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Purification and Molecular Characterization of Protease from *Lacticaseibacillus paracasei* IN17 Isolated from Fermented Fish (Inasua)

Sakina Maya Maulawi[1], Nisa Rachmania Mubarik[2]*, Laksmi Ambarsari[3]

[1]Program of Microbiology, Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University, Bogor 16680, Indonesia
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University, Bogor 16680, Indonesia
[3]Department of Biochemistry, Faculty of Mathematics and Natural Sciences, IPB University, Bogor 16680, Indonesia

*Corresponding author: nrachmania@apps.ipb.ac.id

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Abstract. Protease is a protein degrading enzyme that is widely used in the food fermentation and protein processing industries. An example of a fermented fish product known as inasua, which originates from Maluku in Indonesia, is a proteolytic bacterial source. The aims of this study were to purify, characterize, and analyze the molecular characteristics of the protease produced by *Lacticaseibacillus paracasei* IN17. Proteases from extracellular and intracellular fractions were partially purified using dialysis and ammonium sulfate precipitation. Metal ion effects, kinetic parameters, electrophoretic analysis, and the ideal pH and temperature were all used to assess the enzymatic properties. The extracellular protease enzyme showed an increase in specific activity from 0.079 to 0.182 U mg^{-1} with a yield of 18.9%. Maximum activity was observed at pH 6.0 for the extracellular enzyme and pH 7.0 for the intracellular fraction at 37 °C. Mn^{2+} enhanced the activity, and the kinetics analysis showed higher catalytic efficiency in the extracellular enzyme. An active protein band ~26 kDa was detected, and structural modeling revealed a conserved Ser–His–Asp catalytic triad. This work seeks to expand current knowledge of LAB proteolytic systems derived from non-dairy fermented foods by integrating purification strategies, enzymatic profiling, and gene analysis. This research supports SDGs 3 and SDGs 12 by promoting functional food development and responsible utilization of local microbial resources.

Keywords: 3D structure prediction; lactic acid bacteria; *prtP*; SDS-PAGE.

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INTRODUCTION

Proteases, or protein-degrading enzymes, can be produced by various organisms, ranging from bacteria, fungi, plants, animals, and humans. Microbial proteases are highly valued because of their adaptability to regulated fermentation systems, scalable production, and catalytic efficiency (Kieliszek et al., 2021). These enzymes produce peptides and amino acids during fermentation, which influence nutritional value, flavor, and texture (Ahmad et al., 2018). Therefore, finding novel microbes that produce proteases is still crucial. Proteolytic bacteria can be isolated from a fermented product such as rusip (Amatullah et al., 2023) and tempoyak (Rahayu & Qurbaniah, 2019).

It is commonly known that lactic acid bacteria (LAB) serve a variety of purposes in fermented foods. LAB has proteolytic systems that support growth in substrates high in proteins. LAB species such as *Lactobacillus plantarum, Leuconostoc mesenteroides,* and *Lacticaseibacillus paracasei* were discovered in Inasua, from Central Maluku, Indonesia, a salted fish fermented with palm sap (Mahulette et al., 2018). Inasua isolates have shown antimicrobial activity and biopreservative potential (Dewi et al., 2022; Maulidayanti et al., 2019), suggesting more general functional enzyme synthesis.

Initial screening identified that *Lacticaseibacillus paracasei* IN17 produced the highest proteolytic activity among inasua isolates (Zawawi et al., 2025). The ammonium sulfate precipitation confirmed the activity of extracellular proteases. The fact that the activity

was still limited suggests that more improvement is required. A series of purification steps, such as dialysis, can be performed to increase protease activity (Sun et al., 2019). In addition, intracellular LAB proteases from fermented non-dairy foods remain relatively unknown. Intracellular enzymes of *Bacillus megaterium* have distinct catalytic properties and functional applications (Jeong et al., 2018). The characterization of intracellular proteases limits our understanding of non-dairy LAB proteolytic systems.

Therefore, the purpose of this study is to purify the extracellular and intracellular proteases of *Lacticaseibacillus paracasei* IN17. Also looked at the molecular aspects of the protease-encoding gene (*prtP*) and predicted the 3D structural properties. This work seeks to expand current knowledge of LAB proteolytic systems derived from non-dairy fermented foods by integrating purification strategies, enzymatic profiling, and gene analysis. Furthermore, it aims to support the development of locally sourced microbial enzymes for food processing and sustainable biotechnology.

METHODS

Protease Production

Lacticaseibacillus paracasei IN17 isolated from Inasua, was retrieved from the IPBCC culture collection (Mahulette et al., 2018). The strain IN17 was grown on de Man Rogosa Sharpe agar (MRSA) and confirmed based on colony morphology and Gram staining. A single colony was inoculated into de Man Rogosa Sharpe broth (MRSB) and incubated statically at 37 °C for 8 h. Subsequently, 1% (v/v) of the culture was transferred into 250 mL MRSB supplemented with 1% (w/v) skim milk and incubated at 37 °C for 18 h (Zawawi et al., 2025). Cultures were centrifuged at 12,000 × g for 10 min at 4 °C. The supernatant was collected as a crude extracellular enzyme. The cell pellet was resuspended in 0.1 M phosphate buffer (pH 7.0), disrupted by an ultrasonic cell disruptor (Biobase UCD-650, China) (20 cycles of 20 s pulse and 20 s rest). Sonication solution centrifuged at 12,000 × g for 10 min at 4 °C. The supernatant was used as an intracellular enzyme (García-Cano et al., 2019).

Ammonium Sulfate Precipitation

Crude enzyme extracts were precipitated by adding ammonium sulfate to a final saturation of 20% under constant stirring at 4 °C for 1 h and then incubated overnight at 10 °C. The mixture was centrifuged at 15,000 × g for 10 min at 4 °C, and the resulting pellet was resuspended in 0.1 M phosphate buffer (pH 7.0) (Ullah et al., 2022).

Dialysis Purification

The resuspended enzyme solution was transferred into dialysis tubing (14 kDa cut-off) and dialyzed against 0.01 M phosphate buffer (pH 7.0) at a buffer volume ratio of 1:100 with gentle agitation at 4 °C. The dialysis buffer was replaced three times at 3 h intervals, followed by overnight dialysis. The dialyzed enzyme was collected and stored at 4 °C for further analysis (A. Q. Nguyen et al., 2024).

Protease Activity Assay

Proteolytic activity was evaluated using casein as the substrate, following the procedure described by (Yohanna et al., 2023). The reaction mixture consisted of 250 µL of 1% (w/v) casein prepared in either 0.1 M citrate buffer (pH 6.0) or phosphate buffer (pH 7.0), to which 50 µL of enzyme extract was added. 500 µL of 0.3 M trichloroacetic acid (TCA) was added to stop the reaction after it had been incubated for 10 minutes at 37 °C. To get rid of the precipitated proteins, the mixture was centrifuged for 10 minutes at 4 °C at 15,000 × g. A 600 µL aliquot of the supernatant was mixed with 400 µL of diluted Folin–Ciocalteu reagent (1:2, v/v) and 2 mL of 0.4 M Na_2CO_3. Following incubation at 25 °C for 20 min, absorbance was measured by a UV-Vis spectrophotometer (DLab SP-UV1000, China) at 578 nm.

Protein Concentration

The Bradford colorimetric assay was used to measure the total protein content, and the calibration standard was bovine serum albumin (BSA; 0–1 mg mL^{-1}) (Pulikkottil Rajan, 2024). Absorbance readings were taken at 595 nm. Specific protease activity was subsequently calculated and expressed as units per milligram of protein (U mg^{-1}).

Enzyme Characterization

As previously described by (Sumardi et al., 2019), the effects of temperature and pH on enzyme activity were investigated at temperatures between 20 and 70 °C and pH values between 4 and 10. The enzyme was incubated with Ca^{2+}, Cu^{2+}, $Co2^{+}$, Mg^{2+}, Mn^{2+}, Zn^{2+}, or EDTA at final concentrations of 2 and 5 mM in order to evaluate the impact of metal ions and chelating agents. After that, residual activity was calculated using

(Afriani et al., 2018).

Enzyme Kinetics

The estimation of kinetic parameters was conducted using casein concentrations ranging from 0.25 to 2% (w/v). Reaction velocities were plotted according to the Michaelis–Menten model and further linearized using the Lineweaver–Burk approach. The values of Km and Vmax were calculated by linear regression analysis (Sun et al., 2019).

SDS-PAGE and Zymography

Protein profiling was conducted using a Mini-PROTEAN Tetra Cell electrophoresis system (Bio-Rad, USA) based on the Laemmli system, employing 12% resolving and 4% stacking gels. Twenty microliters of enzyme samples were combined with loading buffer, heated for four minutes at 100 ^{0}C, and then separated electrophoretically using molecular weight markers (Bio-Rad). Coomassie Brilliant Blue R-250 was used to stain the gels following electrophoresis. Zymography was used to detect activity using gels copolymerized with 1% (w/v) casein. Proteolytic bands appeared as clear zones against the stained background (Nur et al., 2023).

Molecular Analysis of Protease-Encoding Gene

A Zymo Research DNA Extraction Kit was used to isolate the genomic DNA from L. paracasei IN17, and a Nanodrop device was used to assess the purity and concentration of the DNA using spectrophotometry. Reference sequences of the *prtP* gene were obtained from the NCBI database and aligned using MEGA X to identify conserved regions. Geneious software was used to design primers. GoTaq Green Master Mix (Promega, USA) was used for PCR amplification under the following thermal profile: 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 50 °C for 45 seconds, and extension at 72 °C for 6 minutes; a final extension step at 72 °C for 10 minutes. Amplicons were visualized on 1.5% (w/v) agarose gels. Sequence data were processed using MEGA X and Geneious, and translation into amino acid sequences was performed via ExPASy. With reference to UniProt ID Q02470, three-dimensional structural models of the PrtP protease were produced using SWISS-MODEL and AlphaFold. PyMOL v3.1.6.1 was used for structural visualization and annotation. Serine protease structure from *Bacillus* (PDB ID: 1AH2) was used for superimposition analysis.

RESULTS AND DISCUSSION

Confirmation of *Lacticaseibacillus paracasei* IN17

The working culture maintained its identity as *L. paracasei* IN17, as demonstrated by the morphological and physiological consistency. The colonies were milky white, round, convex, glossy, and opaque surface when they were cultivated on MRSA (Figure 1A). Microscopic analysis after Gram staining showed Gram-positive rods arranged in short chains (Figure 1B). These characteristics correspond with earlier descriptions of the same isolate (Mahulette et al., 2018; Zawawi et al., 2025). IN17 growth under static incubation demonstrated facultative anaerobic behavior, which is in line with the physiological characteristics of LAB in general. This group's members are able to grow in low-oxygen environments (Kieliszek et al., 2021).

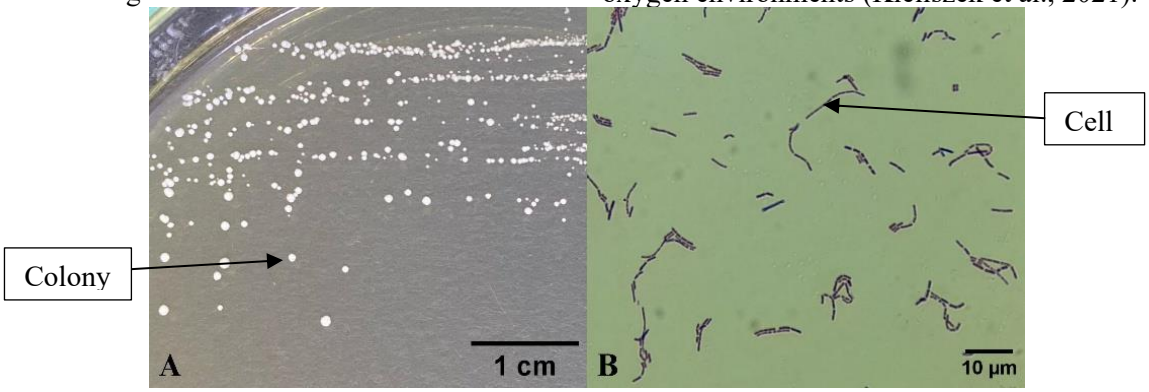

Figure 1. Colony morphology (A) and cell morphology (B) of *L. paracasei* IN17

Table 1. Purification steps for the protease enzyme from IN17.

Purification step	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity ($U.mg^{-1}$)	Purification fold	Yield (%)
Extracellular enzyme (crude)	260	11.725	147.688	0.079	1.00	100.00
Ammonium sulfate 20%	26	1.924	12.259	0.157	1.98	16.41
Dialysis	30	2.213	12.168	0.182	2.29	18.87
Intracellular enzyme (crude)	30	0.923	39.781	0.023	1.00	100.00

Protease Production and Purification

The production volume was scaled up from the previous study's 50 mL to 250 mL. Specific activity increased after fractionation at 20% saturation and continued to improve after dialysis, reaching 0.182 $U \cdot mg^{-1}$ with an overall yield of 18.87% (Table 1). One unit of activity was defined as the amount of enzyme releasing 1 µmol of tyrosine per minute under optimal assay conditions.

IN17 proteases seem to be well-suited for precipitation at 20% ammonium sulfate (Zawawi et al., 2025). Similar findings were made by Silaban et al., (2025), who discovered that protease from fish-derived sources efficiently recovered at 20–30% saturation. Selective precipitation ensues by the salting-out mechanism of $(NH_4)_2SO_4$, which reduces protein solubility by limiting water availability for protein hydration shells (Susanti et al., 2022).

Nevertheless, crude extracts commonly contain a mixture of non-target proteins that dilute catalytic efficiency (Uddin et al., 2025). Following precipitation, dialysis served to remove excess salts and small molecular contaminants, leading to a measurable increase in specific activity. Similar improvements in enzyme purity after dialysis have been documented by (El-Maghrabey et al., 2022). The sequential use of precipitation and dialysis, therefore, proved sufficient to enhance the biochemical quality of IN17 protease prior to further characterization.

In addition to extracellular secretion, IN17 also exhibited intracellular protease activity, although at a lower specific activity (0.023 $U \cdot mg^{-1}$) compared to the extracellular fraction. Because they are less exposed to environmental fluctuations, intracellular enzymes may maintain structural stability despite having a lower numerical value. They are also frequently more concentrated within the cytoplasmic environment (Barros et al., 2020). The proteolytic activity is present in both intracellular and extracellular enzymes from IN17. In lactic acid bacteria, casein is broken down by extracellular proteases into peptides and amino acids. These are then carried into the cell and further broken down by intracellular enzymes (Kieliszek et al., 2021). IN17 exhibits a complete and functionally integrated proteolytic pathway, supporting its ability to adapt in protein rich environments such as fish substrates.

Optimum pH and Temperature of Protease Activity

The extracellular enzyme was most active at pH 6.0 (0.1041 $U \cdot mL^{-1}$), while the intracellular protease performed best at pH 7.0 (0.0311 $U \cdot mL^{-1}$) (Figure 2A). This variation indicates adaptation of the extracellular enzyme to slightly acidic environmental conditions. Whereas the intracellular enzyme activity performs better near cytoplasmic neutrality (Nguyen et al., 2019). This phenomenon is consistent with the proteases from *L. paracasei* FT700 and *L. brevis* R4, which have pH optima at pH 6 and 7, respectively (Anggrahini, 2017; Sun et al., 2019). Each enzyme has a specific pH range that allows for both activity and structural stability (Rachfa et al., 2021). Deviations from the ideal pH range can affect how amino acid residues become ionized, disrupt noncovalent bonds, and influence catalytic activity.

In addition to pH, temperature also affected protease activity. Temperature profiling (20–70 °C) revealed that both extracellular and intracellular proteases were most active at 37 °C, with activities of 0.1050 $U \cdot mL^{-1}$ and 0.0310 $U \cdot mL^{-1}$, respectively (Figure 2B). This aligns with the reported optimum for *L. curvatus* R5 protease at 37 °C (Sun et al., 2019). Lower temperatures reduce molecular kinetic energy, leading to structural rigidity that hinders substrate binding (Delfiana et al., 2023). In contrast, high temperatures can destabilize non-covalent bonds in enzymes, resulting in denaturation and activity loss (Kieliszek et al., 2021).

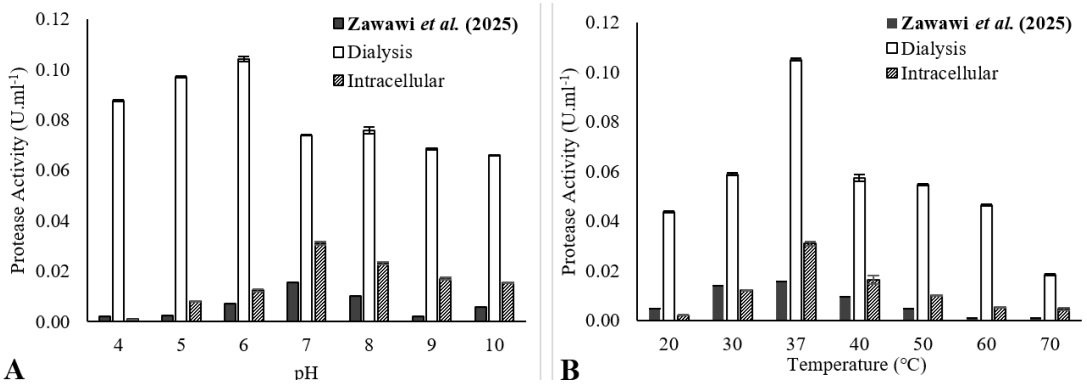

Figure 2. IN17 protease activity at various pH levels (A) and temperatures (B). Error bars represent standard deviation. Optimal protease activity occurs at pH 6 (extracellular) and pH 7 (intracellular), with an optimal temperature of 37 °C

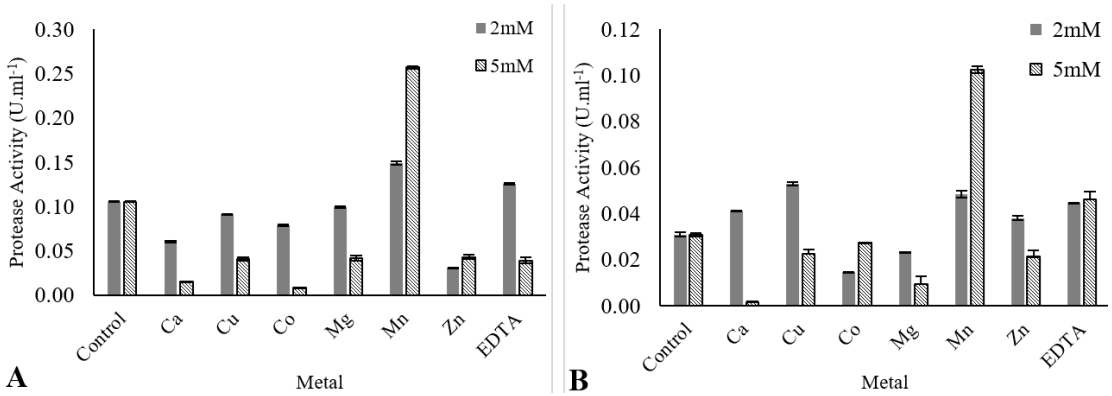

Figure 3. Dialyzed (A) and intracellular (B) IN17 protease activities under various metal cations and inhibitors. Vertical lines (bars) indicate standard deviation

Effects of Metal Ions and Inhibitors

The addition of Mn^{2+} during the IN17 extracellular protease incubation period resulted in a significant increase in activity, with an enhancement of 141.49% at 2 mM and 244.48% at 5 mM (Figure 3A). These findings suggest that Mn^{2+} may function as an effective activator. Mn^{2+} activation has been observed in other proteases (Afriani et al., 2018). Metal binding is thought to stabilize the enzyme's active conformation and improve catalytic efficiency (Herasari et al., 2022). Ca^{2+}, Co^{2+}, and Zn^{2+} significantly reduced activity, whereas Cu^{2+} and Mg^{2+} had moderate inhibitory effects. These findings show that metal ions can either promote or inhibit enzymatic activity, depending on their type and concentration (Ouertani et al., 2019).

The addition of Mn^{2+} resulted in a significant increase in intracellular protease activity, with rises of 156.61% (2 mM) and 332.09% (5 mM) being observed (see Figure 3B). The activity was also increased by Ca^{2+}, Cu^{2+}, and Zn^{2+} ions in addition to Mn^{2+}, confirming earlier research on the function of metal ions in protease activation (Mkhitaryan et al., 2025; Satılmış et al., 2023). It is hypothesised that metal ions stabilise the enzyme's active site, thereby enhancing catalytic efficiency (Herasari et al., 2022), although further investigation is required to elucidate the precise molecular mechanisms involved.

Although the addition of 5 mM EDTA reduced IN17 protease activity to 37.08%. This decrease in value was insufficient to identify the enzyme as a metalloprotease (Sun et al., 2019). Although there was an increase in enzyme activity due to Mn^{2+}, the residual activity observed in the presence of EDTA indicates that this ion is not directly involved in the catalytic site. Therefore, rather than being an essential cofactor, Mn^{2+} is more likely to function as an external activator. A similar phenomenon was observed for a serine protease from *L. casei* NCDO 151, with metal ions increasing activity but strong inhibition occurring in the presence of phenylmethylsulfonyl fluoride (PMSF) (Ji & Agyei, 2020). As a result, further inhibition studies with PMSF are required to determine whether the IN17 protease belongs to the serine protease group.

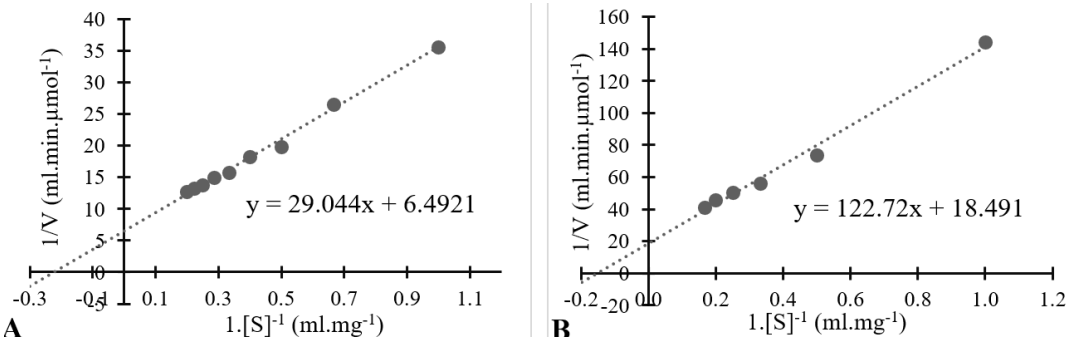

Figure 4. Lineweaver-Burk plots showing the reaction rates of IN17 protease extracellular enzyme (A) and intracellular enzyme (B). Vertical bars represent standard deviation

Enzyme Kinetics

The extracellular (Figure 4A) and intracellular (Figure 4B) proteases had different substrate affinities, according to kinetic analysis. In contrast to the intracellular protease (6.63 mg·mL⁻¹), the extracellular enzyme displayed a lower Km value (4.47 mg·mL⁻¹), suggesting a higher substrate affinity. Affinity at lower concentrations (McDonald & Tipton, 2022). This characteristic suggests that the extracellular protease is better suited to environments with limited substrate availability. The Km value obtained in this study was comparable to that reported by (Sumardi et al., 2019)(4.59 mg·mL⁻¹) and lower than the value described by (Vanessa et al., 2022) (6.83 mg·mL⁻¹).

The maximum reaction rate (Vmax) of the extracellular protease (0.1540 µmol·mL⁻¹·min⁻¹) was nearly threefold higher than that of the intracellular enzyme (0.0541 µmol·mL⁻¹·min⁻¹), indicating superior catalytic efficiency (Sarkono et al., 2017). Although this Vmax was lower than that of a serine protease from *Bacillus* sp. UJ132 (0.33 µmol·mL⁻¹·min⁻¹) (Sumardi et al., 2019), the IN17 protease exhibited moderate activity with favorable substrate affinity.

SDS-PAGE and Zymogram Analysis

SDS-PAGE analysis of the extracellular fraction showed protein bands ranging in size from 20 to 64 kDa (Figure 5A). The difference in band intensity between the extracellular and intracellular fractions was also consistent with the total protein concentration, which was higher inside the cells (Sarkono et al., 2017). The intracellular fraction showed 16 protein bands with a molecular weight range of 23–255 kDa, and a band around 26 kDa appeared to be dominant (Figure 5A).

The results of the zymogram showed that the ~26kDa band in the intracellular fraction was active (see Figure 5B). This size is similar to the serine protease from *Bacillus alcalophilus* (PDB ID: 1AH2), which suggests that the catalytic domain of the enzyme can function well even without the whole non-catalytic domain. In contrast, it appears that no clear zone was detected on the zymogram gel in the extracellular fraction. This could possibly be due to the low protein concentration, which meant that the protease activity was not strong enough to be visualised (Zhang et al., 2019). Nevertheless, the presence of protein bands still indicates that the protein fraction is secreted outside the cell, even though not all of it is enzymatically active.

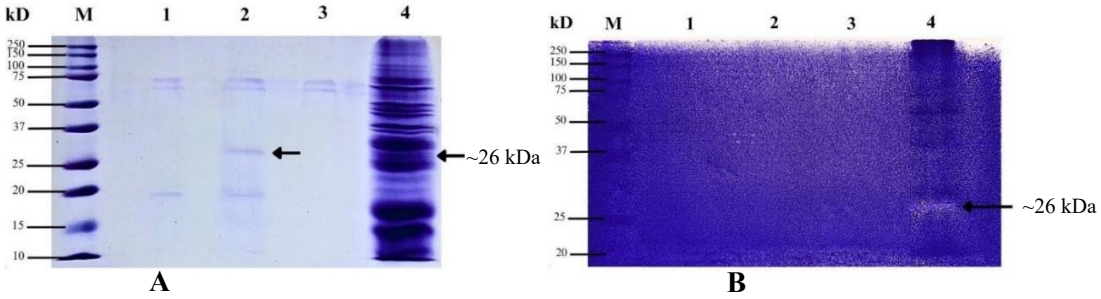

Figure 5. Protease sample analysis using SDS-PAGE (A) and zymogram (B). Protein marker lane M, crude extracellular protease from IN17 lane 1, 20% ammonium sulfate fraction lane 2, dialyzed enzyme lane 3, and crude intracellular enzyme lane 4. The PrtP band is indicated by the arrow.

Molecular Analysis of the *prtP* Gene and 3D Structure Prediction

The amplification of the *prtP* gene from the IN17 genome resulted in the production of a DNA band with a size of approximately 5800 base pairs (bp) (Figure 6A), which is consistent with the expected size of the prtP gene encoding cell-envelope proteinase (CEP). This indicates that IN17 carries a complete *prtP* gene on its genome, as is typical for LAB (Ji & Agyei, 2020). Sequencing produced partial fragments at positions 39–1039 bp (forward primer) and 4422–5761 bp (reverse primer), which showed 91.55% and 97.29% identity to the reference sequence M83946.1, respectively (Figure 6B). These results confirmed that the amplified gene is homologous to *prtP* and validate its use as a basis for structural prediction.

The 3D structure of PrtP was predicted using SWISS-MODEL with the reference sequence M83946.1 (Figure 7A) and validated by superimposition with the AlphaFold prediction (Christensen et al., 2023). The RMSD value of 0.925 Å indicated high structural similarity, far below the 2 Å threshold for reliable alignment (Sargsyan et al., 2017) (Figure 7B).

PrtP contained characteristic CEP domains, including a prepro domain (residues 1–187), catalytic domain (191–697), protease-associated domain (477–548), and LPXTG motif (1867–1871) (Figure 8). The catalytic triad (Asp217, His281, Ser620) was properly oriented, consistent with conserved serine protease mechanisms (Christensen et al., 2023) (Figure 8A).

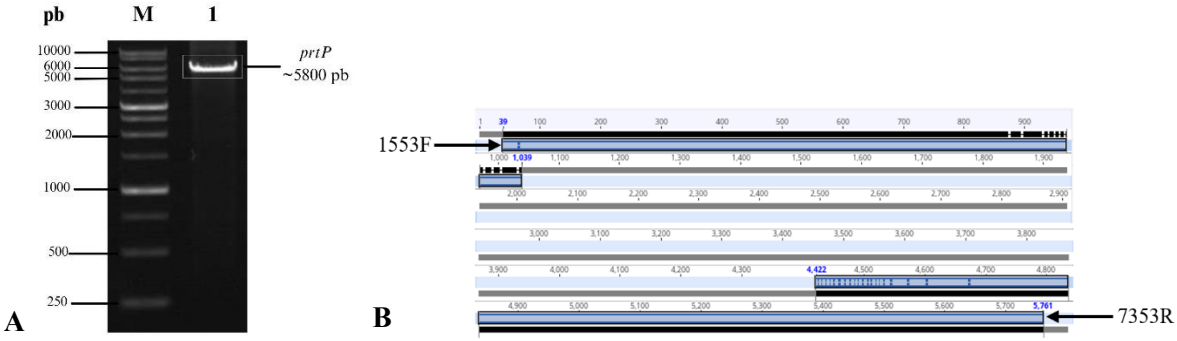

Figure 6. Amplification and sequencing analysis of the *prtP* gene from *Lacticaseibacillus paracasei* IN17. The prtP gene amplicon was electrophoresed on an agarose gel (A). A reference sequence M83946.1 (B) was aligned with partial *prtP* gene sequences at positions 39–1039 bp (forward primer) and 4422–5761 bp (reverse primer). The forward primer is 1553F, and the reverse primer is 7353R.

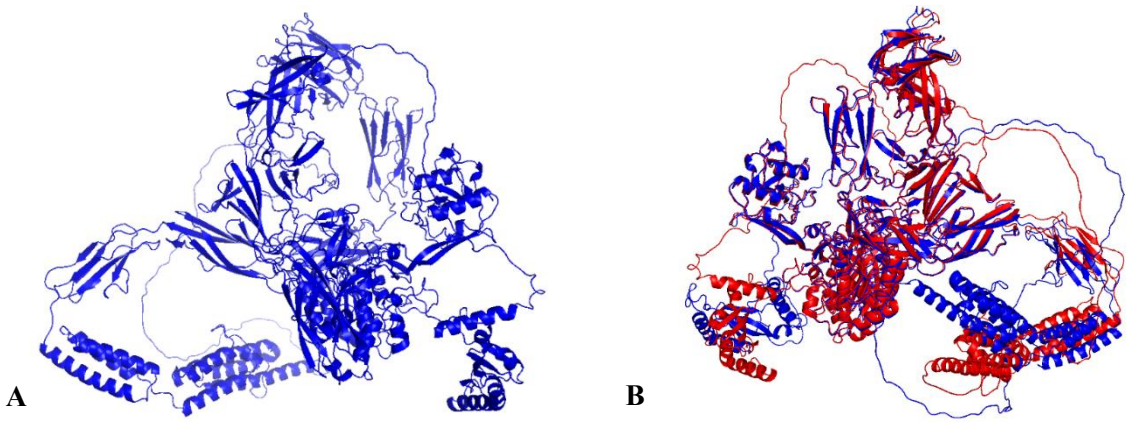

Figure 7. Predicted 3D structure of PrtP IN17 generated using SWISS-MODEL based on NCBI sequence M83946.1 (A). Structural superimposition between the predicted PrtP model (red) and the structure reported by Christensen et al. (2023) (blue) (B).

Additionally, superimposition of PrtP with the sequenced fragments shows structural consistency. Fragment 1553F shows alignment of 934 atoms, with a root mean square deviation (RMSD) of 1.165 Å, as shown in Figure 9A. On the other hand, fragment 7353R shows an alignment of 2210 atoms, with an RMSD of 0.123 Å, as shown in Figure 9B. The results of both experiments show a significant level of structural overlap. However, it was observed that no fragment showed direct compatibility with the catalytic domain.

Structural alignment with the catalytic domain of *Bacillus alcalophilus* protease (PDB ID: 1AH2) resulted in an RMSD of 1.365 Å (Figure 10). This low value indicates strong structural similarity and conservation of the catalytic core. Based on these observations, future research can focus on primers that specifically target the catalytic domain rather than the entire gene. Amplifying a shorter fragment would simplify cloning procedures and improve efficiency during heterologous expression in suitable host systems. Such an approach could support better optimization of protease production.

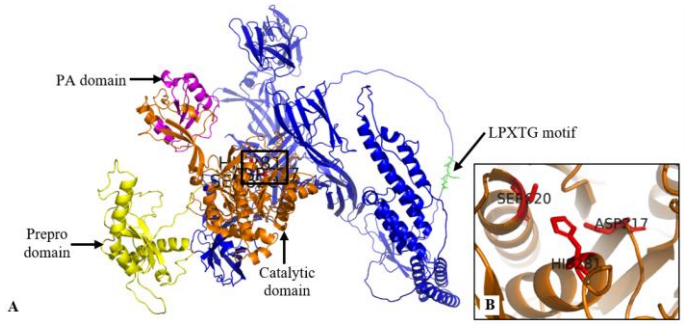

Figure 8. Three-dimensional structure of PrtP protease showing the prepro domain (yellow), catalytic domain (orange), PA domain (magenta), LPXTG motif (green), and catalytic residues (red) (A), and magnified view of the catalytic site of PrtP (B).

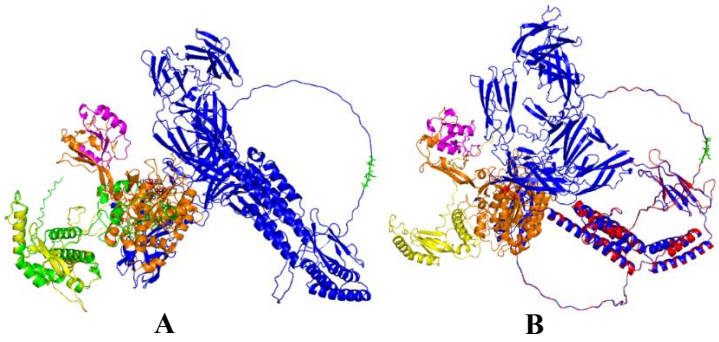

Figure 9. Structural superimposition of the PP domain (yellow) on the PrtP model with the predicted structure of the 1553F fragment (green) (A). Structural superimposition of PrtP with the predicted structure of the 7353R fragment (red) (B).

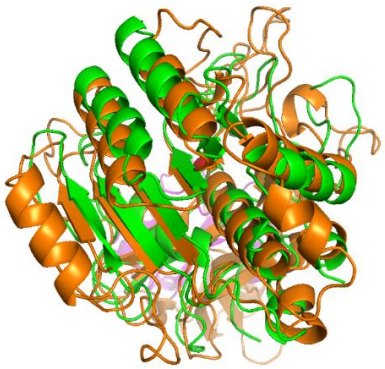

Figure 10. Structural superimposition of the catalytic domain of the PrtP model (orange) with the serine protease structure of *Bacillus alcalophilus* (green, PDB ID: 1AH2).

The serine protease from *Bacillus alcalophilus* (PDB ID: 1AH2) has a molecular size of ~26 kDa, which corresponds to the active band seen in SDS-PAGE and zymogram analyses of the IN17 protease. The non-catalytic domains may not be fully expressed or undergo post-translational cleavage (Scheller & Krebs, 2021). This mechanism is in line with earlier findings that some protease domains are still active even after they are separated from other domains (Harwood & Kikuchi, 2022). The identification of *prtP* in IN17 showed that LAB proteolytic systems are not restricted to dairy environments but also adapted to marine protein substrates.

This study successfully combined biochemical, kinetic, and molecular characterisation to analyse a PrtP-type protease found in a traditional Indonesian fermented fish product, which remains underexplored. This finding broadens current understanding of LAB protease diversity beyond conventional dairy-associated strains into the functional role in non-dairy fermentation systems. The enzyme has the potential to produce peptides with nutritional and functional value, as well as flavor-enhancing compounds in fermented products (Friis et al., 2022). By advancing the creation of functional foods enhanced with health-promoting peptides, this research advances SDGs 3 (Good Health and Well-Being). Also SDGs 12 (Responsible Consumption and Production) by encouraging the sustainable value of fish resources through fermentation techniques based on biotechnology.

CONCLUSION

The protease enzyme produced by *Lacticaseibacillus paracasei* IN17 exhibits extracellular and intracellular activity. Dialysis after ammonium sulfate precipitation increases the specific activity of the enzyme, indicating a successful initial purification step. Optimal protease activity was identified at pH 6.0 for extracellular enzymes and pH 7.0 for intracellular enzymes, both at a temperature of 37°C. Kinetic analysis revealed that the extracellular enzyme had a Vmax of 0.1540 $\mu mol.ml^{-1}.min^{-1}$ and a Km of 4.47 $mg.ml^{-1}$, while the intracellular enzyme showed a Vmax of 0.0541 $\mu mol.ml^{-1}.min^{-1}$ and a Km of 6.63 $mg.ml^{-1}$. The presence of certain metal ions has been demonstrated to increase enzyme activity, with no inhibition observed in the presence of EDTA. SDS-PAGE and zymogram analysis revealed that the ~26kDa protein band exhibited protease activity within intracellular enzymes. Furthermore, the protease-encoding gene (*prtP*) was successfully amplified, yielding an amplicon size of ~5800bp. The structure of PrtP has also been predicted to contain a catalytic domain of ~26kDa. These findings underscore the significant biotechnological potential of *L. paracasei* IN17-derived protease for utilisation in food and industrial enzyme applications. Future research should concentrate on heterologous expression and domain-specific gene amplification to maximize protease production and functional use.

AUTHOR CONTRIBUTION STATEMENT

SMM conceived and designed the study, conducted laboratory experiments, performed data analysis, and drafted the manuscript. NRM supervised the research, contributed to study design and interpretation of results, and critically revised the manuscript. LA contributed to molecular analysis, structural modeling, and data interpretation. All authors reviewed and approved the final manuscript and agreed to be accountable for all aspects of the work.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this paper

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that artificial intelligence (AI) tools were used solely to assist in improving the grammar and language clarity of this manuscript. All research activities, including study design, data collection, data analysis, interpretation of results, and scientific content development, were conducted entirely by the authors. The use of AI was limited to linguistic refinement and did not influence the scientific findings or conclusions of this work.

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