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# Purification and characterization of an extracellular haloalkaline protease produced by the moderately halophilic bacterium, *Salinivibrio* sp. strain AF-2004

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**Abstract**

Among several strains of moderately halophilic bacteria that were isolated from various areas in Iran, strain AF-2004 was selected as the best producer of extracellular protease and was used for further studies. Phenotypic classification and 16S rRNA sequence analysis placed AF-2004 in the genus *Salinivibrio*. Maximal protease production was detected at the end of exponential growth phase in the medium containing 5% (w/v) NaCl. This protease was purified to homogeneity by a combination of acetone precipitation, Q-Sepharose ion exchange and Sephacryl S-200 gel filtration chromatography. The enzyme was a monomeric protease with a relative molecular mass of 38–43 kDa by SDS-PAGE and gel filtration chromatography. The specific activity of the purified enzyme was determined to be 171.6 μmol of tyrosine/min per mg of protein using casein as a substrate. The enzyme exhibited its optimal activity at 65 °C, pH 8.5, and 0–0.5 M NaCl with a high tolerance to salt concentrations of up to 4 M. The protease was identified as a zinc-metalloprotease, which was strongly inhibited by EDTA and 1,10-phenanthroline, and the N-terminal amino acid sequence determined showed high similarity to the zinc-metalloproteases from *Vibrio* species.


*Keywords:* Moderately halophilic bacteria; *Salinivibrio*; Haloalkaline protease; Zinc-metalloprotease

## 1. Introduction

Extracellular proteases are important for the hydrolysis of external proteins and enable the cell to absorb and utilize hydrolytic products [1]. At the same time, these proteases that can be purified easily have been commercially exploited to assist protein degradation in various industrial processes [2]. Despite the fact that many different proteases have been identified and some of them have been used in biotechnological and industrial applications, the present proteases are not sufficient to meet most of the industrial demands. Industrial processes are carried out under specific physical and chemical conditions, which cannot always be adjusted to the optimal values required for the activity and stability of the available enzymes. Therefore, it would be of great importance to have available enzymes showing optimal activities at different values of pH, salt concentrations and temperature. In view of these restrictions, attention to isolation and characterization of proteases from extremophiles is very important. Moderately halophilic bacteria are extremophilic microorganisms that grow optimally in media containing 3–15% NaCl [3] and can be a likely source of such enzymes. They constitute a heterogeneous group of microorganisms, which consist of a great variety of bacteria including haloalkaliphilic species. These organisms require not only high salt but also alkaline pH for growth and enzyme secretion, and their extracellular enzymes must be active under such extreme conditions. While there have been numerous reports on the purification and characterization of extracellular proteases from non-halophilic bacteria, very limited information has been published on proteases from halophilic species. From

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the extreme halophilic group, a few extracellular proteases have been isolated and characterized [4–7], and two extracellular proteases from the moderately halophilic bacteria have been characterized so far [8,9]. Lama et al. have purified and characterized a metalloprotease from the *Salinivibrio* sp. strain 18 AG that was strongly inhibited by phenylmethyl sulfonylfluoride (PMSF) and required 2% NaCl for maximal activity [9]. In this paper, we report the purification and biochemical characterization of a haloalkaline zinc-metalloprotease produced by strain AF-2004, a new moderately halophilic bacterium, phylogenetically related to *Salinivibrio* species. This protease has some different properties from the protease from strain 18 AG that make it very interesting for application in biotechnological processes.

## 2. Materials and methods

### 2.1. Materials

Peptone and beef extract were purchased from DIFCO Laboratories (Detroit, MI, USA). Casein for protease assay, Triton X-100, various inorganic salts, glucose, maltose, sucrose and lactose were from Merck (Darmstadt, Germany). For protease purification, Q-Sepharose (HP) anion exchange resin and Sephacryl S-200 were purchased from LKB Pharmacia Biotechnology (Upssala, Sweden). The specific protease inhibitors (antipain, bestatin, chymostatin, E-64, leupeptin, pepstatin, phosphoramidon, Pefabloc SC, EDTA–$Na_2$, and aprotinin) were obtained from Roche Diagnostics (Mannheim, Germany). Tryptic soy broth (TSB) was purchased from Hi-media (India). All other reagents were from Sigma (St. Louis, Mo, USA). All materials were analytical reagent grade.

### 2.2. Bacterial strain and culture conditions

Strain AF-2004 was isolated from the Bakhtegan lake in the south of Iran (a hypersaline lake with 17% (w/v) total salt) and cultivated aerobically at 32 °C in the basal medium containing (g/l): peptone, 10; beef extract, 10; NaCl, 50; and pH 8.0. For the observation of protease production, bacteria were inoculated on skim milk agar plates containing 10% (w/v) skim milk, 2% agar and 5% NaCl. Plates were incubated at 32 °C for 48 h. Clear zones of skim milk hydrolysis around the colonies gave an indication of proteolytic activity. To determine the effects of culture conditions on protease production, the microorganism was grown in the basal medium supplemented with glucose, maltose, sucrose, lactose (30 mM) and casein (0.5%). Tryptic soy broth (TSB), Luria-Bertani broth (LB) and nutrient broth (NB) were also used to check growth and protease production in these media. Bacterial growth was followed by measuring the absorbance at 620 nm (Shimadzu model UV 160 A).

### 2.3. Purification of the protease

For protease purification, the basal medium (100 ml in 500 ml Erlenmeyer flask) was inoculated with 1% of a stationary-phase culture and incubated at 32 °C with shaking at 220 rpm for 40 h. Supernatant obtained by centrifugation (8000 × *g* for 20 min at 4 °C) of strain AF-2004 culture broth was dialysed against 20 mM Tris–HCl, pH 8.5 containing 50 mM NaCl and 0.5 mM $CaCl_2$ (buffer A) and used as the crude enzyme solution. All the purification steps were performed at 4 °C. At the first step, pre-chilled acetone was added slowly to the solution up to 50% saturation and after discarding the precipitate, further acetone was added to the solution up to 80% saturation with gentle stirring and left for 1 h. The precipitate formed was collected by centrifugation at 12,000 × *g* for 20 min, dissolved in a minimum amount of buffer A and dialysed against the same buffer for 24 h with two buffer changes. The dialysed enzyme preparation was applied to a Q-Sepharose HP column (1.6 cm × 20 cm) equilibrated with buffer A. The column was washed with the same buffer and bound proteins were eluted by applying a linear gradient of 0.05–0.75 M NaCl in the same buffer at a flow rate of 1 ml/min and monitored at 280 nm. Fractions exhibiting protease activity were pooled and concentrated by freeze-drying. Samples were dissolved in a minimal volume of 20 mM Tris–HCl, pH 8.5 containing 0.3 M NaCl and 5 mM $CaCl_2$ (buffer B), and dialysed against the same buffer for 24 h with two buffer changes. The enzyme preparation was finally loaded on a Sephacryl S-200 gel filtration column (1.6 cm × 65 cm), equilibrated with buffer B. Fractions of 3 ml were collected at a flow rate of 0.33 ml/min. The active fractions were pooled and concentrated by ultrafiltration (Centricon, Amicon, USA) and used as the purified enzyme for further analysis. The molecular mass of the purified enzyme was determined using the same column calibrated previously with a range of reference proteins: BSA (67,000 Da), bovine carbonic anhydrase (29,000 Da) and cytochrome C (12,400 Da). Blue Dextran was used to determine the void volume of the column.

### 2.4. Protease assay

The proteolytic activity was assayed by the modified method of Kunitz [10] using casein as the substrate. The reaction was carried out in a tube containing 480 μl of 1% (w/v) casein in 20 mM Tris–HCl buffer (pH 8.5), 50 mM NaCl, 0.5 mM $CaCl_2$, and 20 μl of suitably diluted enzyme at 55 °C for 5 min. The reaction was terminated by adding 500 μl of 10% (w/v) trichloroacetic acid (TCA), kept at room temperature for 15 min and then centrifuged at 14,000 × *g* (Eppendorf 5415 C, Germany) for 8 min. The absorbance was measured against a blank at 280 nm. In some cases (effect of inhibitors), to 500 μl of the TCA precipitated supernatant, 1.5 ml of $Na_2CO_3$ solution and 0.5 ml of 1 N Folin-Ciocalteu reagent were added and mixed thoroughly. The colour developed after 20 min of incubation at 37 °C was measured at 660 nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μmol of tyrosine in 1 min at 55 °C. The specific activity is expressed in units of enzyme activity/mg of protein.

### 2.5. Protein determination

Protein concentration was measured by the method of Bradford [11] using bovine serum albumin (BSA) as the standard. During chromatographic purification steps, protein concentration was estimated by observing the absorbance at 280 nm.

### 2.6. Polyacrylamide gel electrophoresis and zymograms

Native PAGE was performed by the method of Davis [12] in a 7.5% (w/v) polyacrylamide gel with Tris/glycine buffer, pH 8.3. SDS-PAGE was carried out for determination of molecular mass of the protease in a 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate at 4 °C and 10 mA/gel, according to the method of Laemmli [13] after heating the samples at 75 °C for 4 min. A ready to use molecular marker (Fermentase; SM#0661) was used as a standard. Following native and SDS-PAGE, the proteins were stained with Coomassie brilliant blue R-250 (0.2%). Gelatin zymography for proteolytic activity was performed in polyacrylamide slab gels containing SDS and gelatin (0.1%) as a co-polymerized substrate, as described by Heussen and Dowdle [14] with some modifications. After electrophoresis, the gels were rinsed in 2.5% Triton X-100 for 1 h at 25 °C to remove SDS and were incubated under optimal assay conditions (in buffer A and 55 °C) for 10 min to detect the proteolytic activity. The gels were stained in a solution of 0.5% (w/v) amido black 10B. The activity band was observed as a clear colourless area depleted of gelatin in the gel against the blue background. The demonstration of a protease activity band was also done after electrophoresis in native PAGE gel which was placed in 1% BSA in buffer A for 1.5 h at 55 °C and then stained with amido black 10B. A clear band of BSA hydrolysis was observed in the gel.

### 2.7. Protease characterization

#### 2.7.1. Influence of temperature, pH and NaCl

The optimum temperature for enzyme activity was determined by conducting the assay at various temperatures from 25 to 80 °C. The thermostability of the enzyme was measured after pre-incubation of the enzyme in buffer B for 15 and 30 min at various temperatures. The effect of pH on protease activity was determined by incubating the reaction mixture at pH values ranging from 5.0