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Purification and Characterization of an Intracellular Chymotrypsin-Like Serine Protease from *Thermoplasma volcanium*

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An intracellular serine protease produced by *Thermoplasma (Tp.) volcanium* was purified using a combination of ammonium sulfate fractionation, ion exchange, and α-casein agarose affinity chromatography. This enzyme exhibited the highest activity and stability at pH 7.0, and at 50 °C. The purifed enzyme hydrolyzed synthetic peptides preferentially at the carboxy terminus of phenylalanine or leucine and was almost completely inhibited by PMSF, TPCK, and chymostatin, similarly to a chymotrypsin-like serine protease. Kinetic analysis of the *Tp. volcanium* protease reaction performed using *N*-succinyl-L-phenylalanine-*p*-nitroanilide as substrate revealed a K_m value of 2.2 mM and a V_{max} value of 0.045 $\mu mol^{-1} ml^{-1} min^{-1}$. Peptide hydrolyzing activity was enhanced by >2-fold in the presence of Ca^{2+} and Mg^{2+} at 2–12 mM concentration. The serine protease is a monomer with a molecular weight of 42 kDa as estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and zymogram activity staining.

Key words: *Thermoplasma volcanium*; serine protease; chymotrpsin-like protease; *Archaea*; thermophilic

Serine proteases (SPs) are a large class of proteolytic enzymes that have a reactive serine residue (nucleophile), as a part of catalytic triad, in the active center to hydrolyze peptide bonds. SPs execute a diverse array of functions including primary metabolic and regulatory events that extend from the normal physiology of the cells to abnormal patho-physiological conditions, which are most often implicated in protein turn-over, digestion, fibrinolysis, blood coagulation, sporulation, fertilization, immune response, homeostasis, and pathogenesis. Their importance in conducting these essential functions is evident from the ubiquitous occurrence of SPs in all three forms of living organisms, as reviewed in detail by Kalisz,[1] Nduwimana *et al.*,[2] and Rao *et al.*[3] On the other hand, the SP family constitutes an important enzymatic group of biotechnological relevance world wide, and has been used extensively in the detergent, leather, and pharmaceutical industries, and in food and bioremediation processes.[4] Presently, due to their critical position in the physiological and industrial fields, there is growing interest among researchers in the identification and molecular characterization of SP family enzymes from diverse sources, including extremophiles. Several thermophilic and hyperthermophilic *archaea* produce significant levels of intra- and extracellular proteolytic enzymes with high intrinsic molecular stability. Most of these enzymes, which are recognized as the serine type, have been detected in the genera *Pyrococcus* (Pyrolysin and PfpI),[5,6] *Desulfurococcus* (Archaelysin),[7] *Sulfolobus*,[8–10] and *Thermococcus*.[11–13] Genome sequence data revealed even more expansive proteolytic genotypes of these organisms than can be inferred from biochemical analyses.[14] A majority of these organisms are capable of growth on proteinaceous substrates.[15,16] Therefore, the SP enzyme family in *archaea* might be involved in generating a pool of intracellular peptides and amino acids. But the role of proteolysis in the metabolisms of thermophilic *archaea* and the mechanisms through which peptide-based substrates are utilized are less clear, and this is a challenge to continued research from fundamental perspectives. In this study, for the first time we report the purification and biochemical characterization of an intracellular SP from the thermoacidophilic archaeon *Thermoplasma volcanium* GSS1 (DSM 4299). This organism was originally isolated from submarine and continental solfataras at Vulcano Island in Italy.[17] *Tp. volcanium*, which grows optimally at 60 °C and pH 2.0, is a facultatively anaerobic heterotroph.

Materials and Methods

Archaeal strain and growth. Thermoplasma (*Tp.*) *volcanium* GSS1 (type strain 4299) was purchased from DSMZ (Deutsche Sammlung von Microorganismen und Zellkulturen, Braunschweig, Germany). Culture of the organism was grown at 60 °C in liquid Volcanium Medium (pH 2.7),[18] supplemented with glucose (0.5%, w/v) and yeast extract (0.1%, w/v). Renewal of the culture was achieved by subculturing once a week through 1% (v/v) inoculum transfer into fresh medium.

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Preparation of cell-free extract. Tp. volcanium cells from 48 h cultures were harvested by centrifugation at 7,000 × *g* for 15 min at 4 °C. The pellet was washed in unsupplemented growth medium and resuspendend in 1/5 volume (v/v) protease assay buffer (50 mM Tris–HCl, pH 7.5, 5 mM $CaCl_2$). The cells were disrupted by sonication (Sonicator VC 100, Sonics and Materials, Hartford, CT), and the cell debris was removed by centrifugation at 15,700 × *g* for 2 h at 4 °C. The cell-free extract was stored at −20 °C until use.

Protease assays. SP activity was determined by measuring *p*-nitroaniline release from free or N-protected aminoacyl- or peptidyl-*p*-nitroanilides (Sigma, Chemical, St. Louis, MO). The assay mixture (total volume 820 µl) contained 720 µl assay buffer and 50 µl of enzyme solution. After a 5 min preincubation at 55 °C, the reaction was started by adding 50 µl of 10 mM of each oligopeptidyl-*p*-nitroanilide dissolved in dimethylsulfoxide. The absorbance of liberated *p*-nitroaniline was measured at 410 nm, continuously using a UV 1601 A spectrophotometer (Shimadzu, Kyoto, Japan) during a 5 min incubation period, at 55 °C. One unit of peptidyl-*p*-nitroanilide-hydrolysing activity was defined as the amount of enzyme that catalyzed the formation of 1 µmol of *p*-nitroaniline ($\varepsilon_{410} = 8800$ $M^{-1}\,cm^{-1}$) per min.

The caseinolytic activity of the purified enzyme was determined using casein as substrate, according to the method of Anson.[19] The reaction mixture contained 700 µl of 1% (w/v) casein solution (prepared in protease assay buffer, pH 7.5) and 100 µl of purified enzyme. The assay was performed at 55 °C in a time-dependent manner from 0 to 20 min, and terminated by the addition of 900 µl of 15% (w/v) trichloroacetic acid (TCA), which was incubated at room temperature for 15 min. The precipitate was removed by centrifugation at 15,000 × *g* for 10 min, and the supernatant was filtered through Whatman no. 1 filter paper (Whatman International, Maidstone, UK). The absorbance at 275 nm was measured. A control lacking the enzyme was included in each assay. One unit of casein hydrolyzing activity was defined as the amount of enzyme required to produce an increase in absorbance at 275 nm of 0.01 OD under the above-mentioned assay conditions. The proteolytic activity was also determined using bovine serum albumin (BSA) as substrate, following the method described by Foltman *et al.*[20] The enzyme reaction was carried out in a mixture containing 1 ml of 1% (w/v) BSA, prepared in protease assay buffer, pH 7.5, and 100 µl enzyme solution. The assay was performed at 55 °C in a time-dependent manner from 0 to 60 min, and the reaction was terminated by the addition of 3 ml of 15% (w/v) TCA. The resulting precipitate was removed by centrifugation at 3,000 × *g* for 20 min. The absorbance was read at 280 nm using a Shimadzu UV-1601A spectrophotometer. One unit (U) of enzyme activity was defined as the amount of enzyme required to produce an increase in A_{280} equal to 0.01 OD under the above-mentioned assay conditions.

Purification of serine protease by column chromatography. Solid ammonium sulfate was added to the culture supernatant to 80% (w/v) saturation and kept at 4 °C overnight through gentle stirring. The precipitate was collected by centrifugation at 11,000 × *g* for 30 min, followed by dissolution in 1/20 volume (v/v) protease assay buffer, pH 7.5. The crude enzyme in this fraction was filtered through a 0.45 µm Millipore, millex HV filter (Billercia, Boston, MA), and then desalted and concentrated by ultrafiltration using a 5,000 cut-off membrane filter (Vivascience, Sartarious Group, Gloucestershire, UK). After exclusion of ammonium sulfate, the enzyme sample was loaded to an Econo Pac Q ion exchange cartridge (4 × 1.5 cm) (BioRad, Richmond, VA), which was previously equilibrated with the protease assay buffer. The column was washed with 50 ml of the same buffer, and elution was done with 100 ml of a salt gradient of 0–1.0 M NaCl in the same buffer at a flow rate of 2 ml·min^{-1}. Fractions containing activity towards *N*-succinyl L-phenylalanine-*p*-nitroanilide (*N*-Suc-Phe-pNA) were pooled, desalted, and concentrated by ultrafiltration (5,000 cut-off ultrafiltration device). Affinity chromatography was performed on α-casein agarose column (5 × 1.2 cm) (Sigma), which was equilibrated with 20 mM Tris–HCl, pH 8.5, containing 2 mM $CaCl_2$ (Buffer A). Desalted and concentrated active fractions from the previous step were applied to the column and washed with excess Buffer A. The bound protein was eluted from the column in two steps using assay Buffer A plus 1 M NaCl (Buffer B), followed by 25% (v/v) isopropanol in Buffer B, each at a flow rate of 0.6 ml·min^{-1}. Absorbance at 280 nm and protease activity of the fractions (each 1 ml) were determined. Active fractions were pooled and concentrated by ultrafiltration using Centriprep-Microconcentrators equipped with a 5,000 cut-off membrane, and stored in small aliquots at −20 °C until use.

The amount of protein in the samples was calculated using the method described by Whitaker and Granum,[21] according to the following equation: protein concentration (mg/ml) = $(A_{235} - A_{280})/2.51$.

Effect of temperature and pH on enzyme activity. The optimum temperature for the enzyme activity was determined by assaying peptidolytic activity at different temperatures over a range from 40 to 80 °C. The assays were performed using *N*-Suc-Phe-pNA as substrate, and residual activity was expressed as a percentage of the highest enzyme activity measured.

To determine the effect of pH on purified protease, peptidolytic activity was measured at different pH values, using *N*-Suc-Phe-pNA as substrate under standard assay conditions. The pH was adjusted using one of the following buffers (50 mM): 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 6.0–7.0), Tris–HCl

(pH 7.5–9.0), and glycine (pH 9.5–12.0), each containing 2 mM $CaCl_2$. The residual activity was expressed as a percentage of the highest enzyme activity. The pH stability of protease enzyme was determined by preincubating the purified protease at 25 °C in different buffers (pH 6.0–12.0, 50 mM buffers as above) for 1 h. The residual protease activities were then measured as described above.

Effect of protease inhibitors and metal ions on enzyme activity. The effects of various protease inhibitors, such as phenylmethyl sulphonyl fluoride (PMSF), chymostatin and tosyl-L-phenylalanyl-chloromethane (TPCK), ethylenediamine tetraacetic acid (EDTA), ethylene glycol bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and reducing agent, 1,4 dithiothreitol (DTT) (all from Sigma) on the peptide hydrolyzing activity of the purified protease were assessed. The metal ions tested were Ca^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Co^{2+} (in chloride salts) at 4 mM concentration. The protease enzyme (5 µg) was incubated with inhibitors and metal ions for 15 min at room temperature. The residual activity was determined as described before, by measuring the *N*-Suc-Phe-pNA hydrolyzing activity, and expressed as the percentage of the activity measured in the absence of inhibitor and metal ion. The effects of Ca^{2+} and Mg^{2+} ions at different concentrations (in a range 0.5–12.5 mM) on the protease activity of the enzyme were also determined, as described above.

Kinetic measurements. Initial velocity assays were performed by continuous spectrophotometric assay using *N*-Suc-Phe-pNA as substrate. The K_m and V_{max} values of the purified protease were determined from a Lineweaver–Burk plot generated from the initial reaction velocities obtained with substrate concentration in a range of 0.12–1.2 mM. Each assay was carried out in duplicate at 55 °C in protease assay buffer, pH 7.5. The change in absorbance at 410 nm was monitored continuously, and the initial velocity was used for calculation of the kinetic constants.

Electrophoretic analysis. For activity detection by zymogram staining, purified samples were electrophoresed in zymogram-resolving gel containing 7.5% acrylamide, 0.1% sodium dodecly sulfate (SDS), and 0.15% (w/v) co-polymerized gelatin, as described by Makowski and Rampsy.[22] After electrophoresis, the gels were washed twice in 2.5% TritonX-100 solution for 30 min to remove SDS. The proteolytic reaction was carried out in zymogram development solution containing 45% methanol, 10% acetic acid, and 0.1% Coomassie Brilliant Blue R-250. Clear zones against a blue background were observed after several washings in the destaining solution (40% v/v methanol, 10% v/v acetic acid, and 50% distilled water). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out on 10% (w/v) acrylamide gel containing 0.5% (w/v) SDS, as described by Laemmli.[23]

Results and Discussion

Purification of serine protease

The purification steps of the serine protease from *Tp. volcanium* are summarized in Table 1. The crude extract (27 ml) from 1 liter culture was subjected to 80% ammonium sulfate precipitation. The precipitated enzyme sample was dissolved in protease assay buffer and after desalting, applied to a column of anion exchanger. Elution by a linear gradient of NaCl yielded two distinct protein peaks (Fig. 1a). Proteolytic activity against *N*-Suc-Phe-pNA was detected in fractions 117 to 134 covering the second protein peak. In this step a purification fold of 3.6 with a yield of 27.5% and a specific activity of 0.0039 U/mg were achieved.

As shown in Fig. 1b, substrate affinity chromatography on α-casein agarose, the enzyme was efficiently separated from other proteins. This step increased the specific activity to 0.0065 U/mg. The overall purification was approximately 6-fold and the yield was 24% (Table 1). A single distinct peak of protease was eluted from the column with 1 M NaCl only in the presence, not in the absence, of isopropanol. Therefore, like some *Bacillus* serine proteases,[24,25] the binding of *Tp. volcanium* protease to α-casein agarose must be due to a hydrophobic affinity interaction. This phenomenon correlates well with findings from the substrate specificity study, which indicated that the enzyme attacks hydrophobic amino acids residues such as phenylalanine and alanine of various oligopeptidyl *p*-nitroanilide substrates (Table 2).

Enzyme purified to homogeneity by two sequential chromatographies migrated as a single band on SDS–PAGE with an apparent molecular mass of 42 kDa

Table 1. Summary of Purification of Intracellular Serine Protease from *Tp. volcanium*

Purification step	Total protein (mg)	Specific activity $\times 10^{-3}$ (U/mg protein)	Total activity $\times 10^{-3}$ (U)	Yield (%)	Purification (fold)
Crude extract	133.55	1.084	136.62	100.00	1.0
$(NH_4)_2SO_4$ precipitation	18.98	2.014	37.62	27.54	1.9
Ion exchange chromatography	9.72	3.865	37.55	27.48	3.6
Affinity chromatography	5.50	6.450	35.50	23.70	6.0

Unit (U), The amount of enzyme that liberated 1 µmol of *p*-nitroaniline per min.

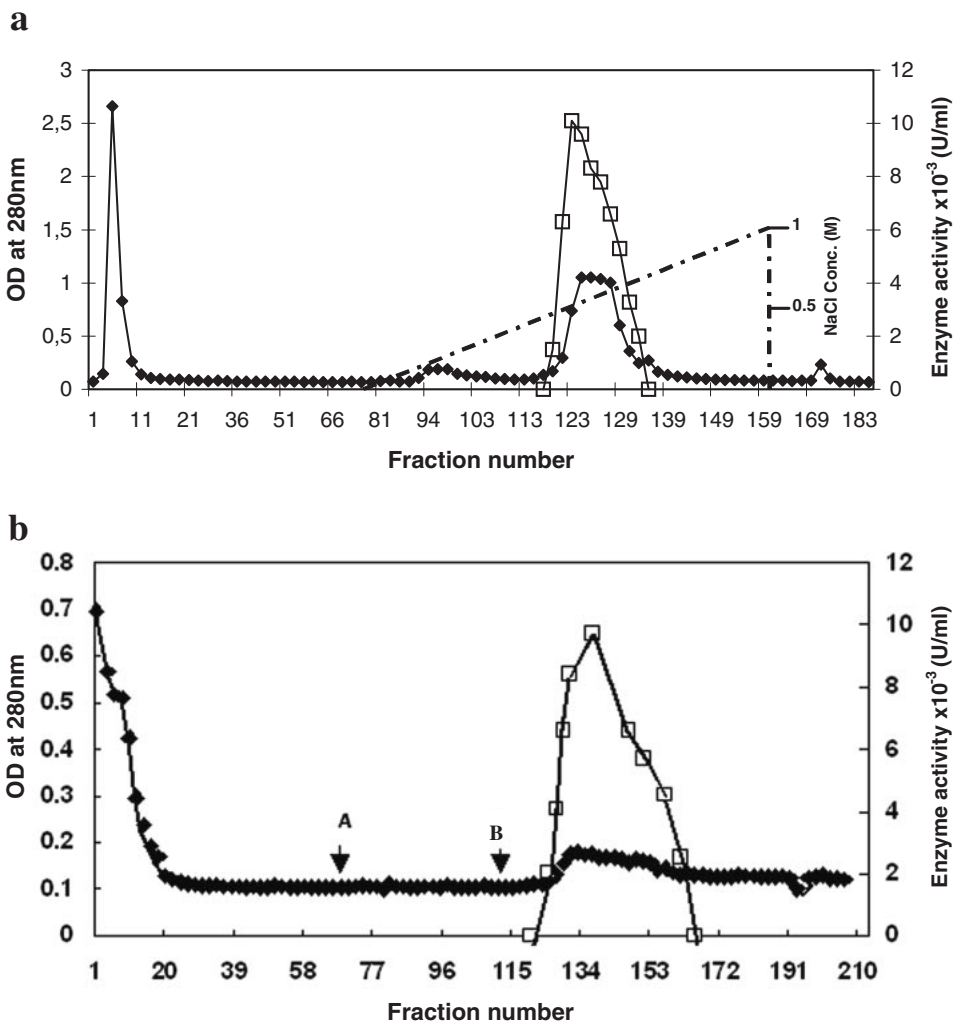

Fig. 1. Purification of the Serine Protease from. *Tp. volcanium.*

(a) Anion exchange chromatography on Econo Pack Q ion exchange column; (b) Affinity chromatography on α-casein agarose column. Arrow A, application of 20 mM Tris·HCl (pH 8.5)-2 mM $CaCl_2$ containing 1 M NaCl; Arrow B, the same buffer with the addition of 25% isopropanol. Symbols: ◆, absorbance at 280 nm; □, proteolytic activity based on *N*-succinyl-L-phenylalanine-*p*-nitroanilide hydrolysis: –·–·–, NaCl. U, the amount of enzyme that catalyzed the formation of 1 µmol of *p*-nitroaniline per min.

(Fig. 2a). Zymogram activity staining also revealed one clear zone of proteolytic activity against a blue background (Fig. 2b). These gel analyses of the purified enzyme indicate that it is a monomer. The molecular mass of the purified protease as estimated by the native gel was 35 kDa, smaller than that determined by SDS–PAGE. This might be due to more compact structure of the native protease, which has been proposed as a means of thermostabilization for some thermophilic proteases through tight packing of hydrophobic residues at the core of the molecule.[26)]

Although the zymogram gel contained 0.1% SDS, the enzyme still displayed activity, indicating that the *Tp. volcanium* protease was resistant to SDS denaturation. As reported earlier, SDS resistance is a property often associated with heat-stable proteases of thermostable *archaea* and *bacteria*.[27)]

The purified enzyme proved to be extremely labile.

Table 2. Hydrolysis of Various Chromogenic Synthetic Peptides and Protein Substrates by the Purified Protease from *Tp. volcanium*

Chromogenic substrates	Relative activity (%)
N-Succinyl-Phe-*p*-nitroanilide	100
Ala-Ala-Phe *p*-nitroanilide	55
L-Leu-*p*-nitroanilide	54
N-Succinyl-Ala-Ala-Ala *p*-nitroanilide	51
N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide	38
N-Succinyl-Ala-Ala-Val *p*-nitroanilide	35
N-CBZ-Arg-*p*-nitroanilide	24
N-Succinyl-Ala-Ala-Val-Ala *p*-nitroanilide	17
Proteins	Activity
Casein	1.800 U*
BSA	0.026 U**

U* (Unit), the amount of enzyme required to produce an increase in absorbance at 275 nm of 0.01 OD under the assay conditions.
U** (Unit), the amount of enzyme required to produce an increase in A_{280} equal to 0.01 OD under the assay conditions.

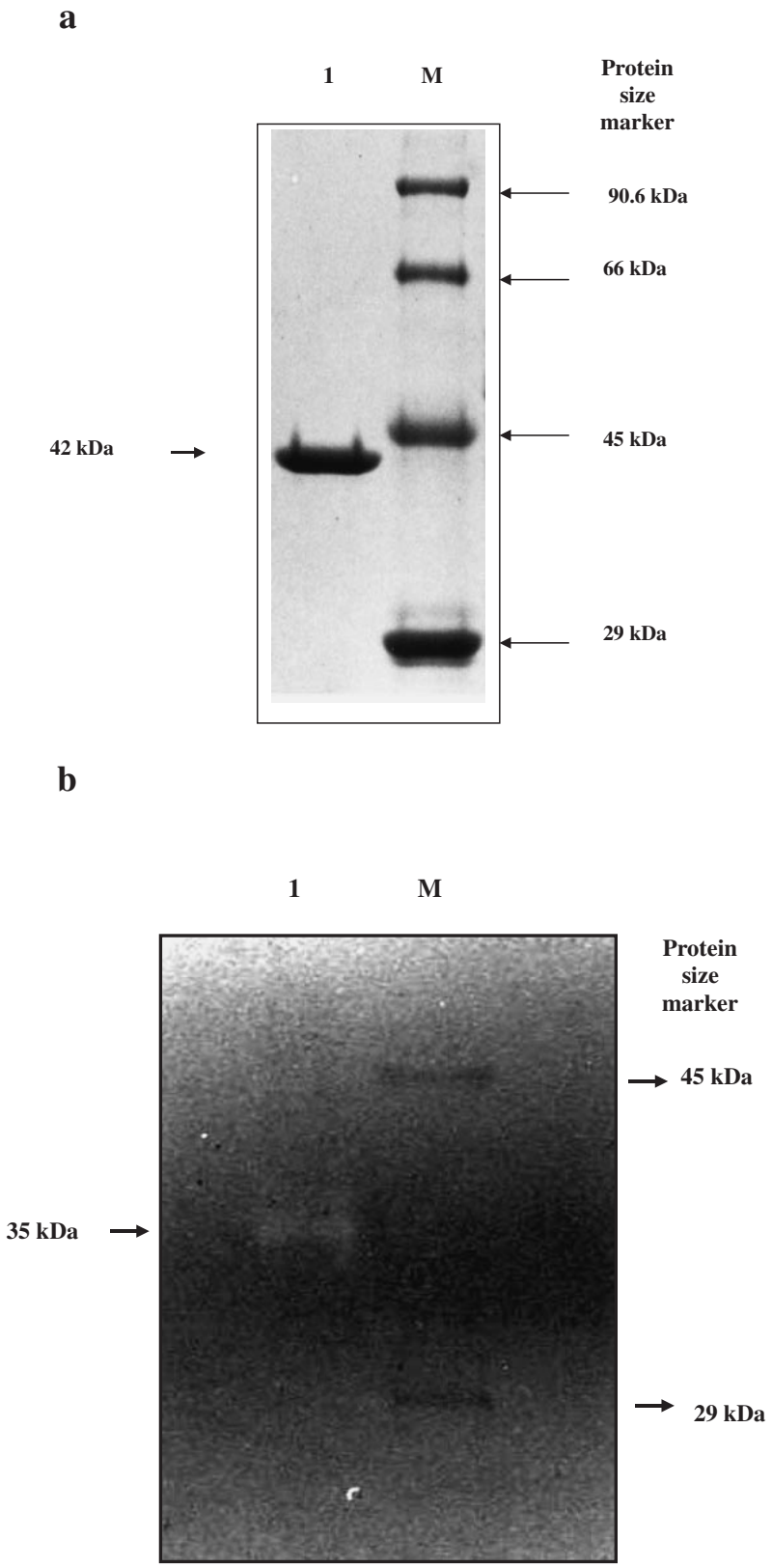

Fig. 2. Purified Serine Protease Activity of *Tp. volcanium* Visualized on SDS–PAGE (a) and Gelatin Zymogram (b).
Lane 1, purified protease; lane M, molecular weight markers: Urease (90.67 kDa), BSA (66 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa).

Activity decreased dramatically on storage on ice, even at −20 °C for 24 h, but the enzyme in crude preparation or 80% ammonium sulfate precipitate was relatively more stable. This might be due to susceptibility of the purified enzyme to autodigestion, as experienced with the other microbial SPs.[12,28,29]

When kinetic analysis of the purified *Tp. volcanium* protease reaction was performed using *N*-Suc-Phe-pNA as substrate, the enzyme showed the classical Michaelis–Menten kinetics. The following kinetic constants were obtained from the double reciprocal plots of the initial reaction rates and substrate concentrations between 0.12–1.2 mM: K_m 2.2 mM, V_{max} 0.045 μmol·ml^{-1}·min^{-1} (Fig. 3). The k_{cat} and k_{cat}/K_m values for *N*-Suc-Phe-pNA were 0.0014 s^{-1} and 6.55 M^{-1}·s^{-1} respectively.

Effects of pH and temperature on activity and stability

The optimum pH of *Tp. volcanium* protease was measured using *N*-Suc-Phe-pNA as substrate, in a series of 50 mM buffers, as explained in "Materials and Methods". The effect of pH on protease activity is shown in Fig. 4. The optimum pH of the purified protease was 7.0. The results indicate that more than 80% of the activity was retained at pHs between 6.0 and 8.0. The enzyme was quite stable in the pH range 7.0–9.5. Although *Tp. volcanium* is an thermoacidophilic archaeon, the purified protease has a neutral pH optimum and is stable over an alkaline range of pH. However, the intracellular enzymes from acidophilic and alkaliphilic microorganisms need not be adapted to extreme growth conditions, since these organisms are capable of maintaining a neutral pH internally.

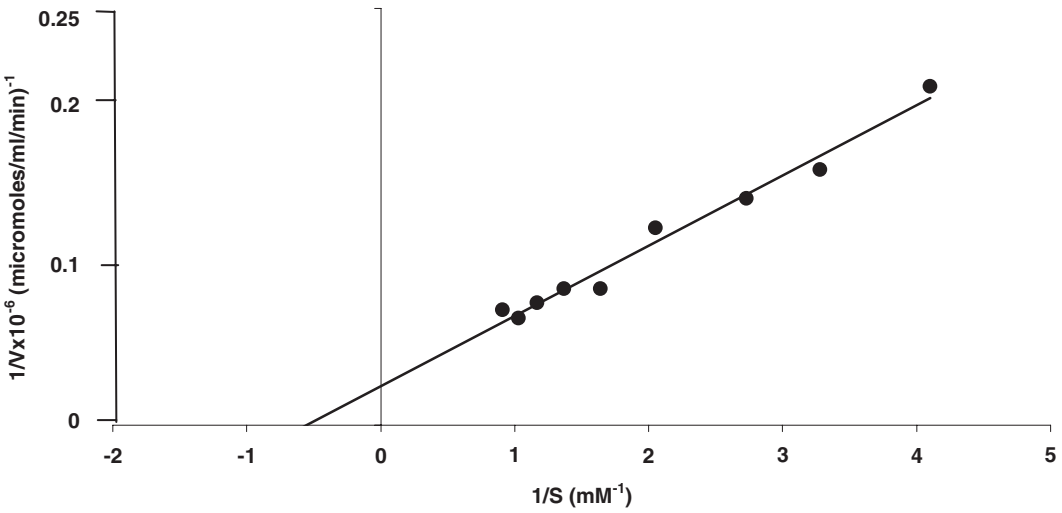

Fig. 3. Lineweaver–Burk Plot Generated from the Initial Reaction Rates Obtained with *N*-Succinyl-L-phenylalanine-*p*-nitroanilide Concentrations in the Range 0.12–1.2 mM and 5 μg of purified *Tp. volcanium* SP Per Assay.

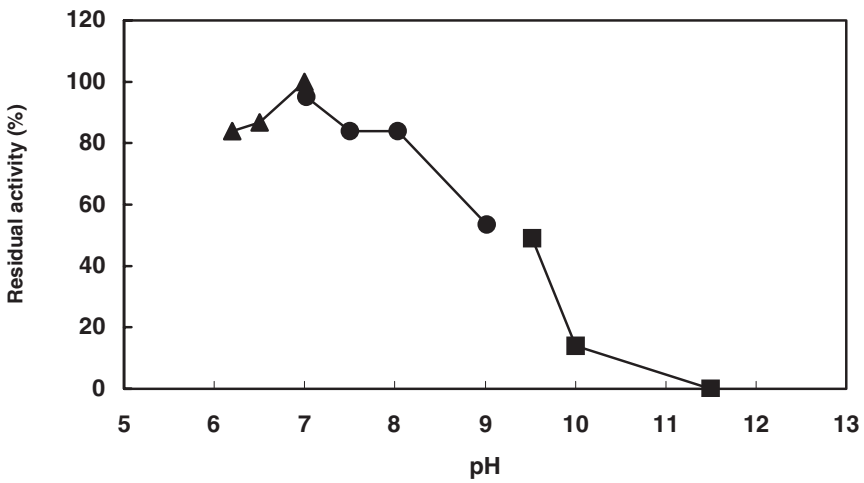

Fig. 4. Effect of pH on Proteolytic Activity of *Tp. volcanium* Serine Protease.

Activity was assayed by the hydrolysis of *N*-succinyl-L-phenylalanine-*p*-nitroanilide in 50 mM MOPS (▲), 50 mM Tris–HCl (●), and 50 mM Glycine buffer (■) under standard conditions.

The effect of temperature on protease activity is shown in Fig. 5. The optimum temperature for *N*-Suc-Phe-pNA hydrolysis was 50 °C, and activity decreased markedly below it. A significant loss (about 40%) in activity was observed at 60 °C, the temperature optimum for the growth of *Tp. volcanium*. This indicates that in thermophilic *archaea*, some form of stabilization (*e.g.*, by extrinsic stabilization factors, such as salts or polyamines) of labile enzymes must occur *in vivo* to make possible cellular functions at high temperatures.[30]

Substrate profile of the protease

A series of oligonucleotidyl-*p*-nitroanilide substrates with various P1 residues was used to test the substrate specificity of *Tp. volcanium* protease. Table 2 summarizes the relative activities of trypsin, chymotrypsin, and elastase with different substrates associated with *Tp. volcanium* protease. *Tp. volcanium* protease hydrolyzed the synthetic peptides preferentially at the carboxy terminus of Phe or Leu, similarly to chymotrypsin-like serine proteases. The enzyme was most active on *N*-Suc-Phe-pNA. Therefore, the activities were expressed relative to that of *N*-Suc-Phe-pNA. The protease displayed hydrolytic activity against other substrates, *N*-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide, Ala-Ala-Phe *p*-nitroanilide, and L-Leucine-*p*-nitroanilide, which are also specific for chymotrypsin-like activity.

On the other hand, fairly high catalytic activity was detected on substrates having Ala at the P1 position, as well. The enzyme efficiently hydrolyzed *N*-Succinyl-Ala-Ala-Ala *p*-nitro-anilide, which is a typical elastase substrate. The other elastase substrates, *N*-Succinyl-Ala-Ala-Val *p*-nitroanilide and *N*-Succinyl-Ala-Ala-Val-Ala *p*-nitroanilide, were also hydrolyzed, but with relatively low efficiency. But the enzyme was not effective in hydrolysis of the substrate with the basic residue at P1 position, and only very low tryptic activity was detected using *N*-CBZ-L-Arginine-*p*-nitroanilide. According to these results, the purified protease preferentially catalyzes the hydrolysis of substrates with non-polar amino acid residues (like L-Ala, L-Leu, and L-Phe) at the P1 site.

Our results also show that *Tp. volcanium* protease was active on natural substrates, BSA, and casein. (Table 2). The enzyme more efficiently hydrolyzed casein than BSA. The initial reaction rates ($\Delta A \cdot min^{-1}$) were 1.8 and 0.026 respectively.

Effects of inhibitors and divalent cations on protease activity

Table 3 shows the effects of various protease inhibitors and divalent cations (at 4 mM) on the activity *Tp. volcanium* protease. Enzyme activity towards *N*-Suc-Phe-pNA was strongly inhibited by the SP inhibitors PMSF, TPCK, and chymostatin. The reducing agent DTT and the Mg^{2+} chelator, EGTA had no effect on the enzyme activity, but 25% of the activity was lost in the presence of the divalent cation chelator EDTA. The fact that EDTA up to a concentration of 10 mM did not lead to complete inhibition of enzyme activity means that this enzyme is not a metalloprotease.

The presence of Ca^{2+} and Mg^{2+} in the assay mixture enhanced the peptide hydrolyzing activity by 2.6- and 2.3-fold respectively. Similarly, Mn^{2+} and Co^{2+} were also shown to have a stimulatory effect on activity, but Ni^{2+} ions exhibited an inhibitory effect, which resulted in a 27% decrease in activity. The stimulatory effects of Ca^{2+} and Mg^{2+} were concentration-dependent, and over a range of 2–12 mM the highest level of enzyme activity was observed (Fig. 6). This result show that *Tp. volcanium* SP requires Ca^{2+} ion for activity as other microbial SPs.[13,31–33] Ions chelated by subtilases at either high or

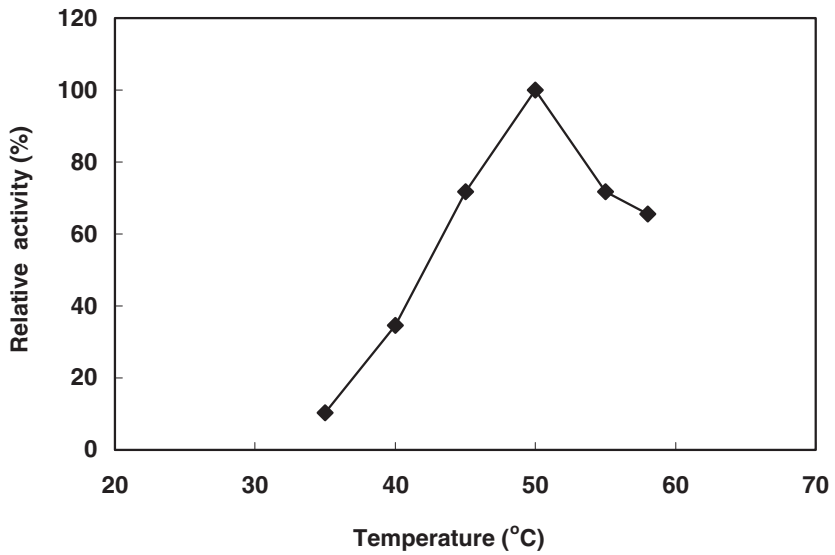

Fig. 5. Effect of Temperature on Proteolytic Activity of *Tp. volcanium* Serine Protease. Activity was based on *N*-succinyl-L-phenylalanine-*p*-nitroanilide hydrolysis.

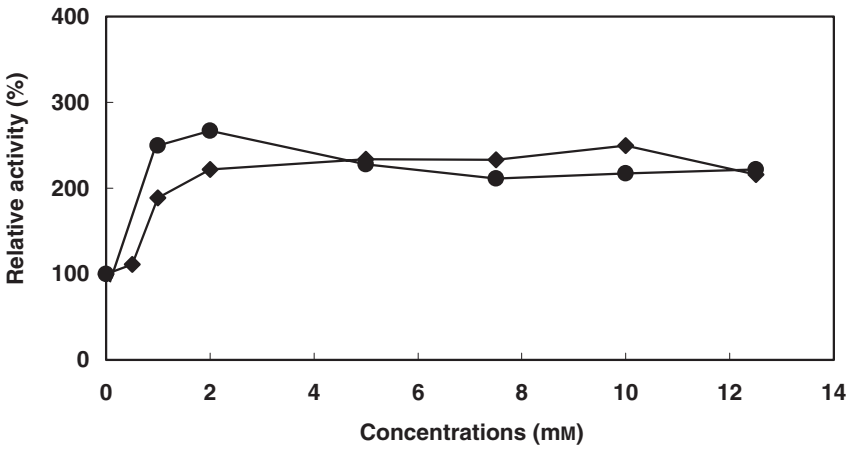

Fig. 6. The Effect Ca^{2+} (●) and Mg^{2+} (◆) Concentration on Proteolytic Activity.
The enzyme assays were carried out in the presence of different concentrations of Ca^{2+} and Mg^{2+}, and the activity in the absence of Ca^{2+} and Mg^{2+} was taken as 100%.

Table 3. Effect of Proteinase Inhibitors and Divalent Cations on the Activity of *Tp. volcanium* Serine Protease

	Concentration	Remaining activity (%)
No Inhibitor	0	100.0
Inhibitors:		
TPCK	1 mM	0
Chymostatin	400 μM	4.5
PMSF	2 mM	5.5
EDTA	10 mM	75.0
EGTA	10 mM	94.0
Reducing agent:		
DTT	20 mM	92.0
Metal Ions:		
No Ion	0	100.0
Ca^{+2}	4 mM	260.0
Mg^{+2}	4 mM	230.0
Mn^{+2}	4 mM	177.0
Co^{+2}	4 mM	167.0
Ni^{+2}	4 mM	73.0

low affinity binding sites might stabilize enzymes by reducing flexibility, acting as salt or ion bridges.[34] The activity of *Tp. volcanium* SP increased significantly when Ca^{2+} was included in the assay mixture. Therefore, in these respects, the loss of enzyme activity in the presence of EDTA seems to be a consequence of Ca^{2+}deprivation.

Thus the purified enzyme can be classified as SP of chymotrypsin-like type, judging from its strong inhibition at effective concentrations of PMSF and chymostatin, as well as its high specificity towards *N*-Suc-Phe-pNA and other substrates, which is typical of chymotrypsin-like proteases. A NCBI database search showed that the *Tp. volcanium* genome contains a number of homologous structural genes for SPs: secretory (Clp P class), membrane-bound SPs, and intracelluler SPs. Our research on the possible association of the serine protease we have isolated with predicted serine proteases of *Tp. volcanium* is still in progress.

Acknowledgments

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