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Purification and molecular weight estimation of protease from a thermophilic *Bacillus* species

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Abstract: An extracellularly produced alkaline protease from a thermophilic *Bacillus* strain was purified using 80% ammonium sulfate precipitation followed by CM-cellulose and DEAE-cellulose ion exchange column chromatography that yield 16.5 fold purification with an increase in specific activity from 417 to 6900 U/mg. Molecular weight of the enzyme was estimated about 62 kDa and clear hydrolytic zone of protease was also observed on zymogram against dark blue background of casein gel.

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INTRODUCTION

Proteases are one of the important groups of industry valuable enzymes that contribute more than 65% in total enzyme market[1]. Protease has numerous applications in different industries such as detergent industry, food and feather processing industry, pharmaceuticals and silk gumming processing industry[2, 3]. Different environmentally benign processes may also be developed by proteases and can be used in various bioremediation procedures including in extraction of oligosaccharides, chitosan, chitin, protein hydrolysates and chitinases[4]. Proteases catalyze the hydrolytic process of protein by cleaving the peptides linkages and yield smaller peptides and amino acids[5]. Several microbial species have been reported for maximum production of protease due to fast growth within short time period and required less space. Microbial species can be genetically modified to alter the properties of enzymes for commercial applications[6]. It is necessary that protease must be stable and active at high temperature and pH as well as in surfactant, solvents and oxidizing agents for different industrial processes[7-9]. Bacterial proteases are mostly produced extracellularly with high concentration and thermal-stable that also survive broad pH range. Proteases from *Bacillus* species have been purified, characterized and substrate specificity, thermal stability, easy down streaming procedures and low cost have been investigated[10, 11]. Such properties increase its utility for industrial applications. Different purification methods such as anion exchange chromatography and gel permeation chromatography have been used for getting more purified enzyme with high purification fold and yield[12, 13].

The aim of the present study was purification of protease isolated from a thermophilic *Bacillus* species. Initially, partial purification was performed by ammonium sulfate precipitation and then for further purification CM-cellulose and DEAE-cellulose ion exchange column chromatography were used to obtain more purified catalytic protein. Molecular mass and purity of the enzyme were examined by SDS-PAGE and Zymography, respectively.

MATERIALS AND METHODS

A thermo-stable protease producing *Bacillus* species was selected from the culture bank of Department of Biotechnology, University of Karachi, Pakistan. Strain was initially preserved on Luria Bertani's (LB) agar slants and maintained by regular sub-culturing on fresh media. Bacterial isolate was grown in 100 ml Luria Bertani's (LB) medium and then transferred to 900 ml same culture broth (250 ml and 1000 ml Erlenmeyer flask) supplemented with 1% casein and incubated at 60°C for 72 hours with orbital rotary shaking at 120 rpm. The culture broth was centrifuged at 5000 rpm for 10 minutes at 4°C. Cell-free filtrate was collected and concentrated by rotary evaporator at 37ºC.

Concentrated cell-free broth was precipitated by gradual addition of $(NH_4)_2SO_4$ in a cold water bath with slow stirring and up to 80% saturation was achieved. Precipitates formed were kept at 4ºC for overnight and then centrifuged at 10,000 rpm at 4ºC for 20 minutes. One gram precipitates were dissolved in 10 ml of 0.05M Tris-HCl buffer containing 5mM $CaCl_2$ with pH 8.0, and centrifuged at 5000 rpm for 10 minutes to remove denatured proteins. Clear supernatant was dialyzed against 500ml of 50 mM sodium acetate buffer containing 5 mM $CaCl_2$ with pH 5.0 and buffer was replaced with 4 hour time intervals to remove residual salt. Then, dialyzed precipitate was applied to a CM-cellulose (20ml bed

volume) column that had been packed and equilibrated with same buffer. Initially equilibration buffer was used to wash the column and then linear gradient of 0-1M NaCl in the same buffer at 20ml/hr flow rate was used. Active fractions were pooled, concentrated using rotary evaporator and desalted by dialysis using aforementioned method. The dialyzed precipitates were then loaded onto a column of DEAE-cellulose (50ml bed volume) equilibrated with same buffer. After being washed with equilibration buffer, bound protein was eluted out with linear gradient of 0-1M NaCl in the same buffer at a flow rate of 20ml/hr. Fractions containing enzyme activity were pooled and stored in small aliquots at -20°C for further studies.

The protease activity[14] was determined in triplicate at 37°C in water bath using casein as a substrate. Initially 0.5ml of sample was incubated with 0.5ml of 1% (w/v) casein solution prepared in 0.1M Tris-HCl buffer having pH 8.0 for 20 minutes at 80°C. Then, reaction was terminated by adding 1.5ml of 0.3M tricholoroacetic acid followed by centrifugation at 10,000 rpm for 10 minutes at 4°C. Optical density was measured at 280 nm by spectrophotometer. One Kunitz unit is defined as the amount of enzyme which causes an increase in absorbance of 0.001 per minute at the wavelength of 280nm.

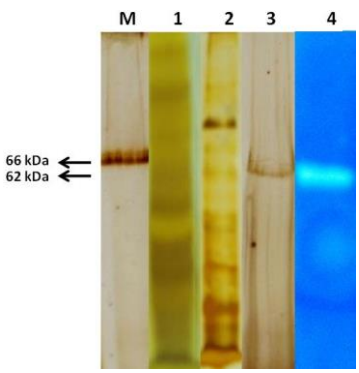

Figure 1: SDS-PAGE and Zymography of the purified protease. Lane M. Molecular marker: 66 kDa-bovine serum albumin. Lane 1. Ammonium sulfate precipitated enzyme. Lane 2. CM-Cellulose purified enzyme. Lane 3. DEAE-Cellulose purified enzyme. Lane 4. Zymogram of purified protease.

Protein was estimated by dye binding method[15]. A standard curve was constructed by preparing different dilutions (10-100µg) from Bovine serum albumin (BSA) stock (100µg/ml). The protein concentration was monitored in term of absorbance at 595nm.

A sample from each purification step was examined for its purity and molecular mass by SDS-PAGE[16] using Bovine serum albumin (BSA) as a marker. 7.5% (w/v) polyacrylamide gels with 25mM Tris, 192mM glycine and 0.1% SDS having pH-8.3 was used in this experiment. Electrophoresis was performed at a constant current of 160V and silver staining was used to stain the gel and visualize the protein band. Hydrolytic activity of protease was analyzed by adding the substrate (1% casein) in 7.5% SDS-PAGE gel before polymerization of gel and then activity of protease[17] was examined in the gel as clear bands against dark blue background after staining with Coomassie brilliant blue[18].

RESULTS AND DISCUSSION

Protease from thermophilic *Bacillus* strain was purified by 80% ammonium sulfate precipitation followed by CM-cellulose and DEAE-cellulose column chromatography. The summary of the purification scheme has been presented in Table 1. Enzyme was purified 13.5 folds with specific activity of 5644 U mg^{-1} after CM-cellulose ion exchange chromatography. The purification by DEAE-cellulose showed 16.5 fold increase with a specific activity of 6900 U mg^{-1}.The overall percent yield of enzyme was found to be 16%. The yield recovery of the protease was declined after every purification step. This might happens due to the elimination of other non-protease proteins during chromatography [19]. Different *Bacillus* proteases have been reported for different % yield and purification fold by using different techniques. Protease from *Bacillus firmus* CAS 7 showed 12.3 % yield with 2.6 purification fold after sephadex G-50 column chromatography[19].

Table 1: Purification scheme of purified protease from *Bacillus* species.

Purification Step	Total Activity (U)	Total Protein (mg)	Specific Activity (U mg^{-1})	Purification (fold)	Yield (%)
Cell free filtrate	386,46	925	417	1	100
$(NH_4)_2SO_4$ precipitation	190500	40	4762	11.4	49
CM-Cellulose	141,12	25	5644	13.5	36
DEAE-Cellulose	62,100	9	6900	16.5	16

The homogeneity of the purified protease was proved by single protein band appeared in SDS-PAGE gel. The molecular weight of the purified protease from *Bacillus* species was observed as approximately 62 kDa and showed clear hydrolytic zone on zymogram (Figure 1). Different molecular

weights have been reported previously for different *Bacillus* species. The purified protease from *Bacillus alveayuensis* CAS 5 indicated about 33 kDa molecular mass[20]. The molecular weights of P1 and P2 protease from *Bacillus megaterium* were resulted as 28 and 25 kDa, respectively[21].

CONCLUSION

In the current study, protease from *Bacillus* species was purified for different commercial applications. CM-cellulose and DEAE-cellulose ion exchange chromatography resulted 16.5 fold purification with an increase in specific activity (6900 U/mg). SDS-PAGE and Zymography of protease were performed that estimated about 62 kDa molecular mass with clear hydrolytic zone.

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