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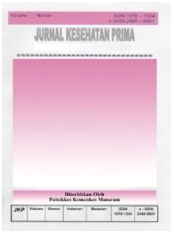

Potential of Crude Protease of *Bacillus Sp.* HSFI-9 ss Anticoagulant Agent and Meat Tenderizer

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Abstract
Some proteases act as thrombolytic enzyme to destroy the abnormal blood clot in the body called thrombus, a leading cause of mortality associated with cardiovascular disease (CVD). In addition, the enzyme may also hydrolyze meat's proteins into simple amino acids causing the meat to become tender. The study aim was to analyze the potential of crude protease *Bacillus sp*. HSFI-9 as anticoagulant agent and meat tenderizer. The anticoagulant test of crude protease HSFI-9 on venous blood samples was performed by measuring the clotting time compared to control (10% EDTA) by the Lee-White method. Protein profiles of beef, chicken and tuna were analyzed before and after immersion in the crude protease of *Bacillus sp*. HSFI-9 in concentration of 30% v/v for 3 hours based on Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) method. The anticoagulant test screening showed that the crude protease of *Bacillus sp.* HSFI-9 was able to prolong the blood clotting time even though, its anticoagulant activity was still less than that of 10% EDTA (a commercial anticoagulant agent used as positive control). The SDS-PAGE results indicated that soaked beef and chicken using crude protease *Bacillus sp.* HSFI-9 could hydrolyze the meats' proteins by denaturing them into smaller molecules indicated by the appearance of new minor protein bands and the disappearance of major ones. It can be concluded that crude protease of *Bacillus sp*. has potential as anticoagulant agent and meat tenderizer.

Keywords: Anticoagulant; *Bacillus sp*. HSFI-9; Bacterial Protease; Meat Tenderizer; Protein Profile

INTRODUCTION

Some proteases are able to destroy blood clots (fibrin) (Kumar, 2019). Fibrin clots can be one of the causes of CVD disease because they can block blood flow (thrombosis) from the heart to the brain acutely (Flora & Nayak, 2019; Netala et al., 2024). In 2019 CVD became the leading cause of death in the world, with a percentage of total deaths of 16%. In Indonesia, CVD is reported to be one of the three highest causes of death with 95.8 deaths per 100,000 population (Muharram et al., 2024; Vaduganathan et al., 2022). CVD can be prevented by treatment therapy to treat blood clots using thrombolytic drugs which include antiplatelet, anticoagulant, and thrombolytic (Alencar et al., 2021; Setiawan et al., 2016).

The enzyme industry has grown rapidly in the fields of food and health. The availability of protease enzymes in the world is still not sufficient, therefore it is necessary to look for other sources of protease enzymes. Proteases can be obtained from plants, animals and microorganisms. Microorganisms are the most potential producers of protease enzymes because they grow fast with cheaper substrates (Octariani et al., 2021; Permatasari, 2016).

The *Bacillus Sp*. HSFI-9 bacterial isolate obtained from the fermentation of the digestive organs of the sea cucumber (*Holothuria scabra*) has been

known to have proteolytic and thrombolytic activity (Ethica et al., 2023). HSFI-9 has the highest secondary metabolite activity among the others (Ethica et al., 2023).

Research on the use of enzymes as meat tenderizer with HSFI-9 has not been reported. furthermore, it is necessary to carry out further research on the ability of crude protease from HSFI-9 bacterial isolates as thrombotic agents by soaking meat to analyze the protein profile. Protease enzymes are enzymes that are able to hydrolyze protein into amino acids which in the food sector can be used as an alternative material for tenderizing meat (Megawati et al., 2021). Based on the research that has been done, tenderness is at the top of the smell and taste of meat (Hidayati et al., 2021). Boiling the meat if it is too long the nutritional content will be denatured or damaged, for this reason it is necessary to process it in a more effective and efficient way, one of which is the immersion method. Immersion can be done using crude or pure protease enzymes (Ha et al., 2013; Rohmah & Fickri, 2020).

In addition, it is also necessary to evaluate the ability of crude protease as a meat tenderizing agent, this ability can be seen from the protein profile of some meats that have been soaked with crude protease from these bacterial isolates and carried out using the SDS-PAGE (Sodium Dodecyl Sulfate Polyacrilamide Gel Electrophoresis) method.

METHOD

The design of this study was an experimental study with the object of study being crude protease isolated from the bacteria HSFI-9. Beef, chicken, tuna purchased in the Pedurungan market for. The data used in this study were primary data and the data obtained are tabulated and then presented in the form of a descriptive narrative. This research was conducted at the Universitas Muhammadiyah Semarang at Microbiology Laboratory, Molecular Biology Laboratory and Hematology Laboratory in January - May 2022.

The tools and materials used were: petri dish, test tube, incubator, electrophoresis chamber, micropipette, power supply, water bath, rotator, mortar cup, centrifiuge, incubator, a set of SDS-PAGE tools and a spectrophotometer. Beef, chicken, tuna fish, human blood, Nutrient Agar media (NA) (Oxoid) , skim milk, Brain heart infusion (BHI) media (Oxoid), Polyacrylamide 30%, Acrylamide and Bisacrylamide (29:1) (electrophoresis grade), TEMED, Ammonium perulfate (APS) 10%, Sodium Dodecyl Sulfate (SDS) 10%, 1.5 M Tris pH 8.8 and 6.8, Staining Coomassie Brilliant Blue, Destaining, Glacial acetic acid 10%, butanol, alcohol 70%, Running buffer 1x, Bovine serum albumin (BSA), Biorad protein assay (BPA), Phosphate buffer saline (PBS) pH 7.4, sterile H2O, buffer sample, protein marker.

1. Isolation of Crude Enzyme Protease

The manufacture of SMA media with the recipe of 7 g of NA is dissolved in 150 mL of aqudest and 10 g of skim milk is dissolved in 100 mL of aquadest. The bacteria HSFI-9 inoculated in Skim Milk Agar (SMA) media were incubated for 1x24 hours at a temperature of 37°C. Colonies grown in SMA media are bred in Skim Milk Broth (SMB) media (SMB media recipe: peptone 3 g/Lx, NaCl 3 g/L skim milk 30 g/L aquadest 300 mL). The suspension is taken supernatant as crude protease and measured absorbance (Jamaludin et al., 2019; Lemenh et al., 2021; Salam Khattab & Al-Nazzal, 2024).

2. Protein isolation (SDS PAGE method)

Samples of 3g mashed meat were treated with soaking crude protease for 3 hours. Samples of 3g of meat were soaked without treatment plus 1X PBS of 7 mL. Treatment samples and no treatment were then carried out meat protein isolation. The sample was centrifuge at a speed of 3000 rpm for 15 minutes, the supernatant was transferred to a new tube. 798 µL

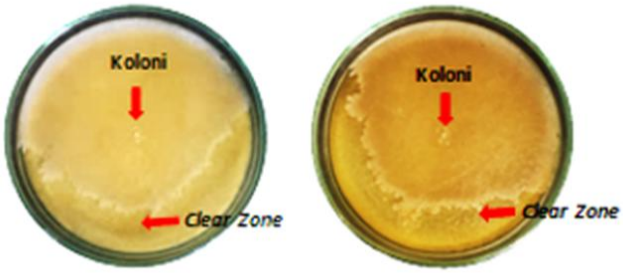
sample was pippetted plus 2 µL of dH2O, samples added with 200 µL BPA. The manufacture of 800 µL dH2O pippetted blanks coupled with 200 µL homogenized by vortex and incubation at room temperature for 15 min. Samples and blanks were read absorbance on a visible spectrophotometer with a wavelength of 595 nm. Samples were analyzed meat protein profiles by the SDS-PAGE method (Nowakowski et al., 2014).

3. Enzyme Anticoagulant Activity Test

Blood samples were treated with the addition of crude proteases of 100 µL, 200 µL, 500 µL and 1000 µL. Tube 1 (1 mL of blood), Tube 2 (1 mL of blood + 100 µL, 500 µL and 1000 µL) Tube 3 (1 mL of blood + 1 mL of EDTA) was then measured the length of blood clotting time using a stopwatch by the Lee and White method (Lailatul, et al., 2024; Rante, et al., 2024).

RESULT AND DISCUSSION

The rejuvenation of the bacteria HSFI-9 in SMA media was measured by the proeolytic activity of colonies and clear zones from day 1 to day 7 shown in Picture 1.



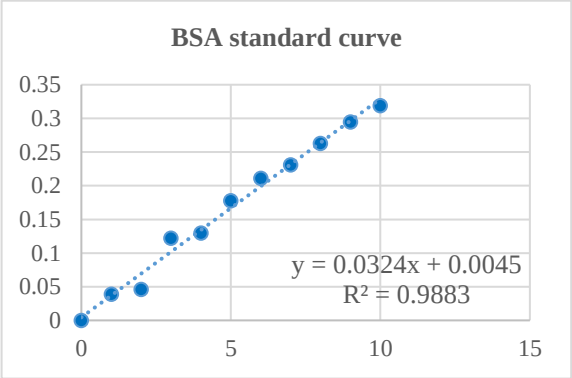
Picture 1. Proteolytic Activity Test On Skim Milk : without staining (A), with lugol staining (B)

Table 1. Proteolytic Index Test Results of Bacteria HSFI-9

Day	Clear Zone Diameter (mm)
1	1,04
2	1,08
3	1,10
4	1,13
Average	1,08

The addition of clear zones is calculated by subtracting the diameter of the clear zone from the diameter of the colony. The bacteria HSFI-9 were rejuvenated in SMB media that had been incubated 1x24 hours at an incubator with a temperature of 37°C shown in Picture 2 (Darmawati et al., 2015; Setiawan et al., 2016).

Crude protease of the bacteria HSFI-9 measured its absorbance using a spectrophotometer with a wavelength of 595 nm. Determination the concentration of crude protease of the bacteria HSFI-9 is calculated using the linear line equation y =0,0324x + 0,0045 (R^2= 0,9883).



Picture 2. BSA standard curve

Table 2. Concentration of Crude Protease HSFI-9

Tube	Absorbance	Consentration (µg/mL)
1	1,705	52
2	1,908	59
3	1,911	59
4	1,993	61
5	1,800	55
6	1,740	53
7	1,986	61

This study aims to determine the protein profile of beef, chicken and tuna soaked in HSFI-9 crude protease for 3 hours using the SDS-PAGE method. Based on the calculations, the protein concentration of the crude protease HSFI-9 soaked meat after being analyzed using spectrophotometry obtained a higher concentration value than the control meat. The addition

of crude protease HSFI-9 to meat will break the peptide bonds in beef and make the meat softer and can increase the protein content of the meat (Purwaningsih, 2017). Protease enzymes are able to degrade proteins or break peptide bonds into simpler protein molecules (amino acids) so as to produce soft meat in connective tissue fibers (Ha et al., 2013).

Determination of the molecular weight (BM) of proteins is carried out by calculating the Rf (Retardation factor) of each band (band) of proteins with the following formula (Darmawati et al., 2015).

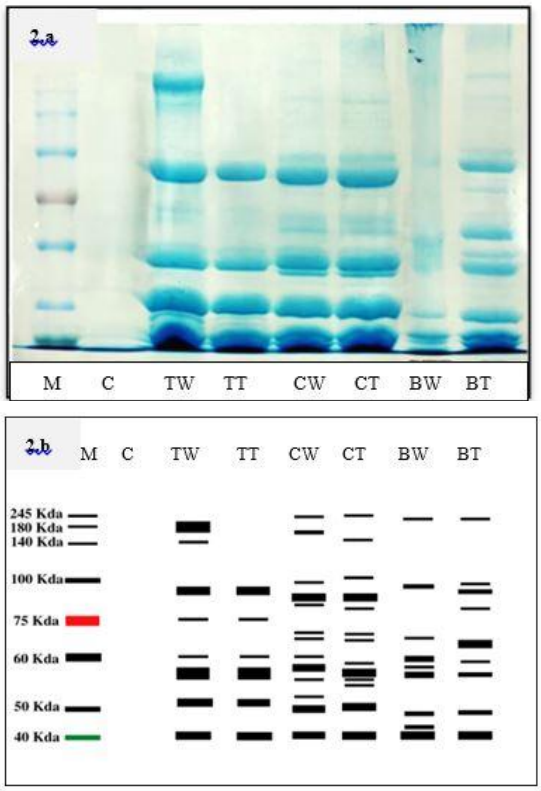
$$RF = \frac{Distance\ of\ movement\ of\ the\ beginning\ to\ the\ first\ band}{Length\ of\ the\ beginning\ to\ end\ band}$$

Table 3. Molecular Weight of samples

Code Sample	Protein Band	Molecular Weight (kDa)
Control (C)	-	-
Tuna Without Treatment (TW)	Mayor	173, 107, 58, 46, 36
	Minor	159, 86, 66
Tuna with treatment (TT)	Mayor	107, 58, 46, 36
	Minor	86, 66
Chicken without treatment (CW)	Mayor	102, 60, 44, 36
	Minor	189, 166, 117, 94, 79, 75, 66, 55, 48
Chicken with treatment (CT)	Mayor	102, 58, 44, 36
	Minor	189, 166, 117, 94, 79, 75, 66, 55,48
Beef without treatment (BW)	Mayor	112, 66, 58, 42, 37,36
	Minor	181, 75, 60
Beef with treatment (BT)	Mayor	107, 72, 58, 42, 36
	Minor	181, 112, 94, 63

From table 3, Crude protease HSFI-9 has neither major nor minor bands. This is because the crude protease has not been purified and has a low protein concentration. Soaking crude protease isolate HSFI-9 for 3 hours on cob meat changed the number of bands. Cob meat without treatment had 8 bands, while after soaking with crude bacterial protease HSFI-9 lost 2 bands, namely 1 major band and one minor band so that it only had 6 bands. Chicken meat with immersion experienced a decrease in molecular weight, the loss of bands with a molecular weight of 166 kDa and 60 kDa with the appearance of new bands with a molecular weight of 159 kDa and 58 kDa which indicated a change in the primary structure. Beef with soaking compared to control there is a decrease in the number of major bands and an increase in the number of minor bands.

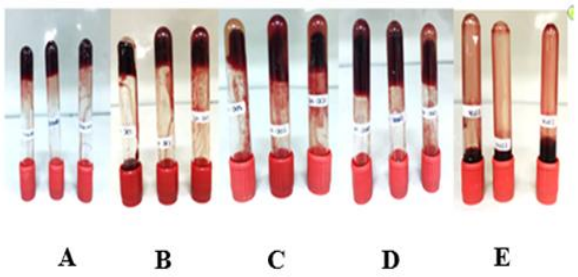
To know the Molecular Weight of the Sample, RF of known value is plotted on a logarithmic graph with a BM (Marker) of known value. Protein profile analysis was performed by the SDS-PAGE method on beef, chicken and tuna before and after soaking, showing the following results:



Picture 3. SDS-PAGE visualization of meat sample protein band representation (a.Raw, b. Chart)

Crude protease HSFI-9 was tested for anticoagulant activity by the Lee-White method.

Anticoagulants are alternative ingredients to prevent blood clots in vitro.



Picture 4. Control (A), Anticoagulant assay with crude protease 100 µL (B), crude protease 500 µL (C), crude protease 1000 µL (D). EDTA 100 µL (E)

The average results of the anticoagulant examination three times are shown in Table 4.

Table 4. Anticoagulant Test Results (Lee and White) with Crude Protease HSFI-9, normal control, and positive control

Tube code	Clotting time (minutes)
Crude 100 µL	10
Crude 500 µL	12,3
Crude 1000 µL	16,3
Normal control	7,8
Positive control	infinite

This study also aims to determine the ability of anticoagulant activity of crude protease HSFI-9 to inhibit blood clotting. anticoagulant test using the Lee-White method, the addition of 100 L of crude blood was able to prolong the clotting time of 10.00 minutes. Blood with the addition of 500 L crude was able to prolong the clotting time by 12.30 minutes. Blood with the addition of 1000 L crude was able to prolong the clotting time to 16.30 minutes. Normal blood clotting period in humans is between 5-10 minutes (Hidayati et al., 2021). Crude proteases do not appear protein bands because the protein concentration is due to the incubation period of the enzyme at the rejuvenation stage on the Skim Milk Broth media must be more than 1 x 24 hours so that the resulting concentration is small in the colorless band protein staining process and crude proteases need to be purified.

CONCLUSION

Based on the results of the study, it can be concluded that the Crude enzyme isolated from the HSFI-9 bacteria was able to soften beef, chicken and tuna that had been soaked for 3 hours. This was indicated by the formation of new bands on the gel samples of beef and chicken and was able to remove 2 bands on tuna meat. Crude protease isolates of HSFI-9 bacteria also has anticoagulant activity because it can prolong the clotting time beyond the normal value.

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