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Stability of Haloalkaliphilic *Geomicrobium* sp. Protease Modulated by Salt

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Abstract—A novel protease from the halophilic bacterium *Geomicrobium* sp. EMB2 (MTCC 10310) is described. The activity of the protease was modulated by salt, and it exhibited remarkable stability in organic solvents, at alkaline pH, and in other denaturing conditions. The structural changes under various denaturing conditions were analyzed by measurements of intrinsic fluorescence and circular dichroism spectroscopy. Circular dichroism showed that the secondary structure of the protease was predominantly α-helical but unfolded in salt-free medium. The structure is regained by inclusion of NaCl in the range of 2-5%. The presence of NaCl exerted a protective effect against thermal, organic solvent, and guanidine hydrochloride denaturation by preventing unfolding.

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Halophiles are a class of organisms growing in salt-rich habitats. They have been classified into extreme and moderate halophiles based on the salt requirement. To avoid exosmosis, their cells have adapted two different mechanisms: accumulation of equivalent concentration of salt in the cytoplasm or accumulation of osmolytes [1, 2]. Their enzyme functions and metabolic activities take place in presence of high salt or osmolytes. Although high salt concentration is known to disrupt normal protein structure and function, halophilic proteins remain integrated and functional under such conditions. This makes them novel for understanding nature's design of protein engineering and unique industrial applications.

From the studies on extreme halophiles and haloarchaea it has become clear that their enzyme possess a higher proportion of acidic amino acids as compared to corresponding mesophilic proteins and lesser proportion of hydrophobic amino acids. The presence of salt exerts charge screening, reduces electrostatic repulsion among acidic surface groups, and enhances hydrophobic interactions favoring a compactly folded structure even at high salt concentration [3]. Proteins folded in a high salt environment are comprised of polypeptide chains and a solvation shell. This includes specific interactions between salt ions, water molecules, and polypeptide, which intervene in solubility and stabilization of subunits [4]. Hydrated salt ions remain more in contact with protein surface than with surrounding solutions by specific interaction with carboxylic group of acidic amino acids. The water molecules bind to the protein through hydrated salt [5]. The charge screening of acidic amino acids plays an important role in stabilizing the proteins at low salt concentrations (below 0.2 M) [6]. At high salt concentrations, the reduced hydrophobicity may contribute towards preventing aggregation. The stabilization of weak hydrophobic core is suggested via salting out [7].

Most of the above understanding has emerged from studies on extreme halophilic and haloarchaeal enzymes viz. *Halobacterium halobium*, *Halobacterium salinarum*, *Natrialba magadii*, etc. [6, 8-10]. Salt activation and stabilization studies among moderate halophiles are still lacking. Only a few enzymes from the moderate halophiles have been characterized for their kinetic behaviour and stability in the presence of salt and solvents [5, 9-12]. Proteases from *Halobacterium halobium* [6], *Natrialba magadii* [8], *Salinivibrio* sp. [13], and *Streptomyces clavuligerus* [14] are reported to exhibited significant salt and solvent stability, but the molecular mechanism of salt activation and stabilization are yet to be understood.

The present study aimed at understanding the protective effect of salt in activity and stability of moderate

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halophiles. The work will have further implication on understanding protein folding in saline environment, which assumes greater significance for expression of halophilic gene in mesophilic hosts for production of industrially important halophilic enzymes [15]. Such information would prove useful for enzyme engineering.

In the present work, a novel alkaline protease from a moderately halophilic *Geomicrobium* sp. has been used as a model system [16]. The protease exhibited salt-dependent resistance against organic solvents and thermal inactivation. Alterations in the structure of the enzyme as a function of denaturants were measured by means of biological activity, circular dichroism (CD), and fluorescence spectroscopy. The results show that the protein requires a high salt concentration to remain intact over extended periods of time, and withdrawal of salt results in the gradual loss of secondary structure and unfolding.

MATERIALS AND METHODS

Reagents. Phenyl Sepharose 6 Fast Flow was purchased from Sigma Chemicals Co. (USA). Casein was a product of Sisco Research Laboratories Pvt. Ltd. (India). Guanidine hydrochloride (GdnHCl) was the product of Spectrochem Ltd. (India). Media components were purchased from Hi Media Laboratories (India). All other chemicals used were of analytical grade.

Bacterial strain. *Geomicrobium* sp. EMB2 (MTCC: 10310), a haloalkaliphilic microorganism, was isolated from water samples collected from Sambhar Salt Lake, India [16]. The culture was maintained in agar slants containing 10% (w/v) NaCl at 4°C and sub-cultured at monthly intervals.

Culture conditions for protease production. Mother culture was prepared by seeding a loopful of slant culture into medium containing (g/liter): peptone, 5.0; yeast extract, 5.0; NaCl, 100.0 adjusted to pH 8.0, followed by incubation at 30°C with constant shaking at 150 rpm (orbital shaker; Orbitech, India). For protease production, 3 ml of 24 h grown culture (OD ~ 0.8) was inoculated in 100 ml production medium containing (g/liter): casamino acid, 6.0; yeast extract, 6.0; peptone, 2.0; $MgCl_2$, 1.0; NaCl, 120.0, and pH 8.5. The inoculated medium was incubated at 30°C and 150 rpm. After 72 h of growth, the cells were harvested by centrifuging at 5500*g* and 4°C for 10 min. The cell-free supernatant filtered through 0.2 μm cellulose acetate membrane filter (Millipore, USA) was used as the crude enzyme source.

Enzyme purification by hydrophobic interaction chromatography. Crude enzyme was ultrafiltered in a stirred cell using a 10-kDa MWCO membrane (Millipore). The protease activity was observed in the retentate fraction. The retentate was further purified by hydrophobic interaction chromatography (HIC) on a Phenyl Sepharose 6 Fast Flow column (1 × 6.5 cm) equilibrated with sodium phosphate buffer (0.1 M, pH 7.5) containing 1 M ammonium sulfate. The retentate supplemented with ammonium sulfate (final concentration 1 M) was loaded onto the column. The column was washed with equilibrating buffer until the washings were free of proteins. The bound proteins were eluted with a decreasing gradient of ammonium sulfate (1-0 M in the equilibrating buffer). Fractions of 1 ml each were collected at a flow rate of 0.5 ml/min using a fraction collector (Bio-Rad, USA) and analyzed for protease activity. Further elution was done by a linear gradient of ethylene glycol (0-50% v/v) in 0.1 M sodium phosphate buffer, pH 7.5. Active fractions were pooled and dialyzed against 50 mM Tris-HCl buffer (pH 8.0) at 4°C. The dialyzed preparation supplemented with 1-20% (w/v) NaCl was used as purified protease for further characterization.

Protease assay. Protease activity was determined as described by Shimogaki et al. [17] using casein as a substrate. Enzyme solution (0.5 ml) was added to 3 ml of substrate solution (0.6% (w/v) casein in 20 mM borax-NaOH buffer, pH 10, containing 1% (w/v) NaCl), and the mixture was incubated at 50°C for 20 min. The reaction was stopped by addition of 3.2 ml of TCA mixture (containing 0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid) and kept at room temperature for 30 min followed by filtration through Whatman No. 1 filter paper. The absorbance of the filtrate was recorded spectrophotometrically (Specord 200; Analytik Jena, Germany) at 280 nm. One unit of protease activity is defined as the amount of enzyme required to produce 1 μg of tyrosine per minute under the assay conditions as described above. A control was run similarly in all the cases, wherein the enzyme solution was first incubated with TCA for 30 min and thereafter the substrate solution was added. Protein concentration was estimated by a dye-binding method [18] using bovine serum albumin as the standard protein.

CD spectroscopy. CD spectra were recorded on a Chirascan spectropolarimeter (Applied Photophysics, UK). The pathlengths of the cuvettes were 1, 0.1, or 0.05 cm, depending on the protein concentration and wavelength region. For each spectrum 5-10 scans were co-added, buffers along with the corresponding salt concentration subtracted, and the spectra smoothed using a mild smoothing function. The α-helix and β-strand content of protease from its CD spectra were determined using the web server K2D2, EMBL (Heidelberg, Germany) [19, 20].

Fluorescence measurements. Fluorescence spectra were acquired on a model FL 3-11 Fluorolog-3 modular spectrofluorometer with single Czerny–Turner grating excitation and emission monochromators having 450-W Xe arc lamp as the excitation source and a PMT as the detector (Horiba-Jobin Yvon, Inc, USA). All the data were acquired using 1 × 1 cm pathlength quartz cuvettes. Spectral response from appropriate blanks was subtracted

before data analysis. All the measurements were taken in triplicate and averaged. Data analysis was performed using SigmaPlot 8.0 software (ASI; Sigma). The excitation wavelength was kept at 280 nm. Emission spectra were recorded between 305 and 400 nm. Baselines were corrected with the corresponding buffers.

Effect of NaCl on structure of the protease. The protease was dialyzed against 50 mM Tris-HCl buffer, pH 8.0, for 18 h at 4°C. The dialyzed protease was reconstituted with various concentrations of NaCl (0-10%, w/v). The protease activities and CD and fluorescence spectra of reconstituted preparations were determined at specified conditions.

Effect of NaCl on organic solvent stability of the protease. The protease was adjusted to different NaCl concentrations (1-10% (w/v) NaCl) and organic solvents were added so as to achieve the final concentration of 50% (v/v). The mixture was incubated at 30°C with constant shaking at 200 rpm. Samples were withdrawn from the aqueous phase after different time interval, and residual protease activity was assayed. The protease incubated without solvent was treated as control. The half-life was calculated from the exponential regression curve. The circular dichroism and fluorescence spectra of the protease were recorded under specified conditions.

Effect of NaCl on thermal denaturation. The thermal stability of the protease (containing 5% NaCl, w/v) was estimated by incubating the protease at different temperatures (30-90°C). Corresponding controls without NaCl were run under similar conditions. Residual protease activity was determined at various time intervals. The circular dichroism and fluorescence spectra were determined as recorded in the previous section.

Protective effect of NaCl during guanidine hydrochloride unfolding. The protease was incubated at 30°C with 6 M guanidine hydrochloride (GdnHCl) in the absence and presence of varying concentrations of NaCl (0-20%, w/v). Aliquots were withdrawn at different time intervals and analyzed for residual protease activity and fluorescence emission spectra. The residual activity in the absence of denaturants was considered as 100%.

All the experiments were performed in triplicate, and variation was within ±5%.

RESULTS AND DISCUSSION

Purification and characterization of haloalkaliphilic *Geomicrobium* sp. protease. *Geomicrobium* sp. protease was purified by ultrafiltration and hydrophobic interaction chromatography. Its purity was checked by SDS-PAGE, which showed a single band corresponding to 38 kDa. The protease was alkaline in nature and stable to detergents, surfactants, and a range of organic solvents [16].

Salt promotes activity and exerts a stabilizing/protective effect on halophilic enzymes. In the present work,

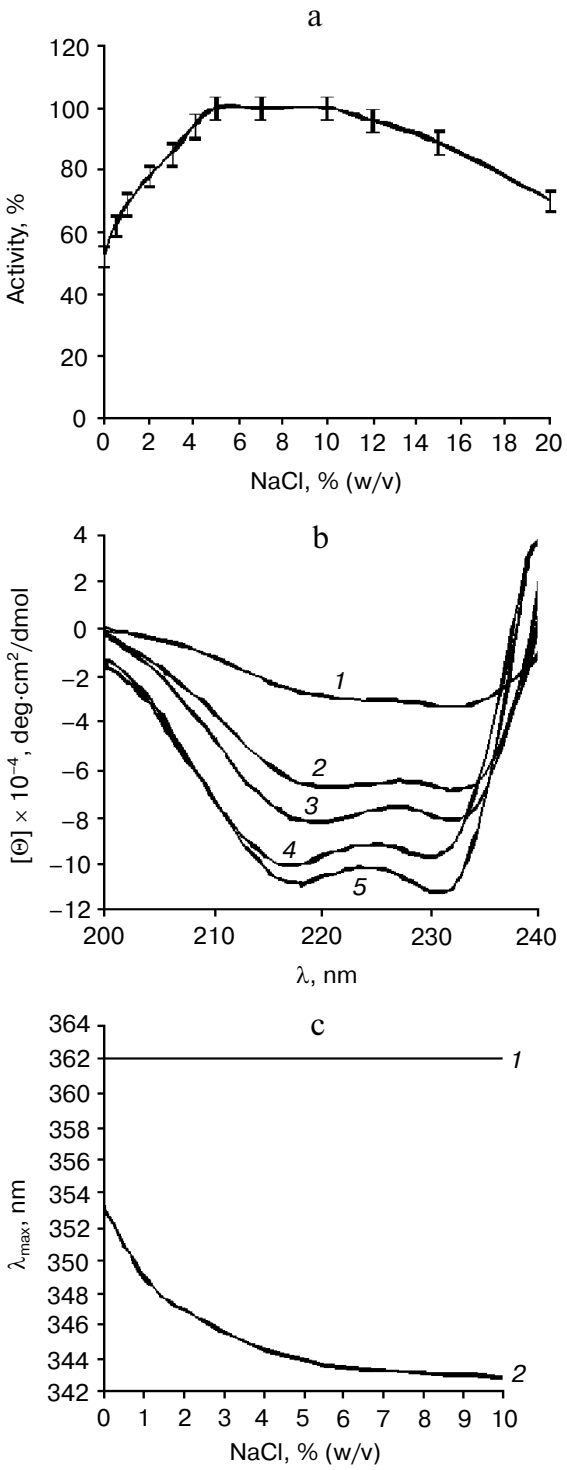

Fig. 1. a) Effect of NaCl on dialyzed *Geomicrobium* sp. protease. Dialyzed protease preparation (having 52% residual activity) was incubated with different concentrations of NaCl for 30 min followed by determination of caseinolytic activity under standard assay conditions. The activity of the undialyzed protease was taken as 100%. b) Far UV-CD spectra of *Geomicrobium* sp. protease in the absence of NaCl (*1*) and with 1% NaCl (*2*), 2% NaCl (*3*), 5% NaCl (*4*), 10% NaCl (*5*). c) Effect of salt concentration on maximum emission wavelength: L-tryptophanamide (*1*); halophilic protease (*2*).

the effect of salt on *Geomicrobium* sp. protease was examined under various destabilizing/denaturation conditions, viz. (i) complete removal of salt by dialysis and gradual addition at different levels, (ii) subjecting the enzyme to high temperature in the presence and absence of NaCl, (iii) incubating the enzyme with solvents of varying log*P* in the presence and absence of NaCl, (iv) denaturation by GdnHCl in the presence and absence of NaCl. The effect was seen by recording protease activity and CD and fluorescence spectra.

Effect of NaCl on protease activity and stability. The effect of dialysis and reactivation of dialyzed preparation by adding NaCl is shown in Fig. 1a. The protease loses half of its activity upon dialysis. Most of the activity (98%) was regained by addition of 5% NaCl. KCl has a similar effect on reactivation. This is understandable as the halophiles achieve osmotic balance by accumulating of salt/organic molecules. Their enzymes therefore, are attuned to function optimal at high salt concentration. Presence of salt is required for their optimum activity and protection against the denaturation [1, 2].

The far UV CD spectra of the protease at different concentration of NaCl are shown in Fig. 1b. In the presence of 5 and 10% (w/v) NaCl, a double minimum near 217 and 230 nm characteristic of the spectral profile of α-helical structure was observed [21]. The effect of NaCl on secondary structure contents is summarized in Table 1. It is evident that at high NaCl concentrations (5 and 10% (w/v)) the protease has predominantly α-helix content. As the salt concentration was shifted away from the optimal, loss of α-helix occurred with simultaneous increase in random coil. Similar behavior of a halophilic protein was seen by Rao et al. [12] in the case of *Haloarcula marismortui*, where maximum α-helix was attained at 25% (w/v) NaCl.

The effect of salt was further examined by fluorescence spectroscopy. In the absence of salt, a red shift with emission maxima at 353 nm was observed (362 nm for free L-tryptophanamide) (Fig. 1c). At high NaCl concentrations (5-10%, w/v) the emission maximum was recorded at 343 nm. These results suggested that the absence of salt causes unfolding of the protease leading to exposure of tryptophan residues, thereby increasing the local polarity in their vicinity, which might be responsible for the increase in the emission wavelength. A similar effect of NaCl on fluorescence of *Halobacterium halobium* protease has been previously documented by Kim and Dordick [6]. They observed a red shift from 339 to 350 nm. When the protein undergoes denaturation, the internal Trp residues become more exposed to the solvent and the maximum emission wavelength tends to become closer to that of free L-Trp-NH_2 [6, 22].

Effect of NaCl on protease activity and stability in organic solvents. The effects of organic solvents of varying log*P* (logarithm of the partition coefficient of a particular solvent between *n*-octanol and water) on the proteolytic activity of *Geomicrobium* sp. protease was checked by caseinolytic activity (Table 2). A number of organic solvents were tested to see the relationship between solvent polarity (log*P* value) and stability of the protease. It was observed that solvent stability and half-life of the protease increased with increasing log*P* value. Dimethyl sulfoxide, toluene, and *n*-hexane were exceptions to some extent. The protease was less stable in the presence of hydrophilic solvents, viz. dimethyl sulfoxide, ethanol, and 1-butanol. The half-life in hydrophobic organic solvents toluene, *n*-hexane, *n*-heptane, *n*-octane, *n*-decane, and *n*-dodecane increased considerably. To see the effect of solvent concentrations on the protease, varying amounts of solvent were tested. The protease was stable up to 75% (v/v) solvent concentrations. Normal mesophilic proteases are usually inactivated or exhibit low rate of reaction in organic solvents [23, 24]. Commercial proteases, e.g. α-chymotrypsin and *A. oryzae* protease, lost significant activity under these conditions [25, 26].

Table 1. Effect of NaCl on secondary structure of halophilic *Geomicrobium* sp. protease

NaCl concentration, % (w/v)	Content, %		
	α-helix	β-strand	random coil
0	25 ± 3	21 ± 3	54 ± 4
1	57 ± 3	5 ± 2	38 ± 3
2	67 ± 4	3 ± 2	29 ± 3
5	70 ± 4	2 ± 1	29 ± 3
10	77 ± 3	2 ± 1	21 ± 2

To check the protective effect of salt, the protease was dialyzed and NaCl was completely removed. The dialyzed protease (NaCl absent) was quite susceptible to all the organic solvents. Additions of NaCl lead to restoration of protease stability against the solvents. The half-life of the protease at 5% (w/v) salt in 50% (v/v) hydrophobic solvents ranged between 25 to 35 days. The protective effect was maximal at 5 and 10% (w/v) NaCl. The presence of 5% (w/v) NaCl is optimal for stability of EMB2 protease [16].

To understand the effect of NaCl in protecting the protease against organic solvents, far-UV CD spectra were recorded in the presence and absence of NaCl (Figs. 2a and 2b). The secondary structure was lost in the dialyzed preparation, i.e. the protease in the absence of NaCl. The protease incubated with solvents dimethyl sulfoxide, *n*-hexane, and *n*-decane also showed lack of secondary structure. However, secondary structure remained intact in the presence of 5% (w/v) NaCl. This clearly confirmed the protective role of salt against sol-

Table 2. Stability of haloalkaliphilic *Geomicrobium* sp. EMB2 protease in organic solvents

Solvent	log*P*	Half-life (days) for 0, 25, 50, 75% solvent concentration			
		0% NaCl	2% NaCl	5% NaCl	10% NaCl
Dimethyl sulfoxide	−1.30	1.0/0.3/0.2/0.2	5.9/3.1/2.4/1.5	24.6/5.3/4.0/3.1	22.5/6.2/5.4/4.3
Ethanol	0.24	1.0/0.2/0.1/00	5.9/1.0/0.7/0.4	24.6/1.8/1.4/0.8	225/2.1/1.6/1.3
1-Butanol	0.8	1.0/0.3/0.2/0.1	5.9/1.9/1.5/1.0	24.6/3.3/2.2/1.5	22.5/3.6/3.1/2.4
Dichloromethane	1.18	1.0/0.4/0.3/0.2	5.9/8.5/5.3/3.4	24.6/13.1/8.2/6.0	22.5/14.3/11.7/8.1
Benzene	2.0	1.0/0.4/0.3/0.3	5.9/13.1/8.5/4.2	24.6/32.5/25.8/21.5	22.5/32.8/29.1/26.3
Toluene	2.5	1.0/0.6/0.4/0.2	5.9/18.7/14.3/9.1	24.6/44.3/39.8/36.4	22.5/44.4/42.1/39.6
n-Hexane	3.5	1.0/0.5/0.4/0.3	5.9/15.3/9.2/5.1	24.6/42.5/36.1/33.7	22.5/41.3/39.0/37.8
n-Heptane	4.0	1.0/0.5/0.4/0.2	5.9/14.7/8.3/4.2	24.6/38.4/32.6/28.1	22.5/38.1/36.1/34.2
n-Octane	4.5	1.0/0.6/0.3/0.2	5.9/15.6/9.2/6.3	24.6/39.1/34.7/30.3	22.5/38.5/35.9/33.6
n-Decane	5.6	1.0/0.7/0.5/0.4	5.9/19.6/14.1/10.6	24.6/40.2/35.7/32.1	22.5/39.3/37.2/35.8
n-Dodecane	6.6	1.0/0.7/0.6/0.5	5.9/20.4/15.1/12.2	24.6/41.6/37.2/34.3	22.5/40.7/38.4/37.1

Note: *P* is a coefficient of distribution of a solvent used between phases *n*-octanol/water.

vent denaturation. Figure 2b shows ellipticity at 217 nm as a function of time. In the presence of 5% (w/v) NaCl and solvents *n*-hexane/*n*-decane, the ellipticity remains rather unchanged with time, thus reconfirming the effect of salt in protecting secondary structure.

The effect of the organic solvents on the conformation of the protein was also investigated by fluorescence spectroscopy. The maximum emission wavelength (λ_{max}) of free L-tryptophanamide and the protease (with and without 5% (w/v) NaCl) were recorded after incubating them with different organic solvents. Differences in maximum emission wavelengths ($\Delta\lambda_{max}$) between λ_{max} of free L-Trp-NH_2 and λ_{max} of the protease under various conditions were recorded (Table 3). When the protein undergoes denaturation, the maximum emission wavelength (λ_{max}) will be closer to that of free L-Trp-NH_2, i.e. the difference in MEW ($\Delta\lambda_{max}$) will become minimal. On the contrary, $\Delta\lambda_{max}$ is increased when the native conformation of the protease is retained. When the protease was exposed to organic solvents in the absence of NaCl, $\Delta\lambda_{max}$ decreased, but it increased if the exposure to organic solvents was done in the presence of 5% (w/v) NaCl. This indicates the protective effect of

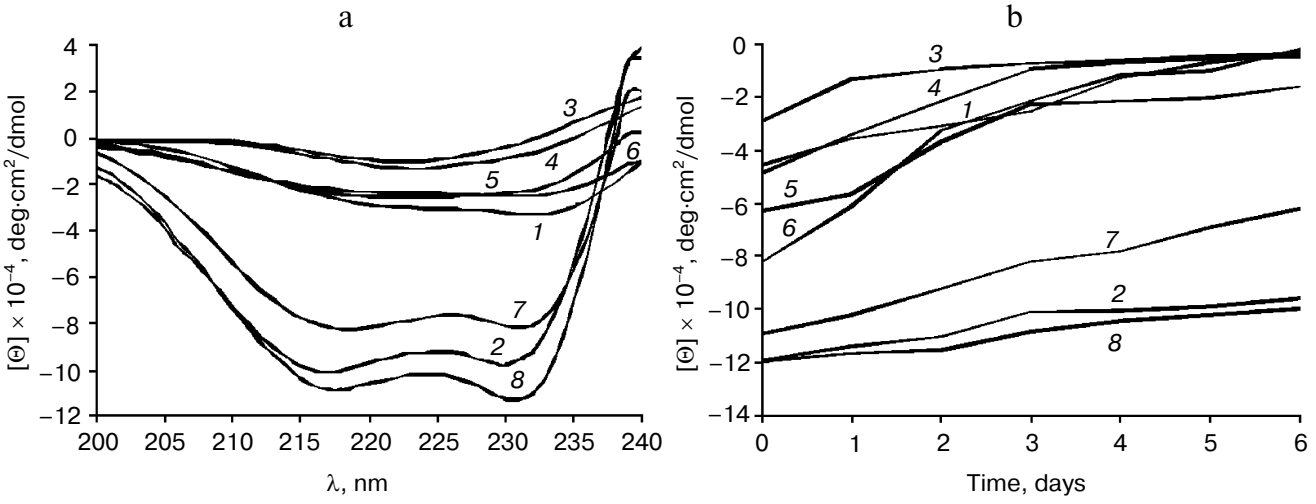

Fig. 2. a) Far-UV CD spectra of *Geomicrobium* sp. protease in the presence of organic solvents. Protease in the absence and presence of NaCl (5%) was incubated with different organic solvents (50%, v/v) for 72 h: *1*) without NaCl; *2*) 5% NaCl; *3*) dimethyl sulfoxide without NaCl; *4*) *n*-hexane without NaCl; *5*) *n*-decane without NaCl; *6*) dimethyl sulfoxide with 5% NaCl; *7*) *n*-hexane with 5% NaCl; *8*) *n*-decane with 5% NaCl. b) CD ellipticity of the protease at 217 nm versus time with different organic solvents in the presence or absence of NaCl. Designations of curves are the same as in Fig. 2a.

NaCl against solvent denaturation. NaCl helped in stabilizing and maintaining native tertiary structure.

Thermal denaturation. The thermal stability profile of EMB2 protease is shown in Fig. 3a. The enzyme activity was completely lost within 1 h of incubation at 50°C in the absence of NaCl, whereas there was no loss in activity when NaCl was present.

To understand the above protective effect of salt, CD spectra were recorded under corresponding conditions. The ellipticity (217 nm) was plotted against incubating the protease at different temperatures (Fig. 3b). For the protease in solution with 5% (w/v) NaCl, ellipticity remained essentially unchanged up to 60°C, whereas in the absence of NaCl the protein showed temperature-dependent reduction in ellipticity values, indicating the loss of secondary structure to be responsible for thermal denaturation.

The fluorescence spectra of free L-tryptophanamide and the protease (with and without NaCl) were recorded after subjecting them to different temperatures and cooling back to 25°C (Fig. 3c). Changes in the maximum emission wavelength demonstrated that the protease without salt was completely denatured at 50°C, as the λ_{max} reached close to λ_{max} of free L-tryptophanamide. In the presence of NaCl, denaturation occurred only at 70-80°C. The protective effect of salt against thermal denaturation was reconfirmed by these studies.

Salt might have increased the activity of water on the surface of the protease, leading to enhanced core hydrophobicity. Improved core packing and enhanced rigidity of proteins are proposed to be the major factors imparting stability at higher temperatures [15, 27].

Guanidine hydrochloride (GdnHCl)-induced denaturation. Guanidine hydrochloride is one of the strongest denaturants used in physiochemical studies of protein folding. At high GdnHCl concentrations most proteins undergo loss of ordered structure and become randomly coiled. The *Geomicrobium* sp. protease was found to be quite sensitive to 6 M GdnHCl and denatured within 2 h in the absence of NaCl. However, when NaCl was added prior to treatment with guanidine hydrochloride it retained 63 and 83% activity at 5 and 10% (w/v) salt, respectively (Fig. 4a). The resistance against denaturation was clearly evident as a function of salt concentration.

Unfolding by GdnHCl caused a strong decrease in tryptophan fluorescence with concomitant red shift of the wavelength maximum [28]. In the absence of NaCl, protease denaturation by GdnHCl was manifested by λ_{max} = 370 nm and 47% fluorescence intensity (I_{max}). Its native form was preserved at 5-15% (w/v) salt, as proven by maximum emission wavelength at 356 nm and highest fluorescence intensity (100%) (Fig. 4b).

A similar effect of salt has been reported in NDP kinase from *Natrialba magadii* [29]. Salt stabilization of a globular protein was observed earlier by addition of 2.5 M NaCl in 434-repressor (1-63) [30]. So far there has been only one report of such resistance of a protease from haloalkaliphilic bacteria [15].

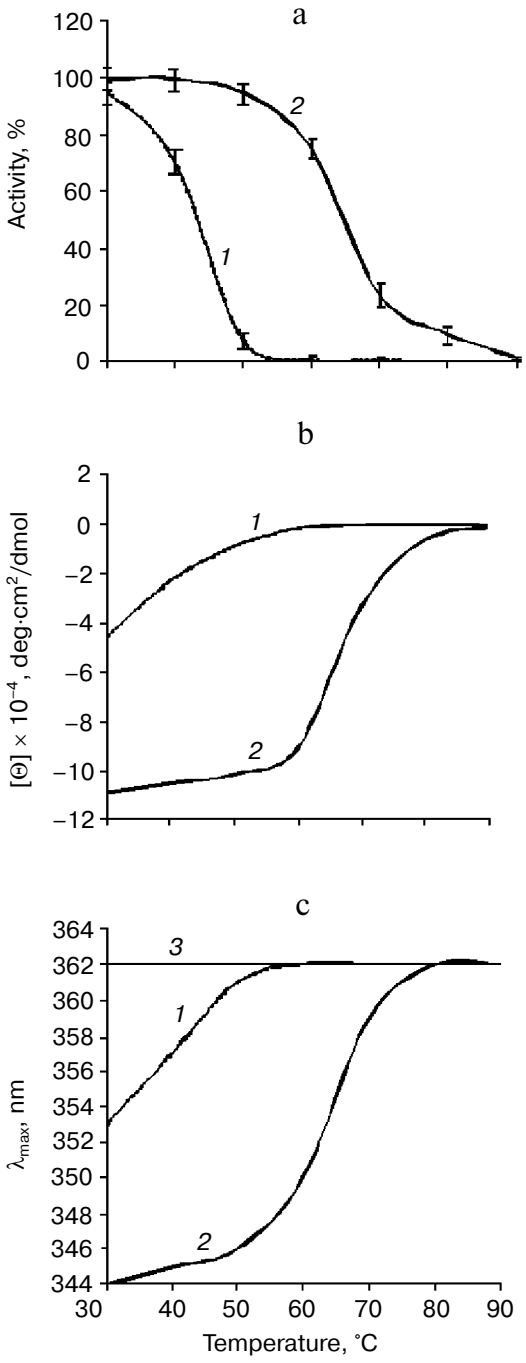

Fig. 3. a) Effect of temperature on *Geomicrobium* sp. protease activity. The protease was incubated at different temperature for 1 h (pH 8.0), and residual caseinolytic activity was recorded in the absence (*1*) and presence (*2*) of 5% NaCl. b) Corresponding CD ellipticity at 217 nm in the absence (*1*) and presence (*2*) of 5% NaCl. c) Protective effect of NaCl on thermal denaturation of *Geomicrobium* sp. (abscissa, λ_{max}; excitation at 280 nm): *1*) no NaCl; *2*) 5% NaCl; *3*) L-tryptophanamide.

In the presence of 5% NaCl (w/v), the *Geomicrobium* sp. protease existed in fully native confirmation. The dia-

Table 3. Fluorescence experiments under various conditions

Conditions	Protease concentration, μg/ml	λ_{max} of L-Trp-NH_2, nm	λ_{max} of protease, nm	$\Delta\lambda_{max}$, nm
– NaCl	75	362	353	9
+ 5% NaCl	75	362	344	18
Heat denaturation	75	362	362	0
Dimethyl sulfoxide	150	356	354	2
Dimethyl sulfoxide + 5% NaCl	150	356	352	5
n-Hexane	150	360	354	6
n-Hexane + 5% NaCl	150	360	345	15
n-Decane	150	361	349	12
n-Decane + 5% NaCl	150	361	341	20

Note: Fluorescence spectra of the protease were recorded after incubation (72 h) with 50% (v/v) organic solvents and in the absence and presence of 5% (w/v) NaCl.

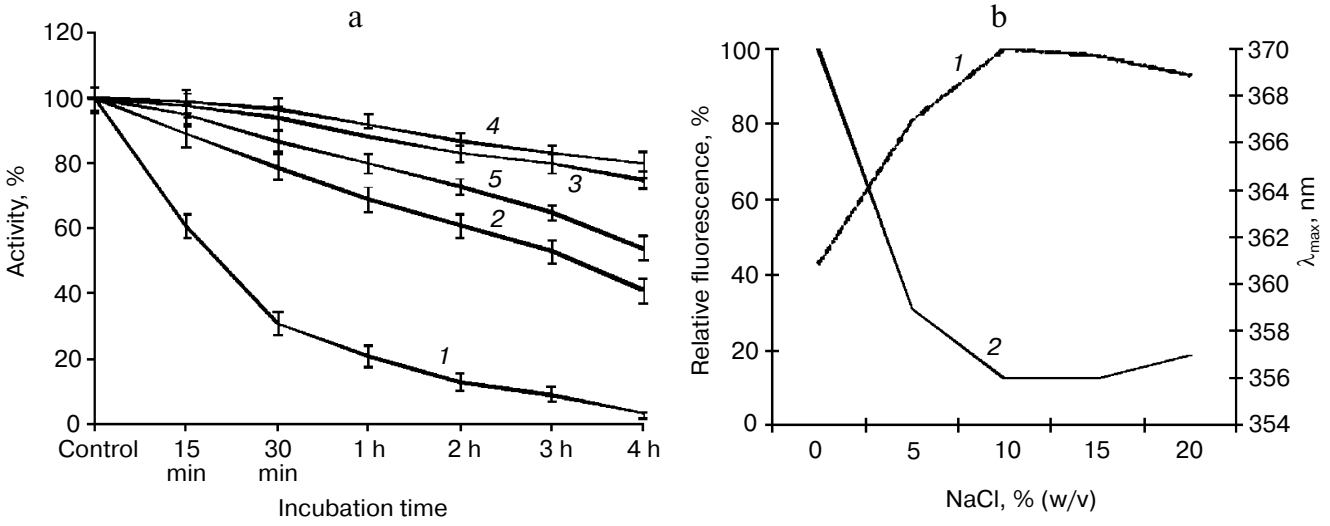

Fig. 4. a) Denaturation of *Geomicrobium* sp. protease by 6 M GdnHCl without NaCl (*1*), at 5% NaCl (*2*), at 10% NaCl (*3*), at 15% NaCl (*4*), and at 20% NaCl (*5*). b) Effect of NaCl on denaturation kinetics in 6 M GdnHCl. Maximum fluorescence intensity (I_{max}) (*1*) and λ_{max} (*2*) of the protease in different conditions (2 h incubation, excitation at 280 nm).

lyzed protease after complete removal of NaCl was partially denatured. The studies confirmed that upon exposure to organic solvent, heat, or GdnHCl, the loss in catalytic activity was due to loss of secondary and tertiary structure of the enzyme. The presence of NaCl stabilized the secondary and tertiary structure. The study has implications in understanding the mechanism of salt stabilization in the case of moderate halophiles.

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