An extremely heat-stable extracellular proteinase (aeropyrolysin) from the hyperthermophilic archaeon *Aeropyrum pernix* K1

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Abstract An extracellular metalloproteinase, which we had designated aeropyrolysin, from the aerobic marine hyperthermophilic archaeon *Aeropyrum pernix* K1 (JCM 9820), was purified by ammonium sulfate precipitation, anionic exchange chromatography, and gel filtration chromatography. The purified enzyme was composed of a single polypeptide chain with a molecular mass of 52 kDa as determined by SDS-PAGE. The proteinase had a broad pH optimum (pH 5–9) with a maximal activity at pH 6–8 for azocasein hydrolysis. The optimum temperature for enzyme activity was 100°C in the absence of 1 mM $CaCl_2$ and 110°C in the presence of 1 mM $CaCl_2$. The enzyme activity was completely inhibited by EDTA and EGTA, indicating that it was a metalloproteinase. The enzyme was highly resistant to the denaturing reagents urea, guanidine-HCl, dithiothreitol, 2-mercaptoethanol and SDS. The enzyme also showed a high activity with the metalloproteinase specific substrate MOCAc-Pro-Leu-Gly-Leu-A_{2pr}(Dnp)-Ala-Arg-NH_2. The enzyme was extremely thermostable showing half-lives of 2.5 h at 120°C and 1.2 h at 125°C in the presence of 1 mM $CaCl_2$. These results indicate that this enzyme is one of the most thermostable extracellular proteinases reported to date.

Key words: Heat-stable; Extracellular proteinase; *Aeropyrum pernix*; Metalloproteinase; Hyperthermophilic archaeon

1. Introduction

Recently, studies on hyperthermophilic archaea have been increasing due to the fact that they are considered an important biotechnological resource [1,4,6]. The enzymes from hyperthermophilic archaea are more thermostable than their counterparts isolated from thermophilic or mesophilic microorganisms [7,8]. In addition, the properties which allow thermostable enzymes to withstand high temperatures also confer resistance to other factors such as detergents and denaturing agents, thereby offering great potential advantages for a range of biotechnological processes [1].

To date, a limited number of proteinases from hyperthermophilic archaea have been reported [7,10,15,17]. The most thermostable proteinases have been purified from the genera *Pyrococcus* and *Desulfurococcus*; some of the reported enzymes are: a cell-envelope associated serine proteinase (pyrolysin) from *P. furiosus* [10] which is probably the most thermostable proteinase reported to date, with a half-life of 4 h at 100°C; an extracellular cysteine protease from *Pyrococcus* sp. KOD1 [20] with a half-life of 60 min at 100°C; a sodium dodecyl sulfate resistant serine proteinase (S66) from *P. furiosus* [2], which has a half-life of 33 h at 98°C; an extracellular serine proteinase (archaelysin) from *Desulfurococcus* sp. strain $Tok_{12}S_1$ [7] with a half-life of 9 min at 105°C; and an intracellular serine proteinase (D.SY) from *Desulfurococcus* sp. strain SY [15] with a half-life of 2.5 h at 98°C. All these proteinases are produced by anaerobic hyperthermophilic microorganisms which have a relatively low cell yield when grown in large scale cultures. Thus, the use of these anaerobic hyperthermophiles as source material for the purification of enzymes presents some problems in obtaining sufficient quantities of cell material for biotechnological applications.

Aeropyrum pernix [22] is the first strictly aerobic hyperthermophilic archaeon that has been described to date. It grows optimally above 90°C with a doubling time of 200 min. Large scale culture can be achieve with reasonable growth yield (1 g wet weight/l) in 1 day. These characteristics make this archaeon a potential biotechnological resource for the purification of new enzymes.

In this study, we report on the purification and characterization of a new heat-stable extracellular metalloproteinase from *Aeropyrum pernix*. The enzyme has been designated aeropyrolysin, derived from the prefix 'aeropyro' (Greek, air and fire) and the suffix 'lysin' (Latin, to cleave).

2. Materials and methods

2.1. Bacterial strain

Aeropyrum pernix strain K1 (Japanese Collection of Microorganism; JCM 9820) was originally isolated from a coastal solfataric thermal vent in Kodakara Island, Japan [22]. Strain K1 is a Gram-negative irregular coccoid and cells grow under strictly aerobic conditions. The optimum cell growth is observed at 90–95°C [22].

2.2. Culture and growth of Aeropyrum pernix strain K1

Cells were grown at 90°C for 42 h (early stationary phase) with shaking (150 rpm) using an air bath rotary shaker (RGS-32.TT; Sanki Seiki, Osaka, Japan) in 500 ml of JXT medium containing Jamarin S, trypticase peptone, yeast extract and thiosulfate [22] in a 2000 ml Erlenmeyer flask. After 42 h of incubation, the culture was centrifuged at 6500×*g* for 30 min at 4°C. The supernatant (20000 ml) was collected and concentrated (100-fold) by ultrafiltration (Minitan system; Millipore Corporation, Bedford, MA).

2.3. Enzyme purification

Unless otherwise stated, all purification steps were carried out at 0–5°C. The concentrated supernatant (200 ml) was taken to 80% saturation with solid ammonium sulfate and the precipitate was collected by centrifugation (12000×*g*, for 30 min at 4°C), dissolved in 50 mM Tris-HCl buffer (pH 8.0), and dialyzed for 12 h with three changes against the same buffer.

The dialyzed enzyme solution was applied to a column of Q 16/10 (HP) Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) after being washed with five bed volumes of the same buffer. The column was eluted with a continuous linear gradient of NaCl (0–1 M) in the buffer at a flow rate of 5 ml/min. The active fractions which eluted around

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0.7 M NaCl were collected and dialyzed against the same buffer. The dialyzed enzyme solution was applied to a DEAE-cellulose column (2.6×10 cm; Whatman Int. Ltd., Maidstone, UK) that had been washed and equilibrated with 50 mM Tris-HCl pH 8.0. Proteins were eluted with a 150 ml linear gradient of 0–1 M NaCl in 50 mM Tris-HCl pH 8.0. The active fractions eluted at 0.6 M NaCl were combined and dialyzed against 50 mM Tris-HCl pH 8.0. The dialyzed enzyme solution was then applied to a Mono Q HR 5/5 column (Pharmacia) equilibrated with 50 mM Tris-HCl pH 8.0. After being washed with 20 bed volumes of the same buffer, the column was eluted with a linear gradient of NaCl (0–1.0 M) in the same buffer at a flow rate of 0.4 ml/min. The active fractions were collected and dialyzed against 50 mM Tris-HCl buffer pH 8.0.

For further purification, the dialyzed enzyme solution (5 ml) was concentrated to 0.2 ml using Ultrafree centrifuge tubes (Millipore) 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. Purified enzyme samples from the active fraction of this column were used to characterize the properties of aeropyrolysin.

2.4. Enzyme and protein assays

Proteinase activity was determined by the hydrolysis of azocasein (Sigma) under the following standard conditions. The reaction mixture consisted of 100 μl of enzyme solution, 500 μl of 0.1% (w/v) azocasein, and 400 μl of 50 mM Tris-HCl pH 8.0. Triplicate mixtures were incubated at 90°C for 20 min and the reaction was initiated by addition of the enzyme solution. The enzyme reaction was stopped by addition of 2 ml of 7% (v/v) perchloric acid and was kept for a further 15–20 min at room temperature. The reaction mixture was then centrifuged (Tomy MRX-150, Tomy Seiko, Tokyo, Japan) for 10 min at 12000×*g* and the absorbance of the supernatant was measured at 430 nm against a blank (complete reaction mixture stopped before incubation). One unit of proteinase activity was defined as giving an absorbance change of 0.1/min.

Protein was determined following the Bradford method [3] using the standard assay kit from Bio-Rad (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as a standard.

2.5. Electrophoresis

The homogeneity of the purified enzyme was examined by SDS-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 [19]. Proteins bands were visualized by Coomassie brilliant blue R-250 staining.

2.6. Molecular mass determination

The molecular mass of the enzyme was determined by SDS-PAGE by comparison with the migration of standard protein markers ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine albumin (66 kDa) and ribonuclease (13.7 kDa) (Pharmacia). The molecular mass of the native enzyme was also determined by gel filtration chromatography on a Superose 12 HR 10/30 column (Pharmacia) using as standard protein markers, aldolase (158 kDa), bovine albumin (66 kDa) and ovalbumin (43 kDa) (gel filtration calibration kit, Pharmacia). Blue dextran was used for calculation of the void volume.

2.7. Effect of temperature on proteolytic activity

The optimum temperature for proteolytic activity was examined in the presence and absence of $CaCl_2$. For the determination 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. The thermal equilibration time was less than 3 min. For temperatures below 95°C test tubes were used.

2.8. Effect of pH on proteolytic activity

The proteolytic activity of the enzyme was measured at 90°C for 20 min at different pH ranges. For this assay, azocasein was dissolved at a concentration of 0.1% (w/v) in volumes of the following buffers: acetate/sodium acetate (pH 2.5–6.0), phosphate (pH 6.0–8.5) and Tris-HCl (pH 6.5–9.0).

2.9. Effect of proteinase inhibitors

Enzyme samples were preincubated at room temperature for 15 min in the presence of the following proteinase inhibitors: 1 mM, 10 mM EDTA (ethylenediamine tetraacetic acid); 1 mM, 10 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 (diisopropyl fluorophosphate); 1 mM, 10 mM phenanthroline; 1 mM *N*-ethylmaleimide; 1 mg/ml soybean trypsin inhibitor; 1 mM, 10 mM iodoacetic acid; 1 mM, 10 mM *p*-(chloromercuri)benzoic acid (Wakenyaku Co., Kyoto, Japan). Remaining activity was determined by azocasein hydrolysis at 90°C for 20 min under the standard condition.

2.10. Substrate specificity

The substrate specificity for proteolytic activity was determined by incubating 5 μl enzyme solution (3.9 μg/ml) with 995 μl of 5 μM MCA and MOCAc labeled peptides (Peptide Institute Inc., Osaka, Japan) in 50 mM Tris-HCl buffer pH 8.0 at 90°C. Samples were removed at 5 min intervals. Proteolytic activity was detected by measuring in a fluorospectrometer (RF-1500, Shimadzu, Kyoto, Japan) the fluorescence of released 7-amino-4-methyl-coumarin (ACM) and 7-methoxycoumarin-4-yl acetyl (MOCAc) at an excitation of 376 nm, emission of 460 nm and excitation 343 nm emission 393 nm, respectively. Catalytic activity was estimated from fluorescence of standard AMC and MOCAc-Pro-Leu-Gly and was expressed as nmol of AMC or MOCAc-Pro-Leu-Gly per min per ml of enzyme solution.

2.11. Effect of denaturing reagents on proteolytic activity

Proteinase activity was measured in the presence of the following chaotropic agents: 4 M, 8 M urea; 1 M, 4 M guanidine-HCl; 1 mM, 5 mM, 10 mM dithiothreitol (DTT); 0.1%, 1%, 5% (w/v) 2-mercaptoethanol; and 1%, 2% SDS. The reaction mixtures (1 ml) containing azocasein and 100 μl enzyme solution were incubated at 90°C for 20 min. The proteolytic activity was measured under the standard condition.

2.12. Thermostability

Purified enzyme (2.5 μg/ml in 50 mM Tris-HCl pH 8.0 containing 1 mM $CaCl_2$) was incubated in sealed 2 ml glass capillary tubes at 90°C, 100°C, 110°C, 120°C and 125°C in a temperature controlled oil bath. Aliquots (500 μl) were removed at intervals, chilled on ice and subsequently the remaining activity was assayed at 90°C under the standard condition. Enzyme solution in 50 mM Tris-HCl pH 8.0 in the absence of 1 mM $CaCl_2$ was assayed as a native enzyme.

3. Results

3.1. Purification of aeropyrolysin

A single proteinase from the culture supernatant of *Aeropyrum pernix* strain K1 was purified by ammonium sulfate

Table 1
Summary of purification of an extracellular proteinase (aeropyrolysin) from *Aeropyrum pernix*

Preparation	Volume (ml)	Total protein (mg)	Total activity (U)	Yield (%)	Specific activity (U/mg)	Purification (fold)
Supernatant	20000	N.D.	N.D.	N.D.	N.D.	N.D.
$(NH_4)_2SO_4$	80	58	130000	100	1961	1
Q Sepharose	40	25	70400	54	2843	1.5
DEAE-cellulose	18	10	219600	169	21960	11.2
Mono Q	5	3.6	128500	99	35694	18.2
Superose 12	2.5	1.3	73750	57	57617	29.3

N.D.: direct determination on supernatant was not done due to detection sensitivity error.

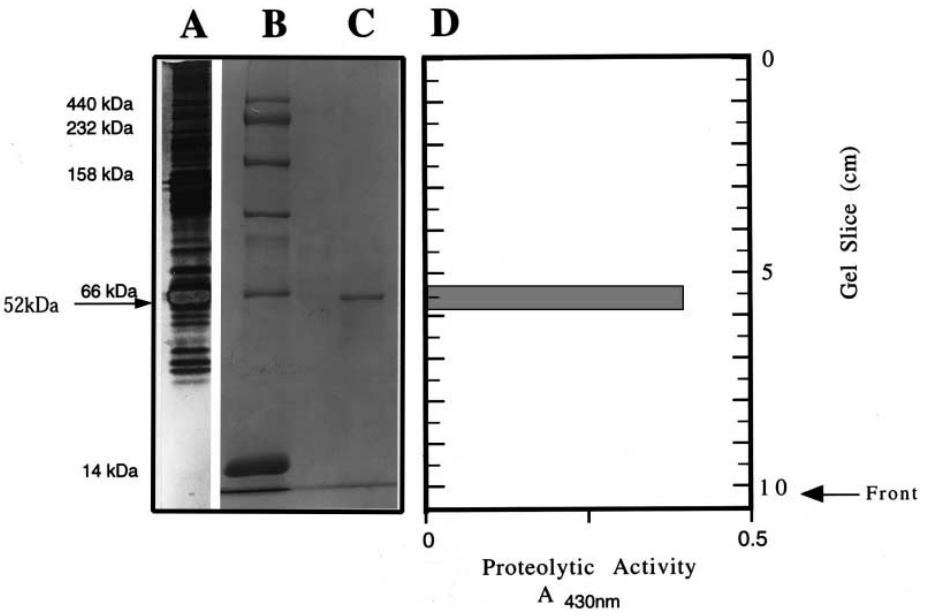

Fig. 1. SDS-PAGE of purified aeropyrolysin (C), crude extract (A) and proteolytic activity of the gel fractions sliced every 3 mm (D). Purified enzyme sample from Superose 6 (3 μg) was loaded on 10% SDS-PAGE and stained with Coomasie brilliant blue R-250. Standard proteins, molecular mass values are indicated (B).

fractionation, Q Sepharose, DEAE-cellulose, Mono Q and Superose 12 gel filtration chromatography as described in Section 2. Measurements of proteinase activity during cell culture indicated that the maximum enzyme production of the enzyme was obtained at the early stationary phase (42 h). Measurements of enzyme activity were not achieved directly in supernatant samples because of the low enzyme concentration and its low detection sensitivity. The purification of the enzyme is summarized in Table 1. The proteinase aeropyrolysin was purified 29-fold with a specific activity of about 57 617 U/mg and a final yield of 57%.

The purified sample of aeropyrolysin, after elution from a Superose 12 column, showed a single protein band on SDS-PAGE, corresponding to the active fraction detected by gel slicing (Fig. 1).

3.2. *Molecular mass determination of purified aeropyrolysin*

The molecular mass of the enzyme determined by SDS-PAGE was 52 kDa (Fig. 1). This result is in good agreement with the estimation of the molecular mass of the enzyme by Superose 12 gel filtration chromatography, which gave a molecular mass of 53 kDa (data not shown). These results indicate that the enzyme is a monomeric protein.

3.3. *Effect of temperature and cations on enzymatic activity*

The effect of temperature on the proteolytic activity of the enzyme was examined in the presence and absence of 1 mM $CaCl_2$ (Fig. 2A). The maximal enzymatic activity was obtained at 110°C in the presence of $CaCl_2$. In the absence of $CaCl_2$, the maximal activity observed was obtained at 100°C. Below 100°C the enzyme activity did not show significant differences either with or without $CaCl_2$. Concentrations higher than 1 mM $CaCl_2$ showed an inhibitory effect on enzymatic activity (data not shown). The effect of divalent cations at different concentrations on the activity of the enzyme were also examined (data not shown). The divalent cations tested

Table 2
Effect of proteinase inhibitors on the activity of aeropyrolysin

Inhibitor	Concentration	Class of inhibitor	Residual proteinase activity (%)
None			100
EDTA	1 mM	Metalloproteinase	0
	10 mM		0
EGTA	1 mM	Metalloproteinase	4
	10 mM		2
Phenanthroline	1 mM	Metalloproteinase	108
	10 mM		104
PMSF	1 mM	Serine proteinase	55
	5 mM		3
DFP	1 mM	Serine proteinase	19
	5 mM		1
Soybean trypsin	1 mg/ml	Serine proteinase	18
N-Ethylmaleimide	1 mM	Cysteine proteinase	109
Iodoacetic acid	1 mM	Cysteine proteinase	100
	10 mM		100
p-(Chloromercuri)benzoic acid	1 mM	Cysteine proteinase	95
	10 mM		80

(Mg^{2+}, Zn^{2+}, and Mn^{2+}) had no effect on enzymatic activity either below or above 100°C.

3.4. pH optimum

The enzyme showed a broad pH optimum (pH 5–9) for the hydrolysis of azocasein (Fig. 2B) with an apparent maximal activity at pH 6–8. The proteolytic activity was markedly decreased below pH 5 and above pH 9.

3.5. Effect of proteinase inhibitors

In order to determine the type of proteinase produced by *Aeropyrum pernix*, different protease inhibitors were tested. The proteinase activity was completely inhibited by 1 mM EDTA and EGTA (Table 2) which are typical metalloproteinase inhibitors. However, phenanthroline did not inhibit the proteolytic activity. The serine proteinase inhibitors PMSF and DFP partially inhibited the enzymatic activity at high concentration. The cysteine proteinase inhibitors *N*-ethylmaleimide, iodoacetic acid and *p*-(chloromercuri)benzoic acid had no effect on the activity. From these results it is suggested that the present enzyme is a metalloproteinase.

3.6. Substrate specificity

The substrate specificity of the purified enzyme was also examined using several fluorescent peptides (Fig. 3). MOCAc-Pro-Leu-Gly-Leu-A_2 (Dnp)-Ala-Arg-NH_2 peptide, which is a specific substrate for metalloproteinase, was efficiently hydrolyzed by the enzyme. On the other hand, hydrolysis of MCA peptides was hardly detected, although a low activity against chymotrypsin and trypsin specific substrates was detected.

3.7. 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 all the denaturing reagents tested at 90°C. In addition, an increase in the enzymatic activity was observed in the presence of high concentrations of SDS.

3.8. Heat stability of the enzyme

Fig. 4 shows the thermostability of the purified proteinase in the presence of 1 mM $CaCl_2$. The proteolytic activity was not lost by heating at 90°C, 100°C and 110°C for over 4 h. The half-life of the enzyme was 150 min at 120°C and 70 min at 125°C. The half-life of the enzyme in the absence of $CaCl_2$ was 20 min at 100°C. Higher concentrations than 1 mM of calcium did not affect the thermostability of the enzyme, but the presence of 1 mM $CaCl_2$ markedly stabilized the enzyme at temperatures over 100°C.

Table 3
Effect of denaturing agents on aeropyrolysin activity

Reagent	Concentration	Proteolytic activity (%)
None		100
SDS	1%	93
	2%	263
Urea	2 M	110
	4 M	100
Guanidine-HCl	1 M	100
	4 M	63
Dithiothreitol	1 mM	92
	5 mM	67
2-Mercaptoethanol	1%	69
	5%	44

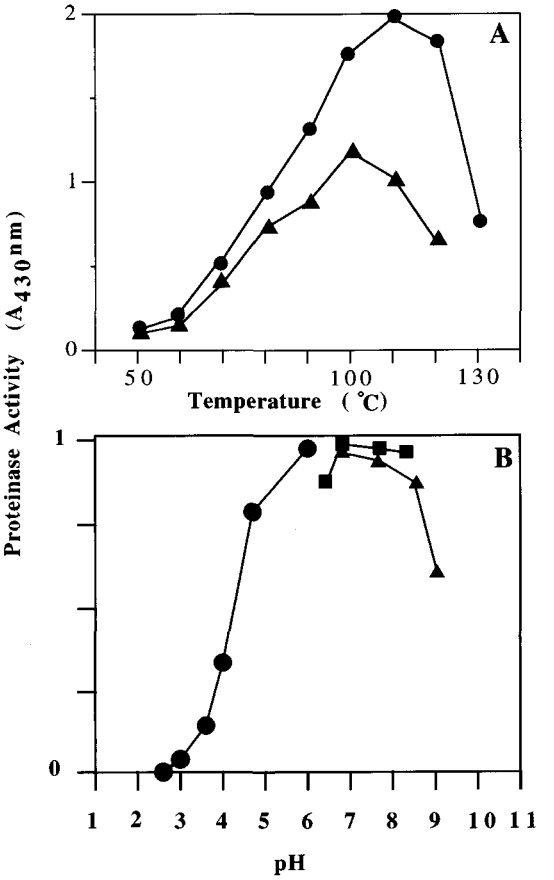

Fig. 2. Effect of temperature (A) and pH (B) on aeropyrolysin proteolytic activity. A: Proteolytic activity was assayed at the indicated temperature in the presence (●) and absence (▲) of 1 mM $CaCl_2$ under the standard condition. B: Proteolytic activity was assayed using the following buffers: 100 mM sodium acetate (●), 100 mM sodium phosphate (■) and 100 mM Tris-HCl (▲) under the standard conditions.

4. Discussion

In general, most of the proteinases purified from hyperthermophiles are composed of a single polypeptide chain, belong to serine enzyme and are active in very broad pH and temperature ranges [2,7,10,15,17,18]. In this study we report on the purification and characterization of an extracellular proteinase, aeropyrolysin, from the hyperthermophilic archaeon *Aeropyrum pernix* K1. Aeropyrolysin is a monomeric metalloproteinase with a broad temperature (70–130°C) and pH profile (pH 5–9) and its optimum temperature for azocasein hydrolysis is 110°C in the presence of calcium ion. The highest optimum temperature for substrate hydrolysis previously reported for pyrolysin was 115°C from *Pyrococcus furiosus* [10] and for archaelysin 100°C from *Desulfurococcus* sp. [7]. Although the temperature for optimal activity of aeropyrolysin is similar to these proteinases, the thermostability of aeropyrolysin is greater than any proteinase described to date. Pyrolysin has a half-life of 3 min at 110°C [10] and archaelysin has a half-life of 9 min at 105°C [7]. Aeropyrolysin showed a half-life of 150 min at 120°C and at 125°C, a temperature

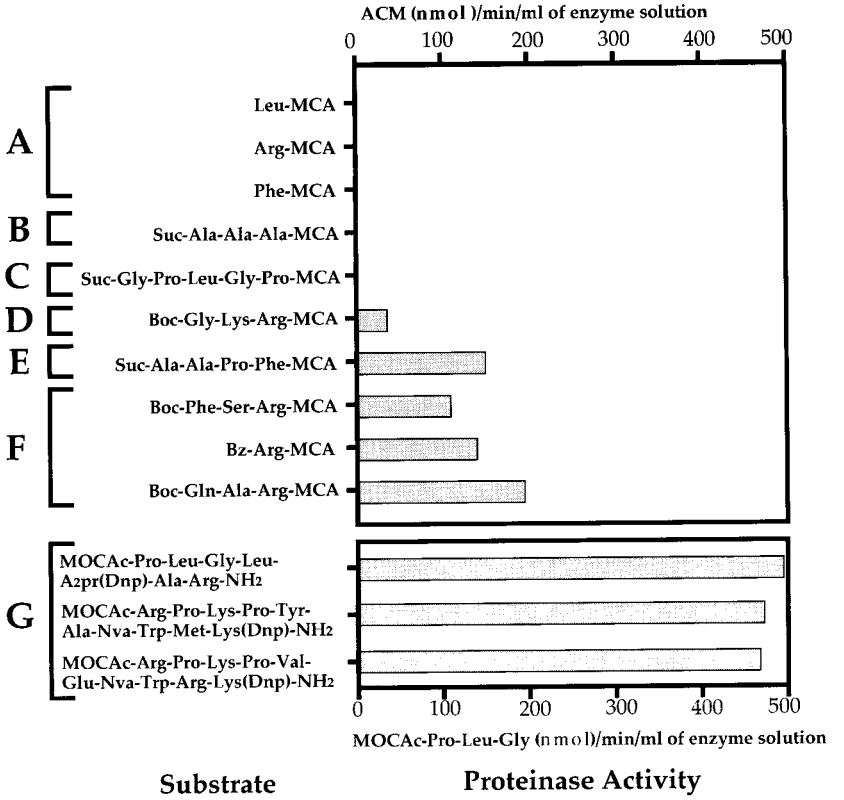

Fig. 3. Substrate specificity of aeropyrolysin. A, B, C, D, E, F and G indicate the substrates for carboxyaminopeptidase, esterase, collagenase-like peptidase, carboxyl side of paired basic residue cleaving enzyme, chymotrypsin, trypsin and metalloproteinase, respectively.

where almost all proteinases are denatured rapidly, aeropyrolysin has a half-life of 70 min.

Aeropyrolysin resembles the proteinases purified from thermophilic bacteria such as the thermolysin from *Bacillus thermoproteolyticus* [11,23], a proteinase from *Thermus* sp. [21], and a proteinase from *Bacillus stearothermophilus* [26]. These enzymes are extracellular metalloproteinases and require calcium for enzymatic activity and stability. However, the optimum temperature for activity and the thermostability of aeropyrolysin are much higher than these proteinases.

The effect of calcium ion on thermophilic proteinases has been widely studied [9,11,12,14,16,25,27]. In most cases calcium has no effect on enzymatic activity but it has an important role for enzyme stability. The role of calcium as stabilizing factor is not always clear, but in many cases it is not replaced by other cations like magnesium or manganese [5]. The activity of aeropyrolysin for azocasein hydrolysis was strongly stimulated by calcium at temperatures above 100°C but below 100°C this effect was slight. Below 100°C, the presence of 1 mM $CaCl_2$ increased the enzyme catalytic activity by about 20%, but above 100°C this increment was higher than 70%. This effect was clearer when the specific substrate MOCAc-Pro-Leu-Gly-Leu-A_2 (Dnp)-Ala-Arg-NH_2 was used (data not shown), where no differences in enzymatic activity were observed only at temperatures below 100°C. From these results it can be suggested that calcium ion plays an important role in the conservation of enzyme conformation and in the catalytic activity at temperatures above 100°C.

The resistance of aeropyrolysin to several denaturing agents is interesting for both mechanistic implications and biotechnological applications. The resistance to urea and guanidine-HCl might indicate that hydrogen bonds play little part in protein stabilization. The enzyme sensitivity to 2-mercaptoethanol might suggest that disulfide-like bonds are involved in preserving enzymatic structure. The increase of activity in the presence of SDS was unexpected; this could be attributed either to the sensitivity of the substrate to SDS, making it

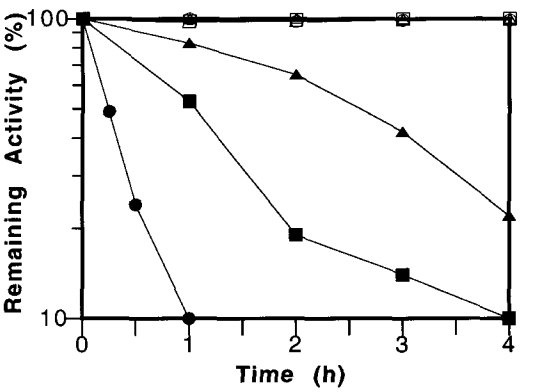

Fig. 4. Heat stability of aeropyrolysin at temperatures between 90°C and 125°C. Purified enzyme (2.5 μg/ml in 50 mM Tris-HCl pH 8 containing 1 mM $CaCl_2$) was incubated at 90°C (○), 100°C (□), 110°C (△), 120°C (▲) and 125°C (■). Native enzyme (●) was used as a control in the abscence of 1 mM $CaCl_2$ at 100°C. Samples of enzyme solution were removed at intervals, cooled, and the residual poteolytic activity was determinated as described in Section 2.

more susceptible to enzyme hydrolysis or to the exposure of the active site of the enzyme.

The unexpected increase of total activity during the purification of aeropyrolysin, clearly observed after DEAE-cellulose column, could be attributed to the removal of an inhibitory agent present in the enzyme solution. For the separation of this unknown agent, the use of DEAE-cellulose column was essential; supernatant samples applied directly to this column also increased the total activity compared to the Q Sepharose column. However, for optimal purification of the enzyme, Q Sepharose column was needed. The presence of an inhibitory agent could also explain the low proteolytic activity in supernatant samples. Considering that aeropyrolysin can be produced by *Aeropyrum pernix* under aerobic conditions at 90°C, with high cell yield and with low risk of contamination by other bacterial strains, that it is highly resistant to denaturing agents, and that it has a thermostability higher than the enzymes purified from anaerobic hyperthermophilic microorganisms, aeropyrolysin is an attractive candidate for various biotechnological applications.

The physiological significance of aeropyrolysin is not clear, but it might play an important role for the heterotrophic *Aeropyrum pernix* K1, allowing it to degrade proteinaceous material in its natural environment. This is strongly suggested by the fact that *Aeropyrum pernix* K1 was able to grow on proteinaceous complex substrates such as yeast extract, trypticase peptone, tryptone or nutrient broth but not on casamino acids [22]. This fact has also been observed in *Pyrococcus furiosus* [13], which can grow either in a defined medium with amino acids as a sole carbon source or in complex media containing casamino acids or tryptone [24]. However, since most of the proteinases previously reported for that archaeon are intracellular enzymes, they are more likely to be related to intracellular protein turnover [2]. Since aeropyrolysin is produced extracellularly, it can be suggested that it plays an important role in the uptake of nutritional substrates from the environment. The characteristics of aeropyrolysin, in addition to their possible physiological role, indicate their importance in enabling *Aeropyrum pernix* K1 to adapt to its extreme environment.

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