Journal of Chromatography B, 889–890 (2012) 61–68

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Purification strategies, characteristics and thermodynamic analysis of a highly thermostable alkaline protease from a salt-tolerant alkaliphilic actinomycete, *Nocardiopsis alba* OK-5

Sangeeta D. Gohel, Satya P. Singh*

Department of Biosciences, Saurashtra University, Rajkot 360005, India

ARTICLE INFO

Article history:
Received 15 June 2011
Accepted 26 January 2012
Available online 3 February 2012

Keywords:
Salt-tolerant alkaliphilic actinomycetes
Thermostability
Alkaline protease
Enzyme purification
Enzyme kinetics
Thermodynamics
Denaturation constant

ABSTRACT

An alkaline protease from salt tolerant alkaliphilic actinomycetes, *Nocardiopsis alba* strain OK-5 was purified to homogeneity by 27 and 13 fold with a yield of 35 and 13% using two-steps and one-step method, respectively. The purification methods involved hydrophobic interaction on phenyl sapharose matrix. The apparent molecular mass was 20 kDa. The temperature optimum shifted from 70 to 80 °C in 4 M NaCl and 30% Na-glutamate, with significant stability at 60–80 °C in Na-glutamate. Deactivation rate constant (K_d) increased and half life ($t_{1/2}$) decreased with the increasing temperatures from 37 to 80 °C. The order of stability was: 30% Na-glutamate > 4 M NaCl > 2 M NaCl > 0 M NaCl. The enzyme was stable even at 80 °C in 30% Na-glutamate with K_d 4.11 and $t_{1/2}$ 168.64 min. The activation energies (E), enthalpy (ΔH^*) and entropy (ΔS^*) for protease deactivation in with Na-glutamate were 31.97 kJ/mole, 29.23 kJ/mole and −211.83 J/mole, respectively. The change in free energy (ΔG^*) for protease deactivation at 60 °C in 30% Na-glutamate was 101.70 kJ/mole. Protease had the highest activity and stability at pH 10–11. While the enzyme was highly resistant against chemical denaturation, it had varied responses to metal ions. Complete inhibition by PMSF confirmed serine nature of the protease. Na-glutamate, H_2O_2, β-mercaptoethanol and different surfactants enhanced the activity.

1. Introduction

Studies on extremophiles led to the search for many enzymes that exhibited much needed features. Besides their biotechnological prospects, the enzymes from extremophiles may also provide unique models to understand the biochemical and molecular basis of the adaptation under extreme conditions. Though enzymes from halophilic archaea and bacteria are fairly well characterized recently [1–4], similar attention has not been focused on haloalkaliphilic actinomycetes [5–9]. While, antibiotics have been the major bioactive compounds from actinomycetes, their ability to produce a variety of enzymes has been explored only in limited sense. The enzymatic spectrum of such organisms, though not systematically explored, appears quite promising as indicated by some preliminary indications. We explored haloalkaliphilic proteases from actinomycetes as these enzymes occupy a pivotal position with respect to their applications and cellular significance.

Property of halophilic proteases severely restricts the choice of purification methods, as they require higher salt for their activity and stability, making most of the conventional procedures unsuitable. The methods used for purification of haloarchaeal proteases include concentration of the enzyme by ethanol precipitation or ultra filtration followed by affinity and gel filtration chromatography. However, many steps make the method cumbersome and adversely affect the yield of the purified enzyme. Therefore, one step purification of the concentrated enzyme by hydrophobic interaction provides a method of choice [10–12].

The activity and stability of enzymes are important parameters to determine the economic feasibility in industrial processes. High stability is generally considered an economic advantage because of reduced enzyme turnover [13]. Before proceeding to develop suitable protease enzyme formulations, accumulating information on the stability of enzymes in different conditions is necessary. Studies on the thermodynamic stability of enzymes have provided fundamental insights into the factors that determine enzyme stability [13,14]. However, for actinomycetes, thermodynamic properties of the purified protease have not been described in the literature. Thus, the present investigation considered different thermodynamic approaches; deactivation kinetics, ΔH^*, ΔS^*, E and ΔG^* to understand the behavior of protease at different temperatures and salts.

Halophilic eubacteria accumulate organic compatible solutes such as sucrose, mannitol, trehalose, glycerol, betaine, proline

* Corresponding author at: Department of Biosciences, Saurashtra University, Rajkot 360005, Gujarat, India. Tel.: +91 281 2586419; fax: +91 281 2586419.
E-mail address: satyapsingh@yahoo.com (S.P. Singh).

1570-0232/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2012.01.031

and glutamate to maintain osmotic balance and functioning of enzymes. Vidyasagar et al. [15] reported the activity of extracellular protease from *Halogeometricum brorinquense* strain TSS101 in presence of compatible solutes; sucrose, mannitol, glycerol and betaine without NaCl. However, no further information is available on the stability of the enzymes in the presence of such solutes. Kinetic studies on the behavior of haloalkaliphilic proteases from actinomycetes in the presence of NaCl and compatible solutes would provide insight on to the unique properties of these enzymes. The stability and activity of proteases under extreme conditions; such as washing pH, high salt, high temperature, presence of surfactants and oxidizing agents would be scientifically and industrially significant. The present investigation was undertaken to establish purification protocol followed by the characteristics and thermodynamic analysis of an extracellular alkaline serine protease produced by salt tolerant alkaliphilic actinomycetes, *Nocardiopsis alba* Strain OK-5.

2. Materials and methods

2.1. Strain, media, cultivation and identification

A Halo tolerant and alkaliphilic actinomycete strain OK-5 was isolated by enrichment culture and standard serial dilution and plating method using salt enriched soil collected from Okha, along the coastal region of Gujarat, India. The media for the screening of protease producing actinomycetes and the cultivation of strain OM-5, contained: 0.5% (w/v) gelatin, 0.5% (w/v) peptone, 0.5% (w/v) yeast extract and 5% (w/v) NaCl. The pH of the medium was adjusted to 9 with 20% (w/v) Na_2CO_3. The growth was carried out aerobically at 37 °C under shake flask conditions at 120 rpm. For 16S rRNA gene sequencing, the genomic DNA of OK-5 was subjected to consensus universal primers designed for 16S rRNA amplification. The PCR product was sequenced by using pair of forward, reverse and internal primers, followed by the sequence alignment and analysis for closest homologous actinomycetes using Mega 3.1 based Neighbor Joining method.

2.2. Protease assay and total protein estimation

Alkaline protease activity was measured by modified Anson–Hagihara's method [16]. The enzyme (0.5 ml) was added to 3.0 ml hammarsten casein (0.6%, w/v, in 20 mM NaOH–Borax buffer, pH 10) and the reaction mixture was incubated at 60 °C for 10 min. The reaction was terminated by the addition of 3.2 ml of TCA mixture (0.11 M trichloro acetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid) and incubated at room temperature for 20 min. The precipitates were removed by filtration through Whatman-1 filter paper and absorbance of the filtrate was measured at 280 nm. One unit was defined as the amount of enzyme liberating 1 μg of tyrosine per minute at 60 °C under the standard assay conditions. Protein concentration was determined by Bradford method [17] using bovine serum albumin as a standard.

2.3. Purification of the protease from OK-5

Hydrophobic interaction chromatography (HIC) is a first column step for proteins that have undergone previous fractionation with ammonium sulfate. The enzyme sample could be stored at low temperature for long period of time in ammonium sulfate. Purification was achieved by a single and two step purification method using HIC on a phenyl sepharose 6 fast flow column (1 cm × 6.5 cm), equilibrated with 0.1 M sodium phosphate buffer (pH 8.0) containing 1 M ammonium sulfate. The crude protease preparation [20 ml crude containing 1 M $(NH_4)_2SO_4$] was loaded directly onto this column for one-step purification, while partially purified protease [1 ml of 80% $(NH_4)_2SO_4$ saturated enzyme] was loaded onto the column in two-step purification. The bound enzyme was eluted by 0.1 M sodium phosphate buffer, pH 8.0 containing a decreasing step gradient of ammonium sulfate from 1000 to 100 mM. Fractions at a flow rate of 0.8 ml min^{-1} were collected by BIO-RAD fraction collector (BIO-RAD, California, USA) and analyzed for protease activity. The final enzyme preparation, from one and two step purification methods were analyzed for the status of purity and molecular weight on sodium dodecyl sulfate polyacrylamide gel electorphoresis (SDS-PAGE) [18].

2.4. Effect of NaCl and Na-glutamate on temperature profile and stability

To determine the effect salt on alkaline protease activity and temperature optima, the reaction mixture (pH 10) was supplemented with 0–4 M NaCl and 30% Na-glutamate followed by the incubation for 10 min at 37–90 °C. The thermal stability of purified enzyme was studied by incubating the enzyme with 20 mM Borex NaOH buffer (pH 10) containing 0–4 M NaCl and 30% Na-glutamate at 37–80 °C for 5 h. The residual activities were measured and expressed as % of the initial activity.

2.5. Estimation of deactivation rate constant

The deactivation rate of alkaline protease was calculated by first order expression:

$$\frac{dE}{dt} = -K_d E \tag{1}$$

So that,

$$\ln\left[\frac{E_t}{E_0}\right] = -K_d t \tag{2}$$

The K_d (deactivation rate constant or first order rate constant) values were calculated from a plot of $\ln[E_t/E_0]$ vs. t at a particular temperature and apparent half lives were estimated using Eq. (3):

$$t_{1/2} = \ln\frac{2}{K_d} \tag{3}$$

2.6. Estimation of thermodynamic parameters for protease deactivation

In order to obtain energies and entropies of protease deactivation, absolute rates of reaction theory were used [19] where the rate of any reaction at a given temperature depends only on the concentration of an energy rich activated complex. Thermodynamic data were calculated by rearranging the Eyring absolute rate equation [20].

The Eyring absolute rate equation is:

$$K_d = \left(\frac{K_b T}{h}\right) \cdot e\left(\frac{\Delta S^*}{R}\right) \cdot e\left(\frac{-\Delta H^*}{RT}\right) \tag{4}$$

where h (Plank constant) = 6.63 × 10–34 J s, R (gas constant) = 8.314 J/K mol, ΔH^* (change in enthalpy), ΔS^* (change in entropy) and K_b (Boltzman constant $[R/N]$) = 1.38 × 10–23 J/K where N (Avogadros no.) = 6.02×10^{-23} mol^{-1}.

To calculate ΔH^* and ΔS^* the Eyring absolute rate equation is rearranged to give:

$$\ln\left[\frac{K_d}{T}\right] = -\left(\frac{\Delta H^*}{R}\right)\left(\frac{1}{T}\right) + \left(\ln\left(\frac{K_b}{h}\right) + \frac{\Delta S^*}{R}\right) \tag{5}$$

ΔH^* and ΔS^* values were calculated from the slope and intercept of a $\ln[K_d/T]$ vs. $1/T$ plot respectively.So that,

$$\Delta H^* = -(\text{slope})R \tag{6}$$

$$\Delta S^* = R\left[\text{intercept} - \ln\left(\frac{K_b}{h}\right)\right] \quad (7)$$

Free energy change (G) for inactivation of protease was calculated by using the following relationship [16]:

$$\Delta G^* = -RT\left[\ln\frac{{K_b}^* T}{{K_b}^* h}\right] \quad (8)$$

Energy of deactivation was estimated using the Arrhenius equation

$$K_d = A\ e\left(-\frac{E}{RT}\right) \quad (9)$$

So that

$$\ln[K_d] = -\frac{E}{RT} + \ln A \quad (10)$$

Energy involved in this process was calculated from the slope of a linear plot of $\ln[K_d]$ vs. $1/T$ [14]. Thermal stability of protease under different salts and pH was determined by incubating the enzyme in the presence of each salt concentrations in sealed tubes at 37–80 °C. Enzyme activity was measured before and after incubation at respective temperature to determine residual activities under each condition. All experiments were conducted in triplicate, results were shown as mean values.

2.7. Substrate specificity and determination of K_m and V_{max}

Protease activity with various protein substrates including BSA, casein, egg albumin and gelatin (0.6%, w/v) was assayed at 60 °C under standard assay condition. The specific protease activity towards casein was taken as a control. K_m and V_{max} were determined by measuring the activity in various concentrations of casein as substrate (0.025–1 g/100 ml) using Lineweaver-Burke plot.

2.8. Effect of pH on protease activity and stability

The effect of pH on alkaline protease activity was determined by assaying the enzyme activity at different pH from 6 to 13, using buffer systems: sodium phosphate (pH 6–8), Tris–HCl (pH 8–9), glycine–NaOH (pH 9–11), borex–NaOH (pH 10–12) and KCl–NaOH (pH 12–13) at 20 mM. Similarly, for enzyme stability, the enzyme was inoculated with respective buffers at 40 °C and activity was measured after 1 h, 6 h and 24 h of incubation. The residual activities were measured by considering the initial activity as 100%.

2.9. Effect of denaturing agents on protease activity and stability

The effect of chemical denaturants such as urea and guanidine hydrochloride on the stability of protease was studied at 0–8 M. The enzyme was incubated with urea at different temperatures for 5 h, followed by the measurement of the residual activities.

2.10. Effect of osmolytes, inhibitors, metal ions, oxidizing agents, reducing agents and surfactants

The reaction mixture was incubated with various osmolytes (5, 10, 20 and 30%): NaCl, KCl, glycerol, mannitol, trehalose, sucrose and Na-glutamate; metal ions (5 mM) Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Sr^{2+}, Ni^{2+}, Ba^{2+}, Hg^{2+}; different inhibitors (1 mM, 5 mM and 10 mM): phenyl methyl sulfonyl fluoride (PMSF), dihiortitol (DTT), ethylene diamine tetraacetic acid (EDTA), thiourea (TU), and *p*-chloromercuribenzoic acid (PCMB); oxidizing and reducing agents (0–50 mM): H_2O_2 and β-mecaptoethanol; surfactants (0.5%): sodimum dodecyl sulfate (SDS), cetyl trimethyl ammonium bromide (CTAB), Tween 80 and Triton X-100; The effect was assessed by considering the control (without respective ingredient) as 100%.

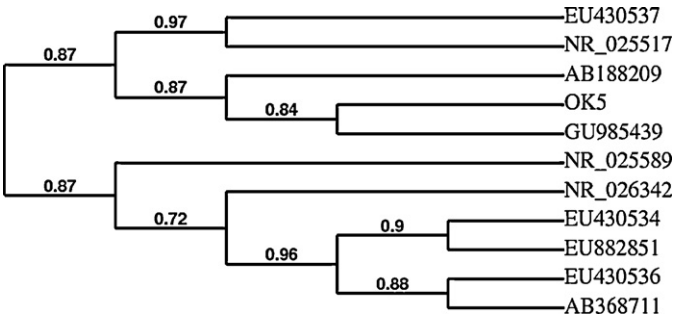

Fig. 1. Phylogenetic tree made in MEGA 3.1 software using Neighbor Joining method.

3. Results and discussion

3.1. Identification of the organism

OK-5, isolated from coastal region of Gujarat (India), was gram positive with filamentous structure. Based on 16S rRNA gene sequence homology and phylogenetic analysis, OK-5 was detected as *N. alba* (GenBank Accession Number: GU985439). The nearest homolog was *Prauseria* sp. (Accession No. AB188209). The 16S rRNA gene sequence of OK-5 has been submitted to the GenBank with the accession number HM560975. Information about other close homologs is displayed in the phylogenetic tree, as majority of the close homologs belonged to the genus *Nocardiopsis* (Fig. 1).

3.2. Enzyme purification

The results of purification of extracellular alkaline protease are summarized in Table 1. The enzyme was purified in active form from the culture filtrate employing hydrophobic interaction chromatography on phenyl sepharose 6FF. Two-step purification method resulted in 27.35 fold purification with a yield of 35% and specific activity at 19014.15 U/mg protein. On the other hand, one-step purification resulted in13 fold purification with a yield of 13% and specific activity at 18589.87 U/mg protein. The results are quite encouraging in the light of the fact that only limited enzymes have been purified form haloalkaliphilic bacteria. Purification of enzymes from halophiles, in general, is quite difficult as salt interferes with chromatographic procedures. For instance, an alkaline protease from *Thermoactinomycetes* with limited purification on DEAE SeparoseCL-6B and Toyopearl 650 column has been reported [11]. SDS PAGE analysis revealed a single band with a molecular mass of 20 kDa (Fig. 2). While the molecular weight was lower than other halophilic alkaline proteases, where it ranged from 40 to 130 kDa [10,12,21,22], the value correspond with some proteases reported in literature [23,24]. The activity staining of the active form of the protease was not successful, as salt interfered with the procedure.

3.3. Effect of NaCl and Na-glutamate on temperature optima and protease activity

The effect of temperature on caseinolytic activity of the purified enzyme was determined in 0–4 M NaCl and 30% Na-glutamate at pH 10. Interestingly, a 10 °C enhancement was observed in temperature optima from 60 to 70 °C in the presence of 4 M NaCl and 30% Na-glutamate, besides maximum activity significantly increased revealing halophilic and thermophilic nature of the extracellular protease from *N. alba* strain OK-5. Evidently, there was NaCl and Na-glutamate dependence for optimal activity at higher temperatures. The temperature optimum of purified protease was 80 °C in 4 M Na-glutamate and 4 M NaCl (Fig. 3), which was higher

Table 1
Purification of OK-5 alkaline protease with salt precipitation and hydrophobic interaction chromatography (using both one and two step purification method).

Purification steps	Total activity (*U*)	Total protein (mg)	Specific activity	Purification fold	Yield (%)
Crude	317 070	456	695.32	1	100
70% NH_4SO_4	217 589.28	37.90	5741.14	8.25	68.62
Two step purification (phenyl sepharose 6FF)	110 852.55	5.83	19014.15	27.34	34.96
Crude	8048.7	9.958	808.26	1	100
One step purification (phenyl sepharose 6FF)	1024.67	0.099	10532.02	13.03	12.95

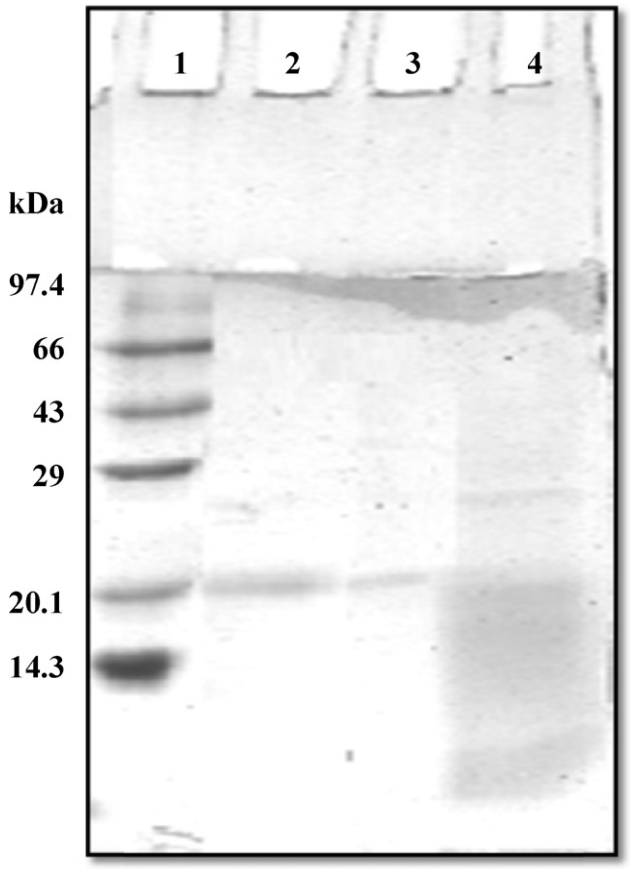

Fig. 2. SDS-PAGE pattern of purified protease using 12% cross linked polyacrylamide gel stained with coomassie brilliant blue. Lane 1: protein marker; lane 2: electophoretic separation of purified protease by two step purification; lane 3: purified protease by one step purification method using hydrophobic interaction chromatography and lane 4: partially purified protease.

than alkaline proteases from *Bacillus subtilis* NCIM 2713 [25] and *Streptomyces clavuligerus* [12]. The optimum temperature for the OK-5 protease was nearly 2 fold higher than those recently reported for haloalkaliphilic bacteria from our laboratory [10,26]. Besides

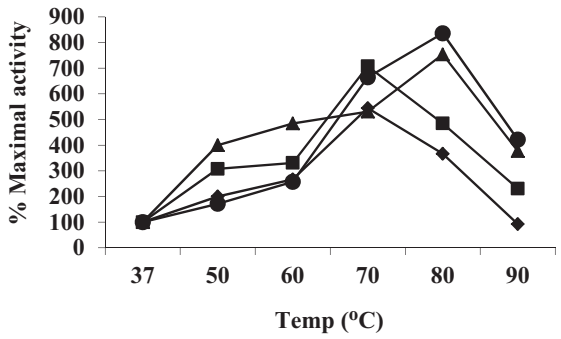

Fig. 3. Effect of 0 M NaCl (♦), 2 M NaCl (■), 4 M NaCl (▲) and 30% Na-glutamate (●) on temperature optima and enzyme activity of purified protease.

the temperature profile, the enzyme had greater stability at higher temperatures, a feature quite desirable for biotechnological applications.

3.4. Thermal stability and deactivation rate constants of protease

Deactivation rates and half-lives of purified protease were calculated at 37–80 °C in the presence of 0–4 M NaCl and 30% Na-glutamate (Fig. 4). The deactivation rate constant (K_d) increased whilst half-life ($t_{1/2}$) decreased with increasing temperatures (Table 2). Highest stability of the enzyme was evident with Na-glutamate followed by 4 and 2 M NaCl. The enzyme was least stable in the absence of NaCl. This was apparent from the K_d and $t_{1/2}$ values at 60 °C in 30% Na-glutamate ($K_d = 0.77 \times 10^{-3}$; $t_{1/2} = 900.19$), 4 M NaCl ($K_d = 1.69 \times 10^{-3}$; $t_{1/2} = 433$), 2 M NaCl ($K_d = 2.37 \times 10^{-3}$; $t_{1/2} = 292.46$) and absence of NaCl ($K_d = 19.15 \times 10^{-3}$; $t_{1/2} = 36.19$). The enzyme was stable even at 80 °C in 30% Na-glutamate with $K_d = 4.11$ and $t_{1/2} = 168.64$ min. The results revealed the role of Na-glutamate in stabilizing the enzyme at higher temperatures. Further, reduction in NaCl from 4 to 2 M led to reduced stability of the enzyme at all temperatures suggesting that a low water activity due to high salt concentrations was necessary for conformational stability. The protease from *N. alba* OK-5 showed highest stability in 30% Na-glutamate followed by 4 M NaCl, while the lowest stability was apparent in the absence and 2 M NaCl. The trends were supported from higher deactivation rate constants (K_d) and lower $t_{1/2}$ values. The effect of salt on the stability of enzyme was related not only to the salt/solute concentration but also to its type. The higher stability in 30% Na-glutamate and 4 M NaCl may be attributed to decrease in unfavorable electrostatic repulsion. Besides, halophilic enzymes have highly negative surface charge with hydrated carboxyl groups shielded by high salt content, which protects against unfolding of the proteins [27,28].

3.5. Thermodynamic parameters for protease deactivation

The thermal denaturation of enzymes is due to the disruption of non-covalent linkages, including hydrophobic interactions, with concomitant increase in the enthalpy of activation [29,30]. The disruptions of enzyme structure leads to increased disorder, randomness or entropy of activation [31]. Thus, investigation of thermodynamic properties of enzyme is necessary to understand its behavior under different physiological conditions (Table 3). The activation energies (E) for protease deactivation calculated in the presence of Na-glutamate and 4, 2 and 0 M NaCl were 31.97, 47.66, 65.34 and 74.08 kJ/mole, respectively. While Change in enthalpy (ΔH^*) and entropy (ΔS^*) for deactivation of protease under same conditions were 29.23, 44.91, 62.59, 71.33, and −211.83, −160.12 J/mole; −152.72, and −69.99 J/mole, respectively. The change in free energy (ΔG^*) for protease deactivation at 60 °C in 30% Na-glutamate and 4, 2 and 0 M NaCl were 101.70, 99.68, 98.59 and 92.81 kJ/mole, respectively (Table 4). Low values of ΔH^* and ΔS^* in Na-glutamate followed by 4 M NaCl revealed a higher thermal stability of protease in the presence of these additives. Moreover, negative values of ΔS^* indicated ordered transition state of protease in the presence of Na-glutamate followed by NaCl.

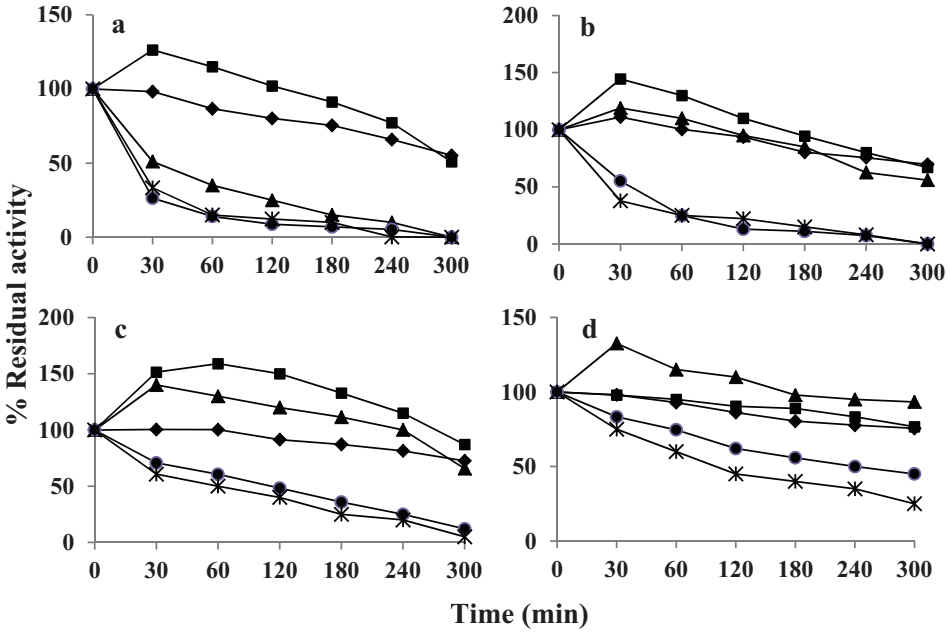

Fig. 4. Thermal stability of purified protease in presence of (a) 0 M NaCl, (b) 2 M NaCl, (c) 4 M NaCl and (d) 30% Na-glutamate at 37 °C (♦), 50 °C (■), 60 °C (▲), 70 °C (●) and 80 °C (∗) temperature.

Table 2
Deactivation rate constant (K_d) and half life ($t_{1/2}$) of purified protease in presence of 0 M NaCl, 2 M NaCl, 4 M NaCl and 30% Na-glutamate at the range of 37–80 °C temperature.

T (°C)	0 M NaCl		2 M NaCl		4 M NaCl		30% Na-glutamate	
	K_d ($\times 10^{-3}$)	$t_{1/2}$ (min)	K_d ($\times 10^{-3}$)	$t_{1/2}$ (min)	K_d ($\times 10^{-3}$)	$t_{1/2}$ (min)	K_d ($\times 10^{-3}$)	$t_{1/2}$ (min)
37	1.55	447.19	1.47	471.52	1.08	641.80	0.99	700.14
50	2.30	301.36	2.01	344.84	1.20	577.62	0.826	839.16
60	19.15	36.19	2.37	292.46	1.69	433.21	0.77	900.19
70	22.4	30.94	18.38	37.71	6.23	111.25	2.50	277.25
80	37.2	18.63	24.31	28.51	8.29	83.61	4.11	168.64

Table 3
Values of ΔH^*, ΔS^* and activation energy for protease deactivation in presence of 0 M NaCl, 2 M NaCl, 4 M NaCl and 30% Na-glutamate at the range of 37–60 °C temperature.

Treatment	ΔH^* (kJ/mole)	ΔS^* (J/mole)	E (kJ/mole)
0 M NaCl	71.33	−69.99	74.08
2 M NaCl	62.59	−152.72	65.34
4 M NaCl	44.91	−160.12	47.66
30% Na-glutamate	29.23	−211.83	31.97

Similar results have been observed for proteases from *Halobacterium* sp. SP(1) [27]; chitinase of *Pantoea dispersa* [14]; ascorbate oxidase of *Cucubita maxima* [32] and amylase of *Bacillus lichiniformis* [33]. The results indicated that high salt/solute concentrations led to a stable conformation of the protease with decreased entropy of unfolding. Further, increase in ΔG^* revealed that the thermal stabilization of protease was due to the higher free energy (functional energy) which enabled the enzyme to resist against unfolding of its transition state [14]. Javed et al. [34] reported thermal stabilization of endoglucanase from *Aspergillus oryzae* cmc-1 due to high free energy. Indeed, the lowest ΔG^* value for the heat labile enzyme corresponds to the largest ΔH^* and ΔS^*, while, conversely, the high ΔG^* corresponds to the low ΔH^* and ΔS^* for heat stable enzyme [35]. Proteases from actinomycetes, however, are not characterized thermodynamically. The substantial stability of the purified protease at higher temperatures in salt, as reflected by thermodynamic analysis, would strengthen the data and add to the novelty of the enzyme.

Table 4
Values of ΔG^* for deactivation of protease in presence of 0 M NaCl, 2 M NaCl, 4 M NaCl and 30% Na-glutamate at the range of 37–80 °C temperature.

T (°C)	ΔG^* (kJ/mole) for deactivation of protease			
	0 M NaCl	2 M NaCl	4 M NaCl	30% Na-glutamate
37	92.69	92.83	93.62	93.85
50	95.63	95.99	97.38	98.38
60	92.81	98.59	99.68	101.70
70	95.23	95.79	98.88	101.48
80	96.60	97.85	101.01	103.07

3.6. Hydrolysis of protein substrate and determination of K_m and V_{max}

The protease had high hydrolytic activity for casein (100%) and poor to moderate hydrolysis of BSA (68%), gelatin (62%) and egg albumin (30%). K_m and V_{max} of extracellular protease for hydrolysis of casein at 60 °C and pH 10, as determined by double reciprocal Lineweaver Burk plot, were 0.50 mg/ml and 3634.12 U/min, respectively. The K_m values indicated the affinity of enzyme towards the substrate. V_{max} is an indication of the catalytic activity of an enzyme which is usually desired to be as high as possible.

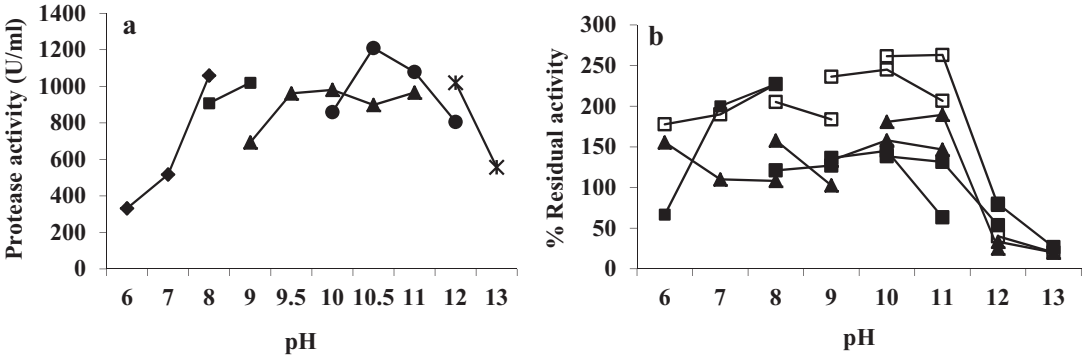

Fig. 5. (a) pH optimum purified alkaline protease and (b) pH stability of purified alkaline protease after 1 h (■), 6 h (▲) and 24 h (□) of incubation. The buffers used were sodium phosphate (pH 6–8), Tris–HCl (pH 8–9), glycine–NaOH (pH 9–11), borex–NaOH (pH 10–12) and KCl–NaOH (pH 12–13).

3.7. Effect of pH on protease activity and stability

The effect of pH on caseinolytic activity of the purified enzyme was determined in the range of pH 6–13 using different buffer systems. Protease exhibited highest activity in the pH range 10–11 (Fig. 5a) with borex–NaOH buffer system. Similarly, maximum stability of enzyme was reported at pH 10–11, reflecting alkaliphilic nature of an extracellular protease from *N. alba* strain OK-5. The pH findings are in accordance with several recent reports showing pH optima of 10–11 [10,12,36,37]. However, OK-5 alkaline protease was stable in broad range of pH 6–12, for 6 h, with significant stability even after prolong incubation (24 h) at pH 10–11 (Fig. 5b).

3.8. Effect of denaturing agents on protease activity and stability

Purified protease was exposed to urea and guanidine hydrochloride (GH) at 2–8 M concentrations (Fig. 6a and b). The enzyme was resistant against 8 M urea and GH and retained nearly 50% of the original activity even after 1 h of incubation at 60 °C. While at 2 M and 4 M urea, the enzyme retained more than 90% of the initial activity. The resistance of OM-6 protease against urea denaturation was in contrast with one of the previous studies from our laboratory, where an alkaline protease from a haloalkaliphilic *Bacillus* sp. was highly sensitive to urea denaturation [26]. The phenomenon of extreme resistance against chemical denaturation appears to be quite rare among the alkaline proteases reported from extremophilic actinomycetes [12]. Urea and guanidine hydrochloride denaturation curves are generally used to obtain an estimate of the conformational stability of proteins by measuring the differences in conformational stabilities between the native (folded) and the denatured (unfolded) states. With respect to the interaction of urea with polar solutes, solvation enhancement is attributed to more favorable hydrogen bond formation between the peptide amide units and urea than with water [38] because urea itself is a soluble amide. It has also been suggested that urea induces an "outside-in" denaturation process of electrostatic character by adhering on the surface of charged residues, leading to a repulsion between residues. The result of the repulsion is an opening to water into the protein interior that will provoke the unfolding. This explains the need of high urea concentration to achieve denaturation [39,40]. Herrera et al. [41] suggested that urea acts indirectly in the denaturation process, decreasing water mobility around the peptide, increasing the ability to form peptide water hydrogen bonds with longer lifetimes. There may be a direct interaction where urea molecules get access to the polar groups of the peptide, thus providing a better peptide solvation than pure water.

3.9. Effect of osmolytes, inhibitors, metal ions, oxidizing and reducing agents and surfactants

The effect of different concentrations of various osmolytes: NaCl, KCl, glycerol, mannitol, trehalsoe, sucrose and Na-glutamate on the activity of purified enzyme in the absence of NaCl was examined (Fig. 7a). The enzyme had varying levels of activities at different concentrations of osmolytes. Among the osmolytes, Na-glutamate (30%) enhanced the activity by 132.43% whereas; sucrose (20%), trehalose (20%) and glycerol (5%) enhanced the activity by 5%, 77% and 20%, respectively. The activity in the presence of Na-glutamate increased with increasing concentrations indicating that osmotic pressure or reduced water activity was important. The

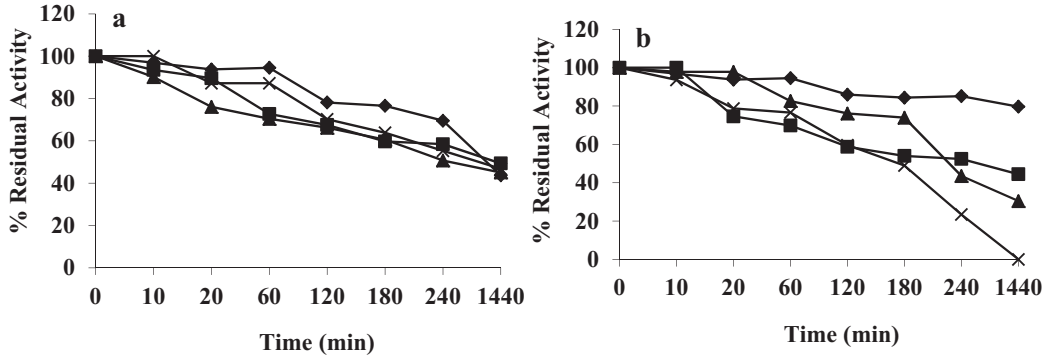

Fig. 6. Denaturation of alkaline protease in presence of urea (a) and guanidine hydrochloride (b) at 0 M (♦), 2 M (■), 4 M (▲) and 8 M (×) concentration up to 5 h of incubation.

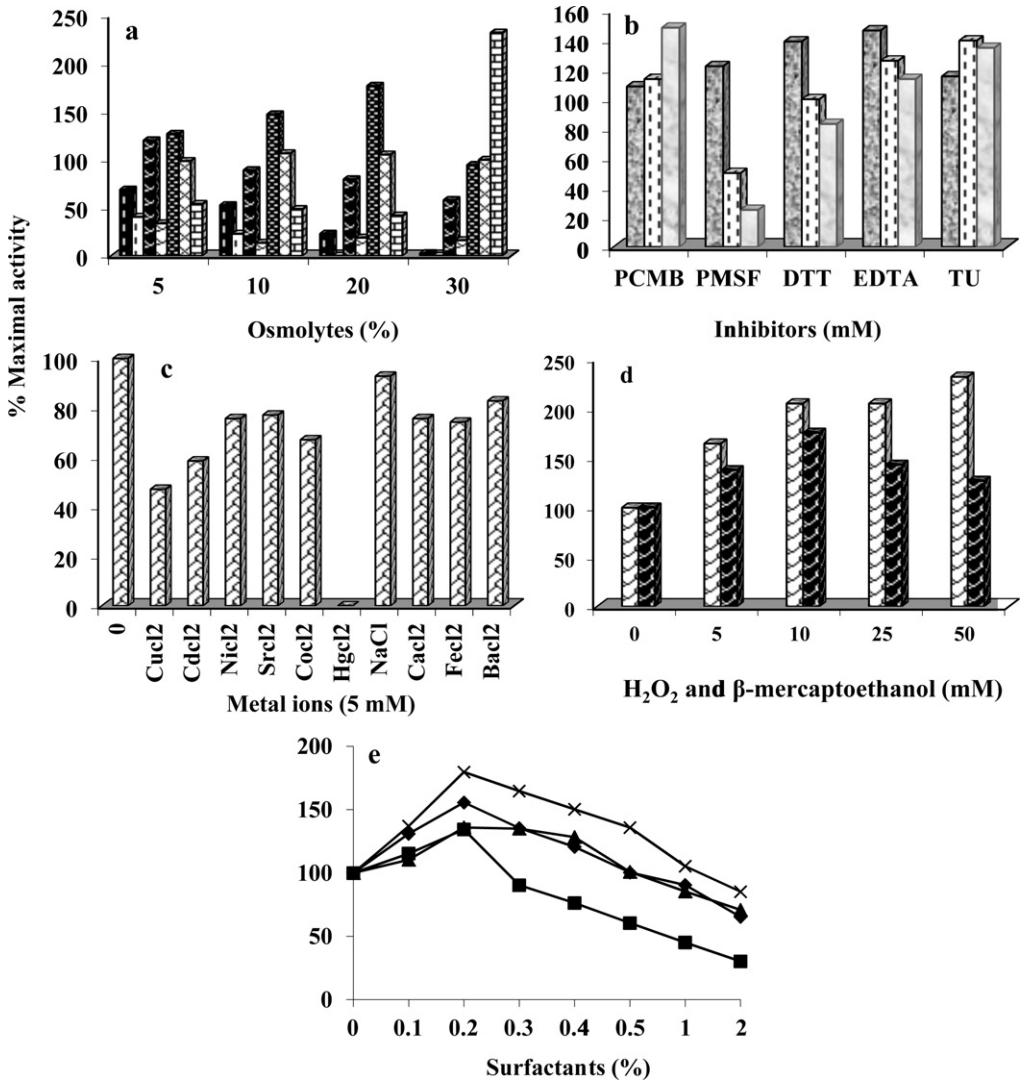

Fig. 7. Effect of (a) osmolytes: NaCl ( ), KCl ( ), glycine ( ), mannitol ( ), trehalose ( ), sucrose ( ) and Na-glutamate ( ); (b) inhibiotors: at 1 mM ( ), 5 mM ( ), and 10 mM ( ) concentration; (c) metal ions: at 5 mM ( ) concentration; (d) Oxidizing and reducing agents H_2O_2 ( ), β-mercaptoehanol ( ) from 0–50 mM concentration and (e) surfactants: SDS (♦), CTAB (■), Tween 80 (▲), Triton X-100 (×) up to 2% concentration.

activity reduced to 25% in 10 mM PMSF, a serine protease inhibitor. On the other hand, no significant effect on enzyme activity was observed by EDTA and DTT indicating the serine nature of the enzyme (Fig. 7b). The results corresponded with the alkaline serine proteases from halophilic and haloalkaliphilic *Bacillus* sp. as well as alkaliphilic *Streptomyces clvuligens* strain Mit-1 [11,26]. The results are also in agreement with an alkaline protease from alkaliphilic *Thermoactinomycetes* sp. HS682 and *S. clvuligens* [12]. None of the metal ions had significant effect on enzyme activity except Hg^{2+}, Cu^{2+} and Cd^{2+} where major loss in activity was evident (Fig. 7c). Hg^{2+} led to the total activity loss of protease activity, a finding which was in the line of a report on two novel halotolerant extracellular proteases form *B. subtilis* strain FP-133 [37]. The effect of H_2O_2 and β-mercaptoethanol was studied on purified protease at 0–50 mM concentrations. The % maximal activity increased to 232 and 175%, even in 50 mM H_2O_2 and 10 mM β-mercaptoethanol, respectively (Fig. 7d). The results indicated that H-bond and disulfide bonds are not directly involved with protein activity or stability [12]. Joo and Chang [37] reported the existence of oxidant stable alkaline protease in a halo-tolerant *Bacillus clausii* l-52. The enzyme had high % maximal activity in different surfactants; SDS (155%), CTAB (134%), Tween 80 (136%) and Triton X-100 (180%) at 0.2% (Fig. 7e). Haddar et al. [23] reported surfactant stable alkaline serine-protease from a newly isolated *Bacillus mojavensis* A21. The high activity in presence of oxidizing agent, reducing agent as well as different surfactants would be attractive features for its applications in detergent and other areas.

4. Conclusion

It was significant to focus on protease purification from rarely explored group of halo tolerant alkaliphilic actinomycetes. The work aimed at the biochemical, thermodynamic and kinetic properties of extracellular protease. Detailed characterization of protease from actinomycetes has not been conducted till date and to the best of our knowledge this would be the first report on the thermodynamic and kinetic parameters of protease from salt tolerant alkaliphilic actinomycetes. Besides, the enzyme had quite high level of activity with significant stability at higher salt, temperature, pH and a range of metal ions. The enzyme also displayed extreme resistance against urea denaturation, oxidizing and reducing agents as well as surfactants. Hence, the findings would provide a base to design the strategies for rational improvements of extremozymes from actinomycets.

Acknowledgments

Sangeeta Gohel greatly acknowledges UGC, New Delhi, India for providing meritorious fellowship. The work was sponsored by Saurahtra University, Rajkot, India.

References

[1] M. Purohit, S.P. Singh, Int. J. Biol. Macromol. 49 (2011) 103.
[2] M.S. Dodia, H.G. Bhimani, C.M. Rawal, R.H. Joshi, S.P. Singh, Bioresour. Technol. 99 (2008) 6223.
[3] B. Kikani, S.P. Singh, Int. J. Biol. Macromol. 48 (2011) 676.
[4] R.K. Patel, M.S. Dodia, S.P. Singh, Process Biochem. 40 (2005) 3569.
[5] J.T. Thumar, S.P. Singh, J. Ind. Microbiol. Biotechnol. 36 (2009) 211.
[6] J.T. Thumar, S.P. Singh, Braz. J. Microbiol. 38 (2007) 1.
[7] W.J. Li, Y.Q. Zhang, P. Schumann, H.H. Chen, W.N. Hozzein, X.P. Tian, L.H. XU, C.L. Jiang, Int. J. Syst. Evol. Microbiol. 56 (4) (2006) 733.
[8] V.J. Mehta, J.T. Thumar, S.P. Singh, Bioresour. Technol. 97 (14) (2006) 1650.
[9] W.J. Li, Y.G. Zhang, Y.Q. Zhang, S.K. Tang, P. Xu, L.H. Xu, C.L. Jiang, Int. J. Syst. Evol. Microbiol. 55 (2005) 1329.
[10] M.S. Dodia, C.M. Rawal, H.G. Bhimani, R.H. Joshi, S.K. Khare, S.P. Singh, J. Ind. Microbiol. Bioechnol. 35 (2008) 121.
[11] A. Gupta, I. Roy, R.K. Patel, S.P. Singh, S.K. Khare, M.N. Gupta, J. Chromatogr. A 1075 (2005) 103.
[12] J.T. Thumar, S.P. Singh, J. Chromatogr. B 854 (2007) 198.
[13] C. Vieille, J. Zeikus, Microbiol. Mol. Biol. Rev. 65 (2001) 1.
[14] V. Gohel, D.C. Naseby, Biochem. Eng. J. 35 (2007) 150.
[15] M. Vidyasagar, S. Prakash, C. Litchfield, K. Sreeramulu, Archaea 2 (2006) 51.
[16] B. Hagihara, The Enzymes, vol. 4, Academic Press, New York, 1958, p. 224.
[17] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[18] U.K. Laemmli, Nature 227 (1970) 680.
[19] H. Eyring, E. Stearn, Chem. Rev. 24 (1939) 253.
[20] S. Cottaz, B. Brasme, H. Driguez, Eur. J. Biochem. 267 (2000) 5593.
[21] T. Miyaji, Y. Otta, T. Nakagawa, T. Watanabe, Y. Niimura, N. Tomizuka, Lett. Appl. Microbiol. 42 (2006) 242.
[22] L. Lama, I. Romano, V. Calandrelli, B. Nicolaus, A. Gambacorta, Res. Microbiol. 156 (2005) 478.
[23] A. Haddar, A. Bougatef, R. Agrebi, A. Sellami-Kamoun, M. Nasri, Process Biochem. 44 (1) (2009) 29.
[24] A. Haddar, R. Agrebi, A. Bougatef, N. Hmidet, A. Sellami-Kamoun, M. Nasri, Bioresour. Technol. 100 (13) (2009) 3366.
[25] Q. Huang, Y. Peng, X. Li, H. Wang, Y. Zhang, Curr. Microbiol. 46 (2003) 169.
[26] R.K. Patel, M.S. Dodia, R.H. Joshi, S.P. Singh, Process Biochem. 41 (9) (2006) 2002.
[27] A. Alolkar, A. Desai, Maharaja Sayajirao University of Baroda, Ph.D. Thesis, 2009, p. 195.
[28] W.A. Joo, C.W. Kim, J. Chromatogr. B 815 (2005) 237.
[29] R.M. Daniel, Enzyme Microb. Technol. 19 (1996) 74.
[30] H. Ohnishi, H. Sakai, Y. Nosoh, T. Sekiguchi, Trends Biotechnol. 19 (1996) 16.
[31] C. Vieille, J.G. Zeikus, Trends Biotechnol. 14 (1996) 183.
[32] S. Porto, S. Porto, H. Cavalcanti, L. Filho, P. Perego, F. Porto, A. Converti, A. Pessoa, Biotechnol. Prog. 22 (2006) 1637.
[33] N. Declerck, M. Machius, P. Joyet, G. Wiegand, R. Huber, C. Gaillardin, Protein Eng. 16 (2003) 287.
[34] R. Javed, H. Rashid, H. Nadeem, M. Riaz, R. Perveen, Appl. Biochem. Biotechnol. 157 (2009) 483.
[35] S. D'Amico, J.C. Marx, C. Gerday, G. Feller, J. Biol. Chem. 278 (2003) 7891.
[36] E. Setyorini, S. Takenaka, S. Murakami, K. Aoki, Biosci. Biotechnol. Biochem. 70 (2) (2006) 433.
[37] H.S. Joo, C.S. Chang, J. Appl. Microbiol. 98 (2) (2005) 491.
[38] D. Tobi, R. Elber, D. Thirumalai, Biopolymers 68 (2003) 359.
[39] A. Wallqvist, D.G. Covell, D. Thirumalai, J. Am. Chem. Soc. 120 (1998) 427.
[40] R.D. Mountain, D. Thirumalai, J. Am. Chem. Soc. 125 (2003) 1950.
[41] A.C. Herrera, K. Nordstrand, K.D. Berndt, L. Nilsson, Biophys. J. 89 (2005) 842.