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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

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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).

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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,

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