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Extracellular Protease Characterization of *Lactococcus lactis* subsp. *lactis* and Its Application on Protein Hydrolysis toward Indonesian Sea Cucumber (*Holothuria* sp.) and *Lorjuk* Shelfish (*Solen* sp.) as an Antioxidant Alternative

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Enzymatic hydrolysis is more efficient compared to other types of protein hydrolysis methods due to the fact that enzymes function as specific catalysts that can produce peptides in a less complex form and avoid non-hydrolytic changes. Extracellular protease from *Lactococcus lactis* subsp. *lactis* (LAC3) has the potential as an agent for enzymatic hydrolysis of marine products, especially for non-fibrillar and non-collagen protein types. Extracellular protease from LAC3 was successfully isolated and was able to hydrolyze sea cucumber and *lorjuk* shellfish. Hydrolyzed products appear to have molecular weights of 4.6 and 15 kDa. The hydrolysates from the enzymatic hydrolysis of the two marine samples have significant antioxidant activities based on the DPPH inhibition assay. The best 3D protein structure modeling was done using the AlphaFold web server.

Keywords: antioxidants, extracellular protease, hydrolysis, LAB, marine products

INTRODUCTION

The Indonesian coastline is ranked as the second longest in the world after that of Canada (Trinanda 2017), thus fostering an ecosystem for Indonesia's abundant marine biota, including sea cucumber (*Holothuria* sp.) and *lorjuk* shellfish (*Solen* sp.). *Holothuria* sp., which is known as sea cucumber, constitutes a group of marine invertebrate animals belonging to phylum Echinodermata and is frequently found in coral reef areas (Husain *et al.* 2017). It is divided into six orders – namely, Dendrochirotida, Aspidochirotida, Dactylochirotida, Apodida, Molpadida, and Elasipoda (Nurwidodo *et al.* 2018). On the other hand, *lorjuk* shellfish is included in the Solenidae family. Both species are known to have several active compounds beneficial in the health sector, especially in the pharmaceutical field. Previous studies identified the use of sea cucumbers as an antibacterial (Siringo

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2017), anticancer (Putram *et al.* 2017), and antioxidant (Senadheera *et al.* 2021). On the other hand, *lorjuk* shellfish have active compounds that are considered antioxidant, antitumor, antiviral, antibacterial, and antifungal (Nurjanah *et al.* 2011).

The proximate content of sea cucumber (*Holothuria* sp.) is as follows: water (87.03%), protein (9.94%), fat (0.54%), ash (1.86%), and carbohydrates (0.64%) (Karnila *et al.* 2011). On the other hand, the proximate composition of *lorjuk* shellfish includes protein (9.79%), carbohydrates (4.95%), fat (0.32%), and ash (2.63%) (Trisyani *et al.* 2021). Since protein dominates the content of both species, practically it has the potential to be used as a hydrolysis substrate to produce protein in simpler forms of amino acids and peptides. Protein hydrolysis works by breaking the peptide chain and transforming proteins into short peptides or free amino acids (Septiana 2014).

Hydrolysis is divided into five types based on the catalyst: [1] pure hydrolysis using water or distilled water during the hydrolysis process, [2] hydrolysis using an acid solution, [3] protein hydrolysis with alkali, [4] protein hydrolysis by alkaline at high temperature, and [5] enzymatic protein hydrolysis (Hutapea and Sitorus 2017). Enzymatic hydrolysis is more efficient in hydrolyzing proteins compared to other methods because enzymes are catalysts, which can produce less complex peptides that easily break down proteins at specific sites and avoid non-hydrolytic changes (Nasution and Abdullah 2019). The resulting hydrolysate generally contains lower molecular weight (MW) peptides and free amino acids (Annisa *et al.* 2017). Furthermore, enzymatically hydrolyzed protein products are recognized to have high solubility and more stable.

Proteases are enzymes that are commonly used as catalysts during hydrolysis and are generally divided into two groups namely exopeptidase and endopeptidase. Exopeptidase is a protease enzyme that catalyzes peptide breakdown at the ends of polypeptide chains, whereas endopeptidase catalyzes the breakdown of peptide bonds inside polypeptide chains (Prasetyo 2016). Several proteases are produced by microorganisms, especially lactic acid bacteria (LABs). Previous studies have carried out enzymatic hydrolysis of skim milk by *L. plantarum* S31 (Budiarto *et al.* 2016), gelatin extracted from Indonesian *Pangasius sutchi*'s bone by *Lactobacillus plantarum* S31 (Atma *et al.* 2021), and *Metapenaeus monoceros* by *Anoxybacillus kamchatkensis* (Mechri *et al.* 2020).

The *Lactococcus lactis* subsp. *lactis* (LAC3) is a LAB that is frequently found in the milk fermentation process. The milk fermentation process occurs because the gene encodes PrtP protease in the cell wall (extracellular protease), which will degrade casein as a substrate outside the cell (Guillot *et al.* 2016). In addition, there is also HtrA protease that plays a role in the maturation of the original protein, propeptide processing, and degradation of misfolded or damaged protein substrates. The HrtA protease is also considered to be a sheddase enzyme, which cleaves its substrate at extracytoplasmic membrane sites (Dalbey *et al.* 2012). This study aims to hydrolyze protein from sea cucumbers and *lorjuk* shellfish using secondary metabolites from LAC3. Then, the resulting hydrolysates were tested for their antioxidant activities to identify the possibility of these hydrolysates as antioxidant alternatives.

MATERIALS AND METHODS

The sea cucumber (*Holothuria atra*) samples were obtained from Talango Island, Sumenep, East Java, Indonesia, whereas the *lorjuk* shellfish (*Solen* sp.) were obtained from Pamekasan, East Java, Indonesia. The hydrolysis enzyme was obtained from the extracellular protease of LAC3, collected from the National Research and Innovation Agency (BRIN), and isolated from buffalo milk fermentation (*dadih*).

Fermentation Growth Curve of *Lactococcus lactis* subsp. *lactis* (LAC3)

The LAC3 liquid culture stock (20 µL) was grown in 5 mL of M17 + 0.5% glucose medium at 30 °C for overnight without shaking. Then, 400 µL of LAC3 cultures were transferred into 100 mL of 0.5% M17 + glucose medium at 30 °C without shaking. Samples (1.5 mL) were obtained from the culture LAC3 every 5 h continuously for 50 h, which were then measured for their OD (OD600nm), pH, and extracellular protease activity.

Purification of Extracellular Protease from *Lactococcus lactis* (LAC3)

Extracellular proteases from the LAC3 were purified by means of precipitation method using ammonium sulfate (Merck, Germany) and gel filtration chromatography using Sephadex G-50 (GE Healthcare). The metabolite of LAC3 (100 mL) obtained from 20 h production was brought to a saturation of 45% by adding ammonium sulfate and incubating overnight at 4 °C. The precipitated protein was harvested by centrifugation at 9300 rpm for 30 min at 4 °C, and the resulting pellet was dissolved in 50mM Tris HCl buffer pH 7.4 by volume (pellet weight x 1000).

Precipitated protease solution was purified using a Sephadex G-50 chromatographic column, which was equilibrated with 10mM Tris HCl buffer (pH 7.4) at 4 °C. The elution samples were collected every 1 mL at a flow rate of 1 mL/min. The protein concentration of collected fractions was analyzed using a BCA kit assay, and the

absorbance was measured at 540 nm. The protease activity of the collected fraction was measured and subjected to a proteolytic activity test. In addition, the purification results were also subjected to cofactor and inhibitor testing to characterize the types of proteases in the extracellular protease from LAC3.

Proteolytic Activity

The proteolytic activity was analyzed quantitatively using casein as a substrate. The protease activity test was done using Cupp-Enyard's method (2008). This test was carried out using a 96-well microplate. A total of 18 µL of the reaction mixture consisting of 6 µL of sample, 6 µL of Tris buffer, and 6 µL of substrate were incubated for 30 min at 37 °C. The enzymatic reaction was stopped by adding 12 µL of trichloroacetic acid to stop the reaction. Tyrosine solutions were used as standards ranging from 0, 10, 20, 30, 40, and 50 mM. Afterward, 143 µL of working reagent sodium carbonate (Na_2CO_3) and copper sulfate pentahydrate ($CuSO_4.5H_2O$) in a ratio of 5:1 v/v and 30 µL of Folin Ciocalteau reagent were added into the reaction mixture, followed by centrifugation for 5 min at 10.000 xg.

Free tyrosine in the supernatant was measured at an absorbance of 540 nm, and the concentration was compared using a standard tyrosine curve. One unit of proteolytic activity was defined as the size of the sample that released 1 mol of tyrosine per minute under experimental conditions.

Isolation of Indonesian Sea Cucumber (*Holothuria atra*) and *Lorjuk* Shellfish (*Solen* sp.) Hydrolysates

Upon proteolytic hydrolysis of sea cucumber and *lorjuk* shellfish using extracellular proteases of LAC3, the proteins were isolated. Isolation of hydrolysates of sea cucumber protein was carried out by the method of Fadhlia (2017). The hydrolysate was immersed in acetone for 24 h with a ratio of 1:2 w/v at 4 °C. The resulting pellets were collected by centrifugation at 5.000 rpm at 4 °C for 30 min, and it is then dried using the vacuum freeze-drying method, in which a sublimation process occurs in the frozen sample, resulting in a dry frozen sample without the melting process. This prevents damage to the proteins in the sample. The sample was dissolved in 5% sodium dodecyl sulfate, and the sample solution was incubated at 85 °C for 10 min then centrifuged at 12,000 rpm for 15 min for further testing.

Isolation of hydrolysates of *lorjuk* shellfish protein was carried out by the method of Widjaya (2021). Protein isolation for *lorjuk* shellfish was carried out by separating the meat and shell, and the meat was then soaked in Buffer A (Tris-HCl, 0.1 M, pH 8.3; NaCl, 2 M; $CaCl_2$, 0.01 M; β-mercaptoethanol 1%; Triton X-100, 0.5%) with a ratio of 5:8. The resulting solution was filtered using a gauze bandage and processed in liquid freeze for 2–3 times using a vacuum freeze-drying method. Then, centrifuged at 6000 rpm at 4 °C for 30 min, and the supernatant was then collected.

Enzymatic Hydrolysis

The sample was adjusted to pH 7. Protease enzyme was added to the sample at a ratio of 1:100 v/v. Hydrolysis was carried out in a water bath shaker at 37 ℃ for 18 h. Enzyme inactivation in the hydrolysis process was carried out by heating at 95 ℃ for 15 min. Supernatant in the form of hydrolysate was collected for further testing by centrifugation at 12.000 rpm at 4 ℃ for 15 min (Budiarto *et al.* 2016).

SDS-PAGE

The sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) method used a separating gel with a concentration of 10% for the lysate sample and 12% for the hydrolysate and 4% stacking gel for both samples. The staining process used Coomassie Brilliant Blue staining and washing using Developer 1 solution (30% methanol + 5% acetic acid) and Developer 2 solution (5% methanol + 7% acetic acid) for 2–3 h until the protein bands appeared clearly. A protein marker with a minimum size of 5 kDa is used to predict the size of the protein bands that appear (Budiarto *et al.* 2016).

Antioxidant Activity Test

The method of Shimada *et al.* (1992) was followed for the antioxidant activity. Twenty microliters (20 µL) of hydrolysate sample was added with 180 µL of 1.1-diphenyl-2-picrylhydrazyl (DPPH) solution (0.2 mM in methanol) mixed in a microplate and incubated in the dark for 30 min. The blank or negative control used was growth medium (M17G), whereas vitamin C was the positive control with several concentrations (100–500 ppm). After completing incubation, the concentration of DPPH was measured using a multi-plate reader (Thermo Scientific) at a wavelength of 540 nm. The color change that occurred is due to a free radical inhibition reaction, during which DPPH is reduced to diphenylpicrylhydrazine, which is a non-radical compound. Percent inhibition was computed based on the following formula:

$$\% \text{ inhibition} = \frac{(\text{Control absorbance } (-) - (\text{Sample absorbance})}{\text{Control absorbance } (-)} \times 100$$

Protein Modelling of the 3D Structure

The protein serine protease from LAC3 was modeled with three modeling methods on webservers such as I-Tasser (https://zhanggroup.org/I-TASSER/), Robetta (www.

robetta.bakerlab.org), and the AlphaFold web server (https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb). The modeling results from the three web servers were compared to determine the best modeling outcome using several validation metrics, including the Ramachandran plot, Errat, and 3D Verify structure (www.saves.mbi.ucla.edu).

RESULTS

Growth and Protease Activity of *Lactococcus lactis* subsp. *lactis* (LAC3)

LAC3 was observed for its growth curve for 50 h by observing its optical density, pH, and proteolytic activity every 5 h. The graph of the growth curve is shown in Figure 1a, where the optical density at 0–5 h shows growth in the lag phase. The exponential phase is shown at 5–10 h, whereas 10–45 h shows the stationary phase and then the start of the death phase at 45–50 h. Protease activity in Figure 1a shows that the 20th hour is when LAC3 exhibits optimal protease activity, which then decreases with prolonged incubation. This aligns with research by Cretenet *et al.* (2011), which explains that LAC3 has optimal activity in culture for 24 h and gradually decreases over 7 d.

Increased protease activity in the stationary phase is due to the formation of lactic acid, which is characterized by a decrease in the pH of the culture media, as shown in Figure 1b. Furthermore, a decrease in pH can also be associated with a decrease in activity after the stationary phase because when the pH decreases it can cause inhibition of cellular function, depletion of nutrients in the growth medium, and inactivation of enzymes due to catabolite repression at low pH (Bhanwar *et al.* 2014).

Purification of Extracellular Protease from *Lactococcus lactis* (LAC3)

The extracellular protease LAC3 grown in media for 20 h was collected by precipitation using ammonium sulfate in a concentration of 45% and then purified using Sephadex G-50 gel. Elution was performed using 25 mM Tris-HCl pH 7.4, which successfully obtained six fractions. Figure 2 shows qualitative and quantitative tests representing proteolytic activity and extracellular protein concentration of LAC3 protease, respectively, that were carried out

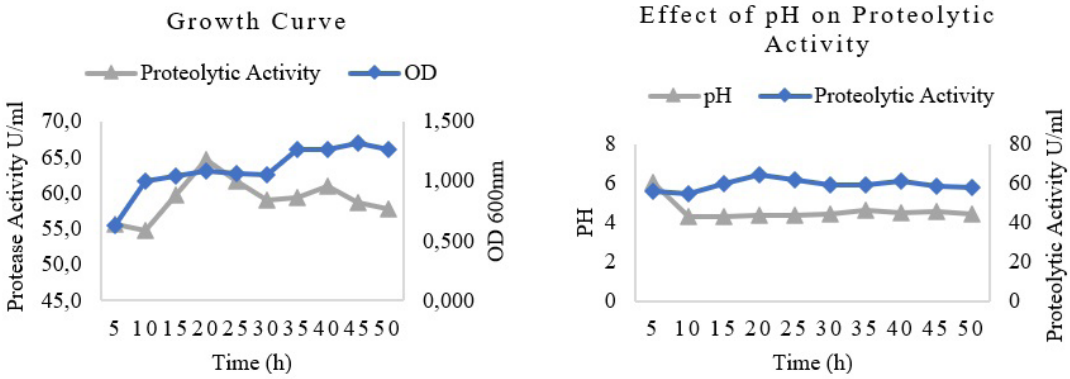

Figure 1. Growth curves of *Lactococcus lactis* subsp. *lactis* (LAC3) cultured at 37 ºC. [a] OD (optical density) with proteolytic activity. [b] pH with proteolytic activity.

Table 1. Summary of partial enzyme purification of *Lactococcus lactis* (LAC3).

Protease in different purification steps	Protein			Enzyme				
	Volume (mL)	Concentration (mg/mL)	Total protein (mg)	Protease activity (U/mL)	Specific activity (U/mg)	Total activity (U)	Yield (%)	Purity (fold)
Crude extract	150	22.9	3441.2	18.9	0.8	2835.0	100	1
Precipitation 45%	0.3	28.2	8.5	16.4	0.6	4.9	86.8	0.7
Dialysis	1.5	18.9	28.4	14.0	0.7	21.0	74.1	0.9
G-50 sephadex	1.3	10.4	13.5	9.5	0.9	12.4	50	1.1

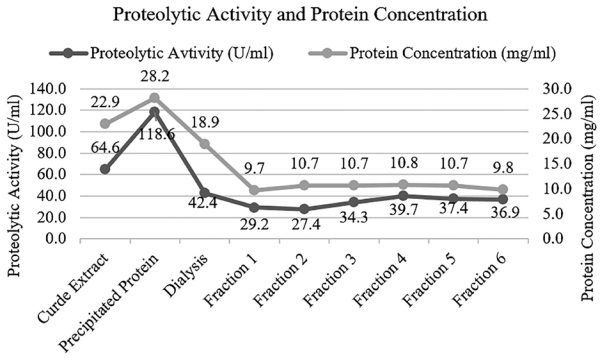

Figure 2. Protease activity and protein concentration of *Lactococcus lactis* subsp. *lactis* (LAC3) at 37 ºC for 20-h incubation.

Table 2. Effect of inhibitors, surfactants, and metal ions on protease activity of *Lactococcus lactis* subsp. *lactis* (LAC3).

No.	Features		Activity (%)
1	Temperature optimum		37 ºC
2	pH optimum		4
3	Surfactants effect	Concentrations	Residual activity (%)*
	Tween 20	2.5%	70.20 ± 0.192
		5%	64.65 ± 0.104
	SDS	2.5%	21.74 ± 0.030
		5%	30.06 ± 0.044
4	Inhibitor effect		
	PMSF	0.1 mM	18.83 ± 0.018
		1 mM	19.60 ± 0.015
	EDTA	0.1 mM	20.04 ± 0.011
		1 mM	17.25 ± 0.025
5	Metal ions effect		
	$MgCl_2$	0.1 mM	18.32 ± 0.005
		1 mM	19.70 ± 0.004
	$CaCl_2$	0.1 mM	20.19 ± 0.012
		1 mM	20.46 ± 0.003
	Control	-	100

*Values are represented as mean ± SD (n = 3)

using a BCA protein assay kit with a standard used as BSA (bovine serum albumin). Fractions 2–5 had quite high proteolytic activity, which could be interpreted as having caseinolytic and gelatinolytic activities but not significantly different.

All fractions were run on SDS-PAGE testing to confirm the presence of purified extracellular protease LAC3 protein, along with zymogram test to determine protein size, which has caseinolytic and gelatinolytic activity, because zymogram gel composed of casein/gelatin, which functions as a substrate. Figure 3 shows SDS-PAGE and zymogram results where several bands at a size of 35.5 kDa are spotted. In SDS-PAGE, all fractions have fairly clear bands at 35.5 kDa; on the other hand, Fraction 2 has clearer bands than other fractions on the zymogram.

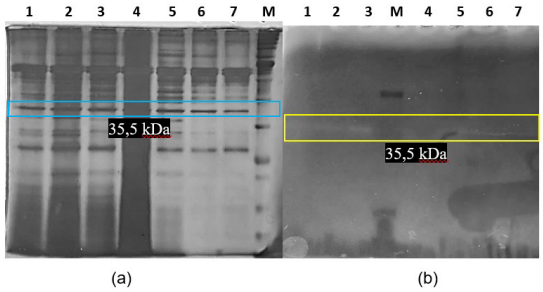

Figure 3. The SDS-PAGE profile (a) and zymogram protein pattern (b) of protease from *Lactococcus lactis*; samples were arranged as follows: [1] dialysis, [2] Fraction 1, [3] Fraction 2, [4] Fraction 3, [5] Fraction 4, [6] Fraction 5, and [7] Fraction 6.

Enzymatic Hydrolysis of *Lactococcus lactis* (LAC3)

Extracellular protease of 20 h of LAC3 culture was used to hydrolyze sea cucumbers and *lorjuk* shellfish. Prior to hydrolysis, sea cucumbers and *lorjuk* shellfish were condensed at pH 7 according to the pH conditions of the enzyme. Qualitative and quantitative measurements of hydrolysate were done by means of protein concentration and SDS-PAGE, respectively. The protein concentration of hydrolysate was measured using a BCA protein assay kit, and the results of hydrolysate of sea cucumber meat had a higher protein concentration than sea cucumber offal, which was 21.2 mg/mL for the sea cucumber meat and 14.2 mg/mL for the sea cucumber offal. Thus, the sea cucumber meat was chosen for following test. On the other hand, *lorjuk* shellfish hydrolysate had the highest protein concentration among others at 31.6 mg/mL.

Hydrolysis of non-fibrillar proteins such as albumin and globulin is commonly controlled by protease, which also selectively hydrolyzes non-collagenous proteins (Sharma *et al.* 2019). Enzymatic hydrolysis results of sea cucumber meat and *lorjuk* shellfish for 18 h were confirmed *via* SDS-PAGE to analyze the size of the hydrolyzed protein. Figure 4a shows the hydrolyzed *lorjuk* shellfish with a MW of 15 kDa. The shellfish had parvalbumin protein with an MW of 12kDa, which can be hydrolyzed by proteases (Elsayed and Apold 1983). Figure 4b shows hydrolyzed sea cucumber meat with a protein size of 4.6 kDa. In a previous study by Chim-chi and colleagues in 2016, they succeeded in hydrolyzing sea cucumber *Isostichopus badionotus* using Alcalase®-Flavorzyme® and obtained hydrolysate with four bands (MW = 19, 14, 6, and 3 kDa).

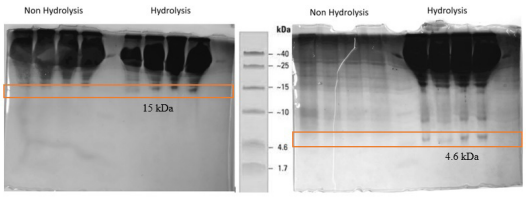

Figure 4. The SDS PAGE profiles of enzymatic hydrolysis by extracelullar protease from *Lactococcus lactis* subsp. *lactis* (LAC3) on *lorjuk* shellfish (a) and sea cucumber (b).

Inhibition of DPPH as Antioxidant Activity Test

Hydrolyzed antioxidant activity of sea cucumber meat and *lorjuk* shellfish was successfully established. The bioactivity of sea cucumbers and *lorjuk* shellfish hydrolysate as antioxidants was measured by their ability to inhibit DPPH free radicals. Table 3 shows the antioxidant activity of hydrolyzed sea cucumbers and *lorjuk* shellfish. In this study, the hydrolysates of sea cucumbers and *lorjuk* shellfish showed relatively similar antioxidant activity results, which can be concluded that higher protein concentration correlates to higher inhibition power to DPPH. This is in line with the findings of research by Theodore and colleagues in 2008, which stated that higher molecular peptides had higher inhibition activity toward DPPH compared to low molecular peptides. The DPPH radical scavenging activity of an antioxidant is based on its ability to donate hydrogen atoms or electrons to stabilize radicals by converting them into non-radical species (Chandrasekara and Shahidi 2011).

Protein Modelling 3D Structure

3D protein structure modeling of serine protease was carried out using three different web servers with the aim of comparing more stable protein structures. Figure 5 shows the visualization of the 3D protein structure of serine protease from LAC3, which is composed of 270 amino acids. The 3D structure of serine protease LAC3 consists of α-helix, β-strand, and random coil structures (Figure 5). Validation of the 3D structure was performed by comparing the Ramachandran plot of the three different protein modeling webservers (Figure 6). The Ramachandran plot score, ERRAT, and verify 3D were also compared among the three protein modeling webservers (Table 4).

The results of 3D protein structure modeling of serine protease from LAC3 using three different web servers showed that the best modeling results were by using the AlphaFold webserver. The AlphaFold web server showed a Ramachandran plot result with a value of 96.1% in the favored region and 0.4% in the disallowed region. The AlphaFold webserver also showed an ERRAT value of 96.02% and a Verify 3D value of 74.07%.

Table 3. Lysate and hydrolysate antioxidant activity of sea cucumber meat (a) and *Lorjuk* shellfish (b). Both represent comparable results that protein concentration influences the level of antioxidant activities.

Sample	% inhibition
DPPH	0.00
Standard vit. C	
100 ppm	31.34
150 ppm	47.15
200 ppm	54.68
250 ppm	73.28
500 ppm	96.34
Sea cucumber meat	
Sample 10 mg/mL	9.54
Hydrolysate 10 mg/mL	10.07
Lorjuk shellfish	
Sample 15 mg/mL	54.12
Hydrolysate 15 mg/mL	55.00

DISCUSSION

The extracellular protease from LAC3 belongs to both serine protease and metalloprotease categories, as observed from the effects of cofactors and inhibitors. It has active sites on serine and lysine amino acids that function as catalysts in hydrolysis (Brandstetter *et al.* 2002). Optical density measures the density level in the culture, and from the 30th–35th hour, there was an increase in OD, possibly due to the accumulation of metabolites and lactic acid, which are detected at a wavelength of 600 nm. LAC3 exhibits a relatively long stationary phase, which is attributed to the fact that during the stationary phase, LAC3 tends to overexpress genes encoding RNA polymerase subunit and murC, which are responsible for maintaining cell walls, making the cells resistant to lysis and, thus, extending the stationary phase (Cretenet *et al.* 2011).

Incubation time and protease synthesis produced by LAC3 greatly affect the production of extracellular protease. In this study, extracellular protease was produced at 20 h of culture, which was in the stationary phase and correlated with an increase in proteolytic activity and a decrease in pH. These results are relevant to other studies, which prove that most extracellular bacterial proteases achieve optimal results in the stationary phase of growth (Ferrero *et al.* 1996; Kaur *et al.* 1998; Bhaskar *et al.* 2007; Palsaniya *et al.* 2012; Budiarto *et al.* 2016). Following the test was the purification of LAC3 extracellular protease, which was collected at the 20th hour of growth.

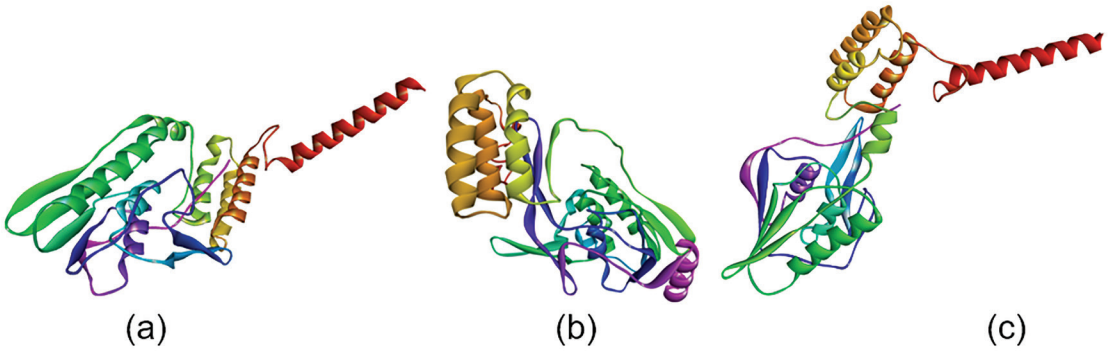

Figure 5. 3D protein structure of serine protease from *Lactococcus lactis* (LAC3). AlphaFold webserver (a), I-Tasser webserver (b), and Robetta webserver (c).

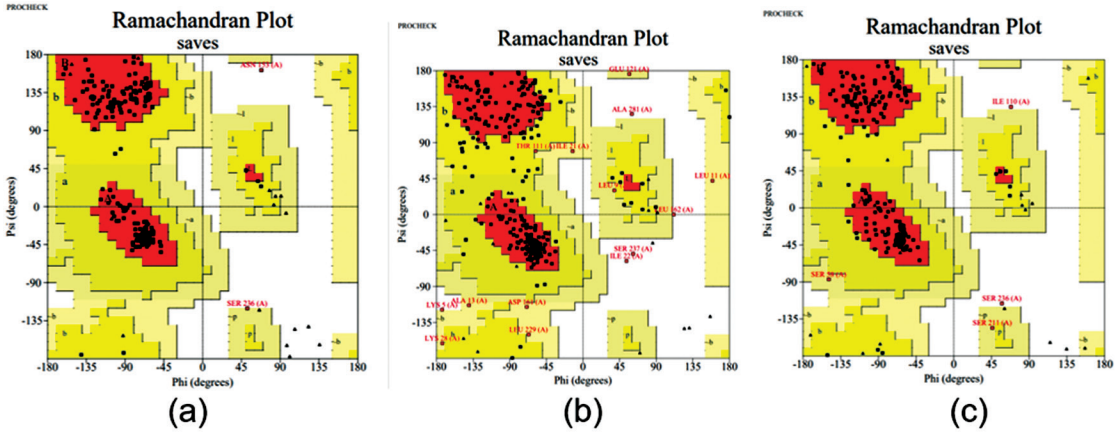

Figure 6. Ramachandran plot of the 3D protein structure of serine protease from *Lactococcus lactis* (LAC3). AlphaFold webserver (a), I-Tasser webserver (b), and Robetta webserver (c).

Table 4. Quality evaluation of serine protease of *Lactococcus lactis* subsp. *lactis* (LAC3) protein structure with ERRAT and Verity 3D webserver.

Webserver	Ramachandran plot (%)				ERRAT (%)	Verify 3D (%)
	Quadrant 1	Quadrant 2	Quadrant 3	Quadrant 4		
AlphaFold	96.1	3.0	0.4	0.4	96.02	74.07
Robetta	90.6	7.7	0.9	0.9	95.03	82.96
I-Tasser	75.2	19.8	3.2	1.8	82.90	63.21

Tween 20 is a non-ionic surfactant that has no critical effect on protease activity and sometimes even increases its activity (Vijayaraghavan *et al.* 2014; Yilmaz *et al.* 2015; El-Gendi *et al.* 2016; Gulmus and Gormez 2019). The addition of PMSF, EDTA, and $MgCl_2$ inhibits protease activity, as shown in Table 2. The indicator for classifying proteolytic enzymes is by examining the sensitivity of proteases to proteolytic inhibitors (Rao *et al.* 1998; Budiarto *et al.* 2016). The activity of the extracellular protease in the LAC3 sample is inhibited by the addition of PMSF (a serine protease-type inhibitor) and EDTA (a metalloprotease-type inhibitor). Therefore, the extracellular protease LAC3 has the potential to be classified as both a serine protease and a metalloprotease. EDTA inhibits protease activity by chelating the metal ions that serve as cofactors and play a role in maintaining stability, which is further enhanced by the addition of metal ions capable of influencing protease activity ($MgCl_2$ and $CaCl_2$). Metal ions affect substrate binding to the enzyme through various mechanisms (Aissaoui *et al.* 2017). Thompson *et al.* (2012) stated that 17% EDTA significantly inhibits the activity of endogenous matrix metalloproteinases.

Several types of alkaline proteases are also inhibited by PMSF. The PMSF inhibits serine residues on the active site of resulting protease enzymes, thereby inhibiting their activity. Admittedly, there is serine alkaline protease in extracellular proteases produced by these microorganisms (Ellaiah *et al.* 2002; Sharma *et al.* 2019).

The Ramachandran plot, ERRAT, and verify 3D are commonly used to assess the stereochemical quality of experimentally determined structures and predictive protein models by analyzing allowed and disallowed dihedral angles (Saravanan and Selvaraj 2017). The quality of protein structure is considered good if the Ramachandran plot has a score of > 90% for the favored area and < 2% for the disallowed area (Sobolev *et al.* 2020). Structures with high resolution generally have ERRAT values above 95% and a Verify 3D value of more than 80%. Better scores on ERRAT and Verify 3D, which are referred to as the "overall quality factor" for non-bonded atom interactions, indicate a higher level of quality (Messaoudi *et al.* 2013).

CONCLUSIONS

Extracellular protease was successfully produced from LAC3 with 20 h of culture incubation. Characterization confirms that the obtained extracellular protease acts as a serine protease and metalloprotease, as well as successfully applied for enzymatic hydrolysis toward sea cucumber meat and *lorjuk* shellfish samples, which produced hydrolysates with MWs of 15 and 4.6 kDa. The hydrolysate also exhibits DPPH inhibitory activities, which can be identified as antioxidant agents. The best 3D protein structure modeling was done using the AlphaFold web server.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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