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

Ayesha Kamran1*, Zainab Bibi2 and Mustufa Kamal1
1Department of Biotechnology, University of Karachi, Karachi, Pakistan
2The Karachi Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi, Karachi, Pakistan

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.

Keywords: Protease, purification, SDS-PAGE, zymography.
Received: August 5, 2014 Accepted: October 17, 2014
*Author for Correspondence: ayesharoomi@hotmail.com

INTRODUCTION

Proteases are one of the important groups of industry valuable enzymes that contribute more than 65% in total enzyme market1. Protease has numerous applications in different industries such as detergent industry, food and feather processing industry, pharmaceuticals and silk gumming processing industry2, 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 chitinases4. Proteases catalyze the hydrolytic process of protein by cleaving the peptides linkages and yield smaller peptides and amino acids5. 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 applications6. 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 processes7, 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 investigated10, 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 yield12, 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 37°C.

Concentrated cell-free broth was precipitated by gradual addition of (NH₄)₂SO₄ in a cold water bath with slow stirring and up to 80% saturation was achieved. Precipitates formed were kept at 4°C for overnight and then centrifuged at 10,000 rpm at 4°C for 20 minutes. One gram precipitates were dissolved in 10 ml of 0.05M Tris-HCl buffer containing 5mM CaCl₂ 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 CaCl₂ 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 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 trichloroacetic 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](image)

**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. 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 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 was examined in the gel as clear bands against dark blue background after staining with Coomassie brilliant blue.

**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. 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.

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

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Activity (U)</th>
<th>Total Protein (µg)</th>
<th>Specific Activity (U mg⁻¹)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell free filtrate</td>
<td>386.46</td>
<td>925</td>
<td>417</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitate</td>
<td>190500</td>
<td>40</td>
<td>4762</td>
<td>11.4</td>
<td>49</td>
</tr>
<tr>
<td>CM-Cellulose</td>
<td>141,12</td>
<td>25</td>
<td>5644</td>
<td>13.5</td>
<td>36</td>
</tr>
<tr>
<td>DEAE-Cellulose</td>
<td>62,100</td>
<td>9</td>
<td>6900</td>
<td>16.5</td>
<td>16</td>
</tr>
</tbody>
</table>

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 mass20. The molecular weights of P1 and P2 protease from Bacillus megaterium were resulted as 28 and 25 kDa, respectively21.

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.

REFERENCES