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RESEARCH ARTICLE

PURIFICATION AND CHARACTERIZATION OF AN EXTRACELLULAR ALKALINE SERINE PROTEASE FROM *Bacillus subtilis* NR18

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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 50^0C and pH 9.0. It was relatively stable between pH 7.0-10.0 and temperature between 40 and 50^0C. Influence of metal ions on enzyme activity revealed that, Ca^{2+}, Mg^{2+} and Mn^{2+} slightly enhanced the enzyme activity; whereas Co^{2+}, Fe^{2+}, Hg^{2+} and Zn^{2+} 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 60 min incubation at 50^0C in the presence of commercial detergents indicating its suitability for application in detergent industry.

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INTRODUCTION

Proteases are one of the most important industrial enzymes and accounting for the 60-65% of total global industrial enzyme market (Trehan, 1997). Of these, 25% is constituted by alkaline proteases, 3% by trypsin, 10% by rennin and 21% by the other proteases (Rao *et al*., 1998; Beg *et al*., 2003; Ellaiah *et al*., 2003). Among these, the alkaline proteases produced by microorganisms are of interest from a biotechnological perspective, and are investigated not only in scientific fields of protein chemistry and protein engineering but also in applied fields such baking, brewing, detergents, leather processing, pharmaceuticals, waste treatment and peptide synthesis (Kim *et al*., 1997; Kumar and Takagi, 1999; Huang *et al*., 2003). The proteolytic enzymes hydrolyse the peptide links of proteins and peptides to form smaller subunits of amino acids and are produced both extracelluraly as well as intracellularly (Gajju *et al*., 1996; Kumar *et al*., 2002). The proteases of industrial importance are obtained from plants, animals and microorganisms. A number of microorganisms such as bacteria (Shalinisen and Satyanarayana, 1993), fungi (Haab et al., 1990), yeast (Ogrydziak and Yamada, 1983), and actinomycetes (Lee *et al*., 1996; Vonothini *et al*., 2008) have been reported for protease production. Among the various proteases, bacterial proteases are the most significant, compared with plant, animal and fungal proteases, and accounting for 20% of the world market with predominant use in detergents especially for alkaline bacterial proteases (Ward, 1985; Lee *et al*., 1992; Shumi *et al*., 2004). Recently, large portions of commercial alkaline proteases are available from the *Bacillus* species (Srinivasan *et al*., 2009). In recent years the use of alkaline protease as an industrial catalyst has increased. These enzymes exhibit high catalytic activity and are economically feasible. In the present study, purification and characterization of an extracellular alkaline serine protease produced by *Bacillus subtilis* isolated from soil are described.

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MATERIALS AND METHODS

Isolation of protease producing bacteria

Soil samples were collected from various locations of Hyderabad, India, and were serially diluted in sterile distilled water. The diluted samples were plated (0.1 ml suspension) onto skim milk agar plates containing casein peptone 5g/l, yeast extract 2.5g/l, glucose 1g/l, skim milk powder 1g/l, agar 15g/l and incubated at 37^0C for 48 h. The colonies with clear zone of skim milk hydrolysis gave an indication of protease producing organisms. Depending upon the zone of clearance,

the isolate NR18 showing the maximum activity on the plates were selected as potential strains and subjected to morphological and biochemical identification. The protease producing bacterial strain was identified according to Bergey's Manual of Determinative Bacteriology (Buchanan *et al*., 1974). The stock culture of the isolate was maintained in glycerol (50% v/v) and stored at -20^{0}C in a deep freezer. The culture was received from the stock in nutrient broth followed by streaking on nutrient agar plates.

Enzyme production

The culture medium used for protease production in this study contained peptone (0.75%), glucose (0.5%), $MgSO_4.7H_2O$ (0.2%), KH_2PO_4 (0.5%), and $FeSO_4.7H_2O$ (0.01%). The pH of the medium (7.5) was adjusted with 1N NaOH or 1N HCl. The culture medium (100 ml in 250 ml Erlenmeyer flasks) was inoculated at 2% with 24 h seed culture and incubated at 30^{0}C for 48 h on a rotary shaker (150 rpm). At the end of the fermentation, the whole fermentation broth was centrifuged at 10000 rpm for 10 min and the clear supernatant was used as the crude enzyme source.

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 4^{0}C, dissolved in minimal volume of 0.1% Tris-HCl buffer (pH 7.0) and dialyzed against the same buffer at 4^{0}C 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 applied on 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.

Protease assay

The protease activity was assayed using casein as the substrate, according to the method of Sarath *et al* (1989). The reaction mixture consisted of 0.25 ml of a 50 mM sodium phosphate buffer (pH 7.8), containing 2% (w/v) casein and 1 ml of the enzyme solution and incubated at 37^{0}C for 30 min. After incubation, the reaction was stopped by adding 3 ml of 10% TCA. The precipitate was then removed by centrifugation at 8000 rpm for 5 min. Next, 1.4 ml of 1M NaOH was added to 1.5 ml of the supernatant and the absorbance measured spectrophotometrically at 280 nm. One unit of protease activity represents the amount of enzyme required to liberate 1 µg of tyrosine under standard assay conditions.

Protein assay

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Gel electrophoresis

The homogeneity and molecular weight of the purified protease was determined by sodium dodecyl sulfate-polyacrylamide 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 acid-methyl 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

Effect of pH on enzyme activity

The effect of pH on protease activity was determined by inoculating the purified protease at different pH levels (5 to 12) under standard assay conditions using casein as the substrate. The pH of the reaction mixture was adjusting to the desired value using the following 50 mM buffers; phosphate (pH 5.0 – 7.0), Tris-HCl (pH 8.0), and glycine-sodium hydroxide (pH 9.0 – 12.0). The pH stability of the protease was determined by pre-incubating the enzyme in the above mentioned buffers for 1 h at 37^{0}C.

Effect of temperature on enzyme activity

The effect of temperature for the protease activity was determined by performing the standard assay procedures at different temperature ranging from 20 to 80^{0}C. To determine the enzyme stability, the enzyme was incubated at different temperatures for 1 h and then relative activities were assayed at standard assay conditions.

Effect of different metals and inhibitors on protease activity

The effect of metal ions on the purified enzyme were determined by treating with different metals ions including Al^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} at concentration of 5 mM for 30 min at room temperature. The protease inhibitors, namely, ethylene diamine tetra acetic acid (EDTA), diisopropyl fluorophosphates (DFP), dithiothreitol (DTT), and phenyl methyl sulfonyl fluoride (PMSF) were also tested against the enzyme under optimum reaction conditions. Aliquots of the protease were pre-incubated with different protease inhibitors at concentration of 5 mM for 30 min at room temperature and the residual activity of the enzyme was assayed.

Compatibility with commercial detergents

The compatibility of alkaline protease in the presence of some commercial laundry detergents (Ariel, Henko, Nirma, Surf Excel, Rin, and Tide) was studied in the presence of 10 mM $CaCl_2$ and 1M glycine. The detergents were diluted in distilled water (7 µg/ml) and incubated with protease for 1 h at 50^{0}C, and the residual activity was determined. The enzyme activity of the control sample (without detergent served as control) was taken as 100%.

RESULTS AND DISCUSSION

The isolate NR18 was characterized as *Bacillus subtilis* based on its morphological, and biochemical analysis (Table 1). The

Table 1. Morphological and biochemical characteristics of the strain *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

Table 2. Summary of the purification profile for alkaline protease from *B. subtilis* NR18

Purification Steps	Total Protein (mg)	Total enzyme activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude	962.5	1463.0	3.7	1.0	100
Ammonium sulfate	363.2	591.0	9.5	1.1	74.5
Sephadex G-100	17.5	173.1	27.3	5.9	37.9
DEAE-sepharose	0.9	100	283.1	13.7	7.3

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

Table 4: Compatibility of alkaline protease activity with commercial detergents in the presence of 10 mM $CaCl_2$ and Glycine at 50^0C

Detergents	Residual activity (%)
None	100
Ariel	81
Henko	87
Nirma	89
Surf Excel	79
Rin	73
Tide	67

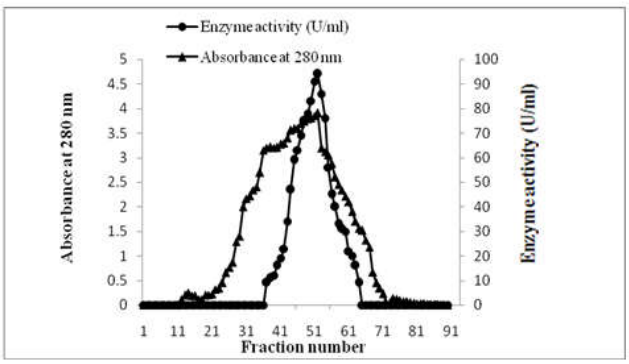

Fig. 1: Purification profile of protease from *B.* subtilis NR18 by DEAE Sepharose CL-6B column chromatography

isolated proteolytic strain was strictly aerobic, motile, Gram-positive, spore forming and rod-shaped. It reacted positively in the catalase and oxidase tests. Therefore, it was designated as *Bacillus subtilis* NR18.

Purification of protease

The protease produced by *B. subtilis* NR18 was concentrated by ammonium sulfate precipitation and purified consecutively with gel filtration and ion-exchange chromatography. The precipitate was adsorbed by a Sephadex G-100 column, and eluted at 0.1M to 1M NaCl in the buffer. The concentrated-active fractions were further purified by a DEAE sepharose CL-6B column chromatography. The elution profile of ion-exchange chromatography is shown in Figure 1. The results of

the purification procedure are summarized in Table 2. After the final purification step, the enzyme was purified 13.7-fold with a recovery of 7.3% and specific activity of 281.1 U/mg of protein. The purified protease appeared as a single protein band in SDS-PAGE and with molecular weight approximately 21 kDa (Fig. 2). Similarly Ramakrishna *et al* (2010) reported the weight of the alkaline protease from the *B. subtilis* MTTC-10110 as 20.5 kDa. Different molecular masses that ranged from 15-35 kDa have been reported for other bacterial alkaline proteases (Adinarayana *et al*., 2003; Ahmed *et al*., 2011; Ramakrishna *et al*., 2011).

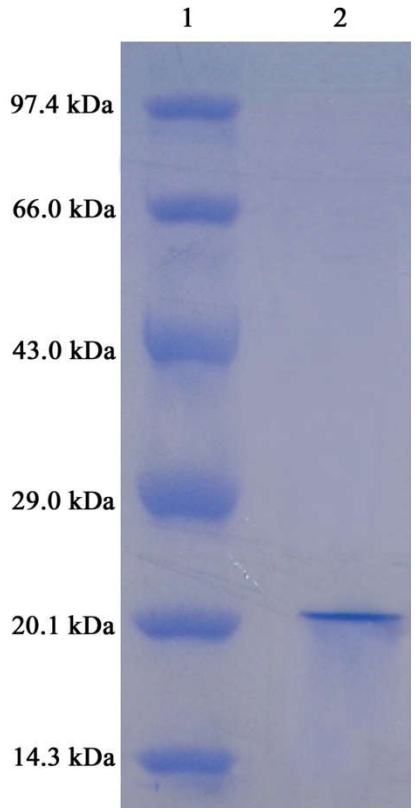

Fig. 2: SDS-PAGE of purified enzyme
Lane 1: Molecular weight marker protein; **Lane 2:** Purified protease

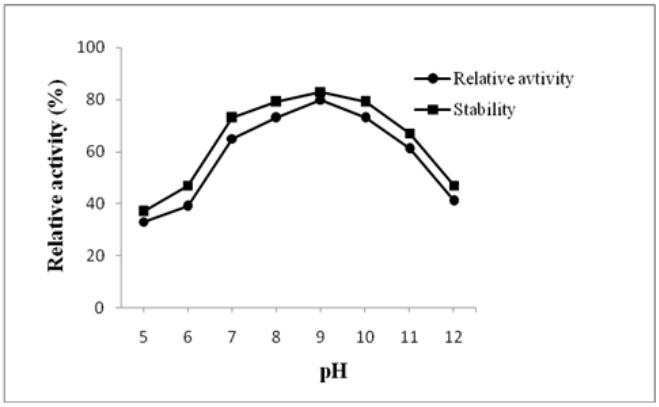

Fig. 3: Optimal pH (•) and stability of pH (■) of purified protease from *B. subtilis* NR18

Effect of pH on enzyme activity

The effect of pH on the activity of protease was studied with various pH from 5 to 12 (Fig. 3). The enzyme was active in the pH range of 7-11, with optimum activity at pH 9.0, suggesting that it is an alkaline protease. It was relatively stable at pHs between 7 to 10. These findings are in accordance with several earlier reports showing pH optimum of 9.0 to 9.5 for protease from *Bacillus* sp., *B. subtilis*, and *B. subtilis* SVR-07 (Saurabh *et al*., 2007; Rao *et al*., 2008; Narsi Reddy *et al*., 2011).

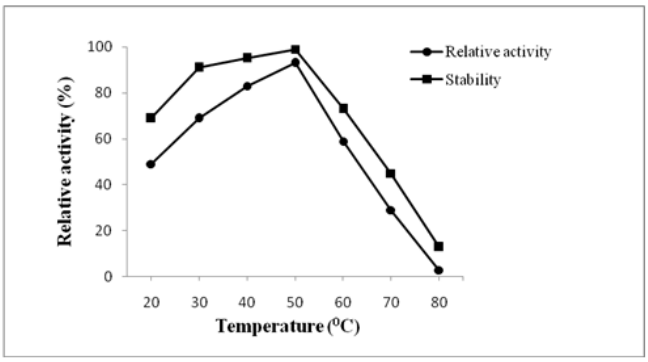

Fig. 4: Optimal temperature (•) and stability of temperature (■) of purified protease from *B. subtilis* NR18.

Effect of temperature on enzyme activity

The protease activity was determined at different temperatures ranging from 20 to 80^{0}C. The optimum temperature was found to be 50^{0}C (Fig. 4), and the activity decreased rapidly above 60^{0}C. Rao *et al* (2008) have also reported similar results. Giri *et al* (2011) reported an optimum temperature of 50^{0}C for alkaline protease from *B. subtilis* VSG-4. The thermal stability of the purified enzyme was kept at various temperatures for 60 min, it was significantly inactivated 60^{0}C and completely at 80^{0}C. The high temperature inactivation may be due to incorrect conformation due to hydrolysis of the peptide chain, destruction of amino acid, or aggregation (Schockker and Van Boekel, 1999).

Effect of metal ions and inhibitors

The influence of various metal ions and inhibitors, at a concentration of 5 mM, on the activity of the enzyme was studied (Table 3). The purified enzyme was slightly activated by metal ions such as Ca^{2+}, Mg^{2+} and Mn^{2+}. It was observed that activity of the enzyme was strongly inhibited by Co^{2+}, Fe^{2+}, Hg^{2+} and Zn^{2+} and was less sensitive to Cu^{2+} and Al^{2+} ions showed a slightly inhibitory effect on enzyme activity. Among the different protease inhibitors, PMSF and DFP were completely inhibiting the enzyme. In this regard, PMSF sulphonates the essential serine residue in the active site of the protease and has been reported to result in the complete loss of the enzyme activity (Gold and Fahrney, 1964). Results were similar to those of Kembhavi *et al* (1993) and Giri *et al* (2011), where the protease was completely inhibited by PMSF. This indicated that the protease produced by *B. subtilis* NR18 belongs to the serine protease group.

Compatibility with commercial detergents

The protease from *B. subtilis* NR18 showed good stability and compatibility with a wide range of commercial detergents at 50^{0}C in the presence of 10 mM $CaCl_2$ and glycine as stabilizer. The enzyme retained more than 50% activity with

most of the detergents tested even 60 min of incubation at 50^0C (Table 4). Bhosale *et al* (1995) reported high activity alkaline protease from *C. coronatus* showing compatibility at 50^0C, in the presence of 25 mM $CaCl_2$ with a variety of commercial detergents. Rajkumar *et al* (2011) studied the stability of protease from *Bacillus* sp RRM1 retained about 41% and 100% of its activity in various surfactants and detergents.

CONLUSION

From the results obtained in this study, a novel extracellular alkaline protease was isolated from *B. subtilis* NR18. The molecular weight of the purified protease was 21 kDa. The enzyme acted optimally at pH 9.0 and 50^0C. The enzyme showed compatibility with various commercial detergents tested in the presence of $CaCl_2$ and glycine. Therefore, these properties make the enzyme suitable for application in detergent industry. It can be concluded that *B. subtilis* NR18 can be industrially exploited for the synthesis of alkaline serine protease and strain improvement studies can be carried out to enhance enzyme production.

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