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Abstract. Cardiovascular diseases (CVDs) are still the leading mortality causes in the last decades. The ailments are multifactorial characterized by excessive clot (thrombus) formation in the blood vessels. Thrombus could be degraded through thrombolysis mechanism by plasmin activated by various fibrinolysis agents including, urokinase, nattokinase, or streptokinase. However, the use of these agents is restricted by relatively high cost, short half-life, allergic reaction, and bleeding effects. The search for more economical and safer thrombolytic (clot lysis) agents are essential to address the underlying problem in CVD therapy. Among Holothurians, *H. scabra* has been known to have the highest protein content making it ideal substrate for protease enzymes including fibrinolytic types with clot lysis properties. However, isolation of a proteases with antithrombotic activities either from tissue or from bacteria of *H. scabra* has not been reported. This study aimed to screen proteolytic and clot lysis activities of crude protease extracts from tissue and bacteria isolated from fermented intestine of *H. scabra*. Crude protease of tissue of *H. scabra* was extracted by cold centrifugation, which activity was measured using UV-spectrophotometer. Crude protease of proteolytic bacteria selected by clear zones on skim milk agar (SMA) medium was isolated from nutrient broth (NB). Extracts showing proteolytic activity were subjected to gravimetry-based clot lysis test. As results, crude proteases isolated directly from the *H. scabra*'s tissue showed low proteolytic activities, thus were no proceed to clot lysis activity test. Crude protease extracted bacteria could show both proteolytic and clot lysis activities. In conclusion, based on this screening study, intestine of *H. scabra* is a rich

source of proteolytic bacteria, some of them could produce crude enzymes showing competitive thrombolysis activities with Nattokinase. Thus, they appeared to have more potentials to be developed as thrombolysis agent than those directly extracted from the organism's tissue.

1. Introduction

Cardiovascular, venous and arterial thrombosis are multifactorial diseases characterized by unrestrained clot formation in the blood vessels [1]. The occurrence of thrombosis is due to failure of homeostasis. This condition could lead to blockage of the blood vessels and during recovery, which could result in blockage of the heart or brain muscle leading to death [2-3]. The world's burden of CVD falls, mainly, on the low and center pay (LMI) nations, representing over 80% of CVD mortality. WHO data says that due to cardiovascular disease, especially stroke and heart disease, about 17 million people are dying each year and predicts that this number will increase to 23.3 million by 2030 [4].

Blood clots have a major protein component, namely fibrin. Blood clots that block blood vessels can be destroyed by fibrinolysis by plasmin. The main process of fibrinolysis is by activating plasminogen into the proteolytic enzyme plasmin. This enzyme changes the shape of the thrombus by destroying the fibrin in the blood clot so that the progression of thrombosis is inhibited [5-6]. Commonly used drugs such as urokinase, tissue-type plasminogen activator (t-PA) and streptokinase, activate plasmin and convert plasminogen to plasmin which degrades fibrin. However, these agents have limitations such as higher cost, shorter half-life, allergic reactions and intravenous administration of these agents are also known to cause bleeding. This has prompted investigators to continue to look for new, safer and less expensive sources of fibrinolytic enzymes for thrombolysis [7-10].

Sea cucumbers are marine invertebrates gaining popularity among researchers in recent decades, not only for their nutritional value, but also for their potential health benefits and therapeutic applications [11]. Sea cucumbers are known organisms producing fibrinolytic proteases with antithrombotic including thrombolysis activity. This is because it is rich in protein, the substrate for the protease enzyme in these organisms, with a content in average between 43.24 - 48.27 % w/w [13]. Of the various species of sea cucumbers found in Indonesia, the sand or white sea cucumbers (*Holothuria scabra*) had been reported to have the highest protein content (79.6% w/w) [14]. However, the isolation of the protease enzyme with fibrinolytic ability from *H. scabra* from both tissue and bacteria of its digestion system has not been published. In this study, blood clot lysis activities of crude protease enzymes from both tissue and bacterial isolates of *Holothuia scabra* digestive tract were evaluated.

2. Materials and Method

2.1. Material

Sample used in this study was a sea cucumber *H scabra*, which was obtained from its captivity located at Lembaga Ilmu Pengetahuan Indonesia Institute for Bio Industry (BBIL LIPI), Kodek Gulf Village, Lombok, West Nusa Tenggara. The size of the 12-mo sea organism was 177 x 4 cm with a weight of 144.01 g. The main reagents used included Nutrient Agar (Oxoid Lot: 2438621), Skim Milk Agar (Oxoid Lot: 4249869), physiological NaCl, casein (Merck, cat. 750344), standard tyrosine (Merck, cat. 1.083710025), Na_2CO_3 (Merck cat. 6392.1000), Folin-reagent Ciocalteau (Merck, cat. 1,0001.0500). The equipment used were Analytical scale (Mettler Toledo), autoclave (Hirayama HVE-50), Laminair Air Flow Cabinet (Dalton), spectrophotometer (Thermo), Micro centrifuge (Bechman Counter), incubator (Mammert), micropipette (Soccorex Calibra 822). Vortex mixer (Genie 2TM 12-82), calipers, micro tubes and other tools used in the laboratory according to work procedures.

2.2. Method

2.2.1 Crude Protease Extraction from Tissue of H. scabra

To obtain crude protease extract, 3 samples of H. scabra "muscle" tissue (from tentacle, ventral and posterior pa) were prepared. As much as 2 g each sample was homogenously blended and then added with 2 ml of Tris HCl 0.05 M (pH = 8.22). The mixture was subjected to centrifugation at 10,000 rpm and 4 °C for 15 min, and the supernatant was set aside as crude protease extract. The extract was subjected to protease activity test.

2.2.2 Activity Test of Crude Protease Extracted from Tissue of H. scabra

The crude enzyme extracts both from tissues of *H scabra* and was then determined for its proteolytic activity based on method reported by Cupp-Enyard [15]. A total of 250 µL of crude extract of the enzyme were added to 250 µL of 1% casein (1 g of casein in 100 ml of K-Phospate buffer 0.05 M pH= 7.5). The mixture was incubated for 30 min. The mixture reaction w terminated by addition of 500µL of 0.4M TCA. A total of 25 L of supernatant filtrate was added with 1.25 ml of 0.4 M Na_2CO_3 and 125 µL of Folin Ciocalteau reagent and incubated at 37 °C for 30 min. The absorbance of the mixture was measured using a spectrophotometer at λ of 600 nm. One unit of enzyme activity is defined as the amount of enzyme required to release 1 µmol of tyrosine on the casein substrate per minute. The linear tyrosine equation was used as a standard curve to be interpolated with the absorbance value obtained [16]

2.2.3 Proteolytic Bacterial Selection from Intestinal Organ of H. scabra

Preparation of bacterial culture from intestine of H. scabra was carried out aerobic and anaerobically following to method reported by Zhang et al. [17]. Next, in a sterile test tube, 1 g of the sample was added with 9 mL of sterile sea water, which then homogenized using a vortex. The resulted suspension was diluted and then inoculated on Skim Milk Agar (SMA) media containing casein protein with incubation at 30 °C for 24-48 to test the ability of bacteria to hydrolyze protein [18]. The bacterial culture that grows and forms a clear zo around the colony is then separated and purified by the quadrant streak method [18-19]. Protease activity was indicated by the presence of a clear circular zone around the colony. The quotient of clear circle diameter and colony diameter is expressed as relative protease activity. This applies also for other common hydrolytic enzyme activites such as lipase, amylase and cellulose from bacteria [20-24]. Each bacterial isolate able to produce clear zones was inoculated on SMA media, one ose and incubated at 30 °C for a week. Hydrolysis Capacity Index calculation was measured on the seventh day of observation on the clear zone to determine the level of ability of each bacterial culture in hydrolyzing the substrate [24].

2.2.4 Crude Protease Extraction from Proteolytic Bacterial Isolates of H. scabra

For quantitative protease activity test, bacterial cultures in a liquid medium containing 20% soy milk and 5% lactose as a protein source substrate were used as a starter in Erlenmeyer. The starter was incubated and shaken on the shaker at a speed of 100 rpm at room temperature for 24 h. A total of 1% starter was then inocu d into 20 ml of the same medium, incubated for 3 days on a shaker at 130 rpm. Next, the culture was centrifuged at 10,000 rpm 4 °C for 15 min to separate the enzyme solution from the substrate particles. The filtrate obtained was regarded as a crude extract of the enzyme [25].

2.2.5 Clot Lysis Activities of Bacterial Crude Proteases

For the gravimetric thrombolysis test, the empty micro-centrifuge tube was weighed and each we t recorded. Blood was taken from healthy volunteers as much as 500 µl, put in a micro centrifugation tube and incubated at 37 ° C for 45 min. After clotting, serum was en so as not to interfere with the blood clot. Weight of the clot was determined as follows: weight of clot = weight of tube wit*h clot - weight of empty tube* [26].

Crude extract enzymes from the tentacle, ventral and posterior muscle tissue H. scabra tissue were pipetted by 100 µl, each was put into a micro-centrifuge tube containing the blood clot (each was

eled). In addition, aquadest was used as a negative control and streptokinase as a positive control. All tubes were then incubated at 37 °C for 90 min and clots lysis was observed [26-27]. Percentage of Clot lysis percentage was calculated based on weight difference of micro-centrifuge tubes obtained before and after clot lysis [26].

3. Results and Discussion

3.1. Sample preparation

Sample preparation in this work was intended for two purposes. First was to obtain crude enzyme extract from muscle tissue of *H. scabra* to detect its proteolytic activity. Second was to isolate proteolytic bacteria from intestine part of the sand sea cucumber, which produce protease. For both purposes, a sand sea cucumber or *Holothuria scabra* was freshly taken and used as sample as (Figure 1). To obtain crude enzyme from muscle tissues, the gut part and the muscle tissue was separated aseptically by dissecting set (Figure 2). Each of the muscle parts (tentacle, ventral and posterior) was grounded separately using sterile mortar and then subjected to centrifugation. Each supernatant was later tested for proteolytic activity using spectrophotometer based on absorbance at λ 600 nm.

Figure 1. A sand sea cucumber or *Holothuria scabra* sample freshly captured from its captivity

Figure 2. Muscle part of *H. scabra* (white color)

This work is the first reporting the cultivation of proteolytic bacteria from gut of *H. scabra*, a rich-protein, sand sea cucumber from Indonesia. To obtain culturable bacteria, the gut part was blended using sterile mortar. The anaerobically prepared sample to obtain culturable bacteria is shown in Figure 2 & 3.

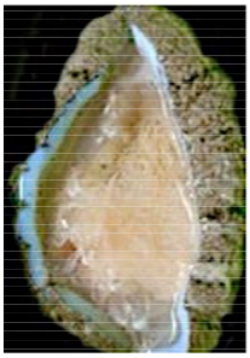


Figure 3. Intestine or gut part of *H. scabra* (orange color)

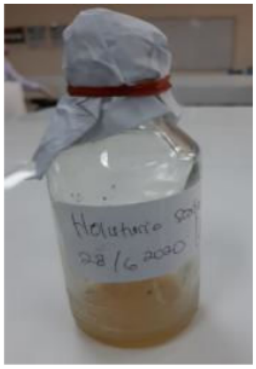


Figure 4. Fermented intestine of *H. scabra* sample as source of culturable bacteria

3.2. Activity Test Results of Crude Protease Extracted from Tissue of H. scabra

Based on linear tyrosine equation obtained from a standard curve made following previous method at λ 600 nm [16], protease activity of crude protease could be calculated using the following equation: Y = 0,0019 X + 0,0034, where Y= absorbance, while X= activity (in U/mL)
The resulted data of proteolytic activity of crude enzyme extracted from tissues based on absorbance at λ 600 nm were shown in Table 1.

Table 1. Activity of crude protease extracted from *H. scabra* tissues with tyrosine as standard

No.	Treatment	Absorbance at 600 nm* (average)				Relative activity (U/mL)
		Tentacle	Ventral	Posterior	Control (tyrosine)	
1	Vortex	0.0791	0.0759	0.0850	0.0994	-1505.1170
2	No vortex	0.0754	0.0897	0.0881	0.0539	3103.3041

*Measurement was performed in 6 repetitions

In most activity test methods using spectrophotometer, homogenization of crude protease extract using vortex was common in order to homogenize samples prior to absorbance measurement. Data in **Table 1** show that activity test results of samples without vortex were positive. However, it could mean that enzyme having proteolytic activity was present in non-homogenous protease crude extract. So, it was assumed that concentration of crude protease extracted from tissue in this work was not so high. It means, proteolytic activity could not be confirmed. Based on this, the result was not proceeded to next step of clot lysis screening.

3.3 Proteolytic Bacterial Selection from Intestine of H. scabra

Unlike result from previous result using intestine of Holothuria leucospilota as sample [17], in this study, culturable bacteria could not be obtained from aerobic cultures after 24-h incubation. The gut bacteria appeared to grow well under anaerobic condition resulting 12 unique colonies, which were subsequently purified and named HSFI-1 to HSFI-12 (HSFI refers to Holothuria scabra Fermented Intestine). Macroscopic characteristics of HFSI-1 to -12 were shown in Table 2. All of the twelve isolates were then tested for their ability to degrade protein on SMA media. This selection step resulted in 10 proteolytic isolates (Table 3).

Table 2. Macroscopic characteristics of bacteria isolated from fermented intestine of sand sea cucumber (*H. scabra*) on NA media

No.	Isolate Code	Bacterial Colony Morphology					
		Shape	Edge	Diameter size (mm)	Colour	Elevation	Consistency
1	HSFI-1	circular	entire	1	cream	convex	smooth
2	HSFI-2	circular	undulate	8	white	umbonate	smooth
3	HSFI-3	circular	entire	3	cream	raised	smooth
4	HSFI-4	irregular	undulate	5	cream	convex	smooth
5	HSFI-5	filamentous	polyform	8	white	crater-form	rough
6	HSFI-6	irregular	undulate	5	cream	convex	smooth
7	HSFI-7	circular	entire	1	white	convex	smooth
8	HSFI-8	circular	entire	4	cream	convex	smooth
9	HSFI-9	circular	entire	8	cream	umbonate	smooth
10	HSFI-10	circular	entire	2	cream	convex	smooth
11	HSFI-11	circular	entire	4	cream	convex	smooth
12	HSFI-12	circular	entire	4	cream	convex	smooth

Note: HSFI (*Holothuria scabra fermentated intestine*)

Table 3. Bacterial colony morphology, proteolytic activity and *clot-lysis* test result data of bacteria isolated from fermented intestine of *H. scabra*.

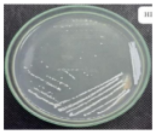
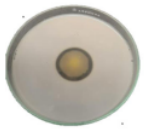
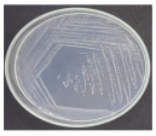
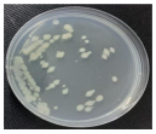
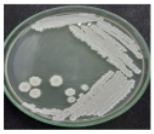
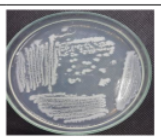
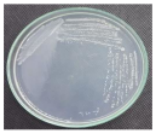
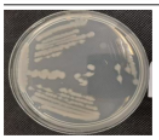
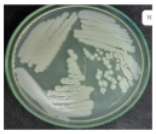
Isolate code	Colony morphology	Proteolytic zone*	Clot lysis (%)
HSFI-1		N.A.	NA
HSFI-2			26.06
HSFI-3			5.24
HSFI-4			19.30
HSFI-5			62.03

Table 4. Continued …

Sample code	Colony morphology	Proteolytic zone*	Clot lysis (%)
HSFI-6			32.94
HSFI-7		N.A	NA
HSFI-8			84.71
HFSI-9			7.78
HSFI-10			31.28
HSFI-11			46.70
HSFI-12			12.47
Aquadest	N.A.	N.A.	0.29
Nattokinase	N.A.	N.A.	56.05

*After 24-h incubation

3.4 *Crude Protease Extraction from Proteolytic Bacterial Isolates*

In this study, crude protease extract was retrieved after centrifugation of each of bacterial inoculum in skim milk broth medium. The supernatant was considered as crude protease, thus 10 extracts from HFSI-1 to -6 and HFSI-8 to -12 could be obtained. The 10 bacterial protease extracts were subjected to clot lysis test as described in previous study [23].

3.5 Clot Lysis Activities of Bacterial Crude Proteases

Visual representation of clot lysis test result on 10 bacterial protease extracts is shown in **Figure 3**. When 100 µl water was added to the negative control (Bl) clot, negligible clot lysis was observed. In other words, no clot lysis was observed in Bl tube. Whereas, tubes to which different crude extracts from different bacterial isolates HFSI-1 to -6 and HFSI-8 to -12 were added, various levels of clot lysis could be visually seen. Percentage of clot lysis obtained after treating clots with water, streptokinase and control group based on gravimetry method is shown in **Table 3**.

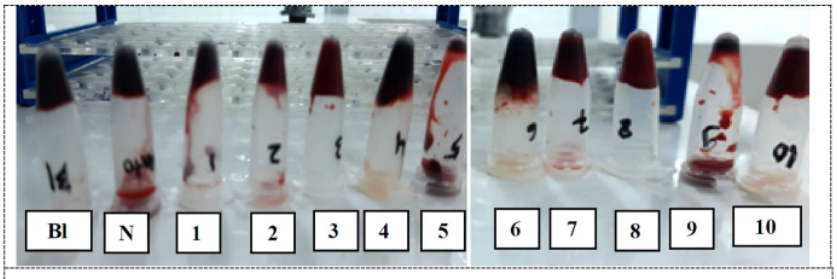


Figure 5. Gravimetry-based thrombolysis test result where lysis of clot was visually observed from each sample. Bl (Blanko) is a negative control to which water was added; N (Nattokinase) is a negative control to which a commercial Nattokinase was added. Tube 1—10 were samples where each of crude proteases from different bacterial isolates, HFSI-1 to -6 and HFSI-8 to -12 was added. After dissolution of the clots, tubes were inverted, so that the remnants of clots could be clearly seen.

Table 5. Clot-lysis activity test results of bacterial crude protease using gravimetry method.

Tube No.	Isolate code	Initial clot weight	Final clot weight	Lysis percentage (%)
1	HSFI-2	0.2467	0.1824	26.06
2	HSFI-3	0.2326	0.2204	5.24
3	HSFI-4	0.2388	0.1927	19.30
4	HSFI-5	0.4128	0.1567	62.03
5	HSFI-6	0.2486	0.1667	32.94
6	HSFI-8	0.2519	0.2323	84.71
7	HSFI-9	0.5776	0.0883	7.78
8	HSFI-10	0.2439	0.1676	31.28
9	HSFI-11	0.2572	0.1371	46.70
10	HSFI-12	0.2526	0.2211	12,47
11	Aqudest	0.2668	0.2650	0.29
12	Nattokinase	0.2462	0.1082	56.05

As seen on **Table 3**, various percentage of clot lysis was observed from each of crude protease extract from 10 bacterial isolates, HFSI-1 to -6 and HFSI-8 to -12. There were two extracts of crude bacterial protease that could show higher clot lysis activity than positive control, Nattokinase coming from HFSI-5 and -8. To confirm the results however, *in-vitro* validation test should be performed either on the

proteolytic and clot lysis activities of these bacterial protease extracts. Moreover, purification and characterization of bacterial proteases from isolate HFSI-5 and -8 is important to do, especially if the aim is to further develop them into prototypes of thrombolysis agent. Overall, results of this study showed that crude proteases of bacteria isolated from fermented intestine of *H. scabra* have the potential as thrombolysis agent more than those extracted directly from the sea cucumber's muscle tissue.

4. Conclusion

Based on this screening study, intestine of *H. scabra* is a rich source of proteolytic bacteria. Some of them could produce crude enzymes showing competitive thrombolysis activities with Nattokinase. Thus, they appeared to have more potentials to be developed as thrombolysis agent than those directly extracted from the organism's tissue.

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