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Potential Application of HSFI-8 Crude Protease as Meat Tenderizer and Anticoagulant Agent

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ABSTRACT: Protease enzymes are enzymes that are able to hydrolyze peptide bonds in proteins into simple compounds. The protease enzyme used was derived from fermented sea cucumber digestive organs (HSFI-8). HSFI-8 uses meat tenderizer as well as an anticoagulant agent. The samples used were beef, chicken and tuna whose protein profile was analyzed using SDS-PAGE. Three types of meat were soaked in HSFI-8 at a concentration of 30% v/v for 3 h. In addition, protease enzymes as therapy for the treatment of CVD. The second sample, human blood, was tested for anticoagulant by Lee-White in vitro. Blood samples were treated with the addition of HSFI-8 as an anticoagulant which measured blood clotting time. The results showed that soaking beef using HSFI-8 was able to break the peptide bonds in proteins which were marked by the addition of minor protein bands. There was no change in the amount of protein bands in chicken and cob meat. The results of the anticoagulant test showed that HSFI-8 was able to prolong blood clotting time in samples given the addition of HSFI-8. Although HSFI-8 did not inhibit the clotting of the 10% EDTA anticoagulant, HSFI-8 was good as an anticoagulant.

KEYWORDS: Protease enzyme, Protein profile, Anticoagulant activity, SDS-PAGE, Lee-White method

I. INTRODUCTION

Proteolytic enzymes or proteases are enzymes that can accelerate the hydrolysis process of peptide bonds in proteins into oligopeptides and amino acids (Fazri et al., 2019). Proteases can be obtained from plants, animals or microorganisms. Microorganisms producing proteases are more potent than those from plants and animals. Microorganisms have fast growth and are able to grow on cheap media so that they are more profitable and potential. Several genera of bacteria that are known to produce proteases include Bacillus, Lactococcus, Streptomyces, and Pseudomonas (Said et al., 2012).

Advances in biotechnology use the use of protease enzymes as an alternative for the food industry, such as meat tenderizer, bread maker, cheese making, beer purifier, and protein hydrolysate (Mayasari, 2015). Peptide bonds in meat proteins can be hydrolyzed by protease enzymes because collagen fibers and muscle fibers are damaged (Kartika et al., 2019). The use of proteases in the non-food sector includes the pharmaceutical field as an anticoagulant therapeutic in overcoming thrombosis, anti-arthritis, antibacterial, and anti-inflammatory (Fuad, 2020). Protease enzymes have the ability to degrade fibrin which is formed during clotting or blood clotting so that it can be used as a therapeutic anticoagulant. Anticoagulant substances can overcome the problem of imbalance in the hemostatic system, which plays a role in regulating bleeding and blood clotting in the event of an injury such as in CVD (Cardio-vascular disease) (Akhtar et al., 2017).

Anticoagulant drugs currently used as treatment for CVD are warfarin and heparin (Al-Saadi, 2013).Based on clinical evidence showing that there are side effects from the use of heparin, such as gastrointestinal bleeding and the potential risk of viral contaminants of animal origin (Rahmawati et al., 2018).Anticoagulant activity test using the Lee-White method. The method is commonly used to determine the length of time it takes for blood to clot (Menantika et al., 2018).

Research conducted by (Hidayati, 2020) obtained the results of research on the fermentation of the digestive organs of sea cucumbers isolates HSFI-8 as a proteolytic bacterium that produces protease enzymes. evidenced by the formation of a clear zone on SMA (Skim Milk Agar) media with the IP (Proteolytic Index) value on the seventh day was 0.64 cm. HSFI-8 bacterial isolate had thrombolytic activity in vitro capable of lysing thrombus which was tested using the gravimetric method.

Research related to the use of protease enzymes as meat tenderizers and anticoagulants still needs to be done, to determine the ability of HSFI-8 protease-producing bacteria isolates as meat tenderizers and safe anticoagulants.

II. METHOD AND MATERIAL

The ingredients used are tenderloin beef, chicken breast and tuna, bacterial strain HSFI-8 isolated from fermented digestive organs of sea cucumbers, Nutrient Agar (Oxoid), skim milk (NZMP), Brain Heart Infusion Powder, Peptone (Oxoid), NaCl (Oxoid), PBS 1X Solution, dH_2O, Biorad Protein assay (BPA), SDS 10% (10 g SDS in 100 ml dH_2O), 10% APS (0.1 g APS in 1 ml dH_2O), Electrode buffer (tris base, glycine, SDS 10%, H_2O), stacking gel (dH_2O, Acrylamide 30%, 1.5M Tris (pH 6.8), SDS 10%, APS 10%, TEMED), Separating gel (Acrylamide 30%, 1.5M Tris (pH 8.8), 10% SDS, dH_2O, TEMED, APS 10%), loading buffer (1M tris or HCl (pH 6.8), DTT, bromophenol blue or BPB, glycerin, sterile distilled water). Materials for staining (Coomassie brilliant blue 0.25 % or 0.1% (R-250), methanol, glacial acetic acid, and aquadest) and materials for de-staining (methanol, aquadest, and glacial acetic acid), Ethanol 96%, EDTA 10%.

Bacterial isolates of HSFI-8 were rejuvenated on Skim Milk Agar (SMA) media, then inoculated in Skim Milk Broth (SMB) media in a centrifuge and the supernatant was taken as crude protease enzyme. Three kinds of meat were treated by immersing crude protease for 3 h, then isolated the meat protein and analyzed the protein profile of the meat using the SDS-PAGE method. Blood samples were treated with the addition of 100 l, 500 l and 1000 l of crude protease and measured the length of time for blood clotting using a stopwatch using the Lee-White method.

III. HSFI

-8 bacterial isolates on SMA medium were measured proteolytic activity by measuring the diameter of the clear zone and the diameter of the colony from day 1 to day 4 shown in Figure 1.

Figure 1. Test of HSFI-8 proteolytic activity on Skim Milk Agar media

Proteolytic index calculated by comparison between the diameter of the clear zone and the diameter of the bacterial growth zone (Efendi et al., 2017).

Table 1. Results of HSFI-8 proteolytic index test on skim milk agar media

Day	Diameter (mm)		
	Proteolytic zone	Colony	IP
1	17	19	1.12
2	25	29	1.16
3	35	42	1.20
4	36	48	1.33
AVG IP	1.20		

HSFI-8 bacterial isolate was inoculated on SMB media and then incubated for 48 h at 37°C. The presence of bacterial growth marked by turbidity is shown in Figure 2.

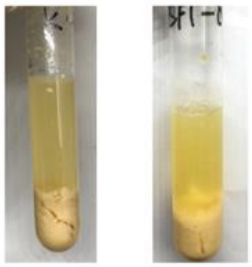

Figure 2. Bacterial inoculation on Skim Milk Broth (SMB) media. Control (1), sample suspension (2)

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HSFI-8 measured its absorbance using a spectrophotometer with a wavelength of 600 nm, to determine the concentration of crude protease HSFI-8 calculated using the linear equation y=ax+b.

Information:

y = Absorbance a = Constant

x = Concentration of crude protease b = Slope

Analysis of the total protein of beef, chicken and tuna after immersing HSFI-8 for 3 h using a spectrophotometer with a wavelength of 595 nm.

Table 2. Total meat protein

Type of Meat	Total protein (µg/ L)	
	Control	Treated
Crude protease HSFI-8	0.98	
Cattle	18.45	25.13
Chicken	17.28	25.04
Tuna	27.09	27.25

According to (Kustia , 2017) determination of protein molecular weight by calculating the Rf (Retardation Factor) of each band with the following formula.

$$Rf = \frac{Jarak\ yang\ ditempuh\ sampel}{Jarak\ yang\ ditempuh\ oleh\ pelarut}$$

Table 3. Molecular weight of beef, chicken, cob

Sample code	Type of Band	MW (kDa)
C8	-	-
STP	Major	114, 73, 59, 46, 44, 39, 32, 29
	Minor	64
ATP	Major	101, 61, 50, 41, 34, 32
	Minor	59, 30
TTP	Major	97, 59, 50, 34, 33, 29, 28
	Minor	79, 44
SP	Major	110, 73, 59, 44, 39, 33, 30, 28
	Minor	61
AP	Major	97, 93, 59, 46, 34, 32, 29
	Minor	54, 28
TP	Major	97, 59, 48, 34, 32, 29, 28
	Minor	79, 42

Note:

C = Crude protease HSFI-8

STP = Cattle without treatment

ATP = Chicken without

TTP = Cob without

SP = Cattle with

AP = Chicken with

TP = Cob treatment

(-) = No molecular

Determination of Molecular Weight (MW) using a known Rf value, plotted on a logarithmic graph with Molecular weight of Marker which value is known. Analysis of protein profiles using the SDS-PAGE method on samples of beef, chicken and tuna soaked in crude HSFI-8 showed the following results:

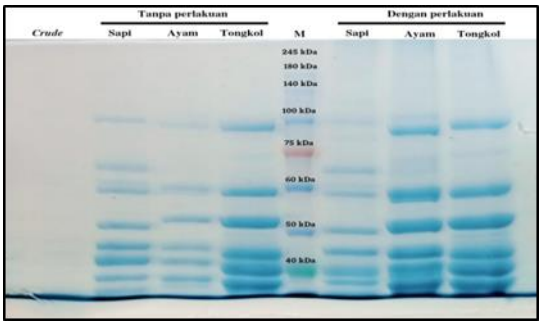

Figure 3. SDS-PAGE results for beef, chicken and milkfish without and with protease immersion treatment

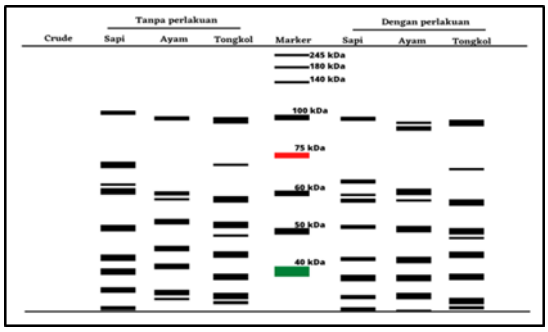

Figure 4. The results of the visualization of SDS-PAGE

Crude protease HSFI-8 were tested for anticoagulant activity using the Lee-White method. Anticoagulants are alternative materials to inhibit blood clotting in vitro (Weliyani et al., 2015).

1 2 3 4 5 6 7

Figure 5. Anticoagulant Test Results

Description:

Tube 1 = Untreated Blood
Tube 2 = Blood plus 96% Ethanol
Tube 3 = Blood plus 10% EDTA
Tube 4 = Blood plus *crude* 100 L
Tube 5 = Blood added *crude* 500 L
Tube 7 = Blood plus *crude* 1000 L
Tube 7 = Blood plus *crude* 100 L
10% EDTA

The average results of the anticoagulant test were repeated five times as shown in table 5.
of anticoagulant test

Sampel code (Tube)	100 µL (mins)	500 µL (mins)	1000 µL (mins)
1	06.04	05.26	06.06
2	06.32	06.18	06.38
3	∞	∞	∞
4	10.58	13.32	17.18
5	∞	∞	∞

Note =
Tube 1 = Blood without Treatment
Tube 2 = blood with 96% ethanol added.
Tube 3 = blood with 10% EDTA added;
Tube 4 = Blood added *Crude* protease HSFI-8
Tube 5 = Blood added with 10% EDTA and *crude* protease HFSI-8
∞ = Infinite

IV. DISCUSSION

Protease enzymes are enzymes that have the ability to hydrolyze or destroy protein biomolecules into smaller and simpler compounds. This study aims to determine the protein profile of beef, chicken and tuna soaked in *crude* protease for 3 h using the SDS-PAGE method. Based on the calculations, the protein concentration of *crude* protease-soaked meat after being analyzed using spectrophotometry obtained a higher concentration value than the control meat. This is due to the protein content in the *crude* protease HSFI-8 which seeps into beef, chicken and tuna, thereby increasing the protein concentration after treatment (Astuti *et al*, 2012).

The mechanism of action of protease enzymes is by breaking protein molecules through protein hydrolysis activity or protein denaturation. The protease enzyme first works by destroying the mucopolysaccharide layer, the matrix of the basic protein substance, then goes to the connective tissue fibers on the inside. During this process, the collagen and myofibril network will be damaged and result in breaking the bonds between the fibers in the meat and the process of breaking the fiber fragments into shorter ones, causing the meat texture to become softer (Lismawati *et al*., 2017).

The results showed that *Crude* protease HSFI-8 did not have major and minor bands (shown in Table 3) due to the impure protease enzyme and known to have low protein concentrations so that the protein bands on SDS PAGE were not visible. Beef with *crude* protease soaking for 3 h did not show any change in the protein profile compared to the control. Chicken meat with *crude* protease soaking for 3 h showed the presence of a new major band with a molecular weight of 93 kDa with thicker characteristics than chicken meat without treatment. This difference was due to the different molecular weights of the proteins inserted into the polyacrylamide gel wells. The protein weight of beef with soaking treatment showed higher protein weight than control chicken meat, while the cob meat with soaking for 3 h did not show any change in protein profile when compared to the control.

In addition to analysis as a meat tenderizer, this study also aims to determine the ability of the anticoagulant activity of *crude* protease HSFI-8 to inhibit blood clotting. anticoagulant test using the Lee-White method, the addition of *crude* able to prolong the clotting time by 10.58 mins. Blood with the addition of *crude* was able to prolong the clotting time by 10.58 mins. Blood with the addition of *crude* was able to prolong the clotting time of 17.18 mins. Normal blood clotting period in humans is between 5-10 mins (Menantika *et al*., 2018).

V. CONCLUSIONS

Crude protease HSFI-8 cannot be used as a meat tenderizing agent, but has activity in inhibiting the blood clotting process because it is able to prolong the blood clotting period beyond the normal value so that it has potential as anticoagulant agent. Further anticoagulant testing as well as enzyme purification need to be carried out in order to produce better proteolytic and anticoagulant activities.

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