Purification Properties of *Bacillus thuringiensis* TS2 Keratinase Enzyme

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**Abstract:** This study was focused on purification and characterization of keratinase from *Bacillus thuringiensis* TS2, isolated from feather dumping soil site. Zymogram of crude enzyme on native-PAGE presented bands with keratinase activity of molecular weights is 41 kDa. In purification steps 2.69 fold purification was achieved after 80% ammonium precipitation of the with 34.42% recovery. In further purification steps a 5.69 fold purified keratinase was recovered by Sephadex G-75 chromatography with 21.23% of recovery. The specific activity of purified enzyme was 970.15.54 U/mg. Kinetic studies indicated that substrate specificity of TS2 keratinase was towards various natural and synthetic proteolytic substrates but inactive against collagen and keratin. These findings suggested that the keratinase may have much industrial application.

**Key words:** Purification • Characterization • Keratinase • Sephadex G-75 • *Bacillus thuringiensis* • Zymogram

**INTRODUCTION**

Keratins are valuable but unavailable fibrous animal proteins. They are components of a range of by-products occurring especially abundantly in slaughterhouses and meat and poultry plants: skin remains, bristle, animal hair, horns and hooves, feathers, etc. These proteins are resistant to physical and chemical environmental factors. They are insoluble in water, weak acids and alkalis, organic solvents and are insensitive to the attack of common proteolytic enzymes such as trypsin or pepsin. Keratin in sensitivity and resistance are closely related to the adaptation of vertebrates to living on the land. High cystine content is the most important property that differentiates keratins from other structural proteins such as collagen and elastin.

Among them, alkaline proteases are of particular interest due to their potential applications in the detergent industry as a cleaning additive [1, 2]. Subtilases, a group of serineproteases which initiate the nucleophilic attack on the peptide (amide) bond through a serine residue at the active site, are one of the most used proteases. Subtilisin, typically have molecular weights of about 20-45kDa. They are secreted in large amounts from many *Bacillus* species. They are widely used in commercial products, for example in laundry and dishwashing detergents, cosmetics, food processing, skincare ointments, contact lens cleaners and for research purposes in synthetic organic chemistry [3, 7].

In spite of the numerous advantages, application of proteases for proteinaceous waste management as well as in dehairing of hides has limitations such as the high cost of production, additional operational costs for concentrating the enzyme and for maintaining controlled conditions of pH and temperature for their optimum action [4]. Also, proteolytic enzyme preparations suitable for use in the leather industry need to be confirmed for their non-specificity towards collagen, or partially purified to make them essentially free of collagenase activity since collagen, the major leather-forming protein, if attacked will reduce the strength of the finished pelt [6]. Isolation and characterization of novel microbes producing robust enzymes with a broad range of pH and temperature requirements as well as the optimization of enzyme production by such organisms to minimize the production cost are important factors that will increase the use of green chemistry in tanneries.

Keratinolytic bacteria, particularly from the bacteria, feather keratin-degrading abilities are observed mostly in strains of *Bacillus licheniformis* [2, 5] less frequently in populations of *Bacillus pumilis*, *B. cereus* and *B. subtilis* [8] and non-spore forming bacteria belonging to, for instance, *Stenotrophomonas* sp.[9]. The industrial demand for highly active preparations of proteases with
appropriate specificity and stability of pH, temperature, surfactants and organic solvents continues to stimulate the search for new enzymes. Microbial proteases, especially from *Bacillus* sp., are the most widely exploited industrial enzymes [6]. The aim of this study was to purify and characterize the keratinase produced by *Bacillus thuringiensis* TS2 isolated from feather dumping site.

**MATERIALS AND METHODS**

**Isolation and Identification of Microorganism:** The soil sample was collected from the feather dumping site at Sivakasi, Tamilnadu, India. The selected isolate shows clear zone around the colony in skim milk agar plates. Then biochemical, carbohydrate test, FAME analysis and 16S rDNA sequences were performed. The organism was submitted at NCBI with accession number FJ377887.

**Keratinase Assay:** This procedure tested the keratinolytic activity of keratinase on azo-keratin. To begin the process, 5mg of azokeratin was added to a 1.5ml centrifuge tube along with 0.8ml of 50mM potassium phosphate buffer (pH 7.5) at 37°C for 1h with constant agitation (900rpm). This mixture was agitated until the azokeratin was completely suspended. A 0.2ml aliquot of supernatant of crude enzyme was added to the azokeratin, mixed and incubated for 15 min at 50°C with shaking. The reaction was terminated by adding 0.2ml of 10% trichloroacetic acid (TCA). The reaction mixture was filtered and analyzed for activity.

The absorbance of the filtrate was measured at 450nm with a UV-160 spectrophotometer. A control sample was prepared by adding the TCA to a reaction mixture before the addition of enzyme solution. The unit of keratinase activity was defined as a 0.01 unit increase in the absorbance at 450nm as compared to the control after 15min of reaction [5].

**Ammonium Sulfate Precipitation and Concentration:** For protein precipitation, solid ammonium sulfate was added slowly to filtrate to give a final (NH₄)₂SO₄ concentration of 48.3% (w/v) and the solution was left overnight at 4°C. After centrifugation at 14000rpm for 15 min. at 4°C, the supernatant was removed and pellet of precipitated proteins was dried at laboratory temperature. Then the pellet which contains keratinase was dissolved in 5ml of double distilled water and was dialyzed against double distilled water for 48 hrs at 4°C. This was further dialyzed against 50% (w/v) PEG in order to concentrate the protein sample [10].

**Sephadex G-75 Chromatography for Keratinase Purification:** The concentrated enzymes fraction (*Bacillus thuringiensis* TS2) was loaded on to Sephadex G-75 column 1cm×50cm equilibrated with 20mM Tris-HCl pH 9.0. The enzyme was eluted by same buffer and fractions of 1.5 ml were collected. For each fraction, the protein content was determined from absorbance at 280nm and enzyme assays was performed. Fractions with protease activity were pooled and further used for enzyme characterization.

**SDS-Polyacrylamide Gel Electrophoresis of Proteins (Laemmli, 1973)**

**Zymogram:** Zymogram was performed by simple modification of the method followed by Xin *et al.* [11]. Purified protease enzyme solution was taken as such (i.e.) not boiled or treated with reducing agent and it was run at 4°C and 20mins through a 10% SDS-polyacrylamide gel containing 0.1% Gelatin. SDS was removed by washing twice for 20 min. with 2.5% Triton X-100. The gel was washed and incubated in mixture of solution containing 50mM Tris HCl (pH-8), 150mM NaCl, 10mM CaCl₂ and 1μM ZnCl₂ at 37°C for 48h. The gel was stained for 20 min in 0.5% Coomassie brilliant blue in glacial acetic acid-isopropanol-distilled water (1:3:6). Washing with distilled water revealed clear area in the shape of bands, where proteolysis of gelatin occurred, on a blue background.

**Determiniation of Kinetic Parameters for Purified Keratinolytic Protease:** The kinetic parameters (Michaelis-Menton constant) Km and maximal velocity Vmax of protease (keratinase) activity were determined from line weaver burk plot optimal assay conditions 55°C, pH 10 at 30 min. for azokeratin concentrations ranging from 0.5mg to 5mg/ml. The evaluation of these graph yielded the kinetic parameters for the keratin hydrolyzing activity of the enzyme (Graph pad Prism 5.04 software).

**RESULTS**

**Purified fractions of Bacillus thuringiensis TS2:** The purification steps for *Bacillus thuringiensis* TS2 are summarized in Table 1 and approximately 2.69 fold purification was achieved after 80% ammonium precipitation of the with 34.42% recovery. In further purification steps, the enzyme was bound to the Sephadex G-75 column which indicates basic protein. The enzyme was finally a 5.69 fold purified Sephadex G-75 chromatography with 21.23% of recovery. The specific activity of purified enzyme was 970.15.54 U/mg (Fig. 1).
Table 1: Summary of Purification Table for *Bacillus thuringiensis* TS2

<table>
<thead>
<tr>
<th>Purification Steps</th>
<th>Enzyme activity (U/ml)</th>
<th>Protein (mg/ml)</th>
<th>Specific activity (U/mg)</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Purification fold</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant</td>
<td>228</td>
<td>1.68</td>
<td>135</td>
<td>840</td>
<td>1,13400</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium Sulphate precipitation</td>
<td>336.7</td>
<td>0.91</td>
<td>370</td>
<td>91</td>
<td>33,670</td>
<td>2.74</td>
<td>36.69</td>
</tr>
<tr>
<td>Sephadex G-75 column chromatography</td>
<td>521</td>
<td>0.65</td>
<td>801.54</td>
<td>32.5</td>
<td>26,050</td>
<td>6.94</td>
<td>25.97</td>
</tr>
</tbody>
</table>

Fig. 1: Specific activity of purified enzyme for TS2

Fig. 2: SDS PAGE analysis of *Bacillus thuringiensis* TS2 by purified keratinolytic enzymes.

**Purification of Keratinolytic Protease:** Ammonium sulphate precipitation was performed as the first step of protease purification. It was done at the levels of saturation of 30, 50 and 80% (W/V). The enzyme activity was obtained only in 50% saturated fraction. The enzyme was purified into homogeneity by gel filtration chromatography using Sephadex G-75. The purity of the enzyme was checked on non-denaturing PAGE which showed a single band where as the crude sample shows several bands. The molecular weight of the purified protease was about 41 KDa, estimated using protein markers of known molecular weight. Crude enzyme and purified protease on zymogram showed a clear zone of proteolytic activity (Fig. 2).

Table 2: Michaelis-Menten constant for TS2

<table>
<thead>
<tr>
<th>Michaelis-Menten</th>
<th>TS1</th>
<th>TS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Best-fit values</td>
<td></td>
<td></td>
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<tr>
<td>Vmax</td>
<td>369.6</td>
<td>265.3</td>
</tr>
<tr>
<td>Km</td>
<td>7.897</td>
<td>5.979</td>
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<tr>
<td>Std. Error</td>
<td></td>
<td></td>
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<tr>
<td>Vmax</td>
<td>32.09</td>
<td>57.12</td>
</tr>
<tr>
<td>Km</td>
<td>0.7196</td>
<td>2.311</td>
</tr>
<tr>
<td>95% Confidence Intervals</td>
<td></td>
<td></td>
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<tr>
<td>Vmax</td>
<td>295.6 to 443.6</td>
<td>153.6 to 417.0</td>
</tr>
<tr>
<td>Km</td>
<td>3.238 to 6.557</td>
<td>2.650 to 13.31</td>
</tr>
<tr>
<td>Goodness of Fit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Degrees of Freedom</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>R square</td>
<td>0.9906</td>
<td>0.9763</td>
</tr>
<tr>
<td>Absolute Sum of Squares</td>
<td>232.2</td>
<td>185.5</td>
</tr>
<tr>
<td>Sy.x</td>
<td>5.387</td>
<td>4.815</td>
</tr>
<tr>
<td>Constraints</td>
<td></td>
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<tr>
<td>Km</td>
<td>Km &gt; 0.0</td>
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<tr>
<td>Number of points</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Analyzed</td>
<td>10</td>
<td>10</td>
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</table>

The purified enzyme was analyzed by SDS PAGE which showed a single band indicating homogenous preparation the molecular weight of protease was determined to be 41 kDa and compared with molecular marker. Zymogram analysis revealed a clear hydrolysis bond against dark background (Fig. 3).
The kinetic parameters (Km and Vmax) were determined at 50°C and pH 10 for Bacillus thuringiensis TS2 for concentrations ranging between 0.5 to 5 mg/ml of azokerain as substrate. The Km and Vmax of purified protease towards TS2 are 5.979 ± 0.17 mg/ml and 265.3 µm/min/mg (Table 2).

**DISCUSSION**

Zymogram analysis of Bacillus thuringiensis TS2 shows the presence of a single band 41 kDa protein. The purified protease was homogenous on SDS-PAGE and its molecular weight was estimated to be 30KDa corresponding with that determined by gel filtration. In addition, the Zymogram analysis of the crude enzyme shows four clear bands indicating the presence of at least four proteases (line 3); however the purified protease shows a unique clear band of casein hydrolysis indicating its homogeneity. The molecular weight of MP Protease was slightly higher than that reported to subtilisin-like protease from Bacillus sp. CK 11-4 (28.5KDa) and lower than that of the alkaline protease from B. licheniformis PWD-1 33 kDa [2,8]. It is nevertheless true that this molecular weight is quite the same of many alkaline proteases from Bacillus sp.

Acetone precipitated enzyme was further purified by Sephadex G-75 column. Enzyme purification up to 50 fold (specific activity of 7266 U/mg) and 30 % yield was achieved. The enzyme was eluted on G-75 gel filtration column at the molecular weight around 58 kDa. Single protein band on non-denaturing PAGE hydrolyzed casein and SDS-PAGE analysis showed that it was a dimeric protein of 30 and 28 kDa subunits pl of the dimeric protein was nearly 8.4 Keratinase from other Bacillus sp. are reported in the range of 24-42 kDa [2,12]. Keratinase from Streptomyces sp. was reported to be 46 kDa [13]. On the contrary, fungal keratinases are of high molecular mass of 130, 148 and >200 kDa protein [14, 15]. Till date, keratinases are reported to be monomer except keratinases from Streptomyces sp. strain 16, KI (203.2 kDa) and KII (100 kDa) which are composed of eight and two subunits respectively [16]. Keratinolytic peptidase from Fervidobacterium islandicum has also been reported to be a homomultimeric keratinase with each subunit of 97 kDa [17,18].

The specific activity of the 13.6 fold purified preparation of Bacillus sp. JB99 was 2989 U/mg. This is the range reported for other thermophilic Bacillus sp. proteases [12]. Similar result observed 2550 U/mg (AH-1010, 23000 U/mg (subtilisin) and 3274 U/mg (FA30-01). The molecular weight of purified protease and Zymogram analysis revealed the presence of a single 29 kDa protein, which is in accordance with already reported Keratinolytic protease from Bacillus sp [13]. The similar report was obtained in Bacillus thuringiensis (TS2). The specific activity of the 2.74 fold purification of the specific activity of the 2.69 fold purified preparation of Bacillus thuringiensis TS2 was 804.54 U/mg. The molecular weight of purified protease and Zymogram analysis revealed the presence of a single 30 kDa protein.

Kinetic parameter (Km and Vmax) of Bacillus thuringiensis TS2 was analyzed. The purified proteases of Bacillus thuringiensis TS2 shown to keratinase enzymes were 5.97 mg/ml and 265.3µm/min/mg respectively. This similar result was observed in the kinetic parameters (Km and Vmax) of purified proteases towards keratin were 3.8 ± 0.5 mg/ml and 15.12 ± 1.6µm/min/mg respectively and towards casein were 3.3 ± 0.4 mg/ml and 15.6 ± 0.9µm/min/mg respectively. It was compared with Km and Vmax of 1.8mg/ml and 11.5µm/min/mg respectively towards casein from Bacillus clausii GMBAE42 [14].

**REFERENCES**


