Homomultimeric protease in the hyperthermophilic bacterium *Thermotoga maritima* has structural and amino acid sequence homology to bacteriocins in mesophilic bacteria

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Abstract A novel homomultimeric protease (>669 kDa), based on 31 kDa subunits, was purified from cell extracts of the hyperthermophilic bacterium *Thermotoga maritima*. This protease exhibits activity toward chymotrypsin and trypsin substrates, optimally at 90°C and pH 7.1, and has a half-life of 36 min at 95°C. Transmission electron microscopy established that the protease consists of a large globular assembly which appears circular from the front view. The function of this protease in *T. maritima* remains unclear, although putative homologs include a 29 kDa antigen from *Mycobacterium tuberculosis* and a 31 kDa monomer of a high molecular weight bacteriocin produced by *Brevibacterium linens* [Valdes-Stauber, N. and Scherer, S. (1996) Appl. Environ. Microbiol. 62, 1283–1286]. The relationship of these mesophilic proteins to the *T. maritima* protease suggests that their antibacterial activity may involve elements of proteolysis, and raises the prospect for anti-microbial ecological strategies in hyperthermophilic niches.

Key words: Proteolysis; Bacteriocin; *Thermotoga*

1. Introduction

Multisubunit proteases have received a great deal of attention over the past decade and appear to be ubiquitous throughout all three domains of life [1]. Bacteria typically contain protease La and members from the Clp family of proteins [2–4]. In contrast, the multicatalytic proteinase (MCP), or proteasome, is conserved throughout the Eukarya [1] and the Archaea [5–8]; related forms have even been found in certain bacteria [9–12]. Multisubunit proteases fulfill essential regulatory, housekeeping, and stress-response roles in the cell [1], and often have multiple activities and effectors [4,13–16]. Although amino acid sequences of the various multisubunit protease subunits may differ, there is often significant homology in the structure and function of these enzymes [1,9,11].

High temperature microorganisms have been examined for various multisubunit proteases in part to provide another perspective, given their proposed evolutionary placement [17,18], on the physiological and regulatory roles of these proteins. Proteasomes have been purified from three thermophilic archaea – *Thermoplasma acidophilum* [7], *Methanosarcina thermophila* [8], and *Pyrococcus furiosus* [5]; homologous amino acid sequences to proteasomes were also apparent in two other archaeal genomes [6,19]. Protease PfpI, initially identified in *P. furiosus* [20], has been identified in the genome sequences of several other archaea, including *Methanococcus jannaschii* [6] and *Archaeoglobus fulgidus* [19]. Although none of the Clp family of proteins has been identified in the archaea, a novel modular proteolytic system (tricorn protease) was discovered in *T. acidophilum* [21]. Proteases produced by hyperthermophilic bacteria, namely those species within the genera *Thermotoga* [22] and *Aquifex* [23], have not been examined to any extent as yet. However, homologous gene sequences in *Aquifex aeolicus* [24] have been identified for the multimeric proteases PfpI, protease La, and ClpP. Thus, the hyperthermophilic bacteria provide another framework, distinct from the archaea, for examining the nature of proteolytic processes.

Here, we report the purification and characterization of a homomultimeric protease from the hyperthermophilic bacterium *T. maritima* [22], which has structural similarities to other proteolytic complexes, but is unrelated to any known protease. This *T. maritima* protease was found to have both structural and gene sequence homology to a bacteriocin from the mesophilic bacterium *Brevibacterium linens* [25,26], which inhibits the growth of certain Gram-positive bacteria [26]. Not only does this finding imply that the mesophilic bacteriocin may be proteolytic but it also suggests that anti-microbial ecological strategies may exist in hyperthermophilic environments, although no evidence for such phenomena has yet been reported.

2. Materials and methods

Unless otherwise specified, all chemicals used are commercially available and are of analytical grade or higher.

2.1. Processing of T. maritima cells

T. maritima (DSM 3109) biomass (103 g wet weight) was grown in continuous culture on RDM-based media [27] and collected under two different growth conditions from unrelated experiments (2 g/l maltose +1 g/l ammonium chloride or 5 g/l glucose+0.5 g/l yeast extract). The cell pellets were resuspended in approximately 190 ml of 50 mM sodium phosphate buffer (SPB) (pH 8), treated with 1 mg/ml lysozyme overnight at 4°C, disrupted in a French pressure cell at 18 000 psi (SLM Aminco, Urbana, IL), and spun at 14 000×*g* for 30 min at 4°C, yielding a crude extract containing 39 mg/ml of protein.

2.2. Column chromatography

All purification steps were carried out at room temperature on a Pharmacia LKB FPLC system (Pharmacia, Uppsala, Sweden). Samples were either clarified with a 0.2 μm filter or centrifuged to remove cellular debris, before being subjected to chromatographic separation. All concentration steps were done with stirred-cell concentrators

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Abbreviations: SPB, sodium phosphate buffer; PPB, potassium phosphate buffer; VKM, *N*-carbobenzyloxy-valine-lysine-methionine; MCA, 7-amino-4-methylcoumarin; A-K, alanine-lysine; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TEM, transmission electron microscopy

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(Amicon, Beverly, MA), using filters of 10 kDa molecular weight cutoff. Assays for the purpose of screening column fractions were done at an incubation temperature of 80°C, using 0.5 mM substrate and 10–15 μl of each fraction.

2.2.1. DEAE chromatography. Cell extract proteins (5.7 g in 145 ml) were loaded at a flow rate of 0.1 ml/min (25–40 ml at a time) to a 120 ml DEAE CL-6B XK 36/40 column (Pharmacia, Uppsala, Sweden) which was previously equilibrated with 50 mM SPB pH 8. After 350 ml of pass-through, a 350 ml linear gradient of 0–0.5 M NaCl, followed by a 50 ml gradient of 0.5–1 M NaCl in 50 mM SPB pH 8, was used to elute the proteins at a flow rate of 1.5 ml/min. The protease activity was followed by assaying with VKM-MCA. The majority of VKM-MCA activity was found in the pass-through.

2.2.2. Hydroxylapatite (HAP) chromatography. The pass-through from the DEAE column (360 mg) was concentrated about 3-fold to 650 ml. This pass-through (538 ml) was loaded at 0.75 ml/min onto an 80 ml hydroxylapatite (Calbiochem, La Jolla, CA) XK 16 column (Pharmacia, 1.6×40 cm), previously equilibrated with 300 ml of 25 mM PPB pH 7. After a 150 ml wash, proteins were eluted at 1.5 ml/min with a 300 ml linear gradient of 25–250 mM PPB pH 7. VKM-MCA activity was high in the pass-through, but also eluted from 70–205 mM PPB.

2.2.3. Affinity chromatography. The HAP fractions that bound (125 mg) were pooled, concentrated to 25 ml and equilibrated to loading buffer (50 mM Tris pH 8, 150 mM NaCl). This pool was loaded (at 0.2 ml/min), 5 ml at a time, on a Pharmacia XK 16/20 column containing 25 ml of benzamidine Sepharose 6B. After washing, the flow rate was increased to 1.6 ml/min, and an 85 ml linear gradient was run from 0–100% elution buffer (10 mM HCl pH 2, 0.5 M NaCl). Fractions eluting at greater than 50% elution buffer were immediately neutralized with 1/10th volume of 100 mM Tris pH 8.5. The column was regenerated as per manufacturer's suggestions. The majority of VKM-MCA activity was detected in the pass-through.

2.2.4. Gel filtration chromatography. The pass-through from affinity chromatography (47 mg) was concentrated to 10 ml before loading (1 ml at a time) on a Superdex 200 HiLoad 16/60 (Pharmacia, Uppsala, Sweden), which was previously equilibrated to 50 mM SPB at pH 7 and 150 mM NaCl. A flow rate of 0.25 ml/min was used. Peaks corresponding to molecular masses of greater than 2000 kDa (47.39 ml) and approximately 780 kDa (72.89 ml) separated cleanly from smaller proteins and each other, and were both found to have VKM-MCA hydrolyzing activity. Blue dextran (2000 kDa) was used to determine void volume (48.25 ml); the following protein standards were used for the calibration curve: thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa) and albumin bovine serum (66 kDa). The work reported here describes the characterization of the multimeric protease which eluted slightly before the highest molecular weight standard, corresponding to $M_r > 669$ kDa.

2.3. Protease activity assays

Proteolytic activity was detected by release of MCA from the carboxyl terminus of N-terminally blocked peptides (Sigma, St. Louis, MO) in 50 mM sodium phosphate buffer, pH 7, as described previously [20]. A Cytofluor 2350 fluorimeter (Millipore Corp., Bedford, MA) was used to follow the increase in fluorescence at 360 nm excitation and 460 nm emission. Substrate concentrations of 0.5 mM were typically used, and enzyme concentrations were adjusted (typically 0.5–5.0 μg per assay), such that the fluorescence measured at a sensitivity setting of 4 was within linear range of the fluorometric plate reader and at least 50% above background levels. At this sensitivity, one unit is equal to approximately 0.0028 μM MCA (0.28 pmol for a 100 μl assay). Temperature optima determinations and substrate specificity comparisons were done in triplicate. Incubation times were 10–20 min. Enzyme was omitted from controls at each substrate concentration and temperature in order to correct for spontaneous hydrolysis of the peptide substrates. Temperature optima experiments were done in a thermal cycler as reported previously [20], and substrate specificity comparisons were done on a 96 U-well microtiter plate with 0.5 mM substrate, in a total volume of 100 μl. Caseinolytic activity was assayed with the EnzChek Protease Assay Kit using BODIPY FL casein (Molecular Probes, Eugene, OR) per manufacturer's guidelines, except that the digestion buffer was replaced with 50 mM SPB pH 7. Green fluorescence was detected with the above fluorimeter at an excitation of 485 and emission of 530 nm. Proteolytic activity toward the substrate A-K (2 mM final concentration) was measured at 80°C in 50 μl of SPB pH 7, using 0.2 μg of purified protein and a 20 min incubation. A scaled-down microtiter plate version of the modified Yemm and Cocking [28] ninhydrin reagent (Sigma, St. Louis, MO) assay was used to detect the increase in amino groups caused by hydrolysis. The ninhydrin assay was also performed on substrate which had been heated without enzyme as a negative control, with enzyme added after quenching the reaction, to account for the presence of extra N-terminal groups.

Fig. 1. Native PAGE (10%) analysis of *T. maritima* VKM-MCA hydrolyzing proteases showing (1) a >2000 kDa membrane/protease assembly and (2) a 780 kDa (est.) homomultimeric protease (estimated from gel filtration column data).

2.4. Sugar assays

Total sugar assays were performed in triplicate using the orcinol-sulfuric acid method [29], which detects 0–20 μg of hexose moieties in 200 μl of sample. Glucose standards of 0.02–0.2 mg/ml were used, and samples were concentrated to approximately 0.4 mg/ml protein before assaying. Glycoprotein was detected as per manufacturer's protocols (Sigma GLYCO-PRO kit) using Schiff's reagent to stain a 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel.

2.5. Lipid assay

Lipid was detected by blotting samples (0.2–20 μg) onto Whatman paper, and exposing it to iodine vapors. Olive oil and cell extracts of *T. maritima* were used as positive controls, while bovine serum albumin and glucose were used as negative controls. Samples containing 0.1 μg of lipid or more changed color almost immediately on contact, while increased sensitivity was attained by extended incubation.

2.6. Total protein determination

Total protein concentration was determined using a bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL) with bovine serum albumin as a standard. The microtiter plate protocol was used, with an incubation time of 30 min at 37°C for concentrations of 0.25 mg/ml and above; a 2 h incubation time was used for lower concentrations, along with 50 μl of enzyme compared to the standard 10 μl. Duplicate readings were taken at 562 nm with an EL 340 Microplate (Bio-Tek Instruments, Winooski, VT), and background, due to buffer, was subtracted. For cases where sugars or other solutes interfered, a Bio-Rad (Bio-Rad Laboratories, Richmond, CA) total protein assay was done or protein concentration was estimated from absorbance at 280 nm.

2.7. Electrophoresis, zymograms, and isoelectric focusing gels

PAGE and SDS-PAGE were run according to standard protocols [30]. Pre-stained high molecular weight markers were purchased from Gibco BRL (Gaithersburg, MD). Zymograms (0.1% gelatin in 10% SDS-PAGE) (Novex, San Diego, CA) were run according to manu-

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                  1                                                  50
    Mtub antigen  MNNLYRDLAP VTEaAWAEIE lEAaRTFKRh IAGRRVVDVs dPgGpvTAAV
Blin bacteriocin  MNNLYRELAP IpgpAWAEIE eEARRTFKRn IAGRRIVDVa GPtGFETsAV
   Tmar protease  MefLkRsFAP lTEkqWqEID nrAReiFKtq lyGRkfVDVe GPyGWEyAAh
       Consensus  MNNLYR-LAP -TE-AWAEIE -EARRTFKR- IAGRR-VDV- GP-G-ETAAV

                  51                                                 100
    Mtub antigen  sTGrliDVka pTnGViahLR aSkPLVrLRv PFTLsRnEID DVERGSkDSD
Blin bacteriocin  tTGhIrDVqs ETsGlqvkqR ivqeyIELRt PFTvtRqaID DVaRGSgDSD
   Tmar protease  plGeVevlsd EnevVkwgLR kSlPLIELRa tFTLdlwElD nlERGkpnvD
       Consensus  -TG---DV-- ET-GV---LR -S-PLIELR- PFTL-R-EID DVERGS-DSD

                  101                                                150
    Mtub antigen  WePVKEAAkK lAfvEDRtIF eGYsAAsIeG IrSaSSNpAl tlPEdPrEip
Blin bacteriocin  WqPVKDAAtt IAmaEDRaIL hGlDAAGIgG IvpgSSNaAV aiPDaveDFa
   Tmar protease  lssleEtvrK VAefEDevIF rGCEksGVkG llS.feerkI ecgstPkDLl
       Consensus  W-PVKEAA-K -A--EDR-IF -G--AAGI-G I-S-SSN-A- --P--P-D--

                  151                                                200
    Mtub antigen  DvIsQALSeL RlaGVDGPYS vLLSaDvYTK VSEtsDHGYP IREHLnR.Lv
Blin bacteriocin  DAVaQALSVL RtvGVDGPYS LLLSsaeYTK VSEstDHGYP IREHLsRqLg
   Tmar protease  EAIvrALSIF skdGIEGPYt LvintDrWin flkeeaghYP lekrveecLr
       Consensus  DAI-QALS-L R--GVDGPYS LLLS-D-YTK VSE--DHGYP IREHL-R-L-

                  201                                                250
    Mtub antigen  dGDIIWAPAI DGAFVltTRG GDFDLqLGtD vaIGYaSHDt DTeRLYLQET
Blin bacteriocin  aGEIIWAPAl EGALlVSTRG GDYELhLGQD LSIGYySHDs ETVeLYLQET
   Tmar protease  gGkIIttPrI EdALVVSeRG GDFkLiLGQD LSIGYedrEk DaVRLFitET
       Consensus  -G-IIWAPAI EGALVVSTRG GDF-L-LGQD LSIGY-SHD- DTVRLYLQET

                  251          266
    Mtub antigen  LTFLcyTaEA SVaLSh
Blin bacteriocin  FgFLalTdEs SVpLSL
   Tmar protease  FTFqvvnpEA lIlLkF
       Consensus  FTFL--T-EA SV-LS-
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Fig. 2. Pile-up showing homology between the *T. maritima* homomultimeric protease and putative homologs in the mesophilic bacteria *M. tuberculosis* (34.8% amino acid sequence identity) *B. linens* (32.5% amino acid sequence identity). Capital letters indicate consensus, bold letters indicate conserved amino acids in all three sequences.

facturer's protocols, with the exception that the electrophoresis was done at 4°C to minimize proteolytic activity, and gels were incubated at 70°C overnight in 50 mM sodium phosphate buffer pH 7. Isoelectric focusing gels were run on a Phast system (Pharmacia, Piscataway, NJ) according to manufacturer's guidelines.

2.8. N-terminal sequence

Protein sequencing was performed at the University of Georgia Molecular Sequencing Facility, Athens, GA. Samples (about 200 pmol) were TCA-precipitated and washed with acetone. The proteins were run on 17% SDS-PAGE, transferred to a Mini ProBlott membrane (Applied Biosystems, Foster City, CA) in 10 mM CAPS buffer, pH 10, 20% methanol using a Semi-Phor transfer apparatus, as directed by the manufacturer (Hoefer Scientific Instruments, San Francisco, CA).

2.9. Blast searching and sequence comparisons

A blastp search was performed using the National Center for Biotechnology Information (NCBI) blast server [31]. Putative homologous sequences were compared using the University of Wisconsin's GCG 8.1 'pileup' program [32], followed by the command 'pretty' implementing the case and consensus functions. The plurality invoked was 2.00, with a threshold of 1.00, and an average weight of 1.00. The percent identity and percent similarity values were calculated by using the UWGCG 8.1 program GAP, with a gap creation penalty of 3.00 and an extension penalty of 0.10.

2.10. Electron microscopy

TEM of negatively stained proteins was performed at the Laboratory for Advanced Electron and Light Optical Methods, College of Veterinary Medicine, NCSU, Raleigh, NC, using a Philips EM 410 microscope. Approximately 4 μl (50–100 μg/ml) of purified protein in 50 mM sodium phosphate buffer plus 150 mM sodium chloride, pH 7, were placed on a 3 mm Formvar-coated grid. Excess solution was removed with filter paper after 5 min, and one drop of 2% sodium phosphotungstic acid (pH 7.25) was used to stain the grid for 1 min. Excess stain was blotted with filter paper, and the grid was allowed to dry before microscopic examination [33]. Photomicrographs were taken at a magnification of 31 000–54 800 and were further enlarged 2.2 times.

3. Results

3.1. Purification

The cell extracts produced from both growth conditions of *T. maritima* contained comparable amounts of proteolytic activity toward the substrate VKM-MCA. Purification by column chromatography was made difficult by the presence of several VKM-MCA hydrolyzing proteases in cell extracts and the tendency for the protein to either not bind at all to columns, or to bind in a non-specific manner. In the first step (DEAE), the protein was present in the pass-through which was very viscous, making it difficult to concentrate and suggestive that there may be some association of the protein with polysaccharides or lipids. The DEAE step produced a five-fold increase in specific activity with a 32% recovery. The

binding of this protein to the HAP column appeared to significantly reduce activity and recovery; subsequent purification steps did not result in any increase in specific activity. Gel filtration chromatography revealed that the protease was larger than any of the standards used (>669 kDa); extrapolation from a calibration curve based on known standards (data not shown) yielded an estimated M_r of 780 kDa. Fig. 1 shows PAGE (native) analysis of the two proteolytic activities identified during purification. As can be seen, the >2000 kDa (est.) protease/membrane assembly in lane 1 has a lower electrophoretic mobility than the 780 kDa (est.) protease in lane 2. The focus here is on the >669 kDa protein, which appears to be purified to homogeneity. Typically, on 10–15% gels, this protease ran into the stacking gel but did not migrate in the separating gel, even after SDS and heat treatment. When TCA-precipitated in preparation for N-terminal sequencing, the protein was difficult to resuspend and quite often appeared to be aggregated; only one type of subunit with a M_r of approximately 29–31 kDa was noted (data not shown).

3.2. Gene sequence analysis

The 15 N-terminal amino acids were sequenced and found to be MEFLKRSFAPLTEKQ. This sequence corresponded to the N-terminus of a 265 amino acid long ORF, assembled from three separate contigs in the *T. maritima* partial genomic sequence obtained from The Institute for Genomic Research [34]. The predicted M_r from amino acid sequence information is 30.5 kDa, while the predicted p*I* is 4.85 [35]. This amino acid sequence yielded two possible homologs [31]: Linocin M18 from *B. linens* (PID g1279217), a homomultimer composed of 31 kDa (266 amino acids) subunits, and a 29 kDa (265 amino acid) antigen from *M. tuberculosis* (PID g2231022). Although there are a number of conserved residues in comparing the three sequences (Fig. 2), it is not obvious what type of active site this protease contains. The *T. maritima* protease is 34.8% identical and 55.3% similar (at the amino acid level) to the *M. tuberculosis* ORF; it is 32.5% identical and 54.0% similar to the *B. linens* ORF. The two mesophilic homologs are 58.1% identical to each other. No homologous genes were identified in genomic sequences of archaea, although a potential homolog was noted in the hyperthermophilic bacterium *A. aeolicus*; this putative protein (PID g2984059) is composed of 281 amino acids with 20–25% identity to the above protein sequences. There is also no apparent sequence homology to any known proteases. It does not appear that post-translational modifications occur since the N-terminus is intact and the calculated molecular weight from the amino acid sequence is close to that predicted by SDS-PAGE.

3.3. Microscopy

Fig. 3A is TEM analysis of the purified multisubunit protease, which was negatively stained. The average diameter of the circular particles was estimated to be 19–20 nm. These pictures are reminiscent of both front and side views (top right of photo) of ClpP [1], although the diameter of ClpP is closer to 11 nm. The globular structures viewed by TEM are the same shape and size of those reported for Linocin M18 [25]. Fig. 3B shows a TEM image of the affinity column pass-through, where circular particles were noted, along with tube-shaped objects, presumably the side view. The latter resemble defective phage tails and are approximately 60–80 nm

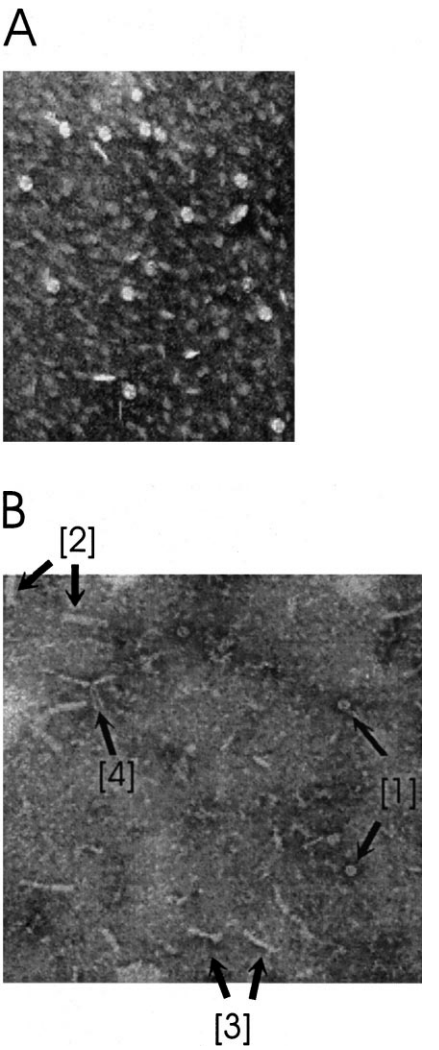

Fig. 3. TEM analysis of *T. maritima* homomultimeric protease. A: S200 pool, taken at 54 800× and photographically enlarged 2.2× further, the average diameter of the multisubunit protease is estimated to be 19–20 nm. B: Affinity pool, taken at 31 000× and photographically enlarged 2.2×. 1: Spherical form of bacteriocin as in A; 2: tail with contracted sheath, average length of 60–80 nm; 3: putative fibers of bacteriocin; 4: (faint) hollow core.

long, which is consistent with phage tail bacteriocins in the contracted state [36]. Occasionally an empty or hollow tube is apparent, as well as smaller diameter fibrous rods. The circular particles seen in Fig. 3A were observed in every step of the purification, from both preparations of crude extracts to the purified product.

3.4. Biochemical analysis

The globular proteolytic assembly did not contain significant amounts of lipid. Total sugar assays estimated that there were potentially hexose moieties in the protein (0.04 μg in a 4 μg protein sample), but glycoprotein analysis was negative. The specific activity of the purified protease toward VKM-MCA is 2.8 nmol MCA released/mg/min. Proteolytic activity was enhanced in the presence of 10 mM Ca^{2+} (1.5 times) and Co^{2+} (1.4 times). EDTA (10 mM) reduced activity by 50% relative to the positive control. The addition of SDS (1%) reduced activity by six-fold. Substrate specificity of the *T. maritima* homomultimeric protease to a variety of MCA-linked peptides revealed the following pattern: AAF-

> AFK > VKM > IIW > LY > GGF > AAPV. Little or no activity was detected on FVR, LLVY or GGR. No activity was noted on casein, even when ATP was added. Zymograms containing gelatin, which were incubated overnight, however, showed multiple faint clearing zones at various sizes (including around 30 kDa) (data not shown). The multiple bands may be due to various forms of the protease resulting from partial unfolding in SDS. Proteolytic activity was optimal between 90–93°C, slightly above the maximum growth temperature (90°C) of *T. maritima*. The protease was most active between pH 6–9 with an optimum slightly above neutral (data not shown). The p*I* could not be determined experimentally, as the protein would not migrate from its initial position in the isoelectric focusing gel. The protease had a half-life of 36 min at 95°C (data not shown).

Lipase and esterase assays were performed, to determine whether this enzyme might be capable of hydrolyzing components of cell envelopes. In the conditions tested, no hydrolysis (at 70°C) of olive oil or ethyl butyrate was detected (data not shown). The main components of the peptide moiety in archaeal cell walls are glutamic acid, alanine, and lysine (all L-isomers) [37]. Peptidase assays on A-K using both the purified protease and *T. maritima* concentrated supernatant were slightly positive, but within experimental error of the assay.

4. Discussion

This report adds to the growing list of multimeric proteases that have been identified in hyperthermophilic and less thermophilic microorganisms. Understanding of the significance and range of proteolytic processes in cells and organisms continues to expand. For example, proteases are implicated in a variety of pathogenic and non-pathogenic medical disorders [38–43], and are also part of the cellular response to DNA damage, foreign protein invasion, stress response, and antigen presentation [44–48]. The possible role of proteases as anti-bacterial agents, as suggested by the homology of the *T. maritima* protease to specific mesophilic proteins, is interesting and bears further examination.

The mechanism of action of the *B. linens* bacteriocin, as well as many others, as an anti-bacterial has not yet been identified. However, there is evidence that similar bacteriocins affect cell walls by affecting peptidoglycan biosynthesis directly [25,49]. Defective phage tails often contain a lytic component [50], so it is possible that proteolytic activity plays a role in bacteriocin function by degrading essential cell envelope proteins. The activity of this multisubunit protease on pseudomureins or mureins present in hyperthermophilic cell envelopes also deserves further investigation.

Although there has been no prior mention of a hyperthermophilic antibacterial agent, viruses have been identified in hyperthermophiles [51], and bacteriocins have been identified in some thermophiles [52,53]. Investigating antimicrobial activity toward hyperthermophiles, especially strict anaerobes, is problematic due to the difficulty in using plating assays, and the inherent error in cell-counting techniques. Preliminary screens comparing growth curves in liquid culture with and without the *T. maritima* protease revealed no significant antagonistic activity against hyperthermophiles (archaea and bacteria), including two members of the Thermotogales (data not shown). Further work is needed to insure the integrity of the putative bacteriocin assembly during purification and to determine its cellular localization which is, perhaps, toga- or membrane-associated.

Hyperthermophilic anti-bacterial agents could lead to some interesting applications as thermostable, protein-based antibiotics [25], if they exhibit antagonistic activity against pathogens. At lower temperatures, the efficacy of such hyperthermophilic bacteriocins may be limited to only the most susceptible organisms, thereby making them more selective. In any case, whether from an ecological or biotechnological perspective, the possibility that proteolysis underlies anti-bacterial activity merits further examination.

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