Purification of Alkaline Serine Protease From Local *Bacillus subtilis* M33 by Two Steps: a Novel Organic Solvent and Detergent Tolerant Enzyme

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Abstract

Alkaline proteases are important from an industrial perspective due to their wide scale applications and obtained from different sources. In this study, an alkaline protease from a newly isolated Bacillus subtilis M33 was purified by ammonium sulfate precipitation and DEAE cellulose anion exchange chromatography with 38.66% yield and 15.50 fold. The results of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and gel chromatography indicate that the molecular weight of the purified enzyme is 39 kDa. The enzyme present pH and temperature optimum of 10.0 and 55°C The purified enzyme has been found to maintain stability over a wide range (pH 8.0-11.0) for 7 days.. Phenylmethyl sulfonyl fluoride (PMSF) which is a specific inhibitor completely inhibited the enzyme activity. However, the increased activity of the enzyme in the presence of 2-mercaptoethanol and dithiothreitol indicates that the enzyme is a thiol-dependent serine protease. The enzyme retained its stability with laboratory bleaches (H2O2), surfactants (Tween 80, Triton X-100, SDS) and organic solvents such as ethanol, toluene, propanol. The enzymatic behavior of the purified enzyme in the presence of some commercial detergents was also evaluated. The two important Michaelis Menten parameters Km and Vm were calculated 0.706 mg/ml, 3000 μM.min-1 respectively.

1. INTRODUCTION

The enzyme family, which cleaves peptides in aqueous media and catalyses peptide synthesis in non-aqueous media, is identified as proteases.

Approximately 500-600 proteases were found as a result of studies on mouse and human genomes (2% genome), most of which are orthologs [1-2]. In metabolism, proteases have roles such as protein fate identification, localization and regulation of activity, arranging protein-protein interactions, organizing new bioactive molecules, contributing to the processing of cellular information, and producing molecular signals. [3]. In addition to its role in metabolism, proteases are frequently used in areas such as food, leather industry, pharmacology and synthetic peptide synthesis [4-8]. A protease family of alkaline proteases whose activity is between pH 6 and 13, and which contains either a serine group at the center or a metallo-type. [9]. Among these, use as laundry detergent additives is head of the industrial applications for alkaline proteases [10-16]. The activity of proteases on fabrics is greatly influenced by factors such as the pH of the detergent, composition of detergent, ionic strength, bleaching agents, and wash temperature. [14]. Many microorganisms are protease producers, with the Bacillus strain secreting the highest activity and highest amount of enzyme among them [11,17]. The main source of commercial alkaline proteases worldwide is B. subtilis. [14]. Instead of water, the use of enzymes as catalysts in aqueous solutions of organic solvents or in organic solvents themselves has many advantages and in the presence of organic solvent, enzymatic synthesis of peptide bonds is promoted. [16]. It is therefore very important to develop techniques for the purification of enzymes that maintain stability in the presence of organic solvents. There is a limited number of studies in the literature on protease purification showing stability in the presence of organic solvents [7,12,16-20].

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From this view, the aim of this study is to purify a new serine alkaline protease from Bacillus subtilis M33 with high stability presence in organic solvents and surfactants in high yield and few purification steps.

2. REAGENTS AND METHODS

2.1. Bacterial strain, Media and Growth Conditions

Bacillus subtilis M33 is original and isolated from Ahlatlıbel soil in Ankara city. [21]. The culture was routinely grown on stocks in nutrient broth containing meat extract, yeast extract, peptone. After the culture medium was incubated at 120 ° C for 20 minutes, cultures were grown in 100 mL of 500 mL erlenmeyer flasks on a rotary shaker (200 rpm) for 24 hours at 37 ° C.

2.2. Test of protease activity and determination of protein content

Protease activity was determined by a slight modification of Takami *et al*. method [20] using casein as the substrate. One unit of protease activity was expressed as an enzyme capable of producing 1 μg of tyrosine per minute under standard assay conditions. All experiments were carried out triplicate and the standart errors of mean values were calculated lower than %3.

In the procedure for determining the protein concentration, the Bradford method, in which bovine serum albümin (BSA) was used as standard, was followed [22]. In the chromatographic process, the amounts of protein in the fractions were evaluated as a function of the absorbance at 280 nm.

2.3. Protease Purification

After 24 h cultivation, the culture medium was centrifuged at $13000 \times g$ for 10 min. The supernatant of the culture was treated with 35-80% graded ammonium sulfate salt. The salt solution was then left overnight at +4 ° C and then the precipitates were collected by centrifugation at $15000 \times g$ for 20 minutes at +4 ° C. After ensuring that your precipitate was dissolved in the least possible volume of buffer 50mM glycine-NaOH buffer (pH 9.5), dialysis was then carried out against the same buffer for overnight with three changes (+4 °C). Dialyzed enzyme solution was loaded on to a DEAE-cellulose column (1,6×20 cm; Bioplot Pharmacia FPLC) preequilibrated with 50 mM glycine-NaOH buffer (pH 9.5). Elution was carried out with the same buffer at a 3ml min-1 flow rate and each fraction was collected every 90 sec. The amount of protein present in each fraction was evaluated by absorbance measurements at 280 nm. Fractions which have high spesific activity were pooled and concentrated for further characterization.

2.4. Evaluation of the Molecular Weight of the Purified Enzyme

Separately from the SDS-PAGE technique, A technique used for molecular weight of the purified enzyme is gel filtration chromatography. After the column was equilibriated with glycine-NaOH buffer purified protease was injected into Superdeks 75 (2.6×60 cm) gel filtration chromatography column. The protein was eluted with the same buffer at a flow rate of 0.3 mL/min.

2.5. Biochemical Characteristics of The Purified Protease

2.5.1. Determination of the pH Optimum and Stability

The assays to find the optimal pH of the purified protease, analyzes were conducted at pH 4.0-12.0, casein as substrate. 50 mM sodium acetate for pH 4.0–5.0, potassium phosphate for pH 6.0–7.0, Tris –HCl for pH 8.0, glycine–NaOH for 9.0, 10.0, 11.0, 12.0 were used. Enzyme pH stability was assessed by incubation with mentioned buffers for 3 hours. For further stabilitiy studies, the purified enzyme pre-incubated in buffers for one week and every 24 hour enzyme assay was applied. The relative activities were quantified under standard assay conditions.

2.5.2. Determination of Optimum Temperature and Thermal Stability

To determine the optimum working temperature of the purified enzyme, the enzyme mixture was incubated at pH 10.0 in glycine-NaOH buffer at various temperatures (20-80 ° C) and enzyme activity was measured afterwards. While assessing the thermal stability of the enzyme, the purified enzyme was incubated at different temperatures for 1 hour followed by the measurement of the remaining activity. (35–80°C).

2.5.3. Behavior Against Some Metal Ions

To investigate efficiency of various metal ions (K^+ ve Na^+, Mn^{2+}, Ba^2, Ca^{2+}, Mg^{2+}, Co^{2+}, $^+$, Zn^{2+}, Cu^{2+}, Ni^{2+}, Sn^{2+} Fe^{3+}, Al^{3+}) on the protease activity was evaluated by pre-incubating at room temperature purified enzyme in a mentioned ion (final concentration 2.0 and 5.0 mM) containing pH 9.0 working buffer solution. The residual enzyme activity was measured at the end of 1 hour incubation.

2.5.4. Behavior of Various Organic Reagents on Stability of Protease

The behavior of pure enzyme against some organic solvents (25%, v/v) in different properties has been tested at 30°C for 60 min. The organic solvent immiscible with water was emulsified with Tween 80 (0.3%, v/v). After 1 h of incubation, residual activity of the enzyme was measured.

2.5.5. Substrate Specificity

The affinity of the enzyme against target substrates such as casein, ovalbumin, gelatin, soy meal and BSA was evaluated. Routine protease activity assay was performed after incubation.

2.5.6. Effect of Protease Inhibitors, Surfactants and Oxidizing Agents

Phenylmethylsulfonyl fluoride (PMSF, 2 mmol L^{-1}), ethylene-diamine tetraacetic acid (EDTA, 2 mmol L^{-1}), mercaptoethanol (0.1%, v/v), Dithiothreitol (DTT) and surfactants such as SDS (0.2% v/v), Triton X-100 (1%, v/v), Tween 80 (1%, v/v), and oxidizing agents such as H_2O_2 (5%, v/v) was used to clarify purified enzyme belonging family. It was investigated by incubating the enzyme for 60 min at 30°C. The enzyme activity assay was applied as described previously. Relative enzyme activity was assessed on the basis of the activity measured without these agents in the medium.

2.5.7. Stability of Commercial Detergents

Different commercially available detergents like Bingo (Hayat Kimya, İstanbul), Ariel (Procter&Gamble), Tode Matik (ABC, İstanbul), Art (Beyaz Kağıt, Adana), OMO (Unilever, Kocaeli), Tursil (Henkel) were used to study the compatibility of the purified protease. The purified enzyme was treated with commercial enzymes (1.0 % w/v) prepared in tap water for 1 hour at 30 ° C and then the enzyme activity was measured. The enzyme activity without detergent is considered 100%.

2.5.8. Determination of Vmax and Km Values of Kinetic Parameters

The kinetic parameters, Vmax and Km, of the purified protease were determined by measuring the enzyme activity at different casein concentrations (0.1–2.0 mg.mL^{-1}) The values of Km and Vmax in the Michaelis-Menten equation were determined by calculating in the Sigma plot enzyme kinetic module assistancy.

3. RESULTS

3.1. Purification and Molecular Weight of Protease

Protease from *B. subtilis* M33 was purified by the two step procedure described in above. Table 1 shows the results of the purification steps of the purified enzyme. After the final purification procedure is complete, it was determined that the enzyme was 15-fold purified, with specific activity of 7040 U mg^{-1}

and yield of 38% with these techniques. When the results of SDS single band and gel chromatography are evaluated together, it can be interpreted that the enzyme is about 39 kDa monomer (Figure 2). This result is consistent with the literatüre reports that provides information that Bacillus genus is mostly protease producers less than 50 kDa. The elution outline of the dialyzed enzyme solutions obtained from DEAE-cellulose column are shown in Figure 1.

Table 1. *Summary of purification of serine alkaline protease from Bacillus subtilis M33 using DEAE cellulose ion exchange chromatography*

| Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification fold
Crude enzyme	67567	149.55	451.80	100.0	1.0
Ammonium sulphate precipitation	36139	31.84	1135.01	53.49	2.51
DEAE cellulose ion exchange	**26125**	**3.73**	**7040**	**38.66**	**15.50**
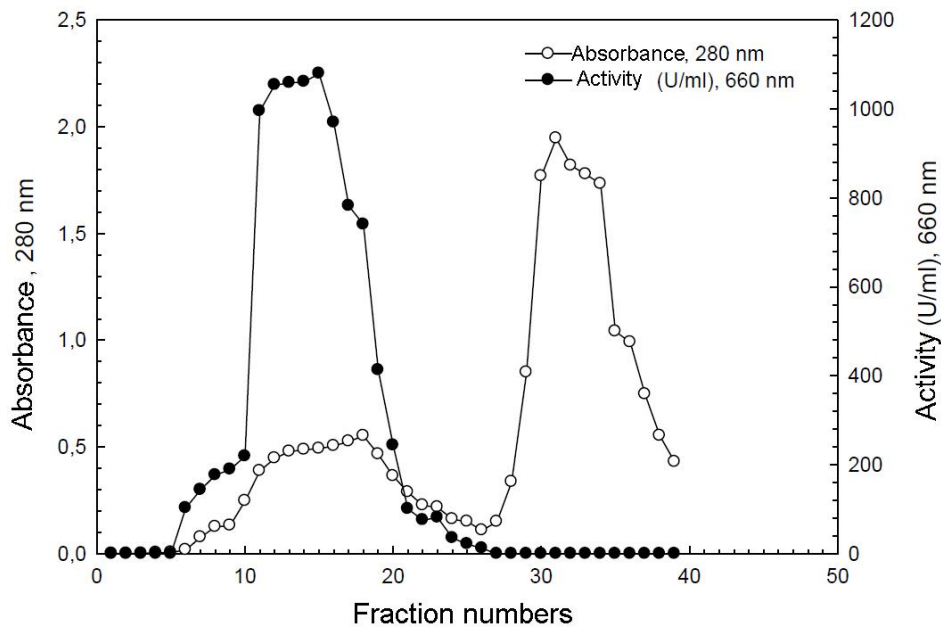
Figure 1. *The elution profile of the dialyzed enzyme solution through the DEAE cellulose columns*
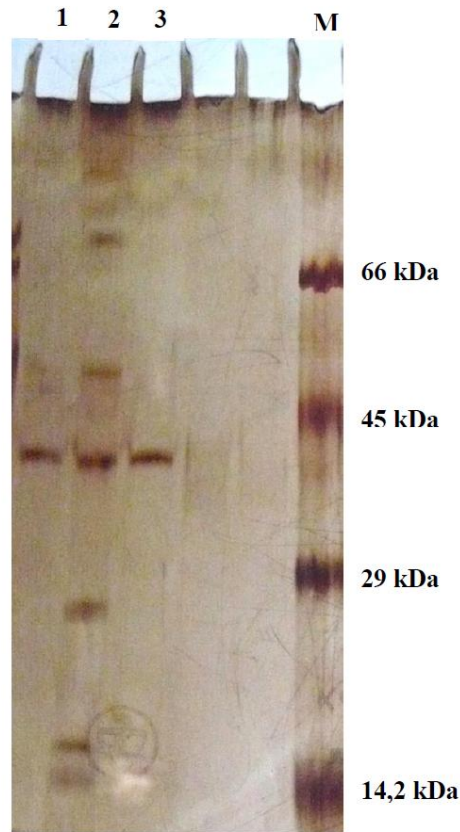
Figure 2. *SDS–PAGE of alkaline protease from Bacillus subtilis M33. Lane M, standard protein molecular mass markers; lane 1,crude enzyme; Lane 2, purified enzyme from ammonium sulphate precipitation; Lane 3, purified enzyme from DEAE anion exchange chromatography*

### 3.2. Different pH Evaluation of Enzymatic Behavior

The pH stability and the behavior exhibited by the purified enzyme are shown in Fig 3. The optimum working pH of the enzyme was determined to be 10 as a result of the analyzes performed at pH 4.0-12.0.
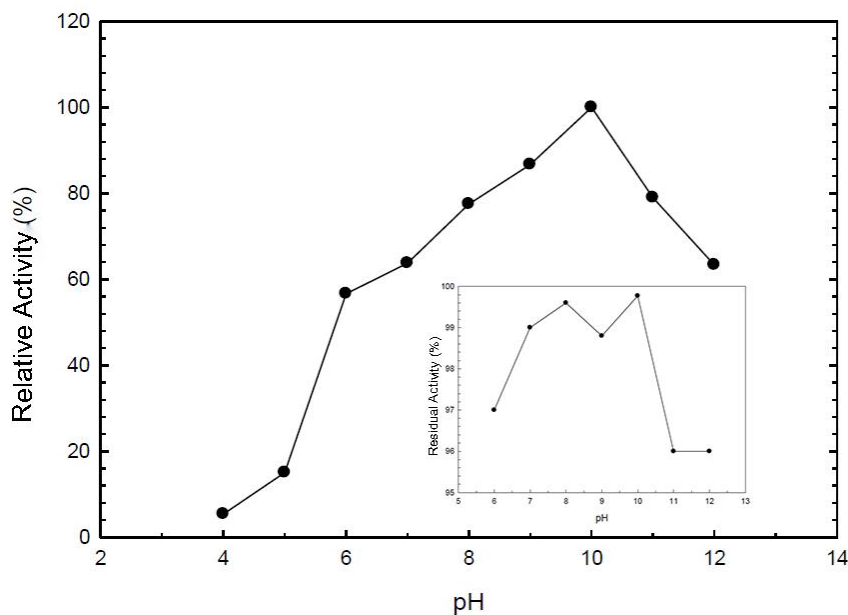
Figure 3. *Effect of pH on activity (A) and stability (B) of protease from B. subtilis M33*

### 3.3. The Behavior of the Enzyme in Different Temperatures

The purified protease shows good activity a wide range 20-60°C and optimum temperature of protease from *Bacillus subtilis* M33 was determined 55°C. The enzyme activity was retained after 3 hours incubation at 40 °C. Besides,eEnzyme has been found to maintain its activity even after 45 minutes at 50°C. However, its activity lost ~ 60% after ten minutes at 60°C (Figure 4).
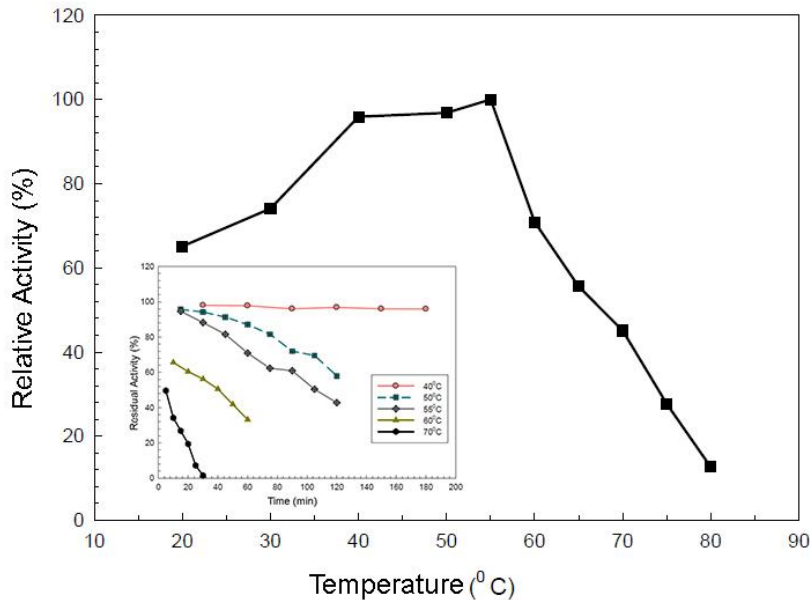
Figure 4. *Effect of temperature on activity and stability of protease from B. subtilis M33*
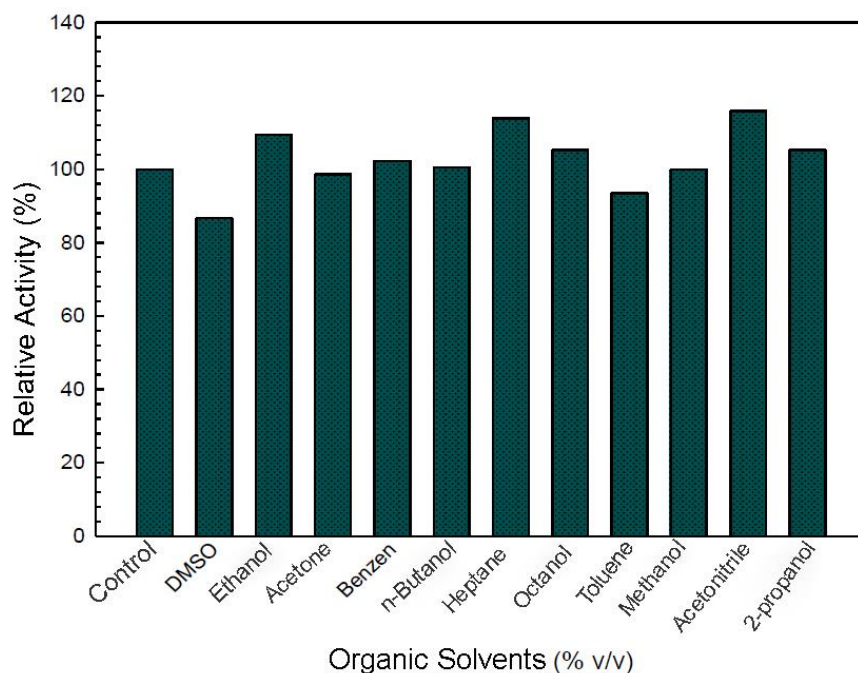
3.4. Results of Metal Ions on Protease Activity

The behavior of the purified enzyme against different metal ions prepared at different concentrations (2 and 5 mM) is shown in Figure 5. While Na^+, Ca^{2+}, Al^{3+} ve Ni^{2+} slightly increased protease activity at both concentrations, heavy metals such as Co^{2+} (2 mM) , Fe^{3+} and Sn^{2+} inactivated enzyme activity significantly Mn^{2+} increased the protease activity prominently up to 50% . The relative activity of the enzyme was improved to approximately 120% by Cu^{2+}. On the other hand, K^+ and Ba^{2+} did not make any contribution or decrease in enzyme activity (Figure 5).
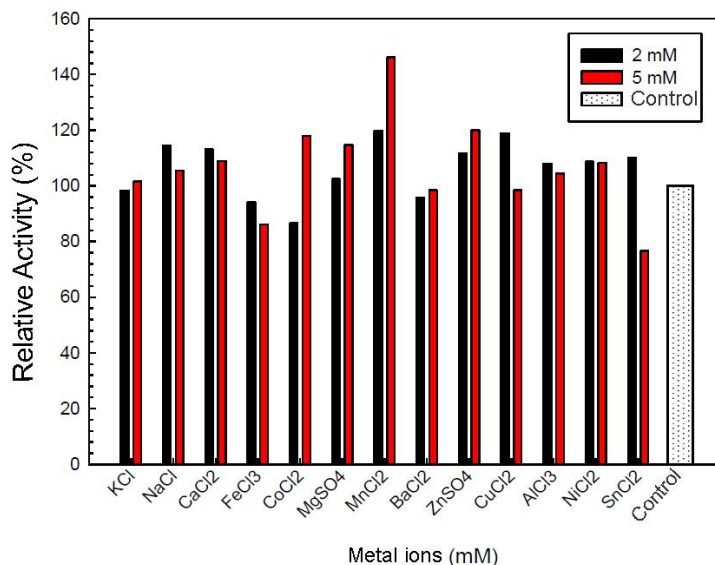
Figure 5. *Effect of various metal ions on activity of the B. subtilis M33 protease*

3.5. Results of Organic Solvents on Protease Activity

Organic solvents such as propanol, acetone, heptane, octanol, methanole, butanol, acetonitrile ,benzene toluene, ethanol, dimethyl sulfoxide (DMSO) have clearly influenced on protease stability from *Bacillus subtilis* M33. It was found that the purified enzyme retained its stability in the presence of most of the organic solvents, but only lost its activity in the DMSO medium by about 15%. Only acetonitrile and heptane were observed to cause a partial increase in enzyme activity for 60 min (Figure 6).

Figure 6. Effect of organic solvents on protease activity

3.6. Results of Inhibitors, Surfactants and Oxidizing Agents on Protease Activity

In order to define the chemical nature of the catalytic sites of the purified protease, the enzyme was tested with various inhibitors. The metalo protease inhibitor, EDTA, did not inhibit the purified protease, but serine protease (2 mmol L−1 and 5 mmol L^{-1} PMSF) inhibitor strongly inhibited the enzyme. (Table2). These results emphasize that the enzyme purified from *Bacillus subtilis* belongs to the serine protease family.
While enzyme activity was slighty increased presence of DTT, mercaptoethanol was significantly (204%) enhanced protease enzyme activity. The relative enzyme activity was determined as 90.68%, in the presence of SDS (sodium dodecyl sulfate) which is an anionic surfactant. Nonionic detergent surfactants (Triton X-100 and Tween-80) and H_2O_2 did not affect enzyme activity seriously.

Table 2. *The effects of some inhibitors, detergent surfactants and oxidizing agents on protease activity*

Chemicals	Concentrations	Relative Activitiy (U/mL)
PMSF	2 mM	5.65
PMSF	5 mM	2.61
EDTA	2 mM	90.02
EDTA	5 mM	79.48
DTT	2 mM	119.68
DTT	5 mM	124.88
2-mercaptoethanol	0.1%	204.98
SDS	0.2%	90.68
Triton X-100	0.1%	105.35
Tween 80	1.0%	108.67
H2O2	5.0%	103.03
Control		100

3.7. Results of Substrate Specificity

Table 3 shows the interest of the purified enzyme to various substrates. The maximum enzyme activity was obtained in presence of the casein (%100). It is emphasized that soybean meal is the least preferred substrate with the lowest activity it possesses. (16.94%)

Table 3. *The activity of protease purified from B. subtilis M33 in the presence of some substrates*

Substrates	Relative Activitiy (U/mL)
Casein	100
BSA	61.22
Ovalbumin	32.85
Gelatin	39.59
Soybean meal	16.94

3.8. Detergent Stability

Some international and local detergent brands were tested to investigate its effect on enzyme activity in the presence of tap water at 30°C. Purified protease was retained its whole activity in the presence of Tode and Art. The enzyme is relatively stable in the presence of commercial detergent called Tursil (86%), but it appears that there is a serious activity decrease in the presence of OMO brand (loss in 60%) within 1 h at 30°C (Table 4).

Table 4. *The effects of some industrial detergents on the activity of purified protease from B. Subtilis M33*

Detergent brand	Relative Activitiy (U/mL)
Bingo	65.26
Ariel	57.54
Tode	103.85
Art	93.51
OMO	39.1
Tursil	86.32
Control	100

3.9. Kinetic Parameters of Purified Enzyme

The two important parameters of enzyme kinetics, Km and Vm, were found to be . 0.706 mg/mL and 3000 μM.min^{-1} respectively, with the assist of the Lineweaver-Burk plot against different casein concentrations (Figure 7).

y= 0.0013+0.0018
R^2 = 0.991511
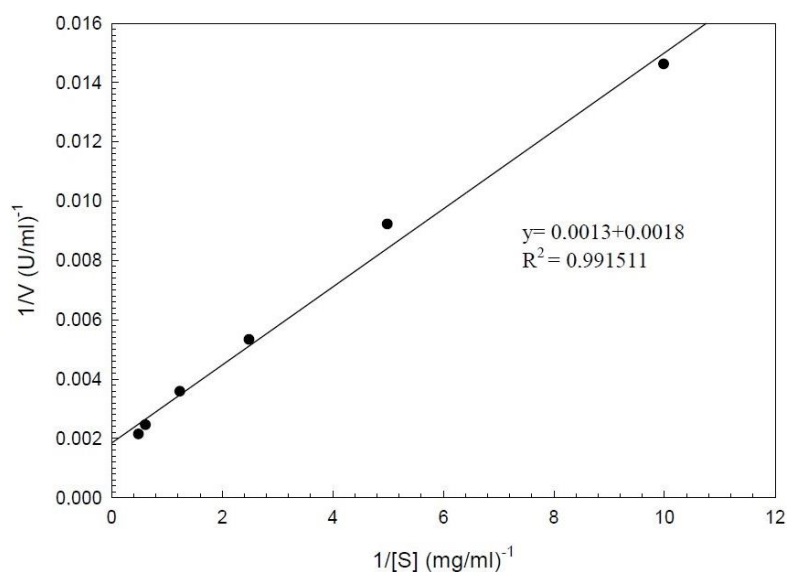
Figure 7. *Lineweaver-Burk graph of the protease enzyme purified from B. subtilis M33 is obtained by casein substrate*

4. DISCUSSION

Alkaliphilic and neutrophilic *Bacillus* species are alkaline protease producers at higher rates. These enzymes have wide application in many fields of industry such as detergent, leather, food, waste treatment, peptide synthesis because of its high catalytic activity, high substrate specificity and increased product capacity [6, 24-25]. Due to proteases are highest value commercial enzyme, it is the driving force for purification of novel proteases from different sources.

In this study, a novel extracellular alkaline protease was purified from *Bacillus subtilis* M33 isolated Ankara Ahlatlıbel (Turkey) soil and characterized. One of the basic criteria for enzyme purification is to isolate the enzyme in as few steps as possible. Purification of protease (35-80%) was carried out in two steps by ammonium sulphate precipitation and DEAE cellulose anion exchange chromatography equilibrated with pH 9.5 glycine-NaOH buffer. Alkali proteases are generally positively charged, therefore do not bind to anion exchange chromatography, such as DEAE cellulose. Gel filtration chromatography and DEAE anion exchange chromatography are among the most preferred methods for the purification of proteases as summarized in Table 5. In present study, the enzyme was found in unbound fractions of DEAE column and was eluted with pH 9.5 glycine-NaOH buffer.

Table 5. *The literature comparison of protease purification with various methods*

Purification method	Recovery (%)	Purification fold	Reference
DEAE cellulose ion exchange	30.16	39	10
DEAE cellulose ion exchange	26.2	18.5	18
DEAE-cellulose ion exchange	29	11	25
DEAE-Sephadex A-50	26.9	---	36
DEAE-Sepharose	37.91	10.48	31
DEAE-Sephadex G-50	18.6	15.6	26
DEAE cellulose anion exchange	38.66	15.50	Present study

Purification of the protease enzyme was also confirmed by the single band in the SDS PAGE stained by silver analysis. The molecular mass of the purified protease enzyme was determined to be about 39 kDa by both SDS PAGE and gel filtration chromatography. This result is consistent with many studies [6, 7, 10, 26-27] in the literature. The activity of many protease enzymes purified from *Bacillus* species is optimal in alkaline environment [4, 26, 28, 29]. One of the prominent parts of this study is that the purified enzyme retains its activity in buffers between pH 7.0-12.0 for a relatively long time. Because proteases used especially in the detergent industry are required to maintain their stability for a long time particularly in alkaline pH environments. The other important parameter for enzymes is to determine the optimum reaction temperature. In this study, the optimum temperature of the purified protease from *B. subtilis* M33 was found 55°C. Similar optimal temperatures for alkaline protease from *Bacillus* sp. have been reported [4, 19, 30]. However, it is also possible to appear proteases in the literature with a lower optimal temperature than present purified protease [31, 32]. In the temperature stability studies of purified protease from *Bacilus subtilis* M33, it was observed that the enzyme retained its activity at 40°C for 3 hours, at 50 and 55°C for about 50 minutes, however as temperature increases, activity decreases partially. This result indicates that the use of the purified protease in moderate temperature conditions is more appropriate. A similar result can be found in the temperature stability of the alkali serine protease purified from *B. mojavensis* A21[15]. In the experiment for identifying the catalytic nature of the purified protease, it was found that the enzyme was strongly inhibited by the serine protease inhibitör, PMSF, and was not influenced by the metalo protease inhibitor -EDTA. Thus, the influence of the PMSF on enzyme activity is an indication that it belongs to the serine alkaline protease family, whereas the enzyme activity is not affected by EDTA means that the enzyme does not need metal cofactors which shows that the purified protease does not belong to the metalloprotease family. EDTA is present in many detergents as a chelating agent due to its ability to soften water and to remove impurities, and it is desirable that the enzymes used in detergent formulations should not be affected by EDTA. It is an advantage that the purified protease exhibits high stability in the presence of EDTA as a detergent additive. Furthermore, the increased activity of the purified protease in the presence of reducing agents such as 2-mercaptoethanol and DTT (dithiothreitol) shows that the purified

protease enzyme is thiol dependent. However, when the activity of protease in the presence of various metal ions was examined, it was determined that Fe^{3+} and Sn^{2+} metals caused inhibition whereas the other metal ions such as Mn^{2+}, Mg^{2+}, Co^{2+} was found to increase the activity of the protease enzyme. The results show that metal ions probably enhance the binding affinity of the protease to the active site of substrate (casein). It is known that alkaline proteases are required for maximum activity to divalent cations such as Ca^{2+}, Mg^{2+} and Mn^{2+}, or their combinations [21]. Literature reports show similarity with present purified protease in particular that Ca^{2+}, Mg^{2+} and Mn^{2+} ions play a positive role in the regulation of the protease enzyme activity [6, 13, 14]. Probably, Ca^{2+}, Mg^{2+} and Mn^{2+} increase the stabilization of the active site of casein while the other metals may have caused denaturation of the active site of the protein. The partially denatured active site reduces enzyme-substrate affinity and thus enzyme activity.

Some organic products with commercial designation are unstable in water and / or are insoluble in water. In this respect, the use of enzymes as catalysts in solutions where organic solvents are used instead of water has many advantages. However, many natural enzymes can easily denatureted in the presence of organic solvents. Therefore, enzymes that maintain their stability in the presence of organic solvents have potential applications in the industry. The stability of the alkaline serine protease purified from *B. subtilis* M33 was examined in presence of some organic solvents and it was found that the enzyme retained its almost whole stability expect DMSO. One of the prominent behaviour of the purified protease is the enzymatic stability exhibited in the presence of organic solvents and it is very important due to organic solvent-stable proteases are needed to the maximum extent, especially in synthetic reactions. The other remarkable point of this study is behavior of the protease in the presence of surfactants and oxidizing agents. The serine alkaline protease purified from *B. subtilis* M33 was found to retain its activity for 1 hour in the presence of anionic substance such as SDS, nonionic substance such as Triton X-100, Tween-80 and oxidants such as H_2O_2. From this results, it can be mentioned that the purified protease may have wide potential applications such as detergent formulations, removal of membrane fouling and removal of hair. The activity of the enzyme in the presence of industrial detergents has also been investigated since the serine alkaline protease purified from *B. subtilis* M33 retains its stability with surfactants and bleaches as discussed above. While maintaining enzyme activity in the presence of detergents such as Tode, Art; after the 1 hour incubation with OMO and Ariel brands have observed loss of enzyme activity dramatically.

Another point that is compatible with the literature is that the substrates of purified serine alkaline protease are most likely to be casein, BSA and ovalbumin, respectively [7, 13, 29]. However, it has been found that the purified enzyme can hardly hydrolyze the soybean meal.

Km (Michaelis-Menten constant) is a value indicating affinity of enzyme to the substrate. The enzyme with a relatively small Km value shows a high affinity for the substrate. The enzyme reaches its maximum rate by saturating at a low substrate concentration. The kinetic parameters Km and Vmax of the purified protease were determined by measuring the protease activity casein as a substrate 0.706 mg/ml, 3000 µM.min-1 respectively. These results show that the purified enzyme has a higher affinity and an effective catalytic role than many studies in the literature [33-36].

5. CONCLUSION

The novel alkaline serine protease was purified from *Bacillus subtilis* M33 by two steps: ammonium sulphate precipitation and DEAE cellulose anion exchange chromatography with relatively high yields which exhibits long-term stability at alkaline pH. The purified protease was stable in presence of organic solvents, anionic, nonionic surfactants and oxidants is a highlight of this study. But in the literature, it is very rare to find these enzymatic features together. In addition, the enzyme maintains its activity in the presence of some commercial detergents. When all these properties are combined, it can be said that the purified serine alkaline protease purified from *Bacillus subtilis* M33 is a candidate for use in detergent formulations.

CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

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