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Purification and characterization of two novel extra cellular proteases from *Serratia rubidaea*

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Abstract

A protease, producing bacterial culture (isolate 'C') was obtained from slaughterhouse waste samples, Hyderabad, India. It was related to *Serratia rubidaea* on the basis of 16S r RNA gene sequencing and biochemical properties. Cultural characters of *S. rubidaea* identified it as a psychrophile secreting protease at 10–30 °C. Single step purification of culture supernatant on sephacryl S-100 column revealed two proteases CP-1 and CP-2. The molecular masses of the enzymes determined by SDS-PAGE and zymography were approximately 97 and 45 kDa, respectively. N-terminal sequencing of CP-1 revealed a novel surface protein of *S. rubidaea* and CP-2 protease has shown 100% homology with protease of *Serratia* sp. A fold purification of 1.5 with 54% recovery was achieved in CP1 and purification of CP-2 resulted in 88% yield with a fold purification of 2. The optimum pH values of CP-1 and CP-2 were shown to be 10 and 8, respectively. The maximum activities for the enzymes were at 40 °C and 30 °C. Both the proteases are inhibited by EDTA indicating that they are metallo proteases. The activity of CP-1 was enhanced with Cu^{2+} that of CP-2 was enhanced with Zn^{2+} and Ca^{2+}. These proteases have stability in presence of detergents, surfactants and solvents. These properties make these proteases an ideal choice for application in detergent formulations, food, leather industries, vaccine and enzyme peptide synthesis.

Keywords: *Serratia rubidaea*; Metalloprotease; Gel-permeation chromatography; Solvent tolerance; Surface layer protein; Detergent activity

1. Introduction

Protease constitutes one of the most important groups of industrial enzymes. Among them, alkaline proteases are one of the most widely studied group of enzymes because of their wide use in many industrial applications such as food, pharmaceutical and leather [1] and with two-third of share in detergent industry alone [2]. Microbial proteases constitute approximately 40% of the total worldwide production of enzymes [3] and bacteria of the genus *Bacillus* produce most commercial proteases used today [4–6]. Alkaline proteases are currently receiving increased attention in view of their inherent stability at high pH, temperature, and in the presence of surfactants, organic solvents and denaturing agents, that enable their use in processes that restrict conventional enzymes. Increasing demand of proteases with specific properties has lead biotechnologists to explore newer sources of proteases.

A considerable attention has been given to the enzymes that are stable in the presence of detergent and solvents and their biotechnological potentials. Reports on few enzymes that are naturally stable and also exhibiting high activities in the presence of solvent and detergent have got significant importance in the present era [7–10]. Several workers have reported proteases from different strains of *Serratia* and *Serratia marcescens* is the most studied organism [11]. Recently an extra cellular detergent and organic tolerant protease producer has been successfully isolated and in this report, we present the purification and characterization of two novel extracellular proteases CP-1 and CP-2 from *Serratia rubidaea*. These were purified in a single step by gel-permeation chromatography and were further characterized.

2. Materials and methods

2.1. Microorganism and taxonomic study

The novel alkaline protease producing strain *S. rubidaea* was isolated from *waste* dump of slaughterhouse, Hyderabad, India. The strain was identified

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according to the methods described in *Bergey's Manual of Systematic Bacteriology* [12], and on the basis of 16S ribosomal DNA sequence [13,14]. The stock culture was maintained on nutrient agar at 4 °C and as a glycerol stock at −20 °C.

2.2. Nutritional factors affecting growth and protease production

For optimization, production of protease by isolate "C" was studied using basal medium ((g l^{-1}) dextrose, 10.0; peptone, 10.0; KNO_3, 0.8; NaCl, 5.0; K_2HPO_4, 0.5; $MgSO_4{\cdot}7H_2O$, 0.5; $CaCl_2$, 1.2), under the following conditions: age of inoculum—2.5 ml of 12 h, temperature: 0–50 °C, with increments of 10 °C, pH 6–12, with increments of one unit, NaCl concentration 0.5–2.0 M. The flasks were incubated at 20 °C for 48 h and cells were removed by cold centrifugation at 8900 × *g* for 10 min. The cell free supernatant was analyzed for protease activity. To test the effect of different carbon sources on the protease production, dextrose in the basal medium was substituted with 1% starch, lactose, sucrose, mannose and maltose.

2.3. Enzyme production

Twenty-four hour culture in basal medium (0.5 ml) was used to inoculate 500 ml flasks containing 100 ml of starch casein broth (Starch 1 g, casein 0.3 g, KNO_3 0.2 g, NaCl 0.2 g, K_2HPO_4 0.2 g, $MgSO_4$ 0.005 g, $CaCl_2$ 0.002 g, $FeSO_4$ 0.001 g, distilled water 100 ml) and incubated for 48 h at 20 °C, 150 rpm. The culture was centrifuged at 8900 × *g* for 10 min. The supernatant obtained was lyophilized and was used as crude enzyme preparation for further steps.

2.4. Purification of proteases by gel-permeation chromatography

One ml of crude enzyme preparation was loaded to a sephacryl S-100 column (3 cm × 100 cm) previously equilibrated with 50 mM phosphate buffer (pH 7.2) and then eluted with the same buffer. Fractions of 0.4 ml each were collected at a flow rate of 0.2 ml/min. Protein concentration of each fraction was determined at absorbance 280 nm and 10 μl aliquots of each fraction was assayed for protease activity. Purity was checked by SDS-PAGE and zymography. Two protease active fractions were pooled independently, named as CP-1 and CP-2 and used for further characterization.

2.5. Assay of proteolytic activity

Protease activity was determined by modified method of Kunitz [15] using casein as a substrate. Fifty microlitres of CP-1 and CP-2 was added to 450 μl of substrate solution (1% (v/v) casein with 50 mM Tris–HCl buffer pH 8.0) and incubated at 30 °C for 30 min independently with respective controls. The reaction was stopped by adding 750 μl of 5% TCA mixture (5% TCA, 9% Na-acetate, 9% acetic acid) followed by 30 min incubation at room temperature and centrifugation (8900 × *g*, 15 min). The absorbance of supernatant was measured at 280 nm. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of tyrosine per minute under the assay conditions. The amount of tyrosine was determined from the tyrosine standard curve.

2.6. Protein measurement

The protein content of the enzyme preparation was estimated by Lowry method [16].

2.7. SDS/native-PAGE and zymography

SDS and native PAGE was carried out for determination of purity and molecular mass of the enzymes by the method of Laemmli [17], in 12% cross linked polyacrylamide gel. Silver staining was used to visualize protein bands on the gels [18]. Protease profile was revealed by zymogram, using gelatin as copolymerized substrate [19]. Proteolytic activity was evident as bands depleted of gelatine. The isoelectric point (p*I*) of purified CP-1 and CP-2 protease was determined by Robertson et al. method [20]. Following SDS-PAGE, the proteins were blotted to a polyvinylidene fluoride membrane excised and the N-terminus was sequenced. The sequence obtained was analyzed with protein database.

2.8. Mass spectrometry

Electrospray ionization mass spectrometry of proteases was carried out on a Mass Spectrometer (Bruker & Ultraflex) and spectrum was acquired using flex control™ 2.2 software (Webster, 2005). The sample was injected in one volume of acetonitrile and two volumes of 0.1 % trifluoro acetic acid.

2.9. HPLC analysis

The purified protease was analyzed by HPLC using a BioSep-SEC-S 2000 size exclusion column (300 mm × 7 mm; Phenomenex, Torrance, CA). Proteins were eluted at 20 °C with an isocratic mobile phase (50 mM Tris–HCl buffer, pH 7.2, 1 ml min^{-1}) and monitored at 280 nm. Apparent molecular masses (AMM) was assigned by comparison with the relative mobility of reference proteins.

2.10. Effects of pH and temperature on enzyme activity and stability

The optimum pH was determined with casein as substrate dissolved in the following buffer systems: acetate buffer (pH 4.0, 5.0, 6.0), Tris–HCl buffer (pH 7), glycine–NaOH buffer (pH 8.0, 9.0), carbonate buffer (pH 10.0–12.0). The pH stability of alkaline protease was determined by dissolving the protease in the different buffers, and incubating the solutions at 20 °C for 4 h. The residual activities were quantified under standard assay conditions.

The optimum temperature was determined for CP-1 and CP-2 proteases at different temperatures (10–80 °C) in carbonate bicarbonate buffer (pH 10.0) for CP-1 and glycine–NaOH (8.0) buffer for CP-2. For determining thermal stability, the enzyme was pre-incubated for 1 h at different temperatures ranging from 10 to 60 °C, and residual activity was measured.

2.11. Effects of different inhibitors, metals, detergents on enzyme activity

To study inhibition of the protease, enzyme was preincubated with inhibitors(1 mM, 5 mM) such as PMSF, leupeptin, pepstatin, *p*CMB, metal ions such as like Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+} Na^{+} and Zn^{2+} (5 mM), detergents such as SDS and Triton X-100 (0.1% (w/v), 0.5% (v/v)) for 1–4 h at 20 °C. Solvent stability studies were carried out by incubating 3 ml of protease solutions in the absence or presence of 1.0 ml of organic solvents, at 30 °C with constant shaking at 160 rpm for 72 h [21]. Subsequently the enzyme assay was performed as described above. The percent residual enzyme activity was calculated with reference to the activity of the enzyme without these supplements.

2.12. Evaluation of washing performance using blood stain

Clean cotton cloth pieces (5 cm × 5 cm) were soiled with blood, allowed to dry and soaked in 2% formaldehyde for 30 min followed by washing with water to remove excess formaldehyde. The stained cloth pieces were incubated with the 100 U/ml of purified proteases CP-1 and CP-2 at 40 °C for time duration of 10–40 min. After incubation, each piece was rinsed with water for 2 min and then dried. The same procedure was done for the control except incubation with the enzyme solution. Ariel a non enzyme based detergent available in Indian market was used (0.5%, w/v, in tap water) in presence and absence of purified enzymes for testing the wash performance.

3. Results

The isolate was identified on the basis of morphological and physiological characteristics, biochemical tests and 16S rRNA sequencing. The isolate was positive for nitrate reduction; H_2S production, catalase and citrate utilization and indole production and these tests were used to differentiate *S. rubidaea* from other *Serratia* sp. The 16S rDNA sequence and their phylogeny is presented in Fig. 1. Interestingly, all the species showed