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Purification and characterization of SDS stable protease from Bacillus safensis strain CK

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ABSTRACT

Proteases are widely used in chemical, medical and food industries. Microbial proteases play an important role in the cellular metabolic processes and have gained considerable attention by industrial communities. Hence, in the present work we have identified and investigated role of novel *Bacillus safensis* strain CK to produce an extracellular protease. The isolated protease was purified by Diethyl amino ethane (DEAE) cellulose column chromatography with 7 fold purity and 8.2% yield. Molecular weight of the protease estimated to be around 40 kDa and showed activity over a wide range of pH, from neutral to alkaline. The purified protease showed 100% and 92% stability in presence of 1% Sodium Dodecyl Sulphate (SDS) and commercial detergent respectively. Thus, *B. safensis* strain CK has potential to produce an extracellular protease, active at neutral and alkaline pH. This protease may be useful in various industrial processes.

1. Introduction

Proteases play an important role in the cellular metabolic processes and have gained significant attention by industrial communities (Rao et al., 1998; Bruins et al., 2001). It has been shown that several animals, plants and microbes are the principal sources of proteases (Shafee et al., 2005). However, production of proteases from plant and animal sources ha some limitations because of climatic reasons and ethical issues respectively (Rao et al., 1998; Shafee et al., 2005). The microbial proteases have two-third share of commercial production in the enzyme market worldwide (Kumar and Takagi, 1999).

Microorganisms of the genera *Pyrococcus, Thermococcus and Staphylothermus*are considered as prominent producers of proteases and metalloproteases (Niehaus et al., 1999). However, the genus *Bacillus* is the center of attraction in biotechnology due to relative ease of isolation from diverse sources like soil, alkaline waters, and the deep sea, and the ability to grow on proteinaceous substrates (Longo et al., 1999). Microbial sources have occupied an unshakable domain in the production of all the three major types of proteases - acidic, neutral, and alkaline. Neutral and alkaline proteases hold great potential for the application in the detergent and leather tanning industries due to the increasing trend of developing eco-friendly technologies (Rao et al., 1998).

At present there is considerable interest in enzymes which should be stable in presence of oxidizing agents and bleaching of detergents (Oberoi et al., 2001). There is always need for the novel enzymes that can enhance the washing performance of the enzyme-based detergents. Currently bleach stability is introduced into the enzyme through site directed mutagenesis and protein engineering by replacement of certain amino acid residues (Wolff et al., 1996). So, present study describes purification and characterization of extracellular SDS stable protease from novel *B. safensis* strain CK.

2. Materials and methods

2.1. Isolation and identification of protease producing microorganism

Soil from slaughter house waste was used as source for screening of protease producing microorganisms. Serially diluted soil sample was spread on medium containing gelatin – 1.2%, peptone – 0.4%, yeast extract – 0.1%, agar – 2% and incubated at 37 °C for 24 h. The identification of the strain was carried out on the basis of morphological, biochemical characteristics and the 16S rDNA gene sequence analysis. All the biochemical tests were performed according to the methods described in *Bergeys Manual of Systematic Bacteriology*. The 16S rDNA sequencing was done at GeNei, Bangalore, India. Sequence comparison with gene sequence database was performed using BLASTn program through the NCBI website (Altschul et al., 1997).

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The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). A phylogenetic tree was constructed with MEGA version 4.0 (Tamura et al., 2007). The sequence has been deposited in the GenBank database under accession no. KJ427751.

2.2. Protease production

The 24 h. fresh culture of isolated *B. safensis* CK was inoculated into broth containing peptone – 0.4%, yeast extract – 0.1%, gelatin – 1.2%. Flasks were incubated at 37 °C for 48 h at static condition. Protease production was monitored at an interval of 12 h.

2.3. Protease assay

Protease activity was quantitatively tested according to method described by Tran and Nagano (Tran and Nagano, 2002). The reaction mixture contained 0.5 ml of (0.2%) gelatin, 1.4 ml 50 mM phosphate buffer (pH- 7.0) and 0.1 ml enzyme incubated for 20 min. The reaction was stopped by addition of 2 ml of (10%) Trichloroacetic acid at room temperature. The released free amino acids were determined by Ninhydrin method at 530 nm (Rosen, 1957). One unit of enzyme activity was defined as the amount of enzyme required to release 1 µg leucine per min under standard experimental conditions.

2.4. Ammonium sulphate precipitation

The fresh culture of *B. safensis* was inoculated into broth. After 48hrs of incubation the supernatant was obtained by centrifugation at 8000 rpm for 15 min at 4 °C. The collected supernatant was subjected to ammonium sulphate precipitation. Ammonium sulphate was added to the supernatant in such amount so as to achieve 30% to 80% saturation. The mixture was kept overnight in refrigerator and precipitate was collected by centrifugation at 8000 rpm for 30 min at 4 °C. The residue was dissolved in the 50 mM sodium-phosphate buffer (pH-7.0) and dialyzed against the same buffer for overnight.

2.5. Purification by ion-exchange chromatography

Purification of protease was carried out by the DEAE-cellulose ion exchange column chromatography. The column was packed with activated DEAE-cellulose equilibrated with 50 mM sodium-phosphate buffer. The height of column was 20 cm with diameter of 2.5 cm. The protein was eluted with the 0.1–0.5 M NaCl gradient and 50 fractions were collected having 5 ml volume with the flow rate of 1 ml min^{-1}. All the purification steps were carried out at 4 °C. The collected fractions were checked for their protein content by taking an absorbance at 280 nm with a UV visible double beam spectrophotometer.

2.6. Characterization of enzyme

2.6.1. SDS-PAGE analysis

Purity of the fraction, showing protease activity, was checked by Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PA GE) by the method of Laemmli (Laemmli, 1970). The bands were visualized by Coomassie Brilliant blue R-250. The molecular weight of protease was determined by comparison with standard molecular marker proteins (Phosphorylase b 98 kDa, Bovine Serum Albumin 66 kDa, Ovalalbumin 43 kDa, Carbonic Anhydrase 29 kDa, Soyabean Trypsin Inhibitor 20 kDa).

2.6.2. Effect of pH and temperature on enzyme activity

The optimum pH was determined by incubating enzyme in wide range of pH from 5 to 12, using 50 mM sodium acetate buffer (pH 5.0), sodium-phosphate buffer (pH 6.0–8.0), glycine- NaOH buffer (pH 9.0–12.0) under standard assay conditions. Enzyme activities were also carried out at different temperatures ranging from 20 °C to 60 °C and optimal temperature was determined. Enzyme activity was expressed as % relative activity.

2.6.3. SDS and detergent stability

Purified enzyme was subjected to study the stability of protease in presence of SDS and commercial detergent. The enzyme assay was performed in presence of various concentrations of SDS such as 0.1%, 0.5%, 1.0% and 1% wheel as per protocol discussed in protease assay section and enzyme activity expressed as % relative activity.

2.7. Statistical analysis

The mean of three determinants were calculated and standard deviation represented in graphs and figures. Analysis of variance was carried out on all data at $p < 0.05$ using Graph Pad software.

3. Results

3.1. Identification of microorganism and phylogenic analysis

Slaughter house soil was screened for protease producing bacteria using medium containing gelatin as a substrate. We got three isolates capable of producing extracellular protease. Among those, isolate showing maximum zone of hydrolysis on gelatin agar plate was considered as potent and hence picked for the further study. The 16S rDNA gene sequence analysis confirmed that the organism belongs to the genus *Bacillus*. It is a Gram positive short rod-shaped non-motile bacterium. The 16S rDNA gene sequence of identified *B. safensis* strain CK was submitted in the GenBank under the accession no. KJ427751. The phylogenetic relation of this isolate *B. safensis* strain CK as shown in Fig. 1.

3.2. Protease purification

The production of protease for 48 h was monitored in the production medium. It was observed that as incubation time increases the enzyme production also enhances up to 24 h. After 24 h incubation production was decreased (Fig. 2). As the *B. safensis* strain CK produces extracellular protease, so the supernatant was subjected for fractional ammonium sulphate precipitation from 30–80%. It was observed that 60% ammonium sulphate fraction showed highest protease activity. Precipitate was dialyzed and loaded on DEAE-cellulose column. It yielded 4 major peaks (Fig. 3), among them, only one showed protease activity. Gradual increase in specific activity of protease was achieved during protein purification (Table 1). At the end of purification 7 fold purity and 8.2% yield of protease was achieved as can be seen in Table 1. Fraction showing protease activity was then subjected for SDS-PAGE. Purified fraction appeared as single band on SDS-PAGE and molecular weight was estimated to be 40 kDa (Fig. 4).

3.3. Effect of pH and temperature on enzyme activity

The pH profile of the enzyme was determined using different buffers of varying pH values. The enzyme was active in a wide range of pH from neutral to alkaline (Fig. 5). The optimum pH for this protease was found to be around pH 7. The protease activity was evaluated at various temperatures. The enzyme was active at 20–50 °C temperature range and with an optimum temperature at 37 °C (Fig. 6).

3.4. SDS and detergent stability

To check the enzyme stability, protease assay was performed in presence of different concentrations of SDS (0.1% to 1%) and 1% commercial detergent Wheel. It was revealed that the enzyme shows