An extremely thermostable extracellular proteinase from a strain of the archaebacterium *Desulfurococcus* growing at 88 °C

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An organism growing at 88 °C that closely resembles *Desulfurococcus mucosus* produced a single extracellular proteinase. We have purified this enzyme and carried out a preliminary characterization. The proteinase, which is a serine-type enzyme, had a molecular mass of 52 000 Da by SDS/polyacrylamide-gel electrophoresis, but only 10 000–13 000 Da by gel-permeation chromatography. Molecular mass values from sucrose-gradient centrifugation were of the same order as those from SDS/polyacrylamide-gel electrophoresis. It had an isoelectric point of 8.7, and was inhibited by di-isopropyl phosphorofluoridate, phenylmethanesulphonyl fluoride and chymostatin. Substrate-specificity studies suggested a possible preference for hydrophobic residues on the C-terminal side of the splitting point. The thermostability of this enzyme is probably greater than any other reported proteinase ($t_{1/2}$ at 95 °C, 70–90 min; $t_{1/2}$ at 105 °C, 8–9 min). Ca^{2+} chelation does not appear to be implicated in stabilization of the protein structure. The stability of the *Desulfurococcus* proteinase was not greatly affected by the presence of reducing reagents (e.g. dithiothreitol), some chaotropic agents (e.g. NaSCN) and some detergents, but activity was lost rapidly at 95 °C in the presence of the oxidizing agent NaBO_3 . Proteolytic activity was readily detected at temperatures up to and including 125 °C, although denaturation was very rapid above 115 °C. A number of Figures supporting some of the findings reported in this paper have been deposited in supplement SUP 50137 (14 pages) at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies may be obtained on the terms indicated in Biochem. J. (1987) 241, 5.

INTRODUCTION

The archaebacteria, a diverse group of organisms set apart from the eubacteria by a range of distinct phenotypic dissimilarities (Woese, 1982), are generally isolated from 'so-called' extreme environments. The Desulfurococcaceae, of which two species (*Desulfurococcus mucosus* and *Desulfurococcus mobilis*) have been isolated, are anaerobic extreme thermophiles growing optimally at 85 °C at pH 5.5–6.0 (Zillig *et al.*, 1982).

As increasing numbers of enzymes have been isolated from organisms capable of surviving at high temperatures, it has become clear that many of the enzymes from these organisms are more thermostable than equivalent enzymes from mesophiles (e.g. Amelunxen & Murdock, 1978). It follows therefore that a search for proteins of very high structural stability would be best directed at organisms capable of active existence under the most thermally extreme conditions, namely the thermophilic archaebacteria.

The mechanisms of stabilization of archaebacterial enzymes are of interest for reasons of both their environmental and evolutionary origins. Many bacterial extracellular proteinases are stabilized by metal ion chelation. The intrinsic molecular stability of *Bacillus subtilis* neutral proteinase (Tajima *et al.*, 1976), thermolysin from *Bacillus thermoproteolyticus* (Tajima *et al.*, 1976) and caldolyisin from *Thermus aquaticus* strain T351 (Cowan & Daniel, 1982; Khoo *et al.*, 1984) are of a similar order, the progressively higher thermostability resulting from Ca^{2+} binding. However, many other

proteins from extreme thermophiles are of high intrinsic thermostability.

To date, the only other archaebacterial proteinase isolated has been from a mesophilic halophile, *Halo-bacterium halobium* (Izotova *et al.*, 1983). We have assigned a trivial name (archaelysin) to the *Desulfurococcus* strain Tok₁₂S₁ extracellular proteinase. The name archaelysin is derived from the prefix 'archae' (Greek, beginning), as in archaebacterium, and the common suffix 'lysin' (Latin, to cleave).

METHODS

Isolation of *Desulfurococcus* strain Tok₁₂S₁

A coccus capable of growing anaerobically at 70–95 °C was isolated from the Tokaanu thermal region of New Zealand (Jasperse-Herst, 1984). The organism closely resembled *D. mucosus* in ultrastructure, anaerobiosis, nutrient requirements and antibiotic-resistance, but possessed a slightly higher optimum growth temperature (88 °C) and differed from the type strain in its propensity to form cell clusters during exponential growth phase and to reproduce by budding (Jasperse-Herst, 1984). Subject to final taxonomic classification, the organism is here referred to as *Desulfurococcus* strain Tok₁₂S₁.

Culture and growth of *Desulfurococcus* strain Tok₁₂S₁

Desulfurococcus strain Tok₁₂S₁ was grown in a modified *Desulfurococcus* medium (Zillig *et al.*, 1982)

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containing 0.1 g of yeast extract (Merck)/l, 2.0 g of tryptone (Baltimore Biological Laboratories)/l, 1.0 g of casein (Hammarsten grade; BDH Chemicals)/l, 1.0 g of cystine (Sigma Chemical Co.)/l, basal salts as described by Brock *et al.* (1972), and 4 ml of aq. 10% (w/v) $\text{Na}_2\text{S}_9\text{H}_2\text{O}$ /l. Resazurin (1 ml of aq. 0.1% solution/l) was added on occasions. The medium (without $\text{Na}_2\text{S}_9\text{H}_2\text{O}$) was boiled, cooled to about 40 °C under a stream of O_2 -free N_2 (New Zealand Industrial Gases, Auckland, New Zealand), the $\text{Na}_2\text{S}_9\text{H}_2\text{O}$ was added, and the pH was adjusted to 6.2 with 5 M-HCl. The medium was then dispensed under a flow of N_2 into 1-litre Schott Duran bottles and autoclaved. These were inoculated by injection with 10 ml of a viable culture (stored for up to 6 weeks at room temperature). Cultures were grown at 88 °C overnight. The proteinase-containing cell-free supernatant was harvested by centrifugation (9000 rev./min in a Sorval RC5B centrifuge with GS3 rotor for 30 min) after a growth period of 16–22 h.

Purification of archaealysin

Fresh cell-free supernatant (approx. pH 6) was cooled to room temperature and passed through a column (Amicon, 5 cm × 3 cm diam.) containing CM-Sephacrose (Pharmacia) pre-equilibrated with 20 mM-sodium acetate/acetic acid buffer, pH 5.0 (20 mmol of sodium acetate dissolved in 500 ml of water, adjusted to pH 5.0 with acetic acid, and diluted to 1 litre). A maximum of 4 litres of cell-free supernatant was applied per 200 ml volume of gel at one time. After loading, the CM-Sephacrose was washed with distilled water until the absorbance at 280 nm of the eluate had returned to the baseline. The proteinase was then eluted with a linear gradient of NH_4HCO_3 (400 ml, 0–200 mM), pH 8.5. The active fractions from the gradient elution were pooled and immediately applied to a column (Pharmacia C25, 6.0 cm × 2.5 cm) of hydroxyapatite (Bio-Gel HTP) pre-equilibrated with 1 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.4. The column was washed with sufficient 1 mM-phosphate buffer (above) to return the absorbance trace to the baseline, and the proteinase was eluted with a linear gradient of phosphate buffer (200 ml, 1–150 mM), pH 7.4. The active fractions were pooled and dialysed for 24 h at 4 °C against 2 × 5-litre volumes of distilled water (3500 Da-cut-off membrane; Spectrum Medical Industries). The salt-free proteinase was then freeze-dried.

Enzyme assay

Proteinase activity was determined by the hydrolysis of azocasein (Sigma Chemical Co.). For this, 900 µl volumes of 0.1% azocasein dissolved in 100 mM-Hepes buffer (40.9 g of Hepes dissolved in 500 ml of distilled water, pH adjusted at room temperature to 8.3 with 10 M-NaOH, and solution diluted to 1 litre) were injected into Plastibrand micro test tubes (capacity 1.5 ml). [Hepes has a $\text{p}K_a/\text{°C}$ of 0.014 (Perrin & Dempsey, 1974). At 85 °C, the substrate pH was 7.5 ± 0.1 .] The assay tubes (normally duplicate with non-enzymic controls) were preincubated at 85 °C, and the reaction was initiated by addition of 10–100 µl of enzyme solution and terminated after a measured incubation period by addition of 500 µl of aq. 15% (w/v) trichloroacetic acid. The assay tubes were allowed to cool for 5–10 minutes, then centrifuged at 12000 g for 3 min in a Runne model 85-1 micro-centrifuge. The absorbance of

the supernatant was monitored at 440 nm. One unit of proteinase activity was defined as giving an absorbance change of 1.0/min.

Protein determination

Protein was determined by the Bradford assay (20–200 µg of protein/ml) or the Bradford micro-assay (1–20 µg of protein/ml) (Bradford, 1976). Bovine serum albumin (Cohn fraction V; Sigma Chemical Co.) was routinely used to generate standard curves.

Electrophoresis

Discontinuous SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) was used for the determination of protein purity and molecular masses. Protein samples were heated in SDS sample buffer (Laemmli, 1970) for 3 min at 120 °C for 20 min at 100 °C. Normal gel slabs (1.5 mm, 3% stacking gel, 10% resolving gel) and similar slabs containing 0.1% casein (Hammarsten grade) were electrophoresed simultaneously in an LKB 2001 vertical electrophoresis unit at 18 °C. Power (90 mA constant current) was supplied by a Bio-Rad 3000/300 power supply. During the 2.5–3 h electrophoresis period, the initial voltage (75 V) increased to approx. 330 V. Immediately after electrophoresis, a zymogram of the casein-free gel was obtained. The polyacrylamide gel was overlaid on an agar slab (1.5% Oxoid purified agar, 0.1% Hammarsten casein, dissolved by boiling in 0.1 M- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.0) for 5 min at room temperature. The polyacrylamide gel was then stained in Coomassie Brilliant Blue R250. The agar zymogram was incubated at 75 °C (wrapped in polyethylene film to prevent drying) for 2–4 h, then stained as above.

Following the procedure of Horie *et al.* (1984), casein-containing gels were washed in 2 × 100 ml volumes of aq. 2.5% (w/v) Triton X-100, then incubated for 12–16 h at 75 °C in 0.1 M-Hepes buffer, pH 8.3 (20 °C). Gels were then fixed in 50% trichloroacetic acid for 30–60 min and stained as above.

For molecular-mass calibration, standard protein markers (carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase b, β -galactosidase and myosin; Sigma SDS/polyacrylamide-gel-electrophoresis protein molecular-mass marker kit) were included in all gels.

Gel-permeation chromatography

High-performance gel-permeation chromatography. Samples (100 µl) of archaealysin dissolved in the eluting solvent (0.3 M-ammonium acetate, pH 6.5) were passed through a 600 mm × 7.5 mm column (TSK G3000 SW; Toyo Soda, Japan) with the aid of a Waters M45 h.p.l.c. pump. The solvent flow rate was set at 1 ml/min. Protein was detected at 280 nm with a Waters model 481 spectrophotometer. The column temperature was monitored continuously to ensure similar chromatographic conditions for samples and standards.

Low-pressure gel-permeation chromatography. Samples (0.5 ml) of archaealysin (approx. 20 µg/ml in 200 mM-NaCl/10 mM-Tris/HCl buffer, pH 7.5) were loaded on to a 1 m column (Pharmacia, K16) containing Sepharose CL-6B (Pharmacia) and eluted with the same buffer. Flow was maintained at 0.4 ml/min with a Pharmacia P1 peristaltic pump. Protein was detected at 280 nm. Protein standards used were cytochrome c (12.4 kDa),

soya-bean trypsin inhibitor (21.7 kDa), ovalbumin (44 kDa) and bovine serum albumin (66 kDa).

Sucrose-gradient centrifugation

Sucrose gradients [10 ml; 6–20% (w/v) sucrose in distilled water] were prepared. Then 100 μ l volumes of distilled water containing 0.5 mg of marker protein (ovalbumin or cytochrome *c*) and approx. 2 μ g of archaealysin were layered on the top of the gradients, which were then centrifuged at 120 000 *g* for 20 h at 4 °C. The contents were removed by piercing the bottom of the tubes, and 0.5 ml fractions were collected. Protein was determined at 280 nm and proteolytic activity was quantified by the hydrolysis of azocasein at 85 °C.

Isoelectric focusing

Isoelectric point (and protein homogeneity) determinations were carried out using 125 mm $\times$ 125 mm pH 3–10 Servalyt-Precotes (Serva). A voltage programme (200 to 2000 V during a 2.5–3 h focusing period) was applied, with the use of a Bio-Rad 3000/300 power supply. The Pharmacia FBE-3000 flat-bed unit was cooled to 10.0 °C with an Edmund Buhler circulating unit. Pharmacia pH 3–10 and pH 5–10.5 isoelectric-point calibration standards were included on each gel. After electrophoresis, a zymogram was obtained as described above. Both zymogram and Precote were stained in Coomassie Blue R250.

pH optimum

Azocasein was dissolved at a concentration of 0.1% (w/v) in volumes of Universal buffer (Johnson & Lindsey, 1939) at pH values from 3 to 11.3. The pH of Universal buffer stocks was adjusted at 85 °C to avoid errors introduced by the effect of complex dpK_a/dT changes. Assays were performed as described above.

Protein hydrolysis

Proteins were dissolved or suspended at concentrations of either 0.1% or 0.2% in 0.1 M-Hepes buffer, pH 8.3 (20 °C). Then 900 μ l volumes were pipetted into Plasti-brand 1.5 ml test tubes and assayed as described above.

When the progress of protein hydrolysis was monitored at temperatures below 100 °C, 6–10 ml volumes of bovine serum albumin (Cohn fraction V, 0.2% in 10 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.4) were sealed in McCartney bottles. After the addition of a sample of archaealysin, 1 ml portions were removed at intervals and injected immediately into vials cooled by liquid N_2 . Once frozen, these were freeze-dried. Electrophoresis was carried out as described above.

For the determination of protein hydrolysis at 100 °C and above, 1 ml volumes of chilled substrate plus enzyme were sealed in 7 ml Hungate tubes and immediately immersed in a heated bath of poly(ethylene glycol). Equilibration times were determined to be less than 60 s. Individual tubes were removed at intervals and plunged into liquid N_2 . The contents were then freeze-dried.

Peptide hydrolysis

The following peptides (obtained from Sigma Chemical Co.) were used as substrates for determining the peptide-bond specificity of archaealysin: serum thymic factor (Glu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn), angiotensin II

(Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) and oxidized insulin B-chain.

The last-mentioned was further purified by reversed-phase h.p.l.c. on a Bio-Rad Hi Pore C_4 column with a linear gradient from 10% to 60% (v/v) acetonitrile (solvent B) in 20 mM- KH_2PO_4 , pH 2.5 (solvent A).

Hydrolyses were carried out in Plasti-brand micro centrifuge tubes at 85 °C. Peptides (1 mg of serum thymic factor, 250 μ g of angiotensin II or 290 μ g of insulin B-chain) were dissolved in 500 μ l of 50 mM- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.0 (pH at 85 °C was 6.8).

Serum thymic factor hydrolysis. A 50 μ l portion of archaealysin (0.004 unit) was added to 1 mg of the preincubated substrate. Then 200 μ l samples were removed at 15 and 60 min and injected into 3 ml of (v/v) 0.1% trifluoroacetic acid at 0 °C to terminate the reaction. The final pH (2–3) was checked with narrow-range indicator paper. These samples (plus a sample of the substrate before addition of the proteinase) were centrifuged at 12 000 *g* for 10 min in a Runne bench-top centrifuge. Supernatants were chromatographed on a Novopak C_{18} reversed-phase column with a linear gradient from 5% to 20% (v/v) acetonitrile (solvent B) in 20 mM- KH_2PO_4 , pH 2.5 (solvent A), at 0.5 ml/min. The chromatography system used two Waters M45 pumps, a Waters model 660 gradient programmer, a Waters model 481 variable-wavelength spectrophotometer (set to detect peptides at 215 nm or 220 nm) and an LKB model 2112 Redirac fraction collector.

Angiotensin II hydrolysis. A 200 μ l portion of archaealysin (0.009 unit) was added to the substrate, and the mixture was incubated for 65 min. The reaction was stopped as described above and the mixture was centrifuged. Separation of peptides was carried out with a Brownlee Spheri 5 C_{18} reversed-phase h.p.l.c. column at a flow rate of 0.8 ml/min with a linear gradient of 15% to 60% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid (solvent B) in aq. 0.1% (v/v) trifluoroacetic acid (solvent A).

Insulin B-chain hydrolysis. A 100 μ l portion of archaealysin (0.007 unit) was added to the preincubated substrate. Samples were removed at 30 and 60 min and treated as for the angiotensin II reaction.

Amino acid analysis. Individual peaks from reversed-phase chromatography were collected and freeze-dried. After the addition of approx. 1 ml of constant-boiling HCl, samples were sealed under vacuum and heated at 110 °C for 12–18 h. Hydrolysed samples were rotary-evaporated to dryness at 40 °C and dissolved in 40 μ l of amino acid analysis buffer, pH 3.5. Amino acid analysis was carried out with a Waters amino acid analyser with a citrate buffer system and a post-column *o*-phthalaldehyde derivative-forming system.

Thermostability

For the determination of thermostability at temperatures below 100 °C, enzyme samples (4–5 ml) were sealed in 7 ml Hungate tubes and immersed in a temperature-controlled water bath. The thermal equilibration time (2–3 min) was generally negligible in relation

Table 1. Purification of archaelysin

For experimental details see the text.

Purification step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Cell-free supernatant	4200	14.7	71.0	0.21	100	1
CM-Sepharose eluate	116	12.1	0.087	139.1	83	662
Hydroxyapatite eluate	46	10.1	0.046	219.6	75	1046

Fig. 1. Elution profiles from successive chromatographic steps in the purification of archaelysin and protein heterogeneity as demonstrated by analytical reversed-phase h.p.l.c.

For details see the Methods section. (a) CM-Sepharose chromatography; (b) reversed-phase h.p.l.c. of active fractions. (c) Hydroxyapatite chromatography; (d) reversed-phase h.p.l.c. of active fractions. Cross-hatched areas indicate the location of proteolytic activity.

to the total incubation period. At intervals, 500 μ l samples were removed by syringe and stored on ice for subsequent duplicate assays.

For temperatures above 100 $^{\circ}$ C, the sealed-capillary-tube procedure described by Barach & Adams (1977) was used.

Analysis of the kinetics of activity loss

Activity data from thermostability experiments were treated as follows. Activity values (units/ml) were converted into residual active enzyme concentrations $[E]_t$. These data were then plotted as $\ln([E]_0/[E]_t)$ versus

Fig. 2. SDS/polyacrylamide-gel electrophoresis of archaealysin

For details see the Methods section. Lane a, sample from CM-Sepharose chromatography; lane b, enzyme from hydroxyapatite chromatography; lanes c and d, zymograms of samples from CM-Sepharose chromatography and hydroxyapatite chromatography respectively.

time ($[E]_0$ = initial enzyme concentration) and $1/[E]_t$ versus time, as tests for first-order and second-order kinetics respectively (Moore, 1962). Rate constants k_1 (min^{-1}) and k_2 ($\text{mol}^{-1} \cdot \text{min}^{-1}$) were obtained from the initial linear portions of these plots, and half-lives were calculated from the equations $\tau_1 = \ln 2/k_1$ or $\tau_2 = 1/k_2[E]_0$ (Moore, 1962).

RESULTS AND DISCUSSION

Purification

In the course of two purification steps (CM-Sepharose ion-exchange chromatography and hydroxyapatite absorption chromatography), a purification of 1046-fold was obtained (Table 1). Most of the extracellular non-enzyme protein would obviously be derived from the casein in the medium (1 g/l). During the period of growth, the initially cloudy medium cleared substantially

as a result of casein hydrolysis, providing a good visual indication of proteinase production. The removal of casein peptide fragments was primarily achieved during the preliminary ion-exchange step (Fig. 1). Subsequent purification steps resulted in a nearly homogeneous enzyme preparation, as demonstrated by SDS/polyacrylamide-gel electrophoresis (Fig. 2, lanes a and b), but the preparation was shown to be less pure by analytical reversed-phase h.p.l.c. (Fig. 1). The zymogram (Fig. 2, lanes c and d) from the SDS/polyacrylamide gel showed a single clear activity band, demonstrating that only one proteinase was excreted in significant amounts by *Desulfurococcus* strain Tok₁₂S₁.

Inhibitors

Archaealysin was fully or partially inhibited by a number of serine-proteinase inhibitors (Table 2). No other significant inhibition was observed. The absence of any effect by tosyl-L-lysyl-chloromethane and tosyl-L-phenylalanyl-chloromethane ('TLCK' and 'TPCK') suggests that the active site of archaealysin is significantly dissimilar to those of trypsin and chymotrypsin. As might be expected, the ester substrates of trypsin and chymotrypsin, tosyl-L-lysine ethyl ester and tosyl-L-phenylalanine ethyl ester (for which the inhibitor chloromethane derivatives are analogous), were not hydrolysed by archaealysin.

The activation of archaealysin by 10 mM-iodoacetate is not readily explained. This effect was still evident, though less significant, at iodoacetate concentrations of 1 mM and 0.1 mM.

Molecular-mass determinations

The molecular mass of archaealysin estimated by SDS/polyacrylamide-gel electrophoresis was 52 ± 2 kDa (mean $\pm$ S.E.M., $n = 6$) (Figure deposited in supplement SUP 50137). However, the molecular-mass values obtained by both high-performance and low-pressure gel permeation chromatography were between 10 kDa and 13 kDa (Figure deposited in supplement SUP 50137). On sucrose-gradient centrifugation, archaealysin exhibited a larger sedimentation coefficient than both cytochrome *c* and ovalbumin (results not shown). Calculations using the sedimentation-ratio method of Martin & Ames (1961) provided estimates for the molecular mass of archaealysin of approx. 43 kDa and 53.9 kDa from

Table 2. Effect of enzyme inhibitors on the activity of archaealysin

Stock concentrates of inhibitors were added to solutions of archaealysin (1–5 $\mu\text{g/ml}$ in 100 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.4) to give the concentrations specified. After incubation at room temperature for 60 min, proteinase activity was determined (see the Methods section). [The following had no inhibitory effect on activity under the conditions specified: hydroxymercuribenzoic acid (10 mM), pepstatin (0.1 mg/ml), elastatinal (0.1 mg/ml), α_1 -antitrypsin (0.5 mg/ml), leupeptin (0.5 mg/ml), soya-bean trypsin inhibitor (0.1 mg/ml), lima-bean trypsin inhibitor (0.1 mg/ml), ovomucoid trypsin inhibitor (0.1 mg/ml), *o*-phenanthroline (10 mM), EGTA (10 mM), tosyl-L-lysyl-chloromethane (5.0 mM), tosyl-L-phenylalanyl-chloromethane (5.9 mM) and dithiothreitol (10 mM).]

Inhibitor	Class of inhibitor	Concentration	Inhibition (%)
Di-isopropyl phosphorofluoridate	Serine proteinase	1 %	100
Phenylmethanesulphonyl fluoride	Serine proteinase	10 mM	100
Chymostatin	Serine proteinase	0.5 mg/ml	80
<i>p</i> -Chloromercuribenzoate	Cysteine proteinase	10 mM	0
Iodoacetate	Cysteine proteinase	10 mM	[60 % activation]
EDTA	Metalloproteinase	10 mM	0

Fig. 3. pH-activity profile for archaealysin

For details see the Methods section. The vertical bars indicate $\pm$ S.E.M. for three determinations.

gradients containing cytochrome *c* and ovalbumin standards respectively. Although this method is relatively inaccurate, the data clearly support the value indicated by SDS/polyacrylamide-gel electrophoresis.

Isoelectric point

The isoelectric point of archaealysin, as determined by comparison with standards of known pI (Figure deposited in supplement SUP 50137), was 8.7 ± 0.1 . A single band on the isoelectric-focusing gel indicated the presence of a single protein. A zymogram showed clear activity bands corresponding exactly to those on the isoelectric-focusing gel.

pH optimum

A plot of proteinase activity over a range of pH values from 3.0 to 11.3 (Fig. 3) gives a pH optimum for the hydrolysis of azocasein of 7.2 ± 0.2 . The 50% activity limits occur at the approximate pH values of 4.9 and 10.0.

Protein hydrolysis: linearity of assay

The rate of hydrolysis of 0.1% azocasein at 85 °C was linear until approx. 10% of the substrate was hydrolysed (Figure deposited in supplement SUP 50137). Analysis of the Michaelis-Menten-type kinetics of azocasein hydrolysis (Fig. 4) showed that the negative deviations from linearity were probably the result of substrate depletion. Marked substrate inhibition was evident at substrate concentrations above approx. 2 mg/ml (0.2%). For routine assays, a substrate concentration of 0.1% was chosen to maximize enzyme activity. This is, however, only about 25% greater than the K_m [0.74 mg/ml; estimated from a Lineweaver-Burk plot (not shown) of the data]. Consequently the decrease in substrate concentration resulting from substrate hydrolysis would rapidly generate a significant decrease in apparent enzyme activity. In order to minimize this effect, assay data were discarded if absorbance values of greater than 0.06 (the upper limit of assay linearity) were obtained.

Fig. 4. Relationship between archaealysin activity and substrate concentration

For details see the Methods section. The vertical bars indicate $\pm$ S.E.M. for three determinations.

Table 3. Hydrolysis of protein substrates by archaealysin

Proteins were dissolved or suspended in 0.1 M-Hepes buffer, pH 8.3 (20 °C), at a concentration of 0.1%. Then 10 μ l volumes of archaealysin [5 μ g/ml in 10 mM- Na_2HPO_4 /NaH₂PO₄ buffer, pH 7.4 (20 °C)] were added, the reaction mixtures were incubated at 85 °C for 10 min, and the reaction was terminated with 15% (w/v) trichloroacetic acid. Absorbance values were monitored at the wavelengths specified.

Substrate	Wavelength (nm)	10 ³ × A
Albumin (bovine)	280	32 ± 3
Albumin (egg)	280	31 ± 4
Azocasein	440	29.5 ± 0.5
Azocoll	510	143 ± 3
Casein	280	108 ± 11
Collagen	280	18 ± 6
Elastin	280	0
Elastin-Congo Red	280	0
Fibrin	280	38 ± 5
Fibrin-Congo Red	540	2 ± 1
Haemoglobin	280	32 ± 5
Keratin	280	0

Protein hydrolysis: influence of temperature

Archaealysin hydrolysed a number of soluble and insoluble proteins but had little apparent effect on keratin and elastin (Table 3).

A comparison of the rates of hydrolysis of several proteins at 37 °C, 85 °C and 120 °C demonstrated that protein (substrate) structure significantly affects reaction rate. Average Q_{10} values for the hydrolysis of casein, azocasein, collagen and azocoll between 37 °C and 85 °C were 2.14, 2.11, 1.78 and 1.95 respectively. From 85 °C to 120 °C, the average Q_{10} value for collagen hydrolysis was 3.17, an indication of its increased susceptibility to hydrolysis after the loss of crystalline structure. This rate

Fig. 5. Changes in protein fragments during the hydrolysis of bovine serum albumin by archaealysin at 37 °C (a), 85 °C (b) and 117 °C (c)

For details see the Methods section.

may be an underestimate, since instability above about 100 °C (see below) would significantly decrease enzyme activity during a 10 min reaction period. This behaviour demonstrates one of the clear advantages of proteolysis at high temperatures (Cowan *et al.*, 1985).

The temperature-dependence of proteinase specificity was apparent when albumin hydrolysis products were separated by SDS/polyacrylamide-gel electrophoresis (Fig. 5a). At 37 °C, well-defined fragments of 70 kDa, 64 kDa, 55 kDa, 48.5 kDa, 42.5 kDa and 28 kDa are visible during the early stages of hydrolysis. Of these, the 70 kDa, 42.5 kDa and 28 kDa fragments are relatively stable to proteolysis, and accumulate throughout the reaction period. It is likely that these are domains of the parent molecule, where the retention of tertiary structure imparts a degree of resistance to further hydrolysis.

At 85 °C and 117 °C, there is evidence of rapid random endopeptidase activity (Figs. 5b and 5c). Limited hydrolysis products (57–65 kDa bands) formed during the first 60 s are rapidly degraded. Of the stable fragments

noted above, only the 43 kDa and 28 kDa bands remain relatively resistant to proteolysis at the higher temperatures.

Hydrolysis of chromogenic peptides

None of the limited range of ester substrate tested (benzoylarginine ethyl ester, tosylarginine methyl ester and acetylarginine ethyl ester) were hydrolysed by archaealysin. The tripeptide *p*-nitroanilide substrate (benzoyl-L-phenylalanyl-L-valyl-L-arginine *p*-nitroanilide) was readily hydrolysed. Unlike a number of other thermophilic serine proteinases (D. A. Cowan, unpublished work), archaealysin did not cleave 3-carboxypropionylalanylalanylalanine *p*-nitroanilide.

Hydrolysis of peptides

Reversed-phase h.p.l.c. separations of the hydrolysis products of serum thymic factor, angiotensin II and insulin B-chain (Figures deposited in supplement SUP 50137) showed that limited cleavage of the peptides had occurred.

In the hydrolysis of serum thymic factor, the major product was a peptide devoid of alanine and lysine, and with only single glutamate/glutamine and serine residues. The obvious explanation of this result is that the Ser-Gln bond was preferentially cleaved, liberating the *N*-terminal tetrapeptide. However, although this pattern of hydrolysis was reproducible, the four *N*-terminal amino acid residues were not recovered either as single amino acids or as an oligopeptide. One possible explanation is that this peptide might not have been eluted from the column under the solvent conditions used.

The hydrolysis of angiotensin II by archaealysin for 65 min at 85 °C yielded four significant products. Amino acid analysis demonstrated that two peaks corresponded to Asp-Arg-Val-Tyr and Ile-His-(Pro)-Phe (proline is not detected when *o*-phthalaldehyde is used as a derivative-forming agent). This is consistent with cleavage of the Tyr-Ile peptide bond. No amino acids were detected in hydrolysates of the other two peaks.

Hydrolysis of oxidized insulin B-chain by archaealysin was limited. After 30 min incubation, the insulin B-chain peak was completely absent, but two major additional peaks were apparent. A further 30 min proteolysis period did not generate any new peaks. The amino acid compositions of the two peaks were consistent with peptides containing amino acids 1–14 and 17–30 respectively. This result is consistent with cleavage at the Ala₍₁₄₎-Leu₍₁₅₎ and Tyr₍₁₆₎-Leu₍₁₇₎ peptide bonds. However, no peak corresponding to the dipeptide Leu-Tyr (or the individual amino acids) was detected.

These results are indicative of a high degree of peptide-bond specificity in archaealysin, but are not entirely consistent. The cleavage sites of angiotensin II and insulin B-chain (Tyr-Ile, Ala-Leu and Tyr-Leu bonds) suggest a strong preference for an uncharged aliphatic side chain on the *C*-terminal side of the cleavage site. However, the cleavage of the Ser-Gln bond in serum thymic factor remains an anomaly, and additional peptide cleavage studies are obviously required for confirmation.

Thermostability

At temperatures between 95 °C and 125 °C in the absence of substrate, archaealysin demonstrated a considerable degree of thermostability (Fig. 6). Activity loss

Fig. 6. Thermostability profiles of archaealysin

Portions of archaealysin [0.1–0.2 $\mu\text{g/ml}$ in 100 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.5 (85 °C)] were incubated at (a) 95 °C, (b) 105 °C, (c) 115 °C and (d) 125 °C and samples were assayed for activity at appropriate intervals (see the Methods section). The vertical bars indicate $\pm$ S.E.M. for two determinations.

Table 4. Effects of low-temperature storage on activity and thermostability of archaealysin

Portions of archaealysin [approx. 1 $\mu\text{g/ml}$ in 100 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.7 (20 °C)] were stored refrigerated (sample I; 6 °C $\pm$ 1 °C) and frozen (sample II; –18 °C $\pm$ 2 °C). At 24 h intervals samples were removed and enzyme activity and thermostability were determined (see the Methods section). Sample II was thawed in running water (18–20 °C) and refrozen after removal of each sample.

Incubation period (h)	Sample I (6 °C)		Sample II (–18 °C)	
	$10^3 \times$ Activity (units/ml)	Half-life at 95 °C (min)	$10^3 \times$ Activity (units/ml)	Half-life at 95 °C (min)
0	35 $\pm$ 1	93	35 $\pm$ 1	93
24	26 $\pm$ 3	75	23 $\pm$ 0.5	60
48	28 $\pm$ 1	72	23	39
72	29 $\pm$ 1	54	17	38
96	32 $\pm$ 1	45	14	38

was a product of protein denaturation rather than autolysis, as indicated by the predominance of first-order kinetics at these temperatures. Half-life values calculated from the first-order rate constants were 70–79 min at 95 °C, 7.7–8.9 min at 105 °C, 0.65 min at 115 °C and less than 10 s at 125 °C. It should be stressed that these values apply only to the particular set of conditions employed (pH, ionic strength, enzyme concentration etc.) and

cannot be accurately extrapolated to other conditions. Nevertheless, the stability of archaealysin apparently exceeds that of most if not all other known proteinases [cf. caldolyisin, $t_{1/2}$ (95 °C) 15 min (Cowan & Daniel, 1982), and thermolysin, $t_{1/2}$ (80 °C) 6 min (Ohta, 1967)].

Unlike most thermostable extracellular proteases, the stability of archaealysin was not diminished in the presence of EDTA or other chelating agents.

Fig. 7. Effect of pH on thermostability of archaelysin at 95 °C

Freeze-dried archaelysin was dissolved in volumes of Universal buffer, pH 5–9 (Johnson & Lindsey, 1939), at a concentration of 2.5 µg/ml. Thermostability data were obtained as described in the Methods section.

Stability at low temperatures

The observation of considerable variations in the thermostability of archaelysin under apparently identical conditions led to a suggestion that storage of enzyme solutions at low temperature might in some way affect subsequent enzyme thermostability. It has been suggested that refrigerated storage of dilute buffered solutions of extremely thermophilic proteinases would negate problems of autolysis. In terms of temperature effects on enzyme activity, storage of such enzymes at 6 °C is equivalent to keeping mesophilic proteinases at about –40 °C.

A solution of archaelysin (approx. 1 µg/ml in 100 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.7) stored for 96 h at 6 °C showed only minor changes in activity (Table 4). However, periodic analysis of thermostability demonstrated a marked ‘aging’ effect, where the half-life at 95 °C decreased by more than 2-fold during a 96 h refrigeration period.

The most likely mechanism for this effect would involve limited autolytic cleavage (‘nicking’ of the protein), probably involving very few peptide bonds, and

apparently not significantly affecting the tertiary structure. Since no change in the level of activity (measured at 85 °C) was noted throughout the refrigeration period, it is probable that the presence of substrate stabilizes the tertiary structure. On heating at 95 °C in the absence of substrate, where reversible $\text{N} \rightleftharpoons \text{D}$ (native $\rightleftharpoons$ denatured) conformational changes would be rapid and significant, any prior cleavage of the polypeptide would tend to limit the extent of renaturation and would be apparent as an increase in thermolability.

Freeze–thaw cycling caused loss of both activity and stability (Table 4). Although the temperature of the sample was above freezing point for only about 4% of the storage period, it is likely that autolysis in the zones of relatively concentrated protein that appear during freezing is responsible for this result.

pH and thermostability

Where autolysis is the primary cause of irreversible activity loss, the reversible suppression of enzyme activity can significantly increase longevity. This can be achieved by adjusting the pH of the environment to a point where activity is minimized (Walsh, 1970). However, activity loss in solutions of archaelysin at 95 °C becomes increasingly rapid at successive unit pH values below 8 (Fig. 7), supporting previous evidence that autolysis is a relatively minor contribution to activity loss at this temperature.

Denaturing reagents and thermostability

The ability of a number of proteinases to function in the presence of organic solvents, denaturing reagents and detergents is well documented (e.g. Cowan & Daniel, 1982) and is interesting for both the mechanistic implications and biotechnological possibilities. The effects of various groups of reagents (e.g. electrolytes, detergents and organic solvents, urea and guanidinium salts) on proteins has been used as an indication of the importance of the various types of intramolecular bonds in generating or maintaining protein stability (Kauzmann, 1959).

The effects of a number of such reagents on the thermostability of archaelysin are shown in Tables 5 and 6. Dithiothreitol, a reagent capable of dissociating disulphide cross-linkages, has little effect on thermostability. Since it might be argued that ‘buried’ cystine

Table 5. Effect of chaotropic agents on the stability of archaelysin

The specified reagents were added to 5 ml volumes of archaelysin [0.5 µg/ml in 75 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.4 (20 °C)]. These were immersed in a water bath at 95 °C, and samples removed at intervals for subsequent assays (see the Methods section).

Agent	Concentration	Probable site of action	Half-life (min)	Kinetics of activity loss
None	—	—	70.3	First-order
Dithiothreitol	10 mM	Exposed cysteine	60.5	Complex
Dithiothreitol/urea	10 mM/6.2 mM	Cysteine/hydrogen bonds	15.2	Second-order
Urea	6.2 M	Hydrogen bonds	24.2	Second-order
Guanidinium chloride	4.4 M	Hydrogen bonds	2.6	Second order
NaBO_3	10 mM	Cysteine, methionine, tryptophan, serine	2.0	Second-order
NaSCN	10 mM	—	77.6	First-order

Table 6. Effect of detergents on the stability of archaealysin

The specified detergents were added to 5 ml volumes of archaealysin (0.1 µg/ml in 150 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.8). These were immersed in a water bath at 95 °C, and samples were removed at intervals for subsequent assay (see the Methods section). Abbreviation: CTAB, cetyltrimethylammonium bromide.

Detergent	Concentration	Half-life (min)	Kinetics of activity loss
None	—	96.7	First-order
SDS	0.1 %	112	First-order
Triton X-100	1 %	280	First-order
	0.1 %	280	Complex
CTAB	1 %	16.3	First-order
	0.1 %	40.3	First-order

residues would be inaccessible to the reagent, dithiothreitol was included in the presence of urea. Although this resulted in a very large decrease in thermostability, it was only marginally greater than that caused by urea alone. These results tend to indicate that disulphide bridges, if present, play little part in protein stabilization.

The effect of urea, and to a greater extent guanidinium chloride, emphasizes the primary role of hydrogen-bonding in the stabilization of tertiary structure. Furthermore, the existence of second-order kinetics of inactivation indicates that the process is certain to be irreversible.

NaBO_3 , like other strong oxidizing agents, will rapidly convert cysteine into cysteic acid (Means & Feeney, 1971). However, it would be expected to oxidize a number of other residues (e.g. tyrosine and serine hydroxy groups) also under the rigorous conditions used. In the absence of cysteine residues (see above) it is likely that the rapid inactivation of archaealysin in the presence of 10 mM- NaBO_3 would, in part, be the result of

Table 7. Stabilization of archaealysin

A protein concentration, before additions, of 0.5 µg/ml in 100 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.4, was used. For further details see the Methods section.

Additive	Concentration	Half-life at 95 °C* (min)
None	—	93
Glycerol	5 %	(45 % in 240 min)
	15 %	(21 % in 240 min)
	50 %	(12 % in 240 min)
Mannitol	5 %	240
	15 %	(36 % in 240 min)
Poly(ethylene glycol) (PEG 4000)	50 %	52
Albumin (bovine)	0.2 %	224
	2 %	280†

* Values in parentheses indicate activity lost in a specified time at 95 °C.

† Substrate inhibition was considerable. This value is based on a calculated initial activity.

modification of the active-site serine hydroxy group. Such a reaction would also be expected to be a second-order process.

The slight stabilization of archaealysin by NaSCN is unexpected. The effect of salts on enzyme thermal stability correlate closely with the classical Hofmeister series (Von Hippel & Wong, 1965), and NaSCN should act as a powerful chaotropic reagent (Mozhaev & Martinek, 1984).

The effects of the neutral, anionic and cationic detergents (Triton, SDS and cetyltrimethylammonium bromide respectively) on the thermostability of archaealysin are widely different (Table 6). There is no simple correlation between the effect on thermostability and the hydrophobicity of the detergents [as indicated by the hydrophile-lipophile balance (HLB) values (Furth,

Fig. 8. Hypothetical mechanisms for the dependence of proteinase inactivation on the pathway of reversible polypeptide unfolding

1980)]. The extremely hydrophobic Triton X-100 (HLB 13.5) resulted in an apparent increase in stability, whereas the relatively hydrophilic SDS (HLB 40) had little effect. Cetyltrimethylammonium bromide, for which no HLB value is available, is probably the most hydrophilic of the three detergents, with a C_{12} chain and a quaternary ammonium ion of high charge density. The interaction of cetyltrimethylammonium bromide with intramolecular hydrogen bonds (in addition to hydrophobic bonding) may provide an explanation of its large destabilizing effect on archaealysin.

Where first-order kinetics of activity loss are apparent (i.e. autolytic degradation is relatively minor), the possibility of reversible denaturation must be considered. The effect on post-incubation enzyme renaturation would result in considerable overestimates of protein thermostability. There is evidence from the zymogram detection of active proteinases after SDS/polyacrylamide-gel electrophoresis (see the Methods section) that archaealysin is readily renatured after removal of SDS, and the results of Table 6 must be considered in this light.

In a general assessment of the data on thermostability, certain trends can be identified. There is some evidence that hydrogen-bonding may be a major contributor to molecular thermostability. Similar conclusions were derived from a determination of the factors responsible for the difference in thermostability between oxyhaemoglobins A and A₂ (Perutz & Raidt, 1975). However, other mechanisms can be equally effective, as demonstrated by several other detailed structural analyses of more and less thermostable proteins. Surface and buried ion pairs respectively have been implicated in the differences in stabilities of various bacterial D-glyceraldehyde-3-phosphate dehydrogenases (Walker *et al.*, 1980) and ferredoxins (Perutz & Raidt, 1975). In a comparison of native and mutant (temperature-sensitive) T4 bacteriophage lysozymes (Grütter *et al.*, 1979), it was concluded that the loss of hydrophobic interactions was a significant factor (see also Yutani *et al.*, 1977). It is now generally agreed that 'differences in thermostability of proteins are, in general, due to subtle changes in one or more types of intramolecular bonding'. This is supported by the fact that the free-energy contribution necessary to increase a protein 'melting' temperature by 10 °C to 20 °C is often only 10–20 kJ/mol (Nojima *et al.*, 1977; Atkinson, 1976) whereas the free energy of stabilization of a single peptide hydrogen bond can range from 2 kJ/mol to 20 kJ/mol (Fersht *et al.*, 1985).

Autolysis versus denaturation

In studying the thermostability of proteolytic enzymes, analyses are made more complex by the presence, often simultaneous, of both thermal denaturation (protein unfolding) and autolytic degradation. In addition, a polypeptide, once unfolded, is highly susceptible to degradation. Furthermore, limited hydrolytic cleavage of a folded polypeptide may promote unfolding or modify the potential for successful renaturation.

Yet, despite this complex set of interacting processes, kinetic analyses can often implicate a single mechanism for activity loss. The mechanisms visualized for first-order and second-order proteinase inactivation (Fig. 8) are dependent on the assumption that proteinase activity is specific for a limited number of peptide bonds. It is proposed that the 'selection' of first-order or second-

Fig. 9. Temperature-activity relationship for archaealysin

Portions (0.9 ml) of 0.2% azocasein in 100 mM- Na_2HPO_4 / NaH_2PO_4 buffer, pH 7.4, were incubated at specified temperatures for 10 min with 100 μl volumes of archaealysin (approx. 20 ng). Samples were then assayed for activity (see the Methods section). The vertical bars indicate $\pm$ S.E.M. for three determinations.

order inactivation is related to the pathway of unfolding (A and B, step 2). (Step 1 is a rapid reversible temperature-dependent conformation fluctuation typical of all proteins.) It is postulated that different unfolding pathways, possibly induced by the disruption of different intramolecular bonds (e.g. guanidinium chloride, cf. SDS), would generate conformers possessing a greater or lesser number of peptide bonds susceptible to autolysis. Further unfolding of conformers of the latter type would be a first-order inactivation (A, step 2). Subsequent degradation of the unfolded polypeptide (A, step 3) might well occur, but would in no way influence the kinetic data. Where the unfolded conformer contained accessible autolysis 'sites', activity loss would obviously follow second-order kinetics (B, step 2).

Increasing enzyme thermostability

Stabilization of enzymes by addition of salts, ligands (including substrates), monosaccharides and polyols, and organic polymers and by other mechanisms has been extensively reviewed (e.g. Klibanov, 1983). Stabilization of proteinases can be a result of either prevention of protein unfolding or a decrease in autolytic degradation (or both).

A number of additives (polyols and substrates) were

Fig. 10. Time course of azocasein hydrolysis by archaealysin in relation to temperature

Portions (1 ml) of 0.1% azocasein in 100 mM- Na_2HPO_4 / NaH_2PO_4 buffer, pH 7.8, containing equal amounts of archaealysin (approx. 0.5 μg) were immersed in temperature-controlled water baths: curve A (∇), 65 °C; curve B ($\blacksquare$), 75 °C; curve C ($\blacktriangle$), 85 °C; curve D ($\bullet$), 95 °C; curve E ($\square$), 105 °C; curve F ($\triangle$), 115 °C; curve G ($\circ$), 123 °C. Duplicates were removed at intervals, the reactions were terminated by addition of 500 μl of 15% (w/v) trichloroacetic acid, and the extent of hydrolysis was measured (see the Methods section).

capable of increasing the apparent thermostability of archaealysin (Table 7). The possibility that enhanced renaturation (see above) is partly responsible for this effect should not be ignored. In addition, the dilution effect at high polyol concentration would be expected to decrease the effect of autolysis, as would the presence of alternative substrate molecules.

Proteolysis at high temperatures and biotechnological implications

A temperature-activity profile of archaealysin (Fig. 9) suggests that, under the specific conditions employed (i.e. 10 min incubation time), temperatures up to at least 100 °C could be used for protein hydrolyses. However, the practical limitations are more evident when the time course of azocasein hydrolysis is monitored (Fig. 10). The rate-limiting effects of substrate depletion are rapidly evident, particularly at temperatures below 95 °C, where the contribution from autolysis and denaturation is minor. The use of a higher substrate concentration would result in lower initial hydrolysis rates because of the effects of substrate inhibition (see Fig. 4). The effects of enzyme thermostability are first evident in the data from the 95 °C incubation. At 105 °C, 115 °C and 125 °C, activity is rapidly lost, with less than 5% of the total substrate degraded to trichloroacetic acid-soluble fragments (ΔA_{440} for complete proteolysis of 0.1% azocasein is approx. 0.6).

The practical consequence of these observations is that under these conditions hydrolyses could be effectively

maintained for maximal periods of 20 min at 95 °C or 10 min at 105 °C. In the presence of higher substrate concentrations or other substances capable of enhancing stability, these limits could certainly be extended.

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