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# Isolation of a chymotrypsinogen B-like enzyme from the archaeon *Natronomonas pharaonis* and other halobacteria

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**Abstract** A protease of a molecular mass of approximately 30 kDa was isolated and purified from the haloalkaliphilic archaeon *Natronomonas* (formerly *Natronobacterium*) *pharaonis*. The enzyme hydrolyzed synthetic peptides, preferentially at the carboxyl terminus of phenylalanine or leucine, as well as large proteins. Hydrolysis occurred over the range of pH from 6 to 12, with an optimum at pH 10. The temperature optimum was 61°C. The enzyme was nearly equally active over the range of salt concentration from 0.5 to 4 M (NaCl or KCl). A strong cross-reaction with a polyclonal antiserum against human chymotrypsin was observed. Enzymatic activity was inhibited by typical serine protease inhibitors. There was significant homology between N-terminal and internal sequences from autolytic fragments and the sequence of bovine chymotrypsinogen B; the overall amino acid composition was similar to that of vertebrate chymotrypsinogens. Evidence for a zymogen-like processing of the protease was obtained. Cell extracts from other halobacteria exhibited similar proteolytic activity and immunoreactivity. The data suggested a widespread distribution of a chymotrypsinogen B-like protease among halo- and haloalkaliphilic Archaea.

**Key words** *Natronomonas pharaonis* · Natronobacteria · Archaea · Serine protease · Chymotrypsinogen · Evolution

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## Introduction

Natronobacteria are members of the extremely halophilic Archaea (the halobacteria), which require not only high NaCl concentrations, but also high pH (up to 11) and low concentrations of $Mg^{2+}$ for growth (Soliman and Trüper 1982; Tindall et al. 1984). Currently, five species of natronobacteria are recognized (see Kamekura et al. 1997 for a recent classification): *Natronobacterium* (now *Natronomonas* [*Nm.*]) *pharaonis*, *Nb. gregoryi*, *Nb.* (now *Natrialba*) *magadii*, *Natronococcus occultus*, and *Nc. amylolyticus*. A further species, *Nb. vacuolata*, has been reclassified as *Halomonas vacuolata*. Although Archaea possess a prokaryotic cell organization, they share numerous properties with eukaryotes (Zillig 1991), such as the genes for transcription and protein synthesis, whereas their metabolic enzymes are more similar to (eu)bacterial counterparts. These early observations were recently amply confirmed by analysis of sequenced bacterial and archaeal genomes (Koonin et al. 1997).

All Archaea investigated so far contain a membrane-bound vacuolar-type (V-) ATPase (EC 3.6.1.34); this enzyme is present in the endomembrane system and some plasma membranes of eukaryotes (for a recent review, see Stevens and Forgac 1997). The archaeal V-ATPase has been proposed to operate also in the reverse mode, i.e., to synthesize ATP in response to a proton or ion gradient (Nelson 1992, 1995), although a direct demonstration is still lacking. When attempting to purify the ATPase complex from *Nm. pharaonis*, we noted loss of ATP hydrolytic activity following storage for about a week. This was in contrast to the well-characterized ATPase from the related haloneutrophilic archaeon *Halobacterium* (now *Halorubrum*) *saccharovorum* (Hochstein et al. 1987; Stan-Lotter and Hochstein 1989), which could be stored for months in the presence of salt, remaining in an active form. We describe here a proteolytic activity from *Nm. pharaonis*, which proved to be an enzyme with remarkable activity in the absence of salt, although it was isolated from an obligate halophile. In addition, the natronobacterial protease was

thermophilic, alkaliphilic, and, surprisingly, highly similar to vertebrate chymotrypsinogen B, including evidence for its enzymatic processing. The presence of such a proteolytic system in halophilic Archaea suggests a role of these prokaryotes in early endosymbiontic events.

## Material and methods

### Bacterial strains and culture conditions

*Natronomonas pharaonis* DSM 2160$^T$, *Natronobacterium gregoryi* DSM 3393$^T$, *Natrialba magadii* DSM 3394$^T$, *Nc. occultus* DSM 3396$^T$, and *Halobacterium halobium (=Hb. salinarium)* DSM 670 were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen), Braunschweig, Germany. *Halorubrum saccharovorum* ATCC 29252$^T$, *Haloferax volcanii*, and *Hf. mediterranei* were gifts from Dr. L.I. Hochstein, NASA Ames Research Center (Moffett Field, CA, USA). Natronobacterial cells were grown at 37°C in alkaline medium (pH 9.5) containing 4 M NaCl as described previously (Tindall et al. 1984). Haloneutrophiles (*Hr. saccharovorum*, *Hb. halobium*, *Hf. mediterranei*, *Hf. volcanii*) were grown in M2 medium (Tomlinson and Hochstein 1976). Samples were taken during various times of growth. Cells were harvested by centrifugation at 10000 × *g* for 25 min. For the examination of extracellular proteolytic activity, the spent medium was concentrated to 1/100 of its original volume in a stirred protein-concentrating cell (Amicon) equipped with a 10-kDa cutoff filter.

### Purification of the protease

From membranes. Cells of *Nm. pharaonis* were suspended in a buffer containing 50 mM Tris-HCl, 4 M NaCl, and 1 mM EDTA, pH 7.4, and passed through a French pressure cell (Aminco) at 27 MPa. Membranes were pelleted by centrifugation and subsequently treated with 0.7% octylglucoside (final concentration) for 3 h. The octylglucoside extract was passed through a molecular sieve column (Sephacryl S300 HR; Pharmacia, Uppsala, Sweden). The active fractions eluted over a range of Ve/Vo (relative elution volume) between 1.8 and 2.3. In a second chromatographic step, using a Superdex 75 (Pharmacia) column, the protease eluted as a narrow peak.

From lysed cells. To the cell pellet from 100 ml culture, 100 ml of distilled water and 2 mg of DNase I were added and stirred for 30 min. This procedure lysed the natronobacterial cells. Following centrifugation, the supernatant was applied to a DEAE CL-6B (Pharmacia) column equilibrated in 100 mM Tris-HCl, pH 7.4. Active fractions were eluted with a linear NaCl gradient and purified further by molecular sieve chromatography on a Superdex 75 column and by ion exchange, using a MonoQ column HR 5/5 (Pharmacia).

The proteases from *Na. magadii* and *Nc. occultus* were prepared by a combination of these methods, starting with the cytoplasmic fraction, following passage of cells through the French press, and dialyzing the samples against water.

### Enzyme and protein assays

Unless noted otherwise, the assay buffer consisted of 1 mM substrate in 3 M NaCl, 50 mM Tris-HCl, pH 9. The following synthetic peptides were used as substrates: Suc-AlaAlaProPhe-pna, Suc-AlaAlaProLeu-pna, Suc-Phe-pna, *N*-methoxy-Suc-AlaAlaProVal-pna, Nα-benzoyl-Arg-pna (all from Sigma); 10–50 µl of sample was added to 3 ml of substrate solution. The absorbance of the liberated nitroaniline was recorded at 405 nm for 10–15 min at a temperature of 55°C, unless noted otherwise. A spectrophotometer DU 650 (Beckman, Fullerton, CA, USA) equipped with a Peltier thermocontroller for temperatures up to 90°C was used. One unit of activity was defined as the amount of enzyme that liberated one µmol of nitroaniline/min, using an absorption coefficient of 1.021 $mmol^{-1} mm^{-1}$ for nitroaniline (Geiger 1984). Bovine pancreatic α-chymotrypsin A and chymotrypsinogen A (both from Worthington, Freehold, NJ, USA) were used for comparisons. Protein substrates were azocoll, fluorescein thiocarbamoyl-casein, and hide powder azure (all from Calbiochem, San Diego, CA USA). Assay conditions for these substrates were as given in the instructions by the manufacturer, except that 3 M NaCl was present in the assay buffers. For the determination of the pH optimum of activity, buffers were made with either 50 mM MES (2-(N-morpholino)-ethane sulfonic acid) (pH 6), TES (N-tris(hydroxymethyl)methyl-2 aminoethane sulfonic acid) (pH 7), Tris (pH 8, 9), or CAPS (3-cyclohexylamino-1-propanesulfonic acid) (pH 10, 11, 12), respectively. Protein concentrations were determined by the method of Lowry et al. (1951), with bovine serum albumin as a standard.

### Storage

The active fractions could be stored for up to 1 week in 100 mM Tris-HCl, pH 7.4 at 4°C without loss of activity. For long-term storage, glycerol was added to a final concentration of 10% before placing the enzyme at −70°C.

### SDS and isoelectric focusing polyacrylamide gel electrophoresis

Samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using either the system of Laemmli (1970) or the Tris-Tricine system (Schägger and von Jagow 1987). Vertical isoelectric focusing (IEF) gels were prepared and run as described previously (Stan-Lotter and Bragg 1986; Stan-Lotter and Hochstein 1989).

### Immunoreactions

Polyclonal antisera, which were raised in rabbits against human chymotrypsin, were purchased from Biogenesis (Poole, England). Immunoreactions were analyzed following transfer of proteins from SDS or IEF gels to polyvinylidene difluoride membranes (BioRad, Hercules, CA, USA) using the standard Western blot procedure (Towbin et al. 1979). Visualization was performed with the enhanced chemiluminescence reagent SuperSignal (Pierce, Rockford, JL, USA); this method has been described elsewhere (Gruber and Stan-Lotter 1997).

### N-terminal sequence analysis and amino acid composition

Protease samples were electroblotted on polyvinylidene difluoride membranes, following SDS gel electrophoresis in the presence of 1 mM thioglycolic acid. Protein bands were identified by brief staining with Coomassie blue and subsequently excised. N-terminal amino acid residues were determined with the protein sequencers model 473 and 467A (Applied Biosystems, Foster City, CA, USA). For determination of cysteinyl residues, protease samples were reduced in the presence of 8.2 M urea and treated with either iodoacetate (Crestfield et al. 1963) or vinylpyridine, using a modification of the procedure described by Lundblad (1995). Amino acid composition was determined with the analyzer model 420 (Applied Biosystems). For sequence comparisons, the SwissProt protein data base was used; sequences were aligned using the program Clustal W.

### Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI TOF)

Protease samples and standard proteins (trypsin inhibitor) were applied in a matrix of gentisic acid (Aldrich, Sigma, Vienna, Austria) in 0.1% trifluoroacetic acid to a metal slide and measurements were performed in a Kompact MALDI III instrument (Kratos Analytical, Manchester, England).

## Results

### Purification of a protease from *Nm. pharaonis*

The enzyme was initially prepared from the membranes of *Nm. pharaonis*. During gel filtration of the octylglucoside extract of membranes on Sephacryl S300HR, proteolytic activity was associated with fractions in a molecular mass range of about 200–300 kDa. Subsequent purification on Superdex 75 yielded a sharp protease peak, which corresponded to an $M_r$ of about 30 kDa. This estimate was confirmed by detection of a major peak of a molecular mass of 30812 daltons, using MALDI-TOF mass spectrometry (data not shown).

A considerable amount of proteolytic activity was always present in the supernatant following sedimentation of membranes; this fraction constituted essentially the natronobacterial cytoplasm. Different batches of cells varied in their ratio of membrane-associated to supernatant-associated protease activity between 1:1 and 1:8, and the maximum purification of the enzyme from membranes was never more than 30 fold. We developed therefore the method for purification of the protease from lysed cells, which proved more efficient. The results of the purification of the protease from water-lysed cells are summarized in Table 1. Purification of the enzyme was 122 fold and the yield was 79%. N-terminal amino acid sequences and immunoblots (see following) confirmed that the enzymes purified from either membranes or lysed cells were identical. One major band of $M_r$ of 30 kDa was present following separation by SDS-PAGE (Fig. 1, lane 4). Autolysis occurred within a day of storage or was already present during preparation, as was apparent by increasing amounts of lower molecular mass fragments (Fig. 1, lane 5). No proteolytic activity was found during any growth phase, when assaying concentrated samples of spent medium. Thus it can be concluded that the protease was not secreted by the cells under the growth conditions used in this work.

### Properties of the protease

The purified protease was assayed with several synthetic peptides as substrates (see Methods). The activity toward Suc-AlaAlaProPhe-pna was 4.5 times higher than toward Suc-AlaAlaProLeu-pna (0.87 units/ml versus 0.19 units/ml). Other peptides were cleaved with less efficiency (as given in % of the activity toward Suc-AlaAlaProPhe-pna): *N*-methoxysuc-AlaAlaProVal-pna, 7.8%; Suc-Phe-pna, 2.3%; Nα-benzoyl-Arg-pna, 0%. Incubation of the protease with the substrates azocoll, fluorescein thiocarbamoyl-casein, and hide powder azure, for 4 h at 55°C yielded about 0.1–0.2 absorbance units/ml; this amounted to about 10% of the activity of a comparable bovine chymotrypsin A solution toward these substrates.

**Table 1.** Purification of the protease from *Natronomonas pharaonis*

| Fraction | Protein (mg) | Protease activity[a] (units) | Specific activity (units/mg) | Purification factor | Yield (%) |
|---|---|---|---|---|---|
| Lysed cells | 68 | 17.1 | 0.25 | 1 | 100 |
| DEAE | 5.5 | 15.9 | 2.89 | 11.5 | 93 |
| Superdex 75 | 0.7 | 14.6 | 21.1 | 83.4 | 85 |
| MonoQ | 0.44 | 13.5 | 30.7 | 121.4 | 79 |

[a] Suc-AlaAlaProLeu-pna was used as substrate in enzyme assays

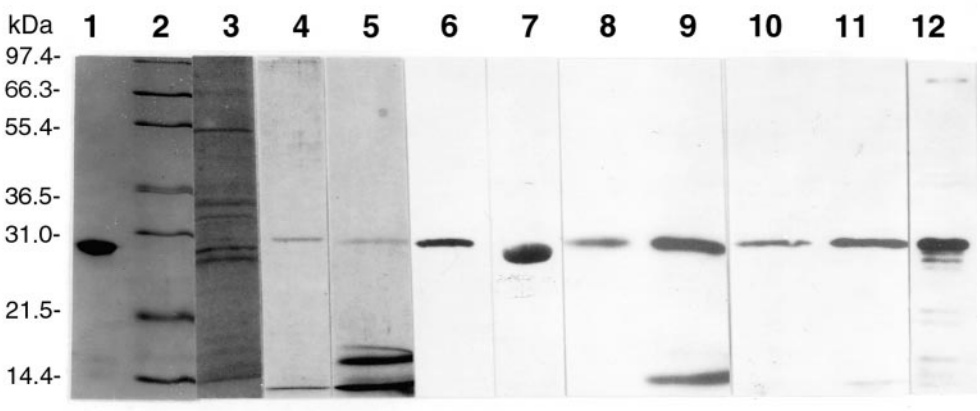


**Fig. 1.** SDS-PAGE and immunoreactions of the protease from *Natronomonas pharaonis* and other halophilic Archaea. Following separation by SDS gel electrophoresis, proteins were stained with Coomassie blue (*lanes 1–5*) or blotted onto polyvinylidene difluoride membranes and incubated with antiserum against human chymotrypsin (*lanes 6–12*). Immunocomplexes were visualized by enhanced chemiluminescence (Gruber and Stan-Lotter 1997). Lanes contain the following samples: *lane 1*, bovine chymotrypsinogen A (2 μg); *lane 2*, molecular markers; *lane 3*, *Nm. pharaonis* lysed cells (20 μg); *lanes 4*, *5*, *Nm. pharaonis* purified protease (see Methods); *lane 6*, *Nm. pharaonis* lysed cells; *lane 7*, bovine chymotrypsinogen A; *lanes 8*, *9*, *Nm. pharaonis* protease (same as in lanes 4 and 5); *lane 10*, *Natrialba magadii* protease; *lane 11*, *Natronococcus occultus* protease; *lane 12*, *Halobacterium halobium* lysed cells. Autolytic protease fragments are visible in lanes 5 and 9. $M_r$ of markers are depicted to the *left*

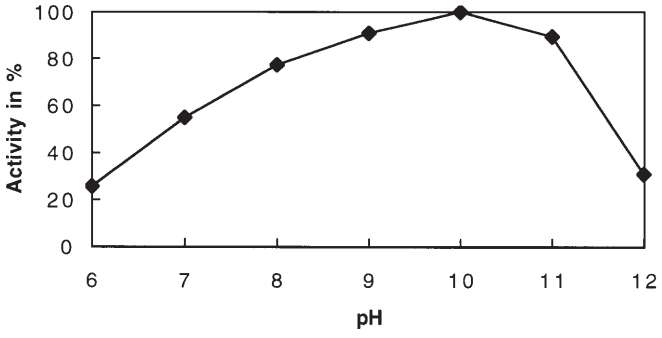


**Fig. 2.** Effect of pH on the protease activity from *Nm. pharaonis*. The enzyme was assayed as described in Methods, except that the assay temperature was 37°C. The substrate was Suc-AlaAlaProLeu-pna

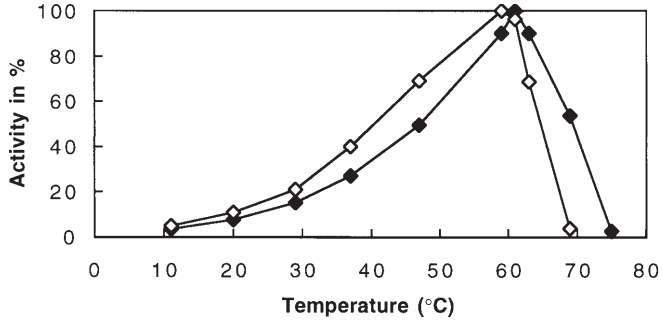


**Fig. 3.** Effect of temperature on protease activity. The protease (*solid diamonds*) from *Nm. pharaonis* and bovine chymotrypsin A (*open diamonds*) were assayed with Suc-AlaAlaProPhe-pna as substrate

The optimum pH of the natronobacterial protease activity was about 10; proteolytic activity was detectable up to pH 12 (Fig. 2). The temperature optimum was 61°C; no activity could be measured beyond 75°C (Fig. 3). The temperature optimum of bovine chymotrypsin A, using the same substrate and buffer, but omitting the NaCl, was 59°C (Fig. 3). Without the addition of salt to the assay buffer (which has a final concentration of about 3 mM NaCl, because of sample transfer) about 50% of the maximum protease activity was measured (Fig. 4). Activity increased up to about 1.5 M salt and remained the same over the range to 4 M. No particular preference by the enzyme for either NaCl or KCl was apparent (Fig. 4). Bovine chymotrypsin(ogen) A was used for the comparisons because bovine chymotrypsinogen B is not available commercially; it is 74% similar in amino acid sequence to bovine chymotrypsinogen B (Smillie et al. 1968). The effect of increasing NaCl concentration on the activity of bovine chymotrypsin A was remarkably similar to the effect on the natronobacterial protease (Fig. 4). Under the assay conditions used here, the specific activity of bovine chymotrypsin A was 34–40 units/mg protein.

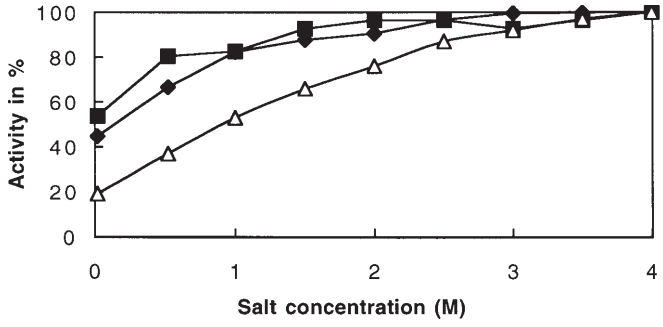


**Fig. 4.** Salt dependence of the protease activity. The protease from *Nm. pharaonis* (*closed symbols*) and bovine chymotrypsin A (*open triangles*) were assayed as described in Methods, except that the buffers contained varying concentrations of NaCl (*squares, triangles*) or KCl (*diamonds*), respectively. Assay temperature was 55°C; the substrate was Suc-AlaAlaProLeu-pna

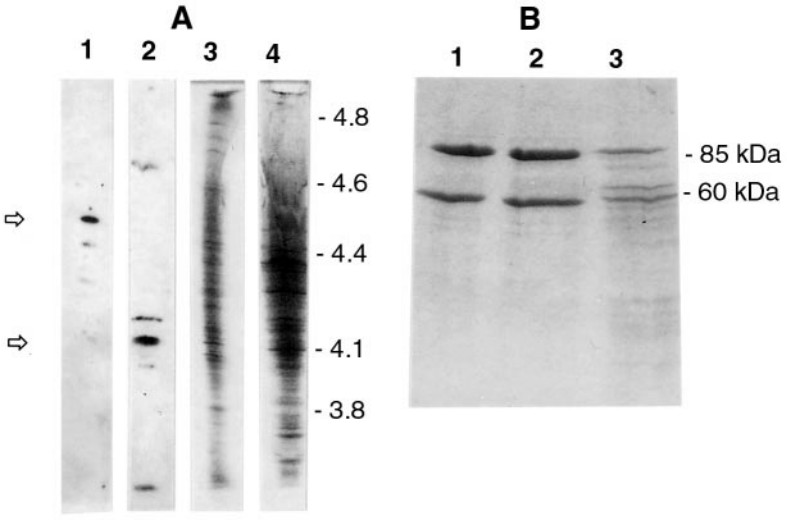


**Fig. 5. A** Immunoreactions of natrono- and halobacterial proteins following separation by isoelectric focusing: 35 μg of protein from lysed cells of *Nm. pharaonis* (*lanes 1, 3*) and *Halorubrum saccharovorum* (*lanes 2, 4*) were applied to an IEF gel. The pH gradient is indicated to the *right*. The gel was stained with Coomassie blue (*lanes 3, 4*). Following drying and rehydration of the gel, immunoreactions (*lanes 1, 2*) were carried out as described in the legend to Fig. 1. **B** Degradation of the membrane ATPase from *Hr. saccharovorum* by the purified protease from *Nm. pharaonis*. The two major subunits of the ATPase (85 kDa and 60 kDa) are shown: 10 μg of ATPase were applied per lane, either untreated (*lane 1*), or following incubation with 1 μg of protease (*lane 3*), or without protease (*lane 2*), respectively, for 18 h

The protease was inhibited by 0.6 mM phenylmethylsulfonyl fluoride (4% activity) and by 50 μM *N*-tosyl-L-phenylalanine chloromethylketone (14% of the activity without the inhibitor). Trypsin inhibitors from lima or soy beans in equimolar concentrations inhibited the protease down to 11% and 35%, respectively, of the activity of the uninhibited enzyme. These data suggested the classification of the protease from *Nm. pharaonis* as a serine protease.

The purified protease from *Nm. pharaonis* reacted in Western blots strongly with a polyclonal antiserum against human chymotrypsin (Fig. 1, lanes 8, 9). The isoelectric point of the enzyme was determined by applying whole cell proteins to vertical isoelectric focusing (IEF) gels (Stan-Lotter and Bragg 1986; Stan-Lotter and Hochstein 1989) and subsequent immunoblotting. Only one major band with an apparent IEP of 4.55 was present in *Nm. pharaonis* (Fig. 5A, lane 1).

## N-terminal sequences and amino acid composition

Sequences obtained from the main band of the natronobacterial protease and from several autolytic fragments of $M_r$ between 9 and 29 kDa are shown in Fig. 6, in comparison with complete sequences from several vertebrate chymotrypsinogen B-type proteases. The natronobacterial protease sequences were determined by three independent laboratories, using preparations from both membranes and lysed cells of *Nm. pharaonis*. The resulting N-terminal sequences (IVNGEDAVPGSGPXQVSLQD and KTPXKLQQATLPIVSNIDXRK) were identical. Of a total of 42 amino acid residues from the protease (Fig. 6; see also following), 41 were identical to residues of bovine chymotrypsinogen B. The amino acid composition of the natronobacterial protease was similar to those of chymotrypsinogen B from bovine pancreas and rat, but much less similar to those of several known halobacterial proteases (Table 2). The cysteinyl content was at least 10 residues per molecule of protease, i.e., about 3.4%, a high level compared to other halobacterial proteins, which are generally low in cysteinyl content at about 0.4% (Soppa et al. 1993), but typical for serine proteases of the trypsin/chymotrypsin family. There was no particularly high excess of acidic amino acids in the natronobacterial protease, although this is a well-known characteristic of many proteins from extremely halophilic Archaea (Table 2; Reistad 1970).

## Distribution of the protease among halobacteria

Other natronobacteria (*Na. magadii*, *Nc. occultus*) showed a similar immunoreactive band of about 30 kDa (Fig. 1, lanes 10, 11), which suggested the presence of a similar enzyme. The immunoreactive protease band could be detected in water-lysed cells of *Nm. pharaonis* before purification (Fig. 1, lane 6), and also in similarly lysed cells of *Hb. halobium* (Fig. 1, lane 12). Several immunoreactive proteins were detected in lysed cells from *Hr. saccharovorum*; most prominent was a band of isoelectric point (IEP) of about 4.2 (Fig. 5A, lane 2). Isoelectric focusing gels of other haloalkaliphiles (*Na. magadii*, *Nb. gregoryi*, *Nc. occultus*) and SDS gels of haloneutrophiles (*Hf. mediterranei*, *Hf. volcanii*) contained immunoreactive bands of IEPs between 4.4 and 4.5 and $M_r$ of 30 kDa, respectively, when probed with the same antiserum (data not shown). Although the identity of these bands with the chymotrypsin-like protease described here has still to be confirmed, the data suggest that halo(neutro)philic Archaea possess a similar enzyme to haloalkaliphiles.

## Evidence for a zymogen-like processing

Bovine chymotrypsin A and B are known to be synthesized as zymogens with an N-terminal peptide of 15 amino acids, which is cleaved by trypsin to yield the active protease (Hess 1971). The N-terminal sequence IVNGEDAVPGSGPXQVSLQD, which was found in the natronobacterial protease (Fig. 6), suggested that cleavage at a conserved

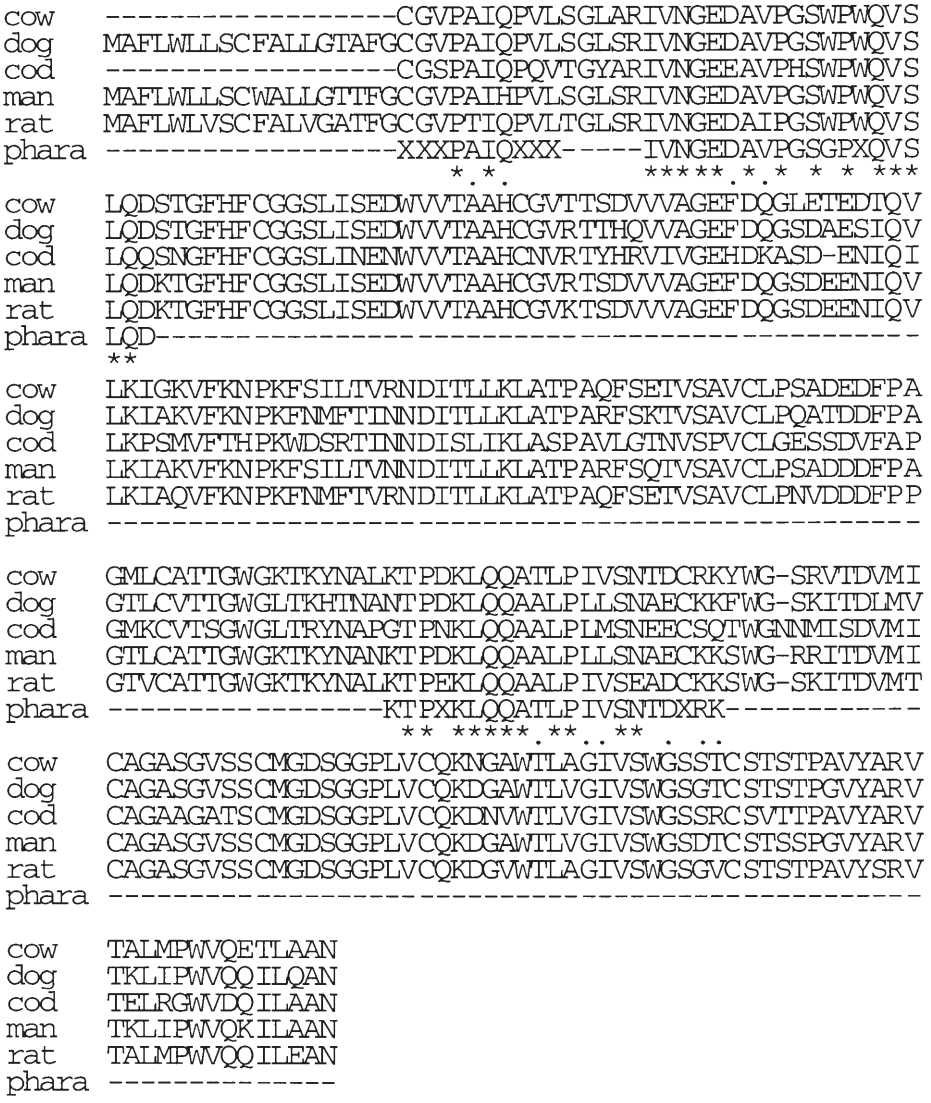


**Fig. 6.** Alignments of partial amino acid sequences of the protease from *Nm. pharaonis* with sequences of vertebrate chymotrypsinogen B. Amino acid residues, which are identical in all compared sequences, are denoted by *asterisks*; similar amino acid residues are indicated by *dots*. *X* indicates a position in the Edman degradation without identification of the amino acid. Organisms and SwissProt accession numbers are cow, *Bos taurus* P00767; dog, *Canis familiaris* P04813; cod, *Gadus morhua* P80646; man, *Homo sapiens* P17538; rat, *Rattus norvegicus* P07338; phara, *Nm. pharaonis*, this study

Arg15 (*Bos taurus* numbering) could have occurred. Another fragment ($M_r$, 19 kDa) contained the conserved short sequence PAIQ (Fig. 6); this was likely an autolytic fragment from a π-chymotrypsin-like equivalent of the protease, where cleavage had occurred between Arg15 and Ile16, and the disulfide bridge between Cys1 and Cys122 was still intact. When storing the crude extract from *Nm. pharaonis* cells at 4°C, we observed an increase of the proteolytic activity within 2 days by about 2.5 fold, which could be ascribed to a gradual processing of the protease. Furthermore, the substrate Nα-benzoyl-Arg-pna was cleaved at the carboxy terminus of Arg by the cell extract to an extent of 11%, when compared to the standard substrate Suc-AlaAlaProPhe-pna (100%); however, the purified protease did not cleave this substrate. This finding suggested the presence of one or more trypsin-like enzymes in the crude extract, which could be responsible for processing. The isolation of a trypsin-like enzyme from *Nm. pharaonis* is currently under way in our laboratory. Taken together, the findings were consistent with the operation of a similar activation mechanism of the natronobacterial protease as is known for chymotrypsinogen.

### Degradation of halobacterial ATPase by the protease

The membrane ATPase from *Hr. saccharovorum* (Hochstein et al. 1987; Stan-Lotter and Hochstein 1989) was incubated with the protease from *Nm. pharaonis* in a ratio of 10:1 (wt/wt) for 18 h at ambient temperature in the presence of 4 M NaCl. Several fragments were formed from the two major subunits of the halobacterial enzyme (Fig. 5B, lane 3), indicating digestion by the protease. This result suggested that the protease is capable of degrading large proteins from halophiles, and thus may have well been responsible for the failure to isolate an intact membrane ATPase from *Nm. pharaonis*.

## Discussion

Serine proteases have been isolated from several halobacteria, such as *Haloferax mediterranei* (Stepanov et al. 1992; Kamekura et al. 1996), *Halobacterium halobium* (Izotova et al. 1983), *Natrialba asiatica* strain 172 P1 (Kamekura et al.

**Table 2.** Amino acid composition of the protease from *Nm. pharaonis*, serine proteases from *Haloferax mediterranei* (Stepanov et al. 1992), *Halobacterium halobium* (Izotova et al. 1983), and *Natrialba asiatica* strain 172P1 (Kamekura et al. 1992), and chymotrypsinogen B from *Bos taurus* and from *Rattus norvegicus*

| | Protease from *Nm. pharaonis* (this study)[a] | Chymo trypsinogen B | | Protease from | | |
|---|---|---|---|---|---|---|
| | | Bovine[b] | Rat[b] | *Hb. halobium* | *Hf. mediterranei* | *Na. asiatica* strain172P1 |
| 1/2Cys | 10.4 | 10 | 10 | 4 | 6 | 5 |
| Asx | 27.8 | 21 | 22 | 69 | 60 | 64 |
| Thr | 25.1 | 22 | 20 | 32 | 31 | 30 |
| Ser | 25.6 | 22 | 21 | 43 | 54 | 48 |
| Glx | 23.8 | 18 | 21 | 34 | 34 | 64 |
| Pro | 15.1 | 14 | 14 | 18 | 15 | 18 |
| Gly | 31.6 | 23 | 23 | 56 | 53 | 58 |
| Ala | 30.6 | 23 | 19 | 47 | 37 | 39 |
| Val | 24.8 | 25 | 27 | 24 | 25 | 25 |
| Met | 3.5 | 4 | 4 | 2 | 2 | 4 |
| Ile | 9.5 | 9 | 11 | 16 | 15 | 12 |
| Leu | 22.6 | 17 | 16 | 28 | 28 | 29 |
| Tyr | 5.1 | 3 | 2 | 14 | 14 | 18 |
| Phe | 9.2 | 7 | 8 | 7 | 6 | 7 |
| Trp | nd[c] | 8 | 8 | 3 | 3 | 4 |
| Lys | 13.2 | 11 | 14 | 5 | 10 | 1 |
| His | 3.9 | 2 | 2 | 3 | 6 | 7 |
| Arg | 7.8 | 5 | 3 | 11 | 8 | 6 |
| Total | 288–290 | 245 | 245 | 416 | 407 | 411 |

[a] means from three determinations
[b] For SwissProt accession numbers, see legend to Fig. 6
[c] nd, not determined

1992; Kamekura and Dyall-Smith 1995), *Hb. salinarium* (Norberg and van Hofsten 1969), *Nc. occultus* (Studdert et al. 1997), and strain TuA4 (Schmitt et al. 1990). All are extracellular proteases, which are secreted into the environment and presumably function in the digestion of protein substrates for nutritive purposes. Their molecular masses are in the range of 40 to 41 kDa, and they appear related to the subtilisin-thermitase family with homologies of 50% or more (Stepanov et al. 1992). All of these are thermophilic, with temperature optima between 55° and 75°C and pH optima in the range of 8.0 to 10.7. One additional protease from *Hb. halobium* was membrane bound and deemed to be a metalloprotease (Fricke et al. 1993).

We describe in this work a serine protease from *Nm. pharaonis*, which was predominantly cytoplasmic and, to some extent, membrane associated, and which was not, under the conditions tested, significantly excreted into the medium. Its physiological role is not known, and it bears striking resemblance to the vertebrate chymotrypsinogens of the B type. This appears to be the first member of the chymotrypsin family from a unicellular organism (Barrett and Rawlings 1993). N-terminal amino acid sequences of the enzyme and some of its fragments with a total of 42 amino acid residues showed 97% identity to bovine chymotrypsinogen B. Its molecular mass was 30 kDa, which is somewhat higher than that of chymotrypsin (26 kDa). Its isoelectric point was 4.55, similar to that of bovine chymotrypsinogen B (IEP 5.2), but significantly different from that of bovine chymotrypsinogen A (IEP 9.1). Other chymotrypsinogen-like properties include its overall amino acid composition, its susceptibility to inhibitors, the occurrence of autolysis, and the evidence for a zymogen-like activation.

Halophilic enzymes are generally dependent on high concentrations of NaCl or KCl and lose activity rapidly when exposed to low-salt conditions (Eisenberg and Wachtel 1987), although a few exceptions, such as the nitrate reductase from *Hf. denitrificans* (Hochstein and Lang 1991) or superoxide dismutase from *Hb. cutirubrum* (May and Dennis 1987), are known that are active in the absence of salt. The protease from *Nm. pharaonis* was active over a wide range of NaCl and KCl concentrations (0.003–4 M); this was surprising in view of the fact that *Nm. pharaonis* is an obligate halophile that cannot grow below 2.2 M NaCl (Soliman and Trüper 1982). On the other hand, we noted remarkable and unexpected activity of bovine chymotrypsin A in the presence of high concentrations of NaCl (Fig. 4). Another similarity between the natronobacterial protease and bovine chymotrypsin A was their temperature optimum of about 60°C under our assay conditions (Fig. 3).

Cellular proteolytic activity of *Nm. pharaonis* had been suspected by us (see Introduction) and by Scharf et al. (1997), who observed degradation of cytochrome *c* during preparation. Figure 5B shows that the protease from *Nm. pharaonis* is capable of digesting the halobacterial ATPase complex under conditions used to prepare the natronobacterial ATPase.

Other halophilic and haloalkaliphilic Archaea appear to possess proteases similar to the one described here from *Nm. pharaonis*, although these enzymes may not be as active or stable. Lysed cells from *Hb. halobium* contained an immunoreactive 30-kDa band; from *Na. magadii*, and

*Nc. occultus* a similarly reactive protease was isolated (Fig. 1). Further indication for the presence of the enzyme was obtained from cells of *Hr. saccharovorum* (Fig. 5A), *Nb. gregoryi*, *Hf. volcanii*, and *Hf. mediterranei* (data not shown). Searching for the enzyme with the bovine chymotrypsinogen B sequence in the whole genome sequences of the non-halophilic Archaea *Methanococcus jannaschii* (Bult et al. 1996) and *Archaeoglobus fulgidus* (Klenk et al. 1997), as well as several of the partial prokaryotic genome sequences, which are available through the Internet (*Deinococcus radiodurans*, *Thermotoga maritima*, *Enterococcus faecalis*), did not produce any matches. No whole genome sequence of a halophilic archaeon has been published yet, but is expected to be available soon for *Hb. salinarum* (http://www.mcs.anl.gov/home/gaasterl/genomes.html). It will be of interest to see if a chymotrypsin-like protease can be detected in this halobacterial genome.

Several proposals have been put forward suggesting that an archaeal host contributed its nucleocytoplasm in symbiotic merging with Eubacteria during eukaryogenesis (e.g., Gogarten et al. 1989; Margulis 1996). The presence of a chymotrypsin-like enzyme in Archaea, including its activating apparatus, would be compatible with this view. In fact, this may be an example of a "molecular system," as pointed out by Woese et al. (1990), which is shared by Archaea and eukaryotes. However, the chymotrypsin-like protease is obviously not present in all Archaea, as deduced from the whole genome sequences.

Except for their status as members of the Archaea, extreme halophiles have not yet been explicitly considered as participants in eukaryogenesis; rather, thermophiles (Margulis 1996) and, recently, methanogens (Martin and Müller 1998) have been suggested as early hosts. The similarities between vertebrate chymotrypsinogens and the protease from *Nm. pharaonis* and other halophiles, including zymogen-like activation, strongly suggest that this group of Archaea or their predecessors should be considered as candidates participating in early fusion events that gave rise to eukaryotic cells.

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