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Functional roles of C-terminal extension (CTE) of salt-dependent peptidase activity of the *Natrialba magadii* extracellular protease (NEP)

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ABSTRACT

Nep (Natrialba magadii extracellular protease) is a halolysin-like peptidase secreted by the haloalkaliphilic archaeon *Natrialba magadii*. Many extracellular proteases have been characterized from archaea to bacteria as adapted to hypersaline environments retaining function and stability until 4.0 M NaCl. As observed in other secreted halolysins, this stability can be related to the presence of a C-terminal extension (CTE) sequence. In the present work, we compared the biochemical properties of recombinant Nep protease with the truncated form at the 134 amino acids CTE (NepΔCTE), that was more active in 4 M NaCl than the non-truncated wild type enzyme. Comparable to the wild type, NepΔCTE protease is irreversibly inactivated at low salt solutions. The substrate specificity of the truncated NepΔCTE was similar to that of wild type form as demonstrated by a combinatorial library of FRET substrates. The enzyme stability, the effect of different salts and the thermodynamics assays using different lengths of substrates demonstrated similarities between the two forms. Altogether, these data provide further information on the stability and structural determinants of halolysins under different salinities, especially concerning the enzymatic behavior.

1. Introduction

Natrialba magadii extracellular protease (Nep) is a halophilic enzyme [from Greek *halos* (salt) and *philos* (affinity)] secreted by the haloalkaliphilic archaeon *Natrialba magadii*. It is a subtilase with great activity in salty and aqueous-organic media as it is highly functional at low water activity environments [1–3]. The highly negative charge of halophilic protein surfaces is responsible for their great water-solubility, also rendering them flexibility at high-salt concentrations. In these conditions non-halophilic proteins tend to aggregate and/or become rigid [4]. Many extracellular proteases have been characterized from archaea to bacteria as adapted to live in hypersaline environments with maximal catalytic activity and stability in the presence of 3.0–4.0 M NaCl [5–12]. Halolysins (the subtilisin-like proteases produced by haloarchaea) possess a distinctive C-terminal extension (CTE) with approximately 134 amino acids residues. The importance of CTE in Haloarcheas subtilases can be related to evolutive adaptability of the halolysins under extreme environments, playing a role on enzyme stability and in substrate recognition, as studied in the halolysin R4 from *Halferax mediterranei* [13] and the halolysin SptA produced by *Natrinema* sp. J7 [12]. The absence of CTE in SptA mutant is responsible for decreased enzymic activity from low to high salts concentrations when tested against larger substrates. However, the effect of CTE in SptA activity against small substrates does not indicate the same correlation. The explanation for this apparent contradiction can be explained by the absence of interactions between small substrates and the protein's CTE. Since halolysins are secreted proteases, that degrade extracellular proteins for nutritional purposes, the presence of an active-site distant region seems to have a physiological importance in small peptides generation from large proteins [3,14].

Recently, we have reported the enzymatic activity, catalytic mechanism, stability and in solution structural behavior of the secreted subtilase (Nep) produced by the haloalkaliphilic archaeon *Natrialba magadii* [15]. In order to better understand the molecular basis of salt adaptation of halophilic proteases and to explore the role of CTE in Nep activity, in the present work we describe the preparation of Nep lacking its C-terminal extension (NepΔCTE) and explore its enzymatic stability activity, substrate specificity and substrate binding compared with the wild type form.

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2. Materials and methods

2.1. Truncated Nep (NepΔCTE)

The DNA coding the halolysin Nep lacking the C-terminal extension (134 amino acids) was PCR amplified using the primers Nep_NdeI_Fw (5-ACGTCTTCATATGACACGTGATACCAATAG-3′) and NepDCt_stop_HindIII_Rv (5′-GGCAAGCTTACGTAGTGACGGCCAG-3′) and high fidelity polymerase (i-Proof DNA polymerase, Bio-Rad) at 60 °C of annealing temperature. The amplicon was gel-purified cloned on pTOPO vector (Invitrogen) according to the manufacturer instructions. The products were transformed into *E. coli* TOP-10 competent cells and plated on LB-Agar plus Kanamycin (50 μg/mL). The recombinant colonies were detected by colony PCR and the sequence was verified by DNA sequencing. The extracted plasmids were excised by digestion with *Nde*I (NEB) and *Hin*dIII (NEB) and the gel product was purified and subcloned into pET-24b (pET-*nep*Δ*ct*). Minipreps of pET-*nep*Δ*ct* were digested with NdeI and Blp1 (NEB), and the purified product was subcloned into the haloarchaeal shuttle vector pJAM under the constitutive promoter rRNAP2 [16]. After a *dam-E. coli* GM33 passage, the demethylated construct pJAM_*nep*Δ*ct* was used to transform *H. volcanii* DS70 sphaeroplasts [15] and was further selected on YPC-agar medium plus Novobiocin (2 μg/mL, Sigma-Aldrich) and skim milk (0.8%).

2.2. Enzyme production and purification

The recombinant proteases NepWT and NepΔCTE were expressed in *Haloferax volcanii* DS70 as previously described [15]. Briefly, *H. volcanii* cells harboring the constructs pJAM-*nep* and pJAM_*nep*Δ*ct* were grown in YPC medium [0.5% (w/w) yeast extract (Difco), 0.1% (w/w) peptone (Oxoid) and 0.1% (w/w) casamino acids (Difco)] [17] plus 2 μg/mL Novobiocin at 42 °C until the stationary phase (OD_{600}~2). Cells were harvested by centrifugation (12,000*g*, 40 min, 4 °C) and the secreted proteins were analyzed by 10% SDS-PAGE. Enzyme purification was carried out by FPLC (AKTA purifier plus Superdex 200 column, GE healthcare) in a 50 mM Tris-HCl pH 8.0 buffer containing 3.0 M NaCl. Nep activity was followed using the fluorescent Suc-Ala-Ala-Pro-Phe-MCA substrate (20 μM) at 37 °C in a 50 mM Tris-HCl pH 8.0 buffer containing 3.0 M NaCl.

2.3. Western blotting assay

H. volcanii cells harboring the different Nep constructs were grown in YPC medium and harvested as described in the previous section (*Enzyme production*). The cell-free media was incubated with 5 mM PMSF (phenylmethylsulfonyl, Sigma-Aldrich) for 30 min 4 °C. Proteins were precipitated with one volume of acetone (100%, 4 °C, 2 h), centrifuged (12,000*g*, 30 min, 4 °C) and washed with acetone (80% × 3; 100% × 1). Protein pellets were dried and suspended 1× Laemni sample buffer. Samples were boiled for 5 min and loaded onto SDS-Page 10% and stained using Coomassie blue R-250 (Bio-Rad) or transferred to Nitrocellulose Membrane (Thermo Scientific). The Nitrocellulose membrane was blocked with 5% BSA in TBS plus 0.01% Tween® 20 for 1 h at room temperature.

After blocking, the membrane were probed with 1/4000 anti-Nep antibody [15] followed by 1/10,000 alkaline phosphatase-conjugated secondary antibody diluted in blocking buffer. Reactive proteins were detected after incubation with NBT and BCIP (Thermo Scientific Pierce, Rockford, IL). Bench Mark Pre-stained Protein Ladder (Invitrogen) was used as molecular mass standard.

2.4. Mass spectrometry analysis

The target NepΔCTE and NepWT bands from SDS-PAGE were Coomassie blue R-250 stained, excised and subjected to *in-gel* digestion with trypsin and followed by peptide mass fingerprinting using MALDI-TOF (Ultraflex III, Bruker Daltonics, Germany), in the mass spectrometry facility CEQUIBIEM, Argentina [18]. Spectra were converted to DTA files and merged to facilitate data base searching using the Mascot search algorithm v2.1 (Matrix Science, Boston, MA) against the mature form of Nep deduced from the Nep gene (AAV66536) [3].

2.5. Peptides

Suc-Ala-Ala-Pro-Phe-MCA was a gift from the Peptide Institute Inc. (Osaka, Japan). The Abz-AAPFSSKQ-EDDnp and the combinatory fluorimetric library Abz-GXXZXXQ-EDDnp (Z constant, excluding cysteine, and X consisting of equimolar mixture of the other amino acids [19] were synthesized by solid-phase peptide synthesis [19–21]. Stock solutions of peptide were prepared in 100% DMSO (dimethyl sulfoxide, Sigma Aldrich) and the concentration measured spectrophotometrically (EDDnp $\varepsilon_{365nm} = 17{,}300\ M^{-1}\ cm^{-1}$).

2.6. Kinetic measurements

Hydrolyses of FRET peptides (Abz-peptidyl-Eddnp) were assayed in a Hitachi F-2500 spectrofluorimeter (Tokyo, Japan). Fluorescence changes were continuously monitored at $\lambda_{ex} = 320$ nm and $\lambda_{em} = 420$ nm. The enzyme concentration was chosen so as to hydrolyze <5% of the amount of added substrate and the inner-filter effect was corrected using an empirical equation [22,23]. The slope was converted into micromoles of substrate hydrolyzed per minute based on a calibration curve obtained from the complete hydrolysis of each peptide. The hydrolysis of the MCA peptide (Suc-AAPF-MCA) were continuously followed at $\lambda_{ex} = 380$ nm and $\lambda_{em} = 460$ nm. The substrates were completely soluble in all used buffer conditions until at least 2-fold the K_m values for their kinetics of hydrolysis. The concentration of DMSO in assay buffers was kept below 1% (v/v). The kinetic parameters k_{cat} and K_m with respective standard errors were obtained through the Michaelis-Menten equation using Grafit®v.5.0 software (Erithacus Software, Surrey, U.K.). The errors were <10% for any of the obtained kinetic parameters in at least three determinations.

2.7. Determination of the substrate cleavage sites

The scissile bonds of hydrolyzed FRET peptides were identified by RPLC/MS (LCMS-2020 EV equipped with the ESI-probe, Shimadzu, Japan) using a Shim-pack CLC-ODS(M)® C18 column (Shimadzu, Japan) [22].

2.8. Salt influence on Nep activity and stability

The influence of NaCl, NaOAc (sodium acetate), Na_2SO_4, NaH_2PO_4, sodium citrate, KCl, $CaCl_2$, $MgCl_2$ and NH_4Cl on the catalytic activity of NepWT and NepΔCTE was investigated over concentration range of 0.5–5.0 M [24–26]. The assays were performed measuring relative activity with (V) or without (V0) salt of hydrolysis of Abz-AAPFSSKQ-EDDnp (10 μM) at 37 °C in 50 mM Tris-HCl, pH 8.0. The effect of NaCl on NepΔCTE stability was analyzed by measuring enzyme activity remaining after incubation in 0.5 M to 4.0 M NaCl at 37 °C, under the same conditions described above.

2.9. Solvent kinetic isotope effects

The solvent kinetic isotope effects (SKIEs) on NepΔCTE activity were measured in 50 mM Tris-HCl, pD 8.0 in the presence of 1.0–3.0 M NaCl. The pD of deuterium oxide solution (Sigma-Aldrich) was calculated from pH-meter readings according to the relationship pD = pH + 0.4 [27].

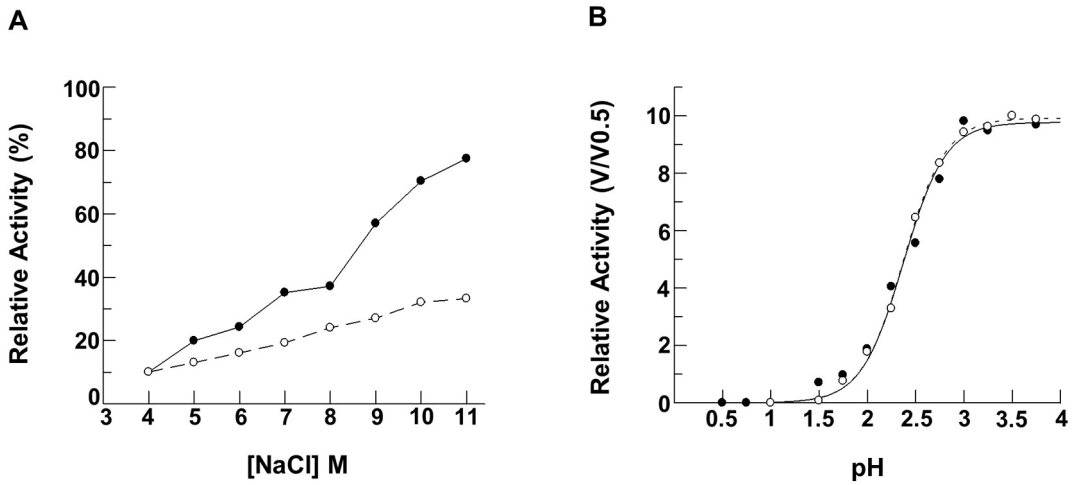

Fig. 1. CTE effect on Nep salt activation. A. NepΔCTE (full circle) protease is more sensitive to salt activation than NepWT (open circle). It is noteworthy that at 4.0 M NaCl the truncated form NepΔCTE is 3-fold more active than NepWT. Relative activities were determined by measuring the initial velocity of hydrolysis of 10 μM Abz-AAPFSSKQ-EDDnp on NepWT and NepΔCTE in the presence of NaCl ranging from 0.5 to 4.0 M. Relative activities were calculated assuming the hydrolysis at 0.5 M of NaCl as 1 (V/V0.5). B. The effect of activation is not due to shifts in pH curve. The CTE truncation (NepΔCTE) does not interfere with the pH profile of at NaCl 3.0 M when compared with NepWT.

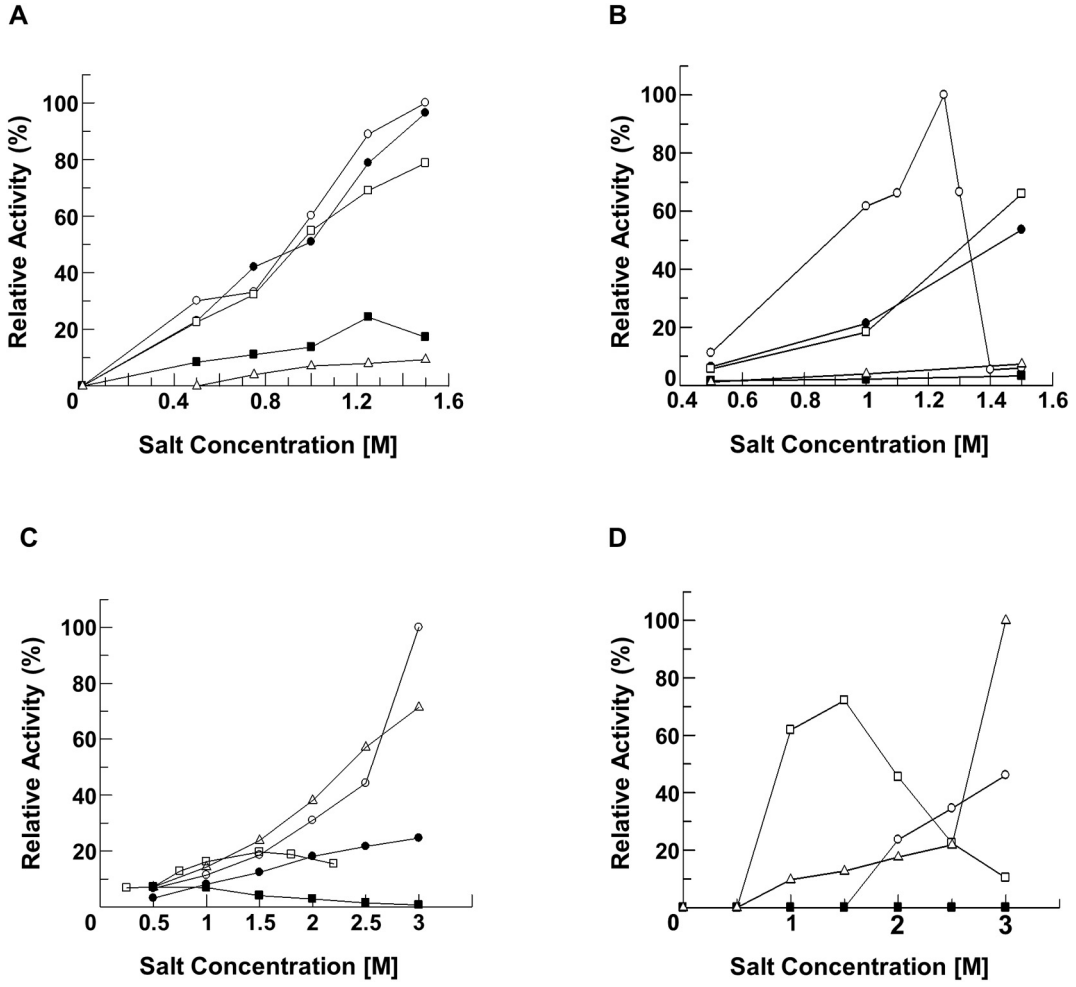

Fig. 2. Effect of Hofmeister anions and cations on NepWT and NepΔCTE activity. Hofmeister anions series on: (A) NepWT and (B) NepΔCTE assayed in presence of (open circle) sodium citrate, (closed circle) Na_2HPO_4, (open square) Na_2SO_4, (closed squares) sodium acetate and (open triangle) NaCl at different concentrations. Effect of Hofmeister cations series on: (C) NepWT and (D) NepΔCTE assayed in presence of (open circle) KCl, (closed circle) NH_4Cl, (open square) $MgCl_2$, (closed square) $CaCl_2$ e (open triangle) NaCl at different salt concentrations.

Table 1
Kinetic parameters comparison between the substrates Abz-AAPFSSKQ-EDDnp and Suc-AAPF-MCA hydrolyzed by NepΔCTE at 0.5–3.0 M NaCl.

Substrates	NepΔCTE kinetic constants at NaCl concentrations											
	0.5 M			1.0 M			2.0 M			3.0 M		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m $(mM.s)^{-1}$	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m $(mM.s)^{-1}$	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m $(mM.s)^{-1}$	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m $(mM.s)^{-1}$
Abz-AAPF↓SSKQ-EDDnp	17.5 ±2.0	57.1 ± 7.4	306.5 ± 52.7	24.6 ± 3.1	42.1 ± 5.3	584.3 ± 104.7	24.9 ± 3.2	34.6 ± 4.4	719.6± 131.5	25.8 ± 3.0	26.1 ± 3.4	988.5 ± 166.8
Suc-AAPF↓-MCA	ND*	ND*	ND*	12.3 ± 0.4	43.7 ± 2.8	281.4 ± 20.4	25.0 ± 0.2	25.6 ± 0.6	978.9 ± 22.8	42.5 ± 1.4	19.7 ± 0.9	2155.7 ± 120.2

*ND stands for non-detected activity.

2.10. Salt effect on Nep activity and stability

The effect of NaCl on the catalytic activity of NepΔCTE was investigated over a concentration range of 0.5 to 3.0 M. The assays were performed measuring the initial velocity of hydrolysis of Abz-AAPFSSKQ-EDDnp (10 μM) at 37 °C in 50 mM Tris-HCl, pH 8.0. The effect of NaCl on NepΔCTE stability was analyzed by measuring the remaining activity for up to 15 days under the same conditions described above.

2.11. Temperature effect on Nep stability

The effect of temperature on NepΔCTE stability was analyzed over a temperature range from 35 °C to 60 °C in 50 mM Tris-HCl buffer (pH 8.0) containing 3.0 M NaCl. Enzyme samples (12 mg/mL) were incubated using a peltier system in a PCR Thermocycler (Bio-rad) for 30 min. The remaining activity was measured at 37 °C under the same conditions described above.

2.12. Determination of inactivation rate constant

Thermal inactivation kinetics of the purified protease was determined by first-order expression:

$$dE/dt = -k_{inat}E$$

so that $\ln[Et/E0] = -k_{inat}t$.

The k_{inat} (inactivation rate constant or first-order rate constant) values were calculated from slopes obtained in ln [Et/E0] or ln [residual activity] versus time plot at a particular temperature. The apparent half-lives were estimated by the equation:

$$t_{1/2} = \ln(2)/k_{inat}$$

The half-life is the time at which the residual activity reaches 50%.

2.13. Temperature dependence of the specificity constant

Temperature dependence of the specificity constant k_{cat} and K_m was determined as earlier described [28]. The hydrolysis of Abz-AAPFSSKQ-EDDnp by NepWT and NepΔCTE were monitored through Michaelis-Menten kinetics in 50 mM Tris-HCl pH 8.0 buffer, either in 0.5 M or 3.0 M NaCl. Temperature corrections were applied to Tris buffers ($\Delta pKa/\Delta T = -0.027$). Activation parameters were calculated from the linear plot of ln [(k_{cat}/K_m)/T] versus 1/T (Eq. (1)).

$$\mathrm{Ln}\,[(k_{cat}/K_m)T] = \ln(R/N_A h) + \Delta S^*/R - \Delta H^*/RT \quad (1)$$

where R is the gas constant (8.314 $J.mol^{-1}\,K^{-1}$), T is the absolute temperature, N_A is the Avogadro number, h is the Plank constant, enthalpy of activation $\Delta H^* = -(\text{slope}) \times 8.314\ J.mol^{-1}$, the entropy of activation $\Delta S^* = (\text{intercept} - 23.76) \times 8.314\ J.mol^{-1}\,K^{-1}$. The free energy of activation ΔG^*, was calculated from Eq. (2).

$$\Delta GT = \Delta H^* - T\Delta S^* \quad (2)$$

The enthalpy, entropy, and free energy of activation associated with the inactivation process were determined by the linear Eyring plots for k_{cat}/K_m obtained in the presence of 0.5 and 3.0 M NaCl.

3. Results

3.1. Expression of a mutant version of halolysin Nep with a C-terminal deletion in H. volcanii

A truncated version of the halolysin Nep which encoded a polypeptide lacking 134 amino acids at the C-terminus was constructed in order to assess the contribution of the C-terminal extension (CTE) on

Table 2
Solvent kinetic isotope effect (SKIE) of Suc-AAPF-MCA hydrolysis by NepΔCTE.

NaCl (M)	Tris 50 mM pH 8.0 (H_2O)			Tris 50 mM pH 8.0 (D_2O)			SKIE
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m $(mMs)^{-1}$	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m $(mMs)^{-1}$	k_{cat} (H_2O)/k_{cat} (D_2O)
1	12.3 ± 0.4	43.7 ± 2.8	281.4 ± 20.4	2.8 ± 0.1	41.5 ± 3.4	68.7 ± 6.2	4.3 ± 0.2
2	25.0 ± 0.2	25.6 ± 0.6	978.9 ± 22.8	7.0 ± 0.9	37.4 ± 0.1	187.3 ± 24.3	3.6 ± 0.4
3	42.5 ± 1.4	19.7 ± 0.9	2155.7 ± 120.2	8.5 ± 0.7	31.1 ± 1.5	273.6 ± 26.7	5.0 ± 0.4

enzymatic properties. The construct was expressed under the constitutive promoter P2 of the pJAM vector in the neutrophilic haloarchaeon *H. volcanii* DS70. Recombinant cells expressing NepWT and NepΔCTE produced clear halos of proteolysis when cultivated in YPC-agar plaques, indicating that both Nep forms were secreted and are active (Fig. S1A) When liquid cultures of *H. volcanii-nepΔct* were grown and the cell-free medium were examined by SDS-PAGE/Western blotting with anti-Nep antibodies, a protein band with lower electrophoretic mobility than Nep evidenced the presence of the truncated protease (Fig. S1B). Cell-free culture medium containing NepΔCTE protease was concentrated and subjected to size-exclusion chromatography (Superdex 200, GE Healthcare) using 50 mM Tris-HCl (pH 8.0) plus 3.0 M NaCl as mobile phase [3,29] (Fig. S1C) and quantified by Bradford assay, resulting in a specific activity of 200 μmol/g.s using Suc-AAPF-MCA as substrate. The purified bands of both Nep forms were excised from SDS-Page were subjected to PMF analysis. The tryptic peptide panels obtained were confronted between both protein forms and also against the contaminants database, showing complete homology. It is noteworthy that the peptide S.AHPGLSNDEL.R (1295.66 Da), corresponding to the C-terminus domain is exclusive of the NepWT and the more intense R.DHLHDTAVDIGLSDDEQGYGR.V (2313.04 Da) was found in both proteins.

3.2. Effect of salt on NepWT and NepΔCTE hydrolytic activity

The main halophilic characteristic of truncated NepΔCTE was determined by measuring the initial velocity of hydrolysis of Abz-AAPFSSKQ-EDDnp at increasing NaCl concentration and then comparing with NepWT. Since at pH 8.0 no activity was observed in absence of salt for both enzymes, 0.5 M NaCl was used as reference.

When we tested the salt concentration effect (NaCl 0.5–4.0 M) in initial velocity the result was a linear dependency for both enzymes (Fig. 1A). Interestingly, the salt activation observed for NepWT was 2.5 times compared with 8 times of activation in the case of NepΔCTE. One possible explanation for differences in enzyme activation could be shifts in pH curve caused by the deletion. This possibility was discarded by performing pH curve for both enzymes at 3.0 M NaCl, resulting in superimposable curves (Fig. 1B).

In order to explore in more details the salt activation of NepΔCTE compared to NepWT, the effects of Hofmeister series was analyzed in concentrations up to 1.5 M, with Na^+ as the common cation (Fig. 2A and B) and Cl^- as the common anion (Fig. 2C and D). It can be noted that sulfate and citrate were the most effective activators for both enzymes with a remarkable 10-fold activity increase. The enzyme was also activated by cations, being sodium and potassium the most effective, followed by NH^{4+} and Mg^{2+}. Curiously, Mg^{2+} showed activation until 1.5 M followed by an inhibitory effect at higher concentrations for the two enzymes.

3.3. Salt effect on NepΔCTE kinetic parameters

Our previous data indicated a complex process for NepWT activation by salts when substrates with different size of were tested [15]. To further address this effect in truncated NepΔCTE, the salt activation profile was determined using the FRET substrate Abz-AAPFSSKQ-EDDnp containing the same non-prime site sequence of Suc-AAPF-MCA but including the prime site sequence SSKQ-EDDnp instead of MCA. This substrate allows the exploration of primed sites of the substrates [30]. It is noteworthy that both wild type and mutant cleaved these peptides after the Phe residue (Phe$^{\downarrow}$MCA or Phe$^{\downarrow}$Ser).

The initial velocity of hydrolysis of the substrate Suc-AAPF-MCA by NepΔCTE was determined in 1.0–3.0 M NaCl. The activation factor was about 10 times comparing the extreme conditions. The same analysis for Abz-AAPFSSKQ-EDDnp showed an activation factor of only 1.5 times considering the same salt range. Furthermore, as previous described, any activity was observed below 0.5 M NaCl when the Suc-AAPF-MCA substrate was tested. This effect can be interpreted from the differences in the substrate sizes, i.e., the presence of prime side residues in the FRET substrate. In fact, the enzyme-substrate interactions at the prime subsites (i.e. carboxy-terminal to site of hydrolysis) were shown to attenuate the favorable effects of salts on the catalytic activity of the halolysins SR5-3 [30]. The effect of environmental salinity on NepΔCTE catalytic activity was further analyzed by measuring the kinetic parameters (k_{cat}, K_m and k_{cat}/K_m) for the hydrolysis of the peptides Suc-AAPF-MCA and Abz-AAPFSSKQ-EDDnp from 0.5 to 3.0 M NaCl. It is clear from Table 1 that the activation of Abz-EDDnp peptide hydrolysis resulted of K_m decrease as NaCl concentration raised. For the MCA peptide this same effect is a result of increases in k_{cat} and decreases in K_m. This result is comparable to the data obtained for NepWT [15].

3.4. Solvent kinetic isotope effect (SKIE) on NepΔCTE kinetic parameters

The presence of proton bridges in rate-determining transition states of general acid-base catalyzed reactions can be assessed by the study of Solvent Kinetic Isotope Effects (SKIEs) [7,29,31,32]. As noted for NepΔCTE (Table 2), the peptide hydrolysis in deuterium oxide (D_2O) buffer occurred slower than in H_2O, with a significant decrease of k_{cat}/K_m and k_{cat} parameters in buffers containing 1.0 and 3.0 M NaCl, which resulted in SKIE values [H_2O/D_2O] varying from 3.5 to 5.0 for k_{cat} and 4.0 to 8.0 for k_{cat}/K_m. These data support the notion that a general acid-base reaction is the rate-limiting step for NepΔCTE catalytic activity and that it is not affected by salt activation. Similar results are observed for NepWT and other serine-peptidases [28,30].

Table 3
Inactivation rate constants (k_{inat}) and half-life ($t_{1/2}$) of NepΔCTE at 0.5–3.0 M NaCl (37 °C) and at 35–60 °C (3.0 M NaCl).

Salt effect			Temperature effect	
NaCl (M)	k_{inat} (h^{-1})	$t_{1/2}$ (h)	Temp (°C)	k_{inat} (h^{-1})
0.5	−1.073	0.64	35	−0.023
1.0	−0.1	6.93	40	−0.017
1.5	−0.05	13.86	45	−0.039
2.0	−0.029	23.9	50	−0.132
2.5	−0.019	36.48	55	−0.429
3.0	−0.006	115.52	60	−1.579

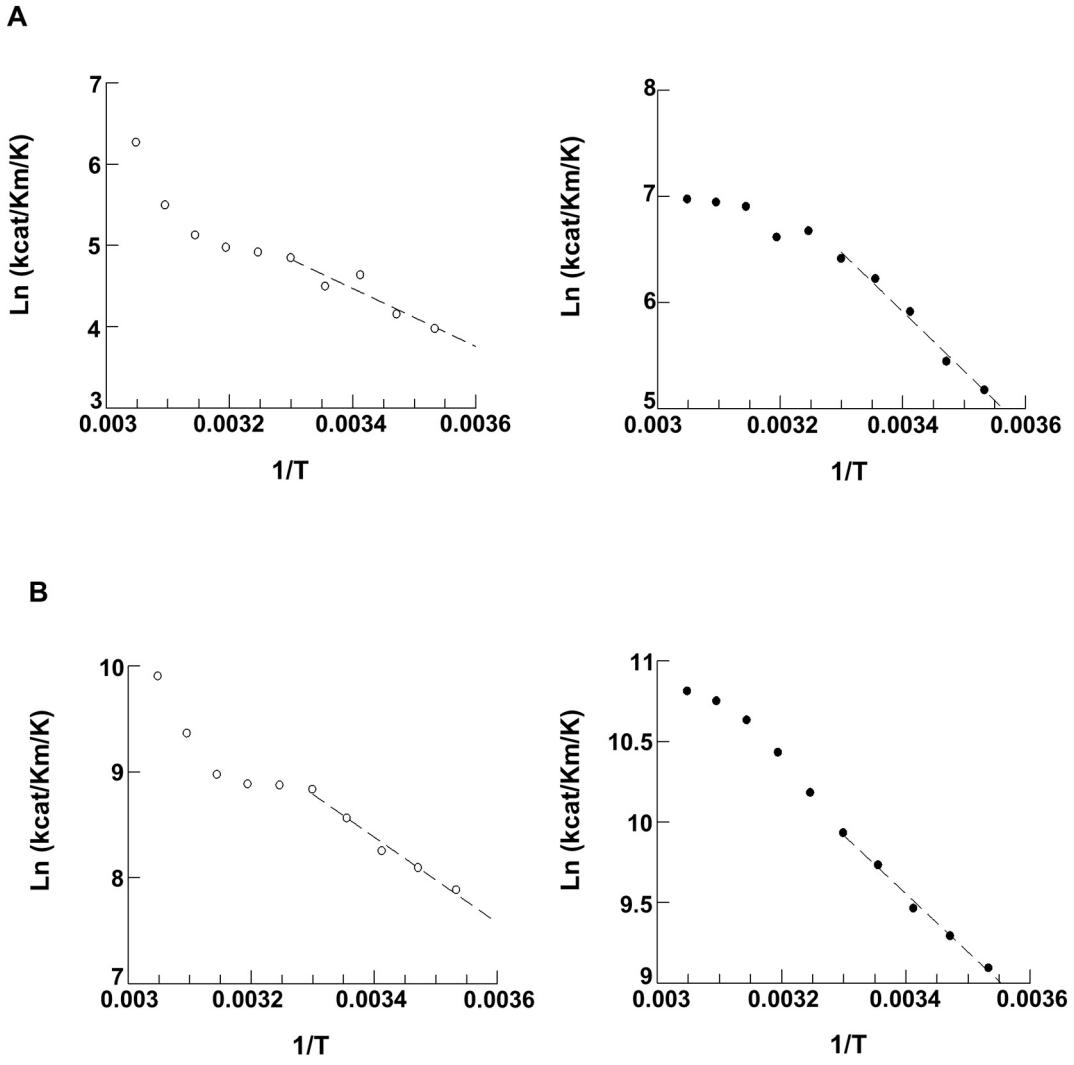

Fig. 3. Eyring plots for the hydrolysis of Abz-AAPFSSKQ-EDDnp by (A) NepWT and (B) NepΔCTE in presence of 0.5 M NaCl (open circle) and 3.0 M NaCl (full circle) over temperature range 10–55 °C. Assays are described in material and methods.

3.5. Salt effect on NepΔCTE stability

The capacity of a protein to resist unfolding in the absence of substrate is a measure of its stability. We evaluated NepΔCTE stability at different salt concentrations (0–3.0 M NaCl) and temperature ranges (35–60 °C), following the enzyme activity at 37 °C (Table 3, Fig. S2). The incubation of NepΔCTE in the absence of salt resulted in complete loss of activity. Further, the inactivation rate constants could not be obtained above 3.0 M NaCl since loss of activity was not observed after 1 month incubation under the same conditions. The inactivation was shown to follow first-order kinetics, where increase in salt concentration decreased inactivation rate constants (k_{inat}). Consequently, this effect increases the half-life from 38 min at 0.5 M NaCl to 5 days at 3.0 M NaCl. The reversibility of the inactivation process by absence of salt showed that the protease lost activity and remained inactive (not recovering activity even in 3.0 M NaCl for 2 days - data not shown), which is compatible with our previous results obtained with NepWT [30].

3.6. Salt effect on temperature dependence of Nep activity

The linear Eyring plots for the k_{cat}/K_m values were obtained in the presence of 0.5 and 3.0 M NaCl over the temperature range of 10–55 °C (Fig. 3). Both wild type Nep and NepΔCTE were stable in this temperature range in the presence of substrate. After 55 °C, however, deviations on linear Eiryng plot led us to use points below this temperature (Fig. 3). These values allowed us to calculate the Gibbs free energy of activation (ΔG_{inat}), that is the association between entropy (ΔS_{inat}) and enthalpy (ΔH_{inat}) [33]. As noted in Table 4, both forms have similar thermodynamic behavior, resulting in high negative ΔG. Our previous results indicate that the NepWT Gibbs free energy does not change from low to high salinity, demonstrating stability and non-protective effect by salt using Suc-AAPF-MCA as substrate [30]. As seen in Table 4, using Abz-AAPFSSKQ-EDDnp as substrate, the changes of ΔS obtained for NepWT from 0.5 M NaCl to 3.0 M may be the result of increase in the typical motion of ES complex, which is not noted in the NepΔCTE form. Considering the entropic term, our

Table 4
Temperature dependence of the specificity constant for hydrolysis of Abz-AAPFSSKQ-EDDnp by NepWT and NepΔCt at 0.5 and 3.0 M NaCl.

	ΔH^* (kJ/mol)	ΔS^* (J/mol/K)	ΔG^* (kJ/mol)
NepWT			
0.5 M NaCl	30.2 ± 1.6	574.8 ± 23.5	−141.1 ± 7.2
3.0 M NaCl	47.4 ± 3.2	644.9 ± 27.7	−144.8 ± 8.8
NepΔCTE			
0.5 M NaCl	33.6 ± 1.7	621.2 ± 21.3	−151.5 ± 6.6
3.0 M NaCl	30.1 ± 2.2	619.9 ± 23.4	−154.6 ± 7.3

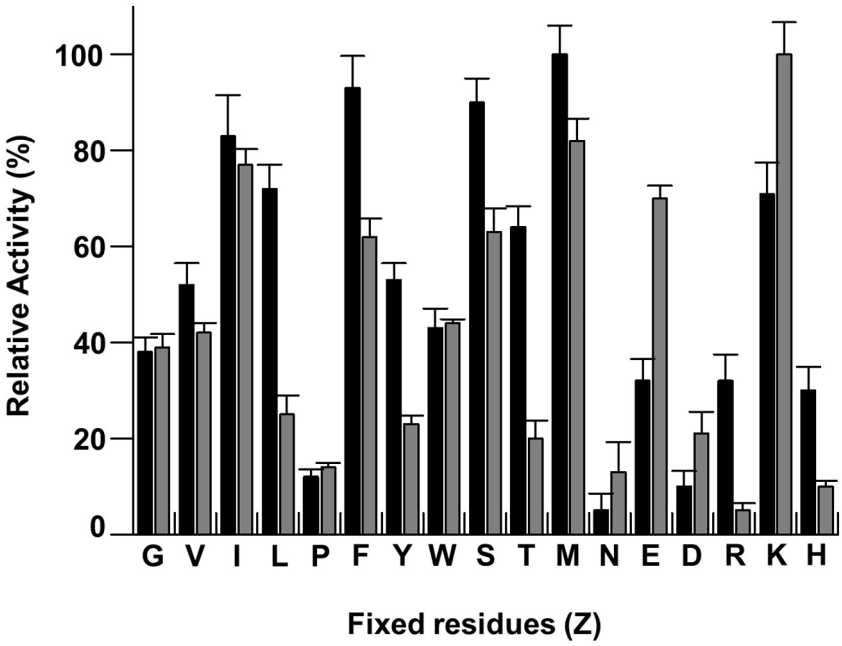

Fig. 4. Contribution of the nominal Z residue in the hydrolysis rate of the FRET combinatory library Abz-GXXZXXQ-EDDnp by NepWT (black squares) and NepΔCTE (grey squares). Assays were carried out in 50 mM Tris-HCl buffer (pH 8.0) plus 3.0 M NaCl at 37 °C by measuring initial velocity. The y axis represents the relative activity. The x axis shows the fixed residue at Z position represented by the one-letter codes. The errors are 10%, each of which represents the average of three determinations.

previous data indicated increase in the system disorder when the NepWT was tested under higher salinities. In the case of the ΔCTE, ΔS changes from 0.5 M to 3.0 M NaCl were not observed.

3.7. Screening of Abz-G-X-X-Z-X-X-Q-EDDnp combinatorial library by Nep

In order to compare the specificity between NepWT and NepΔCTE, we performed the initial velocity in the hydrolysis of synthetic FRET combinatory peptide library based on the Abz-GXX**Z**XX-Q-EDDnp sequence (Fig. 4). This library consists of 19 wells in which the Z position is systematically held constant with each one of the proteinogenic amino acids, and the X positions consist of equimolar mixture of the other amino acids, excluding Z [19]. The contribution of the nominal Z residue in the hydrolysis rate of NepWT and NepΔCTE in the presence of 3.0 M NaCl is shown in Fig. 4. The highest hydrolysis rates were observed with Met, Ile, Phe, Ser and Lys at Z position, while the sub-libraries containing Leu and Thr at Z position were also well hydrolyzed. Though it is not possible to assess the cleavage point of the hydrolyzed sequences in each sub-library, the highest hydrolysis activity with Met and Lys and the low acceptance of the Gly, Pro, Asn and Asp converge to the S_1 preference of serine [34]. Finally, this result clearly indicates that both wild type and ΔCTE forms have comparable behavior in substrate specificity, since the best and worst residues are similar.

4. Discussion

The subtilase group is distributed from archaea and bacteria to higher eukariotes. Among the halolysins subgroup, composed by extreme environment-tolerant proteases, little is known about the biochemical basis of their salt tolerance. The importance of the C-terminus extension in subtilases, even situated far from the catalytic cleft, is related to the characteristic salt tolerance of halolysins. Previous studies showed that the removal of the CTE from halolysin R4 abolished its proteolytic activity in culture supernatants, indicating that the C-terminal has an essential (but yet unknown) function [35]. In the case of SptA, although the deletion of its CTE led to a sharp decrease in measured supernatant activity, the presence of active enzyme indicates that the CTE is not essential for the folding of SptA, not excluding the possibility that the CTE may assist in the correct folding or act in its secretion.

In the case of Nep, our results indicate that the truncated form (NepΔCTE) possess higher hydrolytic activity and activation by higher salt concentration when compared to the wild type, demonstrating that the CTE is not essential. Importantly, for the first time a CTE-truncated halolysin has proved itself more active than the complete enzyme. Further biochemical characterization indicated the same halophilic characteristics for NepΔCTE and NepWT, like the importance of high environmental salinity for catalysis and stability and the same salting-out effect resulting in activation of both forms by kosmotropic salts. The effects of activation by kosmotropic salts of the Hofmeister series are classically explained by their exclusion from the solvation shell of proteins, since their affinity for water is greater than the surface of the protein. Consequently, there is a tendency of conformation changes towards decreasing of surface exposure by mechanism of salting out [36,37].

As well as in SptA, the CTE domain of Nep is not an additional binding site for hydrolysis, since both wild type and ΔCTE show similar substrate specificity. It is also interesting that the environmental salinity did not reflect in thermodynamic changes, as indicated by entropic and free energy Gibbs values. These data indicate that the biochemical characterization of the C-terminal extension in functioning halolysins under different salinities must be better described.

In conclusion, the effects of the CTE portion of the halolysin Nep from *Natrialba magadii* have been demonstrated. The CTE deletion was responsible for increased protease stability and activation by salt, while still maintaining the substrate specificity in some extent. This protease is highly suitable for biotechnological use, since the truncated form is more active and stable than the wild type form.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2018.03.026.

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