Purification and partial characterization of a thermo-halostable protease produced by *Geobacillus* sp. strain PLS A isolated from undersea fumaroles

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**ABSTRACT**

The growing industrial demand for stable proteases has driven this study to purify and partially characterize a protease from a bacterial strain isolated from undersea fumaroles. Phylogenetic analysis of the 400bp conserved area of the 16S rRNA gene indicated that the isolate was related to *Geobacillus thermoleovorans* strain SGAir 0734. Purification by ammonium sulfate precipitation and anion exchange chromatography produced 118-fold purity. The optimum activity (646 U mL\(^{-1}\)) of the pure enzyme was observed at 60 °C, pH 7 and with 5M sodium chloride addition. The enzyme retained 73% of its activity when in 50% n-hexane, while the activity was only 40% of the control when in 50% ethyl acetate. The enzyme was tested for its leather dehairing capability. It only caused a slight dehairing and produced soft leather. The enzyme may be applied in skin rejuvenation, wrinkle-smoothing, and high-quality suede production industries.

1. **Introduction**

Proteases are a group of enzymes that catalyze the hydrolysis of peptide bonds to produce short peptides and amino acids. Proteases may be classified by the role of amino acids on the active site, i.e. serine protease (EC 3.4.21), cysteine/sulfhydryl protease (EC 3.4.22), aspartate protease (EC 3.4.23), metalloprotease (EC 3.4.24) and threonine protease (EC 3.4.25). Proteases can also be grouped according to their optimum activity pH, i.e. acidic, neutral, or alkaline proteases [1]. Based on their catalytic mechanism, proteases are grouped into exopeptidase (E.C. 3.4.11–3.4.19) and endopeptidases (E.C. 3.4.21–3.4.24, 3.4.99). Exopeptidases that hydrolyzed proteins from their termini are further grouped into aminopeptidases and carboxypeptidases based on the end locations of hydrolysis, releasing one, two, or three residues. Meanwhile, endopeptidases hydrolyze proteins in the interior [2].

Proteases are produced from various organisms such as bacteria, yeast, fungi, plants and animals [3]. Bacteria, mainly the *Bacillus* group, are the major source of commercial proteases [4] since they easily produce extracellular enzymes using fermentation technique, thus enable simple downstream processing. The characteristics of proteases from *Bacillus* vary, depending on the conditions of their native habitat. For example, proteases produced by a mesophilic *Bacillus subtilis* isolated from soil sample has optimum activity at 40°C and neutral pH [5]. Meanwhile, a thermophilic *Bacillus toebii* strain LBT 77 isolated from a hot spring has optimum activity at 95°C and pH 13. The activity of the enzyme increases with the addition of 25% acetonitrile, methanol, ethanol, and n-butanol [6]. This shows the versatility of proteases for various uses.

Proteases have different industrial applications, mainly in detergent, food, pharmaceutical and leather processing industries [7]. The benefits of the enzyme are profound in the leather processing industry, which traditionally uses chemicals, employs high temperatures and extreme pH [8]. In addition to reducing the processing time, the use of thermo-halostable proteases that are active at high temperatures and pH will decrease environmental pollution significantly.

Proteases account for 60% of industrial enzymes but their practicality in industries is somehow limited by their low stability under extreme conditions, e.g. high temperature, pH, and salts concentration, as well as the presence of metal ions and organic solvents [9]. Therefore, isolation of microorganisms from extreme habitats is expected to produce proteases with unique characters that could satisfy industrial needs. Their wide uses have made research to find and characterize new proteases from various sources is steadily increasing. The growing research is also driven by a 6% annual increase in thermostable industrial proteases demand [7].

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Considering their significances in industries, this study aims to purify and characterize protease produced by a local microorganism isolated from undersea fumaroles. The isolate was expected to produce unique metabolites, as it was isolated from an environment with extreme conditions (high temperature and salt concentration). The results may be useful to identify novel proteases for industrial applications.

2. Materials and methods

2.1. Microorganism

The microorganism used in the study was isolated from undersea fumaroles of about 100 m off the coast of Pria Laot Sabang village, Weh Island, Aceh Province, Indonesia. The sand from seabed, approximately 15 m below the sea surface, was collected from an area with the highest onsite temperature. The sand was diluted in sterile water and the water was then aseptically spread on a solid Peptone Water (PW) medium. A single colony after 48 h incubation, dubbed Pria Laot Sabang Isolate number A (hereinafter referred to as strain PLS A), was subsequently transferred and further incubated for 48 h in a liquid PW medium. After centrifugation, the pellet was stored in glycerol at low temperature for further use.

2.2. Genotypic identification of strain PLS A

Chromosomal DNA was isolated using Rapidwater™ DNA isolation kit. The 16S rRNA encoding gene was then amplified using a set of primers, i.e. Com 1F (5’-CAGCAGCCGCGTTAACATC-3’) and Com 1R (5’-CCGTCAA TTCCTTTGAGTTT-3’). PCR steps of denaturation, annealing and elongation were conducted at 94°C, 50°C and 72°C for 30 cycles.

The 16S rRNA gene sequencing was performed using Dye Terminator method using Com 1F as the sole primer. Phylogenetic analysis was performed by sequence alignment using data from the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov/blast) employing the Basic Local Alignment Search Tool (BLAST) programme. Some sequences with high homology were selected to construct the phylogenetic tree using Mega 6 Program [10].

2.3. Production and isolation of protease

Strain PLS A from glycerol stock (100 µL) was regenerated on a solid medium consisting of 0.8% peptone, 0.4% yeast extract, 0.2% sodium chloride, 0.25% glucose, 3% agar and dissolved in sterile seawater. The medium was incubated for 24 h at 70°C. A colony from the solid medium was transferred into a liquid medium of similar composition, incubated at 150 rpm and 70°C for 14 h.

Protease production was carried out by growing the cells from the liquid culture (10^6 cells mL^{-1}) in the same medium but with the addition of 2.5% casein as the inducer. The medium was then incubated at 150 rpm at 70°C for 18 h. The extracellular protease was obtained in the supernatant by centrifugation at 10,000×g for 20 min.

2.4. Determination of protease activity and protein concentration

The protease activity was determined by the Anson method [11]. The activity was calculated from the tryosine standard curve (10–100 µg mL^{-1}). A unit of protease activity is defined as the amount of enzyme that converts casein to 1 µmol of tyrosine per minute under experimental conditions. Determination of protein concentration was done by Bradford method [12], using the BSA standard curve (100–1500 µg mL^{-1}).

2.5. Purification of protease

Purification of the protease was carried out initially by precipitation using ammonium sulfate with saturation levels ranged from 0% to 100% (w/v) with 20% interval at cold temperature. The precipitate was centrifuged at 10,000×g for 10 min and dialyzed in 20 mM tris-HCl buffer pH 8. The buffer was changed regularly until no white precipitate was formed in the buffer after the addition of 1 mL of BaCl₂ 0.5M and 1 mL of HCl 0.1N. Protease activity and protein concentration of all saturation levels were determined as described in Section 2.4.

Further purification was performed using DEAE Sepharose anion exchange [13] to the ammonium sulfate fraction showed the highest protease activity. The enzyme was eluted with 0.1 M tris-HCl buffer pH 7 containing NaCl gradient (0.15 M, 0.3 M, 0.6 M and 1.0 M of 40 mL each) at a flow rate of 2 mL min^{-1}. Every 2 mL eluent was collected as one fraction. Elution produced 80 fractions. Protease activity and protein concentration of all fractions were determined as described in Section 2.4.

2.6. Characterization of protease

The effect of temperature on protease activity was studied at 50°C, 60°C, 70°C, 75°C, and 80°C, in phosphate buffer pH 8. The effect of pH on protease activity was tested at pH 5 (sodium acetate 0.2 M), pH 7 (phosphate 0.2 M) and pH 9 (Glycine-NaOH 0.2 M), at optimum temperature. Meanwhile, the effect of NaCl concentrations on activity was examined by adding 2M, 5M and 7M NaCl to the enzyme solution in 1:1 ratio (v/v), incubated at optimum temperature and pH. The final salt concentrations of the solution were 1M, 2.5M and 3.5M, respectively. The effect of organic solvent on the activity was studied by adding analytical grade methanol, ethanol,
Figure 1. Phylogenetic tree showing the relationship of strain PLS A 16S rRNA gene sequence (400 bp) in relation to gene sequences from other microorganisms. The alignment was done by the neighbor-joining method using MEGA6 with 1000 bootstrap replications.

Table 1. Purification results of protease from strain PLS A.

| Purification step                  | Vol. (mL) | Protein (mg mL\(^{-1}\)) | Activity (U mL\(^{-1}\)) | Specific activity (U mg\(^{-1}\)) | Purification fold | % Yield |
|-----------------------------------|-----------|---------------------------|---------------------------|-----------------------------------|------------------|--------|
| Crude extract                     | 1600      | 0.299                     | 22.8                      | 76.2                              | 1.0x             | 100    |
| Ammonium sulfate (20–40%)         | 20        | 0.117                     | 62.2                      | 531                               | 7.0x             | 3.41   |
| DEAE–Sepharose (Fraction 53)      | 8         | 0.005                     | 48.8                      | 9016                              | 118x             | 1.07   |

ethyl acetate and hexane to enzyme solution in 1:1 ratio (v/v), incubated at optimum temperature and pH.

2.7. Application of protease on leather dehairing

Leather dehairing process was performed by the modified method of Vijay Kumar et al. [14]. Cow leather was cut to 5 × 5 cm\(^2\), washed with distilled water, and dried at room temperature. A mixture made from protease and calcium carbonate (15:10 v/w) was sparsely applied on the hairy side of the leather. The treatment was repeated on different pieces of leather using (1) only protease and (2) mixture of lime extract: calcium carbonate (15:10 v/w). Non-treated leather was used as a control. All leathers were incubated at optimum temperature and pH for 6 h and then rinsed with distilled water. The pH was however not controlled. The hair loss, softness and colour of each treatment were visually evaluated.

3. Results and discussion

3.1. Genotypic identification of strain PLS A

The two primers used in this study were able to amplify the conserved area of bacterial 16S rRNA gene to about 400 bp [15]. Sequence alignment to other 16S rRNA genes of the same sequence showed that strain PLS A has a close relationship with *Geobacillus thermoleovorans* strain SGAir0734 (Figure 1), as they were clustered in the same branch. As the alignment was done using truncated genes, classification of strain PLS A could only be done up to the genus level. The sequence was deposited to the GenBank with an accession number MK606068. The sequence of an intact 16S rRNA gene of about 1500 bp is needed to confirm if strain PLS A truly forms a distinct branch.

Strain PLS A was a rod-shaped and Gram-negative bacterium (data not shown). This finding is intriguing because *Geobacilli* are predominately Gram-positive. However, few thermophilic *Geobacilli* have been...
reported to be Gram-positive [16,17]. It has been suggested that hyperthermophilic archaea and bacteria are anciently related and both slowly evolve following different paths. As most of the hyperthermophilic archaea are Gram-negative, hyperthermophilic bacteria may still share similar cell wall structure [18]. This may be the explanation of why strain PLS A was Gram-negative after staining.

3.2. Purification of the protease
The protease activity from strain PLS A in the crude extract was 22.8 U mL$^{-1}$ with a protein concentration of 0.3 mg mL$^{-1}$, giving a specific activity value of 76.2 U mg$^{-1}$ (Table 1). After ammonium sulfate precipitation, the highest specific activity (531 U mg$^{-1}$) was observed in the 20–40% fraction (Table 1) with a purity of 7.0-fold of the crude extract. The high specific activity of the protease at low saturation level is interesting. Several previous studies report that optimum specific activities are achieved at higher ammonium sulfate concentrations, normally in the range of 50–75% [5,19]. Ammonium sulfate concentration up to 90% is sometimes needed [6].

Further purification using DEAE-Sepharose anion exchange chromatography showed that the highest specific activity of the protease (9016 U mg$^{-1}$) was observed in Fraction 53 (NaCl 0.6M) but with only 1.07% yield (Table 1). The purity was 17-fold of the ammonium sulfate precipitate fraction 20–40%.

Methods for proteases purification vary, giving different purity and yields. In most reports, the purification is done by precipitation using ammonium sulfate and dialysis, although few reports use cold organic solvents [20]. Further purification commonly involves column chromatography, using either ion exchange [20] or size exclusion techniques [21]. Occasionally, a combination of chromatography techniques is employed to increase enzyme purity [22].

3.3. Effect of temperature and pH on protease activity
Protease from strain PLS A was stable at a narrow temperature range. Optimum protease activity (283.8 U mL$^{-1}$) was observed at 60°C (pH 8.0). The activity at 50°C (160.3 U mL$^{-1}$) was only 56% of that at 60°C. The activity at 70°C, 75°C and 80°C were 18%, 12% and 8% of that at 60°C, respectively (Figure 2A).

As strain PLS A was isolated from an undersea geothermal area, it was expected to produce thermozymes showing thermal stability. The low protease activity at 50°C could be due to sub-optimum substrate conversion kinetics. Meanwhile, the drop in activity at a temperature above 60°C was very likely due to the breakdown of the hydrogen interactions of amino acids in the protein [23]. Many studies have reported the effect of temperature on the activity of protease from thermophilic Bacillus groups. Optimum temperature ranges from as low as 50°C [5,19,24] to 95°C [6].

Thermostability may be established through unique amino acid composition and sequence, the extent of hydrogen bonds, the ionic interactions between amino acids and the decrease of the hydrophobic surface area [9]. Thermal stability differs amongst thermozymes and is not contributed by a single factor. The stability is affected by internal and external factors. The former includes molecular interactions involving hydrophobic, ionic, hydrogen bonds and metal binding. The hydrophobic interaction within the thermophilic enzymes will orientate the hydrophobic residues in the core of the protein, giving a more rigid structure. The extrinsic factors involve environmental conditions such as protein concentration, substrate, activators, cofactors and the present of specific salts [25]. The sequence and type of amino acids are also considered to increase thermal stability but their role is still elusive. Site-directed mutagenesis of the non-catalytic residues of serine protease from Pseudomonas aeruginosa showed that only two mutants exhibit an increase in thermal stability, while the other six mutants do not although predicted otherwise [26].

The pH study shows that protease from strain PLS A was also stable at a narrow pH range. Protease activity of strain PLS A was optimum (459.4 U mL$^{-1}$) at pH 7 (Figure 2B). Activity at pH 5 was only one-third of that at pH 7. Meanwhile, the activity at pH 8 and 9 were about 62% and 52% of that at pH 7, respectively. The decrease might be due to the changes in the ionic structure of the enzyme caused by protonation or deprotonation of the charged groups [27]. The residual activity was still high at pH above 7. The protease may be classified as a neutral protease [3].

The type of protease from strain PLS A was not determined in this study. The inhibitor investigations by adding some metal ions and inhibitory molecules would beneficial for the identification. Some of the alkaline proteases are metal ion-dependent as their activity is affected by chelating agents such as EDTA [21]. Serine protease and aspartic protease are strongly activated by Ca$^{2+}$. Moreover, most metalloproteases are identified by their increased stability in the presence of Zn$^{2+}$ and Ca$^{2+}$ [2].

3.4. Effect of NaCl concentrations and the addition of organic solvent on protease activity
The effect of NaCl concentrations on the protease activity showed that the highest activity (645.6 U mL$^{-1}$) was obtained after the addition of 5M NaCl (2.5 M solution). The residual activity with the addition of 2M and 7M NaCl was only about 65% and 61%, respectively (Figure 3A). These results suggest that the protease
from strain PLS A was an extreme halophilic protease since it was still active in 2.5–5.2M NaCl [28].

NaCl generally denatures protein by increasing the osmotic pressure around the protein thus affecting structural stability [29]. Proteases from halophiles remain active in the presence of NaCl due to the existence of a large number of acidic amino acids on the protein surface, thereby increasing the electrostatic interaction to stabilize conformation. This class of proteases can catalyze reactions in non-aqueous media, for example for the synthesis of \( \beta \)-carotene, carboxylic acid, oil recycling, and bioremediation [30].

The protease activities in various organic solvents were lower than that of the control. Non-polar solvents (n-hexane) maintained protease activity by up to 73%. Interestingly, residual activity in the semi-polar solvent (ethyl acetate) was only 40%. The activities in methanol and ethanol were higher than that in the semi-polar solvent, i.e. about 63% and 68%, respectively (Figure 3B).

Proteases are reported to have irregular performance in organic solvents. For example, protease activity from \textit{Bacillus alveayuensis} CAS 5 increases in acetonitrile and isopropanol but decreases in methanol, ethanol and n-hexane [19]. Meanwhile, the activity from \textit{Alkalibacillus} sp. NM-Fa4 increases in ethanol, but decreases in methanol [24]. In general, the loss of enzyme activity in organic solvents is caused by disruption to the amino acid side-chain interactions in proteins that alter conformations [31]. Higher residual protease activity of strain PLS A in polar and non-polar solvents than that in semi-polar solvent implies the uniqueness of the sequence, composition, and interaction of amino acids on the enzyme surfaces.

3.5. Protease application to leather dehairing

Visual assessment on the leather dehairing results showed that the leather treated with protease dehaired slightly and produced a soft leather surface (Figure 4A). Meanwhile, the leather dehaired more when treated with a mixture of protease and calcium carbonate (15:10 v/w) and produced soft textured leather with a darker
colour (Figure 4B). A mixture of lime extract and calcium carbonate (15:10 v/w) caused less hair falls and produced leather with hard texture (Figure 4C).

With regards to dehairing activity, the protease from strain PLS A was less superior to other reported proteases. For example, protease from *Bacillus mojavensis* SA gives clean hair pores and smooth grain structure of the skin after enzymatic dehairing treatment [32]. Similarly, protease from *Vibrio metschnikovii* NG155 significantly removes the hair from goat and buffalo hides, giving white surface with clean hair pores, without any damage to collagen layer [8].

The difference in dehairing results might be due to the duration of incubation time, pH and temperature. This study conducted the dehairing process for 6 h at 60°C and uncontrolled pH, while others used alkaline pH at higher temperature with prolonging incubation time [8,32,33]. It has been reported that the hair is much more easily removed from the hides after 24 h incubation when incubated at 37°C than at 25°C. Incubation at 50°C significantly reduces the time for hair removal [32]. However, a complete dehairing of the enzyme-treated hides at room temperature may be achieved by prolonged incubation time [34].

Leather-processing industries typically use proteases in the process of soaking and washing of raw leather. Protease activity at alkaline condition is preferred to assist leather swelling, thus allow easy hair removal. Enzymatic dehairing potential from microorganisms is influenced by the presence of either keratinolytic or collagenolytic activity [35]. Collagenolytic activity is needed for the removal of dye in the treated hides, but it will affect the collagen layer of the hides [33].

4. Conclusions

Although poor in dehairing capability, the characteristics of the protease from strain PLS A were still attractive. The optimum activity at 60°C and in 2.5M NaCl solution indicated that the protease was stable at multiple-extreme conditions. The enzyme retained most of its activity when in a non-polar solvent (n-hexane). Protease from strain PLS A could be used for skin rejuvenation, wrinkle smoothing and producing high-quality suede applications. The enzymatic dehairing process is important to substitute the use of chemical reagents in leather-processing industry, thus reducing environmental pollution.
Disclosure statement

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