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Original Article

Purification and characterization of a harsh conditions-resistant protease from a new strain of *Staphylococcus saprophyticus*

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ABSTRACT

A major road block to the industrial usage of known proteases is their limited stability under harsh conditions. Hence, there is always a need for newer enzymes with novel properties that can further satisfy all industrial demands. This study described a benthic marine bacterium, *Staphylococcus saprophyticus* that secretes an alkaliphilic and broad-temperature active protease (10–80 °C). The protease was successfully purified 42.66-fold using 70–80% ammonium sulfate precipitation and gel-permeable column chromatography. It had a relative molecular mass of 28 kDa on sodium dodecyl sulphate-polyacrylamide gel electrophoresis and retained high activity and significant stability at 60–80 °C, over a wide range of pH (3.0–12.0), inhibitors and metal ions. Furthermore, the enzyme was stable in surfactants (such as sodium dodecyl sulfate), oxidizing agents (such as H_2O_2), bleaching agents (such as zeolite) and hydrophobic solvents (such as benzene, hexanes and hexadecane). These properties support the enzyme's potential as a vigorous biocatalyst for industrial applications.

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Introduction

Protease is a particularly important group of industrial enzymes that account for 60% of the total world enzyme markets (Rao et al., 1998; Raval et al., 2014). It has diverse applications in a wide range of industrial processes including food, detergent, pharmaceuticals, leather and wastewater treatment (Rao et al., 2009; Mesbah and Juergen, 2014). Among the various industrial protease producers, bacteria with their high production capacity and catalytic activity are the most substantial contributors when compared to animals, plants and fungi (Rao et al., 1998; Kumar and Takagi, 1999; Bhunia et al., 2013). However, a major road block to industrial usage of known proteases is their limited function under harsh conditions (Rao et al., 1998; Kumar and Takagi, 1999). Increasing demand for highly active preparations of proteases with appropriate specificity and stability with regard to pH, temperature, metal ions, surfactants and organic solvent continues to stimulate the search for new enzymes (Sellami-Kamoun et al., 2008; Haddar et al., 2009). Many proteases have been documented and some of them are now commercially available (Rao et al., 1998, 2009; Kumar and Takagi, 1999; Dubin, 2002). *Staphylococcus aureus* V-8 protease is a well-known commercial example. This enzyme shows maximum activity at two optimal pH values (4.0 and 7.8) and at 37 °C. The enzyme is active in the presence of many denaturing agents such as sodium dodecyl sulfate (SDS), urea and guanidine-HCl (Arvidson et al., 1973). However, its stability under multiple extreme conditions is rare. Hence, searching for enzymes that have wider versatility and adaptability to multiple harsh conditions is academically and industrially important.

The marine environment is a source of unique microorganisms with great potential for biotechnological exploitation (Podar and Reysenbach, 2006; Sellami-Kamoun et al., 2008; Haddar et al., 2009). Many studies concerning the isolation and characterization of marine bacteria have been carried out and investigations in this field may lead to many new discoveries. This study described the purification and characterization of a novel extracellular protease produced by the newly isolated marine strain *S. saprophyticus*, with a focus on its biochemical properties under harsh conditions.

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Materials and Methods

Isolation of protease-producing strain

Ten marine sediment samples were collected from Koh Jan, Samaesan, Thailand at depths of 9–24 m. Samples (10 g) were mixed with 100 mL of sterile water and the sediment was allowed to settle. The upper phase (100 μL) was spread on marine agar (BD; Difco; Le Pont de Claix, France) and was used for screening. Cultures were maintained at 25 °C for 24 h. Colonies that appeared on the plate were isolated as a single colony by streaking repeatedly and the culture was maintained under the same conditions. Bacteria that secreted protease were screened based on the clear zone surrounding the colonies as shown on skim milk agar (1% (weight per volume; w/v) skim milk powder, 1.5% (w/v) bacto-tryptone, 0.25% (w/v) yeast extract, 0.1% (w/v) glucose and 2.5% (w/v) NaCl, pH 7.2). Strains that showed a maximum ratio of clear zone:colony diameters were selected and assayed for protease activity on a substrate of azocasein (see below). The bacterial strain with the highest protease activity after 24 h of cultivation was selected for further experiments.

Bacterial strain identification

Bacterial strain identification was based on the "API skills Bacterial Identification Method" and 16S rRNA gene sequence analysis (Weisburg et al., 1991). Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was performed using primers designed from the conserved regions at the base positions 22–41 and 1066–1085 located on the *Escherichia coli* 16S rRNA gene (Precigou et al., 2004). Chromosomal DNA (100 ng) prepared using a GF-1 Nucleic acid extraction kit (Vivantis, Selangor Darul Ehsan, Malaysia) was used as a DNA template. PCR was carried out using an initial denaturation step at 95 °C for 10 min which was followed by 30 cycles at 95 °C for 30 s, at 60 °C for 45 s and at 72 °C for 90 s. Final extension occurred at 72 °C for 7 min.

The expected PCR product (~1.1 kb) was purified using a GF-1 Gel DNA recovery kit (Vivantis, Selangor Darul Ehsan, Malaysia) and then was ligated into a pTG19-T vector (Vivantis, Selangor Darul Ehsan, Malaysia) according to the manufacturer's instructions. After transformation into *E. coli* DH5α, plasmids were extracted and purified using a GF-1 Plasmid DNA extraction kit (Vivantis, Selangor Darul Ehsan, Malaysia). A gene insert was verified using sequence analysis (Sanger et al., 1977). Similarity of nucleotide sequence was determined using BLAST (National Center for Biotechnology Information databases, Bethesda MD, USA) and subsequently analyzed using the Ez-Taxon database (Chun et al., 2007). To generate a 16S rRNA gene-based phylogenetic tree, sequences were aligned using the SILVA aligner (Pruesse et al., 2007). Sequence divergence was calculated using the Kimura 2-parameter model (Kimura, 1980) in the MEGA 6 software (Tamura et al., 2013), which was also used to create the neighbor-joining tree and to perform bootstrap analysis (1000 replicates) (Saitou and Nei, 1987). Sequence data were submitted to the GenBank database under accession no. KM370125.

Measurement of protease activity and protein content

Protease activity was measured using azocasein hydrolysis (Meyers and Ahearn, 1997). The reaction mixture consisted of 1% (w/v) of azocasein (125 μL) and 125 μL of enzyme solution. The reaction was incubated for 15 min at room temperature (28–30 °C). Then, the reaction was stopped by the addition of 250 μL of 0.4 M trichloroacetic acid (TCA), and the mixture was allowed to stand at room temperature for 15 min. The sample was centrifuged at 10,000 × *g* for 5 min to remove the precipitate. Thereafter, the supernatant was mixed with 0.4 M Na_2CO_3 (625 μL) and Folin-phenol reagent (125 μL). Reaction was allowed at room temperature for 10 min. After that, absorbance was measured at 660 nm wavelength. A blank was set up following the same procedure, except that the enzyme was added after the addition of 0.4 M TCA. One unit (U) of protease activity was defined as the amount of enzyme liberating 1 μg of tyrosine per minute under the assay conditions. The amount of tyrosine was determined from its standard curve. The protein content was determined using the Bradford method (Bradford, 1976), with bovine serum albumin (BSA) as the standard protein. All experiments were conducted in triplicate and average values with the standard deviation were reported.

Protease purification

A single colony of the bacterial strain was grown in 3 L of Luria-Bertani broth at 30 °C and 250 rpm for 24 h. The culture supernatant was collected using centrifugation at 10,000 × *g* for 10 min. Solid ammonium sulfate was slowly added to the culture supernatant to 70–80% saturation and continuously stirred for 30 min at 4 °C. The precipitate was harvested using centrifugation at 10,000 × *g* for 30 min and subsequently dissolved in 50 mM Tris-HCl buffer (pH 8.0) and dialyzed against the same buffer overnight (4 °C). The dialyzed sample was assayed for protease activity and protein content and loaded onto a Tris-HCl buffer (pH 8.0) pre-equilibrated Sephadex G-75 gel permeable chromatography column. The same buffer was used for elution of protein with a flow rate of 30 mL/h. The ultraviolet absorbance of each fraction was measured at 280 nm. Fractions were assayed for protease activity. Protease active fractions were pooled and concentrated for further characterization.

Purity and the relative molecular mass of protease were estimated using discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (5% stacking and 15% separating gel) (Laemmli, 1970), using High-Range Rainbow molecular weight markers (GE Healthcare, Hatfield, England) as the standard protein markers. Gelatin zymography staining was carried out as previously documented (Anbu, 2013). The relative molecular mass of the native enzyme was approximated using gel-permeable column chromatography as mentioned earlier. A gel filtration calibration kit (GE Healthcare, Hatfield, England) containing cytochrome C (MW = 12,400), carbonic anhydrase (MW = 29,000), albumin (MW = 66,000), alcohol dehydrogenase (MW = 150,000) and β-amylase (MW = 200,000) was used for markers.

Effect of pH on protease activity and stability

The effects of pH on the protease activity and stability were studied at 37 °C over a pH range of 3.0–12.0 with azocasein as a substrate. The buffer systems were sodium acetate (pH 3.0–6.0), potassium phosphate (pH 7.0–8.0), Tris-HCl (pH 7.0–9.0) and sodium carbonate (pH 9.0–12.0). For optimal pH determination, the reaction mixture was incubated at 37 °C for 15 min. The effect of pH on protease stability was studied using 50 mM buffer at the specific pH for 6 h at 37 °C. Aliquots were withdrawn and residual activities were determined under standard assay conditions.

Effect of temperature on protease activity and stability

The temperature effect on the purified protease activity was determined after incubation at specific temperatures (10–80 °C) for 15 min in 50 mM Tris-HCl buffer (pH 8.0). The thermo-stability

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