

Isolation, Purification and Characterization of a Thermophilic Alkaline Protease from *Bacillus subtilis* BP-36

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Abstract

The goal of this research was to isolate and identify the thermostable alkaline protease producing bacteria among several native Iranian microorganisms. At the end of screening program, a *Bacillus subtilis* BP-36 strain producing thermophilic alkaline protease was isolated from a hot spring in Ardebil province. The target enzyme was purified using a one-step Aqueous two-phase systems (ATPS) protocol involving 22% (w/w) polyethylene glycol (PEG)-10,000, and 18% (w/w) citrate with a yield of 39.7%, specific activity of 2600 U/mg and purification factor of 4.8. It was shown to have a molecular weight of 40 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The purified thermophile enzyme was stable in alkaline pH range (9.0-11.0) with the optimum pH of 9.0. It was highly stable at 60 °C and retained 100% activity even after 90 minutes, suggesting that it belong to the family of thermophilous. Collectively, our obtained data revealed that the thermophilic protease produced by *B. subtilis* BP-36 has the potential application in industrial processes under high temperature.

Keywords: Alkaline protease; *Bacillus subtilis*; Isolation; Purification; Thermophilic

Introduction

Proteases constitute one of the most important groups of commercial enzymes accounting for approximately 60-65% of the global enzyme market. These enzymes have diverse applications in a variety of industrial processes involving detergents, brewing, photographic, medical diagnostics, food, pharmaceutical, silk, and leather processing [1, 2]. Proteases can be produced from a wide range of

organisms such as bacteria, yeasts, molds, plants and animals. However, bacterial proteases represent an excellent source of protease enzymes in comparison with others [3, 4]. Among this family of enzymes, thermophilic proteases are of particular interest for bioengineering and biotechnological applications, because of their higher resistance to chemical denaturants and organic solvents. They are also ideal molecular models for elucidating the mechanisms of chemicophysical stability of proteins [5]. Owing to their

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significance, it will be desirable to search new potential thermophilic proteases with novel properties for current and future biotechnological applications. Up to now, many thermophilic proteases have been reported from different organisms such as *Geobacillus* sp.YMTC1049 [6] and *Bacillus lecheniformis* NH1 [7] and so on. The goal of this study was to isolate and screen of new strains producing thermophilic proteases from native Iranian microorganisms [8]. Briefly, in this communication, we describe the purification and partial characterization of a thermostable alkaline protease produced by a thermophilic *B. subtilis*.

Materials and Methods

Materials

For protease purification, tri-sodium citrate and polyethylene glycol (PEG) were purchased from Merck (Darmstadt, Germany). Casein was purchased from Sigma-Aldrich (St. Louis, Mo., USA). The all other chemicals and microbiological media were of laboratory grade and deionized water was used throughout the experiments.

Isolation and Identification of Thermophilic Alkaline Protease Producing Bacteria

For isolation of thermophilic protease producing organisms, 15 soil samples were collected from different hot springs in Ardabil province including Sar-Ein and Khalkhal. 1.0 g of each soil sample was suspended in 10 ml sterilized distilled water and 100 μ l of resulting suspension was transferred to 5 ml of the screening medium which contained nutrient broth with 1% casein. In each case, the culture was incubated at 60 °C and 140 rpm for 72 h. The culture fluid was spread on screening medium agar plate and the protease producers were detected by plating on gelatin agar, which contained 0.5% gelatin, 0.3% peptone and 1% NaCl. The strains showing clear zones were identified as a protease producer. Strain identification was performed based on the morphological and biochemical characteristics [9, 10].

Culture and Growth Conditions for Protease Production

For protease production, the isolated bacterium was grown in a medium (pH=8.0) consisting of 0.5 % (w/w) glucose, 0.75% (w/w) peptone, 0.5 % (w/w) MgSO₄, 0.5% (w/w) KH₂PO₄ and 0.01% (w/w) FeSO₄ in orbital shaking incubator at 60 °C and 140 rpm for 72 h. After

incubation, the culture broth was centrifuged at 4 °C and 3600 rpm for 30 min. The cell precipitate was discarded to obtain the supernatant, which was used as crude protease preparation for further purification [11].

Assay of Protease Activity

Enzyme activity was measured according to the method described by Kunitz *et al.* [12]. The sample contained enzyme solution and 0.5% (w/v) casein in 0.1 M potassium phosphate buffer (pH=8.0) and was incubated at 60 °C for 10 min. This reaction was terminated by the addition of 5% (w/v) trichloroacetic acid (TCA) solution and then centrifuged to remove the resulting precipitate. Protease activity was estimated using a tyrosine standard curve. One unit of alkaline protease activity (U) was taken as the amount of enzyme liberating 1 μ g of tyrosine/min under the assay conditions.

Protein Determination

The concentration of total protein was determined by the method of Lowry *et al.* [13] using bovine serum albumin (BSA) as a standard. A blank without protein was used as the reference and no interference of phase components was observed. Protein concentration during the purification process was estimated by measuring the absorbance at 660 nm.

Purification of Thermophilic Alkaline Protease

Purification of alkaline protease was done using an aqueous two-phase systems (ATPS) technique. ATPS was prepared by adding 2.2 ml of PEG, 1.8 ml tri-sodium citrate dehydrate, 2 ml enzyme crude extract and 4 ml deionized water to make up the final mass of 10 ml. Systems were gently shaken for 1 h and then centrifuged at 3600 rpm for 20 min at 25 °C to complete phase separation. Subsequently, the two-phases became clear and transparent, and the interface was well defined. Samples of the top and bottom phases were carefully extracted and analyzed for enzyme activity and total protein [14].

Enzyme Characterization

Determination of Molecular Weight by Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the purified samples. SDS-PAGE was carried out in the gel made of 12% separating and 5% stacking gels. Protein

solutions were mixed with the treatment buffer and boiled for 3 min. After electrophoresis, the gels were stained with Coomassie brilliant Blue R-250 staining method and destained with 30% methanol and 10% acetic acid [15].

Table 1. Comparison of alkaline protease activity in isolates

Number	Alkaline protease activity (U/ml)
BP-1	2.4
BP-2	4.0
BP-3	4.8
BP-4	4.5
BP-5	4.0
BP-6	4.6
BP-7	5.1
BP-8	6.4
BP-9	6.1
BP-10	5.0
BP-11	3.4
BP-12	4.7
BP-13	5.1
BP-14	3.6
BP-15	4.5
BP-16	3.8
BP-17	4.2
BP-18	4.3
BP-19	4.2
BP-20	4.1
BP-21	3.6
BP-22	3.6
BP-23	5.3
BP-24	6.9
BP-25	6.2
BP-26	5.6
BP-27	3.4
BP-28	2.4
BP-29	2.1
BP-30	3.2
BP-31	4.7
BP-32	5.7
BP-33	6.1
BP-34	6.3
BP-35	3.5
BP-36	7.0
BP-37	4.7
BP-38	5.9
BP-39	4.9
BP-40	4.3

Determination of N-Terminal Amino Acids

The N-terminal amino acid sequence of the enzyme was analyzed with an automated Edman degradation protein sequencer. The phenylthiohydantoin derivatives (PTH-Xaa) were separated and identified using the protein sequencer ppsq-10 (Shimadzu).

Influence of Temperature on Enzyme Stability and Activity

The thermal stability of the protease was tested by incubating of 1 ml enzyme (2.7 U/ml) in 0.1 M potassium phosphate buffer (pH=8.0) at various temperatures ranging from 40 to 90 °C for 1 h [16]. To analyze the temperature effect on protease activity, the proteolytic activity was measured at different temperatures using casein as the substrate for 15 min at pH 9.0. Residual activity was determined under standard assay conditions and compared with the respective controls. All experiments were done triplicate and repeated at least twice.

Table 2. Taxonomic characteristics of isolated bacterial strain

Characteristics	Findings
Shape	Rod
Colony morphology	Large, flat, yellow colored and 0.7-0.8 mm in diameter
Growth at 70oC	+
Gram-stain	+
Methyl red test (MR)	-
Voges-poskauer test (VP)	+
Catalase test	+
Oxidase test	-
Hydrolysis of:	
Casein	+
Starch	+
Gelatin	+
Urea	-
Production of:	
Indole	-
H ₂ S	-
Fermentation of:	
Fructose	A
Lactose	-
Maltose	A
Manitole	A
Sucrose	A

Keys: +, Positive result or growth - , Negative result or no growth A, Acid production

Table 3. Purification steps of thermophilic alkaline protease

Steps	Total protein concentration (g/L/m)	Activity (U/L)	Specific activity (U/mg)	Yield (Y%)	Purification factor (PF)
Crude extract	13	7008	539.07	100	1
ATPS	1.07	2782	2600	39.7	4.8

Influence of pH on Enzyme Stability and Activity

To evaluate the pH stability, 1 ml purified enzyme (2.7 U/ml) was incubated at 4 °C for 10 min in the following buffer systems: 0.1 M sodium acetate (pH 3.0-5.0), 0.1 M potassium phosphate (pH 6.0-8.0), 0.1 M Tris-HCl (pH 8.0-9.0), 0.1 M glycine-NaOH (pH 9.0-11.0) and 0.1 M sodium carbonate (pH 11.0-12.0), respectively [17]. The protease activity was measured under the standard assay conditions after incubation for 24 h. The influence of pH on protease activity was also studied by assaying the enzymatic activity in the above-mentioned buffer. All experiments were done triplicate and repeated at least twice.

Results and Discussion

Screening and Identification of Thermophilic Protease Producing Strains

Isolation and screening of microorganisms from naturally occurring habitats is expected to provide enzymes with novel properties. Proteases are one of the most interesting industrial enzymes produced by a wide range of microorganisms such as bacteria, yeasts and molds. Among bacteria, *Bacillus* strains are specific producers of alkaline proteases [4]. The present work is an attempt to isolate and characterize a thermostable alkaline protease from native Iranian microorganisms. To achieve this goal, several bacterial strains isolated from Iranian hot springs soils were screened for alkaline protease activity. From a large collection, isolates exhibiting a large zone of hydrolysis on skim milk agar plates were selected for further experimental studies. Finally, the isolate BP-36 was selected as the most potent producer of alkaline protease (Table 1). This strain exhibited prominent clear zone around the colonies on milk agar plates indicating that it secreted significant amount of protease. Strain BP-36 was able to grow at temperatures as high as 50 °C. The strain showed large, flat, irregular and yellow colored colonies after 72 h of growth on agar media. It was identified as a *B. subtilis* BP-36 based on biochemical tests (Table 2).

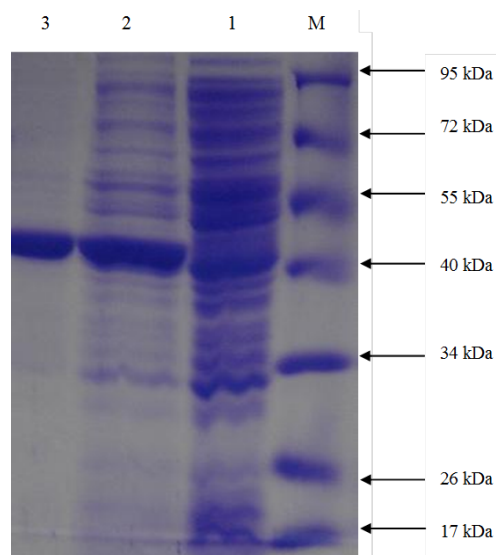


Figure 1. SDS-PAGE pattern of thermophilic alkaline protease from *B. subtilis* BP-36. M: molecular mass markers, Lane 1: supernatant of culture medium; 2: bottom phase of ATPS; 3: top phase of ATPS containing purified enzyme.

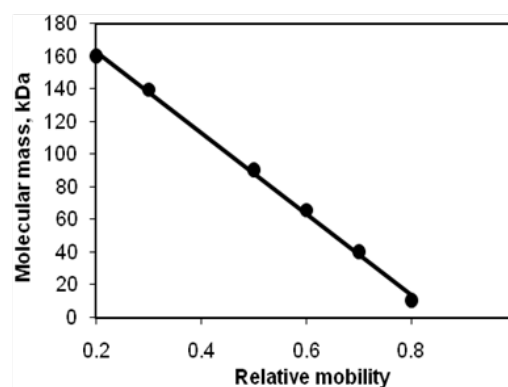


Figure 2. Determination of the molecular mass of alkaline protease of *B. subtilis* BP-36. The molecular mass was determined on the native PAGE using a 6% gel as described in the text. RNA polymerase (160 kDa), lactate dehydrogenase (142 kDa), glutamate dehydrogenase (90 kDa), bovin serum albumin (66 kDa), and cytochrome *c* (12.4 kDa) were used as the molecular mass standards.

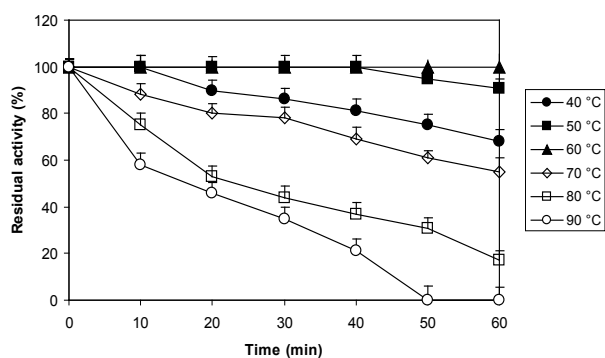


Figure 3. Effect of temperature on the stability of thermophilic alkaline protease. Enzyme samples were incubated in different buffers and aliquots were withdrawn at different time intervals for enzyme assay.

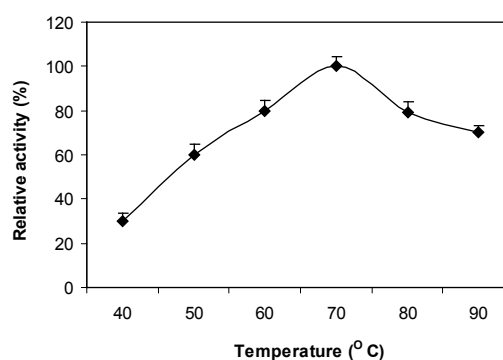


Figure 4. Effect of temperature on thermophilic alkaline protease activity. The activity of the purified enzyme was measured in Tris-HCl buffer (pH 8-9) at 70°C temperatures for 10 min.

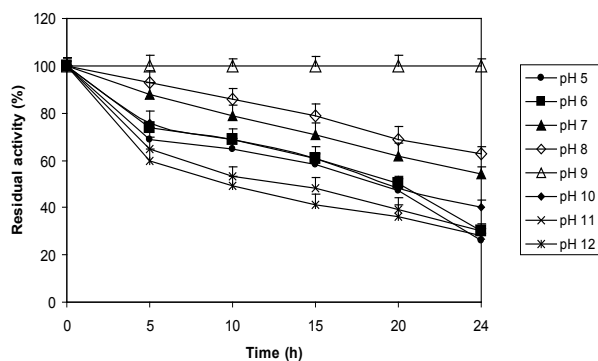


Figure 5. Effect of pH on the stability of the thermophilic alkaline protease at 70°C. Enzyme samples were incubated in different buffers and aliquots were withdrawn at different time intervals for enzyme assay.

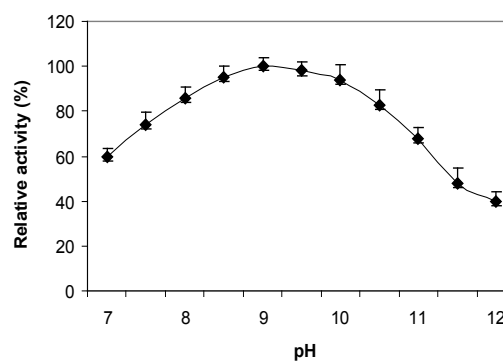


Figure 6. Effect of pH on thermophilic alkaline protease activity at 70°C. The activity of the purified enzyme was measured using Tris-HCl buffer pH 9 at 70°C temperatures for 10 min.

Purification and Molecular Mass Determination of Thermostable Alkaline Protease

In the preliminary experiments, it was found that 100 ml of pH 8.0 medium was better for the protease production by strain BP-36. *B. subtilis* exhibited maximum enzyme production at the late of exponential phase of growth. The optimum growth condition for strain BP-36 was obtained at 70 °C for 3 days. Under this condition, the *B. subtilis* alkaline protease showed its highest activity (7.0 U/ml). A similar result has also been found in *Vibrio fluvialis* TKU005 [3]. The purification of thermostable alkaline protease was carried out based on a one step ATPS procedure, including 22% (w/w) PEG-10,000 and 18% (w/w)

citrate. The results of purification are presented in Table 3. The enzyme was purified 4.8 fold with a yield of 39.7% and a specific activity of 2600 U/mg. The molecular mass of the enzyme was determined to be about 40 kDa by SDS-PAGE (Fig. 1). By gel filtration chromatography, the native molecular mass of the purified protease was estimated to be 40 kDa (Fig. 2). This result was in agreement with that obtained by SDS-PAGE. These results indicated that *Bacillus subtilis* BP-36 alkaline protease is a monomeric protein. The molecular weight of the enzyme of interest was higher as compared to other bacterial alkaline protease where it normally ranged from 15 to 30 kDa [2]. It is noteworthy that bacterial alkaline proteases with molecular mass between 32 and 35 kDa has also been reported [17].

N-Terminal Amino Acid Sequence of Alkaline Protease from Bacillus subtilis BP-36

The N-terminal amino acid sequence of the subunit (15-amino acid residues) of *B. subtilis* enzyme was determined to be MGKISAGTALIISVA. The 15 N-terminal amino acid sequences showed high similarity to those of alkaline protease from *Bacillus* sp. DSM 14392 (MGKIVAGTALIISVA). The Val-5 in *Bacillus* sp. DSM 14392 alkaline protease was replaced with Ser-5 in *B. subtilis* enzyme. The achieved result strongly supported a type of polypeptide catalyzing the alkaline protease reaction.

Influence of Temperature on Enzyme Stability and Activity

The thermal stability of the purified protease was studied by incubating the enzyme at various temperatures of 60 °C, 70 °C, 80 °C and 90 °C for different time periods. As shown in Figure 3, the best thermal stability of the thermophilic alkaline protease was obtained at 60 °C. After incubated for 1 h at 70 °C, *B. subtilis* BP-36 protease remained an activity 85% of that achieved at optimum temperature, and lost 27% of its maximal activity after incubated for 1 h at 90°C. The thermal stability of the target enzyme was similar to the reported results from *Vibrio fluvialis* TKU005 [3] and *Bacillus* strain HUTBS71 [18]. The effect of temperature on the proteolytic activity from 40 to 90°C was also investigated. As can be seen (Fig. 4), the target enzyme showed high activity at 60-90°C, and the highest activity was obtained at 70°C. The optimum temperature was similar to that of alkaline protease from *Geobacillus* sp. YMTC1049 [17] and *B. lecheniformis* NH1 [19]. From these result, it can be inferred that the alkaline protease secreted from *B. subtilis* BP-36 was a thermophilic enzyme.

Influence of pH on Enzyme Stability and Activity

The effect of pH on the stability of alkaline protease from *B. subtilis* BP-36 was tested by incubating the enzyme in different buffers of varying pH from 3 to 12. The pH stability profile of the purified protease is shown in Fig. 5. The target enzyme was stable over broad ranges of pH 5-12, with the highest stability at pH 9.0. As shown in Fig. 5, a sharp decline was seen in enzyme stability at pH below showed 7. This feature is a typical characteristic of alkaline proteases. Our results were similar to the properties of alkaline proteases from *Vibrio fluvialis* [3], *B. proteolyticus* [2] and *Bacillus* sp. B001 [20]. The effect of pH on enzyme activity was

also investigated. The enzyme was active in the range of pH 8.0-11.0 with an optimum at pH 9.0 (Fig. 6). There was a sharp decrease in the activity at pH lower than 9.0, suggesting the alkaline protease of the isolated enzyme. Protease activity decreased significantly above pH 9.0, and was 94, 68 and 40% of maximum activity at pH 10, 11 and 12, respectively. These finding were in accordance with several earlier reports for alkaline protease such as *Vibrio fluvialis* [3], and *Aspergillus oryzae* [21].

In conclusion, we here report the isolation and characterization of a thermophilic alkaline protease from a strain of *B. subtilis* BP-36. The target enzyme was purified 4.8 fold with a specific activity of 2600 U/mg and 39.7% yield. The purified protease was homogenous on SDS-PAGE, and its molecular weight was determined to be about 40 kDa. The enzyme of interest showed an optimum temperature at 60 °C and optimum pH of 9.0. Overall, the results presented in this paper showed that the alkaline protease secreted by *B. subtilis* BP-36 might be a useful source of enzyme for laundry detergents and biotechnological applications.

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