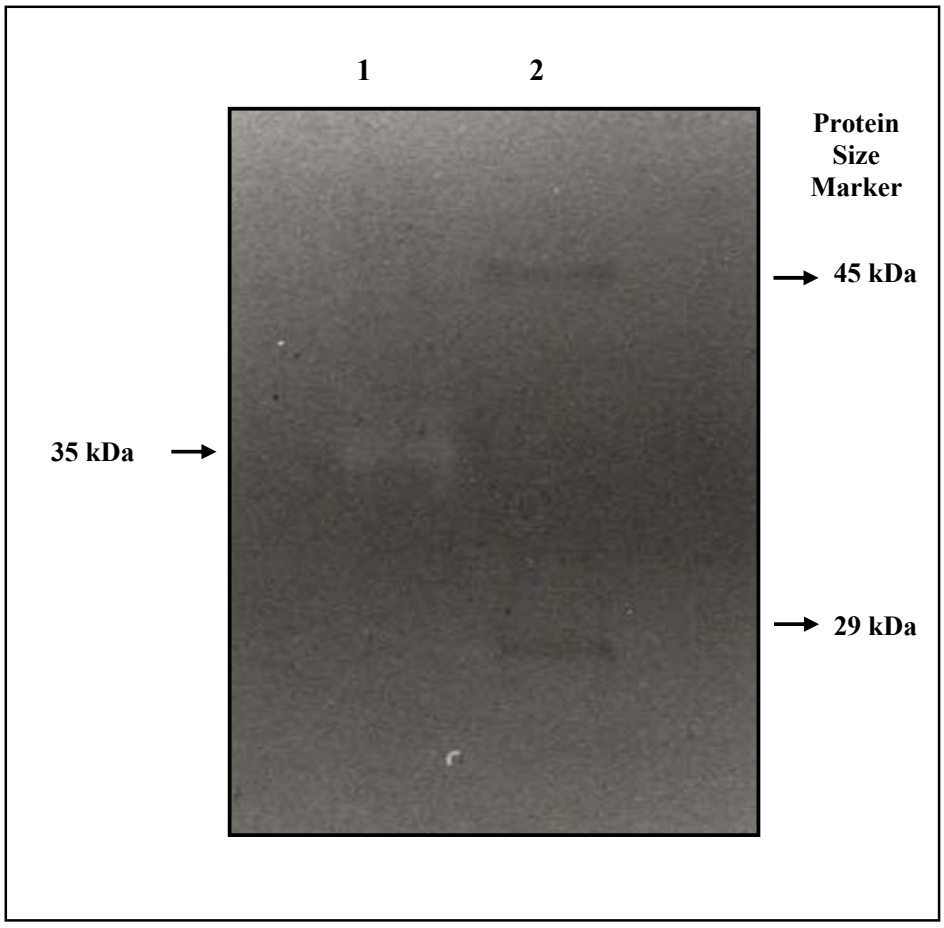


Figure 3.4. Activity gel electrophoresis of the purified *Tp. volcanium* protease. Lane 1. Sample from affinity chromatography, lane 2. Protein molecular size marker (Sigma Chemical Company, Saint Louis, Missouri, U.S.A.).

3.3. Biochemical Characterization of the Purified Serine Protease of *Tp. volcanium*

3.3.1. Substrate Specificity and Kinetic Measurement

The specificity of the *Tp. volcanium* intracellular protease towards various synthetic substrates was examined spectrophotometrically under standard assay conditions. Studies on specificity of the proteolytic activity for amino acid and peptide p-nitroanilide showed that protease was the most active on N-Suc-Phe-pNA, which is a substrate for chymotrypsin-like serine proteases. Therefore, the activities were expressed relative to that of N-Suc-Phe pNA (Table 3.2.). The intracellular protease of *Tp. volcanium* displayed hydrolytic activity against substrates other than N-Suc-Phe-pNA such as N-Suc-Ala-Ala-Pro-Phe-pNA, Ala-Ala-Phe-pNA and L-Leu-pNA which are also specific for chymotrypsin-like activity.

On the other hand, the enzyme exhibited quite high catalytic efficiency for N-Ala-Ala-Ala-pNA which is a typical substrate for elastase. The other elastase substrates such as N-Suc-Ala-Ala-Val-pNA and N-Suc-Ala-Ala-Val-Ala-pNA were also hydrolyzed but with relatively low efficiency. *Tp. volcanium* protease also hydrolyzed the N-CBZ-Arg-pNA, which is the typical substrate for trypsin, with low catalytic efficiency.

The kinetic parameters for the purified *Tp. volcanium* serine protease were determined using N-Suc-Phe-pNA followed the classical Michaelis-Menten kinetics. The Michaelis constants (V_{max} and K_m) were determined from double reciprocal plots of initial rates, as described in the Materials and Methods. The Line weaver-Burk (L-B) plot of the serine protease is given in Figure 3.5**.** The apparent K_m value for N-Suc-Phe-pNA was 2.2 mM and the corresponding V_{max} was 40 μmoles of p-nitroanilide released $min^{-1}.ml^{-1}$.

Table 3.2. Proteolytic activity of *Tp. volcanium* serine proteases on various oligopeptidyl substrates (The activities were determined in the presence of each substrate (0.61 mM) under the assay conditions described before, and were expressed relative to that of N-Suc-Phe-pNA).

Chromogenic Substrates	**Relative Activity (%)**
N-Suc-Phe-pNA	100
Ala-Ala-Phe-pNA	55
L-Leu-pNA	54
N-Suc-Ala-Ala-Ala-pNA	51
N-Suc-Ala-Ala-Pro-Phe-pNA	38
N-Suc-Ala-Ala-Val-pNA	35
N-CBZ-Arg-pNA	24
N-Suc-Ala-Ala-Val-Ala-pNA	17

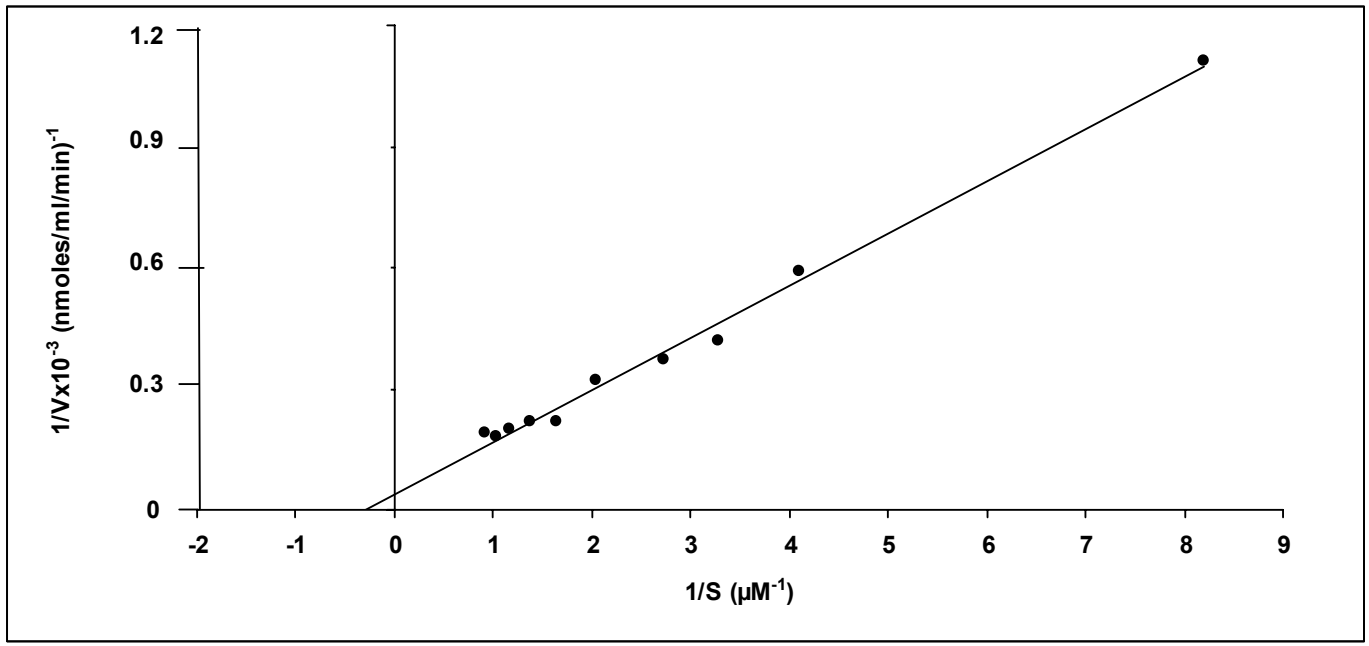


Figure 3.5. Double reciprocal plot of initial reaction rates over the range of 0.122-1.098 mM N-Suc-Phe-pNA, for *Tp. volcanium* serine protease. The assays were carried at 55^{o}C, at pH 7.5, as described in the Materials and Methods.

3.3.2. Proteolytic Activity of *Tp. volcanium* Protease Against Natural Proteins

Also, the hydrolytic activity of the *Tp. volcanium* protease towards natural proteins (such as bovine serum albumin, casein) and modified proteins (such as azocazein) was examined.

Casein hydrolysis was examined in a time dependent manner at 55^{o}C, pH 7.5, under the conditions described in the Materials and Methods. Casein degradation by the *Tp. volcanium* serine protease was the most efficient within the first 1 min of the reaction, over which about 70% of the catalytic activity was displayed (Figure 3.6).

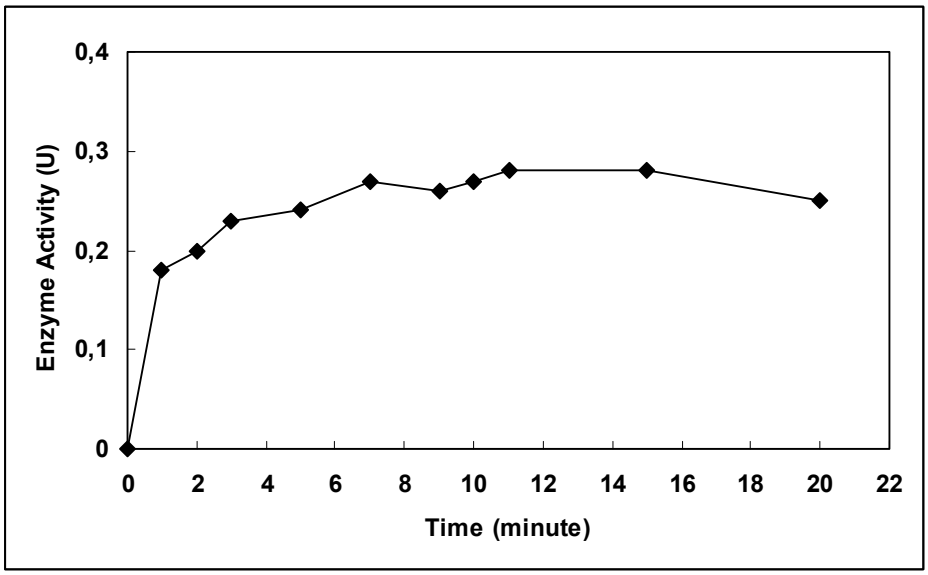


Figure 3.6. Hydrolyzing activity of *Tp. volcanium* against casein.

BSA degradation by the enzyme was relatively less efficient, as compared to casein hydrolysis. For the initial 1 min of the reaction, when casein and BSA were used as substrates, $\Delta Ab.min^{-1}$ was 0.18 and 0.026, respectively (Figure 3.7).

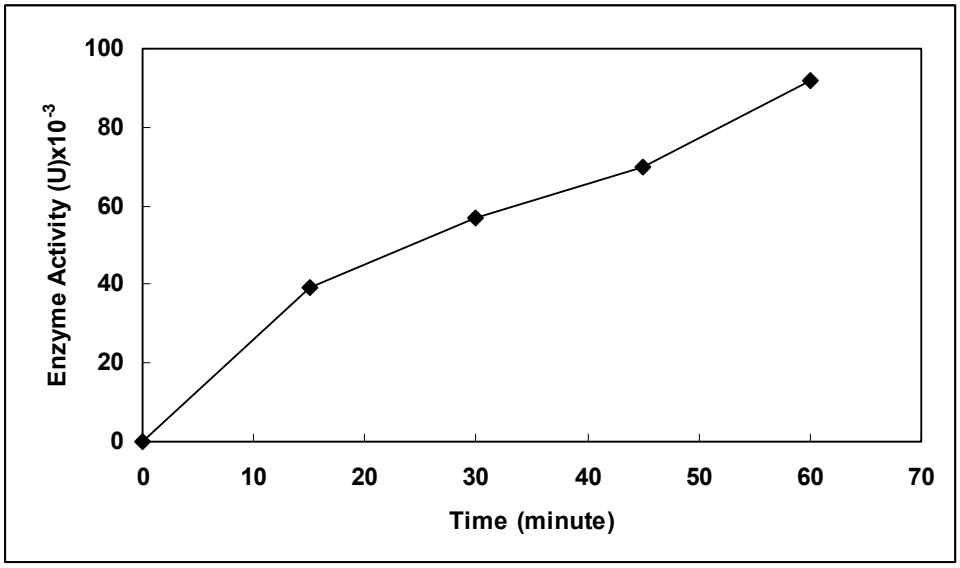


Figure 3.7. Hydrolyzing activity of *Tp. volcanium* against BSA.

3.3.3. Effect of Temperature on Caseinolytic Activity on *Tp. volcanium* Protease

In order to determine optimum temperature for the *Tp. volcanium* protease, the enzyme activity towards azocasein measured between 40 and 80 ^{o}C in protease assay buffer for 5 minute as described in Material s and Methods. Maximum activity was observed at 60^{o}C (Figure 3.8). There was a significant loss in the activity below 50^{o}C, while about 50% has still been retained at 80^{o}C.

3.3.4. Effect of Temperature on N-Suc-Phe-pNA Hydrolysis by *Tp. volcanium* Protease

The effect of temperature on hydrolytic activity of the protease towards N-Suc-Phe-pNA was also examined. The maximum serine protease activity was obtained at 50^{o}C (Figure.3.9).

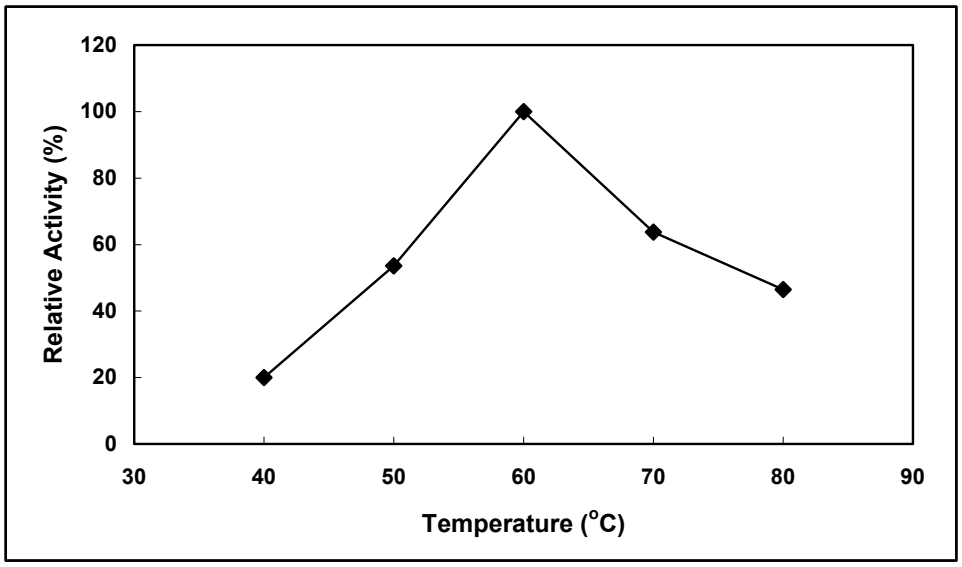


Figure.3.8. Effect of temperature to azocasein hydrolysis .

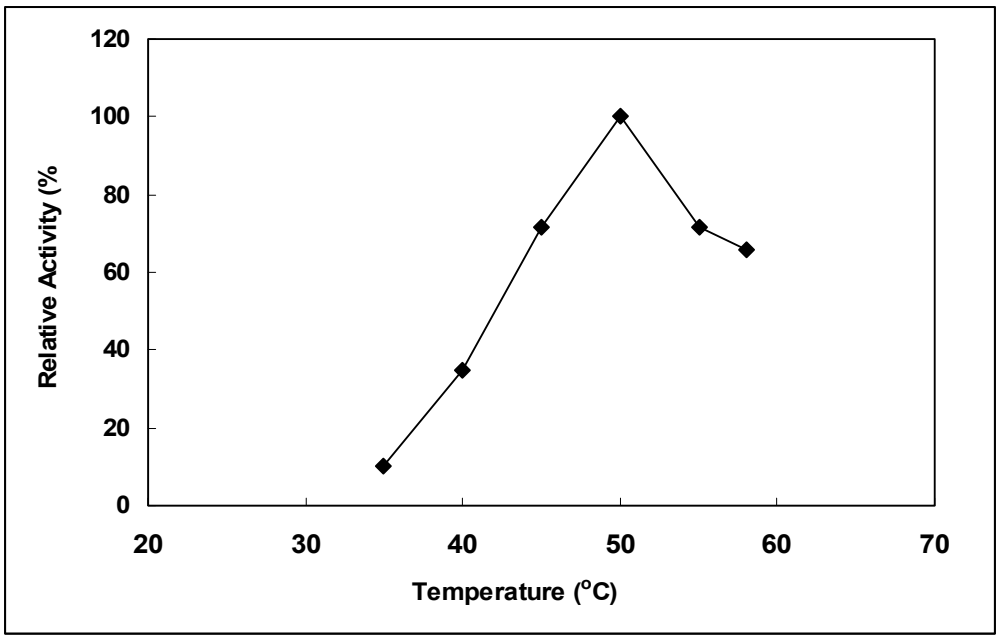


Figure 3.9. Effect of temperature to N-Suc-Phe-pNA hydrolysis.

3.3.5. Effect of pH on Protease Activity

To determine the optimum pH of the enzyme the hydrolytic activity of the enzyme was measured using N-Suc-Phe-pNA as substrate, at different pH, under standard assay conditions. The buffer systems used were 50 mM sodium acetate (pH 5.0-6.0), 50 mM MOPS (pH 6.0-7.0), 50 mM Tris-HCl (pH 7.0-9.0), and 50 mM glycine (pH 9.5-11.5), each containing 2 mM $CaCl_2$. The optimum pH for the activity was pH 7.0 (Figure 3.10). There was considerable activity at alkaline pH, and more than 50% of the activity was retained at pH 9.5. Activities measured in MOPS buffer were comparatively higher than Tris-HCl, at the same pH values. Due to rapid degradation of the substrate at pH<6.0, we couldn't perform the enzyme activity assay within acidic pH range. Stability assays showed that there was significant loss in the activities of the enzyme at pH <6.5 and pH>9.5, after 1 hour incubation in the respective buffer systems (Figure 3.11).

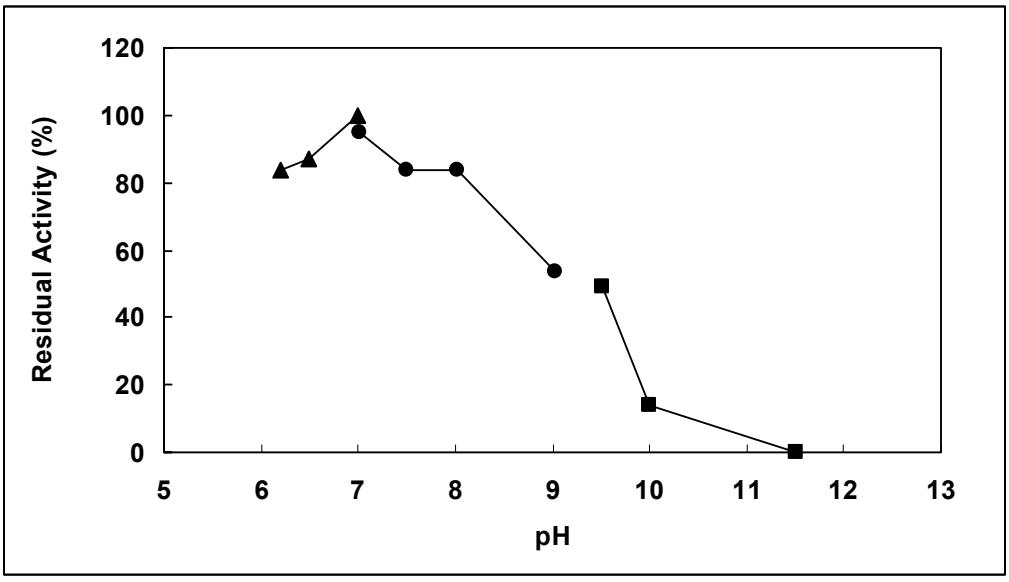


Figure 3.10. Effect of pH on activity of serine protease from *Tp. volcanium.* The protease was assayed in the pH range of 4.0-11.5 using buffers of different pH range (MOPS buffer ▲, Tris-HCl buffer●, Glycine buffer ■).

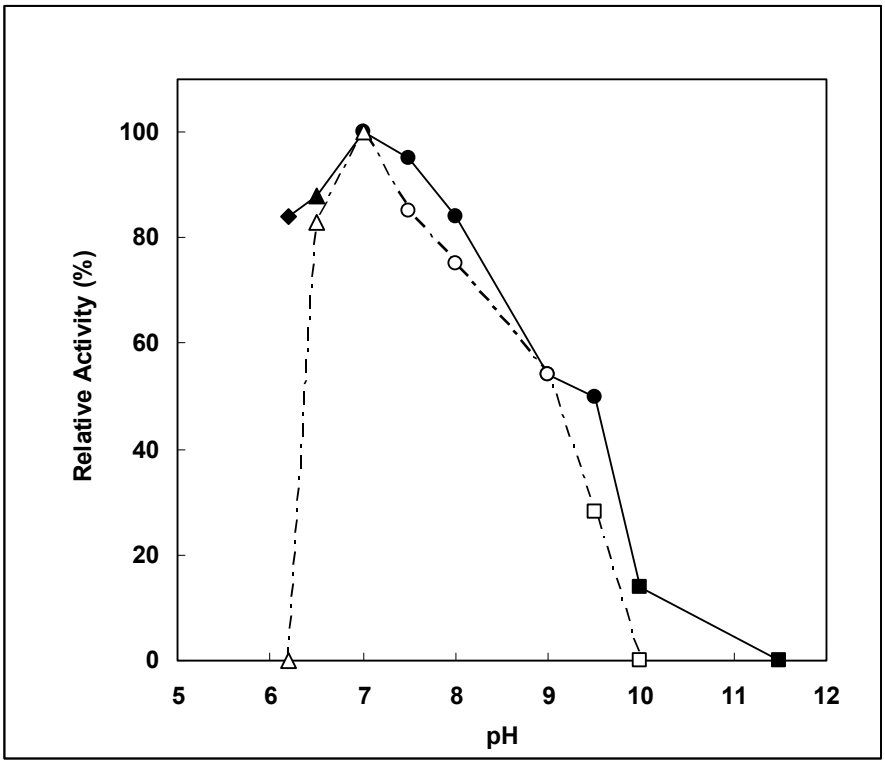


Figure 3.11. Effect of pH stability on activity of serine protease from *Tp. volcanium.* The protease was assayed in the pH range of 4.0-11.5 using buffers of different pH range (MOPS buffer ▲, Tris-HCl buffer ●, Glycine buffer ■) and incubated at room temperature for 1 hour in the same buffer systems (MOPS buffer Δ, Tris-HCl buffer o, Glycine buffer □).

3.3.6. Effects of Inhibitors on the Protease Activity

Inhibition studies primarily give on insight of the nature of the enzyme and nature of the active center. We have studied the effects of various protease inhibitors on the activity of purified protease activity of *Tp. volcanium*. The result of sensitivity of purified *Tp. volcanium* protease to various protease inhibitors are given in Table 3.3

Table3.3. Effects of enzyme inhibitors on the activity of *Tp. volcanium* protease.

Inhibitors	Concentration	Remaining Activity (%)
No Inhibitor	0	100
TPCK	1 mM	0
Chymostatin	400 μM	4.5
PMSF	2 mM	5.5
EDTA	10 mM	75
EGTA	10 mM	94
DTT	20 mM	92

When the protease activity was assayed in the presence of different inhibitors, PMSF strongly inhibited this intracellular enzyme activity. *Tp. volcanium* proteases were also completely inactivated by TPCK. Both PMSF and TPCK are well known inhibitors of serine protease (Beynon and Salvesen, 2001). *Tp. volcanium* intracellular protease was also inhibited by chymostatin which is a specific inhibitor for chymotrypsin.

Effects of metal chelators EGTA, EDTA on *Tp. volcanium* protease activity were also tested. As it is shown in Table 3.3**,** the N-Suc-Phe-pNA hydrolytic activity was not affected by EGTA which is a metal chelator that specifically binds Mg^{2+}. On the other hand about 25% of the activity was lost in the presence of EDTA, metalloprotease inhibitor that binds divalent cations. On the other hand, DTT effect, a reagent capable of dissociating disulphide cross-linkages, on the *Tp. volcanium* protease, was negligible.

3.3.7. Effect of Metal Ions on Protease Activity

The effects of some divalent cations on the protease were investigated by measuring the N-Suc-Phe-pNA hydrolysis of the purified enzyme in the presence of various ions (in chloride salts) at a concentration of 4 mM under the standard assay conditions. The results are shown in Figure 3.12. Among the all ions tested, Ca^{2+} and Mg^{2+} at 4 mM concentrations were the most effective in activating the enzyme resulting 2.6-fold and 2.3-fold increase in the peptide hydrolyzing, respectively. Mn^{2+} and Co^{2+}, at the same concentration exhibited lower stimulation effect. Ni^{2+} had some inhibitory effect, causing 27% decrease in the activity. The effects of Ca^{2+} and Mg^{2+} at different concentration on the enzyme activity were also determined.

3.3.8. Effect of Ca^{2+} and Mg^{2+} Ions *Tp. volcanium* Protease Activity

The effects of Ca^{2+} and Mg^{2+} cations on the protease were assessed by measuring the N-Suc-Phe-pNA hydrolysis activity of the purified enzyme at concentrations of 0.5-12.5 mM under the standard assay conditions (Figure 3.13). Optimum activity was observed at 2 mM $CaCl_2$, and about 2.7 fold increase in the activity was obtained as compared to the absence of Ca^{2+}. There was also stimulation in the activity, at the extent of 2.2-fold, over a range of 2-12.5 mM $CaCl_2$. Similarly, presence of Mg^{2+} in the reaction mixture enhanced the activity of *Tp. volcanium* protease activity. The maximum activity was observed at 10 mM $MgCl_2$, which resulted in 2.5 fold increase in the activity. The activity was enhanced between 2.16-2.5 fold over the range of 2-12.5 mM $MgCl_2$.

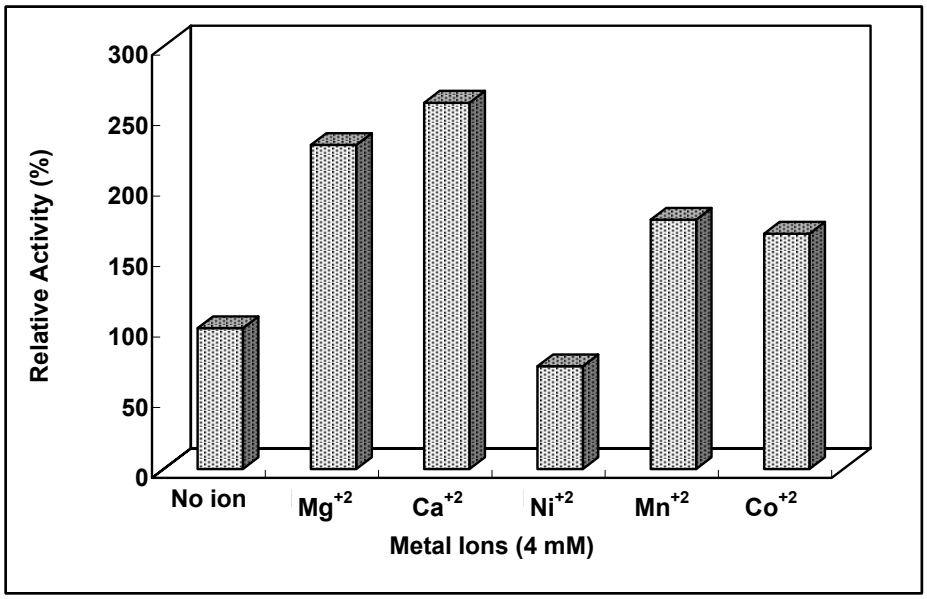


Figure 3.12. The effect of metal ions on the protease activity.

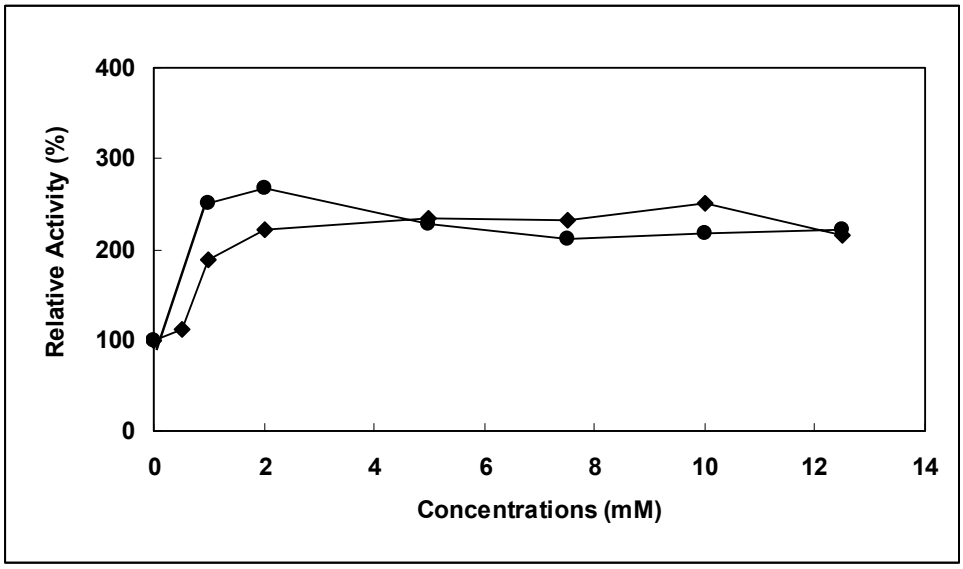


Figure 3.13. The effect of calcium (Ca^{2+}) (•) and magnesium (Mg^{2+}) (♦) on the protease activity.

3.4. Purification of *Tp. volcanium* 20S Proteasome by Colon Chromatography

The purification steps and results of the 20S proteasome produced by *Tp. volcanium* are summarized in Table 3.4. In the first step of purification, the cell free extract from *Tp. volcanium* liquid culture was prepared as described in the Materials and Methods. The extract, then, was subjected to ammonium sulfate precipitation at two steps (40 % and 80 %), which was followed by washing and concentration using ultra filtration. The concentrated enzyme solution was loaded on a Sepharecyl S-300 gel filtration chromatography column. One sharp protein peak corresponding to chymotryptic serine protease activity was eluted from the column and the active fractions of which were pooled (Figure 3.14).

In this step, majority of the eluted proteasome was purified by a factor of 2.4 with 13.02 U/ mg specific activity. The collected active fractions from gel permeation chromatography were washed, concentrated and further purified by ion exchange chromatography using DEAE-Sepharose fast flow column. The elution from the column was achieved with a linear gradient of 0-0.5 M NaCl. The chymotryptic activity was eluted with 0.45 M NaCl, emerging from the column as a distinct peak (Figure 3.15). In this purification step, the enzyme was purified 2.7 fold and the purified enzyme had a specific activity of 14.8 unit mg^{-1} protein. In the next purification step, the pooled and concentrated active fractions from the ion exchange chromatography were subjected to hydroxylapatite column chromatography on a Econo-Pac HTP Cartridge. The active proteasome fractions were eluted with 0.5 M potassium phosphate, in a linear gradient of potassium phosphate (0.1 M-0.6 M). The elution profile is shown in Figure 3.16.

Inthis final step of purification the specific activity of the 20S proteasome preparation was 19.1 U/mg. The progress of the purification process was also fallowed SDS-PAGE (Figure 3.17). After hydroxylapatite column chromatography the α-subunit and β-subunit were detected as unique bands, with estimated molecular sizes of 26 kDa and 21.9 kDa, respectively.

Table 3.4. Purification table for *Tp. volcanium* 20S proteasome.

	Protein			Enzyme				
Purification Step	**Volume (ml)**	**Protein (mg/ml)**	**Total Protein (mg)**	**Activity (U)**	**Total Activity (U)**	**Specific Activity (U/mg))**	**Yield (%)**	**Purification (fold)**
Cell Free Extract	40	4.8	192	26.0	1040	5.4	100	1
$(NH_4)_2SO_4$ Precipitation	4	6.9	27.6	95.4	381.6	13.80	36.7	2.5
Gel Filtration Chromatography	3.7	2.7	10.0	35.2	130.2	13.02	12.5	2.4
Ion Exchange Chromatography	3.7	2.4	8.9	35.6	131.7	14.80	12.7	2.7
Hydroxylapatite Chromatography	2.6	0.9	2.3	17.2	44.7	19.1	4.2	3.5

Unite (U): The amount of enzyme that liberated 1 nmol of p-nitroanilide per minute.

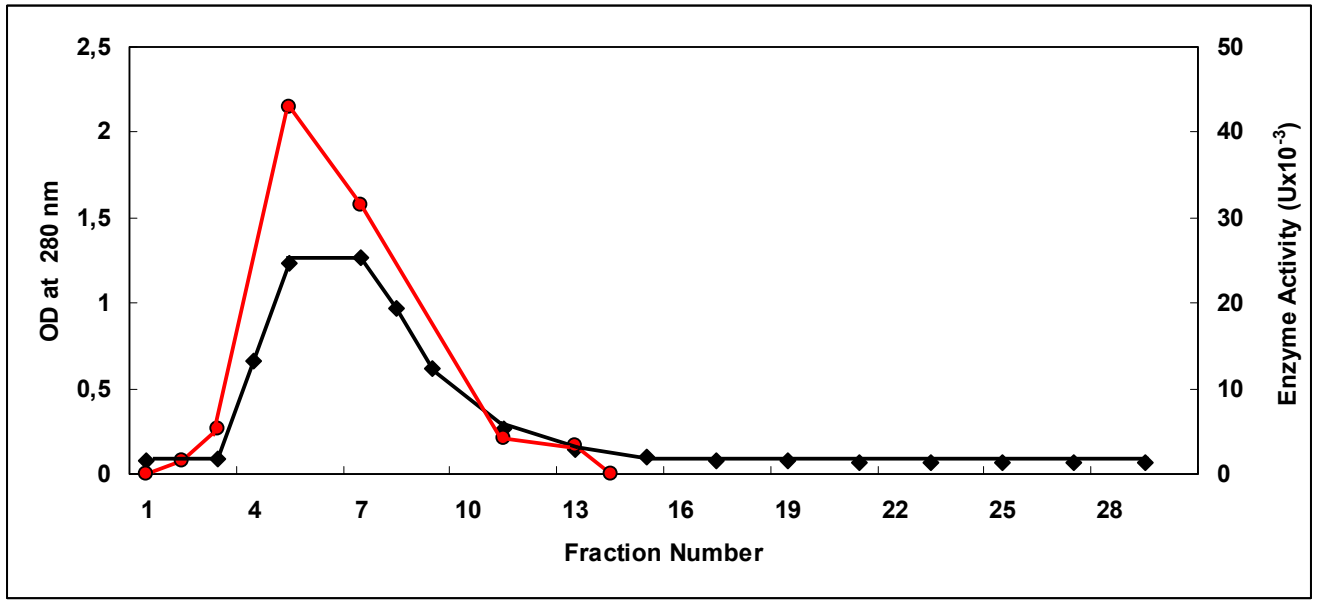


Figure 3.14. Elution profile of *Tp. volcanium* 20S Proteasome by gel filtration chromatography on Sepharacyl S-300 Column. Fractions with chymotryptic activity (from fraction no.3 to fraction no.13) were pooled, then desalted and concentrated by ultrafiltration (–♦– protein, –●– enzyme activity).

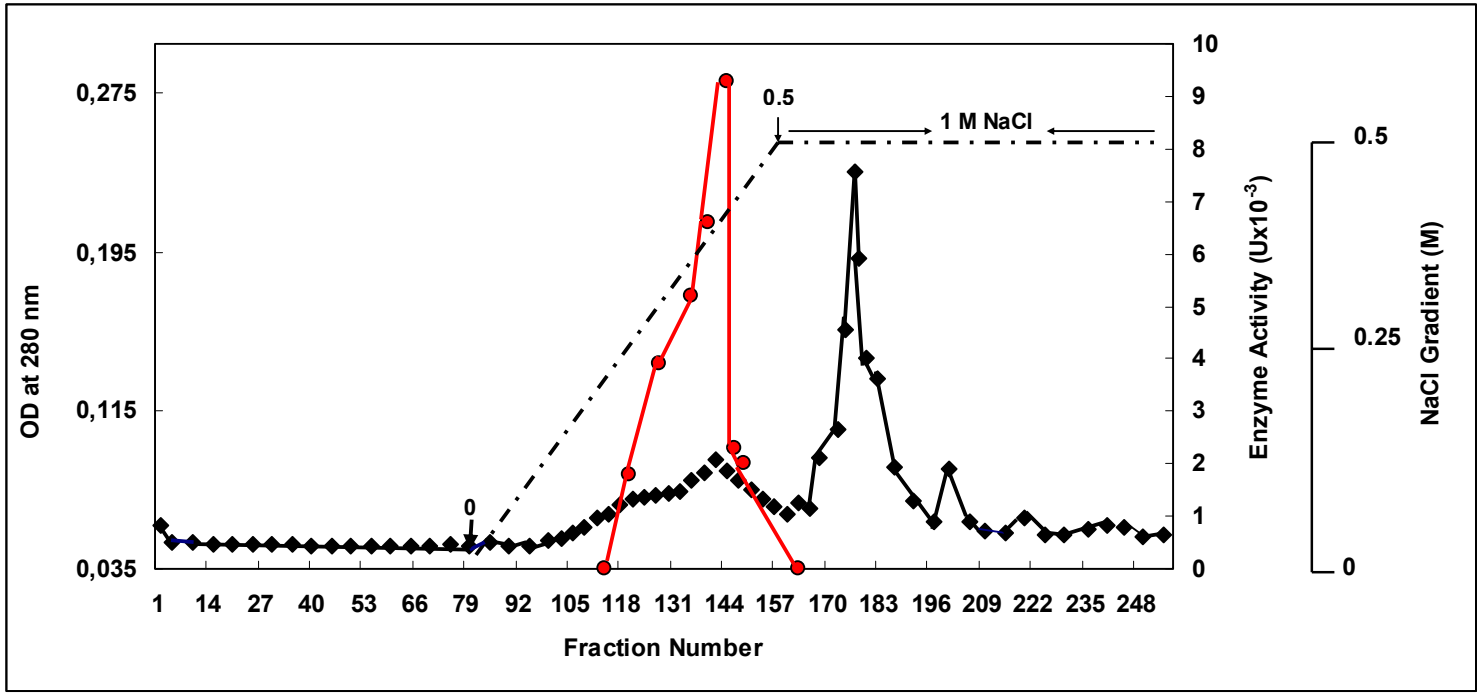


Figure 3.15. Elution profile of *Tp. volcanium* 20S proteasome by ion exchange chromatography on DEAE-Sepharose Fast Flow column. Fractions with chymotryptic activity (from fraction no. 127 to fraction no. 146) were pooled and desalted using ultrafiltration (–♦–protein, –●– enzyme activity, –·–·–NaCl).

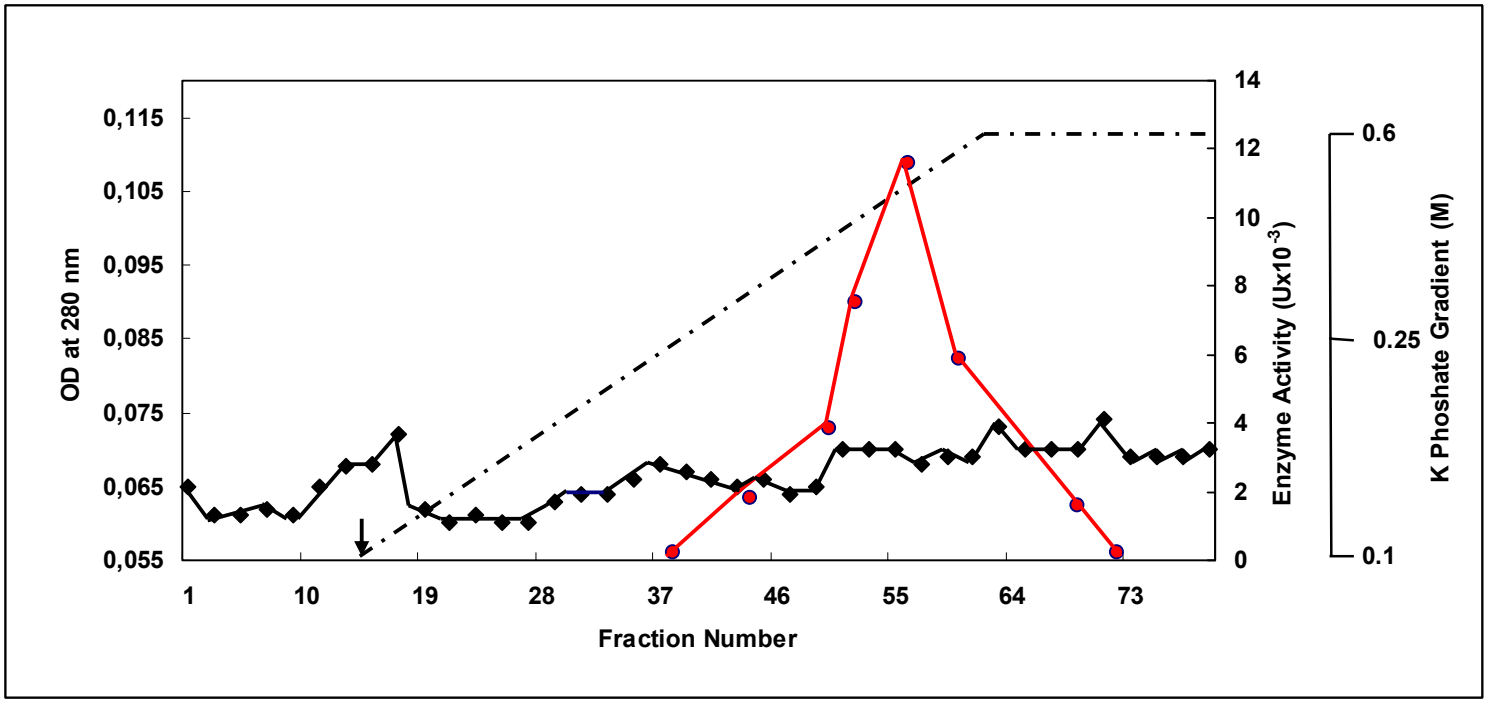


Figure 3.16. Elution profile of *Tp. volcanium* 20S proteosome by hydroxyapatite column chromatography on Econo-Pac HTP cartridge. Fractions with chymotryptic activity (from fraction no. 49 to fraction no. 68) were pooled and desalted using ultrafiltration (–♦– protein, –●– enzyme activity, – – K Phosphate).

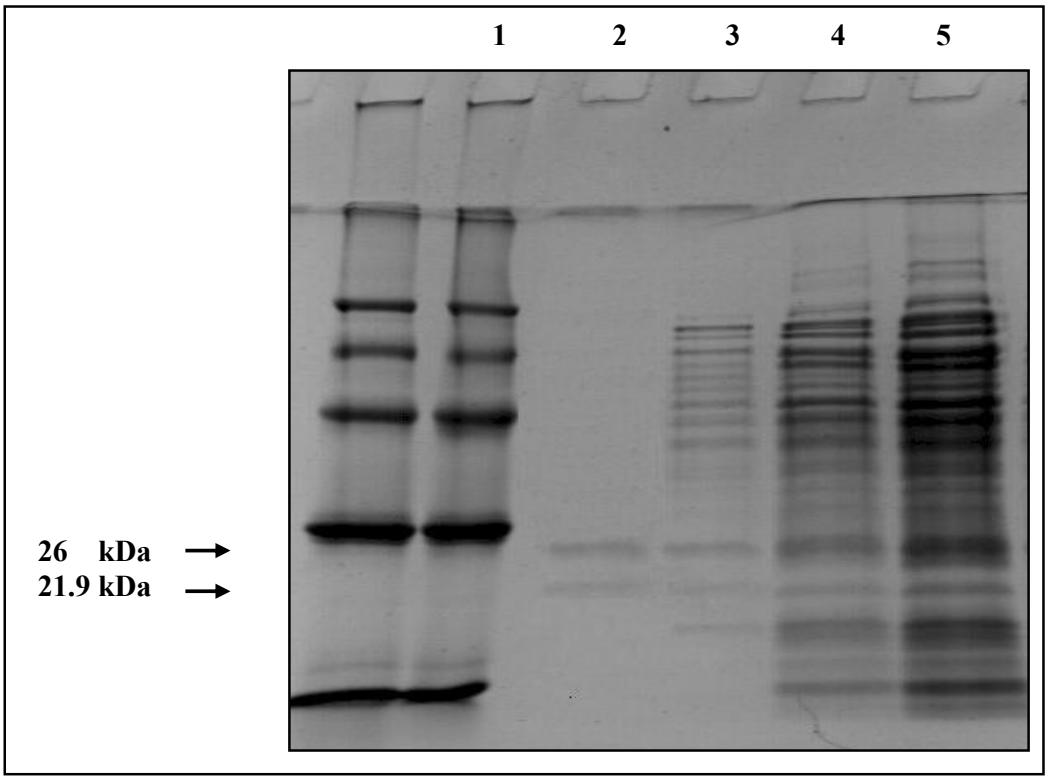


Figure 3.17. SDS-PAGE of the *Tp. volcanium* 20S proteasome and individual α- or β- subunits. Lane 1. Protein molecular size marker (Sigma Chemical Company, Saint Louis, Missouri, U.S.A.), lane 2. α-subunit and β-subunit purified by hydroxylapaptide chromatography; lane 3. Sample from ion-exchange chromatography; lane 4. Sample from gel permeation chromatography; lane 5. Sample from ammonium sulfate precipitation.

3.5. Biochemical Characterization of the Purified 20S Proteasome of *Tp. volcanium*

3.5.1. Substrate Specificity and Kinetic Measurement of *Tp. volcanium* 20S Proteasome

The substrate specificity of the *Tp. volcanium* 20S proteasome was determined at 55^{o}C using various synthetic substrates. The activities were determined in the presence of each substrate (0.61 mM) under the assay conditions described before, and were expressed relative to that of Ala-Ala-Phe-pNA (Table 3.5).

Table 3.5. Multiple peptide hydrolyzing activities of the 20S *Tp. volcanium* proteasome.

Substrates	Relative Activity (%)
N-CBZ-Leu-Leu-Glu-βNA	172
Ala-Ala-Phe-pNA	100
L-Leu-pNA	51.2
N-Suc-Ala-Ala-Val-Ala-pNA	23
N-Suc-Ala-Ala-Val-pNA	24
N-Suc-Ala-Ala-Ala-pNA	7
N-CBZ-Arg-pNA	5
N-Suc-Ala-Ala-Pro-Phe-pNA	6
N-Suc-Phe-pNA	0

The greatest activity was obtained with N-CBZ-Leu-Leu-Glu-βNA, in which the peptide bond was cleaved carboxyl to the acidic glutamate residue (peptidyl-glutamyl peptidase activity, PG). Substantial activity was also obtained with Ala-Ala-Phe-pNA, in which the peptide bond carboxyl to the aromatic phenylalanine residue was cleaved (chymotrypsin-like activity, CL) at 58% of the PG activity. But there was no or very little activity with similar substrates, N-Suc-Ala-Ala-Pro-Phe-pNA and N-Suc-Phe-pNA.

Minor level trypsin-like activity (TL) was detected with peptide substrate N-CBZ-Arg-pNA, which was at 2.9% of the PG activity. The results showed that *Tp volcanium* also cleaved synthetic peptides of which hydrophobic amino acid residues at the carboxyl side of the splitting point such as N-Suc-Ala-Ala-Val-Ala-pNA, N-Suc-Ala-Ala-Val-pNA, and N-Suc-Ala-Ala-pNA (13%, 14% and 4%, of PG activity, respectively).

The kinetic parameters for the purified *Tp. volcanium* 20S proteasome were determined using Ala-Ala-Phe-pNA followed the classical Michealis-Menten kinetics. The Michaelis constants (V_{max} and K_m) were determined from double reciprocal plots of initial rates, as described in the Materials and Methods. The Line weaver-Burk (L-B) plot of chymotryptic activity of *Tp. volcanium* 20S proteasome is given in Figure 3.18. The apparent K_m value for Ala-Ala-Phe-pNA was 1.8 mM and the corresponding V_{max} was 0.83 μmoles of p-nitroanilide released $min^{-1}.ml^{-1}$.

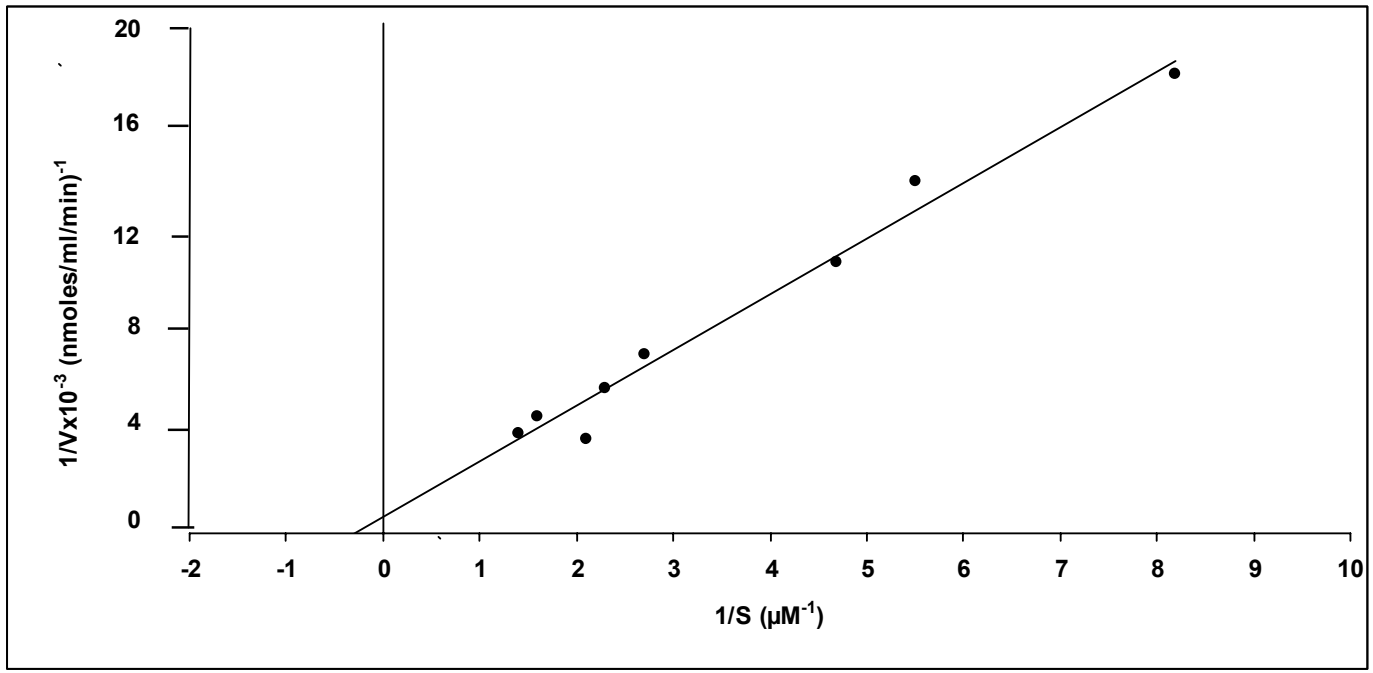


Figure 3.18. Double reciprocal plot of initial reaction rates over the range of 0.122-0.732 mM Ala-Ala-pNA, for *Tp. volcanium* 20S proteasome. The assays were carried at 55^{o}C, at pH 7.5, as described in the Materials and Methods.

3.5.2. Effect of Temperature on the Activity of *Tp. volcanium* 20S Proteasome

The chymotryptic activity of *Tp. volcanium* 20S proteasome activity was assayed at different temperatures between 35°C and 58°C using Ala-Ala-Phe-pNA as the substrate as described in the Materials and Methods. The result showed that the proteasome's optimum temperature for chymotryptic activity is 55°C (Figure 3.19). Activity assays could be not conducted at higher temperatures, due to substantial degradation of the peptide substrate above 58°C.

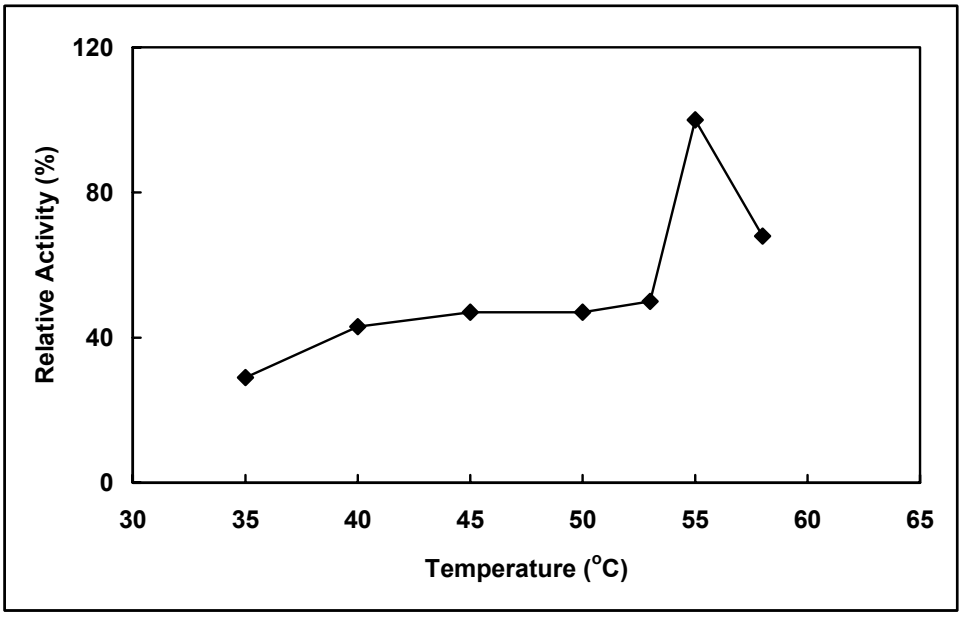


Figure 3.19. Effect of temperature on the activity of the *Tp. volcanium* 20S proteasome. The 20S purified proteasome was assayed for chymotrypsin like activity.

3.5.3. Effect of Mg^{2+} and Ca^{2+} on *Tp. volcanium* 20S Proteasome Activity

The effect of divalent cations Mg^{2+} and Ca^{2+} on the chymotryptic activity of *Tp. volcanium* 20S proteasome was tested. The activity of 20S proteasome was enhanced in the presence of magnesium and calcium salts at ≥125 mM up to 3 fold as compared to control containing no metal ion (Figure 3.20). However, calcium salt at concentrations higher than 1 M resulted in a drastic loss in the enzyme activity which was about 33% at 2 M (Figure 3.21).

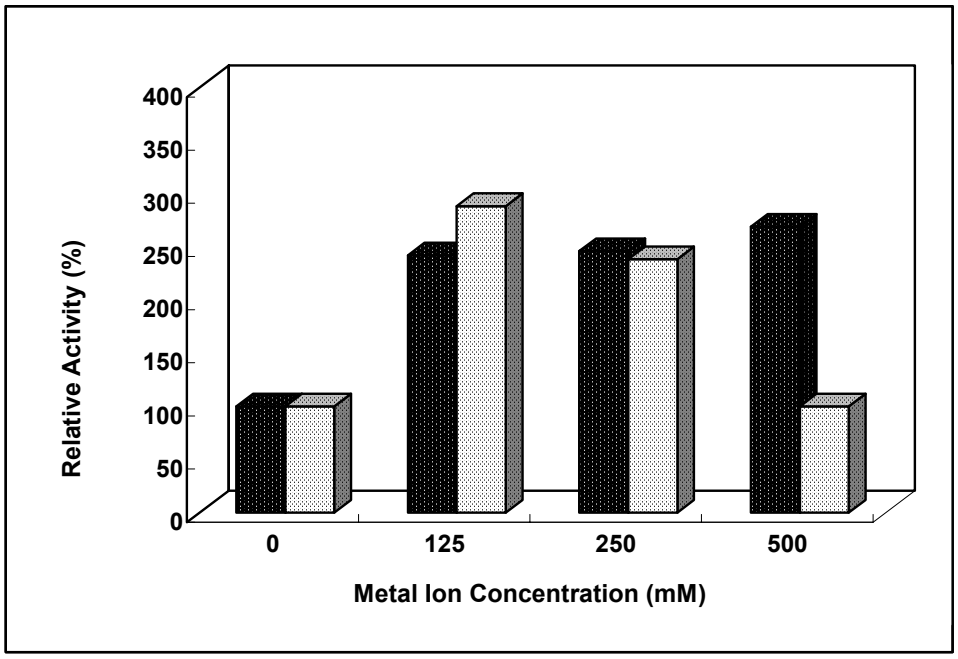


Figure 3.20. Effect of Ca^{2+} (⬚) and Mg^{2+} (▩) on the activity of the *Tp. volcanium* 20S proteasome. The 20S proteasome was assayed for chymotrypsin like activity.

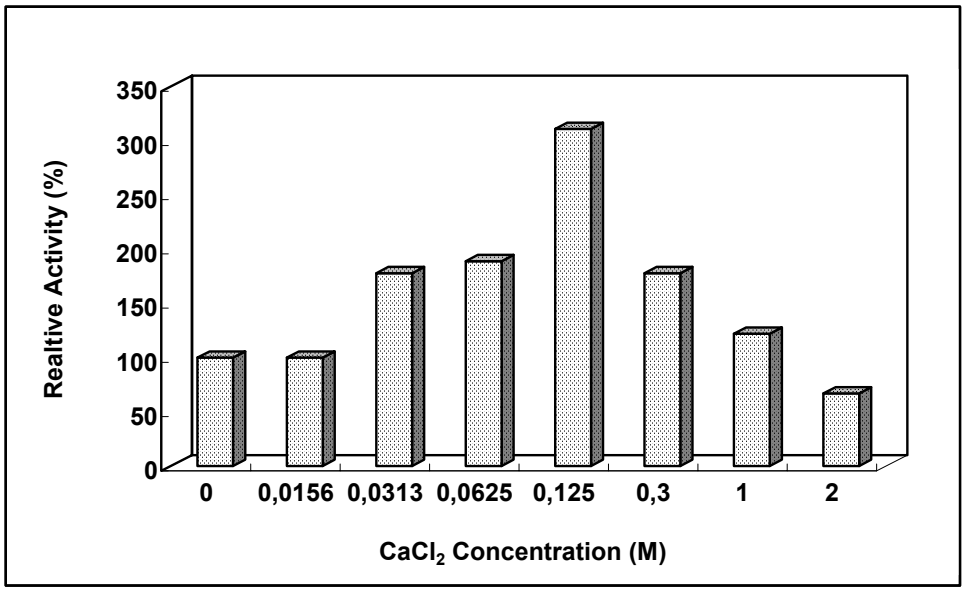


Figure 3.21. The effect of calcium (Ca^{2+}) on the *Tp. volcanium* 20S proteasome.

3.5.4. Effect of Inhibitors on *Tp. volcanium* 20S Proteasome Activity

The effects of specific protease inhibitors on the chymotrypsin-like and trypsin-like activities of the *Tp. volcanium* 20S proteasome were determined (Table 3.6 and Table 3.7 described in the Materials and Methods).

The activity towards Ala-Ala-Phe-pNA was significantly inhibited only by chymostatin which is a specific inhibitor for the chymotrypsin like serine proteases at 400 mg/ml concentration. In its presence, 43 % of the activity was lost. On the other hand, 73% of the trypsin-like activity was lost in the presence of TLCK, which is a specific inhibitor of trypsin.

Table 3.6. Effect of inhibitors on *Tp. volcanium* 20S proteasome chymotrypsin-like activity (Remaining activity were determined by Ala-Ala-Phe-pNA hydrolysis under the standard assay conditions and expressed as the percentage of the enzyme activity of under identical conditions in the absence of inhibitors).

	Concentration	Relative Activity (%)
Control	—	100
TLCK	100 µl	100
Chymostatin	400 µg/ml	57
E64	10 µM	100
PMSF	500 µM	94
Pepstatin	1 µM	100
Leupeptin	100 µM	94
TPCK	500 µM	94
IAA	50 µM	100

Table 3.7 Effect of inhibitors on *Tp. volcanium* 20S proteasome trypsin-like activity.

	Concentration	Relative Activity (%)
Control	—	100
TLCK	100 µl	27
PMSF	500 µM	100

CHAPTER 4

DISCUSSION

Thermophiles offer new opportunities for biotechnological applications as a result of their stability to extreme temperatures (60-110^{o}C). The majority of thermophiles are member of the Archaea, one of the three phylogenetic domains of life, as defined by comparison of the 16S rDNA gene sequences. But also known that numbers of bacteria are member of thermophiles such as *Thermus* and some *Bacillus* species (Hough and Danson, 1999). The enzymes from thermophilic archaea are more thermostable than their counterparts isolated from thermophilic or mesophilic organisms (Cowan *et al*., 1987). In addition, thermostability of enzymes confer resistance to other factors such as detergents and denaturating agents, thereby offering great potential advantages for a range of biotechnological processes.

Serine proteases from thermophilic archaea and thermophilic bacteria are given much attention due to their activity and stability at alkaline pH besides thermostability at high temperature; and these features of proteolytic enzyme provide wide applications in a number of industries (Gupta *et al.,* 2002).

To date a number of alkaline serine proteases have been reported from thermophilic and hyperthermophilic archaea (*Sulfolobus solfataricus*, *Pyroccus abyssi*, strain st 549, *Thermococcus stetteri, Desulforococcus strain* $Tok_{12}D_1$) (Burluni *et al*., 1992, Dib *et al*., 1998, Klingeberg *et al*., 1995, Cowan *et al*., 1987) and thermophilic

microorganisms (*Bacillus thermoruber*, *Bacillus* sp. B18', *Bacillus licheniformis* MIR 29, *Bacillus* sp. PS719, *Bacillus subtilis* CHZ1, *Thermoactinomyces* sp. E79. *Thermus aquaticus* YT-1, *Thermoactinomyces sp*. HS682, *Thermospora fusca, Bacillus* sp. JB-99) (Manachini *et al*., 1988, Fujiwara *et al*., 1993, Ferrero *et al*., 1996, Hutalidok-Towatana *et al*, 1999, Zvauya and Zvidzai, 2001, Lee *et al*., 1996, Matsuzawa *et al*., 1988, Kristjansson *and* Kinsella., 1990, Johnvesly and Naik, 2001). All are extracellular proteases, which are secreted into the environment and presumably function in the digestion of protein substrates for nutritive purposes. Unfortunately, there are not much information about intracellular serine proteases from thermophilic archaea and bacteria. Even several intracellular serine proteases are described from thermophilic archaea (Kocabıyık and Erdem, 2002, Klingeberg, 1991), there are only a few reports obtained about their purification and characterizations. These are heat-stable alkaline protease from *Bacillus stearothermophilus* F1, *Thermoactinomyces* sp. HS682 and hyperthermophilic archaeon *Pyrococcus furiosus* (Rahman *et al*., 1994, Tsuchiya *et al*., 1997 and Halio *et al*., 1997, respectively).

In this study, we have purified and characterized two serine proteases both of, which are cytoplasmic, from thermophilic archaea *Thermoplasma volcanium*. The first one is an independent chymotrypsin-like protease. Even its physiological role is not known; it may be involved intracellular protein turnover so that the bacterium can break down and reassemble peptides depending on metabolic requirements. This capability of recycling amino acids might prove to be an important adaptation of thermophilic archaea to cope with the extreme conditions of their habitats. The second protease activity that has been subject to investigation in this project is a 20S proteasome associated chymotrypsin-like enzyme.

The purification scheme of the *Tp. volcanium* intracellular serine protease involved ammonium sulfate fractionation and anion exchange chromatography which was followed by affinity chromatography on α-casein agarose column. This procedure resulted in a 3.56-fold purification of the enzyme with a final yield of 27%.

Purification was homogeneous as judged by SDS-PAGE (Figure 3.4). We have observed from the results that *Tp. volcanium* intracellular protease was less stable in its purified form than in the cell extract and showed more stability during 80% ammonium sulfate precipitated form and in the presence of NaCl. According to Hensel and Jakob (1994) high ionic strength tends to protect labile amino acid residues from solvent exposure. They demonstrated that heat-induced irreversible changes to some intracellular hyperthermophilic proteins are minimized through the stabilizing influence of novel organic phosphates and potassium ions, which are present at high intracellular concentrations in hyperthermophilic archaea. A polypeptide, once unfoldes, is highly susceptible to degradation and in addition, limited hydrolytic cleavage of a folded polypeptide may promote unfolding or modify the potential for successful renaturation (Cowan *et al*., 1987).

We have found that the highest optimum temperature for azocazein hydrolysis was 60^{o}C and for N-Suc-Phe-pNA hydrolysis was 50^{o}C. This result shows that *Tp. volcanium* serine protease rather thermophilic. With respect to temperature optimum, *Tp. volcanium* serine protease is similar to that of thermostable alkaline proteases or serine proteases from various *Bacillus* spp (Kumar *et al*., 1999, Ferrero *et al*., 1996, Beg and Gupta, 2003, Durham *et al*., 1987, Manachini *et al*., 1988, Kobayashi *et al*., 1996) and those from halophilic archaea (Stan-Lotter, 1999, Giménez *et al*., 2000, Studdert *et al*., 2001). However, this temperature for maximum enzymatic activity of the serine protease is quite lower as compared to the optimum temperature for the serine proteases isolated from various hyperthermophilic archaea. For instance, the optimum temperature for substrate hydrolysis reported for serine protease was 115^{o}C from *Pyroccus furiosus* (Halio *et al*., 1997), 100^{o}C from *Desulfourococcus* sp. (Cowan *et al*., 1987), 95^{o}C from *Pryrococcus abyssi* (Dib *et al*., 1998), 85^{o}C from *Thermococcus stetteri* (Klingeberg *et al*., 1995).

Tp. volcanium is a thermoacidophilic archeabacterium and it grows in acidic pH medium (pH 2.7) as given in Materials and Methods. Interestingly, *Tp. volcanium* serine protease is active in a broad pH range (pH 6.0-8.0) over which displayed more

than 80% of the maximum activity for the hydrolysis of N-Suc-Phe-pNA with an apparent maximal activity at pH 7.0. This result also showed that this protease is very similar to other serine proteases, which were purified from thermophilic and hyperthermophilic archaea (Cowan *et al*., 1987, Blumentals *et al*., 1990, Burlini *et al*., 1992, Klingeberg *et al*., 1995). Generally, commercial proteases from microorganisms exhibit maximum activity in the alkaline pH range of 7.0-11.0 (Rao *et al*., 1998, Kumar and Takagi, 1999, Gupta *et al*., 2002). Thus, these results show that intracellular *Tp. volcanium* protease is an alkaline serine protease. The major draws back of enzymes recovered from thermophiles are their instability at alkaline pH. Due to high activity, in a broad range of pH and stability, this new thermophilic serine protease from *Tp. volcanium* is suitable for several applications in detergent industry, in the leather industry and also in the photographic film industry.

The *Tp. volcanium* enzyme displayed broad substrate specificity, by catalyzing the hydrolysis of N-protected pNA of chromogenic peptide substrates. *Tp. volcanium* protease resembles the chymotrypsin-like proteases that have non-polar substrate-binding pocket, and thus, require an aromatic or bulky nonpolar amino acid such as tryptophan, phenylalanine, tyrosine or leucine. The protease enzyme displayed high activity against N-Suc-Phe-pNA and substantially high affinity for Ala-Ala-Phe-pNA and L-Leu-pNA. The enzyme also showed some elastase-like enzyme activity that preferentially cleaves peptide bonds after valine and alanine, which are present in elastin. We could detect a low trypsin-like protease activity as associated with the purified *Tp. volcanium* protease using N-CBZ-Arg-pNA, the typical substrate for trypsin. Thus, our results showed that *Tp. volcanium* serine protease has a significant component of chymotrypsin-like activity with its high specificity in cleaving at the carboxyl side of both hydrophobic and basic residues in the P1 position.

The protease was also active on a variety of modified (azocasein) or natural proteins (BSA, casein). Among these, *Tp. volcanium* serine protease exhibited the maximum activity against casein. Most of the previous studies also revealed that alkaline serine proteases showed high activity towards casein relative to other native and or modified proteins (Cowan *et al*., 1987, Rahman *et al*., 1994, Kumar *et al*., 1999). It was reported that the ability of serine protease enzyme to act on naturally occurring

proteins, including casein (keratin, albumin, hemoglobin) would help facilitate the release of proteinaceous materials in stains such as those from milk, mud, blood (Hutadilok-Towatana *et al*., 1999).

The inhibition profile of the purified *Tp. volcanium* protease strongly suggested that the purified enzyme can be classified as a serine alkaline protease of chymotrypsin type. Irreversible inhibitor, PMSF is well known to be a good inhibitor of alkaline serine proteases from the studies on serine proteases from mesophilic and thermophilic microorganisms (Cowan *et al*., 1987, Manachini *et al*., 1988, Rahman *et al*., 1994, Kristjansson and Kinsella, 1990, Klingeberg *et al*., 1995, Ferrero *et al*., 1996, Han and Damodaran, 1998, Hutadilok-Towatana *et al*., 1999, Kumar *et al*., 1999, Kim *et al*., 1999, Babé and Schmidt, 1998, Singh *et al*. 2001, Zvauya and Zvidzai, 2001, Joo *et al*., 2002, DeToni *et al*., 2002). PMSF strongly inhibits serine residue at the active site causing complete loss of enzyme activity. Inhibition of the purified *Tp. volcanium* protease by PMSF (94.5% inhibition) indicated that the enzyme contains a serine residue in the active site. We also observed complete inhibition of purified enzyme with chymotrypsin specific irreversible inhibitor; TPCK, which implied the presence of a second catalytically important residue, histidine, in the active site. Efficient inhibition of the purified protease enzyme with another chymotrypsin specific inhibitor, chymostatin (95.5% inhibition), which has Phenylalanine at P1, strongly suggested that the enzyme we have purified from *Tp volcanium* is a chymotrypsin type serine protease. Although this is the first report for the isolation of a thermophilic serine protease from *Tp. volcanium*, serine protease activities were also reported from various other *archaea* mainly thermophilic and halophilic archaea (Stan-Lotter *et al*., 1999, Giménez *et al*., 2000, Studdert *et al.,* 2001).

The use of disulfide-reducing agent, DDT, did not inhibit the protease activity of *Tp. volcanium* protease. It was indicated that enzyme didn't show keratin hydrolyzing enzyme activity (Kumar and Takagi, 1999). The protease from *Tp. volcanium* retained 75% activity and 94% in presence of EDTA and EGTA, respectively. The presence of 10 mM EDTA could not lead to complete inhibition of enzyme activity. This result helps exclude the possibility that this enzyme is a metalloprotease. Partial

inhibition by EDTA indicates that cations may act as cofactor or as stabilizing agents. On the other side, the stability of the enzyme in presence of EDTA is advantageous for use of enzyme as detergent addictive. This is because detergents contain high amounts of chelating agents, which function as water softeners and also assist in stain removal. These agents specifically bind to and chelate metal ions making them unavailable in the detergent solution (Beg and Gupta, 2003).

Among the all divalent metal ions tested, Co^{2+}, $Mn^{2,}$ $Mg^{2,}$ Ca^{2+} were exhibited stimulatory effect on the *Tp volcanium* serine protease activity with increasing degree, in the given order. It has been reported by others that divalent cation like Ca^{2+}, Mg^{2+}, Mn^{2+} or a combination of these cations increased the serine proteases activity (Rao *et al*., 1998, Kumar and Takagi, 1999). Ni^{2+} had some inhibitory effect in the protease activity of *Tp. volcanium* protease. It was reported that the ability of any metal ion to satisfy the binding interactions of the metal-ion binding sites depends on the atomic size, expressed as the effective radius of the hydrated metal ions. It was shown that affinity of the enzyme for divalent cations decreases with decreasing ionic radii, since Ca^{2+}(0.099 nm) which has largest radius among the metal ions tested, was found to be the most efficient at increasing activity in comparison with controls, whereas smaller ions, i.e., Mn^{2+} (0.082 nm), Co^{2+} (0.074 nm), Cu^{2+} (0.073 nm) and Mg^{2+} (0.072 nm) become less effective in that order except for Mg^{2+} (Khoo *et al*., 1984, Hutadilok-Towatan *et al*., 1999).

Generally, Ca^{2+} ion is known to play a role in maintaining the activity of microbial serine proteases. This cation has been known to increase enzyme activity of alkaline serine proteases from *Bacillus* sp. and *Fungi* (Strongin *et al*., 1978, Manachini *et al*., 1988, Rahman *et al*., 1994, Litthauer *et al*., 1996, Toni *et al*., 2002). In addition, the role of Ca^{2+} in the thermostability have been reported for serine proteases from thermophilic microorganisms (*Thermoactinomyces* sp. E79, *Thermus aquaticus*, *Bacillus* sp. JB99, *Bacillus brevis* , *Bacillus* sp. B18') and from mesophilic microorganisms (*Bacillus subtilis*, *Bacillus* NG312, *Bacillus* sp. SSR1, *Pseudomonas* sp. BK7, *Geotrichum candidum* P-5) (Lee *et al*., 1996, Matsuzawa *et al*., 1988; Johnvesly and Naik, 2001, Banerjee *et al*., 1993, Fujiwara *et al*., 1993, Babé and Schmidt, 1998, Sahoo et al., 1999, Singh *et al*., 2001, Lee *et al*., 2000, Litthauer *et*

*al.,*1996). Similarly, activation of serine protease activity by Mg^{2+} was also reported (Kumar *et al*., 1999; Zvidzai and Zvauya, 2001; Tangram and Rajkumar, 2002). It was assumed that the increased proteolytic activity in the presence Ca^{2+}, Mg^{2+} when compared with controls containing no metal ions might be generated from ability of these metal ions to retard enzyme denaturation under the conditions employed (Hudatilok-Towatana *et al*., 1999, Fujiwara *et al*., 1993). The results from these reports suggested that these cations apparently protect the enzyme against thermal denaturation and play vital role probably by acting as a salt or ion bridge between amino acid and consequently maintaining the active conformation of the enzyme at high temperature.

In the second part of our study, 20S proteasome of *Tp. volcanium* with substantial chymotrypsin-like activity was purified to homogeneity and characterized.

The purification course was followed and the molecular mass of the proteasome subunit proteins after hydroxylapaptite chromatography were determined by SDS-polyacrylamide gel electrophoresis. The enzyme was obtained from the hydroxylapatite column chromatography which was the last purification step produced two bands on SDS-PAGE. This result showed that 20S proteasome from the *Tp. volcanium* was found to be composed of two subunits, α and β subunits with relative molecular masses of 26 kDa and 21.9 kDa, respectively, which was in good agreement with the reports on 20S proteasomes from other *archaeabacteria*, *Thermoplasma 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). (Dahlman *et al*., 1989, Maupin-Furlow and Ferry, 1995, Bauer *et al*., 1997).

The peptide hydrolyzing activities of the purified proteasome of *Tp. volcanium* were examined with a variety of chromogenic (βNA and pNA) small-peptide substrate to identify sites of hydrolysis of the peptide chain. The multisubstrate activity of the *Tp. volcanium* 20S proteasome is similar to that of *M. jannaschii* (Wilson *et al*., 2000) and *M. thermophila* (Maupin-Furlow *et al*., 1998), which hydrolyze CBZ-Leu-Leu-Glu-βNA at a rate that is 72% higher than the chymotrypsin like substrate Ala-

Ala-he-pNA. This differs, however, from 20S proteasomes isolated from other *archae* such as *Thermoplasma acidophilum*, *Haloferax volcanii*, *Pyrococcus. furious* and eubacterial *actinomycetes* such as *Rhodococcus erytropolis* and *Streptomyces coelicolor* which exhibit very little PGPH activity (Dalhman *et al*., 1992, Tamura *et al*., 1995, Akopian *et al*., 1997, Bauer *et al*., 1997, Nagy *et al*., 1998, Wilson *et al*., 1999). Synthetic substrate that cleaved by trypsin was hydrolyzed with low efficiency by *Tp. volcanium* proteasome. This low rate of cleavage or no cleavage carboxyl to arginine is common to other archael and eubacterial 20S proteasomes (Akopian *et al*., 1997, Wilson *et al*., 2000, Maupin-Furlow *et al*., 1998, Bauer *et al*., 1997, Dahlman *et al*., 1992, Nagy *et al*., 1998, Wilson *et al*., 1999) but contrasts with eukaryal 20S proteasomes which cleave significantly higher trypsin-like activity (Rivet, 1993).

The effects of some well known serine protease inhibitors on the activity of *Tp. volcanium* 20S proteasome were examined. Chymotrypsin-like activity of *Tp. volcanium* 20S proteasome was significantly inhibited (43%) by chymotrypsin specific serine protease inhibitor chymostatin at 400 µg/ml concentration although inhibition by other serine protease inhibitors PMSF and TPCK was not detectable.

This inhibition pattern suggests that serine protease activity is an important component of *Tp. volcanium* 20S proteasome. Previously reported that proteasome inhibition of proteasome by DFP which is well known serine protease inhibitor and other isocoumarin derivatives, known to act as class-specific inhibitors of serine proteases, potently inhibited proteasome (Orlowski and Michaud, 1989, Bogyo and Wang, 2002). These findings prompted classification of the proteasome as serine proteases. Our inhibition studies with archaea 20S proteasome was also supported that *Tp. volcanium* 20S proteasome is a serine protease.

The chymotrypsin-like activity of *Tp. volcanium* 20S proteasome was not affected trypsin-like serine protease inhibitors (Leupeptin, tosyl-L-lysine-chloromethyl ketone (TLCK), cysteine protease inhibitors (iodoacetic acid (IAA), N-[N-(l-3-trans-carboxyloxirane-2-carbonyl)-L-leucyl]-agmatine L-3-carboxytrans 2,3-epoxypropyl-leucylamido (4-guanidine) butane (E.64), and aspartic acid protease inhibitor

(pepstatin). When we used N-CBZ-Arg-pNA which is a substrate for trypsin, 20S proteasome was strongly inhibited by TLCK.

The optimum temperature for Ala-Ala-Phe-pNA hydrolysis by the *Tp. volcanium* 20S proteasome was 55^{o}C which is substantially lower than the temperature optima reported for the 20S proteasome from *Tp. acidophilum* (85^{o}C to 91^{o}C) (Akopian *et al*., 1997, Dahlmann *et al*., 1992), *P. furiousus* (95^{o}C), (Bauer *et al*., 1997) and *M. thermophila* (70 to 75^{o}C) (Maupin-Furlow *et al*., 1998).

Divalent cations such as Ca^{+2} and Mg^{2+} at concentrations of 125-250 mM stimulated the chymotryptic activity of 20S proteasome of *Tp. volcanium.* However, incubation of the archael 20S proteasome with Ca^{2+} ion at concentrations higher than 500 mM caused significant reduction, even loss of the activity. The stimulating affects of these cations on the other archael 20S proteasomes from *Thermoplasma. acidophilum* and methanoarcheon *Methanosarcina thermophila* were previously reported (Dalhman *et al.*, 1992, Maupin-Furlow *et al*., 1998).

CHAPTER 5

CONCLUSION

Serine proteases from thermophilic archaea and thermophilic bacteria are very important proteases due to their activity and stability at alkaline pH besides thermostability at high temperature; and these features of proteolytic enzyme offering potential advantages for a range of biotechnological applications.

In this study, two novel intracellular serine proteases with chymotryptic activity were purified and characterized from thermoacidophilic archaeon *Tp. volcanium*.

1. The independent chymotryptic-like serine protease was purified (5.5 fold) with a specific activity of 6.0 U/mg from the cell free extract of thermoacidophilic archaeon *Tp. volcanium* by 80% ammonium sulfate precipitation, ion exchange and affinity column chromatography. The presence of purified protease activity was analyzed by gelatin zymography and its MW was estimated as 35 kDa.

2. Purified *Tp. volcanium* displayed maximum hydrolytic activity against N-Suc-Phe-pNA which is specific for chymotrypsin-like serine protease activity. The K_m and V_{max} values for N-Suc-Phe-pNA were 2.2 mM and 40 μmoles/ml/min, respectively. Beside chymotryptic-like substrates, *Tp.*

volcanium purified serine protease has able to hydrolyze chromogenic substrates specific for elastase and subtilisin. The protease has also ability to hydrolyze native proteins such as casein, BSA and modified proteins such as azocasein.

3. The optimum pH and temperature for proteolytic activity with N-Suc-Phe-pNA was around 7.0 and 50ºC, respectively.

4. As well as substrate profile, strong inhibition of the purified enzyme by serine protease inhibitor PMSF and TPCK and chymotrypsin specific serine protease inhibitor chymostatin suggest that the purified protease enzyme from *Tp. volcanium* is a chymotrypsin-like serine protease.

5. Inhibition (25%) of the purified protease by EDTA indicated the requirement for cofactors. Among the all divalent metal ions tested, Co^{2+}, $Mn^{2+,}$ $Mg^{2+,}$ Ca^{2+} exhibited stimulatory effect on the *Tp volcanium* serine protease activity with increasing degree, in the given order. With all these properties, being chymotrypsin-like serine protease permits for this new protease, a wide application in biotechnological processes mainly in detergent industries.

6. *Tp. volcanium* 20S proteasome with chymotryptic activity was also purified from the cell free extract of this archaeon. This protease complex was purified by three successive column chromatography with a 19.1 U/mg specific activity. SDS-PAGE analysis revealed that *Tp. volcanium* 20S proteasome was consisted of an α- (26 kDa) and a β- (21.9 kDa) subunit.

7. *Tp.volcanium* 20S proteasome has multicatalytic activity with variety chromogenic small peptide substrates. 20S proteasome displayed greatest peptidyl glutamyl activity with CBZ-Leu-Leu-Glu-βNA, substantial chymotrypsin-like activity with N-Suc-Phe-pNA and very low trypsin-like

activity with N-CBZ-Arg-pNA. The K_m and V_{max} values for Ala-Ala-Phe-pNA were 1.8 mM and 0.83 µmoles/ml/min, respectively.

8. *Tp. volcanium* 20S proteasome displayed maximum chymotryptic-like activity against Ala-Ala-Phe-pNA at 55^{o}C with pH 7.5 and enzyme activity was enhanced in the presence of magnesium and calcium salts at 125-250 mM. Chymotrypsin-like activity of *Tp. volcanium* 20S proteasome was significantly inhibited (43%) by chymostatin, rather less affected by trypsin-like serine protease inhibitors but not affected by cysteine protease inhibitors and aspartic acid protease inhibitors, at all.

REFERENCES

Akopian, T.N., Kisselev, A.F., Goldberg, A.L. 1997. Processive Degradation of Proteins and Other Catalytic Properties of the Proteasome from *Thermoplasma acidophilum*, *Journal of Biological Chemistry*, 272: 1791-1798.

Anson, M.L. 1938. The Estimation of Pepsin, Trypsin, Papain and Cathepsin with Hemoglobin, *Journal of General Physiology*, 22: 79-89.

Anwar, A. and Saleemuddin, M. 1998. Alkaline Proteases: A Review, *Biosourse Technology* 64: 175-183

Appel, W. 1986. Chymotrypsin: Molecular and Catalytic Properties, *Clinical Biochemistry*, 19: 317-322.

Aqulilar, A. 1996. Extromophiles Research in the Europian Union: from Fundamental Aspects to Industrial Expectations, *FEMS Microbiology Reviews*, 18: 89-92.

Babé, L.M. and Schmidt, B. 1998**.** Purification and BiochemicalAnalysis of WprA, a 52-kDa Serine Protease Secreted by *B. subtilis* as an active complex with its 23-kDa propeptide, *Biochimica et Biophysica Acta*, 1386: 211-219**.**

Banerjee, U.C., Kumar Sani, R., Azmi, W., Soni, R. 1999. Thermostable Alkaline Proteases from *Bacillus brevis* and Its Chracterization as a Laundry Detergent addictive, *Process Biochemistry*, 35: 213-219.

Barber, R.D. and Ferry, J.G. 2001. Archeal Proteasomes, *Methods in Enzymology*, 330: 413-424.

Barthhomeuf, C., Pourrat, H., Pourrat, A. 1992. Collagenolytic Activity of A New Semi-alkaline Protease from *Aspergillus niger*, *Journal of Fermentation Bioengineering*, 73: 233-236.

Barret, A.J., Rawling, N.D., O'Brien, E.A. 2001. The MEROPS Database as Protease Information System, *Journal of Structural Biology***,** 134: 95-102

Barrett, A.J and McDonald, J.K. 1985. Nomenclature: Protease, Proteinase and Peptidase*, Biochemistry Journal*, 237: 935.

Barret, A. J. 1994. Classification of Peptidases, *Methods in Enzymology*, 244:1-18.

Bauer, .W., Bauer, S.H., Kelly, R.M. 1997. Purification and Characterization of a Proteasome from the Hyperthermophilic Archeaon *Pyrococcus furious*, Applied and Environmental Microbiology, 63: 1160-1164.

Baumeister, W., Cejka, Z., Kania, M., Seemuller, E. 1997. The Proteasome: A Molecular Assembly Designated to Confine Proteolysis to a Noncompartment, *Biological Chemistry*, 378: 121-130

Baumeister, W., Walz, J., Zuhl, F., Seemuller, E. 1998. The Proteasome: Paradigm of a Self –Compartmentalizing Protease, *Cell*, 92: 367-380.

Beg, Q. K. and Gupta, R. 2003, Purification and Chracterization of an Oxidation-stable, Thiol-dependent Serine Alkaline Protease from *Bacillus mojavensis*, *Enzyme and Microbial Technology*, 32: 294-304.

Benaroudj, N., Tarcsa, E., Cascio, P., Goldberg, A.L. 2001. The Unfolding of Substrates and Ubiquitin-Independent Protein Degradation by Proteasomes, *Biochimie,* 83:311-318.

Beynon, R.J. and Salvasen, G. Commercially Available Protease Inhibitors. In Beynon R.J. and Bond S.J. 2001. Proteolytic Enzymes 2nd Edition, Oxford University Press Inc., New York, 317-330.

Kirchke, H. and Wiederonsders, B. Chromogenic Peptide Substrates. In Sterchi and Stacker, 1999, Proteolytic Enzymes Tools and Target. Springer, New York, 11-28.

Bloemendal, H., Gerards, W.L.H., de Jong, W.W. 1998. Structure and Assembly of the 20S Proteasome, *Cellular and molecular Life Sciences*, 54: 253-262.

Bluemental, I.I., Robinson, A.S., Kelly, R.M. 1990. Characterization of Sodium Dodecyl Sulfate-Resistant Proteolytic Activity in the Hyperthermophilic Archaebacterium *Pyrococcus furiosus*, *Applied and Environmental Microbiology*, 56 (7): 1992-1998.

Bochtler, M., Dditzel, L., Groll, M., Hartmann, C., Huber, R. 1999**.** The Proteasome*, Annual Review Biophysics Biomolecular Structure*, 28: 295-317.

Bogyo, M. and Wang, E.W. 2002. Proteasome Inhibitors: Complex Tools for a Complex Enzyme, *Current Topics in Microbiology and Immunology*, 268: 185-208.

Bogyo, M., Shin, S., McMaster, J.S., Ploegh, H.L. 1998. Substrate Binding and Sequence Preference of the Proteasome Revealed by Active-site Directed Affinity Probes, *Chemical Biology*, 5: 307-320.

Burluni, N., Magnani, P., Villa, A., Macchi, F., Tortota, P., Guerritore, A. 1992. A Heat-stable Serine Proteinase from the Extreme Thermophilic Archaebacterium *Sulfolobus solfataricus*, *Biochimica et Biophysica Acta*, 1122: 283-292.

Bruins, M.E., Janssen, A.E.M., Boom, R.M. 2001. Thermozymes and Their Applications, *Applied Biochemistry and Biotechnology,* 90:155-186.

Chakrabarti, S.K., Matsumara, N., Ranu, R.S. 2000. Purification and Characterization of an Extracellular Alkaline Serine Protease from *Aspergillus terreus* (IJIRA 6.2), *Current Microbiology*, 40:239-244.

Connaris, H., Cowan, D.A., Sharp, R.J. 1991. Heterogenety of Proteinases from the Hyperthermophilic Archaeobacterium *Pyrococcus furiosus*, *Journal of General Microbiology*, 137: 1193-1199.

Coombs, G.S., Rao, M.S., Olsen, A.J. Dawson, P.E. and Madison, E.L. 1999. Revisiting Catalysis by Chymotrypsin Family Serine Proteases Using Peptide Substrates and Inhibitors with Unnatural Main Chains, *Journal of Biological Chemistry,* 274: 24074.

Cowan, D.A., Smolenski, K.A., Daniel, R.M., Morgan, H.W. 1987. An Extremely Thermostable extracellular Proteinase from a Strain of the Archaebacterium *Desulforococoocus* growing at 88^{o}C, *Biochemistry Journal*, 247: 121-133.

Dahlmann, B., Kuehn, L., Grziwa, A., Zwickl, P., Baumeister, W. 1992. Biochemical Properties of the Proteasome from *Thermoplasma acidophilum*, *FEBS*, 208:788-797.

Dahlmann, B., Kopp, F., Kuehn, L., Niedel, B., Pfeifer, G., Hegerl, R., Baumeister, W. 1989. The Multicatalytic Proteinase (Prosome) is Ubiquitous from Eukaryotes to Archaebacteria, FEBS Letter, 251: 125-131.

Dalev, P.G. 1994. Utilization of waste feathers from poultry slaughter for production of a protein concentrate, *Bioresource Technology*, 48: 265-267.

Darland, G., Brock, T., Samaonoff, W., Conti, S.F. 1970. A Thermophilic , Acidophilic *Mycoplasma* İsolated from a Coal Refuse Pile, Science, 170: 1416-1418.

DeMartino G.N., Proske R.J., Moomaw C.R., Strong A.A., Song X., Hisamatsu H., Tanaka K., Slaugther C.A. 1996. Identification, Purification, and Characterization of a PA700-dependent Activator of the Proteasome, *The journal of Biological Chemistry*, 271 (6): 3112-3118.

DeMartino, G.N. and Slaughter, C.A. 1999. The Proteasome, a Novel Protease Regulated by Multiple Mechanisms, *The Journal of Biological Chemistry,* 274(32):22123-22126.

De Toni, C.H., Richter M.F., Chagas, J.R., Henriques J.A.P., Termignogi, C. 2002. Purification and Characterization of an Alkaline Serine Endopeptidase from a Feather-degrading *Xanthomonas maltophila* Strain, *Canadian Journal of Microbiology*, 48: 242-248.

Dick, L.R., Cruikshank, A.A., Destree, A.T., Greiner, L., McCormack, T.A., Melandri, F.D., Nunes, S.L., Palombella, V.J., Parent, L.A., Plamondon, L., Stein, R.L. 1997. Mechanistic Studies on the Inactivation of the Proteasome by Lactacystin in Cultured Cells, Journal of Biological Chemistry, 272: 182-188.

Dib, R., Chobert, J., Dalgalarrondo, M., Barbier, G., Haertlé, M. 1998. Purification, Molecular Properties and Specificity of a Thermoactive and Thermostable Proteinase from *Pyrococcus abyssi*, Strain st 549, Hyperthermophilic Archaea from Deep-sea Hydrothermal Ecosystem, *FEBS Letter*, 431: 279-284.

Dodson, G. and Wlodawer, A. 1998. Catalytic Triads and Their Relatives, *Trends in Biological Science*, 23: 347-352.

Durham, D.R., Stewart, D.B., Stellwag, E.J. 1987. Novel Alkaline- and Heat-Stable Serine Proteases from Alkoliphilic *Bacillus* sp. Strain GX6638, *Journal of Bacteriology,* 169: 2762-2768.

Erlenger, B.F., Kokowsky, N., Chohen, W. 1961. The preparation and Properties of Two New Chromogenic Substrates of Trypsin, *Archives of Biochemistry and Biophysics*, 95: 271-278.

Feinstein, G., Malemud, C.J., Janoff, A. 1976. The Inhibition of uman Leucocyte Elastase and Chymotrypsin-like Protease by Elastatinal and Chymostatin, *Biochimica et Biophysica Acta*, 429: 25-932.

Ferrero, M.A., Castro, G.R., Abate, C.M. 1996. Thermostable Alkaline Proteases of *Bacillus licheniformis* MIR 29: Isolation, Production and Characterization, *Applied Microbiology and Biotechnology, 1996,* 45: 327-332.

Foltman, B., Szecsi, P.B., Tarasova N.I. 1985. Detection of Proteases by Clotting of Casein After Gel Electrophoresis*, Analytical Biochemistry*, 146: 353-360.

Franzetti, B., Schoehn, G., Garcia, D., Ruigrok, R.W.H., Zaccai, G. 2002. Characterization of the Proteasome from the Extremly Halophilic Archaeon *Haloarcula marismortui*, *Archaea*, 1: 53-61.

Fujiwara, N., Masui, A., Imanaka, T. 1993. Purification and Properties of the Highly Thermostable Alkaline Protease from an Alkaliphilic and Thermophilic *Bacillus* sp., *Journal of Biotechnology,* 30: 245-256.

Gaczynska, M., Osmulski, P.A., Ward, W.F. 2001**.** Caretaker or undertaker? The role of the Proteasome in Aging, *Mechanisms of Ageing and Development*, 122: 235-254.

Garcia-Carreno, F.L. and Del Toro, M.A.N. 1997. Classification of Proteases Without Tears, *Biochemical Education*, 25 (3): 161-167.

Gerards W.L.H., de Jong W.W. Boelens W., Bloemendal H. 1998. Structure and Assembly of the 20S Proteasome, *CMLS Cellular and Molecular Life Sciences*, 54: 253-262.

Giménez, M.I., Studdert, C.A., Sánches, J.J., De Castro, R.E. 2000. Extracellular Protease of *Natrialba magadii*:Purification and Biochemical Characterization. *Extromophiles*, 4: 181-188.

Glickman, M.H. 2000. Getting in and out of the Proteasome, *Cell and Developmental Biology*, 11: 149-158.

Goldberg A.L., Cascio P., Petre B.M., Walz T. 2002. Properties of the Hybrid Form of the 26S Proteasome Containing Both 19S and PA28 Complexes, *The EMBO Journal*, 21 (11): 2636-2645.

Gottesman, S. and Maurizi, M.R. 1992. Regulation by proteolysis: Enegy Dependent Proteases and Their Targets, Microbiology Review, 56 (4): 592-621.

Grammer, T.C. and Blenis, J. 1996. The Serine Protease Inhibitors, Tosylphenylalanine Chloromethyl Ketone and Tosyllysine Chloromethyl Ketone, Potently Inhibit pp70s6k Activation, *Journal of biological Chemistry*, 271 (39): 23650-23652.

Groetrupp, M., Soza, A., Eggers, L., Kuehn, L., Dick, T.P., Schild, H., Rammensee, H.G., Koszinowski, H.U., Kloetzel, M. 1996a. A role for the Proteasome Regulator PA28a in Antigen Presentation, *Nature* 381:166-168.

Groetrupp, M. Soza, A., Kuckelkorn, U., Kloetzel, M. 1996b. Peptide Antigen Production by the Proteasome: Complexity Provides Efficiency. *Immunology of Today*, 17: 429-435.

Groll, M., Ditzel, L., Lowe, J., Stock, D., Bochtler M., Bartunic, H.D., Huber, R. 1997. Structure of 20S Proteasome from Yeast at 2.4 À Resolutions, *Nature*, 386: 463-471.

Gupta, R., Beg, Q.K., Lorenz, P. 2002. Bacterial alkaline proteases: Molecular Approaches and Industrial Applications, *Applied Microbiology and Biotechnology*, 59 (1): 15-32.

Halio, S.B., Bauer, M.W., Mukund, S., Adams, M.W.W., Kelly, R.M. 1997. Purification and Characterization of Two Functional Forms of Intracellular Protease PfpI from the Hyperthermophilic Archaeon Pyrococcus Furiosus, *Appleid and Environmental Microbiology*, 63 (1): 289-295.

Han, X. and Damodaran, S. 1998. Purification and Characterization of Protease Q: A Detergent- and Urea-Stable Serine Endopeptidase from *Bacillus pumilus*, *Journal of Agricultural Food Chemistry,* 46: 3596-3603.

Hensel, R. and Jacob, I. 1994. Stability of Glyceraldehyde-3-Phosphate Dehydrogenases from Hyperhermophilic Archaea at High-temperature, 16 (4): 742-745.

Horikoshi, K. 1996. Alkaliphiles- from an Industrial Point of View, *FEMS Microbiolog Review***,** 18: 259-270.

Hutalidok-Towatana, N., Painupong, A., Suntinanalert, P. 1999. Purification and Characterization of an Extracellular Protease from Alkaliphilic and Thermopholic *Bacillus* sp. PS719, *Journal of Bioscience and Bioengineering*, 87: 581-587.

Hough, D.W. and Danson, M.L. 1999, Extromozymes, *Current Opinion in Chemical Biology*, 3: 39-46

Izotowa, L.S., Strongin, A.Y., Chekulaeva, L.N., Sterkin, V.E., Ostoslavskaya, V.I., Timokhina, E.A., Stepanov, V.M. 1983. Purification and Properties of Serine Protease from *Halobacterium halobium*, *Journal of Bacteriology*, 155 (2): 826-830.

Jacobs, M., Eliasson, M., Uhlen, M., Flock, J. 1985. Cloning, Sequencing and Expression of Subtilisin Carlsberg from *Bacillus licheniformis*, *Nucleic Acids Research*, 13 (24): 8913-8926.

Jäger, S., Groll, M., Huber, R., Wolf, D.H., Heinemeyer, W. 1999. Proteasome β-type Subunits: Unequal Roles of Propeptides in Core Particle Maturation and a Hierarchy of Active Site Function, *Journal of Molecular Biology*, 291: 997-1013.

Johnvesly, B.and Naik, G.R. 2001. Studies on production of Thermostable Alkaline Protease from Thermophilic and Alkaliphilic *Bacillus* sp. JB-99 in a Chemically Defined Medium*, Process Biochemistry*, 37:139-144.

Jolliffe, L.K., Doyle, R.J., Streips, U.N. 1980. Extracellular Proteases Modify Cell-Wall Turnover in *Bacillus subtilis*, *Journal of Bacteriology*, 141(3): 1119-1208.

Joo, H., Kumar C.G., Park, G., Kim K., Paik S.R., Chang C. 2002. Optimization of the Production of an Extracellular Alkaline Protease from *Bacillus horikoshii*, *Process Biochemistry*, 38: 155-159.

Kalisz, H.K. 1988. Microbial Proteinases, *Advances in Biochemical Engineering & Biotechnology*, 36:1-65.

Kawashima, T., Amano, N., Koike, H., Makino, S., Higuchi, S., Kawashima-Ohya.,

Watanabe, K., Yamazaki, M., Kanehori, K., Aramaki, H., Makino, S., Suzuk, M. 2000. Archaeal Adaptation to Higher Temperatures Revealed by Genomic Sequence of *Thermoplasma volcanium*. *PNAS*, 97(26): 14257-14262.

Khoo, T.C., Cowan, D.A., Daniel, R:M., Morgan, H.W.1984. Interactions of calcium and other metal ions with caldolysin, the thermostable proteinase from *Thermus aquaticus* strain T351, Biochemical Journal, 221: 407-413.

Kierszenbaum, A.L. 2000. The 26S Proteasome: Ubiquitin-Mediated Proteolysis in Tunnel, *Molecular Reproduction and Development*, 57: 109-110.

Kim, W., Choi, K., Kim Y., Park, H., Chol. J., Lee, Y., Oh, H., Kwon, I., Lee, S. 1996. Purification and characterization of a fibrinolytic enzyme produced from *Bacillus* sp. strain CK 11-4 screened from chungkook-jangs. *Applied and Environmental Microbiolology*, 62: 2482–2488.

Kim, Y., Lee, J., Sunitha, K., Kim, H., Oh, T. 1999. Minor Thermostable Alkaline Protease Produced by *Thermoactinomyces sp.* E79, *Microbiology and Biotechnology*, 9: 469-474**.**

Kim, Y. and Ahn K. 2001. Theoretical Study of the Role of Low-barrier Hydrogen Bonds in Enzyme Catalysis: A Model of Proton Transfer in Serine Protease, *Theorical Chemistry Acc*, 106: 171-177.

Kirchke, H. and Wiederonsders, B. Chromogenic Peptide Substrates. In Sterchi and Stacker, 1999, Proteolytic Enzymes Tools and Target. Springer, New York, 11-28.

Kisselev, A.F., Akopian, T.N., Goldberg A.I. 1998. Range of Sizes of Peptide Products Generated during Degradation of Different Proteins by Archeal Proteasomes, *The Journal of Biological Chemistry*, 273 (4): 1982-1989.

Klingeberg, M., Hashwa, F., Antranikian, G. 1991. Properties of Extremely Thermostable Proteases from Anaerobic Hyperthermophilic Bacteria, *Applied Microbiology and Biotechnology*, 34: 715-719.

Klingeberg, M., Galunsky, B., Sjoholm, C., Kasche, V., Antranikian, G. 1995. Purification and Properties of a Highly Thermostable, Sodium Dodecyl Sulfate-Resistant and Stereospecific Proteinase from the Extremly Thermophilic Archaeon *Thermococcus stetteri*, *Applied and Environmental Microbiology*, 61 (8): 3098-3104.

Kocabıyık, S. and and Erdem, B. 2002. Intracellular Alkaline Proteases Produced by Thermoacidophiles: Detection of Protease Heterogeneity by Gelatin Zymography and Polymerase Chain Reaction (PCR), *Bioresource Technology*, 84: 9-33.

Kobayashi, T., Hakamada, Y., Hitomi, J., Koike, K., Ito, S. 1996. Purification of Alkaline Proteases from a *Bacillus* strain and Their possible Interrelationship, Applied Microbiology and Biotechnology, 45: 63-71.

Kristjansson M.M. and Kinsella J.E. 1990. Heat Stable Proteinase from *Thermospora fusca*, *International Journal of Peptide Protein Research*, 36(2): 201-207.

Krüger, E., Kloetzel, P., Enenkel, K. 2001. 20S Proteasome Biogenesis, *Biochimie,* 83: 289-293.

Kudrya, V.A., and Simonenko, I.A. 1994. Alkaline Serine Protease and Lectin Isolation from the Culture Fluid of *Bacillus subtilis*, *Applied and Microbial Bacteriology*, 41: 505-509.

Kumar, C.G., Tiwari, M.P., Jany, K.D. 1999. Novel Alkaline Serine Proteases from Alkalophilic *Bacillus* sp: Purification and Some Properties, *Process Biochemistry*, 34: 441-449.

Kumar, C.G. and Takagi, H. 1999. Microbial Alkaline Proteases: From a Bioindustrial viewpoint**,** *Biotechnology Advances*, 17:561-594.

Kitadokoro, K., Nakamura, E., Tamaki, M., Horii, T., Okamoto, H., Shin, M., Sato, T., Fujiwara, T., Tsuzuki, H., Yoshida, N., Teraoka, H. 1993**.** Purification, Characterization and Molecular Cloning of an Acid-specific Proteinase from *Streptomyces fradiae* ATCC 14544, *Biochimica et Biophysica Acta,* 1163:149-157.

Laemmli, U.K. 1970. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4, *Nature*, 227: 680-685.

Lee, E.G., Park, E., Hyun, E. 2000. Purification and Characterization of Two Alkaline Proteases Produced by *Pseudomonas sp*. BK7, *Journal of Microbiology and Biotechnology,* 10: 677-684.

Lee, J., Kim, Y. Kim, H., Park, Y., Oh, T. 1996. Purification and Characterization of a Thermostable Alkaline Protease from *Thermoactinomyces* sp. E79 and the DNA Sequence of the Encoding Gene, *Bioscience Biotechnology and Biochemistry*, 60: 840-846.

Lee, D. and Goldberg, A.L. 1998. Proteasome Inhibitors; Valuable New Tools for Cell Biologist, *Trends in Cell Biology*, 8: 397-403.

Levy, M.R., and Hou, S.C. 1974. Some Properties and Susceptibility to Inhibitors of Partially Purified Acid Proteases from *Plasmodium* Berghei and from Ghost of Mouse Red Cells, *Biochimica et Biophysica Acta*, 334: 423-430.

Litthauer, D.,Louw, C.H ., Toit, P.J.D. 1996. *Geotrichum candidum* P-5 Produces an Intracellular Seine Protease Resembling Cyhmotrypsin, *International Journal of Biochemistry.Cell Biology*, 28(10):1123-1130.

Longworty, T.A., Smith, P.F., Mayberry, W.R. 1972. Lipids of *Thermoplasma acidophilum*, *Journal of Bacteriology*, 112 (3) : 1193-1200.

Löwe, J., Stock D., Jap B., Zwickl, P., Baumeister W., Huber R. 1995. Crystal Structure of the 20S Proteasome from the Archaeon *T. acidophilum* at 3.4 A^o Resolution, *Science*, 268: 533-539.

Lupas, A., Flanagan, J.M., Tamura, T., Baumeister, W. 1997. Self Compartmentalizing Proteases, *Trends in Biological Science*, 22: 399-404.

Manachini, P.L,. Fortina, M.G., Parini, C. 1988. Thermostable Alkaline Protease Produced by *Bacillus thermoruber*- a New Species of *Bacillus*, *Applied Microbiol Biotechnology*, 28: 409-413.

Matsuzawa, H., K. Tokugawa, H.,. Hamaoki, M., Mizoguchi, M., Taguchi, H., Terada, I. Kwon, S., T. Ohta, S. 1988. Purification and Characterization of Aqualysin I (A Thermophilic Alkaline Serine Protease) Produced by *Thermus aquaticus* YT-1, *FEBS,* 171: 441-447.

Mary, E. 1998. Two Empires or Three? *Proceedings National Academic Science, USA*, 95: 9720-9723.

Masui, A., Fujiwara, N., Takagi, M., Imanaka, T. 1999. Feasibility Study for Decomposition of Gelatin Layers on X-ray Films by Thermostable Alkaline Protease from Alkaliphilic *Bacillus* sp. *Biotechnology Techniques*, 13: 813-815.

Maupin-Furlow, J.A., Willson H.L., Kaczowka S.J., Ou M.S. 2000. Proteasomes in the Archaea: from Structure to Function, *Frontiers in Bioscience*, 5: d837-865.

Maupin-Furlow, J.A., Aldrich, H.C., Ferry, J.G. 1998. Biochemical Characterization of the 20S Proteasome from the Methanoarchaeon *Methanosarcina thermophila*, *Journal of Bacteriology*, 180: 1480-1487.

Maupin-Furlow, J.A. and Ferry, J.G. 1995. A Proteasome from the Methanogenic Archeon *Methanasarcina thermophila*, *The Journal of Biological Chemistry*, 48: 28617-28622.

Nagy, I., Tamura, T., Vanderleyden, J. Baumeister, W., De Mot, R. 1998. The 20S *Streptomyces coelicolor*, *Jounal of Bacteriology*, 180: 5448-5453.

Nakamura, T., Yamagata, Y., Ichishima, E. 1992. Nucleotide Sequence of the Subtilisin NAT Gene, *aprN*, of Bacillus subtilis (natto), *Bioscience Biotechnology and Biochemistry*, 56 (11): 1869-1871.

Nduwimana J., Guenet, L., Dorval, I., Blayau M., Le Gall, Y: J., Le Treut. 1995. Proteases, *Annual Biology in Clinique*, 53: 251-264.

Niehaus, F., Bertoldo, C., Kahler, M., Antranikian, G. 1999. Extromophilis as a Source of Novel Enzymes for Industrial Application**,** *Applied Microbiology and Biotechnology*, 51: 711-729.

Orlowski, M. and Wilk, S. 2000. Catalytic Activities of the 20S Proteasome, a Multicatalytic proteinase Complex, *Archives of Biochemistry and Biophysics,* 383: 1-16.

Orlowski, M. and Michaud, C. 1989. Pituitary multicatalytic Proteinase Complex Specificity of Components and Aspects of Proteolytic Activity, *Biochemistry*, 28: 9270-9278.

Paetzel, M. And Dalbey, R.E. 1997. Catalytic Hydroxyl/Amine Dyads within Serine Proteases, *Trends in Biological Science*, 22: 28-31.

Papapostolou, D., Coux, O., Rebould-Ravaux, M. 2002. Regulation of the 26S proteasome Activities by Peptides Mimicking Cleavage Products, *Biochemical and Biophysical Research Communications*, 295: 1090-1095.

Perona, J.J and Craik, C.S. 1997. Evolutionary Divergence of Substrate Specificity within the Chymotrypsin-like Serine Protease Fold, *The Journal of Biological Chemistry*, 272: 29987-29990.

Phadtare S., Rao, M., Despande, V. 1997. A Serine Alkaline Protease from the Fungus *Conidiobolus coronatus* with a Distinctly Different Structure than the Serine Protease Subtilisin Carsberg, *Archives in Microbiology*, 166: 414-417.

Puhler, G., Weinkauf, S., Bachmann, L., Muller, S., Engel, A., Hegerl, R., Baumeister, W. 1992. Subunit Stoichiometry and Three-Dimentional Arrangement in Proteasomes from *Thermoplasma acidophilum*, *The EMBO Journal*, 11: 1607-1616.

Rahman, R.N.Z.A., Razak, C.N., Ampon, K., Basri, M., Yunus, W.M.Z.W., Salleh, A.B. 1994. Purification and Characterization of a Heat-stable Alkaline Protease from *Bacillus stearothermophilus* F1, *Applied Microbiology and Biotechnology*, 40: 822-827.

Rao, M.B., Tanksale, A.M., Ghatge, M.S., Despande, V.V. 1998. Molecular and Biotechnological Aspects of Microbial Proteases, *Microbiology and Molecular Biology Reviews*, 62: 597-635.

Rawlings, D.N. and Barret, A.J. 1993. Evolutionary Families of Peptidases, *Biochemical Journal*, 290: 205-218.

Revee, N.J. 1999. Archaebacteria Then...Archaeas Now (Are There Really No Archael Pathogens?), *Journal of Bactreology,* 181: 3613-3617.

Rivett, A.J. 1993. Proteasomes: Multicatalytic Proteinase Complexes, *Biochemical Journal*, 291: 1-10.

Robb, F.T. and Place, A.R. 1995. Archaea, A Laboratory Manual, Eds. Cold Spring Harbor, Laboratory press, NY, USA.

Rock, K.L and Goldberg, A.L. 1999. Degradation of Cell Proteins and the Generation of MHC class I-presented Peptides, *Annual Review of Immunology*, 17: 739-779.

Sahoo, D.K., Jasvir, S., Gill, N., Devasahayam, G. 1999. Studies on Alkaline Protease Produced by *Bacillus* sp. NG312, *Applied Biochemistry and Biotechnology*, 76: 57-63.

Sambrook, J., Fritsch, E.F., Maniatis T. 2001. Molecular Cloning: a Laboratory Manual 3rd Edition Cold Springer Harbor Laboratory Press, Cold Springer Harbor, New York, USA

Schmidt M., Schmidtke, G., Kloetzel, P. 1997. Structure and Structure Formation of the 20S Proteasome, *Molecular Biology Reports*, 24:103-112.

Seemuller, E., Lupas, A., Zuhl, F., Zwickl, P., Baumeister, W. 1995a. The Proteasome from *Thermoplasma acidophilum* is Neither a Cysteine nor a Serine Protease, *FEBS Letter*, 359: 173-178.

Seemuller, E., Lupas, A., Stock, D., Lowe, J., Huber, R., Baumeister, W. 1995b. Proteasome from *Thermoplasma acidophilum*: A Threonine Protease, *Science,* 268: 579-582.

Segerer, A., Longworthy, T.A., Stetter, K.O., 1988. *Thermplasma acidophilum* and *Thermoplasma volcanium* sp. nov. from Solfatara Fields, *Systematic Applied Microbiology*, 10: 161-171.

Shaw, A., Bott, R., Vonrhein, C., Bricogne, G., Power, S., Day, A.G. 2002. A Novel Combination of Two Classic Catalytic Schemes, *Journal of Molecular Biology*, 230: 303-309.

Singh, J. Vohra, R.M., Sahoo, D.K. 2001. Purification and Characterization of two Extracellular Alkaline Proteases from a Newly Isolated Obligate Alkalophilic *Bacillus sphaericus*, *Journal of Industrial Microbioloogy*, 26: 387-393.

Singh, J., Vohra, R.M., Sahoo, D.K. 1999. Alkaline Protease from a New Obligate Alkalophilic Isolate of *Bacillus sphaericus*, *Biotechnology Letter*, 21:921-924.

Singh, J., Batra, N. Sobti, R.C., 2001. Serine Akaline Protease from a Newly Isolated *Bacillus* sp. SSR1, *Process Biochemistry 2001,* 36:781-785.

Spaltenstein, A., Leban, J.J., Huang, J.J., Reinhadt, K.R., Viveros, O.H., Sigafoos, J., Crouch, R. 1996. Design and Synhesis of Novel Protease Inhibitors. Tripeptide α',β'-epoxyketones as Nanomolar Inactivators of the Proteasome, *Biochemistry*, 35: 1343-1346.

Stalh, M.L. ang Ferrari, E. 1984. Replacement of the *Bacillus subtilis* Subtilisin Structural Gene with an In Vitro-Derived Deletion Mutation, *Journal of Bacteriology*, 158 (2): 411-418.

Stan-Lotter, H., Doppler, E., Jarosch, M., Radax, C., Gruber, C., Inatomi, K. 1999. Isolation of a Chymotrypsinogen B-like enzyme from the Archaeon *Natromomonas pharaonis* and other Halobactreia, *Extromophiles*, 3: 153-161.

Stetter, K.O. 1996. Hyperthermophilic Procaryotes, *FEMS Microbiolog Review*, 18: 149-158.

Stolzing, A. T.and Grune, T. 2001. The Proteasome and Its Function in the Ageing Process, *Clinical and Experimental Dermatology*, 26: 566-572.

Strongin, a. Y., Izotova, S.L., Abramov, Z.T., Gorodetsky, D.I., Ermakova, L.M., Baratova, I.A., Belyanova, L.P., Stepanov, V.M. 1978. Intracellular Serine Protease of *Bacillus subtilis*: Sequence Homology with Extracellular Subtilisins, *Journal of Bacteriology*, 133 (3): 1401-1411.

Studdert, C.A., Seitz, M.K.H., Gil, M.I.P., Sanchez, J.J., De Castro, R.E. 2001. Purification and Biochemical characterization of the Haloalkaliphilic Archaeon *Natronococcus occulltus* Extracellular Serine Protease, *Journal of Basic Microbiology*, 41 (6): 375-383.

Sutherland, K.J., Henneke, C.M., Towner, P., Hough, D.W., Danson, M.J. 1990. Citrate synthase from the Thermohilic an Archaeabacterium *Thermoplasma acidophilum* Cloning and Sequencing of the Gene, *European Journal of Biochemistry*, 194: 839-844.

Tamura, T., Nagy, A., Lupas, F., Lottspeich, F., Cejka, Z., Schoofs, G., Tanaka, K., DeMot, R., Baumeister, W. 1995. The First Chracterization of a Eubacterial Proteasome: the 20S Complex of *Rhodococcus*, *Current Biology*, 5: 766-774.

Tanaka K. 1998. Molecular Biology of the Proteasome, *Biochemical and Biophysical Research Communication*, 247: 537-541.

Tanaka K., and Tsurumi, C. 1997. The 26S Proteasome: Subunits and Fuctions, *Molecular Biology Reports*, 24: 3-11.

Tanahashi, N., Murakami, Y., Minami, Y., Shimbara, N., Hendil, K.B., Tanaka, K. 2000. Hybrid Proteasomes, *Journal of Biological Chemistry*, 275: 14336-14345.

Tanahashi N., Kawahara H., Murakami Y., Tanaka K. 1999. The Proteasome-Dependent Proteolytic System, *Molecular Biology Reports*, 26: 3-9.

Terada, I., Kwon, S., Miyata, Y., Matsuzawa, H., Ohta, T.1990. Unique Precursor Structure of an Extracellular Protease, Aqualysin I with NH2- and COOH-terminal Pro-sequences and Its Processing in *Escherichia coli*, *The Journal of Biological Chemistry***,** 265: 6576-6581.

Thangram, E.B. and Rajkumar, G.S. 2002. Purification of Alkaline Protease from *Alcaligenes faecalis*, *Biotechnology and Applied Biochemistry*, 35:149-154.

Tsuchiya, K., Ikeda, I., Tsuchiya, T., Kimura, T. 1997. Cloning and Expression of an Intracellular Alkaline Protease Gene from Alkalophilic *Thermoactinomyces* sp. HS682, *Bioscience Biotechnology and Biochemistry*, 61: 298-303.

Ulric H.D. 2002. Natural Substrates of the Proteasome of the Proteasome and Their Recognition by the Ubiquitin System, *Current Topics in Microbiology and Immunology*, 268: 137-174.

Vasanta, N., Thompson, L.D., Rhodes, C., Baner, C., Nagle, J., Filpula, D. 1984. Genes for Alkaline Protease and Neutral Proteases from *Bacillus amyloliquefaciens* Contain a large Open Reading Frame Between the Regions Coding for Signal Sequence and mature Protein, *Journal of Bacteriology*, 159 (3): 811-819.

Vieille, C. And Zeius, J.G. 1996. Thermozymes: Identifying Molecular Determinants of Protein Structural and Functional Stability, *Trends in* T*echnology,* 16: 183-190.

Voorhorst, G.B., Eggen, R.I.L., Geerling, A.C.M., Platteeuw, C., Siezen, R.J., Vo, W.M. 1996. Isolation and Characterization of the Hyperthermostable Serine Protease, Pyrolysin, and Its Gene from the Hyperthermophilic Archaeon *Pyrococcus furiosus*, *The Journal of Biological Chemistry*, 271 (34): 20426-20431.

Yamagata, Y., Abe., Fugita., Ichishimata, E. 1995. Molecular Cloning and Nucleotide-sequence of the 90 kDa Serine Protease gene, hspK from *Bacillus subtilis* (natto) No 16, *Current Microbiology*, 31: 340-344.

Young-OK, K., Lee, J., Sunitha, K., Kim, H., Oh, T. 1999. Minor Thermostable Alkaline Protease Produced by *Thermoactinomyces sp.* E79*, Microbiology and Biotechnology,* 9: 469-474.

Yoshimato, T., Oyama, H., Honda, T., Tone, H., Takeshita, T., Kamiyama, T., Tsuru, D. 1988. Cloning and Expression of *Subtilisin amylosacchariticu*s gene. *Journal of Biochemistry*, 103: 1060–1065.

Zhang, Z., Kritchinsky, A., Endicott, S., Realini, C., Renchsteiner, M., Standing, K.G.1999. Proteasome Activator 11S REG or PA28: Recombinant REGα/REGβ Hetero-oligomers are Heptamers. *Biochemistry*, 38: 5661-5658.

Zvauya, R. and Zvidzai, C.J. 2001. Purification of a Protease from an Alkalophilic *Bacillus Subtilis* CHZ1 Isolated From a Zimbabwean Hot Spring, *Journal of Food Biochemistry,* 25:1-13.

Zwickle, P., Grziwa, A., Puhler, G., Dahlmann, B., Lottspeich, F., Baumeister, W. 1992a. A Primary Structure of the Thermoplasma Proteasome and Its Implications for the Structure, Function, and Evolution of the Multicatalytic Proteinase, *Biochemistry,* 31: 964-972.

Zwickle, P., Lottspeich, F., Baumeister, W. 1992b. Expression of Functional *Thermoplasma acidophilum* in *Eschericia coli*, *Federation of Europian Biochemical Societies*, 312: 157-160.

Zwickl, P., Ng, D., Woo, K.M., Klenk, H., Goldberg, A.L. 1999. An Archeabacterial ATPase, Homologous to ATPase in the Eukayotic 26S Proteasome, Activates Protein Breakdown by 20S Proteasomes, *The Journal of Biological Chemistry*, 274:26008-26014.

Walker, B and Lynes, J.F. 2001. Strategies for the Inhibition of Serine Proteases, *Cellular and Molecular Life Sciences*, 58: 596-624.

Walsh, K.A. and Wicox, P.E. 1970. Proteolytic Enzymes, Methods in Enzymology Vol XIX, p. 31.

Ward, D.E., Shockley, K.R., Chang, L.S., Levy, R.D., Michel, J.K., Conners, S.B., Kelly, R.M. 2002. Proteolysis in Hyperthermophilic Microorganisms, *Archaea*, 1: 63-74.

Wilson, H.L., Aldrich, H.C., Maupin-Furlow, J. 1999. Halophilic 20S Proteasomes of the Archaeon *Haloferax volcanii*: Purification, Characterization, and Gene Sequence Analysis, *Journal of Bacteriology*, 181: 5814-5824.

Wilson, H.L., OU, M.S., Aldrich, H.C., Maupin-Furlow, J. 2000. Biochemical and Physical Properties of the *Methanococcus jannaschii* 20S Proteasome and PAN, a Homolog of the ATPase (Rpt) Subunits of the Eukaryal 26S Proteasome, *Journal of Bacteriology*, 182: 1680-1692.

Whitaker, J.R. and Granum, P.E., 1980. An Absolute Method for Protein Determination Based on Difference in Absorbance at 235 and 280 nm, *Analytical Biochemistry*, 109: 156-159.

Woese, C.R., Kandler, O., Wheelis M.L. 1990. Towards a Natural System of Organisms: Proposal for the Domains Archaea, Bacteria, and Eukarya, *Proceedings National Academic Science*, 87: 4576-4579.

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

APPENDIX A

BUFFERS AND SOLUTIONS

TE Buffer pH 8.0

10 mM	TrisHCl
1 mM	EDTA

TAE Buffer pH 8.0

0.04 M	Tris acetate
0.001 M	EDTA

Buffer B pH 5.5

3 M	$NaCH_3COO$

Gel Loading Buffer (6X)

0.25%	Bromophenol Blue
40%	Sucrose

TrisHCl with Sucrose pH 8.0

50 mM	Tris
25%	Sucrose

2X Sample Buffer (1 Lt)

50 mM	Tris-HCL pH 6.8
10% (v/v)	Glycerol
1% (w/v)	SDS
0.01% (m/v)	BPB

Running Buffer (500 ml)

1.51 g	Tris
7.2 g	Glycine
0.5 g	SDS

Zymogram Development Solution (1 Lt)

100 ml	0.5 M	Tris-HCl pH 7.6
100 ml	50 mM	$CaCl_2$
800 ml		sd H_20

2.5% Triton X-100

5 ml TritonX-100 complete to 200 ml with sdH_2O.

Coomassie Blue Staining Solution (1 Lt)

1 g	Coomassie 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 Aceticacid
800 ml	dH_2O

Protease Assay Buffer pH 7.5 (1 Lt)

50 mM	Tris-HCl
2 mM	$CaCl_2$

Buffer A pH 8.0 (1 Lt)

20 mM	TrisHCl
2 mM	$CaCl_2$

Buffer B pH 8.0 (1 Lt)

20 mM	TrisHCl
2 mM	CaCl2
1 M	NaCl

Gel Permeation Chromatography Buffer pH 7.5 (1 Lt)

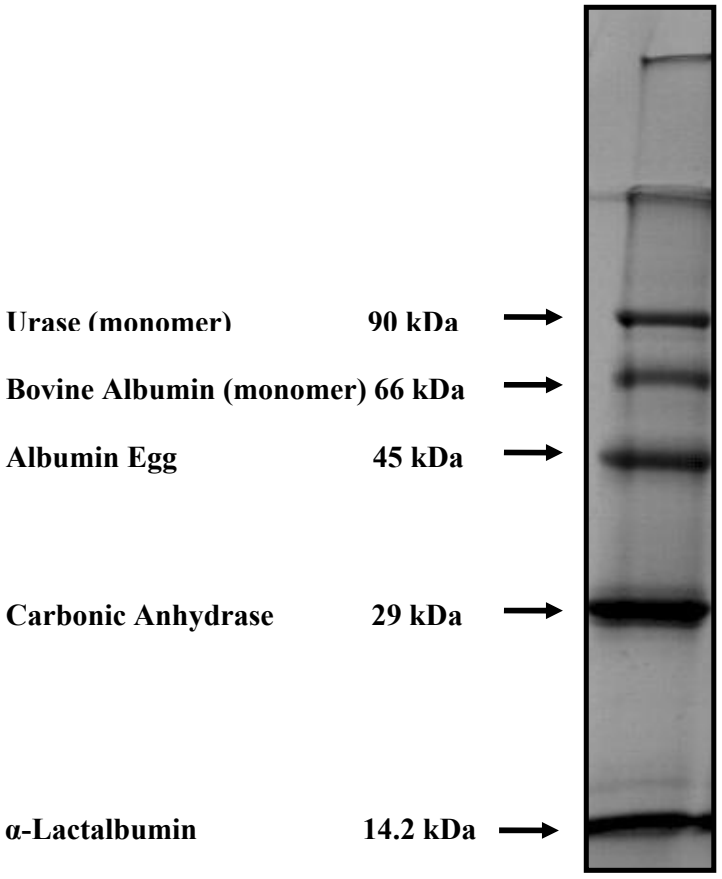
20 mM	Tris HCL
1 mM	EDTA
0.15 M	NaCl

Proteosome Ion Exchange Chromatography Buffer pH.8.0 (1 Lt)

20 mM	TrisHCL

APPENDIX B

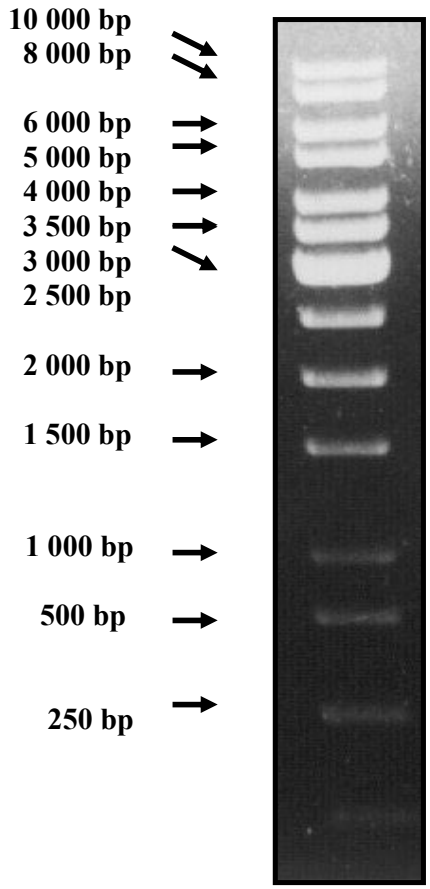
PROTEIN MIX MOLECULAR SIZE MARKER



Protein molecular size marker, Sigma Chemical Company, Saint Louis, Missouri, U.S.A.).

APPENDIX C

DNA 1 KB MOLECULAR SIZE MARKER



Gene ruler 1 kB DNA ladder, MBI, Fermentas, Vilnius, Lithunia.

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Publications

Kocabıyık, S. and Özdemir, İ., Differential Amplification of 16S rDNA Sequences of *Archaea* and *Bacteria* from Solfataric Hot-Springs in Turkey. (Poster) 18th IUBMB Congress, 16-20 July, 2000-11-09 Birmingham, UK. Abstract Book: Abstract No.550

Kocabıyık, S. and Özdemir, İ., Isolation of Thermophilic *Archaea* and *Bacteria* from Natural Hot-Springs in Turkey and Cloning of an *Archaea* Originated Serine Protease Gene in *E.coli*.(Presentation) Turkey Agricultural Research Project Symposium, Abstract Book, p.16-17. TÜBİTAK, Ankara, Turkey, October 26-27, 2000.

Özdemir, İ. and Kocabıyık, S., Purification and Characterization of a Thermostable Serine Protease from *Thermoplasma volcanium*. (Poster) 27th Meeting of the Federation of European Biochemical Societies. Abstract Book, p.193, Lisbon, Portugal, June 30-July 5, 2001.

Kocabıyık, S. and Özdemir, 20S Proteasome Complex from *Thermoplasma volcanium*. (Poster) 27th Meeting of the Federation of European Biochemical Societies, Abstract Book, p.199, Lisbon, Portugal. June 30-July 5, 2001.

İ. Özdemir and S. Kocabıyık. Isolation and Characterization of 20S Proteasome from *Thermoplasma volcanium*. (Poster) 17th National Biochemistry Congress, 24-27 Ankara, Turkey. Abstract Book, P-136, June, 2002.

G. Seyit, İ. Özdemir and S. Kocabıyık. Isolation, Purification and Cloning of Alkaline Serine Proteases from Thermophilic Archaeon, *Thermoplasma volcanium*. (Poster) 17th National Biochemistry Congress, 24-27 June, 2002, Ankara, Turkey. Abstract Book, S-27.

H. Özel, İ. Özdemir, S. Kocabıyık. Cloning and Functional Analysis of Protease Genes from *Thermoplasma volcanium* (Poster) XIXth International Congress of Genetics, 6-11 July, 2003, Melbourne, Australia.

H. Özel, İ. Özdemir, S. Özdoğan, Ç. Cekiç, S. Kocabıyık. Functional Studies on Different Proteases of *Thermoplasma volcanium*. HUPO 2nd Annual&IUBMB XIX Joint World Congress, October, 8-11, 2003.