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Purification and characterization of an alkaline protease by a new strain of *Beauveria* sp

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ABSTRACT

A fungal culture isolated from animal dung was identified as a new strain of *Beauveria* sp MTCC 5184 based on 18S rDNA and ITS nucleotide sequence homology. The fungal isolate secretes alkaline protease active at pH 9 and 50 °C. The alkaline protease from *Beauveria* sp (BAP) was purified to homogeneity with 10.2-folds increase in specific activity and 38.6% recovery. The molecular mass and isoelectric point of the protease were found to be 29 kDa and 9.3, respectively. The N-terminal sequence of the BAP showed only partial homology with subtilisin like proteases from other fungi. The enzyme was stable up to 40 °C and pH 3–11. The protease was inhibited by Cd^{2+}, Hg^{2+} and Mn^{2+}. The activity was totally lost in the presence of 1 mM PMSF suggesting it to be a serine protease. The protease showed maximum activity with casein followed by haemoglobin and BSA. The purified protease is able to separate the endothelial cells and can be used in animal cell culture.

1. Introduction

Proteases constitute a large group of hydrolytic enzymes that cleave peptide bond of protein and degrade them into small peptides and amino acids. As proteases are involved in all the cellular functions, they are found in plants, animals and microorganisms including viruses. Although proteases are widespread in nature, microbes serve as a preferred source of these enzymes. The value of worldwide sales of industrial enzymes in 2006 was estimated to be US $17–2.0 billion. Among the industrial enzymes, 75% are hydrolytic and proteases from plant, animal and microbial sources account for about 60% of total enzyme sales [1].

Current world demand for proteases has led to an interest in microbial proteases because of their rapid growth, low production cost and the ease with which they can be genetically modified to generate high yielding strains with more efficient enzymes with desirable properties required for diverse applications. Because of their broad substrate specificity, they have a wide range of applications such as in leather processing, detergent formulations, food processing industries, peptide synthesis, preparation of protein hydrolysates, resolution of D,L-amino acids, pharmaceutical industry, recovery of silver from waste photographic film, as well as analytical tools in basic research. Most of the commercial proteases are of bacterial origin [2]. Comparatively, studies on fungal proteases are few and limited mainly to species belonging to the genera such as *Aspergillus*, *Conidiobolus*, *Mucor*, *Paecilomyces*, *Penicillium*, *Rhizopus* [3–5]. Reports on proteases from other genera are very few [6–8].

Enzymes of fungal origin are advantageous due to the ease of cell removal during downstream processing. In recent years, the potential use of microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms. Many investigations are focused on the discovery and characterization of novel naturally occurring proteases from sources that have been overlooked [7]. Search for new and more active enzymes has renewed the interest in proteases from fungal strains isolated from diverse and hitherto unexplored habitats. The present paper describes the identification of a novel fungus secreting alkaline protease using molecular techniques. The purification and properties of the purified protease have also been reported.

2. Materials and methods

2.1. Chemicals

Coomassie Brilliant Blue G-250, sodium dodecylsulphate (SDS), β-mercaptoethanol (BME), N,N,N′,N′-tetramethylethylenediamine (TEMED), phenylmethylsulfonyl fluoride (PMSF), N-tosyl-L-lysine chloromethyl ketone (TLCK) and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were purchased from Sigma Chemical Co. (USA). Molecular mass markers, ampholines were obtained from BioRad, India. Enzymes and chemicals for PCR and sequencing were obtained from Bangalore Genie (India). Malt extract, yeast extract and peptone were procured from Hi Media Chemicals, India. All other chemicals used were of analytical grade. Mustard seed cake was obtained from local market.

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2.2. Microorganism

The fungal culture was maintained on MGYP agar slants [4] and sub-cultured once in a month and preserved at 4 °C after growth and sporulation.

2.3. PCR amplification and sequencing of ITS region and 18S of rDNA gene

The fungus was grown in MGYP broth for 48 h at 28 °C, 180 rpm and DNA was isolated from freeze-dried mycelia according to the method described by Lodhi et al. [9] with slight modifications. PCR amplification of 18S ribosomal DNA was performed using primers NS1-F (GTA GTC ATA TGC TTG TCT C), NS8-R (TCC GCA GGT TCA CCT ACG GA) and the internal transcribed spacer (ITS) region using primers ITS1-F (TCC GTA GGT GAA CCT GCG G) and ITS4-R (TCC TCC GCT TAT TGA TAT GC) [10]. The sequencing reactions of PCR product were carried out using *Taq* DNA polymerase dye terminator cycle applying automated DNA sequencing method based on dideoxynucleotide chain termination method using universal primers: NS1 (GTA GTC ATA TGC TTG TCT C), NS2 (GGC TGC TGG CAC CAG ACT TGC), NS3 (GCA AGT CTG GTG CCA GCA GCC), NS4 (CTT CCG TCA ATT CCT TTA AG), NS5 (AAC TTA AAG GAA TTG ACG GAA G), NS6 (GCA TCA CAG ACC TGT TAT TGC CTC), NS7 (GAG GCA ATA ACA GGT CTG TGA TGC), NS8 (TCC GCA GGT TCA CCT ACG GA), ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC).

2.4. Analysis of nucleotide sequence

The nucleotide sequence was analyzed with the NCBI database using BLAST program. The multiple sequence alignment of homologous sequences was done in Clustal X software and sequences were trimmed in DAMBE software. The phylogenetic tree was inferred using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test with 1000 replicates is shown next to the branches [11]. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted in MEGA4 [12].

2.5. Protease production

Spores from 2 to 3 weeks old MGYP slant was used for inoculum development. Enzyme production was carried out in 250 ml Erlenmeyer flasks containing 50 ml GYE (glucose, 1%; yeast extract, 0.3%) medium with 2% (w/v) mustard seed cake as inducer. 48 h old vegetative inoculum (10%, v/v) grown in MGYP was used to inoculate the experimental flasks. Flasks were incubated at 28 °C, 200 rpm for 3–5 days. Samples were removed periodically, centrifuged at 10,000 rpm for 10 min and the clear supernatant was used as crude enzyme.

2.6. Protease assay

Protease as caseinolytic activity was estimated at 50 °C, pH 9 according to Laxman et al. [4]. One unit of activity is defined as the amount of enzyme required to release 1 μM of Tyr/min under the assay conditions.

2.7. Protein determination

Protein was estimated according to the method of Bradford [13] with bovine serum albumin (BSA) as the standard.

2.8. Protease purification

All the purification steps were carried out at 4 °C unless otherwise stated. The crude culture filtrate was subjected to fractional ammonium sulphate precipitation with 10% increments. The pellet from each fraction (10,000 × *g*, 15 min) was suspended in 50 mM phosphate buffer pH 7 and the supernatant was used for the next fractionation step.

Dialyzed ammonium sulphate fraction was loaded on a DEAE-cellulose column (2.5 cm × 25 cm) equilibrated with 50 mM phosphate buffer pH 7. The column was eluted with same buffer and 2 ml fractions were collected. Protein and protease activity in the fractions were estimated.

2.9. High performance liquid chromatography (HPLC)

Purified protease (100 μg) was loaded on HPLC gel filtration TSK G 2000 SW prepacked column (7.5 mm × 600 mm) and eluted with 50 mM phosphate buffer, pH 7 at a flow rate of 0.7 ml/min.

2.10. Polyacrylamide gel electrophoresis and isoelectric focusing

Cathodic and SDS-PAGE were carried out as described by Laemmli [14]. SDS-PAGE was carried out using 6% (w/v) stacking gel and 12% (w/v) separating gel. The molecular mass of the enzyme was estimated using the broad range molecular mass markers (18.8–101.4 kDa).

Isoelectric point of the protease was determined by the modified straight tube method using ampholines in the narrow pH range of 8–10. The gel was taken out from glass tube and cut into 13 pieces of 1 cm each. Protease activity and pH of the fractions were checked.

2.11. Effect of pH on protease activity and stability

Optimum pH was determined by estimating the protease activity at 50 °C and pH values ranging from 5 to 12 (acetate, pH 5; citrate, pH 6; phosphate, pH 7; Tris–HCl, pH 8; carbonate, pH 9 and 10; sodium phosphate–NaOH, pH 11 and KCl–NaOH, pH 12). Stability of protease was examined by incubating the enzyme at 28 °C in buffers at pH values ranging from 3 to 11 for 1 h. Residual activity was estimated as described earlier and expressed as percentage of the initial activity taken as 100%.

2.12. Effect of temperature on protease activity and stability

Optimum temperature was determined by estimating the protease activity at pH 9 and temperatures ranging from 30 to 70 °C for 10 min. Thermal stability was examined by incubating the enzyme at temperatures ranging from 4 to 70 °C for 1 h and the residual activity was measured at 50 °C, pH 9 and expressed as percentage of initial activity taken as 100%.

2.13. Determination of K_m and V_{max}

K_m and V_{max} values of the pure enzyme were determined by measuring the activity with casein concentrations ranging from 1 to 10 mg. Kinetic constants were calculated from Lineweaver-Burk plot.

2.14. Effect of protease inhibitors and chelators

For the determination of protease type, purified protease was pre-incubated with following inhibitors: PMSF, EDTA, iodoacetic acid, TPCK, TLCK, DMSO and benzamidine hydrochloride in 100 mM Tris–HCl buffer (pH 8.0) for 1 h and the residual activity was measured at 50 °C, pH 9. Control without inhibitor was taken as 100%.

2.15. Effect of metal ions

Purified protease was incubated with metal ions added as chlorides of Ca, Cd, Co, Cu, Fe, Hg, K, and Na for 30 min. Protease activity without metal was taken as 100%.

2.16. N-terminal sequencing

The 60 μg of purified protease were loaded on 12% SDS PAGE. Electrophoresis was carried out at 20 mA for 3 h and the semidry electro blotting was done to transfer the protein on polyvinylidene fluoride (PVDF) membrane. The transferred protein was subjected to N-terminal sequencing on a Perkin Elmer sequencer type Procise (Applied Biosystems, Foster City, CA, USA) by Edman degradation method.

2.17. Application of protease in animal tissue culture

The endothelial cells were grown on basal medium-2 (EBM-2). After 48 h of growth, media was removed from plate in a falcon tube. The adherent cells at the bottom of the plate were washed with 2 ml of 1 mM EDTA solution followed by addition of 200 μg of protease in 4 ml of 1 mM EDTA solution and incubated at 37 °C for 2 min and observed under microscope. Cells were flushed out, collected in falcon tube and spinned at 2000 rpm for 2 min. The supernatant was removed and cell pellet was re-suspended in 1 ml fresh medium and transferred to a plate.

The data represented are mean of three independent experiments.

3. Results and discussion

A new strain of *Beauveria* sp was isolated from rabbit dung and deposited with Microbial Type Culture Collection, IMTECH, India under the accession number MTCC 5184. The fungus grows over a pH range of 5–9 with an optimum at 6.5–7.5. The organism grows in the temperature range of 15–35 °C with an optimum at 28 °C.

3.1. Identification and phylogenetic analysis

For identification of the organism, 18S rDNA (1.8 kb) and ITS (0.6 kb) regions were amplified, sequenced and analyzed with the NCBI database using BLAST program. The 18S rDNA sequence showed maximum homology with 18S rDNA of *Beauveria felina* (Fig. 1a). Our analysis indicated that these 18S rDNA genes were clustered into two subclades. All 18S rDNA genes from *B. felina*