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Preliminary Characterization of Intracellular Protease from *Pediococcus acidilactici* Isolated from Stingless Bee Honey

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Introduction: Lactic acid bacteria are known to produce proteases with potential applications in food and biotechnology industries. However, intracellular proteases from lactic acid bacteria isolated from stingless bee honey remain poorly characterized. This study aimed to characterize the intracellular protease produced by *Pediococcus acidilactici* isolated from stingless bee honey. Material and Methods: The bacterial isolate used in this study was previously identified as *Pediococcus acidilactici* based on whole genome sequencing and GenBank database registration. Intracellular protease was obtained through cell disruption. Protease activity was determined using casein as a substrate, and the released tyrosine was quantified using the Folin–Ciocalteu method. The biochemical characteristics of the protease were evaluated under different pH and temperature conditions. Results: The results showed that the intracellular protease exhibited proteolytic activity over a range of pH and temperatures tested. The optimum protease activity was observed at pH 4–5 and a temperature of 37°C. These findings indicate that the intracellular protease from stingless bee honey-derived *Pediococcus acidilactici* has specific biochemical properties and remains active under moderately acidic and physiological temperature conditions. Conclusion: This study provides fundamental information on the enzymatic characteristics of intracellular protease from this bacterium, establishing a baseline for further investigations into its functional properties and potential applications.

Keywords: Lactic Acid Bacteria,*Pediococcus acidilactici*, Protease, Proteolytic, Stingless Bee Honey

Introduction

Lactic acid bacteria (LAB) continue to attract substantial interest due to their established safety profile and therapeutic efficacy as probiotics. According to the WHO, these probiotics are defined as live microorganisms that impart a health advantage to the host when administered in appropriate quantities.[1] To be effective, probiotic must adhere to intestinal epithelial cells to establish themselves in the gut. Thus, probiotics should exhibit antimicrobial properties against harmful microorganisms and possess favorable antibiotic susceptibility profiles.[2] *Pediococcus acidilactici* is widely recognized for its probiotic potential and its capacity to produce metabolites such as bacteriocins, organic acids, and enzymes.[3]

Proteases are important enzymes produced by LAB and function in protein metabolism, nutrient acquisition, and cellular maintenance.[4] These enzymes contribute to the production of bioactive peptides with potential therapeutic applications, such as anti-hypertensive and anti-inflammatory agents in functional foods or supplements. Furthermore, LAB-derived proteases are of

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interest due to their generally recognized as safe (GRAS) status, making them suitable for use in drug development, nutraceutical formulations, and probiotic therapies .[4,5] Most previous studies focus on extracellular proteases, whereas intracellular proteases from LAB, especially those isolated from unique natural environments, remain less characterized.

Stingless bee honey is distinguished by its unique physicochemical environment, including high osmotic pressure, low pH, and complex bioactive compounds, which select for specialized microbial communities adapted to this niche.[6,7] LAB such as Pediococcus acidilactici adapted to this extreme environment are hypothesized to evolve unique metabolic traits and distinct enzymatic machinery compared to strains originating from common sources like dairy products. Acid-tolerance and adaptive evolution studies demonstrate that *P. acidilactici* strains can thrive at low pH, indicating specific biochemical adaptations of their functional enzymes to acidic environments.[8]

While most previous studies on LAB proteolytic systems have focused on extracellular proteases, intracellular proteases remain understudied. These intracellular enzymes are vital for internal peptide processing, cellular stress adaptation, and the liberation of bioactive peptides upon cell lysis.[9] The biochemical characterization of the intracellular protease from this stingless bee honey isolate offers critical insights. The optimal activity observed at acidic pH and 37°C directly reflects the enzyme's evolutionary adaptation to the acidic honey microenvironment, while simultaneously matching the physiological conditions of the gastrointestinal tract and food fermentation matrices.[7,9] Therefore, characterizing this intracellular protease provides essential baseline knowledge regarding its unique ecological adaptation and highlights its physiological relevance for potential applications in functional foods and biotechnologies.

Methods

Bacterial Strain Identification

The bacterial strains used in this study were previously isolated and identified by whole genome sequencing. The genome sequences were deposited in the GenBank database of the National Center for Biotechnology Information under accession numbers NZ_CP184527.1 for *P. acidilactic*i strain LT3 and NZ_CP184530.1 for *P. acidilactici* strain HI5. These strains were obtained from the culture collection of the Research Center for Food Technology and Processing, National Research and Innovation Agency (BRIN), Indonesia[10] and were used for intracellular protease extraction and biochemical characterization in this study.

Bacterial Isolation and Culture

The lactic acid bacteria *Pediococcus acidilactici* HI5 and LT3 were obtained from the Research Center for Food Technology and Processing, National Research and Innovation Agency (BRIN), Gunungkidul, Yogyakarta, Indonesia. These isolates were previously isolated and selected from stingless bee honey. Bacterial cultures were prepared with several modifications method on previous research.[11] The cultures were revived from cryotube stocks containing 40% glycerol (v/v). A total volume of 1 mL of frozen culture was transferred into a sterile 1.5 mL microtube and centrifuged at 15,000 rpm for 2 min. The supernatant was discarded, and the cell pellet was resuspended in 1 mL of de Man, Rogosa, and Sharpe (MRS) broth (Merck KGaA, Darmstadt, Germany). The resuspended culture was inoculated at 5% (v/v) into 5 mL of fresh MRS broth and incubated at 37°C for 24 h under anaerobic conditions. Subsequently, the culture was subcultured by inoculating 5% (v/v) into 40 mL of MRS broth and incubated at 37°C for 24–48 h to obtain sufficient biomass for intracellular protease extraction.

Intracellular Protease Extraction

The bacterial culture was centrifuged at 4,500 rpm for 10 min to collect the cell pellet. The supernatant was discarded, and the pellet was washed with phosphate-buffered saline (PBS) and centrifuged again under the same conditions. The washed pellet was resuspended in a lysis buffer containing 20 mM Tris-HCl, 10% Triton X-100, and 1 M NaCl prepared in lysozyme solution (concentration 1 mg/mL).[12] Cell disruption was performed using a sonicator (Elmasonics S 100 H, ELMA, Jerman) for 90 s in pulse mode (10 s ON and 20 s OFF) to facilitate cell wall lysis. The lysate was then incubated for 30 min to allow complete lysis. After incubation, the mixture was centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant was collected as crude intracellular protease and stored at −20°C until further analysis.

Preparation of tyrosine standard curve

Standard curve was prepared using L-tyrosine at concentrations of 0-1000 µg/mL. An aliquot of each standard solution was treated with 0.1 M sodium carbonate (Na_2CO_3) and Folin-Ciocalteu reagent under the same conditions as the enzyme samples. The mixture was incubated at room temperature for 30 min to allow color development. Absorbance was measured at 578 nm using a microplate reader.[13] A blank containing distilled water was prepared and analyzed under identical conditions. All measurements were performed in triplicate. The standard curve was constructed by plotting absorbance against tyrosine concentration, and the resulting regression equation was used to calculate protease activity.

Protease Activity Assay

Protease activity was determined using casein as substrate according to a modified method from previous study.[14,15] One unit (U) of protease activity was defined as the amount of enzyme that released 1 µg of tyrosine per minute under the assay conditions. In the protease assay, a 1% casein solution was prepared using a 0.1 M citrate phosphate buffer at pH 5.5, serving as the substrate. This solution was subjected to heat denaturation at 50°C for 15 minutes before being allowed to cool. Following this, 120 µL of intracellular crude protease was combined with 360 µL of the previously prepared 1% (w/v) casein and incubated for 10 minutes. The reaction was halted by the addition of 360 µL of 10% (w/v) trichloroacetic acid (TCA) and left to stand at room temperature for 10 minutes. The mixture was then centrifuged at 15,000 × g for 10 minutes, and 150 µL of the supernatant was transferred to a new tube. Next, 500 µL of 0.5 M Na_2CO_3 and 100 µL of Folin–Ciocalteu reagent were added. The mixture was incubated at room temperature for 30 minutes, and the absorbance was measured at 578 nm using a microplate reader.

Determination of optimum pH

The optimum pH was determined at pH 4.0, 5.0, and 6.0 using citrate buffer (pH 4.0 and 5.0) and phosphate buffer (pH 6.0). The reaction mixture was incubated for 10 min at room temperature, followed by TCA precipitation, centrifugation, and color development as described above. All assays were performed in triplicate. Blank samples for each pH condition were prepared using the corresponding buffer without enzyme and treated under the same conditions. Blank measurements were also performed in triplicate and used to correct the absorbance values. Substrate control and enzyme control were also included under the same conditions.

Determination of optimum temperature

The optimum temperature was determined by incubating the reaction mixture at 37, 45, and 50°C at the selected pH condition. After incubation for 10 min, the reaction was stopped with TCA and processed as described above. All assays were performed in triplicate. Blank samples were prepared for each temperature condition using the corresponding buffer without enzyme and treated identically. Blank measurements were performed in triplicate and used as baseline correction. Substrate control and enzyme control were also included.

Statistical analysis

Protease activity was expressed as U/mg protein and analyzed to evaluate the effects of pH and temperature. Data normality was assessed using the Shapiro–Wilk test. Statistical differences among groups were determined using one-way ANOVA followed by Tukey's post-hoc test when significant differences were observed ($p<0.05$). Linear regression analysis was performed to evaluate activity trends. All analyses were conducted using GraphPad Prism version 11.0.0 (GraphPad Software, San Diego, CA, USA).

Result

Tyrosine Standard Curve

The tyrosine standard curve was successfully constructed to provide a reference for protease activity calculations. The results demonstrated a statistically significant linear relationship between tyrosine concentration and absorbance at 578 nm ($p< 0.0001$). As shown in **Figure 1**, the absorbance increased proportionally with concentrations ranging from 0 to 1000.0 µg/mL. The best-fit linear regression equation was determined to be Y = 0.0004107X + 0.05353 (R2 = 0.9618) representing the baseline absorbance. These parameters ensured that the subsequent determination of protease activity, based on the release of tyrosine, was calculated within a validated linear range.

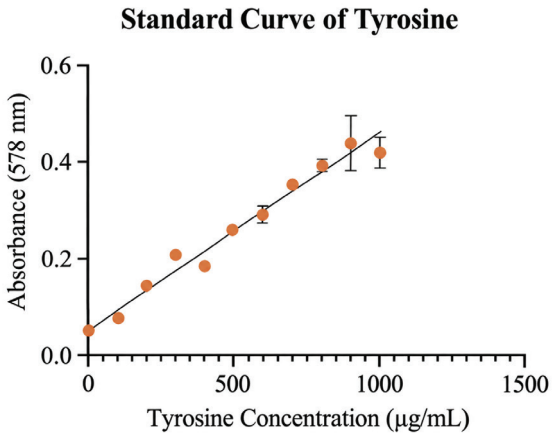


Figure 1. Tyrosine standard curve for protease activity determination measured at 578 nm. Data are presented as mean ± standard deviation (*n*=3).

Determination of Optimum pH on Protease Activity

The determination of the optimum pH was conducted to evaluate the functional stability of the extracellular proteases produced by Pediococcus acidilactici isolates I5 and T3 within a pH range of 4.0 to 6.0. The experimental data revealed that both isolates exhibited distinct enzymatic activity profiles in response to environmental pH fluctuations (**Figure 2**). Isolate I5 showed its maximum protease activity under acidic conditions with an optimum at pH 5.0, yielding an activity value of 5.6567 ± 0.5672 U/mL. Increasing the pH from 5.0 to 6.0 resulted in a drastic decline in enzymatic activity to 0.7667 ± 1.0473 U/mL. Statistical analysis confirmed that the difference in activity between pH 5.0 and pH 6.0 was highly significant (***, $p<0.001$), as shown in **Table 1**. In contrast, isolate T3 demonstrated a more stable activity trend at more acidic levels. The maximum activity for T3 was achieved at pH 4.0 with a value of 4.0467 ± 0.9169 U/mL. As the alkalinity of the medium increased toward pH 6.0, the protease activity of T3 gradually decreased to 2.7767 ± 0.5783 U/mL. However, the comparison between T3 activity at pH 4.0 and pH 5.0 showed no statistically significant difference, indicating a slightly broader pH tolerance range compared to isolate I5 under acidic conditions.

Determination of Optimum Temperature on Protease Activity

The thermal stability of the proteases was assessed at temperatures of 37°C, 45°C, and 50°C to identify the optimum temperature for proteolytic function. Based on the results presented in **Figure 3**, both isolates displayed their peak enzymatic activity exclusively at 37°C, where isolate T3 showed a slightly higher activity compared to isolate I5, with values of 2.320 ± 0.690 U/mL and 2.172 ± 1.316 U/mL, respectively.

In contrast, the thermal stability of the proteases underwent a drastic decline as the incubation temperature increased, as summarized in **Table 2**. Enzymatic activity for both isolates was completely undetectable (Not Detected) when the temperature was raised to 45°C and 50°C. Statistical analysis reinforced these findings by showing a highly significant difference (***, $p< 0.001$) between the activity at 37°C and the higher temperature treatments. These data confirm that the proteases from isolates I5 and T3 are highly thermolabile, where protein denaturation likely occurs as soon as the temperature exceeds the 37°C threshold.

Discussion

The exploration of LAB based probiotics is an increasingly popular area of study, largely because of their positive impact on gut health, such as maintaining

Table 1. Protease activity profiles of *Pediococcus acidilactici* isolates I5 and T3 across various pH levels. Bars represent mean values ± standard deviation (*n*=3)

Environmental Condition	I5 Activity (U/mL)	T3 Activity (U/mL)
pH 4.0	3.4967 ± 0.7655	4.0467 ± 0.9169
pH 5.0	5.6567 ± 0.5672	2.3833 ± 0.9430
pH 6.0	0.7667 ± 1.0473	2.7767 ± 0.5783

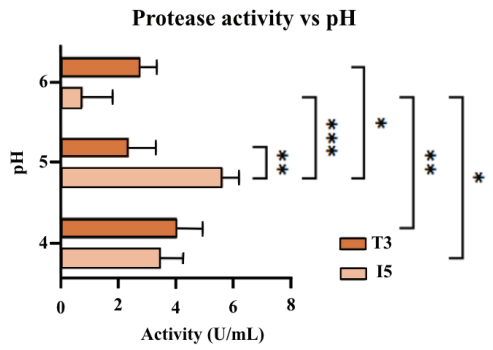


Figure 2. Effect of pH on protease activity. Data represent mean ± SD (*n*=3). Statistical significance was analyzed using one-way ANOVA followed by Tukey's post hoc test (*p < 0.05, **p < 0.01, ***p < 0.001).

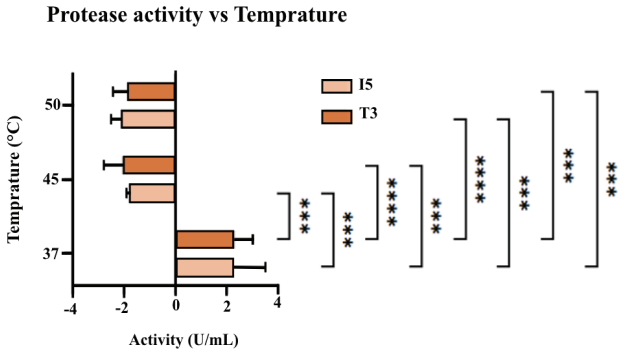


Figure 3. Effect of temperature on protease activity. Data represent mean ± SD (*n* = 3). Statistical significance was analyzed using one-way ANOVA followed by Tukey's post hoc test (***p < 0.001, ***p < 0.0001).

microbial balance and enhancing immune function.[16] Current research trends show a rising demand for natural probiotics. In this regard, LAB has become a preferred choice for improving gastrointestinal health, owing to their robust fermentative traits and survival capacity within the harsh environments of the digestive tract.[17-19] While conventional probiotic strains are predominantly isolated from dairy and non-dairy fermented products, exploring novel ecological niches is essential since bacterial species exhibit distinct habitat preferences. Consequently, this study focuses on isolating and characterizing the probiotic attributes, particularly protease production of *P. acidilactici* derived from stingless bee honey.

Whole genome sequencing of *Pediococcus acidilactici* strains LT3 and HI5 (GenBank NZ_CP184527.1 and NZ_CP184530.1) ensures taxonomic clarity and reproducibility of their enzymatic products. In silico approaches have become indispensable for high-throughput enzyme discovery, facilitating gene identification, structural prediction, and functional annotation. By leveraging genomic data, computational analysis enables precise characterization of protease properties, such as catalytic activity and stability, while significantly reducing experimental costs and enhancing research efficiency.[20,21]

The selection of intracellular proteases in this study was primarily based on in silico analysis, which revealed that the genome of *P. acidilactici* strains harbors genes encoding intracellular proteolytic enzymes. Genome-based annotation and functional prediction indicated the presence of conserved domains associated with cytoplasmic proteases, suggesting their potential role in intracellular protein turnover and peptide generation.[22] Compared to extracellular proteases, intracellular proteases are often involved in essential metabolic processes, including protein quality control, stress response, and the generation of bioactive peptides with potential functional properties.[23] Therefore, the integration of genomic data and bioinformatics analysis supports the targeted exploration of intracellular proteases as promising candidates for further characterization and potential biomedical applications.

As illustrated by the biochemical pH profile in **Figure 2**, the proteases generated by *P. acidilactici* in this study demonstrated stability within the pH range of 4.0-5.0 but became unstable above pH 6.0, classifying them as acidic proteases. The pH stability of the acidic proteases from strains HI-5 and LT-3 aligns with previous findings for *P. acidilactici* isolated from refrigerated meat (pH 4.0)[24] and *P. pentosaceus* from fermented fish (pH 4.0–5.0)[14].

Conversely, these values are lower than those reported in previous study that *P. acidilactici* UL-3 exhibited peak specific proteolytic activity under near-neutral and alkaline conditions.[25] The observed variation in optimum pH between strains HI5 (pH 5.0) and TL3 (pH 4.0) may reflect strain-specific biological diversity. Such differences could be attributed to variations in protease gene expression profiles, amino acid substitutions affecting charge distribution near the

Table 3. Protease activity profiles of *Pediococcus acidilactici* isolates I5 and T3 across various temperature. Bars represent mean values ± standard deviation (*n*=3)

Environmental Condition	I5 Activity (U/mL)	T3 Activity (U/mL)
37°C	2.172 ± 1.316	2.320 ± 0.690
45°C	*ND	*ND
50°C	*ND	*ND

Abbreviation: ND = Not Detected

active site, or differences in post-translational modifications and metabolic regulation between the two strains.

The alteration in the enzyme and substrate's shape and charge characteristics might be responsible for this. A shift in pH modifies the ionization state of amino acids within a protein, which in turn affects the ionic bonds that define the protein's tertiary structure and charge properties. This can lead to the enzyme becoming inactive or changing how it recognizes substrates.[24]

Temperature also plays a crucial role in influencing protease activity. As previous study, enzymes generally exhibit peak activity at a specific temperature, meaning that enzyme activity tends to rise with increasing temperature until it reaches an optimal point.[26] This temperature rise impacts the substrate's conformation, creating obstacles for the substrate's active site as it attempts to enter the enzyme's active site, ultimately leading to reduced enzyme activity.

As illustrated in **Figure 3**, the enzyme exhibited its peak activity at 37°C. However, the complete inactivation observed at 45°C and 50°C highlights a high degree of thermolability. This finding is in agreement with previous study, who reported that proteolytic systems in LAB typically function optimally under mesophilic conditions (30–40°C).[23] A similar result was obtained by the findings where an alkaline protease of *Lactobacillus casei* reached its optimum temperature at 37°C.[27] For comparison with other LAB, the optimal temperature for *Lacticaseibacillus paracasei* protease was reported at 30°C, with an enzymatic activity of 0.0141 U/mL.[13] Conversely, previous research on the proteolytic activity of *Streptococcus lactis* and *Lactococcus lactis* identified a lower optimum temperature of 28°C.[28]

The biochemical profile observed in this study which is characterized by optimal proteolytic activity at 37°C and stability within an acidic pH range of 4.0–5.0 holds significant clinical relevance, particularly for topical infection or wound healing applications. Furthermore, the strict thermolability of these enzymes ensures they operate exclusively within human physiological temperatures (37°C) without the risk of persistent, uncontrolled activity if environmental conditions fluctuate. In compromised epithelial tissues or chronic wounds, the microenvironment is highly dynamic, frequently presenting acidic pH values due to localized inflammation, metabolic shifts, and organic acid accumulation.[29] The capacity of *P. acidilactici* proteases to remain active under these precise physiological conditions suggests their high adaptability to the human body environment. This is supported by previous findings demonstrating that *P. acidilactici* strains maintain robust metabolic and enzymatic activity under acidic constraints as low as pH 3.5 to 4.5.[29,30]

Other studies have confirmed that *P. acidilactici* can grow and metabolize efficiently in acidic environments, which closely mimics the acidic conditions of the stomach. They also remain viable in the presence of bile salts found in the intestine.[31,32] This acid tolerance suggests their enzymes, including proteases, can maintain structural integrity and catalytic activity within the upper gastrointestinal tract. By producing proteolytic enzymes and antimicrobial compounds stable at acidic pH and body temperature, these bacteria can contribute to reducing pathogen colonization, enhancing local gut microbial balance, and supporting mucosal health.

These biochemical traits complement the established antimicrobial potential of *P. acidilactici* against major opportunistic pathogens, including *Staphylococcus aureus*, *Escherichia coli*, and *Listeria monocytogenes*.[32] Mechanistically, the robust performance of these proteases

under acidic conditions may synergize with the strain's overall antimicrobial profile, supporting the healing process by disrupting the protein-rich matrix of bacterial biofilms or facilitating the degradation of necrotic tissue components. Additionally, such targeted proteolytic activity under low pH has been heavily implicated in the enzymatic liberation or enhancement of bioactive components, resulting in improved antioxidant and antimicrobial properties.[33] Consequently, the specific acid-tolerant and mesophilic nature of the intracellular proteases characterized herein establishes a solid biochemical foundation for deploying these LAB-derived enzymes as non-antibiotic therapeutic alternatives to control localized infections and support host tissue repair.

Overall, the alignment between in silico genomic mapping and biochemical characterization confirms that *P. acidilactici* strains LT3 and HI5 harbor unique intracellular proteases with a distinct acidic-mesophilic profile. Their stability under low-pH conditions, coupled with their novel origin from stingless bee niches, underscores their potential as specialized biocatalysts. These findings establish a strong foundation for deploying LAB-derived proteases in targeted biomedical and pharmaceutical applications where acid-tolerance is a prerequisite.

Conclusion

In conclusion, the intracellular protease derived from *P. acidilactici*, isolated from stingless bee honey, exhibits robust catalytic activity under acidic conditions (pH 4.0–5.0) and displays a thermolabile nature. Future studies should further investigate its operational stability by evaluating enzymatic sensitivity toward various metal ions and chemical inhibitors. Furthermore, assessing the direct antibacterial and antibiofilm efficacy of this protease is an essential next step toward translating these findings into viable medical and therapeutic applications.

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Conflict of Interest

The authors declare no conflict of interest.

Authors' Contributions

NF were involved in concepting and planning the research, performed the data acquisition/collection, calculated the experimental data and performed the analysis. AZS aided in interpreting the results and conclusions. NZDS drafted the manuscript and designed the figures.

Ethical Clearance

This study was approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health and Nursing, Gadjah Mada University, Indonesia (Approval No. [KE/FK/1811/EC/2025]). All procedures were conducted in accordance with the ethical standards.

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