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Purification and biochemical characterization of halophilic, alkalithermophilic protease AbCP from *Alkalibacillus* sp. NM-Fa4

Noha M. Mesbah [a,*], Juergen Wiegel [b]

[a] Department of Biochemistry, Faculty of Pharmacy, Suez Canal University, Ismailia 41522, Egypt
[b] Department of Microbiology, University of Georgia, Athens, GA 30602, USA

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ABSTRACT

An extracellular alkaline, halo- and thermostable protease (AbCP) produced by a novel *Alkalibacillus* sp. NM-Fa4, isolated from the alkaline, hypersaline lakes of the Wadi An Natrun, was purified to homogeneity by precipitation with ethanol and anion-exchange chromatography. The molecular weight of the purified protease was 19.7 kDa. AbCP retains proteolytic activity over broad sodium chloride, pH and temperature ranges, with maximal activity at 1 M NaCl, $pH^{45\,°C}$ 9.5 and 50–52 °C. AbCP was resistant to phenylmethylsulfonyl fluoride (2 mM) and ethylene diamine tetra-acetic acid (2 mM), stimulated by the reducing agents dithiothreitol (2 mM) and β-mercaptoethanol (1% v/v) and inhibited with iodoacetic acid (5 mM), suggesting that AbCP is a cysteine protease. AbCP showed stability toward anionic surfactants (sodium dodecyl sulfate), oxidizing agents (H_2O_2), chemical denaturants (urea), and retained most of its activity in the presence of 1% v/v of the non-ionic surfactant Tween 80. The protease was stable in 50% mixtures of ethanol and, to a lesser extent, methanol, and was stimulated by Mg^{2+}, Ca^{2+}, and Fe^{2+}. AbCP shows a broad substrate specificity and hydrolyzes both natural and synthetic substrates. Based on the Lineweaver–Burk plot, the K_m with casein as substrate was 1.3 ± 0.007 mg/mL and V_{max} was 1111 mg/ml/min. The stability of the enzyme under the combined extreme conditions of high salt concentration, alkaline pH and high temperature, in addition to being resistant to chemical denaturants, oxidizing agents, surfactants as well as exhibiting broad substrate specificity makes this enzyme a promising candidate for a variety of biotechnological applications.

1. Introduction

There has been a significant increase in the use of enzymes as industrial catalysts. The global market for enzymes was estimated at $3.3 billion in 2010, and is expected to reach $4.4 billion by 2015, with a compound annual growth rate of 6% over a 5-year period. Proteases constitute the largest product segment in the industrial enzymes market. Proteases (EC 3.4.21-24) are a large group of hydrolytic enzymes that cleave peptide bonds and degrade proteins into smaller peptides and amino acids. Proteases constitute a big share of the enzyme market primarily as detergent additives, and also in the food, organic synthesis and pharmaceutical industries (http://www.reportlinker.com/p0363451-summary/Enzymes-in-Industrial-Applications-Global-Markets.html). Proteases also have clinical and medical applications such as reduction of tissue inflammation and treatment of burns [1,2]. Proteases from different origins have also been used to produce bioactive peptides [3,4].

Extremophilic microorganisms from extreme environments have been an important source of a variety of stable enzymes [5–9]. Many studies have reported on the activity of halophilic proteases, alkaliphilic proteases and thermostable proteases [10–19]. Most halophilic proteases belong to the serine protease family, and are dependent on high salt concentration for structural stability. They display maximal activity at salt concentrations greater than 1.4 M, neutral to slightly basic pH and temperatures between 30 and 45 °C. Alkaline proteases are characterized by high activity at alkaline pH, normally greater than 9.0, and also broad substrate specificity. The optimal temperature for activity is around 60 °C [20]. However, their activity is greatly reduced in the presence of salt concentrations greater than 0.5 M, thereby limiting their applicability in processes necessitating low water activity.

Abbreviations: β-ME, β-mercaptoethanol; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

* Corresponding author. Tel.: +20 1227907957; fax: +20 643230741.
E-mail addresses: noha_mesbah@pharm.suez.edu.eg, noha.mesbah@gmail.com (N.M. Mesbah), juergenwiegel@gmail.com (J. Wiegel).

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Reports on enzymes that have catalytic activity under more than one extreme, such as haloalkaliphilic proteases or alkalithermophilic proteases, are scarce [21]. It is assumed that such polyextremophilic enzymes will have wider versatility and adaptability to multiple harsh conditions commonly employed in different industries, such as low water activity, high temperature, alkaline pH, and the presence of detergents, surfactants and organic solvents. The activity and stability of enzymes are important parameters to determine the economic feasibility in industrial processes. High stability is critical from the economic point of view due to reduced enzyme turnover [11]. Therefore, prior to proposing any novel enzyme preparation, it is necessary to determine the enzyme stability and activity under different and concomitant extreme conditions. Since proteases are the most sought after enzymes by different industries, obtaining pure protease preparations that are both stable and active under multiple extreme conditions such as high salt concentrations, alkaline pH, elevated temperature in addition to the presence of surfactants and oxidizing agents is scientifically and industrially significant.

The goal of the present study was to develop a purification protocol as well as the elucidation of biochemical properties of an extracellular protease, AbCP, from the novel, halophilic, alkalithermophilic *Alkalibacillus* sp. isolated from the alkaline, hypersaline lakes of the Wadi An Natrun, Egypt. AbCP was characterized under different conditions of salt concentration, pH, temperature, surfactants, detergents and oxidizing agents.

2. Experimental

2.1. Organisms

2.1.1. Isolation and partial characterization

Protease producing strains were isolated from mixed water-sediment samples collected from three of the largest lakes of the Wadi An Natrun in North-western Egypt. Samples were collected from Lake Fazda (30°19′43.5′ N, 30°24′29.68′ E), Lake UmRisha (30°20′48.70′ N, 30°23′08.14′ E), and Lake Hamra (30°23′48.28′ N, 30°19′13.39′) during November of 2010.

For isolation of protease producing microorganisms, mixed water-sediment (5 g wet weight) was inoculated into enrichment medium containing, in g L^{-1}: yeast extract, 5; peptone, 5; KH_2PO_4, 1; $MnCl_2$, .018; $FeCl_2$, 0.025; $MgCl_2$, 0.5, KCl, 1; NaCl, 100; Na_2CO_3, 20. The pH of the medium was 9.0 (at 45 °C). Enrichment cultures were incubated at 50 °C for 4 days and then transferred into the same medium three successive times. Pure isolates were obtained by repetitive dilution to extinction in enrichment medium containing 1% (wt./vol.) agar. Pure isolates were maintained on enrichment medium containing 1% (wt./vol.) agar. Pure isolates were screened for extracellular protease production by the protease assay using the Folin–Ciocalteau reagent. The isolate showing the maximum activity was designated strain NM-Fa4, and was used for further studies. Strain NM-Fa4 was isolated from Lake Fazda.

Preliminary taxonomic characterization was based on cultural characteristics and 16S rRNA gene sequencing.

2.1.2. Genomic DNA extraction and purification

Isolate NM-Fa4 was grown in 100 mL of enrichment medium for 4 days at 50 °C. Cells were harvested by centrifugation and washed three times in Tris·EDTA buffer (pH 8.0). Genomic DNA was extracted by the CTAB/NaCl described by Wilson [22].

2.1.3. 16S rRNA gene amplification, sequencing and phylogenetic analysis

The 16S rRNA gene was amplified by PCR using universal primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (50-GGT TAC CTT GTT ACG ACT T-3′), and DreamTaq™ Green DNA polymerase (Fermentas). Sequencing of the PCR products was accomplished using a 3730xl capillary DNA analyzer (Applied Biosystems) operated at the Georgia Genomics Facility (University of Georgia, Athens, GA USA). The complete 16S rRNA gene sequence for strain NM-Fa4 was compared with GenBank entries by BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The complete gene sequence was aligned with closely related sequences using the ClustalX program (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/). Phylogenetic trees were constructed using the PHYLIP software package (http://evolution.genetics.washington.edu/phylip.html). Distances were calculated by using the Jukes–Cantor algorithm of DNADIST, and branching order was determined via the neighbor-joining algorithm of NEIGHBOR. Each tree was a consensus of 100 replicate trees. The 16S rRNA gene sequence was submitted to GenBank under accession number KF537622.

2.2. Protease assay

Protease activity was measured by the method described by Anson [23] using casein as a substrate. Purified enzyme (4–5 μg) was added to assay mixture consisting of 50 mM Tris·Cl, pH 9.0 and 1.7 M NaCl. The reaction (200 μL total volume) was started with the addition of casein (0.2% wt./vol., final concentration). Reaction mixtures were incubated at 50 °C for 20 min. The reaction was stopped by addition of 340 μL of 110 mM trichloroacetic acid solution and centrifuged at 10,000 rpm for 5 min. To 400 μL of supernatant, 800 μL of 500 mM Na_2CO_3 and 200 μL of 0.5 N Folin–Ciocalteu reagent were added and mixed thoroughly. Color developed after incubation at room temperature for 10 min was measured at 660 nm. All assays were done in triplicate. A negative control (no protein added) was run for all assays to correct for non-specific degradation of the substrate. One unit of protease activity was defined as the amount of enzyme yielding 1 μmol of tyrosine per minute at pH 9.0, 50 °C in the presence of 1.7 M NaCl. Specific activity of protein is expressed as the units of enzyme activity per milligram protein.

2.3. Protein determination

Protein concentration of samples was determined by the method of Bradford [24]. Bovine serum albumin was used as a standard. Protein concentration during different steps of purification was determined by absorbance at 254 nm.

2.4. Enzyme purification

2.4.1. Enzyme production and precipitation

Strain NM-Fa4 was grown in enrichment medium for 4 days at 50 °C with constant shaking (225 rpm) in an Innova™ 4000 incubator shaker (New Brunswick, NJ). Cells were separated by centrifugation at 4000 rpm for 20 min. The cell-free supernatant was mixed with 0.8 volume of ice-cold ethanol and incubated at −20 °C overnight. The precipitate was collected by centrifugation. The enzyme was recovered by re-suspending the precipitate in a minimal volume of 50 mM Tris·Cl buffer at pH 9.0. The protein was dialyzed against the same buffer overnight to remove residual ethanol and salts.

2.4.2. Anion exchange chromatography

The dialyzed protein was subjected to anion exchange chromatography on a Q-sepharose column (10 cm × 1.6 cm, GE Healthcare) which had been equilibrated with 50 mM Tris·Cl, buffer, pH 9.0. Protein was fractionated by gradient elution using 50 mM Tris·Cl buffer, pH 9.0, containing an increasing concentration of NaCl (0–2 M). Fractions were collected at a flow rate