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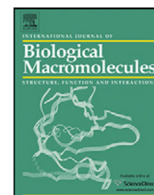

Characterization of solvent stable extracellular protease from *Bacillus koreensis* (BK-P21A)

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ABSTRACT

A total of 18 protease producing bacterial strains were isolated from detergent effluent in South Korea using skim milk agar medium. A strain (BK-P21A) was selected and identified as *Bacillus koreensis* based on morphological, biochemical and molecular characterizations (16S rRNA gene sequence analysis). Optimized culture conditions for the production of protease were pH 8.5, 30 °C, sucrose (2%) and yeast extract (0.2%) during 36 h of incubation. Furthermore, the protease was partially purified by ammonium sulphate precipitation (80%) and again by Superdex 200 10/300 GL and Superdex 75 10/300 GL column chromatography, which resulted in 5.0 fold purification and a yield of 23%. The molecular mass of the protease was estimated to be 48 kDa by SDS-PAGE. The purified enzyme was further characterized and found to be most active at pH 9.0 and 60 °C. The activity of the purified protease was enhanced by $CaCl_2$ and $CoCl_2$, but inhibited by PMSF, which indicated it was a serine type protease. Moreover, the protease was moderately stable in surfactants and 81% stable in H_2O_2. Finally, the enzyme was more active and stable (94–126.5%) in various hydrophilic organic solvents. Considering the stability of protease towards the alkaline pH, high temperature and organic solvents (50%), the enzyme from *B. koreensis* can be used as an alternative biocatalyst for several industrial applications mainly for peptide synthesis in nonaqueous solvents.

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1. Introduction

Proteases constitute the most important industrial enzymes and their applications have recently increased in fields such as baking, brewing, food production, leather processing, pharmaceutical manufacture, and the recovery of silver from photographic film [1,2]. In addition, proteases have other industrial applications including protein processing, peptide synthesis and detergent formulations [2,3]. Proteases can be produced from animals, bacteria, fungi, and plants [1,2]; however, microbial proteases are the most common. Indeed, microbial proteases can be produced in large quantities and represent one of the largest classes of industrial enzymes, accounting for 40% of the total worldwide sales of enzymes by value [4]. *Bacilli are* one of the most important producers of extracellular commercial proteases, because of their high pH and temperature stabilities [5].

Extracellular proteases are secreted into culture medium by many bacteria that differ from one another in their physical and biochemical properties. The practical use of microbial proteases has led to the development of hyperactive strains and the novel biochemical properties of different proteolytic enzymes have been characterized [6,7]. Proteases are particularly important because they are most stable and active at alkaline pH, high temperature and in the presence of surfactants, metal ions, oxidizing agents and organic solvents [8]. Due to industrial demand for proteases, researchers are continuing to identify potent protease producers. Until recently, most solvent stable proteases were isolated from *Pseudomonas* species [9–11], and there have been only a few reports of their isolation from *Bacillus* species [12]. Most *Bacillus* proteases that have been isolated to date are recovered from organisms used in detergent industries [7,13]. Additionally, an organic solvent stable cellulase was recently isolated from *Bacillus* sp. L1 [14]. Normally, enzymes are denatured and unstable in the presence of organic solvents, but some microbes could produce organic stable enzymes [15]. Several methods such as protein engineering, chemical modification and immobilization have been reported for the stabilization of enzymes in the presence of organic acids [16–18]. However, natural organic stable enzymes are useful for many industrial and biotechnological applications that employ organic solvents as reaction media under non-aqueous conditions because they can be used for these applications without any modification to stabilize the enzymes [15]. During peptide synthesis, peptide bonds are hydrolyzed under aqueous conditions and synthesized in non-aqueous media. In this study, I report the production, purification and characterization of solvent stable protease from *Bacillus koreensis* BK-P21A.

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2. Materials and methods

2.1. Isolation and screening of microorganisms for protease

The detergent effluent samples from the detergent industries (Incheon, Republic of Korea) were suspended in sterile water, serially diluted and spread on skim milk agar medium (yeast extract 1 g/L, skim milk powder 10 g/L, agar 18 g/L). The plates were then incubated at 30 °C for 2 days. The organism giving the largest zone was selected for further study and maintained at −80 °C.

2.2. Identification of selected bacterial strain

The selected bacterial strain was identified based on morphological and biochemical characterizations. Briefly, a gram stain was conducted using the Biomerieux system according to the manufacturer's instructions. Biochemical tests were then conducted using the API 50 CHB system according to the manufacturer's protocols (Biomerieux). The identification was further confirmed by sequencing of the 16S rRNA and subsequent comparison of the sequence with those available in the GenBank nucleotide database by using the NCBI BLAST algorithm (www.ncbi.nlm.nih.gov). The DNA was amplified by PCR using the 16S rRNA gene primers: 8-27F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1472R: 5′-TACGGYTACCTTGTTACGACTT-3′.

2.3. Protease production

For optimization, the tryptic soy broth was used with the following composition: casein (17.0 g/L), soybean meal (3.0 g/L), glucose (2.5 g/L), NaCl (5.0 g/L), dipotassium phosphate (2.5 g/L), pH 7.5. In all the cases, Erlenmeyer flasks (250 mL) containing 50 mL of media were inoculated with fresh culture and then incubated with constant shaking at 180 rpm. Aliquots of the cultures were harvested at 12 h intervals during 48 h of incubation and centrifuged at 10,000 rpm for 10 min at 4 °C, after which the clear supernatant was used to estimate the protease activity. The growth of the strain was determined by measuring the absorption at 600 nm. All the experiments were carried out in triplicate and average values were reported.

The effects of pH and temperature were determined in the ranges of pH 7 to 9 and 18 to 35 °C, respectively. The pH of the medium was adjusted before autoclaving. The carbon sources were sterilized separately and aseptically added to the sterilized medium. The carbon sources used were glucose, fructose, maltose, lactose, mannitol, starch, sucrose and xylose. The following organic nitrogen sources were used: corn steep solid, cotton seed flour, gelatin, skim milk, wheat flour and yeast extract.

2.4. Protease assay

Protease activity was measured using a modified version of the method described by Kembhavi et al. [19] with casein as the substrate. Briefly, a 500 μL aliquot of culture supernatant was mixed with 500 μL of 100 mM Tris–HCl buffer (pH 8.0) containing 1% (w/v) casein and incubated for 30 min at 37 °C. The reaction was then stopped by the addition of 500 μL of trichloro acetic acid (20% TCA), after which it was allowed to stand at room temperature for 15 min. and then centrifuged at 12,000 rpm for 15 min to remove the precipitate. Finally, the supernatant was estimated spectrophotometrically at 280 nm. One unit of the protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine in 1 min.

2.5. Protein assay

The protein concentration was determined by the Bradford method [20] using bovine serum albumin (Sigma, USA) as the standard. During column chromatography, the amount of protein was measured in terms of the absorbance at 280 nm. The specific activity was expressed as the enzyme activity per mg of protein.

2.6. Purification of protease

The culture was collected after 36 h of incubation and centrifuged at 10,000 rpm for 10 min. The supernatant of the culture was precipitated by the gradual addition of solid ammonium sulphate with gentle stirring to 80% (w/v) saturation, after which it was allowed to stand overnight at 4 °C and then centrifuged at 15,000 rpm for 30 min. The pellet was subsequently dissolved in Tris–HCl buffer (pH 8.0) and the crude enzyme was concentrated using centrifugal filters (Ultracell 10 K). Next, the concentrated sample was applied to a Superdex 200 10/300 GL gel filtration column by fast protein liquid chromatography (FPLC) using an AKTA FPLC (Amersham Pharmacia Biotech) that had been equilibrated with the same buffer (100 mM Tris–HCl buffer pH 8.0) at a flow rate of 0.5 ml/min. The fractions possessing protease activity were pooled and concentrated. After removal of insoluble substances by centrifugation and filtration, the solution was applied by FPLC to a Superdex 75 GL column that had been equilibrated and eluted with the same buffer. The protease active fractions were pooled and concentrated.

2.7. Electrophoretic analysis and zymogram

The purity of protease and the molecular masses (based on a standard molecular weight marker) were determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli [21] using a 12% separating gel. The protein was stained with a 0.1% solution of CBB R-250 (Sigma, USA).

For zymogram analysis, SDS-PAGE was performed using 12% polyacrylamide containing 0.1% gelatin according to the method reported by Bernal et al. [22], with slight modification. After electrophoresis, the gel was rinsed with 0.25% Triton X 100 and incubated for 1 h at 37 °C in 50 mM Tris–HCl buffer (pH 8.0). Finally, the protease activity was visualized after staining with CBB R-250 (Sigma, USA).

2.8. Biochemical characterization

2.8.1. Effect of pH and temperature on protease activity and stability

The optimal pH and stability of the enzyme were investigated by measuring the activity at 37 °C in buffers with various pH values (phosphate buffer, pH 6.0–7.0; Tris–HCl buffer, pH 8.0–9.0; carbonate buffer, pH 10.0–11.0). The effect of temperature on activity and stability were investigated at a constant pH of 8.0 (30–80 °C).

2.8.2. Effect of metal ions on protease activity

To investigate the effect of metal ions on protease activity, the following metal ions were used (5 mM concentration): $AgNO_3$, $CaCl_2$, $CoCl_2$, $MgSO_4$, $MnCl_2$ and $ZnSO_4$. The samples were incubated for 1 h at room temperature with various metal ions and the residual protease activity was determined.

2.8.3. Effect of protease inhibitors on protease activity

To assess the protease-type, the effect of the following inhibitors were tested: metallo protease inhibitor (ethylenediaminetetraacetic acid (EDTA)), serine protease inhibitor

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