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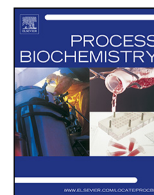

Biochemical and structural characterization of a detergent-stable serine alkaline protease from seawater haloalkaliphilic bacteria

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ABSTRACT

An extracellular protease from a newly isolated seawater haloalkaliphilic bacterium, haloalkaliphilic bacteria Ve_2-20-9_1 [HM047794], was purified and characterized. The enzyme is a monomer with a 37.2 kDa estimated molecular weight. It catalyzed reactions in the pH range 8–11 and performed optimally at pH 10. While maximal activity occurred at 50 °C, the temperature profile shifted from 50 to 80 °C in 1–3 M NaCl. The enzyme's thermal stability was probed using circular dichroism (CD) spectroscopy with NaCl at 50 and 70 °C. The changes in the enzyme's secondary structure were also analyzed using Fourier transform infrared spectroscopy (FTIR). The N-terminal amino acid sequence GKDGPPGLCGFFGCI exhibited low homology with other bacterial proteases, which highlights the enzyme's novelty. The enzyme was labile in anionic surfactant (1% w/v SDS) but showed stability in non-ionic surfactants (Tween 20, Tween 80 and Triton X-100 all 1% v/v), commercial detergents, and oxidizing and reducing agents. The enzyme's excellent stability in commercial detergents highlights its potential as a detergent additive.

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1. Introduction

Over the past few years, the use of enzymes in industrial processes has significantly increased. The global markets for industrial enzymes were close to $3.3 billion in 2010 and are expected to reach $4.4 billion by 2015 (http://bccresearch.blogspot.in/2011/01/global-market-for-industrial-enzymes-to.html). In particular, global financial crises can push optimistic calculations in the opposite direction or, at best, sideways. Due to the looming climate catastrophe, the search for green industrial processes is more urgent and popular, and the search for robust enzymes is a key feature of this urgent effort (http://www.unido.org/en/what-we-do/environment/resource-efficient-and-low-carbon-industrial-production/greenindustry/green-industry-initiative.html).

Proteases are a particularly important group of enzymes that account for 60% of the total worldwide sales of industrial enzymes [1]. Alkaline proteases lead the markets primarily as detergent additives. Microbial alkaline proteases are useful in many industrial sectors, such as leather and feather processing; textile and silk gumming; the production of foods, pharmaceuticals and detergents; and waste water treatment [2]. Because the first alkaline protease, Carlsberg, from *Bacillus licheniformis* was commercialized as a detergent additive in the 1960s [3], multiple *Bacillus*-derived alkaline proteases have been purified and characterized to identify those with significant activity; stability; broad pH, temperature and substrate specificity; low fermentation times and simple downstream processing [4].

With their efficiency of use and varied biotechnological implications, the alkaline proteases with high specificity and stability toward pH, salt, temperature, organic solvents, metal ions and surfactants are in high demand. Screening microorganisms from unexplored natural and manmade environments will significantly facilitate the search for these enzymes. Exploring these habitats will provide access to novel bacteria and their robust enzymes that can act under multiple extreme conditions [5–7]. As part of our ongoing studies on haloalkaliphilic bacteria from the unexplored saline habitats, we have isolated many haloalkaliphilic bacteria and evaluated their enzymatic potential [8–10]. In this report, a haloalkaliphilic bacterium that produces a novel alkaline protease is described. The enzymatic characteristics of catalysis and stability under various extreme conditions are described. Changes in the enzyme's structure in the presence of denaturants were characterized through monitoring of enzyme activity and by CD spectroscopy. The changes in bond angles as well as stretching and bending of various bonds in the purified enzyme were observed using FTIR spectroscopy.

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2. Materials and methods

2.1. The microorganism

A moderately haloalkaliphilic bacterium was isolated from seawater near the Veraval coast lighthouse (latitude 22°53′ N, longitude 70°20′ E) in Gujarat (India). The bacterium was isolated through enrichment culture techniques in complex medium broth (CMB) with 10 g/l glucose, 5 g/l peptone, 5 g/l yeast extract, 5 g/l KH_2PO_4, 30 g/l bacteriological agar, and varying NaCl concentrations (10–20%, w/v) at pH 8–10, which was adjusted by adding individually autoclaved Na_2CO_3 (20%, w/v). The individual colonies were purified through additional streaking on the CMB agar plates. The clear zones around the colonies on gelatin agar plates (30 g/l gelatin, 10 g/l peptone, 10 g/l NaCl and 30 g/l bacteriological agar, pH 8–10) observed after 48–72 h of incubation by adding Frazier's reagent (g/l: $HgCl_2$ 150, HCl 200 ml) indicate extracellular protease production.

The Ve_2-20-9_1 strain was identified through 16S rDNA sequencing. The internally transcribed 16S rDNA (1484 bp) spacer region was amplified using forward (5′-AGA GTT TGA TCC TGG CTC AG-3′) and reverse (5′-GGT TAC CTT GTT ACG ACT T-3′) oligonucleotide primers. DNA amplification was performed using a Master cycler gradient (Eppendorf AG, Hamburg Germany) under the following conditions: initial denaturation at 94 °C for 1 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 2 min and a final extension at 72 °C for 10 min. The PCR-amplified products were sequenced through a custom service (Bangalore Genei, Merck Life Sciences, India). The phylogenetic study was performed using the BLAST tool from NCBI and sequences from the RDP database to identify the nearest homolog.

2.2. Culture, growth and enzyme assay conditions

Protease production was performed in a liquid medium with 30 g/l gelatin, 10 g/l peptone, and 100 g/l NaCl at pH 10. The inocula were routinely grown in CMB medium and transferred into a gelatin medium, which was autoclaved at 120 °C for 20 min. The inoculated medium was incubated at 37 °C with shaking at 180 rpm in 500 ml Erlenmeyer flasks. The cells were harvested at 24 h intervals by centrifugation at 10,528 × *g* for 10 min. The protease activity for the cell-free extracts was estimated. The optimum protease production was observed with the inoculum size 5% v/v at pH 10 and 37 °C after incubating for 89 h. The alkaline protease activity was measured using the Anson-Hagihara method [11]. The enzyme units were determined using tyrosine (0–100 μg) as the standard. The unit activity was defined as the enzyme quantity required to liberate 1 μg of tyrosine/ml of the substrate/minute under the assay conditions using 0.6% w/v casein as the substrate, as described earlier [8–10].

2.3. Enzyme purification

2.3.1. Ammonium sulfate precipitation

The crude protease was fractionated through ammonium sulfate precipitation in two steps: 0–30% and 30–70% w/v saturation. The proteins precipitated through constant stirring at 4 °C overnight. The precipitate was collected by centrifugation at 15,000 rpm for 10 min. The pellets were suspended in a minimum volume of 20 mM NaOH–borax buffer (pH 10); the protease activity was then estimated.

2.3.2. Ultrafiltration and hydrophobic interaction chromatography

The partially purified enzyme was ultrafiltered using an Amicon Ultra 15 centrifugal filter with a 30 kDa MWCO (Millipore, USA). The protease activity was observed in the retentate fraction. The retentate fraction was further purified through hydrophobic interaction chromatography (HIC) on a Phenyl Sepharose 6 Fast Flow column 1 × 30 cm using a Du-flow Protein purification system (Bio-Rad, USA) equilibrated with sodium phosphate buffer (20 mM, pH 8.0) containing 1 M ammonium sulfate. The enzyme sample supplemented with ammonium sulfate (final concentration, 1 M) was loaded onto the pre-equilibrated column. The column was washed with equilibrating buffer until the wash was free of protein. The protein bound to the column eventually eluted with 0 M ammonium sulfate using a decreasing ammonium sulfate gradient (1.0–0 M) in the sodium phosphate buffer (20 mM, pH 8.0) [8]. Two milliliter fractions were collected at the flow rate 0.5 ml/min using a Bio-Frac fraction collector (Bio-Rad, USA) and analyzed for protease activity.

2.3.3. Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Native-PAGE analyses were performed for the purified protease to determine its homogeneity and molecular weight. The samples were individually prepared for SDS- and native-PAGE using Laemmli buffer [12] with and without, respectively, SDS, β-mercaptoethanol, and dithiothreitol on a 15% w/v Tris–glycine stacking and separating gel. While the Native-PAGE samples were not heat treated, the SDS-PAGE samples were incubated at 100 °C for 3 min. The protease molecular weight was estimated using the molecular weight marker #SM1811 (Fermentas, USA). The protein bands were visualized using Coomassie brilliant blue R-250 staining.

2.3.4. Determination of the protease N-terminal amino acid sequence

The lyophilized enzyme (100 μg) was reconstituted in glycine–NaOH buffer-pH 10 and transferred onto a PVDF membrane for protein sequencing using Edman degradation. The protein was sequenced using an ABI Procise 492 protein sequencer (Applied Biosystems, USA) per the standard operating procedures. The amino acid residues were detected as individual signals.

2.3.5. The protein concentration

The protein concentration was determined using the Bradford method with bovine serum albumin as the standard [13]. The protein concentration was also monitored during the elution at 280 nm.

2.4. Enzyme characterization

2.4.1. K_m, V_{max} and K_{cat} studies using the purified protease

The pure enzyme K_m and V_{max} values were determined by measuring the activity with various casein substrate concentrations (0.1–1 g/100 ml). The kinetic constants were calculated using the Lineweaver–Burk plot. The enzyme catalytic constant or turnover number was calculated by dividing the V_{max} value (i.e., the maximal reaction velocity) by $E(t)$ (i.e., the enzyme quantity in moles present in reaction mixture).

$$K_{cat} = \frac{V_{max}}{E(t)}$$

2.4.2. The effect of pH and temperature

The protease activity was monitored over the pH range 8–12 to determine the pH profile. For pH stability, the enzyme was incubated in various buffers at pH 8–12. Enzyme aliquots were withdrawn at regular intervals, and the residual activities were calculated. The enzyme activity at the optimum pH was considered 100%. The buffer systems for pH stability were 20 mM sodium phosphate buffer: pH 7–8, Tris–HCl buffer: pH 8–9, borax–NaOH buffer: pH 10–11, and glycine–NaOH: pH 9–12.

The effect of temperature on protease activity was assessed at 37–80 °C. The thermal stability was determined by incubating the enzyme at temperatures between 37 and 80 °C. The aliquots were periodically withdrawn at 15 min intervals for 2 h, and the residual activities were calculated under the standard assay conditions by taking the activity at the optimum temperature as 100%. The protease assays were performed in triplicate.

2.4.3. The effect of salt on the temperature profile and thermal stability

The effect of NaCl on the temperature profile of the protease was examined by incubating the assay mixture with different NaCl concentrations in the range 0–3 M at 37–90 °C. The residual activities were determined as described above. To determine the influence of NaCl on thermal stability, the enzyme was incubated at 50–70 °C for 2 h with NaCl (1–3 M). Aliquots were withdrawn at definite time intervals, and the protease activity was determined. The residual activities were calculated after the enzyme assay was performed in triplicate.

2.4.4. The effect of cations, inhibitors and additives

We examined the effects of monovalent (Na^+ and K^+) and divalent cations (Ca^{+2}, Mn^{+2}, Mg^{+2}, and Zn^{+2}) at 10 mM; the inhibitors (10 mM) phenylmethylsulfonyl fluoride (PMSF), p-chloromercuribenzoate (PCMB), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), and thiourea; the oxidizing agent hydrogen peroxide (10 mM); and the reducing agent β-mercaptoethanol (10 mM). The enzyme preparations with the additives/denaturants were pre-incubated at 37 °C for 30 min for the stability studies. The residual activities were measured at 50 °C and pH 10 in triplicate. The activities were referenced to the activity for samples without additives (100%).

2.4.5. The effect of surfactants and detergents

To determine the effect of surfactants and detergents on the enzyme activity, the reaction mixture was supplemented with various detergents at 1% w/v. For the enzyme stability assays, the enzyme was incubated with the anionic and non-ionic detergents SDS, Tween-20, Tween-80 and Triton X-100 as well as the commercial detergents Wheel Active, Aerial, Rin Supreme, Surf Excel, Nirma Super and Tide (all at 1% v/v). Concentrations of all liquid detergents and surfactants used were 1%v/v, while that of powder detergents was 1% w/v. The enzymes already present in the commercial detergents were denatured at 70 °C for 1 h. Aliquots were withdrawn at regular intervals over a period of 24 h, and the residual activities were calculated at 50 °C, pH 10.

2.5. Circular dichroism (CD) spectroscopy for the Ve_2-20-9_1 protease

Circular dichroism (CD) spectra were recorded using a JASCO J-810 CD spectropolarimeter (JASCO Corporation, Tokyo, Japan) and cuvettes with the path lengths 1, 0.1, or 0.05 cm, depending on the protein concentration and wavelength region. For each spectrum, 5–10 scans were collected and averaged co-added, the buffers and corresponding salt concentrations were subtracted, and the spectra were smoothed using a mild smoothing function. Far-UV CD spectra were collected over

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