Exoproduction and Biochemical Characterization of a Novel Thermophilic Serine Protease From *Ornithinibacillus Caprae* L9^T With Hide-dehairing Activity

Xiaoguang Li

Sichuan University

Qian Zhang

Sichuan University

Longzhan Gan

Sichuan University

Guangyang Jiang

Sichuan University

Yongqiang Tian (✉ yqtian@scu.edu.cn)

Sichuan University <https://orcid.org/0000-0001-5729-209X>

Bi Shi

Sichuan University

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Abstract

BLAST, basic local alignment search tool; BBD, Box-Behnken design; BSA, bovine serum albumin; CGMCC, China General Microbiological Culture Collection Center; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylene-diamine-tetraacetic acid; EGTA, ethylene glycol-bis (β -aminoethyl ether)-N,N,N',N'-tetraacetic acid; GBDP, genome blast distance phylogeny; HE, hematoxylin and eosin; KCTC, Korean Collection for Type Cultures; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LB, Luria-Bertani; β -ME, β -mercaptoethanol; NR, non-redundant protein database; PMSF, phenylmethylsulfonyl fluoride; RSM, response surface methodology; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SD, standard deviation; TCA, trichloroacetic acid; TSB, tryptic soytone broth; TYGS, type strain genome server; UV-Vis, ultraviolet-visible.

Introduction

China, one of the major leather-producing countries, is well known for its most active and potential leather trading markets worldwide. According to the China Leather Industry Association data, around 529 million square meters of genuine leather was produced in China in 2019. The leather industry mainly uses hides and skins, the livestock by-products, as the raw materials for processing. Leather processing involves many physical and chemical treatments such as soaking, dehairing, bating and tanning (Ward 2011). The dehairing of skin/hide is considered as the most polluting process (Kandasamy et al. 2012). The traditional hair-removal methods require the use of several chemical depilatory agents, such as sodium sulfide, sodium bisulfide and calcium chloride (Ockerman and Basu 2014), which contribute to environmental pollutions, including the generation of noxious gases and solid wastes (Kanagaraj et al. 2015; Thanikaivelan et al. 2004). Therefore, the introduction of more eco-friendly methods is highly desirable. In this regard, microbial proteases could fill this niche (Paul et al. 2016).

Enzymatic dehairing, which has been proved to be the most cost-effective and eco-friendly process, exerts tremendous potential in obtaining high-quality leather (Wang et al. 2009). This phenomenon has increased the attention toward the exploitation of enzyme-producing microorganisms (Barzkar 2020). So far, a variety of extracellular proteases have been reported and characterized from different microorganisms, including fungi like *Paecilomyces marquandii* (Gradisar et al. 2005), and bacteria, such as *Bacillus subtilis* (Dettmer et al. 2013), *Lactobacillus curvatus* (Sun et al. 2019), *Nocardiopsis dassonvillei* (Sharma et al. 2020) and *Streptomyces koyangensis* (Ben Elhoul et al. 2015). Interestingly, most of the reported extracellular proteases are serine proteases (Brandelli 2007; Jaouadi et al. 2013), while a few belong to metalloproteases (Wang et al. 2008; Chandrasekaran et al. 2016). As a member of the proteolytic enzyme family, serine proteases exert a wide range of biotechnological applications, including skin dehairing (Jaouadi et al. 2013), feather degradation (Jagadeesan et al. 2020), stain washing (Haddar et al. 2009), food production (Kostyleva et al. 2016), silk degumming and sericin degrading (Suwannaphan et al. 2017). Of the reported proteases to date, the extracellular serine proteases were preponderantly secreted by *Bacillus* strains. However, little attention has been paid to the rare strains, especially the species of *Ornithinibacillus*.

Ornithinibacillus species are generally Gram-stain-positive, aerobic, rod-shaped and moderately halophilic bacteria from the family *Bacillaceae* (Li et al. 2020). Over the past decades, a total of 12 strains have been isolated and placed in the genus *Ornithinibacillus* (Parte et al. 2020). Despite these studies, no protease from *Ornithinibacillus* strains has been reported so far. Therefore, further research on the proteases secreted by *Ornithinibacillus* species is highly necessitated to expand the existing industrial enzyme toolbox and achieve potential applications. In the previous study, an extracellular protease-producing bacterium, *Ornithinibacillus* sp. L9^T, was isolated from a goatskin and scientifically classified as *Ornithinibacillus caprae* (Li et al. 2020). In addition, strain L9^T could produce a transparent hydrolysis ring on milk plates supplemented with 100 g/L NaCl. Herein, the extended research was set out to enhance the extracellular protease production from *O. caprae* L9^T by optimizing the medium components and fermentation conditions. Simultaneously, the crude enzyme was investigated for the biochemical and molecular characteristics, and used as a biocatalyst for dehairing hides to provide a theoretical foundation for further application in the leather industry.

Materials And Methods

Microorganism and reagents

The moderate halobacterium *O. caprae* L9^T was isolated from a goat hide obtained from a local farm in Chengdu, south-west China (Li et al. 2020). The bacterium was deposited in the laboratory of industrial biotechnology, the Korean Collection for Type Cultures (KCTC, Korea) and China General Microbiological Culture Collection Center (CGMCC, China) with accession number L9, KCTC 43176 and CGMCC 1.17659, respectively.

Unless specified, all the chemical reagents used in this experiment were of analytical. Casein, gelatin and collagen were purchased from Sangon Biotech (China). Keratin from wool, azocasein, bovine serum albumin (BSA), Folin & Ciocalteu's phenol reagent and protein marker were obtained from Tokyo Chemical Industry Co., Ltd. (Japan), Sigma-Aldrich (American), BioFroxx (Germany), Solarbio (China) and Takara (Japan), respectively. All other tested chemicals, surfactants, organic solvents and media components were obtained from several commercial companies.

Genomic analysis

The genome sequence data has been uploaded to the type strain genome server (TYGS) (Meier-Kolthoff and Goker 2019) to determine the genome-based phylogeny of strain L9^T. Meanwhile, the DNA sequences were performed BLASTX search against the non-redundant protein database (NR) (Li et al. 2002) in the National Center for Biotechnology Information to assess the variety of genes in the L9^T strain.

Inoculum preparation and protease production

The pure culture of bacterium L9^T was precultured in LB broth medium supplemented with 100 g/L NaCl [used to inoculate (2%, v/v) all fermentation medium for protease production]. After the fermentation was

completed, the crude enzyme extract was obtained by removing the cell biomass through high-speed centrifugation for 5 min at 4 °C, and stored at 0 °C for further analyses.

Determination of protease activity

Protease activity was measured using casein as a substrate according to the national standardization administration commission GB/T 23527-2009 with minor modifications. The reaction was performed in an Eppendorf tube with a total volume of 2 mL, containing 0.2 mL of appropriately diluted enzyme solution and 0.2 mL casein (20 g/L) dissolved in Tris-HCl (pH 7). The mixed components were incubated at 70 °C for 10 min, and the reaction was stopped using 0.4 mL of 65.4 g/L trichloroacetic acid (TCA). After shaking evenly, the unreacted casein precipitate was removed by centrifugation at 12000 rpm for 5 min. Later, 0.4 mL of clear supernatant was mixed with 2 mL of 42.4 g/L Na₂CO₃ and 0.4 mL of 1 M Folin & Ciocalteu's phenol reagent. The mixture was incubated for 20 min at 40 °C and cooled under running water. Then the absorbance was monitored at 680 nm against the control using a UV-Vis spectrophotometer (UV-1100, Mapada, China). The assay was conducted in triplicate, and a control was run in parallel in which the substrate was added after the addition of TCA. One unit of protease activity (U/mL) was designated as the amount of enzyme required to release 1 µg of tyrosine per minute under specified assay conditions.

Optimization of protease production by strain L9^T

The effect of fermentation process variables on the extracellular protease derived from *O. caprae* L9^T was investigated using the one-variable-at-a-time approach and response surface methodology (RSM). Three independent variables (C source, N source and initial pH), with the most significant effect on protease production, were selected. Then, the optimum levels and interaction of these three factors for enhancing the protease production were evaluated by RSM using Box-Behnken design (BBD). Each factor was investigated at three different levels: low (-1), central (0) and high (1) (Table S1). The experimental matrix was designed using DesignExpert 8.0.6 (Stat-Ease Inc., Minneapolis, USA), consisting of 17 experimental runs in which 5 runs were repeated at the central level (Table S2). The proteolytic activity (U/mL) was recorded as a response, and the response surface model was constructed and analyzed.

SDS-PAGE, zymography and peptide identification

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the crude enzyme was performed using a PAGE gel preparation kit (BaiHe, China) according to the method described by Laemmli (Laemmli 1970) under denaturing conditions.

The caseinolytic activity was confirmed by zymography analysis according to the method described by Garciacarreno et al. (1993) with minor modifications. The sample was mixed with an equal volume of 2×SDS non-reducing loading buffer, and then electrophoresed. The gel was soaked twice in 50 mM Tris-HCl pH 7 buffer containing 2.5% (v/v) Triton X-100 with agitation to remove SDS. Subsequently, the gel was submerged in Tris-HCl buffer for 40 min to remove Triton X-100. Later, the hydrolysis reaction

occurred inside the gel during incubation at 40 °C for 12 h in Tris-HCl containing 10 g/L casein. At last, the gel was stained with the dye solution composed of 25% (v/v) ethanol, 8% (v/v) acetic acid and 1 g/L Coomassie Brilliant Blue R-250 for 12 h. The appearance of a white band on the dark blue background indicated the presence of protease activity.

Later, the white band was extracted from the gel, and subjected to reduction, alkylation, trypsin digestion and desalination. The obtained peptide mixture was analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) coupled with a TripleTOF 5600 system at Sangon Biotech. All mass spectra were acquired in the m/z 350–1350 mass range, and the obtained peptide sequences were searched against the GenBank database using ProteinPilot software (version 4.5) with the Paragon algorithm.

Protease biochemical characterization

Temperature optima and thermal stability

The optimum temperature of protease activity was evaluated at 30–80 °C for 10 min using Tris-HCl buffer (pH 7), and the maximum value of enzyme activity was set as 100%. Subsequently, the enzyme solution was pre-incubated at 30–80 °C for 1 h, and the residual enzymatic activities were measured at the optimum temperature to evaluate the thermal stability of the protease. All the analyses were conducted in triplicate.

pH optima and stability

For optimum pH assay, the crude enzyme solution was diluted in the following buffer system 50 mM of glycine-HCl (pH 2–4), sodium acetate-acetic acid (pH 5–6), Tris-HCl (pH 7–9), glycine-NaOH (pH 10–11) and KCl-NaOH (pH 12–13). The proteolytic activity was determined at each pH under the optimum temperature. Later, the enzyme solution was mixed with different pH buffers (pH 2–13) at 25 °C for 1 h to explore the enzyme stability and residual activity at different pH conditions. The unincubated enzyme activity measured by Tris-HCl buffer (pH 7) was considered as 100%.

Effect of NaCl on enzyme activity and stability

The fermentation supernatant was dialyzed using a dialysis bag (molecular weight cut-off = 8000 Da) against 50 mM Tris-HCl pH 7 buffer for 4 h at 25 °C, and the dialysis buffer was updated hourly to ensure the reliability of the experimental results. Later, the enzyme solution was amended with NaCl at final concentrations of 0–220 g/L, and the effect of NaCl on hydrolytic activity against casein was measured. The salt-containing enzyme solution was preincubated at 25 °C for 1 h prior to determination of residual activities to assess its NaCl stability. The enzyme solution without NaCl served as the control and was considered as 100%.

Effect of chemical agents on protease activity

The effect of chemical agents on protease catalysis was investigated by preincubating the crude enzyme at 25 °C for 1 h with each reagent. Organic solvents, such as glycerol, methanol, benzene, acetone, ethanol, n-hexane, isopropanol and ethanediol were provided at the working concentrations of 5 and 10% (v/v), respectively. Similarly, the surfactants, such as Tween 20, Tween 80, Triton X-100, dimethyl sulfoxide (DMSO) and H₂O₂, were evaluated at 1% (v/v). Other classes of chemical agents were used at a final concentration of 5 mM; protease inhibitors: phenylmethylsulfonyl fluoride (PMSF), ethylene-diamine-tetraacetic acid (EDTA) and ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA); reducing agents: dithiothreitol (DTT) and β-mercaptoethanol (β-ME). The crude enzyme solution incubated in the ultrapure water was used as a control and considered as 100% enzyme activity.

Effect of different metal ions on protease activity

The effect of different metal ions were determined at 5 mM concentration; monovalent cations: Li⁺, K⁺ and Ag⁺; divalent cations: Sr²⁺, Mn²⁺, Fe²⁺, Co²⁺, Cu²⁺, Zn²⁺, Ca²⁺, Ba²⁺ and Mg²⁺; trivalent cations: Fe³⁺ and Cr³⁺. The enzyme solution was incubated with the respective metal ion solution at 25 °C for 1 h, and the residual activity was measured following the optimal conditions. The enzyme solution treated with ultrapure water served as the control.

Substrate specificity

Substrate specificity was determined under standard assay conditions using different protein substrates, including casein, gelatin, collagen, keratin, azocasein and BSA. All the substrates were added to a final concentration of 10 g/L.

Dehairing performance of *O. caprae* L9^T protease

The fresh goatskins, cowhides and rabbit skins were procured from a local farm, and required consent was obtained from the farmer to use these animal parts for experiments. Some pieces of skin with hair were cut with a sharp knife from the same area of the obtained hides, then rinsed with tap water several times and drained at 25 °C. Most hides were independently placed into a flask containing 100 mL diluted crude enzyme (600 U) and 30 mg/g Na₂S, respectively, while the rest of the hides were submerged in 100 mL of Tris-HCl buffer (pH 7) containing 20 g/L NaCl that served as a control. All the flasks were incubated in a shaker incubator for 24 h at 150 rpm and 38 °C. Afterward, the application prospect of crude enzyme in the leather industry was evaluated through dehairing efficacy and histological examination.

About 1 cm² pelts from the hides treated with Tris-HCl buffer, Na₂S and enzyme, were incised and fixed with 40 g/L paraformaldehyde (Servicebio, China) for 24 h. The immobilized samples were dehydrated by ethanol, embedded with paraffin wax and cut by microtome. Then the slices were stained by hematoxylin and eosin (HE) and Masson's trichrome staining, and observed using a microscope (Olympus, Japan).

Statistical analysis

All experiments were performed in triplicate, and the control experiments were proceeded under the identical conditions. The statistical data analysis was done using the software OriginPro version 8.5, and the results were presented as mean $\pm$ standard deviation (SD).

Nucleotide sequences accession numbers

The 16S rRNA gene sequences and the whole genome of strain L9^T have been submitted to the GenBank database under accession MN577409 and WOCA000000000, respectively.

Results And Discussion

Genomic characteristics

The family *Bacillaceae* was first proposed by Fischer, and it now includes more than 100 recognized genera (Parte et al. 2020), including *Virgibacillus*, *Oceanobacillus* and *Ornithinibacillus*, the neighbors of the phylogenetic evolution. Additionally, in the phylogenomic tree (Fig. 1), the species of the genera *Oceanobacillus* and *Ornithinibacillus* are intertwined with each other, specifying that their genomes have some identical sequences or are highly similar. To date, a few *Oceanobacillus* species have been isolated and reported to secrete protease (Li et al. 2017; Pandey et al. 2012; Thanapun 2013), lipase, amylase and carboxymethyl cellulase (Rohban et al. 2009; Seghal Kiran et al. 2014). Thus, as the twin brother of *Oceanobacillus*, the *Ornithinibacillus* species might also have the ability to produce enzymes. A total of 3944 coding DNA sequences of strain L9^T obtained from homology analyses were annotated by the NR database, including at least 25 enzyme-encoding genes. The most abundant was serine protease, followed by metalloprotease, lipase, amylase and glycosidase. The diversity of enzyme genes provides theoretical support for further research on strain L9^T. Furthermore, a zone of clearing formed by strain L9^T was overtly observed on the milk agar plates, and the protease activity was detected at 60.97 U/mL in the TSB medium supplemented with 100 g/L NaCl.

Statistical optimization of protease production

Response surface methodology for optimizing experimental design

The significant independent variables [yeast extract (A), urea (B) and initial pH (C)] were used to determine the optimum levels of these parameters based on the single-factor experimental results (depicted in Supplementary Material). The response values of 17 experiments are listed in Table S2. Later, the DesignExpert software was employed for regression analysis and significance tests. A quadratic model was generated and applied to predict the maximum protease production (Y). The model was as follows:

$$Y \text{ (U/mL)} = 246.15 - 12.97A - 32.89B - 7.51C + 0.35AB - 37.30AC - 8.68BC - 42.35A^2 - 68.29B^2 - 77.57C^2$$

As summarized in Table 1, the variance analysis results of the regression model indicated that the model was significant as it was obvious from the high model *F*-value (= 107.4) and very low probability value (<

0.0001). Additionally, the model terms of A, B, C, AC, A², B² and C² were significant (*p*-value less than 0.05), whereas the interaction between urea and yeast extract was not significant (*p*-value greater than 0.1). This result indicated that the combination of urea and yeast extract had no significant effect on protease production by *O. caprae* L9^T. The 'lack of fit *F*-value' of 3.56 implied that the lack of fit was not significant relative to the pure error. Moreover, the good fits were realized, and the variability of most of the test data was explained by the model, with the correlation coefficient (R²) of 99.28% and adjusted R² of 98.36%. The adequate precision was 28.942 (> 4), which was desirable, confirming the feasibility of this model to predict protease production by strain L9^T.

The interaction effects and optimum values of a combination of the three independent factors for maximum protease production by *O. caprae* L9^T were represented by three-dimensional response surface graphs and contour plots (Fig. 2). With the increase of yeast extract in the medium, the caseinolytic activity increased first and then decreased (Fig. 2a and 2b). Fig. 2b depicts the shape of the contour to be ellipse, indicating a significant interaction between yeast extract and pH.

Validation of the optimized process conditions

Based on the regression equation, three repeated verification tests were performed by setting yeast extract, urea and initial pH at the predicted levels of 14.3 g/L, 3.8 g/L and 9.0, respectively. The maximum protease activity of 251.12 U/mL was predicted by the model, which was comparable to the experimental results (255.86 ± 0.71 U/mL), confirming the model's authenticity. After RSM optimization, the protease yield of *O. caprae* L9^T increased by 319.65% than the unoptimized medium and original fermentation conditions.

Gel electrophoresis and molecular analysis

The crude extracellular enzyme produced by strain L9^T was analyzed through SDS-PAGE and zymogram. As depicted in Fig. 3, a single protein band was obtained with the cell-free supernatant, and its proteolytic activity was confirmed by zymogram using casein as a substrate. The molecular weight of L9^T protease was estimated as 26 kDa, which was consistent with some literature reports. Earlier, Deng et al. (2010) reported the characterization of a 28 kDa high-alkaline serine protease from *Bacillus* sp. B001. Two detergent stable serine proteases named protease BM1 and BM2 with molecular masses of 29 and 15.5 kDa, respectively, were purified from *Bacillus mojavensis* A21 (Haddar et al. 2009).

The unique protein band was extracted and detected by mass spectrometry. The offline data was analyzed by ProteinPilot, and a total of 27 peptide sequences were obtained. Afterward, the quality control of peptide fragment was performed based on the unused ProtScore ≥ 1.3, and 9 peptides (Table S3) with the reliability of no less than 95% were screened out. These peptides exhibited high homology with S8 family serine peptidases, suggesting that the extracellular enzyme of strain L9^T might belong to serine protease family and provides a direction for further research.

Biochemical characterization of the protease

Effect of pH, temperature and NaCl on enzymatic activity and stability

As depicted in Fig. 4a, the protease was active over a wide range of pH (2–13) with the nadir activity at pH 2. More than 80% of the protease activity was retained at pH 3–13. Besides, the pH stability profile (Fig. 4b) showed that the extracellular protease was highly stable in the pH range of 3–13. The enzyme remained 57.54 and 88.64% of its activity at pH 2 and 13, respectively, after 1 h incubation at 25 °C. The protease secreted by *O. caprae* L9^T has broader pH activity and stability than the previously reported proteases (Ibrahim et al. 2015; Deng et al. 2010). Herein, the protease exhibited a typical characteristic of the serine protease (wider pH action), suggesting it to be a good candidate for industrial applications such as use in the detergents (Ibrahim et al. 2015), tanning processes (Kanagaraj et al. 2015) or biocontrol agent (Darwesh et al. 2020).

The proteolytic activity was detected at all test temperatures (30 to 80 °C), and the maximum activity was observed at 70 °C (Fig. 4c), revealing the thermophilic nature of the protease. The crude protease showed only 5.4% of the maximal activity at 30 °C and exhibited a few activities till the enzyme assay at 80 °C. These results were consistent with several studies, including an alkaline serine protease produced by *N. dassonvillei* OK-18 (Sharma et al. 2020), showing the maximal activity at 70 °C, and protease BM2 secreted by *B. mojavensis* A21 (Haddar et al. 2009), showing the maximal activity at 60 °C. Thermostability studies have revealed that L9^T protease is quite stable between 30 to 45 °C, retaining 95.75 and 9.02% of the initial activity after 1 h of incubation at 50 and 65 °C (Fig. 4d), respectively. Nevertheless, at 70–80 °C, the enzyme fully denatured and lost activity within 60 min.

The effects of NaCl on enzyme activities towards casein were determined in the salinity ranging from 0–220 g/L at 70 °C. The protease exhibited the optimum activity at 20 g/L NaCl (Fig. 5). The protease retained 80.31, 55.23 and 32.49% of its activity at NaCl concentrations of 80, 140 and 220 g/L, respectively, suggesting that high salt concentration suppresses the enzyme activity. Notably, the proteolytic activity measured at different salt concentrations remained unchanged before and after heat preservation, indicating the stability of this protease at all tested NaCl concentrations for 1 h. These results were in accordance with the recent report of alkaline serine protease from *Bacillus* sp. NPST-AK15 (Ibrahim et al. 2015), showing the maximum activity at 15 g/L NaCl and highly stability in NaCl up to 200 g/L. These findings demonstrate that L9^T protease is a slightly halophilic enzyme (different from extreme halophilic protease), which could be useful for certain biotechnological processes depending on salinity.

Effect of various metal ions on protease activity

Table 2 summarizes the proteolytic activity of the crude enzyme under incubation with different metal ions. Ag⁺, Ca²⁺ and Sr²⁺ significantly enhanced the enzyme activities at a rate of 104.46, 104.40 and 120.37% than the control at a final concentration of 5 mM. In consistent with the present study, many investigators have reported the stimulating effect of Ca²⁺ on serine proteases (Ben Elhoul et al. 2015;

Jagadeesan et al. 2020; Jaouadi et al. 2013). Gong et al. (2015) has reported that Sr^{2+} increased the keratinase activity by 10%. The above-mentioned results suggest that Ag^+ , Ca^{2+} and Sr^{2+} could maintain the stability of the active site of L9^{T} protease, which has a selective preference for Sr^{2+} ions at a certain concentration as an inducer. The addition of Li^+ , K^+ , Ba^{2+} , Mg^{2+} , Mn^{2+} and Co^{2+} had a little effect on the enzyme activities. Other ions, including Fe^{2+} , Fe^{3+} , Cu^{2+} and Zn^{2+} , significantly inhibited the enzyme activities, and a loss of 70% in the activity was observed with Fe^{3+} . Moreover, under the same conditions, Fe^{3+} ions are more likely to deactivate enzyme than Fe^{2+} , which is presumably attributed to the fact that Fe^{3+} easily absorbs electrons from the enzyme surface through strong oxidation, thereby destroying the enzyme stability. Herein, the enzyme was moderately affected by Cr^{3+} at 5 mM, revealing that the protease might act as an adjuvant to the chrome tannage and bating processes in leather industry.

Effect of chemical substances on protease activity

The effects of various organic solvents on protease were appraised to examine the potential application. Results showed that the crude enzyme was considerably stable in the presence of the tested solvents (Table 3), particularly at a concentration of 5% (v/v). Upon incubation with 10% (v/v) of glycerol, benzene and n-hexane for 1 h, the enzyme also displayed high stability, with respective residual activity of 99.50, 100.76 and 95.70%. This remarkable stability exhibited by L9^{T} protease in common organic solvents supported the potential candidacy of it as a biocatalyst for the synthesis of peptide and ester.

Later, the effects of surfactants on the extracellular protease were evaluated, and the corresponding results are presented in Table 4. The stimulatory effects were observed in the presence of some nonionic surfactants, such as Tween 20 (163.41%) and Tween 80 (115.01%), which were higher than previous reports (Jagadeesan et al. 2020; Ibrahim et al. 2015). On the contrary, the inhibitory effect of Triton X-100 was observed at a concentration of 1% (v/v). The enhanced activity of the enzyme in presence of DMSO reflect the fact that DMSO is an amphipathic molecule that easily penetrates the enzyme with low toxicity, and 99.94% of the activity was maintained (data not shown) at 10% concentration. The strong anionic surfactant sodium dodecyl sulfate (SDS) at 10 g/L inhibited the protease activity up to 76.98%, which was consistent with most of the previous conclusions reporting that SDS denatures and inactivates the enzyme (Ibrahim et al. 2015; Suwannaphan et al. 2017). Furthermore, the L9^{T} protease was moderately stable against H_2O_2 , retaining 88.18% of its initial activity at a concentration of 1%. All these prominent features ensure that the enzyme is compatible with certain detergent formulations.

Moreover, the effect of various inhibitors and chelators on L9^{T} protease activity was examined (Table 4). The protease remained unstimulated against thiol reagents, such as DTT and β -ME at a concentration of 5 mM, suggesting that L9^{T} protease is a thiol-independent enzyme, and the thiol group does not directly participate in the catalytic reaction (Ben Elhoul et al. 2016). On the contrary, the protease activity was partially inhibited by the chelators EDTA and EGTA, which confirmed that L9^{T} protease is not a member of the metalloprotease family but uses certain cations as stabilizers (Deng et al. 2010; Jellouli et al. 2009).

Notably, L9^T protease showed no activity in the presence of 5 mM PMSF, confirming the enzyme to be a serine protease (Jagadeesan et al. 2020).

Substrate specificity profile of the L9^T protease

The effect of various substrates on L9^T protease activity is summarized in Fig. S4. The highest activity was observed against casein, but a relatively high activity was observed against azocasein. However, no activity was observed toward gelatin, collagen and keratin. Therefore, the absence of collagenase activity indicates the potential application of L9^T protease in hair removal in the leather industry without damaging the skin collagen.

Hide dehairing ability of the crude enzyme

The dehairing efficacy of the crude enzyme from *O. caprae* L9^T on hides was evaluated by touch-visual tests and histological analyses. The pelt with hair, traditional dehaired pelt, and enzymatic dehaired hide are depicted in Fig. 6. The results elucidated that both the traditional sodium sulfide depilation and enzymatic dehairing could effectively remove the hair from goatskins, cowhides and rabbit skins; however, the control hides, incubated under the same conditions, showed no sign of hair removal. Notably, the enzymatic dehaired pelts were white in color and displayed smooth grain surface, soft touch and clean hair pores; while, organoleptic tests showed that the skins obtained after chemical dehairing were dark brown or yellow in color, wrinkled and hard in touch. Sodium sulfide dissolved in water could produce large amounts of hydroxide, and the disulfide bond of the hair breaks under the action of strong alkali to complete the hair removal process (Sujitha et al. 2018), which leads to the production of toxic gas H₂S. In contrast, as a biologically active catalyst, the crude enzyme cannot directly act on hair shafts; instead, it may hydrolyze the mucinoid, mucin, albumin and globulin in raw skin tissue, thereby destroying the bond between the hair follicle and the hair root, the hair bulb and the hair papilla, to achieve the purpose of hair removal (Sujitha et al. 2018; Yates 1972).

The histological images of control, Na₂S and crude enzyme treated goatskins stained with HE and Masson's trichrome staining are depicted in Fig. S5. The results demonstrated that compared with the blank control, the epidermis was removed from the goatskins via two different treatments; and the collagen fiber structure of the enzymatically dehaired pelt was more regular and intact compared with the Na₂S treatment. It could be observed from Masson's trichrome staining images that the collagen components appeared in blue color, and the non-collagen substances appeared in deep red color. This result suggested that the enzyme treatment could well maintain the inherent collagen component in the dermal structure, while part of the damaged collagen existed in the fur treated with Na₂S. Overall, the crude enzyme exhibited no collagenase activity, and was advantageous over chemical depilatory agents.

In this study, the crude protease completely dehaired the skins from goat and rabbit (Fig. 6a and 6c) in 24 h without any chemicals. This result strengthened the hypothesis that this protease could be beneficial to protect the environment and reduce energy waste (Jaouadi et al. 2013; Kandasamy et al. 2012).

Moreover, the reasons for incomplete enzymatic depilation of cowhide (Fig. 6b) might be attributed to the fact that the thick cowhide leads to slower enzyme penetration and lack of mechanical pulling force. It is noteworthy to mention that the microbial enzymes with excellent dehairing ability are stable in an alkaline environment, especially between pH 8 and 10 (Jaouadi et al. 2013; Bouacem et al. 2016). Fortunately, L9^T protease meets these criteria and hence, is considered as a potential dehairing candidate in improving leather quality.

Conclusions

In the present study, a novel thermophilic and slightly halophilic serine protease from *O. caprae* L9^T was reported. The halophile exhibited a higher protease production capacity (255.86 U/mL) under the following conditions: 72 h of fermentation time, initial pH 9, culture temperature 37 °C, and the modified medium containing 14.3 g yeast extract, 3.8 g urea, 130 g NaCl and 1 L distilled water. The crude enzyme appeared as a single band on SDS-PAGE with a molecular mass of 26 kDa. The optimum temperature for caseinolytic activity was 70 °C. Furthermore, the protease activity was enhanced by metal ions Ca²⁺, Sr²⁺, Ag⁺ and 20 g/L NaCl. The enzyme exhibited excellent stability toward surfactants, organic solvents and a wide range of pH from 3 to 13. Further studies confirmed that the crude protease has an excellent ability to remove hair in a short time without damaging the collagen. In summary, the protease could be a propitious candidate for various industrial applications.

Declarations

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Author contributions XL performed the experiment and wrote the manuscript. QZ revised the grammar and typography of the manuscript. LG and GJ provided guidance on response surface analyses. YT supervised and guided the experiments. BS critically reviewed the structure of manuscript.

Compliance with ethical standards

Conflicts of interest The authors declare that there are no conflicts of interest.

Ethical statement This article does not contain any studies with human participants and/or animals performed by any of the authors.

References

1. Barzkar N (2020) Marine microbial alkaline protease: An efficient and essential tool for various industrial applications. *Int J Biol Macromol* 161:1216–1229

2. Ben Elhoul M, Zarai Jaouadi N, Rekik H, Bejar W, Boulkour Touiou S, Hmidi M, Badis A, Bejar S, Jaouadi B (2015) A novel detergent-stable solvent-tolerant serine thiol alkaline protease from *Streptomyces koyangensis* TN650. *Int J Biol Macromol* 79:871–882
3. Ben Elhoul M, Zarai Jaouadi N, Rekik H, Omrane Benmrads M, Mechri S, Moujehed E, Kourdali S, El Hattab M, Badis A, Bejar S, Jaouadi B (2016) Biochemical and molecular characterization of new keratinolytic protease from *Actinomadura viridilutea* DZ50. *Int J Biol Macromol* 92:299–315
4. Bouacem K, Bouanane-Darenfed A, Zarai Jaouadi N, Joseph M, Hacene H, Ollivier B, Fardeau ML, Bejar S, Jaouadi B (2016) Novel serine keratinase from *Caldicoprobacter algeriensis* exhibiting outstanding hide dehairing abilities. *Int J Biol Macromol* 86:321–328
5. Brandelli A (2007) Bacterial Keratinases: Useful Enzymes for Bioprocessing Agroindustrial Wastes and Beyond. *Food Bioprocess Tech* 1:105–116
6. Chandrasekaran M, Chandrasekar R, Chun SC, Sathiyabama M (2016) Isolation, characterization and molecular three-dimensional structural predictions of metalloprotease from a phytopathogenic fungus, *Alternaria solani* (Ell and Mart) Sor. *J Biosci Bioeng* 122:131–139
7. Darwesh OM, Ali SS, Matter IA, Elsamahy T, Mahmoud YA (2020) Enzymes immobilization onto magnetic nanoparticles to improve industrial and environmental applications. *Meth Enzymol* 630:481–502
8. Deng A, Wu J, Zhang Y, Zhang G, Wen T (2010) Purification and characterization of a surfactant-stable high-alkaline protease from *Bacillus* sp B001. *Bioresour Technol* 101:7111–7117
9. Dettmer A, Cavalli É, Ayub MAZ, Gutterres M (2013) Environmentally friendly hide unhairing: enzymatic hide processing for the replacement of sodium sulfide and delimig. *J Clean Prod* 47:11–18
10. Garcíacarreno FL, Dimes LE, Haard NF (1993) Substrate Gel-Electrophoresis for Composition and Molecular-Weight of Proteinases of Proteinaceous Proteinase-Inhibitors. *Anal Biochem* 214:65–69
11. Gong JS, Wang Y, Zhang DD, Zhang RX, Su C, Li H, Zhang XM, Xu ZH, Shi JS (2015) Biochemical characterization of an extreme alkaline and surfactant-stable keratinase derived from a newly isolated actinomycete *Streptomyces aureofaciens* K13. *Rsc Adv* 5:24691–24699
12. Gradisar H, Friedrich J, Krizaj I, Jerala R (2005) Similarities and specificities of fungal keratinolytic proteases: comparison of keratinases of *Paecilomyces marquandii* and *Doratomyces microsporus* to some known proteases. *Appl Environ Microbiol* 71:3420–3426
13. Haddar A, Agrebi R, Bougatef A, Hmidet N, Sellami-Kamoun A, Nasri M (2009) Two detergent stable alkaline serine-proteases from *Bacillus mojavensis* A21: purification, characterization and potential application as a laundry detergent additive. *Bioresour Technol* 100:3366–3373
14. Ibrahim ASS, Al-Salamah AA, El-Badawi YB, El-Tayeb MA, Antranikian G (2015) Detergent-, solvent- and salt-compatible thermoactive alkaline serine protease from halotolerant alkaliphilic *Bacillus* sp NPST-AK15: purification and characterization. *Extremophiles* 19:961–971
15. Jagadeesan Y, Meenakshisundaram S, Saravanan V, Balaiah A (2020) Sustainable production, biochemical and molecular characterization of thermo-and-solvent stable alkaline serine keratinase

- from novel *Bacillus pumilus* AR57 for promising poultry solid waste management. *Int J Biol Macromol* 163:135–146
16. Jaouadi NZ, Rekik H, Badis A, Trabelsi S, Belhouli M, Yahiaoui AB, Ben Aicha H, Toumi A, Bejar S, Jaouadi B (2013) Biochemical and molecular characterization of a serine keratinase from *Brevibacillus brevis* US575 with promising keratin-biodegradation and hide-dehairing activities. *PLoS One* 8:e76722
 17. Jellouli K, Bougatef A, Manni L, Agrebi R, Siala R, Younes I, Nasri M (2009) Molecular and biochemical characterization of an extracellular serine-protease from *Vibrio metschnikovii* J1. *J Ind Microbiol Biotechnol* 36:939–948
 18. Kanagaraj J, Senthilvelan T, Panda RC, Kavitha S (2015) Eco-friendly waste management strategies for greener environment towards sustainable development in leather industry: a comprehensive review. *J Clean Prod* 89:1–17
 19. Kandasamy N, Velmurugan P, Sundarvel A, Rao JR, Bangaru C, Palanisamy T (2012) Eco-benign enzymatic dehairing of goatskins utilizing a protease from a *Pseudomonas fluorescens* species isolated from fish visceral waste. *J Clean Prod* 25:27–33
 20. Kostyleva EV, Sereda AS, Velikoretskaya IA, Nefedova LI, Sharikov AY, Tsurikova NV, Lobanov NS, Semenova MV, Sinitsyn AP (2016) A new *Bacillus licheniformis* mutant strain producing serine protease efficient for hydrolysis of soy meal proteins. *Microbiology* 85:462–470
 21. Laemmli UK (1970) Cleavage of structure protein during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
 22. Li W, Jaroszewski L, Godzik A (2002) Tolerating some redundancy significantly speeds up clustering of large protein databases. *Bioinformatics* 18:77–82
 23. Li X, Zhang S, Gan L, Cai C, Tian Y, Shi B (2020) *Ornithinibacillus caprae* sp nov, a moderate halophile isolated from the hides of a white goat. *Arch Microbiol* 202:1469–1476
 24. Li Y, Wu C, Zhou M, Wang ET, Zhang Z, Liu W, Ning J, Xie Z (2017) Diversity of Cultivable Protease-Producing Bacteria in Laizhou Bay Sediments, Bohai Sea, China. *Front Microbiol* 8:405
 25. Meier-Kolthoff JP, Goker M (2019) TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. *Nat Commun* 10:2182
 26. Ockerman HW, Basu L (2014) BY-PRODUCTS | Hides and Skins, in: Dikeman, M, Devine, C (Eds), *Encyclopedia of Meat Sciences* (Second Edition) Academic Press, Oxford, pp 112–124
 27. Pandey S, Rakholiya KD, Raval VH, Singh SP (2012) Catalysis and stability of an alkaline protease from a haloalkaliphilic bacterium under non-aqueous conditions as a function of pH, salt and temperature. *J Biosci Bioeng* 114:251–256
 28. Parte AC, Sarda Carbasse J, Meier-Kolthoff JP, Reimer LC, Goker M (2020) List of Prokaryotic names with Standing in Nomenclature (LPSN) moves to the DSMZ. *Int J Syst Evol Microbiol* 70:5607–5612
 29. Paul T, Jana A, Mandal AK, Mandal A, Das Mohapatra PK, Mondal KC (2016) Bacterial keratinolytic protease, imminent starter for NextGen leather and detergent industries. *Sustain Chem Pharm* 3:8–22

30. Rohban R, Amoozegar MA, Ventosa A (2009) Screening and isolation of halophilic bacteria producing extracellular hydrolyses from Howz Soltan Lake, Iran. *J Ind Microbiol Biotechnol* 36:333–340
31. Seghal Kiran G, Nishanth Lipton A, Kennedy J, Dobson AD, Selvin J (2014) A halotolerant thermostable lipase from the marine bacterium *Oceanobacillus* sp PUMB02 with an ability to disrupt bacterial biofilms. *Bioengineered* 5:305–318
32. Sharma AK, Kikani BA, Singh SP (2020) Biochemical, thermodynamic and structural characteristics of a biotechnologically compatible alkaline protease from a haloalkaliphilic, *Nocardiopsis dassonvillei* OK-18. *Int J Biol Macromol* 153:680–696
33. Sujitha P, Kavitha S, Shakilanishi S, Babu NKC, Shanthi C (2018) Enzymatic dehairing: a comprehensive review on the mechanistic aspects with emphasis on enzyme specificity. *Int J Biol Macromol* 118:168–179
34. Sun F, Sun Q, Zhang H, Kong B, Xia X (2019) Purification and biochemical characteristics of the microbial extracellular protease from *Lactobacillus curvatus* isolated from Harbin dry sausages. *Int J Biol Macromol* 133:987–997
35. Suwannaphan S, Fufeungsombut E, Promboon A, Chim-anage P (2017) A serine protease from newly isolated *Bacillus* sp for efficient silk degumming, sericin degrading and colour bleaching activities. *Int Biodeter Biodegr* 117:141–149
36. Thanapun T (2013) Screening and characterization of protease-producing *Virgibacillus*, *Halobacillus* and *Oceanobacillus* strains from Thai fermented fish. *J App Pharm Sci* 3:025–030
37. Thanikaivelan P, Rao JR, Nair BU, Ramasami T (2004) Progress and recent trends in biotechnological methods for leather processing. *Trends Biotechnol* 22:181–188
38. Wang R, Min C, Haiming C, Li ZQ (2009) Enzyme unhairing- an eco-friendly process. *J Soc Leath Tech Ch* 93:51–55
39. Wang SL, Hsu WT, Liang TW, Yen YH, Wang CL (2008) Purification and characterization of three novel keratinolytic metalloproteases produced by *Chryseobacterium indologenes* TKU014 in a shrimp shell powder medium. *Bioresour Technol* 99:5679–5686
40. Ward OP (2011) Proteases, in: M. Moo-Young (Ed.), *Comprehensive Biotechnology* (Second Edition), Academic Press, Burlington, pp 571–582
41. Yates JR (1972) Studies in depilation. Part X. The mechanism of the enzyme depilation process. *J Soc Leather Trades Chem* 56:158–177

Tables

Table 1. Analysis of variance for the regression equation

Source	Sum of squares	df	Mean squares	<i>F</i> -value	<i>p</i> -value
Model	74551.59	9	8283.51	107.40	< 0.0001**
A-Yeast extract	1346.02	1	1346.02	17.45	0.0042**
B-Urea	8652.39	1	8652.39	112.18	< 0.0001**
C-pH	451.79	1	451.79	5.86	0.0461*
AB	0.48	1	0.48	0.01	0.9392†
AC	5565.63	1	5565.63	72.16	< 0.0001**
BC	301.41	1	301.41	3.91	0.0886
A ²	7552.10	1	7552.10	97.91	< 0.0001**
B ²	19637.99	1	19637.99	254.61	< 0.0001**
C ²	25334.82	1	25334.82	328.47	< 0.0001**
Residual	539.90	7	77.13		
Lack of fit	392.94	3	130.98	3.56	0.1256†
Pure error	146.97	4	36.74		
Cor total	75091.49	16			

F, *F* ratio; *p*-value, probability value; df, degree of freedom.

*, Significant ($p < 0.05$); **, very significant ($p < 0.01$); †, not significant ($p > 0.1$).

$R^2 = 0.9928$; adjusted $R^2 = 0.9836$; adequate precision = 28.942.

Table 2. Effect of different metal ions on the protease activity

Metal ions	Final Concentration (mM)	Relative activity ($\pm$ SD) (%)
Control	-	100.00 $\pm$ 0.79
Li ⁺	5	90.19 $\pm$ 0.97
K ⁺	5	97.71 $\pm$ 2.49
Ag ⁺	5	104.46 $\pm$ 1.27
Fe ²⁺	5	84.85 $\pm$ 1.25
Cu ²⁺	5	85.32 $\pm$ 1.40
Zn ²⁺	5	78.39 $\pm$ 2.47
Ca ²⁺	5	104.40 $\pm$ 2.12
Mg ²⁺	5	97.77 $\pm$ 2.00
Ba ²⁺	5	93.31 $\pm$ 2.92
Mn ²⁺	5	96.36 $\pm$ 2.13
Co ²⁺	5	95.54 $\pm$ 0.35
Sr ²⁺	5	120.37 $\pm$ 3.10
Fe ³⁺	5	28.55 $\pm$ 3.16
Cr ³⁺	5	84.15 $\pm$ 1.64

Table 3. Effect of organic solvents on L9^T protease stability

Solvents	Relative activity ($\pm$ SD) (%)	
	5% (v/v)	10% (v/v)
Control	100.00 $\pm$ 0.1	-
Acetone	88.44 $\pm$ 5.31	85.09 $\pm$ 3.72
Benzene	100.76 $\pm$ 2.24	95.83 $\pm$ 6.36
Ethenediol	99.12 $\pm$ 2.28	93.37 $\pm$ 2.10
Ethanol	90.90 $\pm$ 0.77	74.92 $\pm$ 4.05
Glycerol	88.76 $\pm$ 3.03	99.50 $\pm$ 1.22
n-Hexane	91.16 $\pm$ 1.91	95.70 $\pm$ 3.94
Isopropanol	91.98 $\pm$ 1.37	71.64 $\pm$ 4.18
Methanol	97.22 $\pm$ 3.90	89.77 $\pm$ 2.22

Table 4. Effect of various surfactants, oxidizing agent, alkahest and inhibitors on crude serine protease from *O. caprae* L9^T

Chemical substances	Working concentration	Relative activity ($\pm$ SD) (%)
Control	-	100.00 $\pm$ 3.06
Surfactants		
Tween 20	1% (v/v)	163.41 $\pm$ 2.23
Tween 80	1% (v/v)	115.01 $\pm$ 1.13
Triton X-100	1% (v/v)	90.34 $\pm$ 3.32
SDS	10 g/L	23.02 $\pm$ 1.18
Oxidizing agent		
H ₂ O ₂	1% (v/v)	88.18 $\pm$ 1.18
Alkahest		
DMSO	1% (v/v)	110.22 $\pm$ 1.70
Inhibitors		
DTT	5 mM	96.96 $\pm$ 0.73
β -Me	5 mM	95.05 $\pm$ 2.23
EDTA	5 mM	30.44 $\pm$ 2.77
EGTA	5 mM	49.85 $\pm$ 0.60
PMSF	5 mM	0

Figures

Figure 1

The phylogenomic tree based on genome sequences of *O. caprae* L9^T and the related type species using the genome blast distance phylogeny (GBDP) method. The numbers above branches are GBDP pseudo-bootstrap support values > 70% from 100 replications.

Figure 2

Three-dimensional response surface graphs and contour plots of extracellular protease production by *O. caprae* L9T elucidating the interaction between: a, yeast extract and urea; b, yeast extract and pH; c, urea and pH.

Figure 3

SDS-PAGE (12.5%) of extracellular protease from *O. caprae* L9T. Lane 3, cell-free supernatant; lane M, molecular weight marker; lane 6, crude enzyme zymogram.

Figure 4

Effect of pH and temperature on the protease activities from *O. caprae* L9T. a, Relative activities at different pH values; b, pH stability of the protease; c, relative activities at different temperatures; d, thermal stability of the protease. Results represent means of three experiments, and vertical bars indicate standard error of the mean.

Figure 5

Effect of various concentrations of NaCl (0–220 g/L) on activity and stability of the dialysed L9T protease. Each value represents mean $\pm$ SD, $n = 3$.

Figure 6

Dehairing performance on goatskin (a), cowhide (b) and rabbit skin (c) of crude enzyme from *O. caprae* L9T.

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