Partial Purification of Protease by A Novel Bacterium, *Bacillus cereus* and Enzymatic Properties

Yeni İzole *Bacillus cereus* Proteazının Kısmi Saflaştırılması ve Enzimolojik Özellikleri

Research Article / Araştırma Makalesi

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ABSTRACT

Proteases (E.C. 3.4.X.X) catalyze the hydrolysis of peptide bonds in proteins. Microbial proteases are classified based on their mode of action and biocatalytic mechanisms. They participate in most aspects of cell nutrition, physiology, and regulation, and in microbial pathogenesis. Protease production was detected from 15 bacteria isolated from soil samples and the one showed the highest protease activity was selected. The strain was identified and determined as Bacillus cereus by 16S rRNA phylogenetic analysis. After optimization of protease production from the novel medium, the Michaelis-Menten kinetics was studied. Temperature, pH and, time parameters of protease incubation was determined and maximum temperature was detected at 50oC as 5.15 IU/ml. The optimum pH range of the enzyme was in between pH 7-9. The crude enzyme was approximately 2-fold purified by dialysis.

Key Words
Protease, Production, Optimization, *Bacillus cereus*.

ÖZET

Proteazlar (E.C. 3.4.X.X) proteinlerdeki peptid bağlarının hidrolizinden sorumlu enzimlerdir. Mikrobiyal proteazlar, etkide bulundukları bölge ve biyokatalitik mekanizmalarına göre sınıflandırılırlar. Bu proteazlar hücre beslenmesi, fizyolojisi, düzenlenmesi ve mikrobiyal patogenezde rol oynamaktadırlar. Toprak örneklerinden izole edilmiş 15 bakterinin proteaz aktiviteleri saptanmış ve en yüksek aktiviteye sahip suş seçilerek stoklanmıştır. Bu suşun 16S rRNA filogenetik analizi yapılarak *Bacillus cereus* olduğu saptanmıştır. Proteaz üretiminin optimizasyonu sonrasında bu suşun Michaelis-Menten kinetiği hesaplanmıştır. Proteaz inkübasyonunun sıcaklık, pH ve zaman parametreleri araştırılmış ve maksimum sıcaklık aktivitesi 50°C'de 5.15 IU/ml olarak saptanmıştır. Enzimin optimum pH aralığı 7-9'dur. Ham enzim, diyaliz yöntemiyle yaklaşık olarak 2 kat saflaştırılmıştır.

Anahtar Kelimeler
Proteaz, Üretim, Optimizasyon, *Bacillus cereus*.

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INTRODUCTION

Proteases (E.C. 3.4.X.X) catalyze the hydrolysis of peptide bonds in proteins [1]. Microbial proteases are classified based on their mode of action and biocatalytic mechanisms. They participate in most aspects of cell nutrition, physiology, and regulation, and in microbial pathogenesis. [2].

Today, proteases account for approximately 40% of the total enzyme sales in various industrial market sectors [3] and the total industrial enzyme market in 2009 is expected to reach nearly $2.4 billion [4]. The major microorganisms that were found as industrial protease producers exist in bacteria such as genera Clostridium, Bacillus and Pseudomonas [5]. Although variant microorganisms were used to produce protease, genus Bacillus were so far the most important group of enzymes produced commercially [6]. Bacterial strains are generally more used as they offer higher activities compared to yeasts and tend to have neutral or alkaline pH optima and are often thermostable. Only about 2% of the world's microorganisms have been tested as enzyme sources [4]. This shows the importance to continue screening of enzymes from novel bacteria and fungi.

This work was undertaken to produce protease from a novel strain and detection of the protease characteristics. Therefore 15 bacterial strains were isolated from soil samples collected at different regions of Turkey, the one showed the highest protease activity was selected and identification of the novel strain investigated. After optimization of protease production, temperature, pH and effect of time parameters of protease was determined. The crude protease was partially purified by dialysis.

MATERIALS AND METHODS

Isolation and Identification

Soil samples were collected from different regions of Turkey and 15 bacterial strains were isolated. Strains were assayed for proteolytic activity and a strain showed the highest proteolytic activity was selected. Besides biochemical and morphological tests, the novel Bacillus strain then was identified by 16S rRNA phylogenetic analysis.

Medium and Incubation

Nutrient Broth (5 g peptone and 3 g meat extract, pH 7.0, Merck) was used as the common growth media as well as stock media. Incubation was carried out at the growth medium at 30°C at 150 rpm for 18 hours. After incubation the culture media centrifuged at 7200 rpm for 10 minutes to obtain the cell free supernatant (CFS). The protease activity studies were carried out from the CFS.

Protease Assay

Proteolytic activity was carried out according to Casein-Pholine Method [7]. Culture media was santrifugated at 7200 rpm for 10 minutes and CFS was used as enzyme source however 1% casein (solved in 0.1 M phosphate buffer, pH 7.0) was used as substrate. 1 ml of enzyme and 1 ml of substrate was incubated at different temperatures for 60 minutes. The reaction was terminated by adding 3 ml of trichloroacetic acid (TCA). One unit of protease activity was defined as the increase of 0.1 unit optic density at 1 hour incubation period. Total protein amount of CFS was measured by Lowry Method [8].

Characteristics of Protease and Partial Purification

Temperature and pH characteristics were determined respectively. 10 to 70°C of incubation temperature and acidic, neutral and alkaline pH conditions were measured. The maximum and optimum values were determined. The effect of time to protease was also studied. The effect of caseine substrate concentration to protease activity was detected and Km value was calculated by Michaelis-Menten kinetics. The partial purification of the crude protease was carried out by dialysis.

RESULTS

Fifteen bacterium strains were isolated from collected soil samples. Their protease activity was detected and the strain showed the highest proteolytic activity was selected. The novel strain was determined as Bacillus sp. according to the biochemical and morphological analysis. 16S rRNA phylogenetic analysis was performed to determine the species of the novel isolate and was identified as Bacillus cereus.

Enzyme localization was detected as extracelular. Before optimization the novel Bacillus cereus isolate could produce 5.5 IU/ml protease at 30°C and 7.0 pH and after it could produce 9.56 IU/ml protease at 30°C and 6.4 pH. The results showed that optimization was succesful at range of 173.8%.

Protease assay options was differentiated, the temperature and pH options were studied. Protease assay was carried out between 10-70°C and optimum temperature range of the protease was detected between 40-60°C and maximum activity was measured at 50°C as 5.15 IU/ml (Figure 1).

The pH conditions were carried out as pH 5, 7 and 9, respectively 6.35 IU/ml, 10.20 IU/ml and 8.50 IU/ml protease activities detected. Maximum activity of the protease is on neutral pH conditions but it showed high activity on alkaline pH conditions as well (Figure 2).

Incubation period of protease assay was detected between 30 – 120 minutes and it showed that by time protease activity increased linearly (Figure 3).

According to Michaelis-Menten equation protease enzyme isolated from novel Bacillus cereus showed affinity to it's substrate which was caseine and Km value was measured as 3.5 x 10^{-3} (Figure 4).

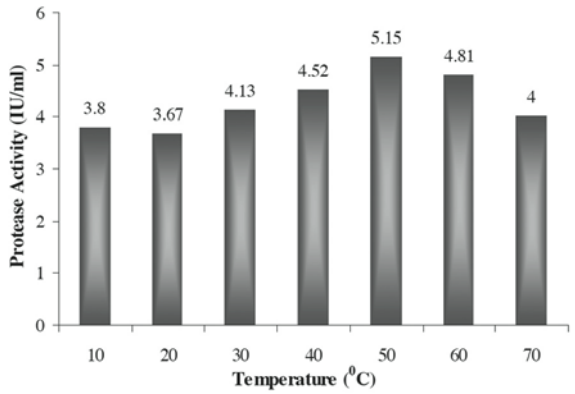

Figure 1. Effect of temperature on protease activity (Standart Deviation is 0.547227, Standart error is 0.500705).

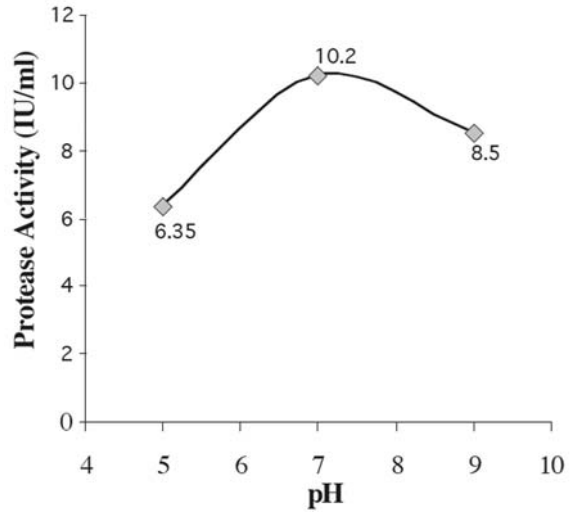

Figure 2. Effect of pH on protease activity (Standart Deviation is 1.929378, Standart error is 2.265778).

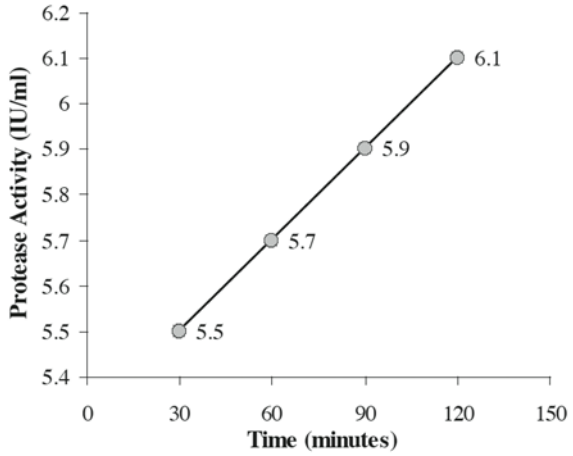

Figure 3. Incubation period of protease assay (Standart Deviation is 0.25819889, Standart error is 3.72529E-09).

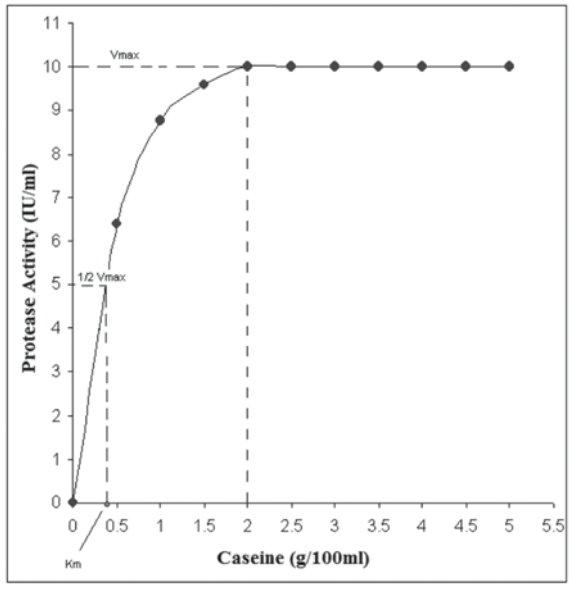

Figure 4. Km value according to Michaelis-Menten equation.

Table 1. Partially purification of protease.

	Total Protein (mg/ml)	Protease Activity (IU/ml)	Specific Activity (IU/mg protein)	Enrichment
Crude Protease	6.376	8.56	1.343	1.00
Protease after dialysis	3.066	7.60	2.479	1.85

The crude protease was purified 2-fold by dialysis partially (Table 1).

DISCUSSION

Production of enzymes from bacterial sources has been affected by the conditions of growth and the production media. In this study, Nutrient Broth was used as growth and production media. Cihangir, 1987, Upton and Fogarty, 1977, also used the same medium for the production of protease and they described Nutrient Broth is a suitable medium for production [9,10].

Although many potent strains are on market for enzyme production, scientists prefer studying on new isolates because they could be alternative for commercial use. Mehrotra et al isolated 52 novel bacteria from saline-alkaline soil samples [11]. They produced alkaline protease from one of which had the highest protease activity by *Bacillus sp*. Dube et al worked on 25 proteolytic bacteria isolated from lake sediment samples of Antarctica [12]. Hawumba et al isolated two proteolytic thermophilic aerobic bacterial strains from Burnaga hot springs in western Uganda [13]. Many studies such as those showed that researches will continue to isolate alternative strains for production of enzymes as well as proteases.

Proteolytic activity was carried out according to Casein-Pholine Method [7]. Caseine, milk and hemoglobin are frequently used substrates for detection of protease activity. In this study 1% caseine solved in 0.1 phosphate buffer (pH 7.0) was used as substrate. Aikat and Bhattacharyya used a mixture of enzyme solution (0.1 ml), 2% casein solution (2 ml), and 0.6 ml of glycine-NaOH buffer (0.2 M, pH 8.0) and incubated at 37°C for 30 min [14]. Bjurlin et al used Azurine-crosslinked casein as substrate [15]. Kanekar et al used 0.625g% casein as a substrate but also tyrosine for estimation of protease activity [16]. To stop reaction, various ratios of TCA were used in different studies such as Mehrotra et al used 3 ml of 10% TCA [11], Abdel-Naby et al used 2 ml of 15% TCA [17] and Yang et al used 5 ml of 0.19 M TCA [18].

Optimum enzyme-substrate reaction, temperature range was between 40-60°C, and 7.0-9.0 pH. Maximum enzyme-substrate reaction temperature was detected at 50°C, and maximum pH was at 7.0. Durham et al detected that optimum protease activities from alkalophilic *Bacillus sp*. GX6638 were between 8.0-12.0 pH and at 65°C [19]. Gupta et al found out that the protease produced from *Bacillus sp*. had optimum activity at 60-70°C, pH: 10.0 [20].

The relationship between substrate concentration and protease by *Bacillus cereus* was investigated. Km value was computed as 3.25×10^{-3} g/ml. Km is the required substrate concentration for 50% of the enzyme activity shown. And Km value of the protease shows Michaelis-Menten-type kinetics [21,22]. The low Km value describes that protease enzyme has much affinity to the casein substrate. Crude enzyme preparation produced by novel Bacillus cereus, partially purified as 1.85 fold by dialysis. Bono et al performed 1.2 fold purification after dialysis [23].

This study showed that the protease produced by novel Bacillus cereus can be used commercially because growth conditions are simple, it can maintain it's activity up to 60°C and highest activity at neutral and alkaline pH.

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