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Purification and characterization of a thermostable, haloalkaliphilic extracellular serine protease from the extreme halophilic archaeon *Halogeometricum borinquense* strain TSS101

MALASHETTY VIDYASAGAR,[1] S. PRAKASH,[1] CAROL LITCHFIELD[2] and K. SREERAMULU[1,3]

[1] *Department of Biochemistry, Gulbarga University, Gulbarga-585 106, Karnataka, India*

[2] *Department of Environmental Science & Policy, George Mason University, 10900 University Blvd., PW1 MS 4D4, Manassas, Virginia, 20110*

[3] *Corresponding author (ksramu@rediffmail.com)*

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Summary A novel haloalkaliphilic, thermostable serine protease was purified from the extreme halophilic archaeon, *Halogeometricum borinquense* strain TSS101. The protease was isolated from a stationary phase culture, purified 116-fold with 18% yield and characterized biochemically. The molecular mass of the purified enzyme was estimated to be 86 kDa. The enzyme showed the highest activity at 60 °C and pH 10.0 in 20% NaCl. The enzyme had high activity over the pH range from 6.0 to 10.0. Enzymatic activity was strongly inhibited by 1 mM phenyl methylsulfonyl fluoride, but activity was increased 59% by 0.1% cetyltrimethylammonium bromide. The enzyme exhibited relatively high thermal stability, retaining 80% of its activity after 1 h at 90 °C. Thermostability increased in the presence of Ca^{2+}. The stability of the enzyme was maintained in 10% sucrose and in the absence of NaCl.

Keywords: calcium chloride, cetyltrimethylammonium bromide, halophilic serine protease, metal ions, osmolytes, protease inhibitors.

Introduction

The haloarchaea are aerobic, extreme halophiles that grow optimally in medium containing 15–25% NaCl (Kushner and Kamekura 1988), although most species can grow over a wide range of salinities. Many extreme and moderate halophiles have been isolated and investigated for possible biotechnological applications, including the production of compatible solutes, enhanced oil recovery and the degradation of industrial pollutants in saline habitats (Hough and Danson 1999, Madern et al. 2000, Margesin and Schiner 2001, Schiraldi et al. 2002, Van den Berg 2003, Gomes and Steiner 2004). Additionally, halophiles produce exoenzymes of potential commercial value (Thongthai and Sontinanalert 1991, Adams and Kelly 1995). Some extracellular proteases from halophiles have maximal activity at near neutral pH (Norberg and Hofstein 1969, Izotova et al. 1983, Kamekura and Seno 1990, Schmitt et al. 1990, Stepanov et al. 1992). A few extracellular proteases from haloalkaliphilic archaea have been characterized (Studdert et al. 1997, Gimenez et al. 2000), and their activity has been found to depend on both a high salt concentration and a high pH. Here, we report on the purification and characterization of a haloalkaliphilic extracellular protease from the extreme halophilic archaeon *Halogeometricum borinquense* strain TSS-101.

Materials and methods

Organism

We isolated *H. borinquense* strain TSS101 from solar salterns of Tuticorin, Tamilnadu, India. The organism strain was identified based on 16S rRNA sequencing, membrane lipid analysis and physiological properties. *Halogeometricum borinquense* is a gram negative, extremely pleomorphic and rod-shaped organism forming pink pigmented colonies. Strictly aerobic, *H. borinquense* grows at pH 6.0–10.0, temperatures of 25–45 °C and NaCl concentrations of 15–25%. Optimal growth occurs at pH 7.0–7.5, 40 °C and 20% NaCl. *Halogeometricum borinquense* lipids include glycerol diether moieties, indicative of an extreme halophilic archaeon. A high salt requirement, resistance to kanamycin, penicillin and chloramphenicol (which inhibit growth of halophilic eubacteria) and susceptibility to bacitracin, place this microorganism in the family Halobacteriaceae, order Halobacteriales (Montalvo-Rodriguez et al. 1998). The *H. borinquense* strain TSS101 has been deposited at the National Centre for Industrial Microorganisms, Pune, India (Accession No. NCIM 5238). The 16S rRNA sequence has been deposited in the NCBI database (Accession No. DQ375808).

Growth conditions and media composition

The archaeon *H. borinquinse* strain TSS101 was cultured aerobically at 40 °C in a 250-ml flask containing 50 ml of growth

medium (200 g l^{-1} NaCl, 10 g l^{-1} $MgCl_2$, 5 g l^{-1} KCl, 3 g l^{-1} trisodium citrate and 10 g l^{-1} peptone; pH 7.2) and shaken at 200 rpm.

Enzyme purification

Purification was carried out at room temperature (25 °C). The microorganism was grown at 40 °C for 4 days, centrifuged at 10,000 *g* for 10 min and the supernatant retained as the source of extracellular enzyme. One liter of crude enzyme was concentrated to 20 ml with a membrane protein concentrator (MWC 50 kDa Nalgene). The concentrated enzyme was precipitated by the drop-wise addition of 10 ml of chilled absolute ethanol with constant stirring. After 2 h at –10 °C, the precipitate was recovered by centrifugation at 16,000 *g* for 20 min. The precipitate was resuspended in 5 ml of 50 mM glycine-NaOH buffer (pH 10.0) containing 20% NaCl and dialyzed in the same buffer. The dialyzed sample was passed through a sephacryl S-200 gel permeation column (1.6 × 60 cm) equilibrated with 50 mM glycine-NaOH buffer (pH 10.0) containing 20% NaCl. The flow rate was 0.2 ml min^{-1} and 2 ml fractions were collected and analyzed for protein content and azocaseinase activity. The active fractions were pooled, concentrated and passed through a G-75 column (2 × 30 cm) equilibrated with 50 mM glycine-NaOH buffer (pH 10.0) containing 20% NaCl and re-analyzed. Aliquots of the purified enzyme were inactivated with 5 mM of the serine protease inhibitor, phenylmethylsulphonylfluoride (PMSF) (1 h, 25 °C), and exhaustively dialyzed against water containing 1 mM PMSF to remove salts. The dialyzed sample was examined by sodium dodecyl sulphate (8%) polyacrylamide gel electrophoresis (SDS-PAGE, Laemmli 1970). Total protein was determined by the method of Lowry et al. (1951), with crystalline bovine serum albumin (BSE, Sigma Chemical) as the standard.

Azocaseinase assay

Azocaseinase activity was determined by a modification of the method of Brock et al. (1982). One ml of 0.4% azocasein (Sigma-Aldrich) was dissolved in 0.1 M glycine-NaOH buffer (pH 10.0) containing 20% NaCl in Eppendorf tubes (1.5 ml) and pre-incubated at 60 °C. The reaction was initiated by the addition of 50–100 µl of enzyme solution. After incubation at 60 °C for 15 min, the reaction was terminated by the addition of 500 µl of 20% (w/v) trichloroacetic acid (TCA). The tubes were incubated at room temperature for 30 min, and then centrifuged at 10,000 *g* for 5 min. The absorbance of the supernatant at 450 nm was determined. One unit (U) of azocaseinase activity was defined as the amount of enzyme that produced an absorbance change at 450 nm of 0.01 min^{-1} under standard assay conditions. All assays were performed in triplicate.

Substrate specificity

Protease activity was determined by measuring the amount of *p*-nitroaniline released from the substrate benzoyl-DL-arginine *p*-nitroanilide (BAPNA) prepared in glycine/NaOH buffer (pH 10.0) containing 20% NaCl. The esterolytic activity was determined with benzoyl-L-arginine ethyl ester (BAEE), *p*-tosyl-L-arginine methyl ester (TAME) and benzoyl-L-tyrosine ethyl ester (BTEE) (Sigma) as described by Strongin et al. (1978) with slight modification. Briefly, the reaction mixture consisted of 0.1 ml of enzyme solution in 100 mM glycine-NaOH, pH 10.0, 20% NaCl, 100 mM $CaCl_2$ and 1 ml of the substrate. Activity was monitored as the increase in absorbance at 254 nm.

Effect of pH, temperature and NaCl

Protease activity of the purified enzyme was measured at pH 5.0–11.0 at a temperature of 60 °C in 20% NaCl (w/v), with azocasein as a substrate. A plot of relative activity against pH was constructed to determine the optimum pH for the reaction. The effect of temperature on protease activity of the purified enzyme was measured at 30, 40, 50, 60, 70, 80 and 90 °C at pH 10.0 in 20% NaCl (w/v), with azocasein as substrate. The protease activity of the purified enzyme was measured at NaCl concentrations of 0, 5, 10, 15, 20, 25 and 30% (w/v) at 60 °C and pH 10.0.

Thermal stability

The purified enzyme was incubated at various temperatures from 30 to 95 °C for 1 h at pH 10.0, and the azocaseinolytic activity subsequently assayed at 60 °C and pH 10.0 in the presence of 20% NaCl (w/v).

Effect of metal ions and inhibitors

The effects of various metal ions, detergents and inhibitors were tested on the activity of purified enzyme at 60 °C in glycine-NaOH buffer (pH 10.0) containing 20% NaCl (w/v), with azocasein as a substrate. The enzyme was preincubated with 1 mM of metal ions, such as K, Ba, Co, Cu, Mg, Mn, Hg, Zn and Ca (100 mM), for 10 min under standard conditions. Activity was measured as described above. The increase or decrease in enzyme activity was calculated relative to a control.

Effect of osmolytes

The activity of the purified enzyme in the absence of NaCl was measured in the presence of various concentrations (w/v) of the osmolytes betaine, sucrose, glycerol and mannitol in the absence of NaCl, at pH 10.0 and at 60 °C, with azocasein as substrate.

Results

Results of the purification of the protease secreted by *H. borinquense* strain TSS101 are summarized in Table 1. The active extracellular protease was isolated from the culture filtrate by alcohol precipitation and gel permeation chromatography. Purification of halophilic protease was performed in the presence of 20% NaCl, so that the enzyme would maintain a high specific activity against azocasein. The protease was purified 116-fold, with a final yield of 18%. The specific activity of the purified enzyme was 350 U mg^{-1} protein. Analysis of the purified enzyme by SDS–PAGE revealed a single band with a

Table 1. Purification of a serine protease of *Halogeometricum borinquense* strain TSS101.

Purification step	Volume (ml)	Protein concentration (mg ml^{-1})	Specific activity (U mg^{-1} protein)	Purification fold	Yield (%)
Culture medium supernatant	1000	5.6	3	1	100
Protein concentrator Nalgene (50 kD)	20	15.6	30	10	55
Ethanol precipitate	5	42	35	12	44
Gel permeation Sephacryl S-200	20	0.9	294	97	31
Gel permeation G-75	15	0.6	350	116	18

molecular mass of 86 kDa as determined by SDS-PAGE (Figure 1). The molecular mass of the active enzyme as estimated by GSK 2000 gel filtration column was 78 kDa.

The enzyme exhibited greatest activity in the pH range of 6.0–10.0, with an optimum pH of 10.0 (Figure 2). Activities at pH 5.0, 11.0 and 12.0 were 10, 70 and 45%, respectively, of the activity at pH 10.0. The enzyme was stable over a broad pH range of 6.0–10.0 at 60 °C (data not shown), indicating that it is an alkalophilic enzyme.

In the presence of calcium ions, the optimum temperature for enzyme activity was 60–70 °C (Figure 3). However, in the absence of calcium, there was a distinct optimum at 60 °C with a 25% loss in activity at 70 °C. Sixty percent of the optimal activity was retained in the 70–90 °C range. Less than 40% of the activity remained at 37 °C with or without calcium and 95 °C in the absence of calcium. Thus, the enzyme was alkaliphilic and moderately thermostable (Figure 4).

The enzyme was stable at 60 °C for 2 hours and could be stored for 30 days at 4 °C without loss of activity in the presence of 20% NaCl. In absence of NaCl, all activity was lost. Over the range of 0–30% (w/v) NaCl, maximal protease activity was observed in the presence of 20–25% NaCl (Figure 5). At an NaCl concentration of 30%, activity was 65% of the maximum.

Among the metal ions tested, only Ca^{2+} stimulated (21%) the activity of the enzyme (Table 2; Figure 6). Protease activity was completely inhibited by Cu^{2+} and Zn^{2+}. The thermostability of the enzyme was increased by 10 °C in presence of Ca^{2+}. Other metal ions like Co^{2+}, Ba^{2+}, Hg^{2+} and Mg^{2+} had no significant effect on the activity of the enzyme. The enzyme was completely inhibited by 1 mM PMSF, a serine protease inhibitor, but not by 1,10-phenanthroline (Table 3). Neither dithiothreitol (DTT) nor 2-mercaptoethanol affected enzyme activity. Inhibitors of sulfhydryl protease (PCMB, $HgCl_2$), metalloprotease (EDTA) and aspartic protease (pepstatin) were also without effect on the activity of the enzyme. These results are consistent with the enzyme being a serine protease. The enzyme retained 53% of maximal activity at 0.1% SDS, but at 0.5% SDS, all activity was lost. Cetyltrimethylammonium bromide (0.1%) enhanced activity by 59%.

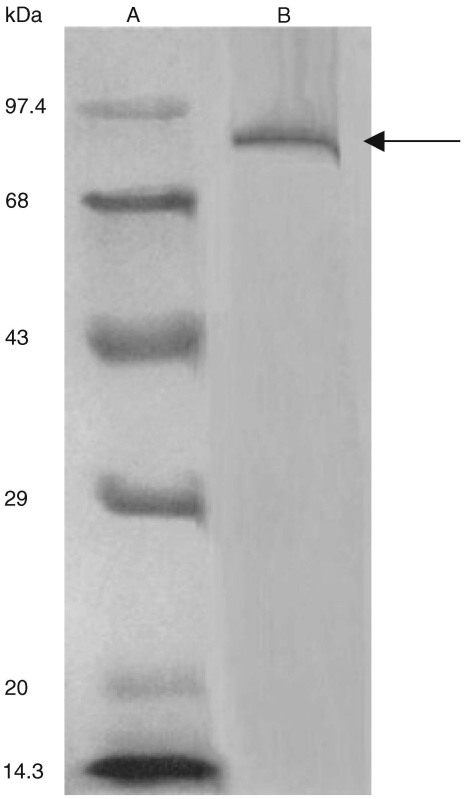

Figure 1. The sodium dodecyl sulfate polyacrylamide gel electrophoresis of the purified protease from *Halogeometricum borinquense* strain TSS101. Lane A shows relative molecular mass standards: phosphorylase b (97.4 kDa); bovine serum albumin-(68 kDa); ovalbumin (43 kDa); carbonic anhydrase (29 kDa); soyabean trypsin inhibitor (20 kDa); and lysozyme (14.3 kDa). Lane B shows the purified protease (86 kDa, indicated by arrow).

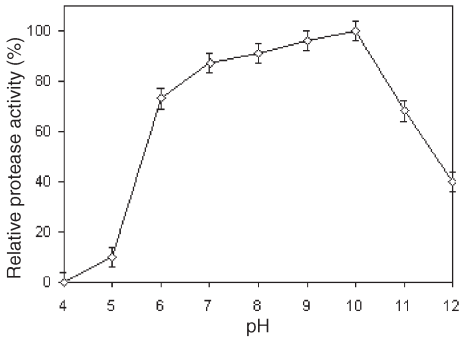

Figure 2. Activity of purified protease from *Halogeometricum borinquense* strain TSS101 at different pH values. Azocaseinolytic activity was measured at 60 °C in the presence of 20% NaCl in the indicated buffers at a concentration of 0.1 M. Each value represents the mean ± SE of three independent experiments.

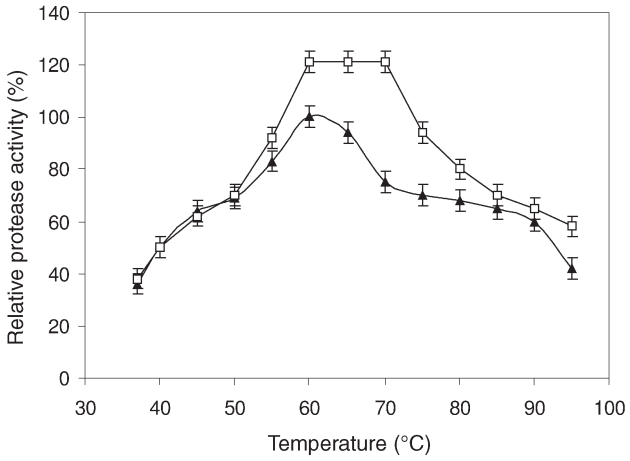

Figure 3. Effect of temperature on purified protease activity. Azocaseinolytic activity was determined in 0.1 M glycine-NaOH buffer (pH 10.0) with 20% NaCl at each temperature after incubation for 15 min with 100 mM Ca^{2+} (□) and without 100 mM Ca^{2+} (▲). Each value represents the mean ± SE of three independent experiments.

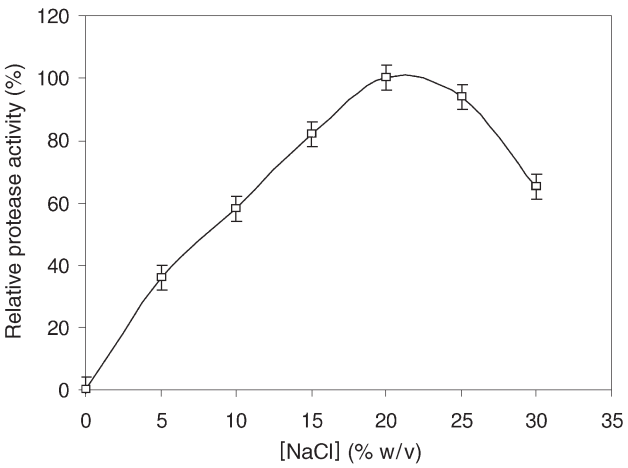

Figure 5. Effect of NaCl concentration on purified protease activity. Azocaseinolytic activity of protease was determined in 0.1 M glycine-NaOH buffer (pH 10.0) at 60 °C in the presence of the indicated concentrations of NaCl, after incubation for 15 min. Each point represents the mean ± SE of three independent experiments.

The effects of different concentrations of the osmolytes betaine, glycerol, mannitol and sucrose on the activity of the purified enzyme in the absence of NaCl were examined. The enzyme was stable and retained 100% activity in sucrose (10%) and betaine (20%), indicating that osmotic pressure or reduced water activity is important in maintaining enzymatic activity (Figure 7).

The protease was active on a variety of modified substrates (azocoll and azocasein) or natural proteins (BSA and casein). The protease exhibited the highest activity with azocoll and azocasein (Table 4). Results with synthetic substrates confirmed that trypsin-like activity was absent, as BAPNA, a chromogenic trypsin substrate, was not hydrolyzed. Likewise, neither of the ester substrates of trypsin, BAEE and TAME, nor the ester substrates of chymotrypsin, BTEE, tested were hydrolyzed by the enzyme.

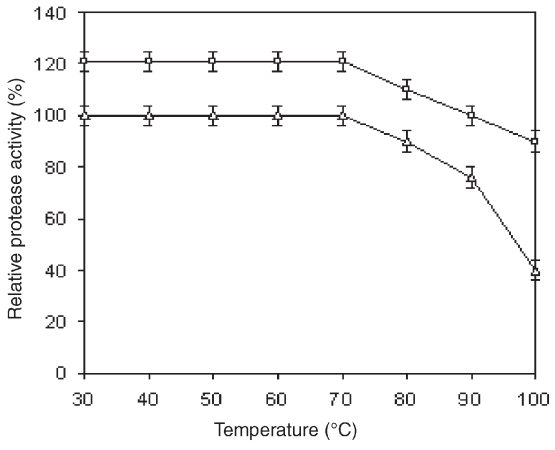

Figure 4. Effect of temperature on the stability of purified protease. The enzyme in glycine-NaOH buffer (pH 10.0) with 100 mM Ca^{2+} (□) and without 100 mM Ca^{2+} (△) was preincubated for 1 h at the specified temperatures. The residual enzymatic activity was measured under the standard conditions. Each value represents the mean ± SE of three independent experiments.

Discussion

In the purification of the highly haloalkaliphilic, thermostable protease produced by *H. borinquense* strain TSS101, precipitation by ethyl alcohol was important in the removal of contaminating proteins and exopolysaccharides. Studdert et al. (1997) similarly purified the halophilic serine protease from *Natronococcus occultus*.

The molecular mass of *H. borinquense* TSS101 protease estimated by SDS-PAGE was higher than that estimated by gel filtration (Figure 1). The difference between the estimates is likely connected with the resistance of the halophilic protease (an acidic protein) to denaturation in the presence of SDS. The enzyme presumably binds less anionic detergent compared with the marker protein isolated from nonhalophilic organ-

Table 2. Effects of metal ions on purified protease activity.

Metal ion	Concentration (mM)	Relative activity (%)
$ZnCl_2$	1	0
$HgCl_2$	1	80
$MnCl_2$	1	12
$MgCl_2$	1	62
$CaCl_2$	100	121
$CuCl_2$	1	0
$BaCl_2$	1	78
$CoCl_2$	1	68
$NaSO_3$	1	96
KCl	1	100

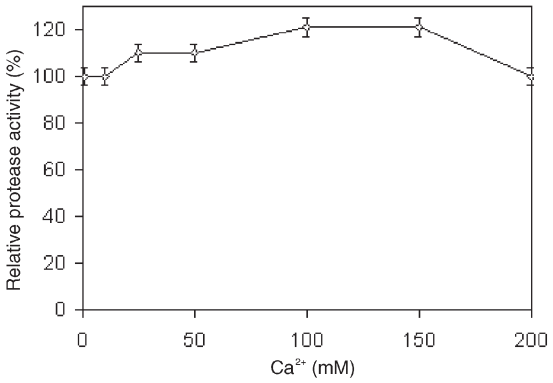

Figure 6. Effect of Ca^{2+} on purified protease activity. The enzyme was incubated with different concentrations of Ca^{2+} at 60 °C, 20% NaCl and pH 10.0, and the relative activity was measured. Each value represents the mean ± SE of three independent experiments.

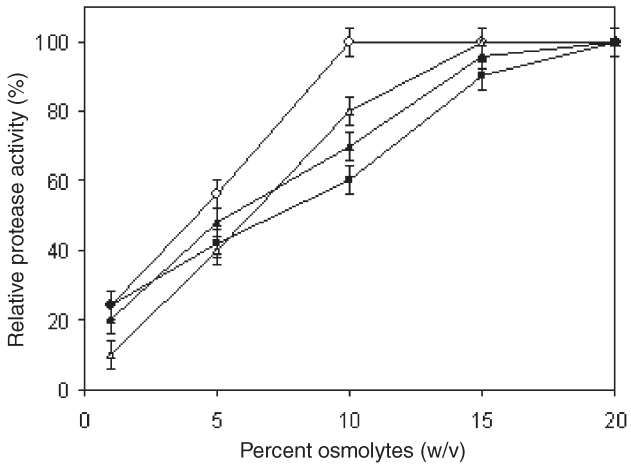

Figure 7. Effects of osmolytes on the purified protease activity in the absence of NaCl. The enzyme was incubated in the various concentrations of osmolytes: sucrose (◇); mannitol (▲); glycerol (△); and betaine (■) without NaCl in standard assay conditions. Each value represents the mean ± SE of three independent experiments.

isms. This led to reduced mobility of the SDS-protease complex and to an overestimation of the enzyme's molecular mass. A difference in molecular mass estimated by SDS-PAGE and gel filtration was reported in the case of a serine protease purified from *Halobacterium salinarum* (Izotova et al. 1983). Different molecular masses for different halophilic proteases have been reported by gel filtration: 130 kDa for *Natrinococcus occultus* halophilic protease (Studdert et al. 2001); 42 kDa for halolysin 172P1 (Kamekura and Seno 1990); 49 kDa for *Natronobacterium* sp. (Yu 1991); 60 kDa for *Halobacterium halobium* protease TuA4 (Schmitt et al. 1990); and 45 kDa for *Natrialba magadii* (Gimenez et al. 2000).

A minimum of 1 M NaCl is required for enzyme stability. Similar observations have been reported for halolysin 172P1 (Kamekura and Seno 1990), the halophilic protease produced by *Natrialba magadii* (Gimenez et al. 2000), and the haloalkaliphilic serine protease produced by *N. occultus* (Studdert et al. 2001), but not for the extracellular protease of *Halobacterium halobium* which became completely inactivated after 10 days under the same conditions (Izotova et al. 1983). In the presence of 20% (w/v) NaCl, the enzyme was stable from 30 to 70 °C without loss of activity and retained 80% activity at 90 °C for 1 h. Thermostability studies of proteases reported in the literature show stable enzymes with a half-life of 1 h at 50 °C (Bhosale et al. 1995), 25 min at 60 °C or, for the serine protease, subtilisin Carlsberg, with a half-life of 2.5 min at 60 °C (Durhan et al. 1987). By comparison, our results show a much greater thermal stability at 70 °C. In the conditions of an industrial laundry, where alkaline proteases are commonly used for 10 min at 60 °C, the high thermostability of the protease from *H. borinquense* might be advantageous.

The thermostability of the enzyme increased by 10 °C in the presence of Ca^{2+} (Figure 4). Consistent with this finding, 10 mM EDTA, a chelating agent for calcium ions, caused a 68% reduction in enzyme activity (Table 3). Activation and stabilization by Ca^{2+} has also been observed in bacterial serine proteases (Kelly and Fogarty 1976, Strongin et al. 1978, Ward 1983) and an extracellular protease, caldolysin, produced by the thermophilic bacterium, *Thermus aquaticus* (Cowan and Daniel 1982). The protease from *H. borinquense* is a serine protease as shown by its complete inactivation by PMSF, a

Table 3. Effects of chelators, detergents and inhibitors on purified protease activity.

Inhibitor	Concentration	Relative activity (%)
PMSF	1 mM	3
EDTA	1 mM	65
EDTA	10 mM	38
SDS	0.1%	53
Triton x-100	1%	80
CTAB	0.1%	159
2-Mercaptoethanol	1 mM	81
Urea	8 M	44
1,10-Phenanthroline	10 mM	76
DTT	10 mM	81
PCMB	1 mM	80
Pepstatin	1 mM	82

Table 4. Substrate specificity of purified protease. Specific activity values are means of triplicate determinations ± SD.

Substrate	Specific activity (U mg^{-1})
Casein 0.1% (Hammerstein)	262 ± 5.291
BSA 0.1%	180 ± 5.033
Azocasein 0.4%	350 ± 3.464
Azocoll 0.1%	1248 ± 7.571
BAPNA	0
BAEE	0
TAME	0
BTEE	0

common inhibitor of these enzymes (Morihara 1974, Ward 1983). This protease is more stable in anionic detergents than in cationic detergents, with activity enhanced 59% by CTAB.

Osmolytes stabilized the protease in the absence of added NaCl (Figure 7). Halophiles respond to increases in osmotic pressure in different ways. The accumulation by halophilic bacteria of compatible organic solutes, such as glycine, betaine, sugars, polyols, amino acids and ectoines, helps them to maintain an internal environment isotonic with the growth medium (Galinski 1993, Danson and Hough 1997, Da Costa et al. 1998). These substances also help to protect cells and enzymes against stress due to high temperature, desiccation and freezing (Galinski 1993, Danson and Hough 1997, Mevarech et al. 2000). However, to date, these compatible organic solutes are known to serve as significant osmolytes only among halophilic bacteria. In general, Haloarchaea use K^+ as the internal inorganic compatible solute.

The enzyme was unaffected by a specific trypsin inhibitor. Moreover, it was unable to hydrolyze trypsin substrates (Table 4), the characteristics of the enzyme are thus not those of a trypsin-type protease. Similarly, proteases from *B. stearothermophilus* F1 (Rahman et al. 1994) and *B. thermoruber* (Manachini et al. 1988) also fail to show trypsin-like activity. The pH and temperature stability in presence of such high salt concentration of the investigated protease from *H. borinquense* strain TSS101 suggest that it may have potential application in the formulation of detergents and in the fermentation of seafood.

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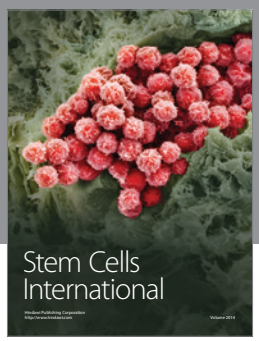

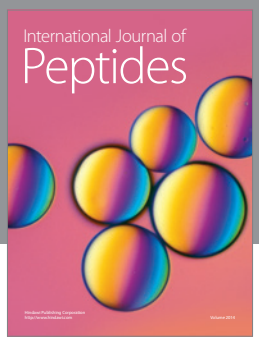

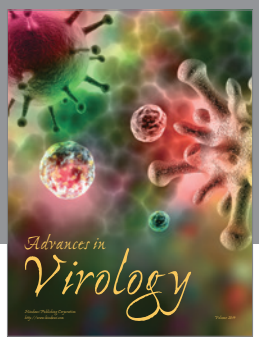

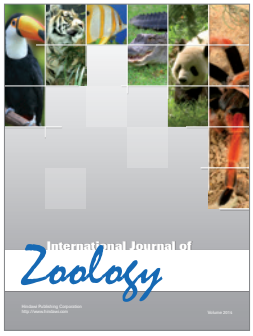

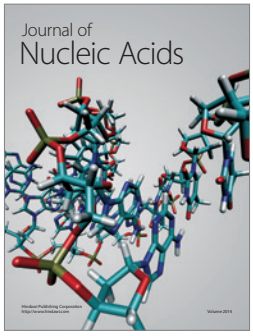

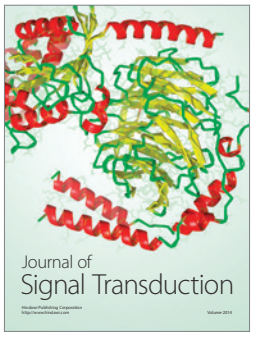

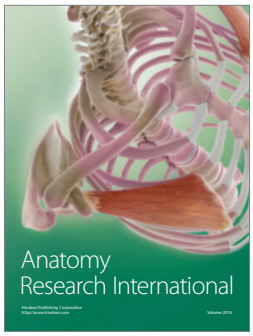

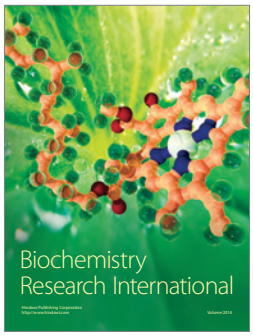

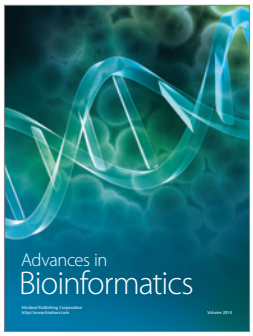

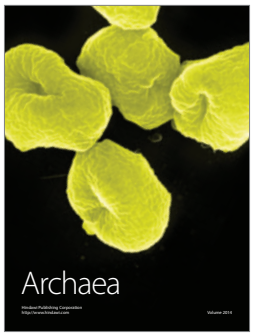

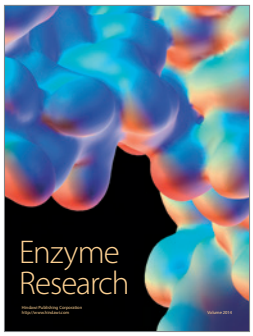

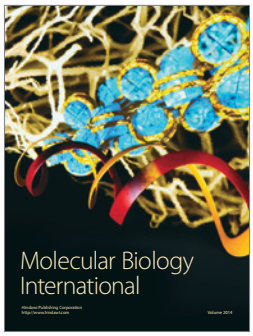

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