International Biodeterioration & Biodegradation 65 (2011) 29–35

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Characterization of alkaline thermostable keratinolytic protease from thermoalkalophilic *Bacillus halodurans* JB 99 exhibiting dehairing activity

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ARTICLE INFO

Article history:
Received 8 March 2010
Received in revised form
21 April 2010
Accepted 21 April 2010
Available online 29 October 2010

Keywords:
Keratinase
Thermoalkalophilic
Alkaline protease
Bacillus sp. JB 99
Dehairing

ABSTRACT

A thermostable alkaline protease produced from *Bacillus* sp. JB 99 exhibited significant keratinolytic and dehairing activity. The enzyme was purified by ammonium sulphate precipitation followed by CM-cellulose and Sephadex G-100 chromatography and resulted in 13.6 fold purification with 23.8% of recovery. The specific activity of purified enzyme was 2989.6 U mg^{-1}. Purified protease had a molecular weight of 29 kDa and appeared as a single band. Gelatin zymogram analysis also revealed a clear hydrolytic zone, which corresponded to the band obtained with SDS-PAGE. The optimum pH and temperature for the keratinolytic activity was pH 11.0 and 70 °C respectively and half life of protease was 70 °C for 4 h. N-terminal amino acid sequence of purified enzyme exhibited extensive homology with other thermostable alkaline proteases and inhibition by PMSF indicated serine type of protease. The K_m and V_{max} of protease for keratin substrate were 3.8 ± 0.5 mg ml^{-1} and 15.1 ± 1.6 μm min^{-1} mg^{-1} and casein were 3.3 ± 0.4 mg ml^{-1} and 15.6 ± 0.9 μm min^{-1} mg^{-1} respectively. The enzyme efficiently dehaired buffalo and goat hide without damaging the collagen layer, which makes it a potential candidate for application in leather industry to avoid pollution problem associated with the use of chemicals in the industry. The enzyme also degraded chicken feathers in presence of reducing agent which can help poultry industry in management of keratin-rich waste and obtaining value added products.

1. Introduction

Microbial alkaline proteases (E.C.3.4.21) are important constituent of commercial enzyme preparations, accounting for more than 60% of the total industrial enzyme market. Alkaline protease produced from thermophilic and alkalophilic *Bacillus* which can withstand high temperature, pH, chemical denaturing agents and non aqueous environments have attracted a great deal of attention due to their multitude industrial application such as fertilizers, detergent, leather and pharmaceutical (preparation of ointments) industries and also in biotechnological application such as peptide synthesis, wastes management from various food-processing industries, silver recovery from used X-ray or photographic films and proteinaceous fodder from waste feathers or keratin-containing materials (Rao et al., 1998; Gupta et al., 2002; Gupta and Ramnani, 2006). Due to these properties, proteases exhibiting keratinolytic activity also play a significant role in waste management of leather industry and poultry production. The use of enzyme-based products is currently being explored in many areas of leather making process, with increasing importance in dehairing process, eliminating the need for sodium sulfide which otherwise contribute 40% BOD and 50% COD to waste effluent water (Dayanandan et al., 2003; Thanikaivelann et al., 2004). Keratinases (alkaline proteases) are robust enzymes which are active over a wide range of temperature and pH; sequence analysis of keratinases indicates their relatedness to subtilisin family and their close link to serine or metalloprotease, which are generally bacterial in origin. This family of proteases are specific for aromatic or hydrophobic amino acid residues. They are most active around pH 10, with a molecular weight range of 10–30 kDa and isoelectric point near pI 9.0. This class of protease is produced by various *Bacillus* species like *Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus halodurans* (Rao et al., 1998; Takami et al., 1999; Kim et al., 2001; Gupta and Ramnani, 2006). Keratinases are a particular class of proteolytic enzymes that display the capability of degrading insoluble keratin substrates. These enzymes are gaining importance in the last years, as several potential applications have been associated with the hydrolysis of keratinous substrates. The conversion of feathers to feather meal (which is used as supplement in animal feed) requires high energy input. Keratinolytic proteases are useful in upgrading the nutritional value of feather meals (Onifade et al., 1998; Grazziotin et al., 2006). Among bacteria, several genus have been shown to produce keratinase, they include *Stenotrophomanas* (Yamamura et al., 2002), *Chrysobacterium* (Riffel et al.,

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doi:10.1016/j.ibiod.2010.04.013

2003), *Microbacterium arborescens* (Thys et al., 2004), *Bacillus* (Gupta and Ramnani, 2006; Janice et al., 2007; Corrêa et al., 2010; Lateef et al., 2010). However, there are very few reports on protease exhibiting keratinase activity from thermoalkalophilic *Bacillus* species with high pH and temperature stability. Keratinase from *Bacillus* sp. particularly *B. licheniformis* and *B. subtilis* have been extensively studied due to their effectiveness to degrade feather (Manczinger et al., 2003; Gupta and Ramnani, 2006). We have previously reported the production of thermostable alkaline protease from thermoalkalophilic *Bacillus* sp. JB 99 using pigeon pea waste as a novel substrate which are stable at pH 10.0–11.0, at temperature 60–80 °C, in the presence of detergents and metal ions (Johnvesly et al., 2002). There are hardly any reports on leather dehairing and keratinolytic activity of thermoalkalophilic *Bacillus* sp. except for *B. halodurans* AH-101 (Takami et al., 1999), *Bacillus pseudofirmus* AL-89 (Gessesse et al., 2003) and *B. pseudofirmus* FA 30-01 (Kojima et al., 2006). Because of increasing demand of enzyme in leather industry and management of feather waste, there is need for new proteases. In the present investigation, we report the molecular identification of *Bacillus* sp. JB 99 and purification, characterization of thermostable alkaline protease from *Bacillus* sp. JB 99. In addition to purification, N-terminal amino acid sequence and application of alkaline protease in degradation of chicken feathers and dehairing of goat and buffalo skin were also studied.

2. Materials and methods

2.1. Bacterial strain, maintenance and molecular identification

The thermoalkalophilic *Bacillus* sp. JB 99, used in the present investigation was isolated in our laboratory from sugarcane molasses, biochemically characterized at the Institute of Microbial Technology (IMTECH), Chandigarh, India. The culture maintenance procedure and characterization was carried out as described previously (Virupakshi et al., 2005). The Molecular identification of the strain was confirmed by 16S rRNA gene sequence analysis. The 16S rRNA gene was sequenced at National Centre for Cell Science (NCCS), Pune, India. The DNA sequence was compared to National Centre for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) and it was submitted to the database (Accession number DQ406675).

2.2. Enzyme production

The keratinolytic protease-producing *Bacillus* sp. JB 99 was grown in 500 ml of Erlenmeyer flask containing optimized medium consisting of ($g\,l^{-1}$): chicken feather 5.0; yeast extract, 2.5; peptone, 5.0; $NaNO_3$, 5.0; K_2HPO_4, 5.0; NaCl, 10.0; $MgSO_4 \cdot 7H_2O$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.2; pH 10.0 was adjusted with sodium carbonate, which was autoclaved separately and added to the above medium just before inoculation. Chicken feathers were washed with tap water and cut into sections of $5mm^2$. About 5% of 16 h old culture was used to inoculate the flasks, which were incubated at 55 °C for 48 h with agitation of 220 rpm for enzyme production.

2.3. Enzyme assays

Keratinolytic and caseinolytic activities were determined by using keratin and casein as substrates. A total of 2 ml of reaction mixture containing 1 ml of casein 1% (w/v) dissolved in 50 mM glycine–NaOH buffer pH 11.0 and 0.9 ml of glycine–NaOH buffer was pre-incubated at 70 °C. Reaction was initiated by the addition of 100 μl of suitably diluted enzyme solution and kept at 70 °C. The reaction was terminated after 20 min by adding 2 ml of 10% TCA. The reaction mixture was centrifuged at 12,000 × g for 10 min and absorbance was observed at A_{280}. Keratinolytic activity was studied using 1% keratin (Himedia, India) as a substrate in place of casein in the above reaction mixture. One Unit of caseinolytic and keratinolytic activity was defined as the amount of enzyme required to release 1 μg of tyrosine per ml under experimental condition. Collagenase activity was measured by incubating the enzyme with 4 mg of azocoll (Sigma Aldrich, USA) in 1 ml of 50 mM glycine–NaOH buffer (pH 11.0) at 70 °C for 1 h with constant agitation on a rotary shaker at 250 rpm. The samples were centrifuged at 12,000 × g for 10 min and the absorbance of the supernatant was measured at 520 nm (Jenssen et al., 1994). One unit is defined as the amount of enzyme required to increase 0.1 absorbance per minute at 60 °C.

Protein concentration was determined according to the methods of Bradford (1976) using bovine serum albumin as standard.

2.4. Purification of keratinolytic protease

All subsequent purification steps were carried out at 0–4 °C. The culture broth was centrifuged at 10,000 × g for 10 min (Sorvall, RC5B Centrifuge) and the clear supernatant exhibiting keratinolytic protease activity was precipitated at 80% saturation of ammonium sulphate. The resulting precipitate was collected by centrifugation at 12,000 × g for 15 min. The precipitate was dissolved in a minimal volume (15 ml) of 20 mM Tris–HCl buffer pH 9.0 and dialyzed for 18 h against three changes of same buffer (1 l).

2.4.1. CM-cellulose chromatography

The resultant dialyzed enzyme fraction was applied to a CM-cellulose column (1.5 × 9 cm) previously equilibrated with 20 mM Tris–HCl buffer pH 7.0. The column was initially eluted with a two-bed volume of the buffer to wash out unabsorbed proteins. The adsorbed proteins were then eluted with a linear gradient of 0–0.5 M NaCl in same buffer. The eluted fractions (1.5 ml each) with protease activity were pooled and concentrated in tube concentrator (6–8 kDa MWCO, Merck).

2.4.2. Sephadex G-100 chromatography

The concentrated enzyme fraction was loaded on to a Sephadex G-100 column (1 cm × 50 cm) previously equilibrated with 20 mM Tris–HCl pH 9.0. The enzyme was eluted by using same buffer and fractions of 1.5 ml were collected. For each fraction, the protein content was determined from absorbance at 280 nm and enzyme assays was performed. Fractions with protease activity were pooled and further used for enzyme characterization.

2.5. SDS-PAGE, zymography analysis and N-terminal amino acid sequencing

The molecular weight and homogeneity of the purified alkaline protease was analyzed by SDS-PAGE electrophoresis. The SDS-PAGE (12%) was performed according to Laemmli (1970). Proteins were visualized by staining with Coomassie brilliant blue R-250. Gelatin zymogram analysis was performed as described with modification (Heussen and Dowdle, 1980). The polyacrylamide (12%) gel with separated proteins was washed for 30 min at 4 °C in a 50 mM glycine–NaOH buffer at pH 11.0 containing 2.5% Triton X-100 to remove SDS and to renature protein in the gel, The washed gel was layered on a 1% agarose gel plate containing 1% gelatin in 50 mM glycine–NaOH buffer pH 11.0. After incubation at 50 °C for 90 min, the plate containing gelatin was developed with 15% of $HgCl_2$ in 20% HCl (v/v) to visualize zones of hydrolysis corresponding to protease activity. The N-terminal amino acid sequencing of PVDF membrane blotted protein was performed by Automated Edman degradation method using an Applied Biosystem model 494 Procise sequencer (Indian Institute of Technology, Mumbai, India).