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Purification and Characterization of Heat-Tolerant Protease Produced by *Bacillus polyfermenticus* SCD

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A protease produced by *Bacillus polyfermenticus* SCD was purified and characterized as a new detergent material. The protease was purified from supernatant produced by *B. polyfermenticus* SCD, by ammonium sulfate precipitation, ion-exchange chromatography on a DEAE-Sephadex A-50, and finally gel filtration chromatography on Sephadex G-50. The molecular mass of this enzyme was 44 kDa based on SDS-PAGE. The optimum temperature and pH were 50°C and pH 8.0. The ranges of its stability to the pH and temperature were 7.0 to 9.0 and under 40°C, respectively. The enzyme was highly stable in the presence of the surfactants like Triton X-100 (0.1%), showing a 2-fold increase in its proteolytic activity. However, the enzyme was slightly inhibited by the chelating agent EDTA (1 mM). The enzyme has a maximum activity at 50°C and the activity can be increased by surfactants such as Triton X-100 and Tween 80.

Keywords: *Bacillus polyfermenticus* SCD, protease, purification, surfactant, Triton X-100, Tween 80

Introduction

Microorganisms represent an excellent source of enzymes, including proteases, because of their broad biochemical diversity. Microbial proteases represent one of the three largest groups of industrial enzymes and account for approximately 60% of the total enzyme sales in the world [30]. These enzymes have numerous applications in industrial production, including food processing, leather processing, pharmaceutical and biomediation processes, and in the textile industry to remove protein-based stains [6, 22].

Proteases produced by *Bacillus* species are the most important group of secondary metabolites that are widely exploited [19]. Proteases constitute approximately 65% of the total worldwide production of enzymes [9] and bacteria of the genus *Bacillus* produce most of the industrial proteases used today [5, 7, 32]. For application of protease to the detergent industry, it would be of great importance to have available enzymes with optimal activities at different salt concentrations, temperatures, and pH values [20, 23, 31]. The increasing demand for proteases with specific properties has led biotechnologists to explore newer sources of proteases.

B. polyfermenticus strains have been reported to be effective for the treatment of long-term intestinal disorders because their endospores can successfully reach the target intestine [10]. In the past, many studies have reported on the properties of *B. polyfermenticus*, including its industrial utility and safety [2, 11, 27], capacity to inhibit carcinogen-induced DNA damage [25], antioxidant effects [4], anticarcinogenic and antigenotoxic effects [26], and probiotic potential [12]. However, there are no studies to date regarding the use of any proteases from *B. polyfermenticus*.

The aim of this study was to purify and characterize a protease produced by *B. polyfermenticus* SCD as a detergent additive.

Materials and Methods

Bacterial Strain and Culture Conditions

B. polyfermenticus SCD (*B. polyfermenticus* KCCM10104) was stored at −70°C in tryptic soy broth (TSB; Difco, USA) to which

20% (v/v) glycerol was added. Cultures were grown in 1,000 ml of nutrient broth (NB; Difco) as a working volume in 2,000 ml flasks. The temperature was maintained at 37°C for 48 h and the agitation speed was 150 rpm [14].

Protease Assay

Protease activity was determined by a modified Anson-Hagihara's method using casein as the substrate [8]. The enzymatic reaction mixture consisted of 1 ml of protease solution, 1 ml of 0.6% (w/v) azocasein (Sigma-Aldrich Co., USA), and 1 ml of 100 mM sodium phosphate buffer (pH 7.0). The reaction was initiated by the addition of protease solution and incubated at a defined temperature for 10 min. The reaction was stopped by the addition of 2.5 ml of 5% (w/v) trichloroacetic acid (TCA) followed by a 30 min incubation at room temperature and centrifugation (20,760 ×*g*, 20 min). Enzymatically hydrolyzed casein was measured by a modified Folin-Ciocalteu method, with casein treated with inactive protease as a blank. One unit of protease activity was defined as the amount of protease that liberated 1 μg of tyrosine per minute at 37°C. Protease units were measured using tyrosine (0−100 mg) as a standard curve [36].

Protein Measurement

The amount of protein was determined by the method of Lowry using bovine serum albumin (BSA) (0−5 mg/ml) as the standard. In addition, during the enzyme purification with column chromatography, the protein elution profile was monitored spectrometrically at 280 nm [17].

Ammonium Sulfate Precipitation

Ammonium sulfate was added to the cell-free culture supernatant at up to 60% saturation, and the precipitate was allowed to form at 4°C for 24 h. The precipitate was collected by centrifugation at 20,760 ×*g* for 20 min at 4°C and resuspended in 0.1 M phosphate buffer (pH 7.0) and dialyzed for overnight against the same buffer with the sample:buffer ratio of 1:1,000 (v/v) [1, 20].

DEAE-Sephadex A-50 Column Chromatography

The ammonium sulfate fraction was put on a column (3 × 40 cm) of diethylaminoethyl (DEAE) Sephadex A-50 (Sigma) that was equilibrated with 50 mM phosphate buffer (pH 7.0). The column was washed with the same buffer and eluted with a linear gradient of 0 to 1 M NaCl in 50 mM sodium phosphate buffer (pH 7.0). The fractions with protease activity were collected and dialyzed against 10 mM sodium phosphate buffer (pH 7.0) at 4°C [1, 18]. The enzyme solution was concentrated and stored at −20°C until used. This dialyzed fraction was made up to a known volume and referred to as partially purified protease. The activity of purified protease was assayed as described above.

Sephadex G-50 Chromatography

For gel filtration chromatography, the dialyzed material was loaded onto a gel column (Sephadex G-50; Sigma-Aldrich Co., USA) previously equilibrated with 10 mM sodium phosphate buffer (pH 7.0). The column was then washed with 250 ml of the same buffer, and bound proteins were eluted with the same buffer at a flow rate of 0.5 mg/ml. Fractions were collected, and positive fractions were pooled together, lyophilized, and resuspended in the same buffer [24].

Polyacrylamide Gel Electrophoresis

PAGE was carried out to determine the molecular mass of the protease with a 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS) at 4°C and 20 mA/gel, after heating the samples at 90°C for 1 min [16]. Following SDS-PAGE, the proteins were stained with Coomassie brilliant blue R-250 (0.2%). The molecular mass of the protease was determined by comparing with the mobility of standard molecular mass marker proteins: myosin (250 kDa), phosphorylase (148 kDa), bovine albumin (98 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin (22 kDa), and lysozyme (16 kDa) (Sigma-Aldrich Co., St. Louis, MO, USA).

Influence of Temperature on Protease Activity

To investigate the effect of temperature on protease activity, the protease activity was measured in the temperature range of 0−90°C at pH 7.0 for 10 min. Prior to the assays, the enzyme sample, substrate solution, and 0.1 M sodium phosphate buffer (pH 7.0) were pre-incubated at the desired temperature for 2 min [30].

Effects of pH on Protease Activity

The effect of pH on protease activity was investigated using 0.1 M acetate buffer (pH 4, 5, and 6), 0.1 M phosphate buffer (pH 6, 7, and 8), and 0.1 M glycine-NaOH buffer (pH 8, 9, and 10) at the range of pH 4−10 at 50°C, previously determined for 10 min. Substrate solution of azocasein was prepared in the respective buffers. The assay procedure was the same as described above [34].

Effects of Inhibitors and Surfactants

The effects of inhibitors and surfactants on protease activity was were under standard assay conditions where the assay cocktail was supplemented with EDTA (1 mM), Tween 80 (0.1%), and Triton X-100 (0.1%). The effects were assessed relative to a control [35].

Statistical Analysis

Triplicate experiments were done in this study. Analysis of variance (ANOVA) for data was performed by using the SPSS software 8.0 (Statistical Package for the Social Sciences). Significance of differences was defined as $p < 0.05$.

Results and Discussion

Protease Production

During growth in nutrient broth, the protease activity reached 41.8 units/ml within 48 h, when cell growth reached

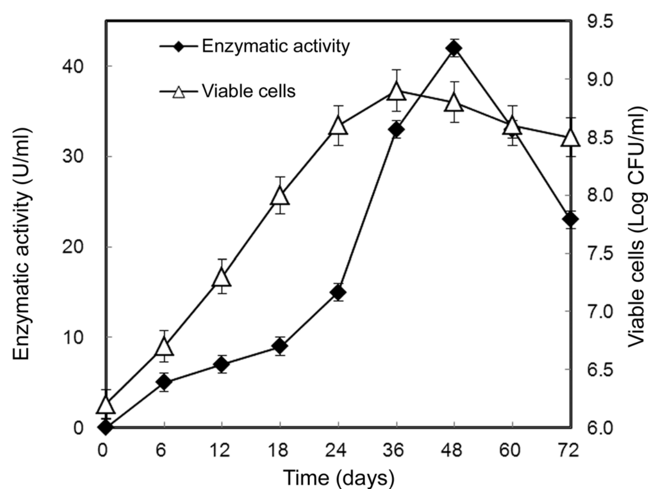

Fig. 1. Time course of protease production by *Bacillus polyfermenticus* SCD.

△: Cell number, ▲: enzyme activity.

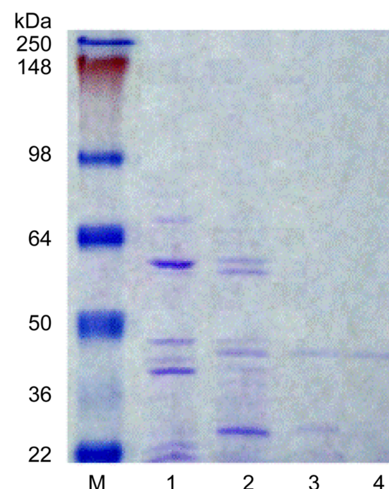

Fig. 2. Analysis of protease purification by 10% SDS-PAGE.

Lane M, Molecular mass marker; Lane 1, Crude broth; Lane 2, Fraction after 60% ammonium sulfate precipitation; Lane 3, Fraction after ion-exchange chromatography with DEAE Sephadex-A50; Lane 4, Fraction after gel filtration chromatography on Sephadex G-50.

the late log phase or early stationary phase (Fig. 1). An alkaline protease from marine yeast produced protease activity within 30 h when the cell growth reached the mid-log phase [3]. The content of protease decreased because of the utilization of protein by the organism. The decline in protease activity upon prolonged incubation may be due to autolysis of the enzyme.

Purification of Protease

The protease was purified by ammonium sulfate fractionation followed by ion-exchange and gel filtration chromatographies. A summary of the purification of the protease is shown in Table 1. The ammonium sulfate fractionation was an effective step in the purification process, leading to 28.2-fold purification (Table 1). Ion-exchange chromatography with DEAE Sephadex-A50 enhanced the specific activity to 2,973.1 units/mg protein, achieving 58.4-fold purification. During the last step of purification using gel filtration chromatography, the specific activity of the purified protease was 3,845.3 units/mg protein, with a fold purification of 75.5% and a recovery of 31.3% from the spent medium.

Polyacrylamide Gel Electrophoresis

Partial purification of the protease after ion-exchange chromatography with DEAE SephadexA-50 was successfully achieved, as shown by a double band corresponding to 32 and 44 kDa on SDS-PAGE.

Bacillus spp. have many kinds of extracellular protease during culturing [13, 23]. Kim *et al.* [13] presented that *B. cereus* has two proteases with molecular masses of approximately 38 and 36 kDa. Nilegaonkar *et al.* [23] reported that partially purified protease from *B. cereus* MCM B-36 contained multiple proteases of molecular masses 45 and 36 kDa. In this study, the purified enzyme appeared as a single band on SDS-PAGE, corresponding to a molecular mass of 44 kDa after gel filtration chromatography with DEAE Sephadex G-50 (Fig. 2).

Effect of Temperature on Protease Activity

The effect of temperature from 0 to 90°C on protease activity in 0.1 M sodium phosphate buffer (pH 7.0) is shown in Fig. 3. The optimum temperature for protease

Table 1. Purification of protease from *Bacillus polyfermenticus* SCD.

Procedures	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Fold purification
Culture supernatant	6,800	346,000	50.9	100.0	1.0
60% $(NH_4)_2SO_4$	142	204,000	1,436.6	58.9	28.2
DEAE Sephadex A-50	52.1	154,900	2,973.1	44.8	58.4
DEAE Sephadex G-50	28.2	108,400	3,845.3	31.3	75.5

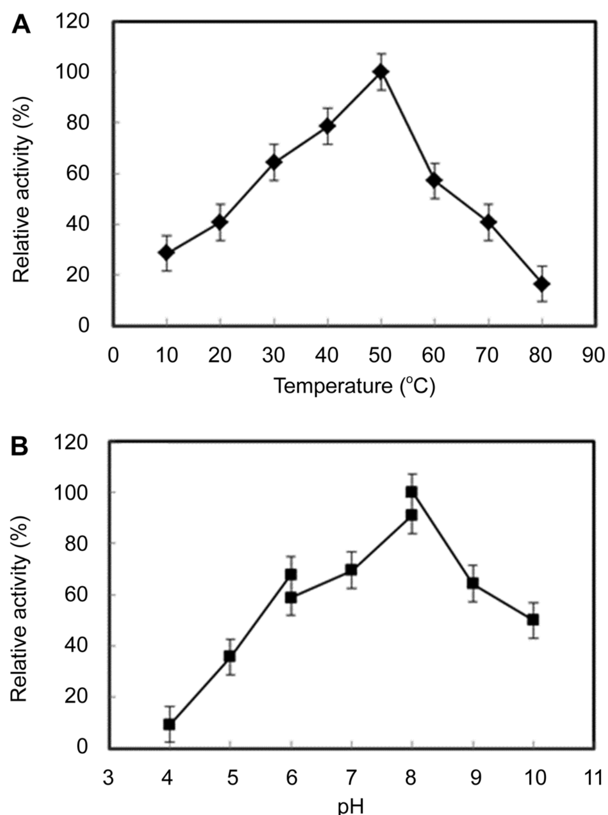

Fig. 3. Effects of temperature (**A**) and pH (**B**) on protease activity.

The protease assay was carried out at pH 7.0 and at different temperatures in the range of 10−80°C. Activity at 50°C was referred to as 100% and the relative activity was calculated for other temperatures. pH optima were determined by incubating the enzyme with the substrate at different pH values at 50°C: acetate buffer (pH 4.0−6.0); phosphate buffer (pH 6.0−8.0), and glycine-NaOH buffer (pH 8.0−10.0).

activity was 50°C, beyond which there was a rapid decline. In an earlier report, Raju *et al*. [29] reported that the protease from *Bacillus* species was active in the temperature range of 20−50°C, with optimum activity at 37°C. Thus, the protease of *B. polyfermenticus* SCD in this study is more thermotolerant than the proteases referred to above.

Effect of pH on Protease Activity

The activity of the protease at the range of pH 4.0−10.0 was determined after the enzyme was incubated at 50°C for 10 min. The protease showed high activity at pH 7.0−9.0, and the highest activity was obtained at pH 8.0 (Fig. 3). The effect of pH on the protease activity shows that the protease is a type of alkaline protease active in a broad range of pH values. Preliminary studies on the extracellular de-hairing protease secreted by *Bacillus* sp. showed that it has dual pH maxima at 7.5 and 9.0 [29]. These findings are in accordance with several earlier reports showing pH optima of 8.0−9.5 for fungal proteases such as those from *Aspergillus fumigatus*, *A. parasiticus*, and *A. clavatus* CCT2759 [21, 33, 34].

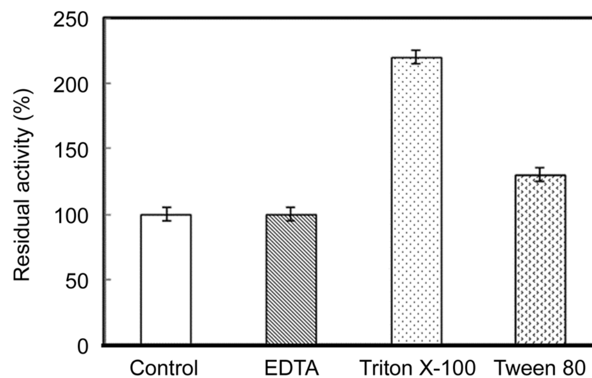

Fig. 4. Effects of inhibitors and surfactants on protease activity.

The standard protease assay was supplemented with EDTA (1 mM), Triton X-100 (0.1%), and Tween 80 (0.1%). The effects of these additives were assessed relative to the control. Percent residual activity was calculated by considering the activity of control to be 100%.

Effects of Inhibitors and Surfactants on Protease Activity

The effects of inhibitors and surfactants on the purified protease is detailed in Fig. 4. The protease was highly stable in the presence of the surfactants such as Triton X-100 (0.1%), showing a 2-fold increase in proteolytic activity. The enzyme was slightly inhibited by the chelating agent EDTA (1 mM). The high activity of the protease in the presence of EDTA is very important for its potential application as a detergent additive because most detergents contain such chelating agents. This was similar to results obtained by Kim *et al*. [15] and Rahman *et al*. [28].

Despite the fact that many different proteases have been identified and some of them have been used in biotechnological and industrial applications, the presently available proteases are not sufficient to meet most industrial demands. Industrial processes are carried out under specific physical and chemical conditions, which cannot always be adjusted to the optimal values required for the activity and stability of the available enzymes. From this study, it appeared that protease produced from *B. polyfermenticus* SCD has a single molecular mass unlike other *Bacillus* species, so it would be more economical and convenient to apply it to industrial production for the detergent industry than those produced from other strains. The stability of the protease in detergent appears to be an attractive feature for its use in industrial

applications. In general, the optimum temperature of most proteases in nature is at the range of 30−40°C, but enzymatic activity at higher temperature such as 50°C can increase the yield of proteolysis industrially. In addition, detergents and EDTA as major detergent ingredients tested in this study have no significant inhibitory effects on the protease produced from *B. polyfermenticus*. Therefore, these results suggest that protease produced from *B. polyfermenticus* SCD is a potential material as a detergent additive for laundry and may be useful in other industries such as textile or leather processing.

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