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Purification and characterization of an intracellular heat-stable proteinase (pernilase) from the marine hyperthermophilic archaeon *Aeropyrum pernix* K1

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Abstract A novel intracellular serine proteinase from the marine aerobic hyperthermophilic archaeon *Aeropyrum pernix* K1 (JCM 9820) that we designated pernilase was purified by ammonium sulfate precipitation, anionic-exchange chromatography, affinity chromatography, and gel filtration chromatography. The purified enzyme was composed of a single polypeptide chain with a molecular mass of 50 kDa as determined by SDS-PAGE. The proteinase had a broad pH profile (pH 5–10) with an optimum pH of 9.0 for peptide hydrolysis. The optimum temperature for enzyme activity was 90°C. The enzyme was strongly inhibited by diisopropyl fluorophosphate (DFP) and phenylmethyl sulfonylfluoride (PMSF), suggesting that it corresponds to a serine proteinase. The enzyme was highly resistant to the reducing agents dithiothreitol and 2-mercaptoethanol but sensitive to the denaturing reagents guanidine-HCl and urea and also to the detergent sodium dodecyl sulfate (SDS). Pernilase showed high substrate specificity for Boc-Leu-Gly-Arg-MCA peptide. Thermostability of this enzyme showed half-lives of 85 min at 100°C and 12 min at 110°C.

Key words Archaeon · Marine · Hyperthermophilic · Serine proteinase · Heat stability

Introduction

Since the discovery of the first extremely thermophilic organism with a temperature maximum at 85°C (Brock et al. 1972), various extremely thermophilic microorganisms have been reported from geothermal areas, terrestrial and marine solfataric springs, hot springs, and submarine hydrothermal vents all over the world. These thermophilic microorganisms represent the upper temperature limit for life (Huber et al. 1990). The most extremely thermophilic organisms reported to date, growing at temperatures above 110°C, belong to the archaeal genera *Pyrolobus*, *Pyrodictium*, and *Methanopyrus* (Stetter 1982; Stetter et al. 1983; Huber et al. 1989; Pley et al. 1991; Kurr et al. 1991; Blochl et al. 1997).

In recent years much research effort has been directed toward the isolation and characterization of enzymes from hyperthermophilic Archaea. Interest in these enzymes has increased, both because of their biotechnological potential for novel applications (Cowan et al. 1985; Cowan 1992) and because of the need for a better understanding of their intrinsic heating and denaturing resistance properties and the stabilization mechanisms that enable hyperthermophilic Archaea to adapt to their extreme environments. The heat stabilization mechanism of extremozymes has been one of the most challenging and yet unresolved problems in biochemistry and biotechnology (Cowan et al. 1985; Adams et al. 1995; Cowan 1995).

A number of hyperthermophilic Archaea have been found to produce heat-stable enzymes (Stetter 1988). These include amylolytic, cellulytic, and proteolytic enzymes (Bragger et al. 1989; Koch et al. 1990, 1991; Klingeberg et al. 1991; Laderman et al. 1993). Heat-stable proteinases have been detected from the genera *Pyrococcus* (Eggen et al. 1990; Connaris et al. 1991; Snowden et al. 1992; Morikawa et al. 1994; Vooshorst et al. 1996; Halio et al. 1997), *Thermococcus* (Klingeberg et al. 1991, 1995), and *Desulfurococcus* (Cowan et al. 1987; Hanzawa et al. 1996). All these proteinases are produced in anaerobic conditions, and they share characteristics of high thermoactivity and high thermostability at temperatures near 100°C, as well as high resistance to detergents and denaturing agents.

Aeropyrum pernix K1 is the first reported strictly aerobic hyperthermophilic archaeon that grows optimally above 90°C. This archaeon is a heterotrophic microorganism that utilizes proteinaceous complex compounds such as tryptone, yeast extract, and trypticase as nutrients during

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aerobic respiration. These proteinaceous complex compounds supply most of the carbon, energy, and nitrogen demand (Sako et al. 1996). We have previously reported that this strain produces one of the most heat-stable extracellular metalloproteinases reported to date (Sako et al. 1997). *Aeropyrum pernix* K1 would certainly appear to be an excellent source for enzymes that are stable at temperatures above 100°C. Research toward the possible biotechnological applications is still in its infancy.

In this study we report on the purification and characterization of an intracellular heat-stable serine proteinase from the aerobic hyperthermophilic archaeon *Aeropyrum pernix* K1. This proteinase has been designated as pernilase, derived from the prefix "pernix" (Latin, adj., nimble) and the suffix "ase," a general term for enzymes.

Materials and methods

Strain and growth conditions

The strain used in this study is *Aeropyrum pernix* K1 (Japanese Collection of Microorganisms; JCM 9820) previously isolated by Sako et al. (1996) from a coastal solfataric vent located in Kodakara Island, Kagoshima, Japan. Cells were grown under aerobic cultivation at 90°C as described by Sako et al. (1997). Cells were harvested at 24 h by centrifugation and washed three times with 50 mM Tris-HCl (pH 8.0) buffer. The cell pellet was frozen at −85°C before enzyme purification.

Enzyme assays

Enzyme activity was determined by the hydrolysis of the peptidyl-MCA (α-(4-methyl-coumaryl-7-amide)) substrate, Boc-Leu-Gly-Arg-MCA (Peptide Institute, Osaka, Japan), under the following standard conditions. The reaction mixture consisted of 5 μl of 2 μg/ml enzyme solution, 5 μl of 1 mM Boc-Leu-Gly-Arg-MCA, and 990 μl of 50 mM Tris-HCl (pH 9.0). Proteinase activity was assayed in triplicate at 90°C for 10 min, and the reaction was initiated by the addition of the enzyme solution to the preheated reaction mixture. Controls without enzyme solution were tested to determine stability of the substrate in all reaction conditions examined. The enzyme reaction was stopped by the addition of 10 μl of 10 mM diisopropyl flurophosphate (DFP) and was kept for a further 10 min on ice. Catalytic activity was detected as described by Odagami et al. (1993). Catalytic activity was estimated from fluorescence of the standard 7-amino-4-methyloumarin (AMC) and was expressed as nanomoles of AMC per min per ml of enzyme solution. One unit of proteinase activity was defined as giving an fluorescence change of 100/min.

Boc-Leu-Gly-Arg-MCA substrate was used in this study because it has high stability at a wide range of temperature (50–130°C) and at a wide range of pH (4.0–10.4), as indicated by controls in the absence of enzyme solution. In addition, the high specificity of the enzyme to this substrate allowed the identification of this enzyme in crude extract during purification. Azocasein was first used as substrate to identify the presence of proteolytic activity in crude extract and also during purification.

Protein was determined following the Bradford method (Bradford 1976) using the standard assay kit from Bio-Rad (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard.

Enzyme purification

The purification of Pernilase was achieved at 5°C as follows: Cells (13 g) were suspended in 50 mM Tris-HCl (pH 8.0) buffer and ruptured by five passages through a French press (5501-N; Ohtake Seisakusho, Tokyo, Japan) at 1500 kg/cm^2. The suspension was centrifuged at 22 000 g for 20 min and the supernatant was used as the crude extract. Solid ammonium sulfate was added to the crude extract to reach 80% saturation. This solution was then centrifuged at 12 000 g for 30 min, and the pellet was dissolved in 50 mM Tris-HCl (pH 8.0) and dialyzed three times against the same buffer. The dialyzed protein solution was applied to a column of Macro-Prep 50 Q (2.6 cm × 20 cm; Bio-Rad) equilibrated with 50 mM Tris-HCl (pH 8.0). Proteins were eluted with a 200-ml linear gradient from 0 to 1 M NaCl in 50 mM Tris-HCl (pH 8.0). The active fractions eluted at 0.6 M NaCl were combined and dialyzed against 20 mM phosphate buffer (pH 6.8). The enzyme solution was loaded onto a column of hydroxylapatite (2.6 cm × 15 cm; Nacalai tesque, Kyoto, Japan) equilibrated with 20 mM phosphate buffer (pH 6.8).

Proteins were eluted with a 100-ml linear gradient in phosphate buffer to 500 mM (pH 6.8). The absorbed active fractions, eluted at 400 mM phosphate buffer, were pooled and dialyzed against 50 mM Tris-HCl (pH 8.0). This fraction was then applied to a Mono Q HR 5/5 column (Pharmacia, Uppsala, Sweden) equilibrated with 50 mM Tris-HCl (pH 8.0). A linear gradient of 0 to 1.0 M NaCl in the same buffer was used to elute the bound proteins. The active fractions were collected and dialyzed against 50 mM Tris-HCl buffer (pH 8.0). For further purification and determination of molecular weight, the dialyzed enzyme solution was concentrated to 0.2 ml using 10 000 MW cutoff membrane centrifuge tubes (Ultrafree-MCUFC3LGC00; Millipore, Bedford, MA, USA) and washed with 200 mM Tris-HCl (pH 8.0). The concentrate was applied to a Superose 12 HR 10/30 column (Pharmacia) equilibrated with 200 mM Tris-HCl (pH 8.0). As the molecular weight standard, a HMW gel filtration calibration kit (Pharmacia) was used.

Electrophoresis

The homogeneity of the purified enzyme was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a vertical slab of 10% acrylamide. The enzyme sample was treated at 100°C for 3 min according to Laemmli (1970). Proteins bands were visualized by Coomassie brilliant blue R-250 staining. The pro-

teolytic activity of the band was examined by gel slicing as indicated by Sako et al. (1997).

Effect of temperature on proteolytic activity

The optimum temperature for proteolytic activity was examined under the standard condition at different temperatures. For determinations at temperatures above 90°C, enzyme solutions (1 ml) were sealed in 3-ml glass capillary tubes and incubated in a temperature-controlled oil bath at intervals.

Effect of pH on proteolytic activity

The proteolytic activity of the enzyme was measured at 90°C for 10 min at different ranges of pH. Five microliters of enzyme solution (2 μg/ml) and 5 μl of 1 mM Boc-Leu-Gly-Arg-MCA peptide were mixed with 990 μl of the following buffers: 50 mM acetate/sodium acetate (pH 3.0–6.0), 100 mM sodium phosphate (pH 6.0–8.0), and 50 mM Tris-HCl (pH 7.0–11.0).

Effect of proteinase inhibitors

Enzyme samples were preincubated at room temperature for 15 min in the presence of the following proteinase inhibitors: 1 mM EDTA (ethylenediamine tetraacetic acid); 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid]; 1 mM, 5 mM PMSF (phenylmethylsulfonyl fluoride); 1 mM, 5 mM DFP (diisopropylfluoro phosphate); 1 mM, 10 mM phenanthroline, 1 mM *N*-ethylmaleimide; and 1 mg/ml soybean trypsin inhibitor, 1 mM iodoacetic acid, 1 mM *p*-(chloromercury) benzoic acid (Wako Pur. Chem., Tokyo, Japan). The remaining activity was determined as indicated (in Enzyme assays) for the standard condition.

Substrate specificity

The substrate specificity for proteolytic activity was determined by incubating 5 μl enzyme solution with 5 μl of 5 μM MCA-labeled peptides (Peptide Institute) in 990 μl of 50 mM Tris-HCl buffer (pH 9.0) at 90°C for 10 min. The enzyme reaction was stopped by the addition of 10 μl of 10 mM DFP inhibitor. Proteolytic activity was detected by measuring, in a spectrofluorophotometer (RF-1500; Shimadzu, Kyoto, Japan), the fluorescence of released 7-amino-4-methyl-coumarin (AMC) at an excitation of 380 nm and emission of 460 nm. Catalytic activity was estimated from fluorescence of the standard AMC and was expressed as nmoles of AMC per min per ml of enzyme solution.

Effect of denaturing reagents on proteolytic activity

Proteinase activity was measured, under the standard condition, in the presence of the following denaturing agents: 4, 8 M urea; 1, 4 M guanidine-HCl; 1, 5, 10 mM dithiothreitol (DTT); 0.1%, 1%, 5% (w/v) 2-mercaptoethanol; and 1%, 2% SDS.

Thermostability

Purified enzyme solution (2 μg/ml in 50 mM Tris-HCl, pH 9.0) was incubated in sealed 2-ml glass capillary tubes at 90°C, 100°C, 110°C, and 120°C in a temperature-controlled oil bath. Aliquots of 5 μl were removed at intervals, chilled on ice, and subsequently the remaining activity was assayed at 90°C under the standard condition.

Results

Purification of pernilase

An intracellular proteinase from the cell extract of *Aeropyrum pernix* strain K1 was purified by ammonium sulfate fractionation, Macro-Prep 50 Q, hydroxylapatite, Mono Q, and Superose 12 gel filtration chromatography as described in Materials and methods. The procedure for purification of the enzyme is summarized in Table 1. The proteinase, pernilase, was purified about 28 fold with a specific activity of about 34 835 U/mg and a final yield of 16%. During purification steps, a number of substrates were examined to determine if the azocasein hydrolytic activity observed was produced for more than one protease in the crude extract. This method allowed us to separate at least two different proteases after the Macro-Prep 50 Q column. The protease purified and reported in this study showed activity for both azocasein and Boc-Leu-Gly-Arg-MCA substrates.

Table 1. Summary of purification of an intracellular proteinase (pernilase) from *Aeropyrum pernix* K1

Preparation	Volume (ml)	Total protein (mg)	Total activity (U)	Yield (%)	Specific activity (U/mg)	Purification fold
Crude extract	100	2100	2643589	100	1259	1.0
$(NH_4)_2SO_4$	50	1710	2102140	80	1229	1.0
Macro-Prep 50 Q	40	1100	1609640	61	1463	1.2
Hydroxylapatite	26	198	939800	36	4746	3.8
Mono Q	10	33	655876	25	19875	15.8
Superose 12	4	12	418021	16	34835	27.7

The purified sample of pernilase, after elution from gel filtration (Superose 12 column), showed a single protein band on SDS-PAGE, corresponding to the active fraction detected by gel slicing (Fig. 1). The molecular mass of the enzyme determined by SDS-PAGE was 50 kDa (Fig. 1). Elution value after Superose 12 showed same molecular mass as obtained by SDS-PAGE, suggesting that the enzyme corresponded to a monomeric enzyme.

Effect of temperature on enzymatic activity

Examination of the effect of temperature on the proteolytic activity of the enzyme (Fig. 2) showed that maximal enzymatic activity was obtained at 90°C. At temperatures below 90°C, enzymatic activity decreased considerably, and at temperatures near 60°C activity was hardly detected. On the other hand, at temperatures above 90°C the enzyme was less sensitive, and 77% of the activity was retained at 110°C. The effect of divalent cations at different concentrations on the activity of the enzyme was also examined (data not shown). The divalent cations tested (Ca^{2+}, Mg^{2+}, Zn^{2+}, and Mn^{2+}) had no effect on the enzymatic activity.

pH optimum

The enzyme showed a broad pH profile (pH 5.0–10.0) for the hydrolysis of Boc-Leu-Gly-Arg-MCA peptide (Fig. 3). The proteolytic activity was remarkably decreased below pH 6.0 and above pH 9.0. The optimal pH of the enzymatic activity was around 6.5 to 9.0, but the highest value for peptide hydrolysis was obtained at pH 9.0. Controls without enzyme

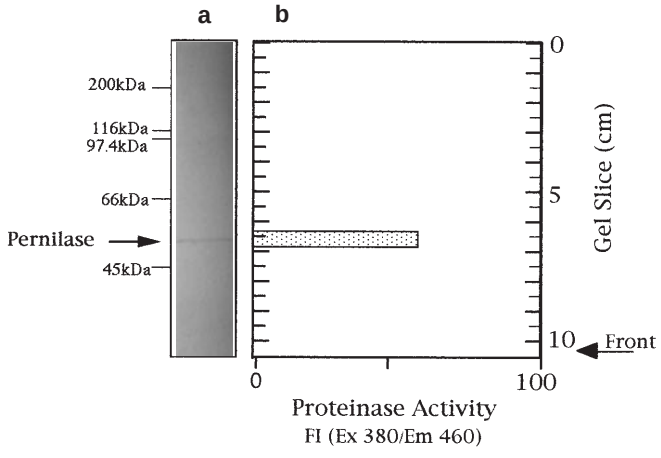


Fig. 1a,b. SDS-PAGE of purified pernilase from *Aeropyrum pernix* K1 (**a**) and proteolytic activity of the gel fractions sliced every 3 mm (**b**). Purified enzyme sample from Superose 12 (3 μg) was loaded on 10% SDS-PAGE and stained with Coomasie brilliant blue R-250. (**a**) Standard proteins; molecular mass values

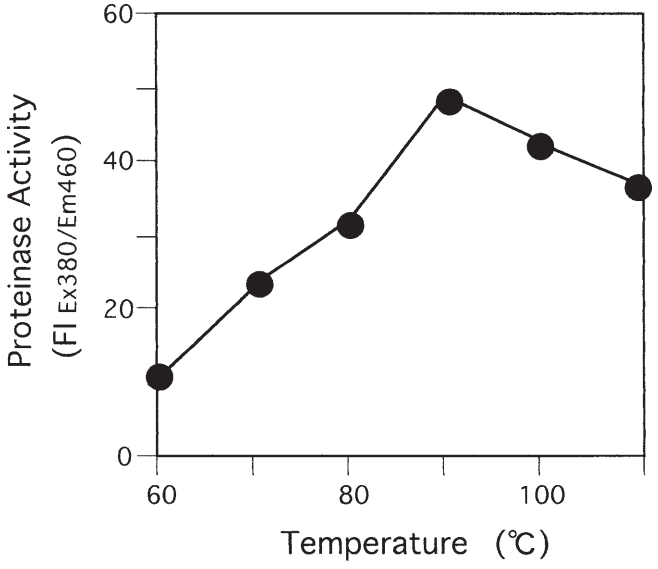
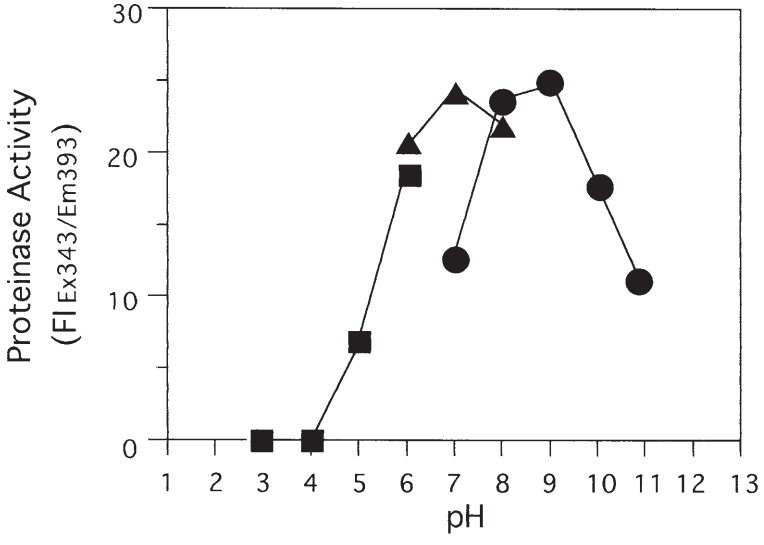


Fig. 2. Effect of temperature on pernilase proteolytic activity assayed at indicated temperatures as described in Materials and methods

Fig. 3. Effect of pH on proteolytic activity of pernilase assayed using the following buffers: 50 mM sodium acetate (*squares*), 100 mM sodium phosphate (*triangles*), and 50 mM Tris-HCl (*circles*) under standard conditions



solution showed that the MCA substrate used was highly stable to the different pH conditions used in this study.

Effect of proteinase inhibitors

To characterize the type of proteinase produced by *Aeropyrum pernix,* several protease inhibitors were tested. The proteinase activity was strongly inhibited by the typical serine proteinase inhibitors PMSF and DFP. The rest of the inhibitors showed only partial or no inhibition (Table 2).

Substrate specificity

Substrate specificity of the purified enzyme was examined with several fluorescent peptides (Fig. 4). Boc-Leu-Gly-Arg-MCA peptide was efficiently hydrolyzed by the enzyme. A preference for the arginine residue was observed.

Effect of denaturing agents on the enzymatic activity

The activity of the enzyme in the presence of different concentrations of denaturing agents was examined (Table 3). The enzyme showed significant resistance to low and high concentrations of dithiothreitol and 2-mercaptoethanol. However, the activity of the enzyme was highly affected by the presence of SDS, urea, and guanidine-HCl.

Table 2. Effect of protease inhibitors on pernilase proteolytic activity

Inhibitor	Concentration	Residual activity (%)
None		100
EDTA	1 mM	70
EGTA	1 mM	60
Phenanthroline	1 mM	66
Phenylmethylsulfonyl fluoride (PMSF)	1 mM	13
	5 mM	9
Diisopropylfluoryl phosphate (DFP)	1 mM	12
	5 mM	1
Soybean trypsin inhibitor	1 mg/ml	60
N-Ethylmaleimide	1 mM	100
Monoiodoacetate	1 mM	100
4-Chloromercuribenzoate	1 mM	100

Thermostability of the enzyme

The thermostability of the purified enzyme was examined at different pH values, and the optimum pH for highest stability was 9.0 (data not shown). Figure 5 shows the thermostability of the purified proteinase at pH 9.0. Pernilase was extremely stable at 90°C, retaining 100% of activity after 4 h of incubation. Longer incubations to determine the half-life at this temperature were performed; however, no loss of activity was observed for more than 8 h (data not shown). At 100°C, the half-life of the enzyme was 85 min and it was 12 min at 110°C. At 120°C, the enzyme was rapidly inactivated.

Discussion

We recently reported on the production of an extracellular metalloproteinase, aeropyrolysin (Sako et al. 1997) by the archaeon *Aeropyrum pernix* (Sako et al. 1996). The experiments reported in this paper led to the identification of a different intracellular serine proteinase, pernilase, from the same archaeon. Studies on the detection of pernilase in the extracellular medium showed that this enzyme is not excreted extracellularly as is aeropyrolysin (data not shown). In addition, aeropyrolysin and pernilase differ in their general biochemical characteristics such as substrate specificity, resistance to denaturing agents, and the requirement of calcium for thermostability.

The inhibition by 1 mM of PMSF and DFP support strongly that pernilase is a serine proteinase. The enzyme is resistant to dithiothreitol (10 mM) and 2-mercaptoethanol (1%) but sensitive to urea (8 M) and guanidine-HCl (4 M). These results may suggest that hydrogen bonds play an

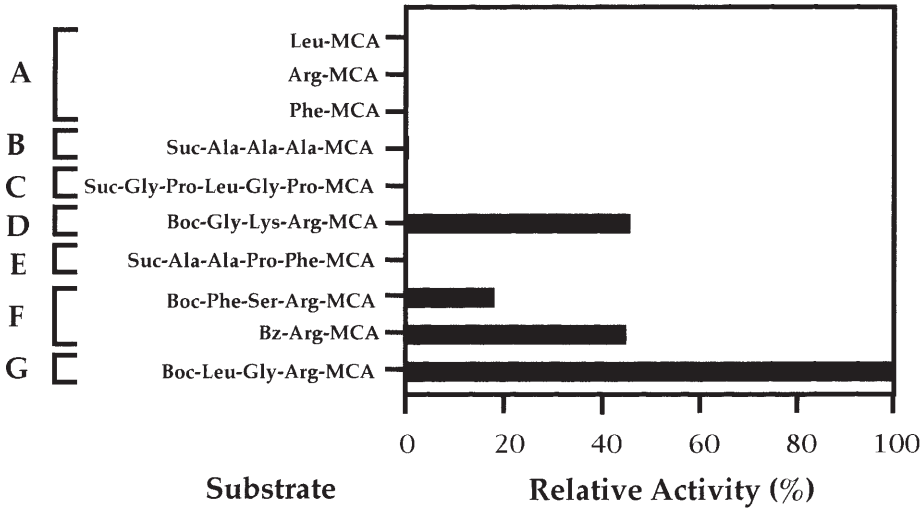


Fig. 4. Substrate specificity of pernilase. *A*, *B*, *C*, *D*, *E*, *F*, and *G* indicate the substrates for carboxyaminopeptidase, esterase, collagenase-like peptidase, carboxyl side of paired basic residue-cleaving enzyme, chymotrypsin, trypsin, and horseshoe crab clotting enzyme, respectively

Table 3. Effect of denaturing agents on pernilase activity

Reagent	Concentration	Proteolytic activity (%)
None		100
SDS	1%	9
	2%	3
Urea	4M	54
	8M	12
Guanidine-HCl	1M	5
	4M	0
Dithiothreitol	1mM	80
	5mM	81
	10mM	94
2-Mercaptoethanol	0.1%	56
	1%	53
	5%	32

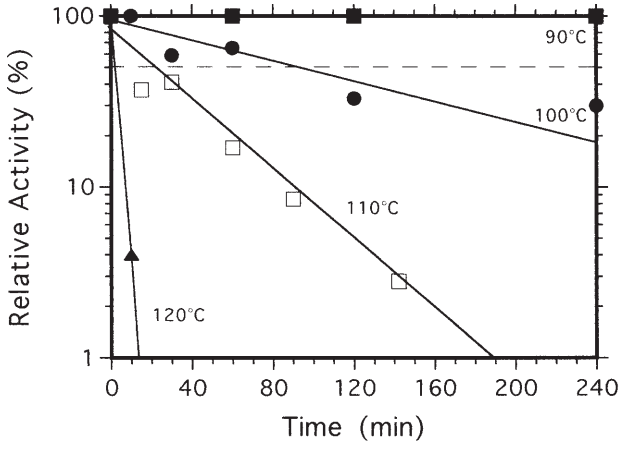


Fig. 5. Heat stability of pernilase at temperatures between 90°C and 120°C. Purified enzyme (2 μg/ml in 50 mM Tris-HCl, pH 9.0) was incubated at 90°C (*solid squares*), 100°C (*circles*), 110°C (*open squares*), and 120°C (*triangles*). Samples of enzyme solution were removed at intervals, chilled, and residual poteolytic activity determined

important role in the stabilization of the enzyme and that disulfide bonding is not involved in preserving proteolytic activity. These observations have also been reported by Cowan et al. (1987) for the serine proteinase archaelysin, isolated from the anaerobic archaeon *Desulfurococcus* sp., in which disulfide bridges seem to play an insignificant role in stabilizing the enzyme. The addition of calcium and other metal ions did not affect either the activity or the stability (data not shown) of pernilase. However, the addition of chelators such as EGTA and EDTA (1 mM) produced a decrease of activity, suggesting that metal ions might have an important role in the preservation of the enzyme conformation.

The optimal temperature of the enzyme for substrate hydrolysis (90°C) and the thermostability data indicated that pernilase is an extraordinarily heat-stable proteinase. The half-lives of the enzyme, 85 min at 100°C and 12 min at 110°C, are comparable to one of the most thermostable serine proteinase reported to date, pyrolysin, from the anaerobic archaeon *Pyrococcus furiosus*, which has a half-life of 3 min at 110°C (Eggen et al. 1990). Pyrolysin is a cell envelope-associated proteinase and has been classified as having serine-type protease activity (Eggen et al. 1990). The temperature optimum is 115°C and it has a broad pH range, between 6.5 and 10.5. The molecular weight of pyrolysin is not clear because the enzyme shows a multiple band pattern in gel (Eggen et al. 1990; Halio et al. 1997). Pernilase also resembles some characteristics of archaelysin (Cowan et al. 1987). Archaelysin is also a serine-type enzyme with a similar molecular weight of 52 kDa. Thermostability of this enzyme is lower than that of pernilase (half-life at 95°C, 70–90 min; at 105°C, 8–9 min), and denaturation is very rapid above 115°C. The chelation of calcium does not appear to be implicated in stabilization of the protein structure in archaelysin.

All the hyperthermostable proteinases reported to date are produced by anaerobic hyperthermophilic microorganisms, and although cells might be produced in relatively large scale using bioreactors, the cultivation characteristics of these microorganisms, such as strict anaerobiosis, sulfur dependence, and other features (Volkl et al. 1994), are still a barrier for biotechnology. *Aeropyrum pernix* is the first strictly aerobic hyperthermophilic archaeon that has been described to date (Sako et al. 1996). It grows optimally above 90°C with a doubling time of 200 min. Large-scale culture can be achieved with reasonable growth yield (1 g wet weight/l) in 1 day without H_2S production. These characteristics make this archaeon a potential biotechnological resource for the purification of new enzymes.

Considering that pernilase can be produced by *A. pernix* under aerobic conditions at 90°C with high cell yield and with low risk of contamination by other bacterial strains, has a high thermostability, is resistant to chelating agents, and is resistant to temperatures above 100°C, this enzyme is an attractive candidate for various biotechnological applications.

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References

Adams MW, Perler FB, Kelly RM (1995) Extremozymes: expanding the limits of biocatalysis. Biotechnology 13:662–668

Blochl E, Rachel R, Burggraf S, Hafenbradl D, Jannasch HW, Stetter KO (1997) *Pyrolobus fumarii*, gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113°C. Extremophiles 1:14–21

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Bragger JM, Daniel RM, Coolbear T, Morgan HW (1989) Very stable enzymes from extremely thermophilic archaebacteria and eubacteria. Appl Microbiol Biotechnol 31:556–561

Brock TD, Brock KM, Belly RT, Weiss RL (1972) *Sulfolobus*: a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. Arch Microbiol 84:54–68

Connaris H, Cowan DA, Sharp R (1991) Heterogeneity of proteinases from the hyperthermophilic archaeobacterium *Pyrococcus furiosus*. J Gen Microbiol 137:1193–1199

Cowan DA (1992) Biotechnology of archaea. Trends Biotechnol 10:315–323

Cowan DA (1995) Protein stability at high temperatures. Essays Biochem 29:193–207

Cowan DA, Daniel RM, Morgan HW (1985) Thermophilic proteases: properties and potential applications. Trends Biotechnol 3:68–72

Cowan DA, Smolenski KA, Daniel RM, Morgan HW (1987) An extremely thermostable extracellular proteinase from a strain of the archaebacterium *Desulfurococcus* growing at 88°C. Biochem J 247:121–133

Eggen R, Geerling A, Watts J, M de Vos W (1990) Characterization of pyrolysin, a hyperthermoactive serine protease from the archaebacterium *Pyrococcus furiosus*. FEMS Microbiol Lett 71:17–20

Halio SB, Bauer MW, Makund S, Adams MW, Kelly R (1997) Purification and characterization of two functional forms of intracellular protease PfpI from the hyperthermophilic archaeon *Pyrococcus furiosus*. Appl Environ Microbiol 63:289–295

Hanzawa S, Hoaki T, Jannasch H, Maruyama T (1996) An extremely thermostable serine protease from a hyperthermophilic archaeum, *Desulfurococcus* strain SY, isolated from a deep-sea hydrothermal vent. J Mar Biotechnol 4:121–126

Huber R, Kurr M, Jannasch HW, Stetter KO (1989) A novel group of abyssal methanogenic archaebacteria (*Methanopyrus*) growing at 110°C. Nature (Lond) 342:833–834

Huber R, Stoffers P, Cheminee JL, Richnow HH, Stetter KO (1990) Hyperthermophilic archaebacteria within the crater and open-sea plume of erupting Macdonald Seamount. Nature (Lond) 345:179–181

Klingeberg M, Hashawa F, Antranikian G (1991) Properties of extremely thermostable proteases from anaerobic hyperthermophilic bacteria. Appl Microbiol Biotechnol 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 extremely thermophilic archaeon *Thermococcus stetteri*. Appl Environ Microbiol 61:3098–3104

Koch R, Spreinat A, Lemke K, Antranikian G (1991) Purification and properties of a hyperthermoactive amylase from the archaebacterium *Pyrococcus woesi*. Arch Microbiol 155:291–310

Koch R, Zablowsky P, Spreinat A, Antranikian G (1990) Extremely thermostable amylolytic enzyme from the archaeobacterium *Pyrococcus furiosus*. FEMS Microbiol Lett 71:21–26

Kurr M, Huber R, Koning H, Jannasch HW, Frickie H, Trincone A, Klistjansson JK, Stetter KO (1991) *Methanopyrus kandleri* gen. and sp. nov. represents a novel group of hyperthermophilic methanogens, growing at 110°C. Arch Microbiol 156:239–247

Laderman KA, Davis BR, Krutzsch HC, Lewis MS, Griko YV, Privalov PL, Anfinsen CB (1993) The purification and characterization of an extremely thermostable amylase from the hyperthermophilic archaebacterium *Pyrococcus furiosus*. J Biol Chem 268:24394–24401

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond) 227:681–685

Morikawa M, Izawa Y, Rashid N, Hoaki T, Imanaka T (1994) Purification and characterization of a thermostable thiol protease from a newly isolated hyperthermophilic *Pyrococcus* sp. Appl Environ Microbiol 60:4559–4566

Odagami T, Suzuki S, Takama K, Azumi K, Yokosawa H (1993) Characterization of extracellular protease produced by the marine putrefactive bacteria, *Alteromonas haloplanktis*. J Mar Biotechnol 1:55–58

Pley U, Schipka J, Gambacorta A, Jannasch HW, Fricke H, Rachel R, Stetter KO (1991) *Pyrodictium abyssi*, new species represents a novel heterotrophic marine archaeal hyperthermophile growing at 110°C. Syst Appl Microbiol 14:245–253

Sako Y, Chavez PC, Ishida Y (1997) An extremely heat-stable extracellular proteinase (aeropyrolysin) from the hyperthermophilic archaeon *Aeropyrum pernix* K1. FEBS Lett 415:329–334

Sako Y, Nomura N, Uchida A, Ishida Y, Morii H, Koga Y, Hoaki T, Maruyama T (1996) *Aeropyrum pernix* gen. nov., sp. nov., a novel aerobic hyperthermophilic archaeon growing at temperatures up to 100°C. Int J Syst Bacteriol 46:1070–1077

Snowden LJ, Blumentals II, Kelly RM (1992) Regulation of proteolytic activity in the hyperthermophile *Pyrococcus furiosus*. Appl Environ Microbiol 58:1134–1141

Stetter KO (1982) Ultrathin mycelia-forming organisms from submarine volcanic areas having an optimum growth temperature of 105°C. Nature (Lond) 300:258–260

Stetter KO (1988) Hyperthermophiles – physiology and enzymes. J Chem Technol Biotechnol 42:315–317

Stetter KO, Koning H, Stackebrandt E (1983) *Pyrodictium* gen. nov., a new genus of submarine disc-shaped sulphur reducing archaebacteria growing optimally at 105°C. Syst Appl Microbiol 4:535–551

Volkl P, Markiewicz P, Stetter KO, Miller JH (1994) The sequence of a subtilisin-type protease (aerolysin) from the hyperthermophilic archaeum *Pyrobaculum aerophilum* reveals sites important to thermostability. Protein Sci 3:1329–1340

Vooshorst WG, Eggen RI, Geerling AC, Platteeuw C, Siezen RJ, M de Vos W (1996) Isolation and characterization of the hyperthermostable serine protease, pyrolysin, and its gene from the hyperthermophilic archaeon *Pyrococcus furiosus*. J Biol Chem 271:20426–20431