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Partial Purification and Characterization of Protease Enzyme from *Bacillus subtilis megatherium*

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Abstract

Bacteria of genus *Bacillus* are active producers of extracellular proteases, and characteristics of enzyme production by *Bacillus* species have been well studied. The aim of this experimental study is isolation and partial purification of protease enzyme from the *Bacillus subtilis megatherium* bacteria species. Protease enzyme is obtained by inducing spore genesis of bacteria from *Bacillus* species on suitable media. The partial purification was reali-zed by applying successively ammonium sulfate precipitation, dialysis, DEAE–cellulose ion exchange chromatography to the supernatant. In this study, the effect of substrate concentration, reaction time, the effect of inhibitor and activator on the optimum pH, optimum temperature, pH stability, and temperature stability was determined. Molecular weight of the obtained enzyme was investigated by SDS-PAGE. In this study, the specific activity of the supernatant, which was partially purified from *Bacillus subtilis megatherium* bacteria, was 10.4 U/mg, specific activity of supernatant was 13.5 U/mg after 80% ammonium sulfate fractionation. The final enzyme preparation was 1.1-fold purer than the crude homogenate. Molecular weight of the protease was determined, and it was found that the weight of enzyme was 45 kDa by using SDS-PAGE.

Index Entries: Enzyme; protease; *Bacillus subtilis megatherium*; purification; isolation.

Introduction

There has been growing interest in microbial proteases that are of commercial importance. Of these, proteases produced by alkalophilic microorganisms are investigated not only in the scientific areas but also find wide application in the laundry, dishwashing, food, and other such industries. In recent years, the potential use of thermostable alkaline enzymes has increased in a wide range of other biotechnological applications such as silver recovery, in feeds, and peptide synthesis. These enzymes are currently receiving increased attention in view of their inherent stability

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at such high pH and temperature values, and in the presence of surfactants, organic solvents, and denaturing agents, which enable their use in processes that restrict conventional enzymes *(1).*

Bacillus subtilis is well known for the production of industrially important enzymes such as amylase and proteases. Bacteria of genus *Bacillus* are active producers of extracellular proteases and characteristics of enzyme production by *Bacillus* species have been well studied. The production of protease depends on the availability of both nitrogen and carbon sources in the medium; either an excess or a low level of nitrogen may cause an inhibition of the biosynthesis of protease by species of *Bacillus*. Production of protease may also be inhibited by levels of carbon source *(2).*

Dormant spores of various *Bacillus* species carry out no detectable endogenous metabolism or macromolecular biosynthesis. Protein synthesis early in germination of *Bacillus subtilis megatherium* spores is dependent on endogenous amino acid reserves, which are generated by degradation of dormant spore protein *(3).*

In this study, the authors attempted to isolate bacteria from soils and optimized then for protease production with a promising strain. Different bacterial strains were isolated from soil and screened for their ability to produce protease and only one potential producer were examined and was identified as *Bacillus subtilis megatherium.*

Materials and Methods

The compounds obtained from Sigma Chemical Co., UK were Bacto Tryptone, Bacto Soytone, Bacto Agar, peptone, beef extract, ammonium sulfate, manganese chloride, calcium chloride, sodium carbonate, sodium bicarbonate, and DEAE–cellulase.

Manganese sulfate, acetic acid, tricholoro acetic acid, sodium acetate, boric acid, sodium hydroxide, casein, ammonium sulfate, hydrochloric acid, sodium chloride, ethyl alcohol, EDTA, TRIS, sodium dodecylsulfate, ammonium persulfate, glycerol, and tin sulfate were purchased from Merck Chemical Co., Germany. Acrylamide, bis-acrylamide, and glysine were obtained from Biorad Chemical Co. France.

Isolation, Screening, and Identification of Protease Producing Strains

Different bacterial strains were isolated from a waste discharge area of industry, and they were screened for their ability to produce protease. Samples collected from different factories in Turkey were analyzed for their content of microorganisms, especially for the genus *Bacillus*; 200 μL of each sample was spread onto different nutrient agar plates containing (g/L): peptone, 5; meat extract, 3; agar, 15 (pH adjusted to 7.0) and cultured at 37°C for 24 h. Some colonies were detected. Each of these colonies was then transferred to new agar plates and were grown under same conditions.

First, the purity of the strain was checked. Then by Gram-staining test, they were examined under the microscope if they have the shape of a spore-forming rod and also if they are Gram positive. Those that had the appropriate characteristics were further grown on agar plates to have adequate amounts of bacteria *(4)*.

Specific identification of bacterial strains was done with API 50 CH test kit (Biomérieux, France), which is a standardized system associating 50 biochemical tests for the study of the carbohydrate metabolism of microorganisms. The API 50 CH strip consists of 50 microtubes used to study fermentation of substrates belonging to the carbohydrate family and its derivatives (heterosides, poly alcohols, uronic acids). The fermentation tests were inoculated with a suspension of microorganisms prepared with the suitable API CHB/E medium containing (g/L): ammonium sulfate, 2; yeast extract, 0.5; tryptone, 1; disodium phosphate, 3.22; monopotassium phosphate, 0.12; phenol red, 0.18, and trace elements, 10 mL. This medium served to re-hydrate the substrates. During incubation, fermentation was revealed by a color change in the tube, caused by the anaerobic production of acid and detected by the pH indicator present in the medium.

All the bacteria from the culture plate were picked up. Then, in a sterile solution of NaCl (0.85%) all bacteria were suspended to prepare a heavy suspension (S). To another tube of 5 mL NaCl (0.85%) solution, drops were added from suspension (S) to have a turbidity equivalent to 2 McFarland units. Number of drops was recorded as (n) number. The medium used to inoculate the strips (API 50 CHB/E medium 10 mL) was then inoculated, adding twice the number of drops ($2n$) from the suspension (S). Each tube in the test strip was inoculated with the bacteria-medium suspension and incubated at 37°C for 48 h.

Strips were read after 24 and 48 h of incubation. A positive test corresponded to an acidification revealed by the phenol red indicator contained in the medium changing to yellow. A positive test may become negative at the second reading. This was because of the production of ammonia from peptone, which neutralizes the acids. Such tests were recorded as positive. The results recorded as + or − were entered in the identification software. Only one bacterial strain was identified as *Bacillus subtilis megatherium.*

From these strains, to find out the most protease producers, an API ZYM enzymatic activity research system was used. Using the same inoculation principle as API 50 CH, these strips containing 20 different substrates were inoculated with a dense suspension of organisms to re-hydrate the enzymatic substrates. The base of these strips was made of non-woven fibers containing synthetic substrates. This allowed an enzymatic reaction to take place even if the substrates were insoluble. The metabolic end products produced during the incubation period were detected through colored reactions revealed by the addition of reagents. Next, experiments were carried out by the most protease producers of the only strain detected by this test.

Production of Enzyme

The cultivation of isolates and was carried out in 1 L Erlenmayer flasks containing 200 mL nutrient broth (peptone 5 g, meat extract 3 g, 10 mg $MnSO_4 \cdot H_2O$ for 1 L media) as liquid media. The isolates were cultivated for 20–22 h at 37°C with agitation of 200–250 rpm.

Partial Purification of Protease

After incubation, the culture broth was centrifuged at 4°C and 20,000*g* for 20 min. The supernatants were collected. The enzyme in the cell-free supernatant was precipitated between 40 and 80% saturation of ammonium sulfate. They were left for 30 min after addition of salt, and the resulting precipitates were collected by centrifugation at 20,000*g* for 30 min. The precipitates were then dissolved in a minimal volume of 50 m*M* Tris-HCl–5 m*M* $CaCl_2$ (pH 7.5). These solutions were dialyzed overnight against 500 mL of the same buffer at 4°C. These solutions were studied with DEAE–cellulose ion exchange chromatography. The supernatant that was obtained after ammonium sulfate saturation, dialysis, and centrifugation was loaded to a DEAE–cellulose ion-exchange chromatography column (2×60 cm) that had been preequilibrated with borax–NaOH buffer. The protein was eluted with the same buffer using a fraction size 10 mL at the rate of 1 mL/min by three times. Fractions containing the majority of the protease activity were pooled for activity assay. The activity of protease enzyme at the end of each step was measured by spectrophotometric method *(9)*.

Experimental Procedures

Protease Assay

Protease activity was determined spectrophotometrically by measuring at 275 nm by two different methods.

Method 1: Protease activity was determined at 30°C in this method. The reaction condition was prepared by tubes containing 2.5 mL milk casein substrate solution in water bath for 5 min, and the reaction was carried out by adding 0.5 mL enzyme solution for 10 min. After 10 min, the reaction was stopped by adding 2.5 mL TCA solution, the solution was stored in a water bath for 20 min per sample, without enzyme but mixture was used as blank solution *(7)*.

Method 2: Protease activity was determined at 35°C in this method. Tubes containing 3 mL substrate solution were incubated in the water bath at 35°C for 5 min and the reaction was carried out by adding 0.5 mL enzyme solution for 10 min. At the end of this period, the reaction was stopped by adding 3.2 mL TCA solution, the solution was stored in water bath for 10 min and absorbance values were measured at 275 nm for protease activity by rate of casein hydrolysis (*8*).

One unit of activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine in 1 min at 30°C (1 U/mL = 1 µg tyrosine/min · mL).

A tyrosine standard was prepared by dissolving different amounts of tyrosine in TCA solution.

Protein Determination

Protein concentration was determined with 2,2′-bichinolyl-4,4′-dicarbonic acid (BCA), Na_2-salt at 570 nm *(13)* in a Bio-Rad Model 3550 UV Microplate Reader (Hercules, CA, USA). Bovine serum albumin was used as a standard.

Stability of Protease and Activity Profiles

Optimum pH, optimum temperature, pH stability, temperature stability, effect of substrate concentration, effect of time, effect of activators, and effect of inhibitors were determined for partial purified protease enzyme by using casein solution as substrate.

Effect of Temperature on Enzyme Activity and Stability

The temperature stability was determined by measuring the residual activity. Enzyme solution (50 µL) was incubated in 950 µL of 0.05 *M* Tris-HCl buffer (pH 7.5) for 30 min at different temperatures ranging from 0 to 70°C. Then they were put on ice and relative activities were assayed at standard assay conditions.

The activity of partially purified enzyme was assayed at the same temperature range for 10 min, 0.6% casein solution was preincubated at respective temperatures for 5 min before the enzymatic assay. Activity was determined by using Method 2.

Effect of pH on Enzyme Activity and Stability

The optimum pH was determined at 30°C with casein solution prepared in Britton–Robinson (B-R) buffer ranging from pH 4 to pH 10.

pH stability was also determined at the same pH range in the same B-R buffer systems; 50 µL enzyme solution was incubated with 150 µL B-R buffer for 20 h at 30°C. Then it was diluted with 0.05 *M* Tris-HCl buffer (pH 7.5) to 1 mL. For the reference value, 50 µL enzyme was left at +4°C for 20 h in 150 µL of 0.05 *M* Tris-HCl buffer (pH 7.5), then diluted with the same buffer to 1 ml. Activity was determined by using Method 2.

Substrate Concentration and Time on Enzyme Activity

Substrate concentration is an important factor in determining the degree of enzyme reaction *(12)*. Casein substrate solution concentrations were prepared as 1.2%, 0.6%, 0.3%, 0.15%, and 0.075%. Activity was assayed for every substrate concentration in 10, 30, 50, and 70 min.

Table 1
Protein Purification of Partial Purified Protease Enzyme from *Bacillus subtilis megatherium*

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold
Crude homogenate	10878	136111	12.5	1
40% saturation	9359	106921	11.4	—
80% saturation	7950	106915	13.5	1.1

Effect of Activator and Inhibitor on Enzyme Activity

The effects of some divalent cations on the activity of protease enzyme were also analyzed. Mn^{+2}, Fe^{+2}, and Ca^{+2} are used in the study; 10^{-3} *M* $MnCl_2$, $FeCl_2$, $CaCl_2$ solutions were prepared and 100 µL activator solution and casein substrate solution completed 2.5 mL. Solution was stored in a water bath at 30°C for 5 min and 0.5 mL enzyme solution was added and the reaction stopped after 1 min by using 2.5 mL TCA solution.

The effects of EDTA and Cu^{+2} were investigated. For this purpose, 10^{-3} *M* $CuCl_2$ and EDTA solutions were prepared and 100 µL inhibitor solution and casein substrate solution completed 2.5 mL. Solution was stored in water bath at 30°C for 5 min and 0.5 mL enzyme solution was added and the reaction stopped after 1 min by using 2.5 mL TCA solution.

Determining Molecular Weight of Partially Purified Enzyme

The molecular weight of partial purified protease enzyme from *Bacillus subtilis megatherium* bacteria was analyzed by using SDS-PAGE. The standards containing materials (β-galactosidase: 116 kDa; bovine serum albumin: 66.2 kDa; ovalbumin: 45 kDa; lactate dehydrogenase: 35 kDa; endonuclease Bsp 981: 25 kDa; β-lactoglobulin: 18.4 kDa; lysosyme: 14.4 kDa) were used as protein markers in the method.

Results and Discussion

Purification of Protease

Protease enzymes from only one *Bacillus* species were partially purified with 80% ammonium sulfate precipitation followed by dialysis. BSMP (*Bacillus subtilis megatherium* protease) was loaded onto a DEAE–cellulose ion exchange column. These results are recorded in Table 1. In this study, all the activity of partial purified protease from BSM was lost after DEAE–cellulose ion exchange column, so this step did not work. Also, the protease activity was determined spectrophotometrically by measuring at 275 nm by two different methods. Comparison of these activity assays is given in Table 2.

Table 2
Comparing Activity Assays for Partial Purified Protease Enzyme from *Bacillus subtilis megatherium*

After 80% fractionation	Total protein (mg)	Total activity (U)	Specific activity (U/mg)
Method 1	11454	150947	13.5
Method 2	10723	133756	12.5

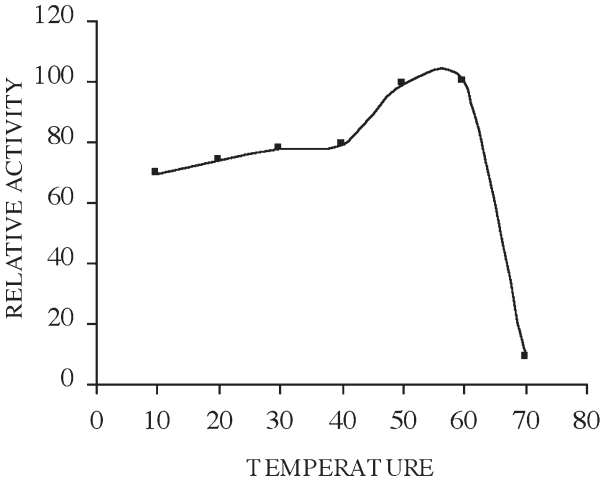

Fig. 1. Effect of temperature on the activity of partially purified protease from *Bacillus subtilis megatherium.*

Characterization of Protease

Optimum pH and *T* values and pH and thermal stabilities of BSMP were determined.

The optimum temperature for BSMP was found to be 60°C as shown in Fig. 1. These optimum *T* values are higher than other recorded proteases that have optimum temperature values at 40°C (*2*), 45°C (*5*), and 50°C (*6*). The thermal stability studies suggested that enzyme from BSMP was stable between 30 and 60°C as shown in Fig. 2.

Optimum pH of protease enzyme from BSMP was estimated 9.1 as shown in Fig. 3. pH stability of protease enzyme from BSMP decreased after pH 10 as shown in Fig. 4.

On the other hand, it was more stable on acidic conditions such as pH 4–6 when compared with the other protease but there is an increase in the activity of BSMP under alkaline conditions, such as pH 8.

Substrate concentration is an important factor in determining the degree of enzyme reaction.

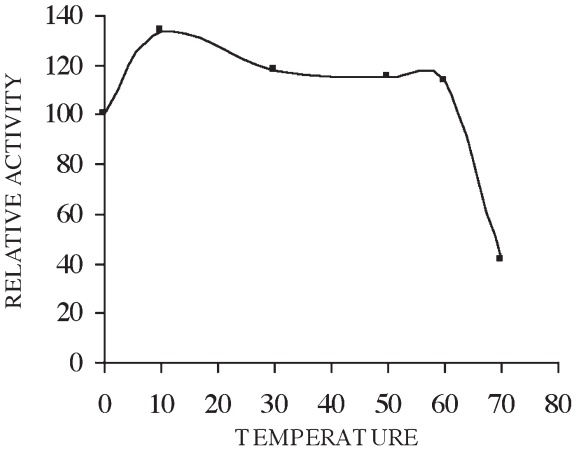

Fig. 2. Thermostability of partially purified protease from *Bacillus subtilis megatherium.*

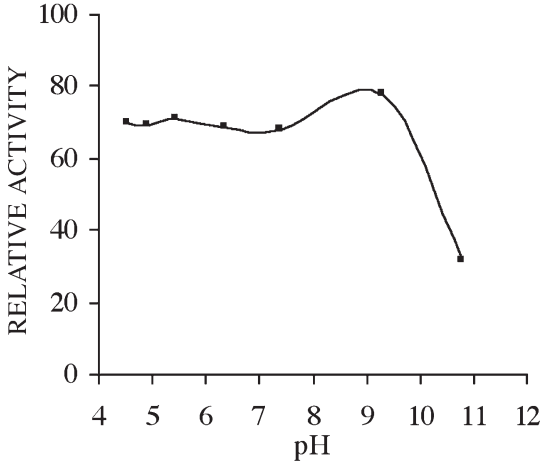

Fig. 3. Effect of pH on the activity of partially purified protease from *Bacillus subtilis megatherium.*

The results of our study were shown for BSMP in Table 3. Generally, casein is used as a substrate for protease studies reported in the literature. The activity of protease enzyme from BSM increased with both increment in reaction time and substrate concentration also, for enzyme from *Bacillus* spp.; specific activity increased until 70 min for all concentration values. The activity was stable after 70 min. This led us to believe that 0.6% casein solution concentration could be used in further studies.

The molecular weight of the enzyme obtained as a result of the conducted SDS-PAGE was found to be approx 45 kDa for BSM bacteria species. Comparing the molecular weights of this enzyme, it is also evident

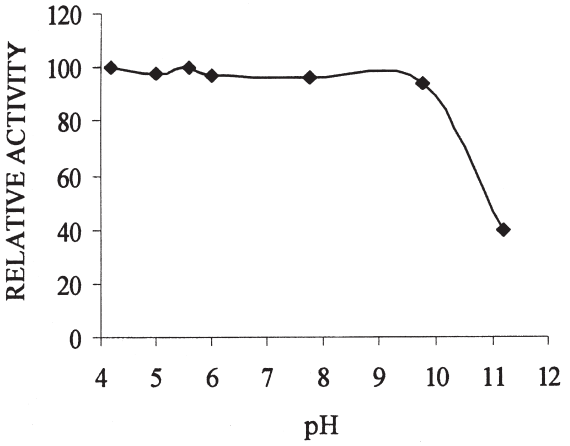

Fig. 4. pH stability of partially purified protease from *Bacillus subtilis megatherium.*

Table 3
Effect of Substrate Concentration and Time on the Activity of Partially Purified Protease from *Bacillus subtilis megatherium*

Substrate concentration (%)	Reaction time (min)			
	10	30	50	70
0.075	51.1	38.6	22.5	52
0.15	41.4	37	46.3	54.1
0.3	46	40.1	40.8	55
0.6	45.7	45.4	41	52
1.2	68.1	48.3	20.7	55.9

Activity values are U/mL.

Table 4
Effect of Activator on the Activity of Partially Purified Protease from *Bacillus subtilis megatherium*

Substrate concentration (%)	Relative activity (%)	Relative activity (%) ($MnCl_2$)	Relative activity (%) ($FeCl_2$)	Relative activity (%) ($CaCl_2$)
0.6	100	111	97	106
0.3	100	114	107	93
0.15	100	106	90	86
0.075	100	115	109	93

Table 5
Effect of Inhibitor on the Activity of Partially Purified Protease from *Bacillus subtilis megatherium*

Substrate concentration (%)	Relative activity (%)	Relative activity (%) ($CuCl_2$)	Relative activity (%) (EDTA)
0.6	100	79	77
0.3	100	98	78
0.15	100	110	90
0.075	100	102	90

that they are different—the protease examined by the other researchers has a molecular weight of 45–66, while the enzyme isolated in the present study has a molecular weight of 45.

The presence of Mn^{+2} ions increased the protease activity more than Fe^{+2} and Ca^{+2} ions for BSM as shown in Table 4. The presence of Mn^{+2} ion increased the protease activity for *Bacillus* bacteria species and EDTA produced a partial inhibitory effect for protease enzyme from *Bacillus* spp. bacteria and $CuCl_2$ was able to decrease the velocity of the reaction very strongly for protease enzyme from BSM bacteria. It was estimated that EDTA solution was an effective inhibitor for protease enzyme from BSM as shown in Table 5. It may be concluded that BSM is a metalloenzyme, as suggested by inhibition with EDTA.

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