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Process Biochemistry 41 (2006) 208–215

PROCESS BIOCHEMISTRY

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Purification and characterization of a salt, solvent, detergent and bleach tolerant protease from a new gamma-Proteobacterium isolated from the marine environment of the *Sundarbans*

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Received 5 April 2005; received in revised form 10 September 2005; accepted 18 September 2005

Abstract

In this communication we report purification and characterization of a single salt, solvent, detergent and bleach tolerant alkaline serine protease produced by a truly marine bacterium. Based on the 16S rRNA gene sequence, absence of polyhydroxybutyrate accumulation, growth with very high NaCl levels as well as with hydrocarbons as sole carbon source, the isolate was classified as a new bacterium belonging to the gamma-Proteobacteria family. A 69-fold purification (specific activity 791.7 U/mg protein, unit expressed as μmole of tyrosine liberated per minute) was achieved by a three-step purification procedure. The enzyme is active over a broad range of pH (6.0–11.0), the optimum being at 9.0. This protease enzyme shows activity from 30 to 70 °C and Ca^{2+} increase the thermostability. This enzyme exhibits appreciable activity in presence of up to 30% NaCl and is highly stable even after 18 h pre-incubation with 35% (w/v) NaCl. While Ba^{2+} and Ca^{2+} enhance the enzyme activity, heavy metals like Co^{2+}, Zn^{2+}, Hg^{2+} inactivate the enzyme. The enzyme is completely stable in presence of laboratory detergents (Tween 80 and Triton X-100), oxidizing agents, reducing agents, commercial detergents and bleaches (hydrogen peroxide and sodium perborate) after 1 h of pre-incubation. Water miscible and immiscible organic solvents like ethylene glycol, ethanol, butanol, acetone, DMSO, xylene and perchloroethylene enhance as well as stabilize the enzyme activity.

Reflectance measurements during wash performance studies show that our enzyme is capable of almost complete removal of recalcitrant blood and egg stains in both wet and dry wash operations. Enzymatic activity against a wide variety of substrates indicates that our enzyme can be investigated for a range of commercial applications especially for soy protein and gelatin hydrolysis in the food processing industry as well as for the dehairing process in the leather industry in addition to catalyzing hydrolysis reactions at high salt concentrations.

Keywords: Marine bacteria; Serine protease; Gamma-Proteobacteria; *Sundarbans*

1. Introduction

Proteases encompass a group of enzymes, which hydrolyze peptide bonds in aqueous environment and synthesize peptide bonds in non-aqueous environment. These enzymes account for about 60% of total worldwide sales of enzymes [1] and have application in food, pharmaceuticals, detergent, leather industry, basic research and for extraction of silver from used X-ray films [2–4]. Alkaline protease comprises 30% of the total worldwide enzyme production [5] and is particularly suitable for the detergent industry because of its stability at high pH and activity in presence of different surfactants.

Microbial proteases constitute approximately 40% of the total worldwide production of enzymes [6] and bacteria belonging to the genus *Bacillus* produce most commercial proteases used today [7–12]. Increasing demand of proteases with specific properties has lead biotechnologists to explore newer sources of proteases [13–16]. Marine microorganisms represent one such novel source. Due to their unique natural habitat these microorganisms show distinct physiological characteristics, metabolic patterns and nutrient utilization as compared to their terrestrial counterparts. Therefore, the enzymes from marine microorganisms should be expected to possess unique properties [17,18]. However, till date few

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doi:10.1016/j.procbio.2005.09.010

proteases from truly marine bacteria have been purified and characterized [19].

The *Sundarbans*, the world's largest tidal mangrove forest, lies on the delta of the *Ganges*, *Brahmaputra* and *Meghna* rivers on the Bay of Bengal. The biotechnological applications of the microbial biodiversity of the *Sundarbans* remained unexplored till we reported the isolation of a novel antimicrobial agent [20]. In this paper we report, for the first time, purification and characterization of a single salt, solvent, detergent and bleach tolerant alkaline serine protease having a broad substrate specificity produced by a truly marine bacterium.

2. Materials and methods

2.1. Microorganism

2.1.1. Isolation and characterization

The microbial isolate, designated as DGII, isolated from the sediment of the Lothian Island (Lat. 20°50′N, Long. 88°19′E) at a depth of 1 m was used in the study. The isolate was maintained by routine culture on Marine Agar 2216 media (all units in g/l; peptone 5.0, yeast extract 1.0, $FeCl_3$ 0.1, $MgCl_2$ 8.8, NaCl 19.45, Na_2SO_4 3.24, $CaCl_2$, $6H_2O$ 1.8, KCl 0.55, $NaHCO_3$ 0.16, KBr 0.08, $SrCl_2$ 0.034, H_3BO_3 0.022, Na_2SiO_3 0.004, NaF 0.0024, NH_4NO_3 0.0016, Na_2HPO_4 0.008, agar 15; pH 7.0–7.2). Gram characteristics of the isolate and polyhydroxybutyrate accumulation have been studied by Gram staining and by staining with Sudan Black, respectively. Growth in the presence of hydrocarbons as sole sources of carbon (1% benzene and cyclohexane) was observed in a basal medium [21]. Growth with varying levels of NaCl in the Marine Broth 2216 medium was studied. Further taxonomic characterization was done based on the nucleotide sequence of the 16S rRNA gene.

2.1.2. Chromosomal DNA extraction and purification

The isolate was grown in 100 ml of Marine Broth 2216 media for 48 h at 35 °C. The biomass was harvested by centrifugation at 10,000 rpm for 10 min and washed twice in sterile Tris-EDTA buffer (10:1 molar ratio, pH 8.0) and approximately 100 mg wet biomass was used for DNA extraction. The extraction and purification of DNA were carried out by the phenol chloroform method [22,23].

2.1.3. DNA amplification, sequencing and phylogenetic analysis

Two primers were selected for use in PCR amplification experiments, forward primer 5′-AGA GTT TGA ACA TGG CTG-3′(S-D-Bact-0027-a-S-18) and reverse primer 5′-CTA GCG ATT CCG ACT TCA-3′ (S-D-Bact-1327-a-A-18) [24]. The reaction mixture for PCR amplification contained 10 × PCR buffer 5 μl, dNTP 4 μl, Taq DNA polymerase 1 μl, 45 pmol of each primers (Genei, Bangalore, India) and 50 ng of bulk DNA. Amplification was performed in a thermal cycler (GeneAmp 2400 PCR system, PE-Biosystem, California, USA) for an initial denaturation at 94 °C for 5 min followed by 40 cycles of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 2 min and a final extension at 72 °C for 7 min. Amplified DNA was purified by spin column method (WIZARD PCR Prep DNA purification system, Promega, Madison, Wisconsin, USA). Sequencing of the amplified DNAs and phylogenetic analysis of the sequence data was done using the computational techniques described by Saha et al. [20]. A phylogenetic tree was constructed by using the neighbor joining DNA distance algorithm [25].

2.2. Enzyme production

The culture was grown in Erlenmeyer flasks (500 ml) containing 100 ml of Marine Broth 2216 media grown for 48 h at 35 °C on a shaker at 180 rpm. The culture was centrifuged at 5000 rpm for 10 min and the supernatant was used as crude enzyme for further study.

2.3. Protease assay

Protease activity was assayed by using casein as a substrate. The reaction mixture containing 100 μl Tris-chloride buffer (100 mM, pH 8.5), 100 μl of 1% casein solution and 200 μl of suitably diluted enzyme solution was incubated at 40 °C for 30 min. The reaction was terminated by addition of 400 μl of 10% trichloroacetic acid solution and the non-hydrolyzed casein was precipitated by centrifugation at 10,000 rpm for 10 min. Peptide concentration of the supernatant was determined by measuring absorbance at 280 nm using tyrosine as a standard. One unit of proteolytic activity was defined as the amount of the enzyme required to liberate 1 μmol of tyrosine per minute at pH 8.5 and temperature 40 °C.

2.4. Protein assay

Protein was measured by the method of Lowry [26] with bovine serum albumin as standard. During chromatographic purification steps protein concentration was estimated as a function of its absorbance at 280 nm.

2.5. Purification of protease

2.5.1. Acetone precipitation

To the culture filtrate of DGII pre-chilled acetone was added slowly up to 60% saturation with gentle stirring and left for 4 h at 4 °C for complete precipitation. The precipitate was collected after centrifugation at 12,000 rpm for 15 min at 4 °C, air-dried and re-dissolved in a minimum amount of 20 mM Tris-chloride buffer (pH 8.0). Finally, the insoluble material was removed by centrifugation at 15,000 rpm for 20 min at 4 °C and the supernatant was collected.

2.5.2. Anion exchange chromatography

The precipitated enzyme solution was loaded at a flow rate of 0.2 ml/min to a DEAE-cellulose (Sigma–Aldrich, St. Louis, USA) column (2 cm × 7 cm), pre-equilibrated with 20 mM Tris-chloride buffer of pH 8.0 and the eluted fractions (1 ml each) were collected. After loading the sample the column was washed with the same buffer until OD_{280} of the effluent was zero. The bound proteins were then eluted with a concentration gradient of sodium chloride (0–0.5 M NaCl) in the same buffer and fractions of 2 ml each were collected at a flow rate of 0.2 ml/min. Protein concentration of each fraction was determined from OD_{280} and 50 μl of aliquots of each fractions were assayed for protease activity. Fractions with high specific activity were pooled and then concentrated by lyophilization.

2.5.3. Sephadex gel filtration

One milliliter of partially purified enzyme was loaded to a Sephadex-G-100 (Fluka Chemicals, Buchs, Switzerland) column (1.25 cm × 35 cm) pre-equilibrated with 80 mM of Tris-chloride buffer (pH 8.5) and then eluted with the same buffer. Fractions of 2 ml each were collected at a flow rate of 2.5 ml/h. Protein concentration of each fraction was determined from OD_{280} and 50 μl of aliquots of each fraction were assayed for protease activity. The active fractions were pooled, concentrated by lyophilisation and purity checked by SDS-PAGE.

2.6. Electrophoresis and zymography

SDS-PAGE was performed using 5% stacking gel and 12% resolving gel by the method of Laemmli [27] to monitor the purification process. Detection was done by the sensitive silver staining method.

Substrate polyacrylamide gel electrophoresis (Substrate-PAGE) was performed to detect the band with protease activity. Discontinuous substrate SDS-PAGE was performed with a 4% stacking gel, except that 0.1% heat-denatured azocasein was incorporated into the 12% separation gel. A 1% stock of azocasein in separation gel buffer was boiled for 20 min prior to addition to the gel. Sample was mixed with equal volume of non-reducing sample buffer and was not boiled prior to application. Electrophoresis was done at a constant current of 15 mA. After electrophoresis, gel was washed twice for 1 h each with 100 ml of 2.5% Triton X-100 in water to replace SDS and separation buffer in the gel. Incubation of the washed gel at 40 °C for 3 h in 50 mM Tris–HCl (pH