ISSN : 0974 - 7435 Volume 7 Issue 1

Trade Science Inc.

BioTechnology

An Indian Journal

FULL PAPER

BTAIJ, 7(1), 2013 [34-40]

Purification and characterization of protease enzyme from native isolate *Bacillus subtilis* and its compatability with commercial detergents

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ABSTRACT

An alkaline serine protease producing strain was isolated from local soil samples and identified based on morphological and biochemical characteristics as *Bacillus subtilis* NR18. The enzyme was purified in three step procedure involving ammonium sulfate precipitation, followed by gel filtration and ion-exchange chromatography. Through the process 13.7-fold increase in purity with a specific activity of 283.1 U/mg proteins was obtained. The molecular weight of the purified enzyme was found to be 21 kDa by SDS-PAGE. The enzyme was most active at 60C and pH 9.0. It was relatively stable between pH 7.0-10.0 and temperature between 40 and 60°C Influence of metal ions on enzyme activity revealed that, Ca2+, Mg2+ and Mn2+ slightly enhanced the enzyme activity; whereas Co2+, Fe2+, Hg2+ and Zn2+ strongly inhibited the enzyme activity. Among the protease inhibitors that were tested, the PMSF and DFP completely inhibited the enzyme activity, indicating that the protease is a serine protease. The enzyme retained more than 50% activity after incubation at different time intervals at 60°C in the presence of commercial detergents indicating its suitability for application in detergent industry.

KEYWORDS

Bacillus subtilis;
Alkaline serine protease;
Purification characterization.

INTRODUCTION

Enzymes are biocatalysts synthesized by living cells. They are protein in nature (exception- RNA acting as ribozyme) colloidal and thermo labile in character and specific in their action. Isolated enzymes were first used in detergents in the year 1941, their protein nature proven in 1926 and their large scale production in 1960s. Industrial enzymes business is steadily growing due to improved production technology engineered enzymes properties and their application fields. More than 3000 enzymes, which catalyze a wide variety of chemical reactions, are known. Although Louis Pasture recognized that fermentation is catalyzed by enzymes. Among six classes of enzymes, hydrolases (Proteases, Amylases, Cellulases and Xylanases) have wide range of biotechnological applications.

PROTEASE

Proteases are the single class of enzymes which occupy a pivotal position with respect to their applica-

tions in detergents, pharmaceuticals, brewing leather, food industry, peptide syntheses and waste treatment. Proteases represent one of the largest groups of industrial enzymes. Proteases ate obtained from plant, animal and microbial sources. Microbial proteases have more advantages when compared to plant and animal. Proteases form the microbes are easy to manipulate for getting highly stable enzymes, shorter time required for production and purification steps. From plants papain and ficin are important proteases, trypsin and chemotrypsin form animals and alkaline proteases form microorganisms like *Bacillus sp*, Microbacterium, *Pencillium sp, Nocardia, Aspergillus sp* etc.

Types of proteases

Serine proteases, Subtilisins, Cysteine proteases, Aspartic proteases Metallo proteases.

Applications of proteases

Proteases have large variety of applications, mainly in the detergent and food industries. Proteases are envisaged to have extensive applications in leather treatment and in several bioremediation processes. The worldwide requirement of enzymes for individual applications varies considerably. Proteases are used in pharmaceutical industries for preparation of medicines such as ointments for debridement of wounds. Etc. Proteases that are used in the food a detergent industries are prepared in bulk quantities and used as crude preparative. Whereas. Those used in medicine are produced in small amounts but require purification before they can be used.

MATERIALS AND METHODS

Collection of soil sample

Soil samples were collected from Local Garden, Hyderabad, A.P, India. The samples were collected in sterilized bottles for isolation of *Bacillus subtilis*.

Isolation of bacteria

The bacteria strain was isolated by serial dilution plate method described as follows.

1 gm of garden soil samples were taken into 9 ml of sterilized water blanks to get 10^{-1} dilutions. From these dilutions, 1ml was transferred to another 9ml sterile water and thus six dilutions (10^{-1}-10^{-6}) were maintained. 1 ml of aliquots was transferred from six dilutions to the corresponding labeled Petri plates. Triplicates were maintained for each dilution. The molten and cooled nutrient agar medium was poured into the Petri plates and rotated gently for proper distribution of the inoculum suspension with the medium. After solidification, the plates were incubated at 27 ± 1ºC for 48 hours. After incubation, the colonies were separated, sub cultures were maintained.

Maintenance of pure cultures

The identified colonies were sub cultured on nutrient agar slants and preserved at 4ºC. Sub-culturing was performed at one month interval.

Plate hydrolysis assay for production of protease

Plates were incubated at 37°C for 24 hours. A clear zone of skim milk hydrolysis gave an indication of protease producing organisms. Depending upon the zone of clearance, strain *Bacillus subtilis* was selected for further experimental studies. The isolated proteolytic strain was a spore-forming gram-positive rod, identified as *B. subtilis*, and it was designated as *B. subtilis*.

Extracellular protease production: Production of protease from *B. subtilis* was carried out in a medium containing the following: casein 2gms; peptone 5 gms; NaCl 5 gms and pH was adjusted to 8 and maintained at 37°C for 48 hours in a shaker incubator (140 rpm). After the completion of fermentation, the whole fermentation broth was centrifuged at 10000 rpm at 4°C, and the clear supernatant was recovered. The crude enzyme supernatant was subjected to further studies.

Protein assay

Protein was measured by the method of Lowry et al with bovine serum albumin (BSA) as the standard. The concentration of protein during purification studies was calculated from the absorbance at 750 nm.

Assay of protease Protease activity was assayed according to Anson using casein as the substrate [18]. The reaction mixture (7.0ml) contained 5.0 ml of Tris-Hcl buffer pH 8.5, 1.0 ml of 0.5% casein, and 1.0 ml of enzyme. After 30 min, of incubation at 37C, the reaction mixture is terminated by addition of 0.5 ml of 10 % TCA and kept in ice for 10 min, and contents are filtered through whatts man No-5 filter paper. To 2ml of

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filtrate, 5.0 ml of 0.2M Alkaline sodium carbonate and 1.0 ml of Folin & ciocalteu's phenol reagent was added and incubated at 37C for 30 min and absorbency was measured at 660 nm in UV Spectrophotometer (Anson et al).

Enzyme purification

The protease was purified by ammonium sulfate precipitation,gel filtration (Sephadex G-100) and ion-exchange chromatography (DEAE Sepharose CL-6B). The cell-free culture supernatant (crude enzyme) was precipitated with ammonium sulfate saturation (between 50% and 70% of saturation). The precipitate was collected by centrifugation at 13000 × g for 15 min at 40C, dissolved in minimal volume of 0.1% Tris-HCl buffer (pH 7.0) and dialyzed against the same buffer at 40C overnight. The dialysate was applied to a Sephadex G-100 colum (1.6 × 36 cm) equilibrated with Tris-HCl buffer, pH 7.8. The column was eluted at a flow rate of 1ml/min with a 1:1 volume gradient from 0.1M to 1M NaCl in the same buffer. The active fractions were pooled and appliedon a DEAE sepharose CL-6B column chromatography (2.6 ×20 cm) that had been equilibrated with 0.1 mM phosphate buffer (pH 7.8) containing 0.1M NaCl and then eluted at a flow rate of 1 ml/min. The active fractions were collected and lyophilized.

Gel electrophoresis

The homogeneity and molecular weight of the purified protease was determined by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Protein bands were visualized by staining with 0.2% Coomassie brilliant blue R-250 and destained by washing overnight with mixture of acetic acidmethyl alcohol-water (5:5:1 v/v). The molecular weight of the purified protease was determined by SDS-PAGE using a standard protein marker (Genei, India).

Characterization

(a) Effect of pH on enzyme activity

The activity of the crude and purified protease was measured at different pH values in the absence and presence of 10mM $CaCl_2$. The pH was adjusted using the following buffers (0.05M): phosphate (pH 5.0-7.0), Tris-HCl (pH 8.0), and glycine-NaOH (pH 9.0-12.0). Reaction mixtures were incubated at 40°C for 30 minutes, and the activity of the enzyme was measured. The purified enzyme was diluted in different relevant buffers (pH 5.0-12.0) and incubated at 40°C for 2 and 20 hours for enzyme stability. The relative activity at each exposure was measured as per assay procedure.

(b) Effect of temperature on enzyme activity and stability

The activity of the crude and purified enzyme was determined by incubating the reaction mixture at different temperatures ranging from 30°C to 90°C in the absence and presence of 10mM $CaCl_2$ for 30 minutes. To determine the enzyme stability with changes in temperature, purified enzyme was incubated at different temperatures (60°C, 70°C, and 80°C) in the presence of 10mM$CaCl_2$, and relative protease activities were assayed at standard assay conditions.

(c) Effect of various metal ions on protease activity

The effects of metal ions (eg, Ca^{2+}, Mg^{2+}, Al^{+3}, Co^{+2}, Cd^{+2}, Fe^{+3}, Na^{+}, Zn^{2+}, Hg^{2+}, and Cu^{2+} [5mM]) were investigated by adding them to the reaction mixture. Relative protease activities were measured.

(d) Detergent stability

The compatibility of *Bacillus subtilis* protease with local laundry detergents was studied in the presence of 10mM $CaCl_2$ and 1M glycine. Detergents used were Nirma (Nirma Chemical, India); Henko (Henkel Spic, India); Surf, Surf Excel, Super Wheel, Rin (Hindustan Lever Ltd, India); and Ariel (Procter and Gamble, India). The detergents were diluted in distilled water (0.7% wt/vol) and incubated with protease for 3 hours at 60°C, and the residual activity was determined. The enzyme activity of a control sample (without any detergent) was taken as 100%.

(e) Washing test with protease preparation

Application of protease (5000 U/mL) as a detergent additive was studied on white cotton cloth pieces (6 × 6 cm) stained with ink. The stained cloth pieces were taken in separate flasks. The following sets were prepared and studied:

1. Flask with distilled water (100 mL) + stained cloth (cloth stained with Ink).
2. Flask with distilled water (100 mL) + stained cloth

(cloth stained with Ink) + 1 mL wheel de-tergent (7 mg/mL).

3. Flask with distilled water (100 mL) + stained cloth (cloth stained with Ink) + 1 mL wheel de-tergent (7 mg/mL) + 2 mL enzyme solution.

The above flasks were incubated at 60°C for 15 minutes. After incubation, cloth pieces were taken out, rinsed with water, and dried. Visual examination of various pieces exhibited the effect of enzyme in removal of stains. Untreated cloth pieces stained with ink were taken as control.

RESULTS AND DISCUSSION

Bacterial culture

Figure 1 : *Bacillus subtilis* on nutrient agar medium.

Casein hydrolysis

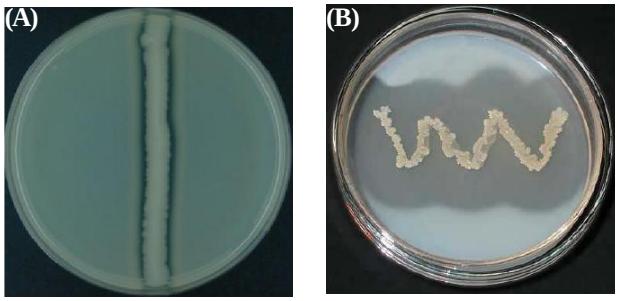


Figure 2 : (A)&(B) The zone of casein hydrolysis of *B. subtilis*.

Purification of extracellular protease of *B. subtilis* NR18

(a) Sephadex G-200 gel filtration chromatography

The protein pellet obtained after 60% saturation with ammonium sulphate was dissolved in 0.1M Tris-HCl buffer and loaded onto a column of Sephadex G-200 (1.5 × 24 cm) equilibrated with Tris-HCl buffer, pH 7.8. The elution profile of gel filtration chromatography is shown in (Figure 3).

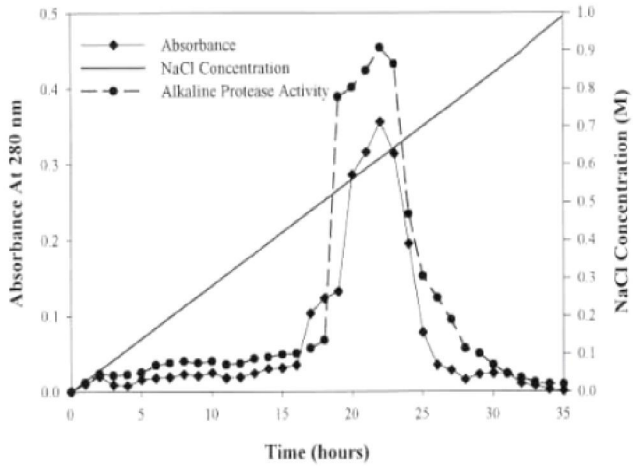
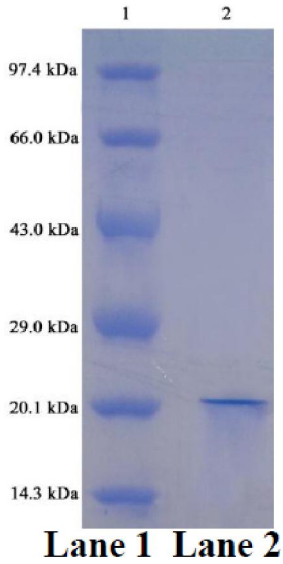


Figure 3 : Elution profile of *B subtilis* protease by sephadex G-200 column.



Lane 1 : Molecular marker Lane 2 : Purified protease (21 kda).

Figure 4 : SDS-PAGE showing the purified protease.

From the elution profile, it was observed that the protease was eluted as a well-resolved single peak of caseinase activity coinciding with a single protein peak at NaCl concentration of 0.6M. Fractions (19-23) with protease activities were pooled, dialyzed, and concentrated by lyophilization and used for further studies. The summary of purification steps involved for protease is presented in (TABLE 1).

SDS-PAGE of culture filtrate and purified protease from *B. subtilis* NR18

When the ammonium sulphate precipitation and purified protease were analyzed by SDS-PAGE, 6 bands were observed in the case of the ammonium sulphate

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precipitation, while purified protease showed a single band, indicating a homogeneous preparation. The molecular weight of the protease was determined by comparison of the migration distances of standard marker proteins. Depending on the relative mobility, the molecular weight of the protein band was calculated to be 21 kd, which coincided with the with the band of α-lactalbumin marker protein (Figure 4).

TABLE 1 : Summary of the purification profile for alkaline protease from *B. subtilis* NR18.

PURIFICATION STEPS OF ALKALINE PROTEASE					
Purification Step	**Total Enzyme Activity (U)**	**Total Protein (mg)**	**Specific Activity (U/mg)**	**Purification Fold**	**% Recovery**
Crude enzyme	50400	4920	10.2	1.0	100
(NH4)2SO4 precipita-tion, dialyzed	36250	3000	12.1	1.2	72.0
Sephadex G-200	3760	17.6	213.6	21.0	7.5

Effect of pH on enzyme activity

For the determination of the pH optimum, phosphate (pH 5.0-7.0), Tris-HCl (pH 8.0), and glycine-NaOH (pH 9.0-12.0) buffers were used in the absence and presence of 10mM $CaCl_2$. The highest protease activity was found to be at pH 10.0 using glycine-NaOH buffer (Figure 5).

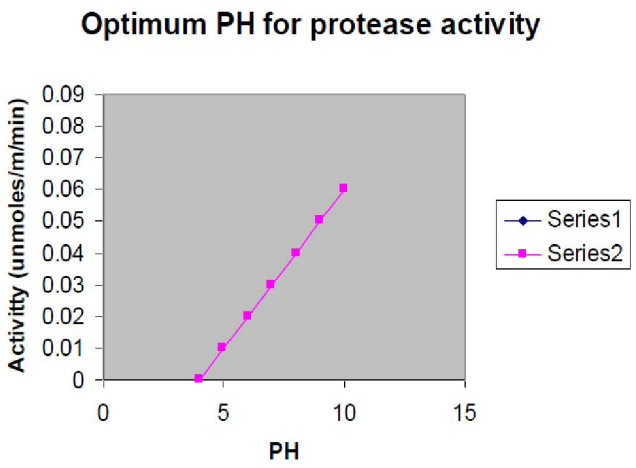

Figure 5 : Optimum pH for protease activity.

Effect of temperature on enzyme activity

The activity of the crude and purified enzyme was determined at different temperatures ranging from 30°C to 90°C in the absence and presence of 10mM $CaCl_2$. The optimum temperature recorded was at 60°C for protease activity. The enzyme activity gradually declined at temperatures beyond 60°C (Figure 6). The thermal stability of the purified protease was tested at different temperatures of 60°C, 70°C, and 80°C for different periods (50 to 350 minutes) in the presence of 10mM $CaCl_2$. The PE-11 protease had a half-life of 250 and less than 50 minutes at 70°C and 80°C, respectively. The enzyme was almost 100% stable at 60°C even after 350 minutes of incubation (Figure 6).

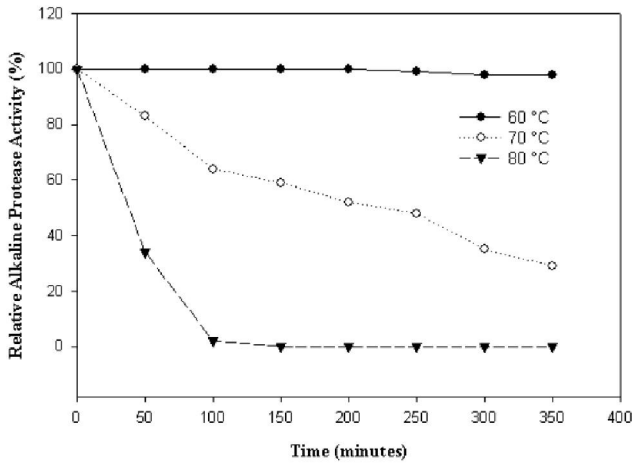

Figure 6 : Effect of temperature on the stability of the enzyme in the presence of 10mM $CaCl_2$.

TABLE 2 : Morphological and biochemical characteristics of the bacteria *B.subtilis* NR18.

Tests	Results
Shape of the cell	Rod
Gram reaction	G^{+ve}
Spore formation	+
Motility	+
Oxidase test	+
Catalase test	+
Voges-proskauer test	-
Methyl red test	+
Indole test	-
Citrate utilization test	+
H_2S production	-
Milk test	+
Hydrolysis of glucose	+
Hydrolysis of starch	+
Hydrolysis of sucrose	+

(+)=Positive; (-)=Negative

Effect of metal ions

Most of the metal ions tested had a stimulatory effect (Ca^{2+}, Mg^{2+}, and Mn^{2+}) or a slight inhibitory effect (other ions) on enzyme activity (TABLE 3). Some

of the metal ions such as Ca^{2+}, Mg^{2+}, and Mn^{2+} increased and stabilized the protease activity of the enzyme; this is possible because of the activation by the metal ions. These cations also have been reported to increase the thermal stability of other *Bacillus* alkaline proteases. These results suggest that concerned metal ions apparently protected the enzyme against thermal denaturation and played a vital role in maintaining the active confirmation of the enzyme at high temperatures. Other metal ions such as Zn^{2+}, Cu^{2+}, Hg^{2+}, Co^{2+}, Na^{+}, Cd^{2+}, Al^{3+}, and EDTA did not shown any appreciable effect on enzyme activity.

TABLE 3 : Effect of various metal ions and inhibitors (5 mM) on the activity of alkaline protease of *B. subtilis* NR18.

Metal ions/inhibitors	Relative activity (%)
Control	100
Al^{2+}	89.5
Ca^{2+}	127.3
Co^{2+}	11.6
Cu^{2+}	96.0
Fe^{2+}	19.3
Hg^{2+}	23.5
Mg^{2+}	113.7
Mn^{2+}	123.1
Zn^{2+}	27.3
EDTA	87.0
DFP	0
DTT	71.6
PMSF	0

Compatibility with detergents

Besides pH, a good detergent protease is expected to be stable in the presence of commercial detergents. The protease showed excellent stability and compatibility in the presence of locally available detergents (Nirma, Wheel, Henko, Surf, Surf Excel, Ariel, and Rin). Protease from B subtilis showed stability and compatibility with a wide range of commercial detergents at 60°C in the presence of $CaCl_2$ and glycine as stabilizers. Our protease showed good stability and compatibility in the presence of Wheel followed by Surf excel (TABLE 4). The enzyme retained more than 50% activity with most of the detergents tested even after 3 hours incubation at 60°C after the supplementation of $CaCl_2$ and glycine. The compatibility of alkaline protease was studied with Wheel in the presence of 10mM $CaCl_2$ and 1M glycine for different periods (0.5 to 3 hours) at 60°C. The enzyme retained about 67% activity after 1.5 hours in the presence of Wheel at 60°C and was almost inactivated after 3 hours in the absence of any stabilizer (Figure 7). However, the addition of $CaCl_2$ (10mM) and glycine (1M), individually and in combination, was very effective in improving the stability, where it retained 52% activity even after 3 hours. So it was used as an additive in detergent, to check the contribution of the enzyme in improving the washing performance of the detergent. The supplementation of the enzyme preparation in detergent (ie, Wheel) could significantly improve the cleansing of the ink stains (Figure 7). Washing test with protease preparation.

TABLE 4 : Compatibility of alkaline protease activity with commercial detergents in the presence of 10mM CaCl2 and glycine at 60°.

Relative Residual Alkaline Protease Activity (%)								
Time (hour)	Control	Nirma	Wheel	Henko	Surf	Surf Excel	Ariel	Rin
0.0	100	100	100	100	100	100	100	100
0.5	96	90	95	92	91	93	88	87
1.0	94	87	92	89	87	90	85	83
1.5	91	85	89	86	83	87	82	81
2.0	87	79	82	79	75	80	74	72
2.5	80	69	73	68	63	70	61	59
3.0	76	58	65	58	51	56	52	53

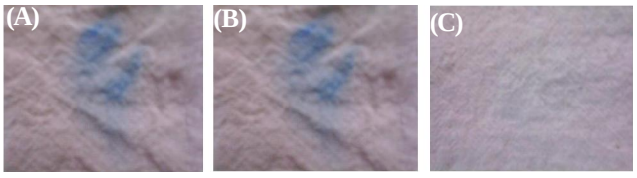

Figure 8 : Washing performance of protease from *B. subtilis* in the presence of detergent(Wheel). (A): Cloth stained with ink (B): Ink-stained cloth washed with detergent only (C): Ink-stained cloth washed with detergent and enzyme.

CONCLUSION

The alkaline protease isolated from *B subtilis* is a thermostable serine protease. It is stable at alkaline pH, at high temperatures, and in the presence of commercial detergents and is compatible with commercial and local detergents. These properties indicate the possibilities for use of the protease in the deter-gent industry. This enzyme can be exploited commercially.

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