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Extracellular protease of *Natrialba magadii*: purification and biochemical characterization

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Abstract A serine protease secreted by the haloalkaliphilic archaeon *Natrialba magadii* at the end of the exponential growth phase was isolated. This enzyme was purified 83 fold with a total yield of 25% by ethanol precipitation, affinity chromatography, and gel filtration. The native molecular mass of the enzyme determined by gel filtration was 45 kDa. *Na. magadii* extracellular protease was dependent on high salt concentrations for activity and stability, and it had an optimum temperature of 60°C in the presence of 1.5 M NaCl. The enzyme was stable and had a broad pH profile (6–12) with an optimum pH of 8–10 for azocasein hydrolysis. The protease was strongly inhibited by diisopropyl fluorophosphate (DFP), phenylmethyl sulfonylfluoride (PMSF), and chymostatin, indicating that it is a serine protease. It was sensitive to denaturing agents such as SDS, urea, and guanidine HCl and activated by thiol-containing reducing agents such as dithiotreitol (DTT) and 2-mercaptoethanol. This protease degraded casein and gelatin and showed substrate specificity for synthetic peptides containing Phe, Tyr, and Leu at the carboxyl terminus, showing that it has chymotrypsin-like activity. *Na. magadii* protease presented no cross-reactivity with polyclonal antibodies raised against the extracellular protease of *Natronococcus occultus*, suggesting that although these proteases share several biochemical traits, they might be antigenically unrelated.

Key words Archaea · Haloalkaliphile · *Natrialba magadii* · *Natronococcus occultus* · Serine protease.

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Introduction

Archaeal halophiles dominate in hypersaline environments (>15% NaCl). *Natrialba magadii* (formerly *Natronobacterium magadii*) belongs to the haloalkaliphilic group, which are particularly interesting because they are among the most alkaliphilic organisms thriving in the pH range 8.5–11 and NaCl concentration about 20% (Tindall et al. 1980).

Halobacteria have developed an efficient metabolism for the utilization of proteins, peptides, and amino acids. From the haloneutrophilic group, several extracellular proteases have been isolated and characterized (Norberg and v. Hofsten 1969; Izotova et al. 1983; Schmitt et al. 1990; Stepanov et al. 1992). They are serine proteases dependent on high salt concentrations for activity and stability and are moderately thermophilic. One membrane-bound metalloprotease has been also reported for *Halobacterium halobium* (Fricke et al. 1993). Halolysin, a subtilisin-like protease produced by the strain 172 P1 (recently designated *Natrialba asiatica* strain 172 P1), has been purified and characterized, and the gene has been cloned and expressed in *Haloferax volcanii* (Kamekura and Seno 1990; Kamekura et al. 1992). Proteases from haloalkaliphilic Archaea have been less investigated and may have distinct properties. An extracellular serine protease from the haloalkaliphilic strain A2 (unidentified but presumably from the genus *Natronobacterium*) was partially purified and characterized (Yu 1991). We isolated and characterized extracellular and cell-associated proteolytic activities from *Natronococcus occultus* (Studdert et al. 1997; Herrera Seitz et al. 1997). Partial amino acid sequences were obtained from the secreted protease, and they did not show significant similarity to other proteins; this fact, in addition to its high native molecular mass compared to other extracellular proteases, suggests it might be a novel enzyme (Studdert et al., submitted for publication). Recently, the isolation of a cytoplasmic chymotrypsin-like protease from *Natronomonas pharaonis* cells was reported (Stan-Lotter et al. 1999).

As they remain less characterized compared to the nonhalophilic proteases, halobacterial proteases represent an underutilized resource from the standpoint of fundamental biochemistry and enzymology as well as biotechnology. We report here the purification and biochemical characterization of an extracellular serine protease produced by *Natrialba magadii* cells at the end of the exponential growth phase. Comparison with the extracellular proteases of another haloalkaliphilic archaeon, *Natronococcus occultus*, is also presented.

Materials and methods

Strain and growth conditions

Natrialba magadii cells (formerly *Natronobacterium magadii* ATCC 43099) were grown at 37°C in Erlenmeyer flasks on a rotary shaker at 150 rpm in darkness using a modification of the medium described by Tindall et al. (1984). The medium (pH 10) contained (in g/l): NaCl, 200; Na_2CO_3, 18.5; yeast extract (Gibco BRL), 20; sodium citrate, 3; KCl, 2; $MgSO_4 \cdot 7H_2O$, 1; $MnCl_2 \cdot 4H_2O$, 3.6×10^{-4}; and $FeSO_4 \cdot 7H_2O$, 2×10^{-5}. Growth was monitored by the increase in optical density at 600 nm (OD_{600}). Samples were taken at various times during growth. Cells were harvested by centrifugation at 10000 × *g* for 10 min, and the supernatant (culture medium) was stored at 4°C for determination of extracellular proteolytic activity.

Determination of proteolytic activity

Routinely, azocasein (prepared as described by Kirtley and Koshland 1972) was used as substrate. The reaction mixture contained 0.5% (w/v) azocasein, 0.1 M sodium borate buffer (pH 8), 1.5 M NaCl (unless otherwise indicated), and enzyme solution. Incubations were performed at 45°C and stopped by adding 1 volume of cold 10% (v/v) trichloroacetic acid. The assay tubes were left on ice for 15 min and centrifuged for 15 min at 3000 × *g*. Acid-soluble products were detected in the supernatant by measuring OD_{335}. One unit of activity (U) was defined as the amount of enzyme that produced an increase of 1 in OD/h under the conditions just described.

For proteolytic activity determination with synthetic oligopeptides, the reaction mixture contained 0.2 mM substrate, 0.1 M Tris-HCl buffer (pH 8), 1.5 M NaCl, and 0.4 U of purified enzyme in a final volume of 75 µl. Incubations were performed at 45°C and stopped by adding 0.33 M sodium citrate (pH 5) or 2 mM PMSF (phenylmethylsulfonyl fluoride) for pNA substrates (Sigma, St. Louis, MO, USA) and peptidyl MCA (α-4-methyl-coumaryl-7-amide) substrates (Sigma), respectively. The degradation product of peptidyl MCA was meassured fluorometrically at 460 nm with an excitation at 360 nm in a Turner 450 fluorometer, while liberation of pNA from peptidyl pNA was determined at 410 nm in a Beckman DU 530 spectrophotometer.

Protein determination

Protein concentrations were determined by the bicinchoninic acid procedure (Smith et al. 1985) using bovine serum albumin as a standard.

Protease purification

Natrialba magadii extracellular protease was purified from stationary-phase cultures. All the purification steps were performed at room temperature. One volume of cold absolute ethanol was gradually added to the culture medium, and protein was allowed to precipitate for 1 h on ice. Precipitated material was pelleted at 5000 × *g* for 10 min and was then resuspended in 1/10 volume of 50 mM Tris-HCl buffer (pH 8), 4.5 M NaCl, and recentrifuged to eliminate insoluble material. The ethanol-precipitated protein was loaded onto a bacitracine-Sepharose 4B column (25 cm^3) (Sigma) equilibrated in 50 mM Tris-HCl buffer (pH 8), 4.5 M NaCl. Contaminating proteins were removed with the same buffer containing 5 M NaCl, and the proteolytic activity was eluted (at 1 ml/min flux) with 50 mM Tris-HCl buffer (pH 8) plus 15% (v/v) ethanol and 4.5 M NaCl. Fractions with proteolytic activity were concentrated by ultrafiltration (Amicon, 10000) and applied onto a Sephacryl S-200 gel filtration column (Pharmacia, Uppsala, Sweden) (74 × 1.6 cm) equilibrated in 50 mM Tris-HCl buffer (pH 8) containing 3 M NaCl. The column flux was 0.5 ml/min and the fraction volume 2 ml. For estimation of the native molecular mass of the protease, the Sephacryl S-200 column was calibrated with proteins of known molecular mass: β-amylase (200 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), and soybean trypsin inhibitor (20 kDa).

Polyacrylamide gel electrophoresis

Samples were electrophoresed in 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS-PAGE) and 4 M betaine (Sigma) at 4°C and 10 mA/gel, according to the method of Laemmli (1970). Samples previously dialyzed against 0.1 M sodium borate buffer (pH 8) plus 4 M betaine were mixed with 5× Laemmli sample buffer without diothiothreitol (DTT) and electrophoresed without boiling. Proteins were stained with Coomassie brilliant blue R-250 (CBB).

Western blotting

Proteins were fractionated in polyacrylamide gels as described and then transferred onto nitrocellulose membranes at 190 mA for 30 min in a Trans-Blot SD-cell (Bio-Rad). The transfer solution contained 48 mM Tris, 39 mM glycine, and 20% (v/v) methanol (pH 9.2). Nonspecific binding sites on the blotted membrane were blocked in 25 mM Tris-HCl (pH 7.4), 0.03% (v/v) Tween-20, 5% (w/v) skimmed milk, and 0.02% sodium azide (v/v) (blocking

buffer) at room temperature for 2 h. The blocked membranes were incubated with specific antiserum diluted 1:5000 in blocking buffer at room temperature for 16 h with gentle shaking and then washed in blocking buffer. The membranes were incubated with affinity-purified antirabbit IgG antibody conjugated with alkaline phosphatase (Sigma) diluted 1:5000 in the same buffer for 1 h. After extensive washing, the membranes were immersed in alkaline phosphatase buffer (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$), containing 0.33 mg nitroblue tetrazolium and 0.01 mg 5-bromo-4-chloro-3-indolyl phosphate per milliliter.

Results

Production of extracellular proteolytic activity during growth

To investigate the occurrence of extracellular protease during growth, samples were taken at different times, and the azocaseinolytic activity of the cell-free medium was measured. No extracellular proteolytic activity was detected during exponential growth; however, it was produced and accumulated in the culture medium at the end of the exponential growth phase, reaching higher values (20–25 U/ml) during the stationary phase (Fig. 1).

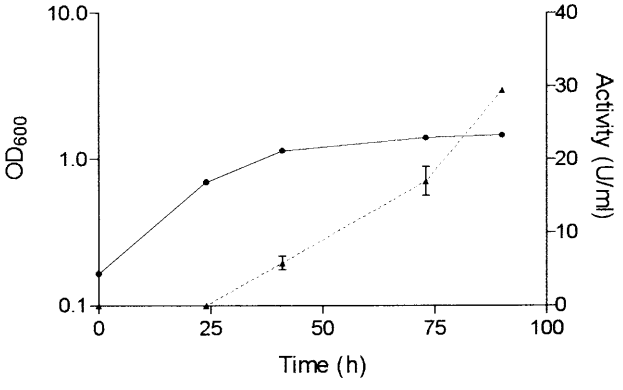

Fig. 1. Time course of protease secretion by *Natrialba magadii*. Cells were grown in the medium described by Tindall et al. (1980) as described in Materials and methods. Samples were taken at different times for determination of cell growth (OD_{600}) (●) and extracellular azocaseinolytic activity (▲)

Protease purification

Natrialba magadii extracellular protease was purified from stationary-phase cultures by ethanol precipitation, affinity chromatography, and gel filtration. Results of the purification of *N. magadii* protease are summarized in Table 1. The protease was purified 82.5 fold with a total yield of 25%. Only one sharp peak of azocaseinolytic activity was eluted from the Sephacryl S-200 column (not shown), suggesting that this protease may be the major casein-degrading enzyme produced by *Na. magadii* during the stationary phase. The relative molecular mass of this protease was 45 kDa, as determined by Sephacryl S-200 gel filtration. To assess homogeneity, the purified protease was analyzed by SDS-PAGE in the presence of 4 M betaine. High concentrations of betaine were included in the gel because we have previously observed that it partially prevents autolysis of halophilic proteases under conditions of low ionic strength (Studdert et al. 1997; Herrera Seitz et al. 1997). Following electrophoresis, one lane of the gel was stained with CBB, and another lane containing the purified protease was sliced every 3 mm from top of the gel and assayed for azocaseinolytic activity. As shown in Fig. 2A, a major protein

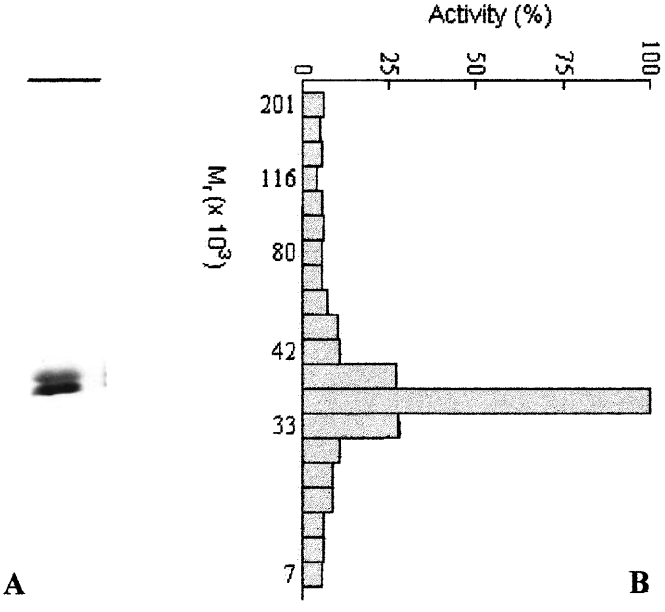

Fig. 2A,B. SDS-PAGE of purified extracellular protease of *Natrialba magadii* (**A**) and azocaseinolytic activity of the gel fractions sliced every 3 mm (**B**). The purified enzyme was loaded on a 10% SDS polyacrylamide gel containing 4 M betaine and stained with Coomassie brilliant blue (CBB). Molecular mass values, kDa

Table 1. Purification of *Natrialba magadii* extracellular protease

Fraction	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell-free stationary-phase culture	535.0	49.60	10.8	100	1.0
Ethanol-precipitated material	209.6	2.01	104.0	39	9.6
Bacitracin-Sepharose 4 B	179.7	0.22	816.8	34	75.6
Sephacryl S-200	133.6	0.15	890.7	25	82.5

band with an apparent molecular mass of 36 kDa was observed after SDS-PAGE and CBB staining, corresponding to the active fraction detected by gel slicing (Fig. 2B). When the purified protease was labeled with (1,3,-^{3}H)-DFP (DFP, diisopropyl fluorylphosphate) and analyzed by SDS-PAGE and fluorography, a single band of 36 kDa was detected (not shown), demonstrating that this protein band corresponds to a serine protease.

Effect of temperature, salt concentration, and pH on activity and stability

To establish the optimal conditions for determination of proteolytic activity, we first tested the effect of different salt concentrations and temperatures on the azocaseinolytic activity of the ethanol-precipitated fraction. Identical results were obtained with the purified protease. The highest enzyme activity (measured in 3 M KCl and pH 8) was obtained at 60°C (Fig. 3A); however, it was not stable under these conditions. Therefore, 45°C was used for subsequent determinations because the enzyme activity was stable for more than 1 h at this temperature. No proteolytic activity was detected at 70°C. Maximal protease activity was measured at concentrations of 1–1.5 M NaCl or KCl (at 45°C and pH 8); however, it was undetectable at less than 0.5 M (Fig. 3B). The enzyme was active at a broad pH range (6–12) for azocasein hydrolysis (1.5 M NaCl, 45°C) (Fig. 3C), and showed highest activity between pH 8 and 10. Considering these results, the standard conditions for determination of azocaseinolytic activity were established as 1.5 M NaCl, 45°C, and pH 8.

Stability of the protease under various conditions was also examined (Fig. 4). To analyze thermal stability, samples were incubated for 2, 5, and 24 h in 3 M NaCl and pH 8 at different temperatures (30°, 40°, 45°, 50°, and 60°C); then, azocaseinolytic activity under standard conditions was measured (Fig. 4A). *Na. magadii* protease retained 80% and 40% of initial activity after 2 and 5 h, respectively, of incubation at 30°–45°C. No further loss of activity was observed up to 24 h. At higher temperatures (50°–60°C), the enzyme was rapidly inactivated, and 50% and 30% residual activity, respectively, was detected after 2 h. Protease stability at different salt concentrations (0.5, 1, 2, or 3 M NaCl or KCl) was determined after preincubation of the enzyme at 4°C for various times. As shown in Fig. 4B, the protease was more stable in NaCl than in KCl at all the salt concentrations tested and, in both cases, the percent of residual activity increased at higher salt concentrations (100% of residual activity in 1–3 M NaCl or 3 M KCl after 1 week at 4°C). After 24 h in 0.5 M NaCl or KCl, 30% and 50% loss of the initial activity, respectively, was observed.

The effect of pH on enzyme stability was also investigated. The protease was preincubated at different pH values in the presence of 3 M NaCl at 4°C. The protease was stable in a broad pH range (6–12) for at least 2 weeks (Fig. 4C).

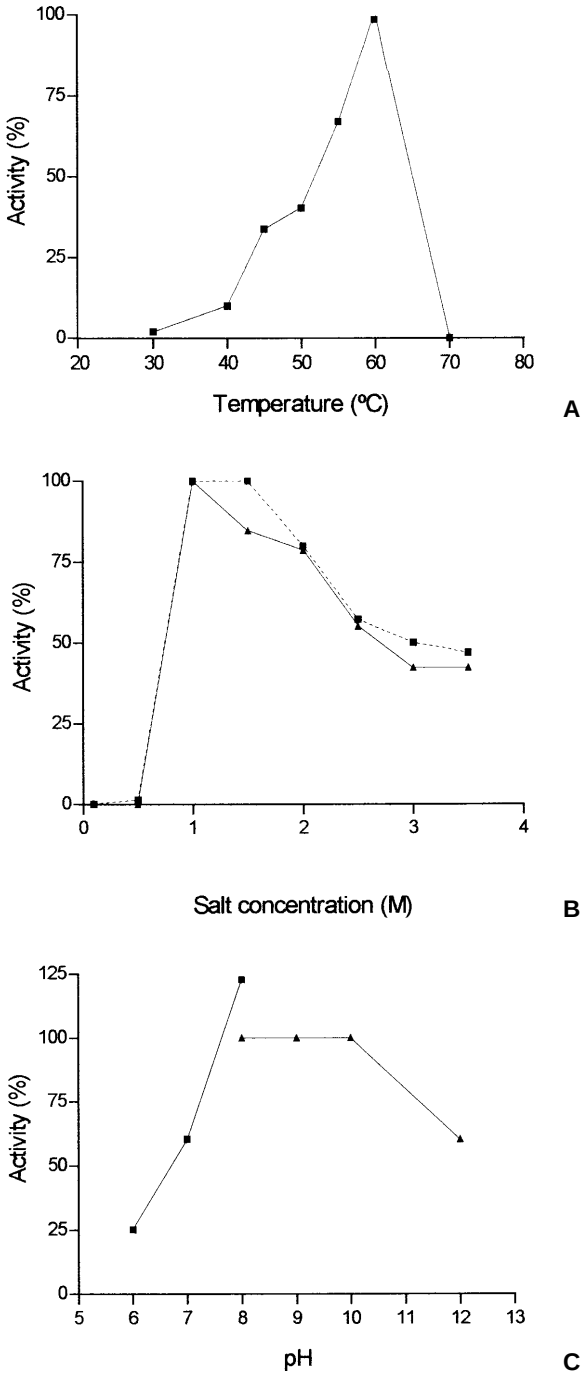

Fig. 3A–C. Effect of temperature, salt concentration, and pH on the extracellular protease of *Natrialba magadii*. Azocaseinolytic activity was assayed under the following conditions: (**A**) at temperatures between 30° and 70°C, pH 8, and 1.5 M NaCl; (**B**) at different concentrations of NaCl (■) and KCl (▲), pH 8 at 45°C; (**C**) at pH values between 6 and 12 in bis-Tris-propane (■) or Tris-glycine buffer (▲), both containing 1.5 M NaCl at 45°C

Effect of protease inhibitors

The effect of protease inhibitors was tested on the azocaseinolytic activity of the purified protease. Table 2 shows that *Na. magadii* protease was inhibited by the

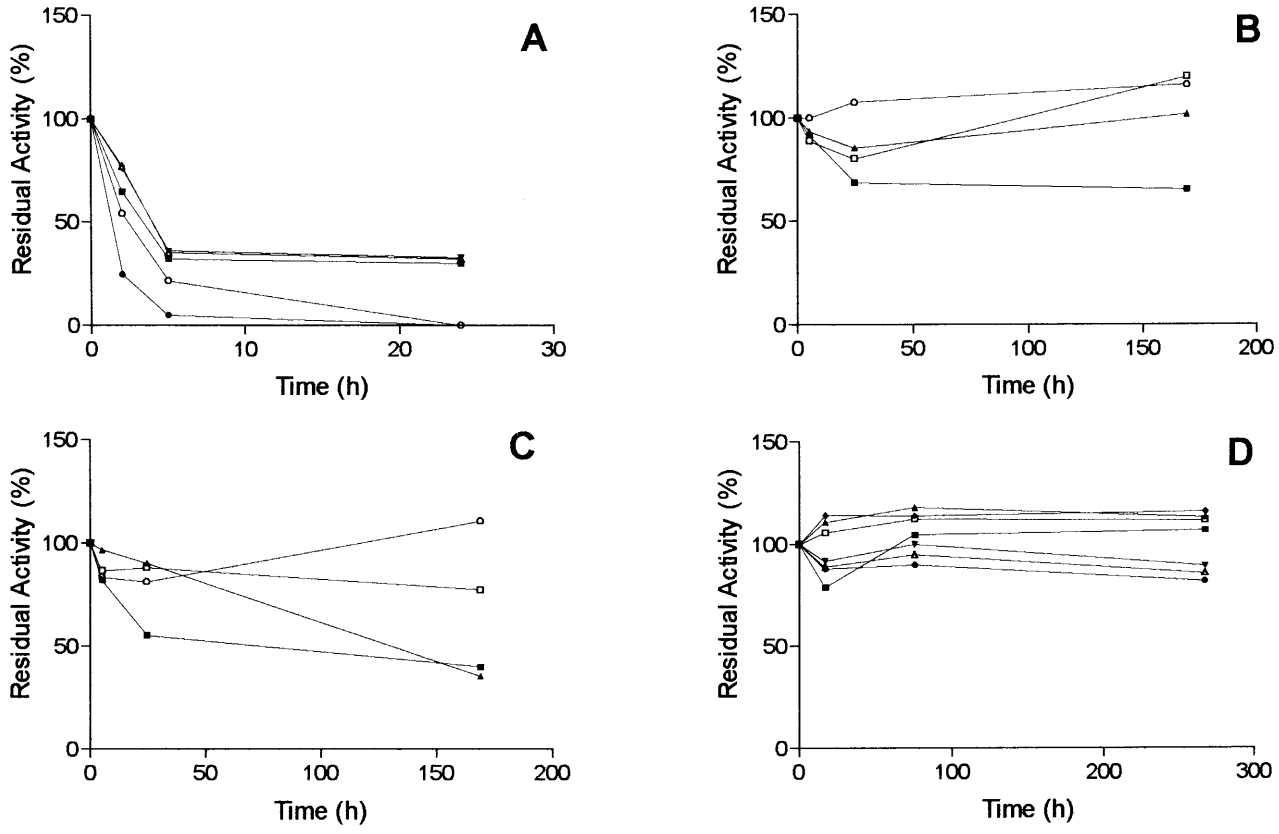

Fig. 4A–D. Effect of temperature, salt concentration, and pH on protease stability. Azocaseinolytic activity under standard conditions was determined after incubation at (**A**) 30° (■), 40° (▲), 45° (▼), 50° (○), and 60°C (●) in 3 M NaCl and pH 8; (**B**) 0.5 (■), 1 (▲), 2 (○), and 3 M NaCl (□) at pH 8 and 4°C; (**C**) 0.5 (■), 1 (▲), 2 (□), and 3 M KCl (○) at pH 8 and 4°C; (**D**) pH 6 (■), 7 (▲), 8 (▼), and 9 (◆) in bis-Tris-propane buffer and pH 10 (□), 11 (□) and 12 (□) in Tris-glycine buffer, at 3 M NaCl and 40°C

Table 2. Effect of protease inhibitors on the extracellular protease of *Natrialba magadii*

Inhibitor	Residual activity (%)
None	100.0
Leupeptin (0.1 mM)	73.0
SBTI (100 μg/ml)	100.0
Chymostatin (0.1 mM)	0.2
DFP (30 μM)	18.0
PMSF (1 mM)	0.1
EDTA (10 mM)	91.8
E-64 (30 μM)	100.0
Pepstatin (8 μM)	100.0

Purified enzyme was preincubated with the indicated concentration of inhibitor in 0.1 M borate buffer (pH 8), 1.5 M NaCl for 30 min at room temperature; azocaseinolytic activity was then determined at 45°C, and residual activity was expressed as percent of activity of the uninhibited enzyme

Table 3. Substrate specificity of the extracellular protease of *Natrialba magadii*

Substrate	Proteolytic activity (%)
N-Succinyl-Ala-Ala-Phe-MCA	100.0
N-Succinyl-Leu-Leu-Val-Tyr-MCA	60.9
Boc-Val-Leu-Lys-MCA	0
Val-Pro-Arg-MCA	25.9
N-Succinyl-Ala-Phe-Lys-MCA	10.0
N-*t*-Boc-Phe-Ser-Arg-MCA	41.3
N-CBZ-Gly-Gly-Leu-*p*-nitroanilide	100.0
N-CBZ-Val-Gly-Arg-*p*-nitroanilide	17.8

Proteolytic activity was determined by incubating the purified enzyme with 0.2 mM substrate in 0.1 M Tris-HCl (pH 8), 1.5 M NaCl at 45°C for 0, 10, and 20 min; 100% of activity was assigned to the substrates that presented the highest MCA and *p*-nitroanilide liberation

serine protease inhibitors PMSF, DFP, and chymostatin (0.1%, 18%, and 0.2% of residual activity, respectively), whereas inhibitors of metallo-, cysteine, and aspartyl proteases (EDTA, E-64, and pepstatin) and the serine protease inhibitors leupeptin and SBTI had no significant effect.

Substrate specificity

Natrialba magadii extracellular protease degraded large proteins such as gelatin and casein. To assess substrate specificity, activity against synthetic peptides was tested under standard conditions. As summarized in Table 3,

Table 4. Effect of denaturing agents on *Natrialba magadii* extracellular protease

Reagent	Concentration	Residual activity (%)
None		100.0
SDS	2%	24.3
Urea	4 M	0.7
Urea	8 M	0.0
HCl guanidine	1 M	67.1
HCl guanidine	4 M	0.0
DTT	1 mM	166.0
DTT	10 mM	210.0
2-Mercaptoethanol	0.1%	244.0

Cleavage of Succ-Ala-Ala-Phe-MCA substrate (Sigma) was measured at 1.5 M NaCl, pH 8, and 45°C in the presence of the indicated concentrations of each reagent; 100% was assigned to the activity in absence of denaturing reagent

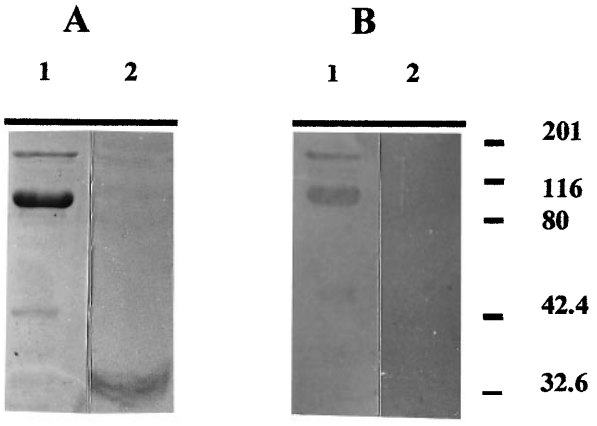

Fig. 5A,B. SDS-PAGE (**A**) and Western blot (**B**) of *Natronococcus occultus* and *Natrialba magadii* extracellular proteases. The purified enzymes (10 μg protein) were preincubated with 1 mM PMSF (phenylmethylsulfonyl fluoride) for 30 min at room tempetature, electrophoresed in a 10% polyacrylamide gel containing 4 M betaine, and then stained with CBB (**A**) or transferred to a nitrocellulose membrane and incubated with polyclonal antibodies against *Natronococcus occultus* extracellular protease (**B**). *Lane 1*, *N. occultus* protease; *lane 2*, *Natrialba magadii* protease. Molecular mass values, kDa

peptides containing Phe, Tyr, and Leu at the P1 position were degraded more efficiently than those with basic amino acids such as Arg or Lys. These results indicate that *N. magadii* protease has chymotrpsin-like proteolytic activity.

Effect of denaturing agents

The activity of the protease against Suc-Ala-Ala-Phe-MCA in the presence of different concentrations of denaturing agents was determined. The enzyme was activated by thiol-reducing agents such as DTT and 2-mercaptoethanol. However, it was negatively affected by SDS, urea, and guanidine-HCl (Table 4).

Comparison with *Natronococcus occultus* extracellular protease

We have previously purified and characterized an extracellular protease secreted by another haloalkaliphilic archaeon, *Natronococcus occultus* (Studdert et al. 1997). To further compare this enzyme with *Natrialba magadii* extracellular protease, protein profiles by SDS-PAGE as well as immunoreactivity with polyclonal antibodies against *Natronococcus occultus* protease were examined. Protein profiles of purified extracellular proteases from both Archaea showed a different pattern. *N. occultus* protease showed a major protein band of 90 kDa and minor species of 120 kDa and lower molecular mass (Fig. 5A, lane 1), whereas the main protein band of *Natrialba magadii* protease had an apparent molecular mass of 36 kDa (Fig. 5A, lane 2). When *N. magadii* protease was probed with polyclonal antibodies against *Natronococcus occultus* extracellular protease, no cross-reaction was observed (Fig. 5B), suggesting that these two proteins may not be antigenically related.

Discussion

Several proteolytic enzymes have been isolated and characterized from haloneutrophilic Archaea (Norberg and v. Hofsten 1969; Izotova et al. 1983; Stepanov et al. 1992; Schmitt et al. 1990). All these enzymes are extracellular serine proteases, some of them related to the subtilisin family; they are moderately thermophilic and dependent on high salt concentrations for activity and stability. The best characterized extracellular protease of this group is halolysin, a subtilisin-like serine protease secreted by the strain 172 P1 (recently classified as *Natrialba asiatica*) (Kamekura and Seno 1990). Haloalkaliphilic Archaea predominate in environments of elevated salt concentrations and high pH (10–11); therefore, their proteins may have distinct properties compared to the neutrophilic ones. Proteolytic enzymes from haloalkaliphilic Archaea have been described by Yu (1991), by our laboratory (Studdert et al. 1997; Herrera Seitz et al. 1997) and by Stan-Lotter et al. (1999).

In this study we report on the purification and biochemical characterization of an extracellular protease of the haloalkaliphilic archaeon *Natrialba magadii*. As are many proteolytic enzymes, it is secreted at the late exponential growth phase (see Fig. 1). Although we have not investigated its physiological role, it may be involved in the degradation of exogenous proteins for nutrition purposes. This enzyme was purified 82.5 fold with a total yield of 25% (see Table 1). Only one sharp peak of azocaseinolytic activity was eluted from the gel filtration on a Sephacryl S-200 column, suggesting that it may be the main protease secreted by *N. magadii* cells during the stationary phase.

Natrialba magadii protease was inhibited by PMSF, DFP, and chymostatin (see Table 2), indicating that it is a serine protease. It hydrolyzed large proteins such as casein and gelatin and synthetic peptides, preferentially at the carboxyl terminus of Phe, Tyr, or Leu (see Table 3). These

results show that *N. magadii* protease is a chymotrypsin-like serine protease.

As well as many halophilic enzymes, the extracellular protease of *Na. magadii* depends on high salt concentrations for its enzymatic activity and stability. Maximal azocaseinolytic activity was attained at 1–1.5 M NaCl or KCl, and it was undetectable at lower salt concentrations (Fig. 3B). As expected for an extracellular enzyme, this protease was more stable in the presence of NaCl than in KCl at all the concentrations tested. The enzyme retained full activity for months when stored at 4°C in the presence of 3 M NaCl (Fig. 4B). This property was also observed for halolysin 172 P1 (Kamekura and Seno 1990) and for the extracellular protease of *Natronococcus occultus* (Studdert et al. 1997) but not for the extracellular protease of *Halobacterium halobium*, which was completely inactivated after 10 days under the same conditions (Izotova et al. 1983). The optimum temperature for *Natrialba magadii* extracellular protease was 60°C (Fig. 3A); however, stability was favored at lower temperatures (30°–45°C) (see Fig. 4A). The enzyme was active and stable in a broad pH range (6–12) and showed the highest activity for azocasein hydrolysis at a pH of 8–10 (Figs. 3C, 4D).

The effect of denaturing and reducing agents on *Na. magadii* protease was also examined (see Table 4). The proteolytic activity was inhibited by SDS, urea, and HCl guanidinium, indicating that hydrogen bonds may be important in maintaining enzyme activity. Similar observations have been reported for several hyperthermophilic proteinases (Cowan et al. 1987; Chavez Croocker et al. 1999). On the other hand, the protease was activated by low concentrations of thiol-reducing agents such as DTT and 2-mercaptoethanol. Enzyme activation by low concentrations of thiol-reducing agents has been previously described for some metallo- and serine peptidases (Wolz 1999). These results suggest that *Na. magadii* extracellular protease may be a thiol-dependent serine protease.

We have previously purified and characterized an extracellular protease from another haloalkaliphilic archaeon, *Natronococcus occultus* (Studdert et al. 1997; Studdert et al., unpublished results). Even though it shares some biochemical features with *Natrialba magadii* protease (salt concentration, pH and temperature optima, and sensitivity to protease inhibitors), they seem to be different enzymes, as suggested by the following experimental evidence. (i) The native molecular mass of *Natrialba magadii* protease, estimated by gel filtration, is 45 kDa (this work), while that of *Natronococcus occultus* protease is 130 kDa (Studdert et al., unpublished results), and (ii) protein profiles of these proteases showed a different pattern as determined by SDS-PAGE with 4 M betaine (Fig. 5A). The apparent molecular mass of the major protein bands in *N. occultus* and *Natrialba magadii* proteases were 90 and 36 kDa, respectively. These protein species corresponded well to active fractions when they were analyzed in gelatin zymograms (data not shown) or activity determination by gel slicing (see Fig. 2). In either case, minor additional protein bands of higher and lower molecular mass were also detected because of autodigestion of these enzymes during electrophoresis (Fig. 5A,B). Autolysis of halophilic proteases under low ionic conditions has been previously reported (Izotova et al. 1983; Studdert et al. 1997; Studdert et al., unpublished results). (iii) Polyclonal antibodies against *Natronococcus occultus* extracellular protease did not crossreact with *Natrialba magadii* protease, suggesting that they are antigenically unrelated (Fig. 5B).

Although *Natrialba magadii* protease seems to be different from the *Natronococcus occultus* enzyme, it may be more closely related to other halophilic proteinases such as halolysin 172 P1 and R4, secreted by strain 172 P1 and by *Haloferax mediterranei* strain R4, respectively, because their native molecular mass (40–45 kDa), salt dependence, substrate specificity, and sensitivity to protease inhibitors are quite similar. Amino acid sequencing of *Natrialba magadii* protease is currently in progress in our laboratory. Information on its primary structure as well as the molecular characterization of the gene will allow a more extended comparison with other halobacterial and eubacterial proteinases, and also will contribute to elucidation of the molecular mechanisms of protein export in Archaea.

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