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Research Paper

Characteristics and Substrate Specificity of Semi-Purified Bacterial Protease of *Bacillus thuringiensis* HSFI-12 with Potential as Antithrombotic Agent

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Abstract
Commercial proteases, such as Nattokinase (NK), Staphylokinase (SAK), and Streptokinase (SK) play an important role in the destruction of thrombus, the main cause of death in cardiovascular disease. The latest technology combining enzymes with certain drugs is the target of new research in the thrombolytic area. The first step is to develop protease from *Bacillus thuringiensis* HSFI-12 bacteria as an antithrombotic agent, characterization of the bacterial enzyme is necessary. This study aims to determine the specificity of protease from *Bacillus thuringiensis* HSFI-12 to explore its potential as an antithrombotic agent in terms of anticoagulant and fibrinolytic activities. The molecular weight and specificity of bacterial protease were determined with a zymographic method with casein as substrate. *Bacillus thuringiensis* HSFI-12 was first cultured on Nutrient Agar (NA) media and then on Skim Milk Agar (SMA) media. The obtained crude protease from Skim Milk Broth (SMB) was then concentrated as dialysate. Both crude and dialysate proteases were tested for their specific activity, as well as anticoagulant and fibrinolytic activities. Next, the dialysate's molecular weight and specificity on the casein substrate were investigated using the zymographic method. As result, protease activity in crude form is lower than that in dialysate, which was 0.5570 ± 0,004 U/mL and 2.1767 ± 0,005 U/mL, respectively. The molecular weight of the obtained bacterial protease was between 117 – 133 kDa and the enzyme is capable of degrading casein as shown on the zymogram. Overall, both crude and dialysate proteases of *Bacillus thuringiensis* HSFI-12 show potential as an antithrombotic agent for exhibiting anticoagulant and antiplatelet activities. Yet, it could not exhibit direct fibrinolytic activity implying the possibility that the enzyme plays a role as a plasminogen activator, which can dissolve fibrin by activating plasmin.

Keywords
Anticoagulant, *Bacillus thuringiensis* HSFI-12, Enzyme Purification, Protease, Zymography

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1. INTRODUCTION

Cardiovascular disease (CVD) is a major cause of global death and a contributor to the decreased quality of life (Mensah et al., 2019). In 2017, CVD caused about 17.8 million deaths worldwide, of which in Europe more than 3.9 million deaths per year or equivalent to 45% of deaths that occurred (Wilkins et al., 2017; Mensah et al., 2019). CVD is caused by several factors, one of which is acute myocardial infarction (Mensah et al., 2019), occurring when coronary blood flow decreases suddenly after thrombus occlusion of an atherosclerotic plaque (Satoto, 2014). Thrombus is a clot that forms when blood turns from a liquid to a solid. Thrombus is an intravascular aggregate of fibrins and platelets (with sometimes the presence of white and red blood cells), usually in stacked layers, attached to the walls of blood vessels or valves (Michaud, 2019). Thrombus formation is the result of two interdependent mechanisms; platelets and protein clotting factors (Sperling et al., 2018). Platelets, together with endothelial cells and circulating coagulation proteins (including plasma proteins), are crucial mediators of vascular hemostasis and thrombosis (Koupenova et al., 2017; Talens et al., 2019).

To overcome platelet and fibrin aggregates, there will be activation of the fibrinolytic system where plasminogen is activated into the main active product, namely plasmin. Plasmin is a protease, which will work to destroy fibrin and blood clots. Plasmin itself is a protease binding to lysine residues in fibrin and then cleaves fibrin at the binding site (Parry and Squire, 2017).

Proteases are a large group of enzymes with six different classes including Serine, Glutamic Cysteine, Aspartic, Threonine, and Metalloproteases. These enzymes are described

as molecular scissors required in various cellular physiological processes such as hemostasis, blood coagulation cascade, and fibrinolysis (Philipps-Wiemann, 2018). Since they are also involved in broad pathophysiological conditions, their therapeutic applications become wide (Razzaq et al., 2019).

Proteases play an important role in the destruction of thrombus. So, the absence of enzymes can cause the balance of the homeostatic process to be disturbed. The latest technology combining enzymes with certain drugs is the target of new research (Purwani, 2018). Thrombosis therapy currently available includes anticoagulants and thrombolysis. Anticoagulants are given to prevent coagulation of the aggregated platelets. Thrombolysis is used to lyse thrombus that has formed through a fibrin clot dissolution process called fibrinolysis or thrombolysis (Wang et al., 2017).

Antithrombotic drugs are agents intended to degrade thrombus, which can be grouped into three different types based on their different actions. The first is an antiplatelet drug, which limits the migration or aggregation of platelets. The second is an anticoagulant drug, which limits the ability of the blood to clot. The third is a thrombolytic drug (including fibrinolytic drugs), which acts to dissolve clots after they have formed (Li et al., 2020).

Among bacterial strains producing thrombolytic proteases previously isolated from the intestine of sea cucumber *Holothuria scabra* is *Bacillus thuringiensis* HSFI-12. The in vitro thrombolytic activity of *Bacillus thuringiensis* HSFI-12 was found to be competitive when compared to commercial protease Nattokinase, known as a standard antithrombosis agent (Zafrida et al., 2022, Dewi et al., 2022). However, its potential to be an antithrombotic agent needs confirmation and retesting. Therefore, the antithrombotic activity testing included testing of anticoagulant activity using two procedures, namely the clotting time of the Lee and White method as well as the Eustrek method by Giemsa staining is important. Furthermore, testing of fibrinolytic activity and characterization of the bacterial protease by looking at molecular weight and specificity for certain substrates using the zymography method is required (Hidayati et al., 2021a).

2. EXPERIMENTAL SECTION

2.1 Materials

The materials used were: HSFI-12 bacterial culture, previously isolated from sea cucumber Rusip by Hidayati et al. (2021b), Nutrien Agar media (Oxoid, UK), Skim Milk Agar media (Oxoid, UK), Skim Milk Broth media, NaCl (Merck, Germany), blood, anticoagulant EDTA, sterile distilled water, methylene blue (Himedia, India), ddH$_2$O, Sodium Dodecyl Sulfate (Himedia, India), Tris-HCl 0.5 M pH 6.8, Tris-HCl 1M pH 8.8, acrylamide (Merck, Germany), 10% APS (Merck, Germany), 10% glycerol (Merck, Germany), TEMED (Merck, Germany), 200 kDa marker protein (Bio-Rad, California), casein substrate (Sigma Aldrich, USA), Coomasive blue (Merck, Germany).

2.2 Methods

2.2.1 Subculture of *Bacillus thuringiensis* HSFI-12

Refreshment of bacterial isolates was carried out by growing a loopful of HSFI-12 bacterial colony on Nutrien Agar (NA) growth media. The media was incubated in an incubator (Memmert, Germany), at 37°C for 24 h (Birolli et al., 2021). The growing colonies were then observed under an optical microscope (Olympus, Japan) for their uniform cell morphology (Purwaningrum et al., 2021).

2.2.2 Proteolytic Activity Assay of Protease of *Bacillus thuringiensis* HSFI-12

A proteolytic activity test was carried out by growing a loopful of HSFI-12 isolate on Skim Milk Agar (SMA) growth media. The media was incubated at 37°C for 24 h, then observed for the formation of a clear zone around the growth of the colony (Wardani and Nindita, 2013).

2.2.3 Fibrinolytic Activity Assay of Protease of *Bacillus thuringiensis* HSFI-12

On the Fibrin Agar (FA) medium, 4 wells were made and 100 l mL of sterile distilled water was added to each well, Nattokinase standard, crude, and dialysate respectively. The fibrin media was incubated at 37°C for 24 h (Hidayati et al., 2021c). Fibrinolytic activity was indicated by the presence of a clear zone around the well and was determined by measuring the fibrinolytic enzyme activity index by calculating the diameter of the clear zone divided by the diameter of the colony (Archna et al., 2015).

2.2.4 Anticoagulant Activity Assay of Protease from *Bacillus thuringiensis* HSFI-12

Anticoagulant activity test in vitro was carried out by two methods, clotting time observation, and microscopic Eustrek methods. Five clean test tubes with a diameter of 7-8 mm were prepared, labeled from numbers 1-5, and placed in a tube rack. An mL of blood was put into each of the test tubes, then 100 μL of EDTA (10%) was added to tube 1 as a positive control, and bacterial protease was put into tubes 3-5 as samples. No EDTA was added to tube 2 as a negative control. A hundred mL of crude enzyme/dialysate was added to it. in tube 3,300 μL into tube 4,500 μL into tube 5 using a micropipette and mixed using a vortex. When mixing samples and enzymes using a vortex (Thermolyne, USA), a stopwatch was run to determine the time of freezing that occurs. A total of 20 μL of the sample was smeared on a slide, then stained with Giemsa stain and observed under a microscope (Olympus, Japan) at 400x magnification. All of these steps were then repeated as duplicates.

2.2.5 Partial Purification of Bacterial Protease

The crude protease was concentrated using the salting out method with 70% ammonium sulfate which was left overnight at 4°C. The concentrated enzyme precipitate was separated by centrifugation (EDLAB Clinical, Indonesia) at 15,000 rpm for

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10 mins. (Zilda et al., 2014). The precipitate obtained was then dialyzed by placing it in a cellophane bag, then soaking it in 0.05 and 0.025 M phosphate buffers at pH 7 and stirring using a stirrer at 4°C for one night. Dialysis was stopped when all of the ammonium sulfate salt had been removed from the membrane by testing it with BaCl and HCl solutions; A few drops of 0.1 M $BaCl_2$ and 0.1 M HCl were added to the buffer solution outside the cellophane bag. Sulfate ions (SO_4^{2-}) will form a white precipitate of $BaSO_4$ (Wardani and Nindita, 2013).

2.2.6 Specific Activity Test of Crude and Dialysate Protease from *Bacillus thuringiensis* HSFI-12

Specific activities of bacterial protease in the form of crude extract and dialysate were measured based on the method of Bergmeyer and Grab (1984) with modifications. Crude extract and dialysate samples were prepared by mixing 250 mL of 0.05 M citrate buffer at pH 6, 250 mL of 2% casein, and 50 mL of enzyme (crude or dialysate) solution, then incubated at 60°C for 10 mins. A total of 500 mL of 10% TCA and 50 mL of distilled water were added to each of the samples, then incubated again at 60°C for 10 mins. The mixtures were then centrifuged at 4°C at 10,000 rpm for 5 mins. The supernatant was separated, and 300 mL of the supernatant was reacted with 1 mL of 0.5 M sodium carbonate and 200 mL of Folin's reagent, then incubated again at 60°C for 20 mins. The absorbance was measured at λ = 578 nm (Zilda et al., 2014). The protease activity of the crude enzyme and the dialyzed enzyme was calculated using this equation:

$$Y = 0.7724x + 0.0000 \quad (1)$$

Note: Y = absorbance; X = activity (in U/mL)

2.2.7 Determination of Molecular Weight and Specificity of Protease by Zymography

Determination of molecular weight, confirmation of active protein bands that have protease activity, and substrate specificity were determined using the zymographic method. In determining the molecular weight and specificity of the enzyme to the casein substrate, polyacrylamide gel was used. In the 1st well, 5 μL of protein marker was filled, and the 2nd and 3rd wells were filled with 10 μL of sample and buffer with a ratio of 1:4. Prepared enzyme samples and protein markers were inserted into the comb hole (well) as much as 20 μl/well. Protein separation by zymography was carried out by electrophoresis (Bio-Rad, USA) with an electric current of 100 V. The zymogram gel was observed using Coomassie blue after being renatured in Triton X-100 by shaking for 1 h and incubated (digestion) using 0.05 M phosphate buffer pH 8 at 60°C overnight. After the staining process, the gel was immersed in a de-staining solution containing 50% methanol, 10% glacial acetic acid, and distilled water (Agrebi et al., 2009). The protease band appears as a clear zone surrounded by a dark blue color on the gel (Zilda et al., 2014).

3. RESULT AND DISCUSSION

Our works were grouped into 3 purposes. The first is to determine the potential of the protease from *Bacillus thuringiensis* HSFI-12 as an antithrombotic agent in terms of its anticoagulant and fibrinolytic activity. The second is to determine the molecular weight of the bacterial protease by zymography. The third is to determine the specificity of the bacterial enzyme against casein and fibrin substrates based on the zymographic method. For all of these objectives, refreshed colonies of *Bacillus thuringiensis* HSFI-12 were needed (Purwaningrum et al., 2021). Refreshment by subculturing on NA media was done and the bacterial pure colonies were isolated. The uniform morphology of bacterial cells was confirmed after Gram-staining as displayed in Figure 1.

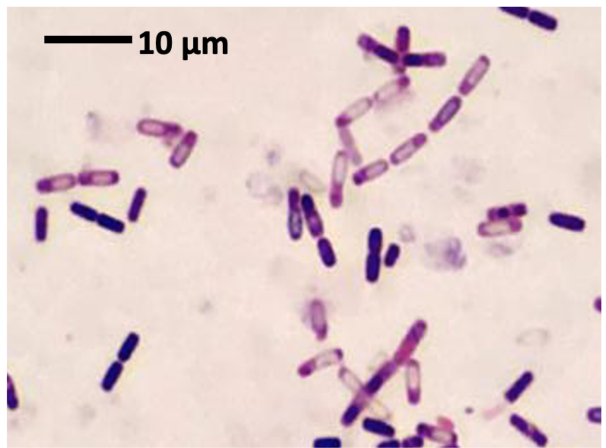

Figure 1. Bacterial Cell morphology *Bacillus thuringiensis* HSFI-12 Observed under Optic Microscope after Gram Staining. It can be seen that the bacterial cells are gram-positive and have endospores.

3.1 Qualitative Proteolytic Activity Assay of Protease from *Bacillus thuringiensis* HSFI-12

Testing the ability of *Bacillus thuringiensis* HSFI-12 was carried out at 37°C based on the assessment of the clear zone formed during the preliminary test. The clear zone which was incubated at 37°C showed a wide area, so it was judged that the bacteria could work optimally in producing protease enzymes at that temperature. The use of 37°C temperature was also reported by Birolli et al. (2021).

Observations for proteolytic activity tests were made for each of the 7 days. Various diameter sizes around the colony for daily measurements were obtained (Table 1). The variety in diameter indicates the different performance of bacteria to produce extracellular proteases capable of degrading casein contained in SMA media by day. Data in Table 1 showed that on day 7, *Bacillus thuringiensis* HSFI-12 produced the largest clear zone and colony diameters.

The proteolytic index curve (Figure 2) shows *Bacillus thuri-*

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Table 1. Representative and the Average Values Hydrolysis Capacity Index of *Bacillus thuringiensis* HSFI-12 for 7 Days on Skim Milk Agar (SMA) Media. Measurements Were Repeated in Triplicates

Day	Appearance	Hydrolysis Capacity Index
1		2.57 ± 0.03
2		2.00 ± 0.07
3		1.65 ± 0.04
4		1.48 ± 0.05
5		1.40 ± 0.04
6		1.39 ± 0.07
7		1.40 ± 0.10

ngiensis HSFI-12 has the highest Hydrolysis Capacity Index (HCI) value at 24-h of incubation, where the index value began to decrease after 48 h, as displayed in Figure 2. This is likely due to the decreased ability of bacteria to hydrolyze casein substrates as the bacteria get older, resulting in a reduction in metabolism speed.

Bacillus thuringiensis HSFI-12 appeared to have unstable protease activity by day, with activity protease being highest at 72-h of incubation and began to decrease at 96-h of incubation. This can be improved by optimizing the optimum condition of the medium, pH and temperature. The proteolytic activity of a bacterium has a correlation with the formation of the virulence factor of the bacterium (Pallett et al., 2019).

3.2 Crude Protease Production and Partial Purification

Separately, colonies growing on NA separately were also planted on Minimum Synthetic Skim Milk Broth (MSMB) media.

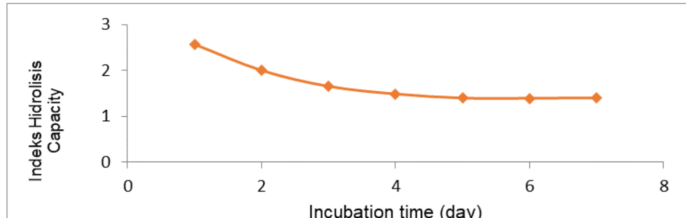

Figure 2. The Proteolytic Index Curve Showing the Average of Hydrolysis Capacity Index (HCI) Values of *Bacillus thuringiensis* HSFI-12 by Day from Day-1 to Day-7. Tests were done in duplicates

Crude protease was obtained as supernatant after centrifugation of MSMB bacterial culture at 10,000 rpm (Figure 3).

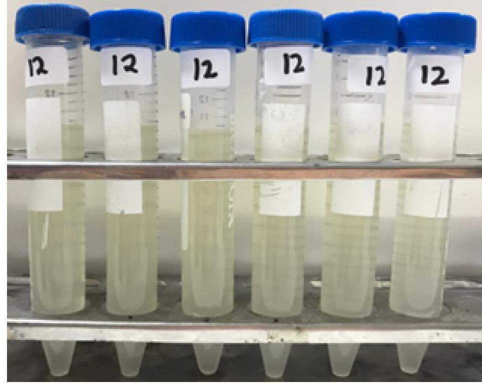

Figure 3. The Extracellular Crude Protease of *Bacillus thuringiensis* HSFI-12 Obtained from the Production Medium was Centrifuged at 10,000 rpm.

3.3 Fibrinolytic Activity Test of Protease from *Bacillus thuringiensis* HSFI-12

Results of the fibrinolytic activity test on protease secreted by *Bacillus thuringiensis* HSFI-12 showed that a clear zone was only formed in wells filled with Nattokinase as a positive control (data not shown). This indicated that the bacterium had no specific activity on fibrin substrates. This presumably shows that the HSFI-12 strain was able to utilize proteins other than fibrin or non-fibrinolytic. This means, in terms of fibrinolysis action, protease of *Bacillus thuringiensis* HSFI-12 most likely acts as PA (plasminogen activator), which indirectly cause fibrin degradation. The bacterial protease can still be grouped into thrombolytic protease although it is a non-fibrinolytic one. Fibrinolytic enzymes refer to those that directly use fibrins as a degradation substrate (Akhtar et al., 2017).

3.4 Partial Purification of Protease

In this study, partial purification of the enzyme was carried out in two steps. The first is protein precipitation using ammonium salt. Protein precipitation can be done by adding salt which will later remove the remaining impurities in the precipitation by

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dialysis to obtain semi-pure enzymes. Precipitation of crude extract (crude enzyme) was done by adding ammonium sulfate into the supernatant that has been obtained. Precipitation was carried out using ammonium sulfate salt with a saturation of 70% (Koshy and De, 2019).

The enzyme sample which has been added with ammonium sulfate was left overnight at 4°C. The concentrated enzyme precipitate was separated by centrifugation at 15,000 rpm for 10 minutes. The precipitate formed after centrifugation was dissolved in a small amount of 0.05 M phosphate buffer solution pH 7.0. A buffer solution with this pH was used because at that pH, the enzyme seemed more stable than other pHs (Zilda et al., 2014).

3.5 The Specific Activity of Crude and Dialysate Protease from *Bacillus thuringiensis* HSFI-12

The protease activity was measured by the Bergmeyer method (Zafrida et al., 2022). The principle of measuring protease activity using the Bergmeyer method is the hydrolysis of the substrate by proteases into amino acids and peptides. The substrate used was casein. Casein is a milk protein consisting of a phosphoprotein that binds to calcium to form a calcium salt called calcium calcinate. Amino acids produced from the hydrolysis of casein by proteases are separated from proteins that have not been hydrolyzed using trichloroacetate acid (TCA). Amino acids and peptides will be dissolved with TCA, while proteins that have a large molecular weight will precipitate (Zilda et al., 2014).

Based on the linear tyrosine equation obtained from the standard curve made following the previous method at $\lambda = 660$ nm, the protease activity of the crude enzyme and the dialyzed enzyme can be calculated using Equation 1.

The data generated from the proteolytic activity of crude enzymes and dialysates obtained from the bacterium *Bacillus thuringiensis* HSFI-12 measured at $\lambda = 660$ nm by spectrophotometer is shown in Table 3. As seen in Table 3, crude protease produced by *Bacillus thuringiensis* HSFI-12 showed quantitatively much lower activity (0.5570 U/mL) than its dialysate (2,1767 U/mL). The higher activity of protease dialysate showed that the partial purification process through ammonium salt precipitation and dialysis performed could remove impurity components or other proteins from the enzyme (Zhu et al., 2021). So, when reacted with casein as a substrate and TCA, only protease enzymes will hydrolyze amino acids and peptides. This may result in an increase in absorbance value and thus improve the measured activity.

3.6 Anticoagulant Activity Test of Protease from *Bacillus thuringiensis* HSFI-12

Anticoagulant activity assay was carried out by comparing clotting times of the positive control of blood with 10% EDTA solution, negative control of blood with no treatment (as an artificial thrombus), and samples of blood with treatments of crude and dialysate protease added in various volume variations. The use of this negative control as a thrombus model was previously reported by Hidayati et al. (2021a) and Fuad et al. (2021) Visual appearances of anticoagulation performance of both crude and dialysate proteases along with controls were displayed in Figure 4. The measured blood clotting time (CT) is displayed in Table 2.

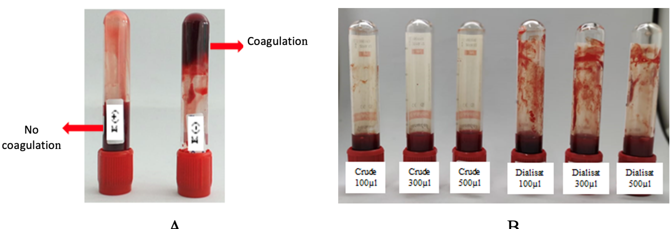

Figure 4. Representative Visual Observation Results of Anticoagulation Test (A). Control (+) Containing Blood with EDTA 10% Treatment (left) and Control (-) Containing Blood without Treatment (B). Samples containing blood treated with crude and dialysate protease of *Bacillus thuringiensis* HSFI-12 in varied volume of 100, 300, and 500 μL. The measurements were performed in 5 times.

As seen in Figure 4, all blood samples treated with varied volumes of both crude and dialysate proteases showed no coagulation activities, and so did the positive control. Only untreated blood in the negative control tube showed coagulation. These results showed that qualitatively, both crude and dialysate proteases had anticoagulation activities. The differences in results however were found on the calculated CT.

Anticoagulant activity of protease obtained from *Bacillus thuringiensis* HSFI-12 was quantitatively demonstrated by its ability to extend CT values compared to negative and positive controls. Data in Table 3 showed that treatment involving the addition of crude protease in the test blood can extend CT up to 10-16 mins, while treatment with the addition of dialysate protease can extend CT up to 12-21 mins. Overall, the dialysate performs better than crude protease in terms of preventing the blood to coagulate. The best anticoagulation activity of all belongs to EDTA 10%. However, there is still a possibility that by further purification or increased volume, dialysate protease of strain HSFI-12 could have much better anticoagulation activities.

Figure 5 and Figure 6 show the results of blood coagulation tests, which were conducted as microscopic observations aiming to see the state of the blood cells after bacterial protease treatment in varied quantity. As seen in both figures, all blood samples treated with both crude and dialysate protease show that the erythrocytes did not crenate. In all treated samples, there was no platelet aggregation and the erythrocytes were evenly distributed. On the contrary, untreated samples show erythrocyte crenation as well as platelet aggregation. This shows the potential of the bacterial protease to be used as a reagent supporting blood morphology tests, regardless of whether in the form of crude extract or as dialysate.

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Table 2. The Protease Activity of Crude Enzymes and Dialysates Extracted from *Bacillus thuringiensis* HSFI-12 with Tyrosine as Standard. Tests were repeated in 5×.

Protease Sample	Absorbance at λ = 660 nm			
	Active Sample	Inactive Sample	Total (Y)	Enzyme Concentration (x) U/ml
Crude	1.407 ± 0.006	0.943 ± 0.002	0.464 ± 0.004	0.5570 ± 0.004
Dialisate	2.220 ± 0.007	0.505 ± 0.003	1.715 ± 0.005	2.1767 ± 0.005

Table 3. The Test Results of Crude Anticoagulant Activity and Protease Dialysate from *Bacillus thuringiensis* HSFI-12 based on the Lee-White Method

Enzyme Concentration (μL)	Lee-White Anticoagulant Activity Time (mins) *			
	Control (-)	Control (+)/ EDTA	Crude Protease	Dialysate Protease
100	NA	NA	10 ± 0.24	12 ± 0.26
300	NA	NA	14 ± 0.37	17 ± 0.12
500	NA	NA	16 ± 0.32	21 ± 0.27

*) Note: Control (-) = negative control (without enzyme treatment); control (+) = positive control (only EDTA 10%); all tests were repeated in 5×. NA: Not Applicable.

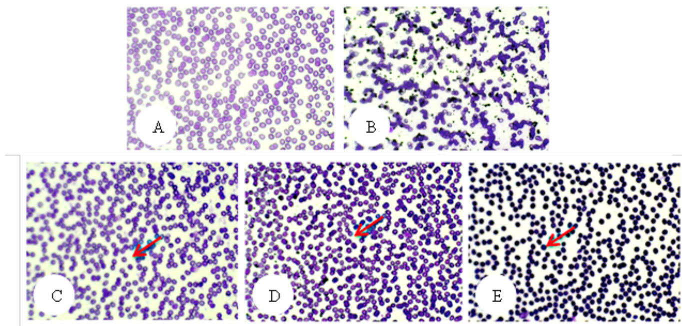

Figure 5. The Results of Blood Coagulation were Observed Microscopically to See the State of theBblood Cells (A), Positive Control (EDTA 10%) (B), Negative Control (no treatment). In smear preparations with the addition of crude protease of *Bacillus thuringiensis* HSFI-12 in varied quantity of 100μl (C), 300μl (D), and 500μl (E), the erythrocytes did not crenate, there was no platelet aggregation and the erythrocytes were evenly distributed.

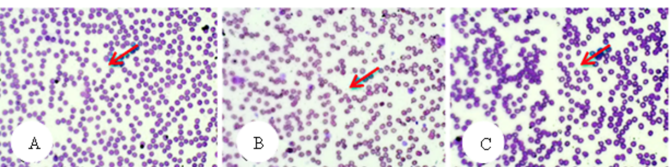

Figure 6. The Results of Blood Coagulation were Observed Microscopically to See the State of Blood Cells. In smear preparations treated with the addition of dialysate as much as 100μl (A), 300μl (B), and 500μl (C), the erythrocytes did not crenate, there was no platelet aggregation and the erythrocytes were evenly distributed.

3.7 Determination of Molecular Weight and Specificity of Protease by Zymography

Determination of molecular weight, confirmation of active protein bands that have protease activity, and substrate specificity were determined using the zymographic method. The dialysis enzyme (dialysate) which will be characterized using zymography is first mixed with the sample buffer at a ratio of 1:4.

As displayed in Figure 7, the presence of protease on the zymogram can be seen as the clear zone showing that casein as the substrate is degraded by the enzyme. It could also be seen from the figure that the molecular weight of the obtained bacterial proteases was between 117–133 kDa. This value is close to those previously reported. Fathimah and Wardani (2014) found that cysteine protease isolated from Moringa leaf has a molecular weight of 156 kDa, Meanwhile, Baehaki and Budiman (2011) reported the molecular weight of metalloprotease secreted by a bacterium isolated from Indralaya swamp is between 131–138 kDa.

Overall, the potential of protease of *Bacillus thuringiensis* HSFI-12 (both in crude and dialysate forms) as an antithrombotic agent in terms of anticoagulant is confirmed by this study. The bacterial protease, however, did not show direct fibrinolytic activities. It was proven by the fact that the bacterial protease was unable to utilize fibrin as a substrate (Hidayati et al., 2021a; Hidayati et al., 2021b). This shows that the thrombolysis activ-

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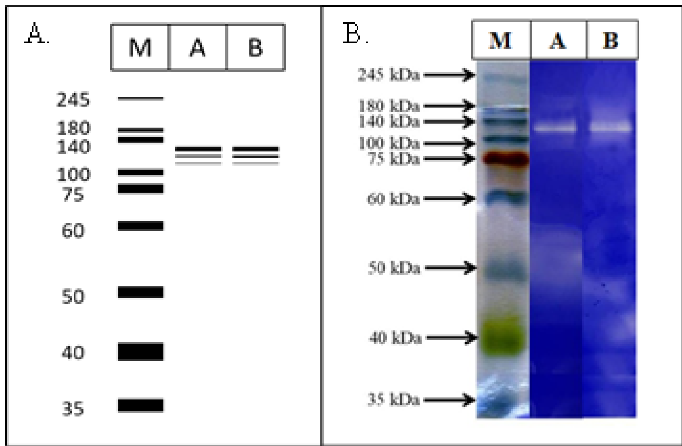

Figure 7. The Zymogram Shows the Molecular Weight Marker (M) as Standard Protein, Crude Protease (A) and Dialysate Protease of *Bacillus thuringiensis* HSFI-12 (B). The clear zone on the zymogram indicates the ability of crude and dialysate protease to degrade the casein substrate.

ity of protease of strain HSFI-12 likely comes as a plasminogen activator (PA), which action will activate plasmin leading to the cleavage of fibrins (Akhtar et al., 2017). Based on the results, the molecular weight and specificity of bacterial protease were also successfully determined with zymographic method. The obtained zymogram also underline that casein is among the suitable substrate of protease of strain HSFI-12.

Further tests to confirm the potential of purified protease as an antithrombotic agent are prospective to be done in vivo. This is also as stated by Dewi et al. (2022). Further purification can be done by subjecting protease dialysate obtained in this study to an ion exchange or gel filtration chromatography (Zilda et al., 2014). Such a higher level of purification will likely increase enzyme purity leading to higher both specific activity as well as the anticoagulation performance of the bacterial protease studied.

4. CONCLUSION

Both crude and dialysate protease from *Bacillus thuringiensis* HSFI-12 have the potential as an antithrombotic agent for demonstrating anticoagulant and antiplatelet activity, yet it could not demonstrate any fibrinolytic activity. The specific, as well as anticoagulant activities of the dialysate, were generally higher than the crude protease. The bacterial protease consists of several subunits with molecular weights between 117–133 kDa and is capable of degrading casein as substrate.

5. ACKNOWLEDGMENT

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