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A novel extracellular subtilisin-like protease from the hyperthermophile *Aeropyrum pernix* K1: biochemical properties, cloning, and expression

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Abstract A novel extracellular serine protease designated Pernisine was purified to homogeneity and characterized from the archaeon *Aeropyrum pernix* K1. The molecular mass, estimated by SDS-PAGE analysis and by gel filtration chromatography, was about 34 kDa suggesting that the enzyme is monomeric. Pernisine was active in a broad range of pH (5.0–12.0) and temperature (60–120 °C) with maximal activity at 90 °C and between pH 8.0 and 9.0. In the presence of 1 mM $CaCl_2$ the activity, as a function of the temperature, reached a maximum at 90 °C but at 120 °C the enzyme retained almost 80% of its maximal activity. Activity inhibition studies suggest that the enzyme is a serine metalloprotease and biochemical data indicate that Pernisine is a subtilisin-like enzyme. The protease gene, identified from the sequenced genome of *A. pernix*, was amplified from total genomic DNA by PCR technique to construct the expression plasmid pGEX-Pernisine. The Pernisine, lacking the leader sequence, was expressed in *Escherichia coli* BL21 strain as a fusion protein with glutathione-*S*-transferase. The biochemical properties of the recombinant enzyme were found to be similar to those of the native enzyme.

Keywords Archaeon · Expression · Extracellular proteases · Heat stability · Hyperthermophilic

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Introduction

Hyperthermophiles represent the most extensively studied group among extremophiles (Horikoshi 1997), and have been isolated all over the world from geothermal areas, terrestrial and marine solfataric fields, hot springs, and submarine hydrothermal vents. These microorganisms are able to grow at very extreme temperatures ranging from 80 °C up to 113 °C (Blochl et al. 1997), and generally belong to the domain *Archaea*.

The enzymes isolated from them have been recognized to be of high interest both for clarifying the molecular mechanisms at the base of their stability and activity at high temperature (Robb and Clark 1999), and for their peculiar properties, such as resistance to organic solvents, detergents, etc., that are of interest for potential biotechnological applications (Adams and Kelly 1998; Vieille and Zeikus 2001).

Among hydrolases, heat-stable proteases have received much attention. Heat-stable proteases have been isolated from the genera *Pyrococcus* (de Vos et al. 2001), *Thermococcus* (Klingeberg et al. 1991, 1995), *Desulfurococcus* (Cowan et al. 1987; Hanzawa et al. 1996), *Sulfolobus* (Fusek et al. 1990), *Staphylothermus* (Mayr et al. 1996), and *Aeropyrum* (Sako et al. 1997; Chavez Croocker et al. 1999). All these microorganisms are able to grow on proteinaceous substrates, and peptide requirement has been shown for the genera *Desulfurococcus* (Jannasch et al. 1988) and *Pyrococcus* (Snowden et al. 1992).

The majority of proteases from hyperthermophiles belong to the family of subtilisin-like proteases (subtilases), that are further classified into six families: subtilisin, thermitase, proteinase K, lantibiotic peptidase, kexin, and pyrolysin (Siezen and Leunissen 1997). The subtilisin family has been the most extensively studied in terms of structure and function (Wright et al. 1969; Jain et al. 1998).

Nearly all subtilases are synthesized in the cells as a precursor called preprosubtilisin, in which presequence

and prosequence are attached to the N-terminus of the mature protein. The presequence is a signal peptide that leads the secretion of prosubtilisin across the cytoplasmic membrane, while the prosequence is believed to act as an intramolecular chaperone that guides the correct folding of the protein, and is then removed by autoproteolysis from the active enzymatic form.

This study reports the purification to homogeneity and characterization of a novel extracellular protease isolated from *Aeropyrum pernix* K1, a strictly aerobic hyperthermophile, growing optimally above 90 °C (Sako et al. 1996). The protease, designated as Pernisine, is an endopeptidase belonging to the family of subtilisin-like serine proteases, stable at high temperature, and active in a broad range of pH values.

Its gene, identified from the sequenced genome of *A. pernix* K1 (Kawarabayasi et al. 1999), has been expressed in *Escherichia coli,* and the recombinant enzyme has been purified and characterized.

Materials and methods

Strain and growth conditions

The strain used in this study is *A. pernix* K1 (JCM 9820) kindly provided from the Centre of Applied and Microbiological Research, UK. Cells were grown under aerobic conditions as described by Sako et al. (1997). The *E. coli* strains Dh5α [*sup*E 44 Δ *lac*U169 (Φ80*lacZ* ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1] and BL21 (DE3) [*hsd*S *gal*(λ*cIts*857 *ind*1 Sam7 *nin*5 *lac*UV5-T7 gene1)] were used for transformation. The plasmid pUC19 (Amersham Pharmacia Biotech) was used for cloning and sequencing procedures.

Enzyme isolation and purification

The culture medium (20 l) devoid from cells was collected and reduced to 200 ml by ultrafiltration (Minitan System; Millipore, Bedford, Mass., USA). To the concentrated solution solid ammonium sulfate was added to reach 90% saturation and kept for 30 min with stirring at 4 °C. After centrifugation at 12,000 *g* for 30 min at 4 °C, the pellet formed was resuspended in 50 mM TRIS-HCl pH 8.0 and dialyzed against the same buffer at 4 °C for 16 h with three changes of buffer. The enzyme solution was then loaded onto an anion exchange column of DEAE-cellulose "DE52" (2×24 cm; Whatman), equilibrated with 50 mM TRIS-HCl pH 8.0. Bound proteins were eluted by a 400-ml linear gradient from 0 to 1 M NaCl in the equilibration buffer at a flow rate of 1.2 ml/min. The fractions of interest, which eluted at 0.45 M NaCl, were pooled, dialyzed extensively against 50 mM TRIS-HCl pH 8.0, and concentrated by polyethylene glycol (MW 12,000; Fluka) for further use.

This enzymatic solution was then applied to a prepacked ion-exchange Uno Q-12 column (BioRad) adapted to an HPLC system (LKB) equilibrated with 50 mM TRIS-HCl pH 8.0. The column was eluted by a linear gradient from 0 (buffer A) to 1 M NaCl (buffer B) in equilibration buffer at a flow/rate of 2 ml/min (at $t = 0$, buffer B = 0%; at $t = 20$ min, buffer B = 0%; at $t = 60$ min, buffer B = 50%; at $t = 65$ min, buffer B = 100%). The active fractions eluted at 0.5 M NaCl were pooled and dialyzed against 50 mM sodium acetate pH 5.5. This protein solution was then loaded onto a column of Mono Q HR 5/5 (Pharmacia) adapted to an HPLC system (LKB) and equilibrated with 50 mM sodium acetate pH 5.5. After washing with 20 bed volumes of the same buffer, proteins were eluted by a linear gradient of 0–0.5 M NaCl in 50 mM sodium acetate pH 5.5 at a flow rate of 1 ml/min.

Active fractions, eluted at 0.3 M NaCl, were combined and dialyzed against 50 mM TRIS-HCl pH 8.0 containing 0.3 M NaCl. After concentration to 0.4 ml by polyethylene glycol, the enzymatic solution was then applied to a "Bio-Silect SEC 125" gel filtration chromatography column (300×7.8 mm; BioRad), equilibrated with 50 mM TRIS-HCl pH 8.0. The elution was performed by 50 mM TRIS-HCl pH 8.0 containing 0.3 M NaCl at a flow rate of 0.5 ml/min. Purified enzyme samples from the active fractions were used to characterize the properties of the enzyme.

Isolation of the *A. pernix* subtilisin gene

Homology search and multiple alignments were performed using the BLAST program (Altschul et al. 1990). DNA restriction maps were achieved by the DNA Strider program (Douglas 1994). Genomic DNA from *A. pernix* K1, which was prepared according to Sako et al. (1996), was used as template in PCR assays. Oligonucleotides Ape0263F (5′-GGGGTGGCTGTAGTAACTGGTGTAATT-3′) and Ape0263R (5′-CCCAATAGTCGAACTCTGCCGTCA-3′) were designed to bind at the start site and at the stop codon, respectively, of the ORF 0263. Both primers contained additional bases (underlined) at the 5′ terminus to create compatible ends to *Sma*I sites of the cloning vector. Thirty cycles of PCR were performed on thermal cycler PCR (Hybaid) using *Taq* polymerase (Finnzymes). The reaction conditions were 94 °C/5 min; 94 °C/30 s, 50 °C/45 s, 72 °C/1 min ×30, with a final extension at 72 °C/10 min. After treatment with T4 polynucleotide kinase to remove the 3′-A-base protruding produced by *Taq* polymerase, the 1.2-kb amplified DNA was then cloned into *Sma*I site of pUC19 and this ligation mix was used to transform *E. coli* Dh5α cells. Colonies selected on LB agar containing 0.1 mg/ml ampicillin grew at 37 °C and recombinant plasmids were screened by restriction analysis. Positive clone was sequenced using ABIPRISM system.

Construction of expression plasmid and induction assays

The gene encoding Ap-subtilisin was amplified using oligonucleotides Ape0263pro (5′-GGGGGATCGGCCGCTGGGGCTAG-CA-3′), which annealed at the predicted start of the proregion of the enzyme, and Ape0263R as reverse primer. Ape0263pro was designed to have an enzyme lacking the leader sequence. *Sma*I sites (*underlined*) were introduced to subclone the amplified gene in frame with glutathione-*S*-transferase into pGEX-3X expression vector (Amersham), to construct plasmid pGEX-Ap-subtilisin. This plasmid was used to transform the *E. coli* strain BL21 (DE3). The recombinant cells were grown at 37 °C on LB medium supplemented with 0.05 mg/ml ampicillin with vigorous shaking to an $A_{600\ nm}$ of 0.6. Protein expression was induced by addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and the growth was continued for an additional 4 h. Cells were then harvested by centrifugation at 4,000 *g* at 4 °C for 10 min.

Purification of recombinant Ap-subtilisin

Cells were harvested by centrifugation at 4,000 *g* at 4 °C for 15 min, resuspended in 10 mM Na_2HPO_4 pH 7.2, and disrupted by addition of 1 mg/ml lysozyme. After centrifugation at 15,000 *g* at 4 °C for 30 min, the soluble fraction was collected and subjected to heat treatment at 65 °C for 20 min. The enzymatic solution was then applied onto a hydroxylapatite column (Bio-Gel HTP; BioRad) equilibrated with 10 mM Na_2HPO_4 buffer pH 7.2. After washing with 70 ml equilibration buffer, bound proteins were eluted by increasing the ionic strength of the buffer stepwise (10, 50, 100, 200 mM Na_2HPO_4 buffer pH 7.2). The fractions containing proteolytic activity were pooled, dialyzed against 50 mM TRIS-HCl pH 8.0, and concentrated by polyethylene glycol for further use. Glycerol (20%) was added as stabilizing agent.

Electrophoresis and zymograms

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970) using 12.5% polyacrylamide gels. Before electrophoresis, proteinase samples were treated with 1 mM phenylmethylsulfonyl fluoride (PMSF) and heated at 100 °C for 5 min. Protein bands were visualized by Coomassie brilliant blue R-250 staining. The protein content was estimated according to the method of Bradford (1976), using bovine serum albumin as standard.

Proteolytic activity was detected by using an overlay of SDS-polyacrylamide gel containing 0.1% casein according to de Vos (2001). Proteolytic bands were visualized as clear bands after 1 h incubation at 90 °C against a dark background after Coomassie brilliant blue R-250 staining.

Enzyme assay

Proteolytic activity was determined using azocasein (Sigma) as substrate, according to Sako et al. (1997) with some modifications. The reaction mixture, containing 0.150 ml 50 mM TRIS-HCl pH 8.0, 0.250 ml 0.1% (w/v) azocasein in the same buffer, and 0.100 ml enzyme solution, to a final volume of 0.500 ml, was incubated at 90 °C for 20 min. The assays were performed in duplicate. The reaction was stopped by the addition of 1 ml 7% perchloric acid and the solution was kept at 5 °C for 20 min. After centrifugation at 15,000 rpm for 10 min in a microcentrifuge (microCentrifugette 4214; ALC), the absorbance of the supernatant was measured at 366 nm, against a blank (complete reaction mixture stopped before incubation) using a spectrophotometer "DU640" (Beckman).

One unit was defined as the amount of enzyme which yielded an increase in A_{366} of 0.10 O.D. in the standard assay conditions.

Purified Pernisine was tested for the degradation of chromogenic *p*-nitroanilides (N-Suc, N-succinyl; pNA, *p*-nitroanilides). Synthetic chromogenic substrates were from Sigma and Bachem, Switzerland, and their hydrolysis was monitored spectrophotometrically at 405 nm by the release of *p*-nitroaniline against a blank without enzyme (Suzuki et al. 1997). Purified enzyme (1.5 μg) was added to the assay mixture containing 0.1 mM substrate, 1 mM $CaCl_2$ in 50 mM TRIS-HCl buffer pH 8.0 in a final volume of 1 ml. Hydrolysis was monitored by noting the increase in absorbance at 405 nm.

Other natural proteins such as hemoglobin, casein, and ovalbumin were also tested as substrates (Murao et al. 1993). The enzyme (1.5 μg) was incubated at 90 °C for 20 min, and enzymatic reactions were stopped by adding 1 ml 7% (v/v) PCA and kept at 5 °C for 10 min. After centrifugation (12,000 *g*, 10 min), the recovered supernatant was assayed for protein content according to Lowry et al. (1951). Azocoll degradation was also investigated according to Chavira et al. (1984).

Effect of pH on proteolytic activity

The effect of pH on proteolytic activity was determined by hydrolysis of azocasein at 90 °C for 20 min. The buffers used were the following: 0.1 M sodium acetate (pH 4.0–6.0), 0.1 M sodium phosphate (pH 6.0–8.0), 0.1 M TRIS-HCl (pH 7.0–10.0), and 0.1 M glycine-NaOH (pH 8.0–12.0).

Effect of temperature on enzyme activity

The optimum temperature for proteolytic activity was examined at temperatures ranging from 50 °C to 120 °C using azocasein as substrate. The mix contained 0.1 ml purified enzyme (1.5 μg), 0.250 ml 0.1% azocasein, and 0.150 ml 50 mM TRIS-HCl pH 8.0. Controls without enzyme solution were tested to determine stability of the azocasein in all reaction conditions examined. The effect of 1 mM $CaCl_2$ on the activity was also tested.

Thermostability

Two milliliters of the purified enzyme solution (3 μg/ml) in 50 mM TRIS-HCl pH 8.0 containing 1 mM $CaCl_2$ was incubated at 90, 100, 110, and 120 °C in sealed tubes in a temperature controlled oil bath. An aliquot of the enzyme (0.200 ml) was recovered at intervals of 1 h, cooled, and the remaining activity was assayed at 90 °C for 20 min by hydrolysis of azocasein. The thermostability of the purified enzyme solution (3 μg/ml) in 50 mM TRIS-HCl pH 8.0 was also determined.

Effect of proteinase inhibitors on enzymatic activity

The purified enzyme solution (1.5 μg) in 50 mM TRIS-HCl pH 8.0 (0.5 ml final volume) was preincubated at room temperature for 15 min with specific inhibitors and the residual proteolytic activity was determined at 90 °C using azocasein as substrate. The following protease inhibitors were used: 1 mM EDTA (ethylenediaminetetraacetic acid); 1 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid); 1 mM, 5 mM phenanthroline; 1 mM, 5 mM *p*-(chloromercuri)benzoic acid; 1 mg/ml trypsin inhibitor; 1 mM, 5 mM PMSF; 1 mM TLCK (*N*-tosyl-L-lysine chloromethyl ketone); 1 mM TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone); 0.001 mM, 0.01 mM aprotinin.

Effect of denaturing agents on proteolytic activity

The enzymatic activity of the purified enzymatic solution (1.5 μg) in 50 mM TRIS-HCl pH 8.0 (0.5 ml final volume) was assayed at 90 °C in the presence of the following denaturing agents: 1%, 5% 2-mercaptoethanol; 1 mM, 5 mM dithiothreitol (DTT); 2 M, 4 M urea; 2 M, 4 M guanidine-HCl; 0.2%, 0.5%, 1%, 2% SDS.

Molecular mass determination

The apparent molecular mass of the enzyme was determined by gel filtration chromatography on an HPLC column of "Bio-Silect SEC 125" (BioRad) using a standard gel filtration calibration kit (BioRad): thyroglobulin 670 kDa, IgG 158 kDa, ovalbumin 44 kDa, myoglobin 7 kDa, vitamin B12 1.3 kDa. The molecular mass was also estimated by SDS-PAGE by comparison with migration of standard protein marker, broad range (New England Biolaboratories).

Results

Purification of Pernisine

A novel extracellular proteinase, Pernisine, was purified from the growth medium of *A. pernix* K1 by ammonium sulfate precipitation, DEAE-cellulose, Uno Q-12, Mono Q HR 5/5, and Bio-Silect SEC 125 gel filtration chromatography as described in Materials and methods and summarized in Table 1. One peak showing proteolytic activity was obtained by Mono Q anion exchange column. The 30-fold purified and homogenous enzyme had a specific activity of 3,585 U/mg using azocasein as substrate. The molecular mass of Pernisine was estimated to be 34 kDa by SDS-PAGE analysis (Fig. 1 *lane b*), and this result was confirmed by gel filtration chromatography, suggesting that the enzyme is monomeric. Activity staining on zymogram gel showed that

Table 1 Summary of purification of Pernisine from growth culture medium of *Aeropyrum pernix*

Preparation	Volume (ml)	Total protein (mg)	Total activity (U)	Yield (%)	Specific activity (U/mg)	Purification (fold)
$(NH_4)_2SO_4$	80	68.0	8,000	100	118	1.0
DEAE-cellulose	60	40.0	12,000	150	300	2.5
Q-12	20	6.0	6,160	77	1,027	8.7
Mono Q	4	2.4	5,304	66	2,210	16.0
SEC 125	4	1.1	3,944	49	3,585	30.0

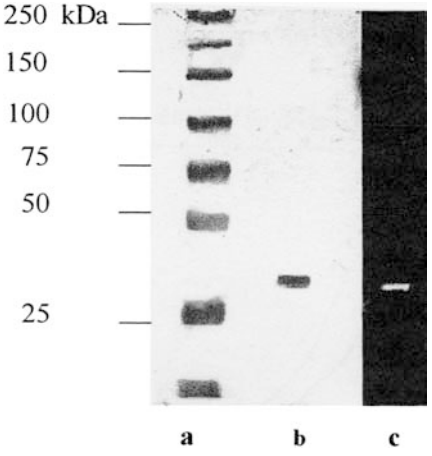

Fig. 1 SDS-PAGE analysis of purified Pernisine. *Lane a* Protein marker. *Lane b* Electrophoretic separation of purified Pernisine on 12.5% SDS-PAGE and staining with Coomassie brilliant blue R-250. *Lane c* Activity staining of Pernisine using 0.1% casein overlay. After incubation at 90 °C for 1 h, the polyacrylamide support was soaked in Coomassie brilliant blue R-250 for detection

purified Pernisine contains one active band (Fig. 1 *lane c*).

Effect of temperature on enzymatic activity

The enzyme was active at temperatures ranging from 50 °C to 120 °C. As shown in Fig. 2b, the maximal activity was observed at 90 °C. However, in the presence of 1 mM $CaCl_2$ at the same maximal temperature the enzyme showed higher activity, and this activity at 120 °C was 80% of the maximal. An increase in $CaCl_2$ concentration did not affect further the enzyme activity.

Thermostability

The stability of the enzyme to heat was examined in 50 mM TRIS-HCl pH 8.0 containing 1 mM $CaCl_2$. The results showed that 1 mM $CaCl_2$ stabilized the enzyme for up to 4 h at 120 °C. Without the addition of calcium, Pernisine was stable at 90 °C, retaining 100% of activity after 4 h of incubation (Fig. 3), and had a half-life of 60 min at 100 °C, 40 min at 110 °C, and 30 min at 120 °C.

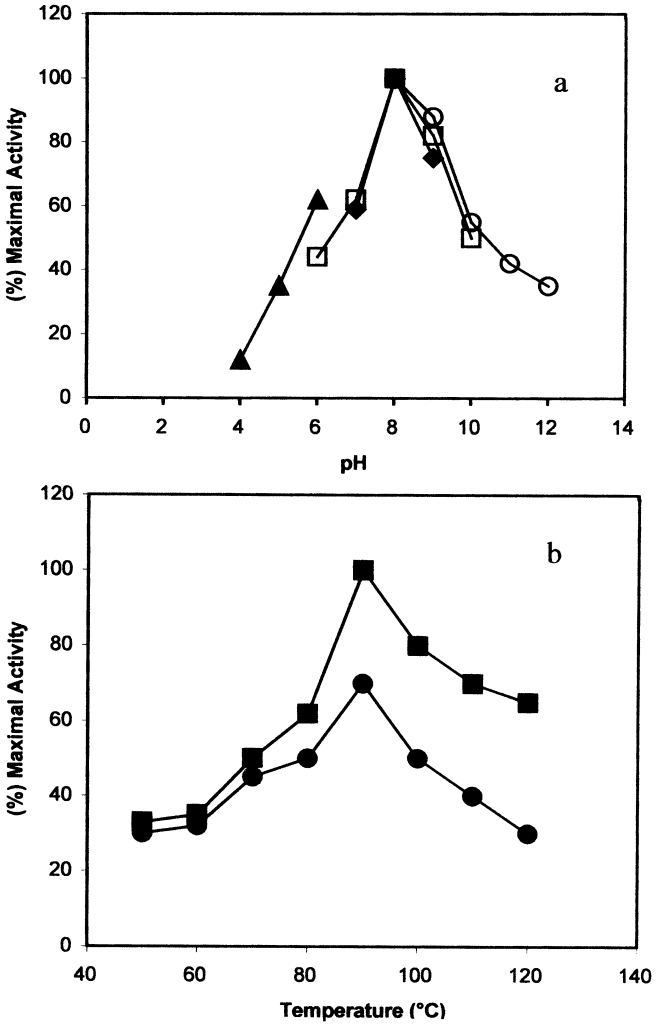

Fig. 2 **a** Effect of pH on proteolytic activity. Purified enzyme (1.5 μg) was incubated at 90 °C in the presence of the following buffers: 0.1 M sodium acetate (*solid triangle*), 0.1 M sodium phosphate (*solid diamond*), 0.1 M TRIS-HCl (*open square*), 0.1 M glycine-NaOH (*open circle*). **b** Effect of temperature on Pernisine activity. Purified enzyme (1.5 μg) was incubated in 50 mM TRIS-HCl pH 8.0 containing 1 mM $CaCl_2$ (*solid square*) and in 50 mM TRIS-HCl pH 8.0 (*solid circle*)

Effect of pH on enzymatic activity

Figure 2a shows the enzymatic cleavage of azocasein as a function of pH in the range between 5.0 to 12.0 at 90 °C. The maximum activity was reached at pH 8.0 under standard assay conditions. A relative activity of

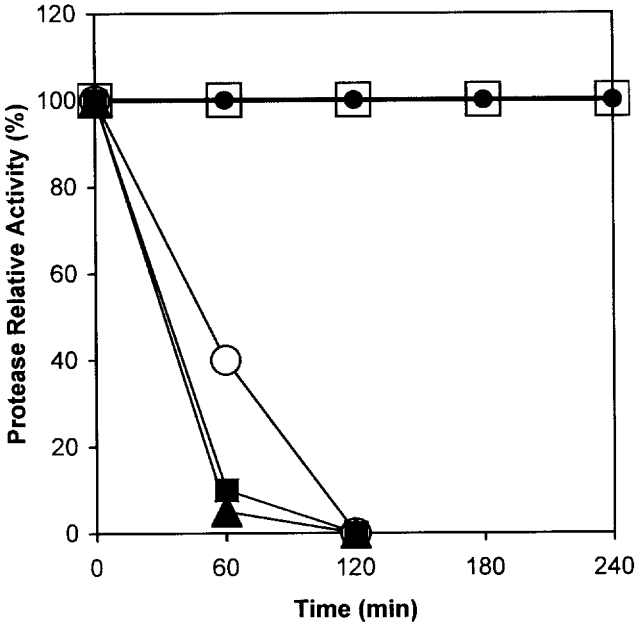

Fig. 3 Heat-stability of Pernisine at temperatures between 90 °C and 120 °C. Purified enzyme (3 μg/ml) was incubated at 90 °C (*solid circle*), 100 °C (*open circle*), 110 °C (*solid square*), 120 °C (*solid triangle*) in 50 mM TRIS-HCl pH 8.0. *Open squares* refer to enzyme in 50 mM TRIS-HCl pH 8.0 containing 1 mM $CaCl_2$ incubated at 90 °C, 100 °C, 110 °C, and 120 °C up to 4 h. Samples of enzyme solution were removed at 1-h intervals, chilled, and residual proteolytic activity determined at 90 °C by azocasein hydrolysis

about 50% was observed at pH 6.0, while at pH 10.0 and at pH 12.0 it was estimated to be about 55% and 40%, respectively.

Effect of inhibitors

In order to classify it, the Pernisine was challenged with different protease inhibitors. As shown in Table 2, the most effective inhibitors were PMSF, soybean trypsin inhibitor, and aprotinin that are typical serine proteinase inhibitors. In contrast TPCK and TLCK, respectively chymotrypsin and trypsin-like inhibitors, did not affect the activity. Metal chelators such as EDTA and EGTA inhibited the enzyme. Nevertheless, the zinc chelator 1,10-phenanthroline, usually used as a classic indicator for inhibiting metalloproteinases, had no effect on it. The cysteine-inhibitor *p*(-chloromercuri)benzoic acid partially affected proteinase activity. The data obtained suggest that Pernisine is a serine metalloproteinase.

Effect of denaturing agents on enzymatic activity

Pernisine activity was assayed in the presence of sulfhydryl and reducing agents (Table 3). When used at 5% final concentration, 2-mercaptoethanol reduced the activity by 80%, while DTT was less effective. The enzyme assayed in the presence of 4 M urea and 4 M guanidine-HCl showed a residual activity of 44% and 14%, respectively. Significant resistance was reported toward SDS, and the enzyme retained 20% proteolytic activity at 2% SDS, when assayed against a blank containing SDS. Nevertheless, when the assay was performed against a blank without SDS, residual activity was 190%, as described for aeropyrolysin (Sako et al. 1997).

Substrate specificity

The substrate specificity of Pernisine was investigated with a series of chromogenic peptide substrates. Pernisine had no activity on *N*-α-benzoyl-D-Arg-pNA, *N*-α-benzoyl-D-Tyr-pNA, and *N*-Suc-Ala-Ala-Ala-pNA used to detect trypsin, chymotrypsin, and elastase activity, respectively. However, Suc-Ala-Ala-Pro-Phe-pNA and Suc-Ala-Ala-Pro-Leu-pNA were efficiently hydrolyzed by Pernisine, with a percentage of 100% and 20%, respectively, suggesting that the enzyme preferably cleaves hydrophobic residues at the P1 site (nomenclature according to Schechter and Berger 1967), similarly to subtilisin BPN′ (Wells et al. 1987) and other subtilisin-like enzymes. Aminopeptidase activity was not detected. Among the proteins tested, casein showed the

Table 2 Effect of inhibitors on enzymatic activity

Inhibitor	Concentration	Class of inhibitor	Residual activity (%)
None	–	–	100
EDTA	1 mM	Metalloproteinase	10
EGTA	1 mM	Metalloproteinase	6
Phenanthroline	1 mM	Metalloproteinase	100
	5 mM		100
	10 mM		100
p-(Hydroxymercuri) benzoic acid	1 mM	Cysteine proteinase	89
	5 mM		40
	10 mM		20
Soybean trypsin	1 mg/ml	Serine proteinase	0
PMSF	1 mM	Serine proteinase	10
	5 mM		4
TLCK	1 mM	Serine proteinase	100
TPCK	1 mM	Serine proteinase	100

highest degree of susceptibility with 100% of hydrolysis, while in the case of hemoglobin, ovalbumin, and bovine serum albumin it was about 50%. Azocoll hydrolysis was also observed.

Identification of the Pernisine gene

On the basis of the estimated molecular weight of the Pernisine, a computational analysis of the sequenced genome of *A. pernix* was performed, and an ORF identified as 0263 was retrieved. The deduced amino acid sequence (440 residues) was compared to sequences deposited in a data bank using the BLAST program, and results revealed a strong similarity sequence to subtilisin-like proteases, showing an identity of 59% to *Thermococcus kodakaraensis* subtilisin, 36% for subtilisin from *Pyrobaculum aerophylum*, and 27% to pyrolysin from *Pyrococcus furiosus*. Furthermore, a high percentage of homology was found to subtilisins of mesophilic origin (Fig. 4). As reported for other subtilases, *A. pernix* putative enzyme consists of a presequence, a putative prosequence, and a putative catalytic domain of 36, 83, and 321 amino acid residues, respectively. The putative presequence was identified as a secretion signal by the Signal P program, version 2.0 worldwide server. The first amino acid of the mature form is Ala as suggested by multiple amino acid sequence alignment. So the gene was amplified from genomic DNA and cloned as reported in Materials and methods.

Table 3 Effect of denaturing agents on proteolytic activity

Reagent	Concentration	Residual activity (%)
None	–	100
2-Mercaptoethanol	1%	66
	5%	20
DTT	1 mM	90
	5 mM	71
Urea	2 M	86
	4 M	44
Guanidine-HCl	1 M	31
	4 M	14
SDS	0.2%	100
	0.5%	40
	1.0%	23
	2.0%	21

Expression and purification of recombinant Pernisine

To obtain Pernisine, the expression plasmid pGEX-Pernisine was constructed. Pernisine lacking presequence was expressed as a fusion protein to glutathione-*S*-transferase in *E. coli* BL21 DE3 strain. The recombinant *A. pernix* protease was expressed in soluble form with high proteolytic activity for hydrolysis of 0.1% azocasein at 90 °C. When the crude extract containing 5 mM PMSF was analyzed on 12.5% SDS-PAGE, the protein expressed was not distinctively observed in Coomassie stained PAGE gel (data not shown); nevertheless, when no PMSF was added, small peptides were evident as degradation products. Therefore it was assumed that proteolysis occurred because of the presence of Pernisine.

The enzyme concentration was about 2 mg/l of culture and was purified 38-fold to a specific activity of 3,000 U/mg. The molecular mass of the purified protease estimated by SDS-PAGE (35.2 kDa) and by gel filtration chromatography (35.16 kDa) is in agreement with the molecular mass determined for the native enzyme. Detection of proteolytic activity on 0.1% casein overlay revealed the presence of a single band of activity corresponding to the native one.

The properties of recombinant Pernisine were determined and results showed that the recombinant enzyme maintained the same characteristics as the native protease.

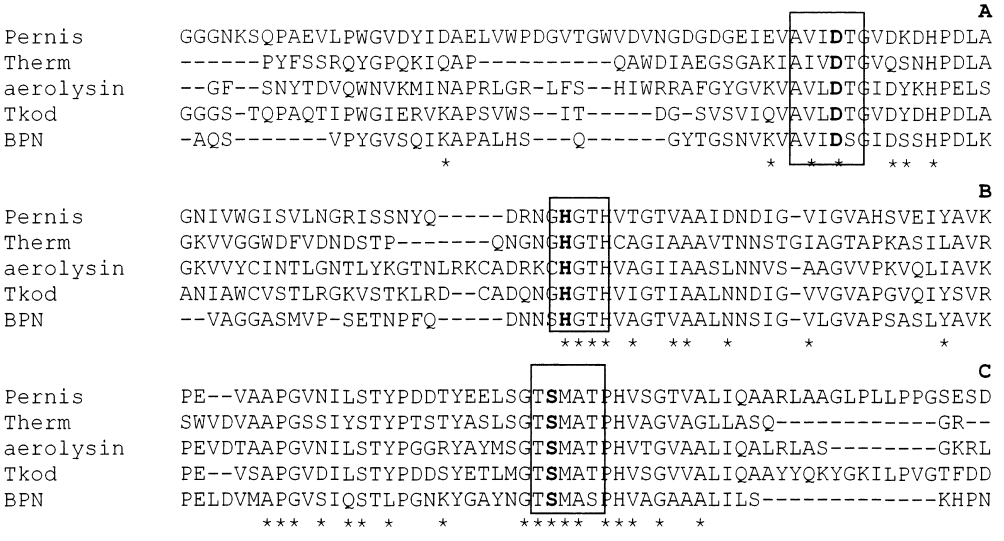

Fig. 4 Catalytic domain sequence alignment of *A. pernix* protease (*Pernis*), thermitase (*Therm*), *Pyrobaculum aerophilum* protease (*aerolysin*), *Thermococcus kodakaraensis* subtilisin (*Tkod*), and subtilisin BPN (*BPN*). *Boxed residues* correspond to conserved amino acids around catalytic residues D, H, S that are indicated in *bold*

Discussion

Pernisine was identified as a member of the subtilisin family. The presence of extracellular proteolytic activity in *A. pernix* was previously reported by Sako et al. (1997) who described the biochemical characterization of aeropyrolysin, a 52-kDa molecular mass metalloprotease. In this study we report the isolation of a new 34-kDa extracellular protease from *A. pernix*, designated Pernisine. The gene of this serine protease has also been cloned and successfully expressed in *E. coli* producing a protein in its active form. Based on biochemical data of native Pernisine (low molecular weight, inhibition assays, substrate specificity) together with the deduced amino acid sequence of the cloned gene, it is proposed that Pernisine be considered a new member of the subtilisin-like enzyme family. Among hyperthermophilic serine protease sequences isolated to date, *T. kodakaraensis* subtilisin and *P. aerophylum* aerolysin have been shown to belong to the subtilisin family, unlike pyrolysin from *P. furiosus* and stetterlysin from *T. stetteri* that have been classified as members of the pyrolysin family according to subtilase classification (Siezen and Leunissen 1997). *Aeropyrum pernix* putative catalytic domain shows 59% identity with *T. kodakaraensis* subtilisin (Kannan et al. 2001), and 41% with aerolysin from *P. aerophylum* (Volkl et al. 1994), while the percentage of identity is only 34% with *Haloferax mediterraneii* (Kamekura et al. 1996) and 27% with pyrolysin from *P. furiosus* (Voorhorst et al. 1996). A high percentage of identity is also encountered with subtilisins of mesophilic origin, such as subtilisin from *Bacillus subtilis* LG12 (Schmidt et al. 1995) that produced a score of 42%.

In spite of the increasing number of genes encoding subtilases found in Archaea, the expression experiments have not always been successful. So far, halolysin R4 from *H. mediterranei* (Kamekura et al. 1996) and a subtilisin-like protease from *T. kodakaraensis* have been expressed in active molecules (Kannan et al. 2001). Pernisine was cloned as a fusion protein with GST. Normally, thermophilic enzymes that are fused to GST are inactive at their optimum temperature because at elevated temperatures the mesophilic GST portion of the fusion is denatured and precipitates. The GST portion is generally removed by cleavage with thrombin. In the case of Pernisine protease, our data suggest that the GST is removed along the proregion maturation, which is not dependent on temperature activation.

The role of the calcium ions seems to be linked to the stabilization of the enzyme more than needed for its activity. In fact the binding of calcium at specific sites, as demonstrated for other subtilases (Siezen et al. 1991) increases the stability of these enzymes reducing the flexibility of the molecule and hence the denaturation and/or its role of autolysis.

Analysis of the purified enzyme in native and denaturing conditions showed that Pernisine is composed of a single polypeptide chain with a molecular mass of 34 kDa. However, upon storage at 4 °C in the absence of $CaCl_2$ a smaller band of 24 kDa with proteolytic activity was observed on SDS-PAGE (data not shown). This event was thought to depend on the autolysis of the enzyme in which Ca^{2+} ions play a key role in preserving enzymatic structure.

On the basis of inhibition assays Pernisine was defined as a serine metalloprotease. In fact, activity is strongly inhibited in the presence of 1 mM PMSF and other serine proteinase inhibitors but also by chelator agents such as EDTA and EGTA which produce 90% and 94% inhibition, respectively. As reported by other authors, many enzymes classified as serine proteinases have been shown to be susceptible to EDTA. They include proteases isolated from the fungi *Aspergillus fumigatus* (Frosco et al. 1992), *A. flavus* (Kolattukudy et al. 1993), *A. niger* (Barthomeuf et al. 1989), *Aureobasidium pullulans* (Donaghy and McKay 1993), *Trikoderma koningii* (Manonmani and Joseph 1993), as well as thermophiles and mesophiles of bacterial origin such as the genus *Thermus* (Matsuzawa et al. 1988; Peek et al. 1992), *Burkholderia pseudomallei* (Lee and Liu 2000), *Xanthomonas maltophila* (Debette 1991), and the well-studied proteases from *Bacillus* spp. Among the hyperthermophilic serine proteases, pyrolysin was shown to be susceptible to EDTA (Eggen et al. 1990). The effect of EDTA and EGTA may be attributed then to the destabilization of the enzyme structure by removal of calcium ions and therefore the loss of activity was due to enzyme denaturation rather than inhibition.

The effect of EDTA on Pernisine was also investigated by analysis on 12.5% SDS-PAGE gel. The data reveal that no proteic band is observed on the gel after electrophoresis, suggesting that Pernisine is subject to an autolytic mechanism determined by chelation of Ca^{2+} by EDTA (Wells and Estell 1988) as reported for subtilisin and other subtilisin-like enzymes (Reichard et al. 1990; Frosco et al. 1992; Abraham and Breuil 1996). Thus, removal of calcium by chelation increases the flexibility of the protein, and therefore its rate of autolysis (Siezen et al. 1991).

Four calcium binding sites have been found by crystal structure determinations of subtilisins, thermitase, and proteinase K, and from sequence alignments and homology modeling (Siezen and Leunissen 1997) it was predicted that Ca1 (strong) and Ca3 (weak) sites are most common in members of the subtilase family, whereas Ca2 (medium strength) site is less common. The weak Ca4 site has been found in proteinase K. Amino acidic sequence analysis of Pernisine revealed that presumably two calcium binding sites, Ca1 and Ca3, might be present. The Ca1 site requires coordination from side chain ligands of residues 2 and 41 and from several side chains of residues 76–81 in the Ca^{2+} embranching loop. Preliminary data suggest that Pernisine Q^2 and S^{78}

residues are replaced by E^2 and D^{78} residues. Likewise, the amino acids that form the Ca3 site (K^{170} to V^{174} and E^{195} to D^{197}) at which the Ca^{2+} ion binds with lower affinity, are relatively well conserved in the Pernisine sequence.

More investigations at structural level will contribute to clarify the mechanisms involved in protein stabilization.

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