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In Vitro Anticoagulant Activity of Crude Protease of *Bacillus tequilensis* HSFI-5

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Abstract

Bacillus tequilensis HSFI-5 is a food-grade bacterial isolate obtained from the fermented intestine of *Holothuria scabra* (sand sea cucumber). Strain HSFI-5 had been reported to be able to produce proteases, which had shown several characteristics of an antithrombotic agent, i.e., fibrinolytic and clot-lysis activities. However, its anticoagulation activity test is yest to be done. This study aimed to determine the anticoagulant activity of the crude protease HSFI-5 *in vitro*. The study design was a completely randomized design with a sample size of 90 calculated using the Federer formula. The material used was crude protease from *B. tequilensis* in skim milk broth. Prothrombin time (PT), activated partial thromboplastin time (aPTT), and plasma recalcification time (PRT) were carried out to test the anticoagulant activity. Citrated platelet poor plasma samples were divided into positive control, normal control, direct examination with crude enzyme in volumes of 50 and 100 µL and pre-incubation at 37ºC for 5, 10, and 15 min with crude enzyme volumes of 50 and 100 µL. The data normality was tested with the Kolmogorov-Smirnov test and the different tests were analyzed by one-way ANOVA with the Post hoc LSD test. The results of one-way ANOVA both on PT, aPTT, and PRT examinations showed that there was a significant difference between the treatment groups ($p<0.05$). The longest results of PT, aPTT, and PRT are positive controls, and the shortest results are normal controls for PT, and 15' 50 group for aPTT and PRT. It is clear that crude protease B. tequilensis HSFI-5 exhibits anticoagulant as well as thrombolytic action, raising the possibility that it could function as an antithrombotic drug.

Keywords

Crude Protease, *Bacillus tequilensis* HSFI-5, Anticoagulant.

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INTRODUCTION

Coagulation is a process involving several components, such as blood vessels, platelet cells, and blood coagulation factors. Normal coagulation pathways are a collaboration between clot formation and clot decay processes. An imbalance of the coagulation system can occur in an illness that causes bleeding or thrombosis (1,2). One of the diseases causing blood coagulation disorders is coronary vascular disease (CVDs). Data from the World Health Organization (WHO) states that 17.9 million people (32%) in the world die every year due to CVDs. As many as 85% of people with CVD die from heart attacks and strokes. The risk of venous thromboembolism (VTE) increases in the first 3 months after the occurrence of an ischemic stroke (3,4). Ischemic stroke can occur due to a sudden reduction in blood flow to the brain, leading to reduced neurologic function. Blood flow is reduced as a result of thromboembolic blockage. After 4 to 14 days from the time of commencement, antithrombotic therapy may be given (5,6). The antithrombotic potential of bacterial proteases can be explored in order to minimize costs (7).

Antithrombotic activities of bacterial proteases encompass mainly three characteristics: fibrinolysis, clot lysis and anticoagulant. The anticoagulant activity of bacterial proteases has been reported in several studies (7,8). Proteases with fibrinolytic and thrombolytic activities had also been reported, mostly from *Bacillus subtilis* (9,10). *Bacillus tequilensis* HSFI-5 is a bacterial isolate obtained from the fermented intestine of the sand sea cucumber, *Holothuria scabra*. The sea cucumber was isolated from sand sea cucumber from Kodek Gulf Village, Lombok, West Nusa Tenggara. Strain HSFI-5 was found to have the ability to produce fibrinolytic protease, which is an important characteristic of an antithrombotic agent (11,12). Another important characteristic of the protease of *B. tequilensis* that underlines its potential as an antithrombotic agent as clot lysis activity. This study represents novelty as the anticoagulant activity of the protease from *B. tequilensis* HSFI-5 has not yet been tested. Such studies are important as a basis for further exploring bacterial protease activity through in vivo antithrombotic and toxicity assays.

The commonly used antithrombotic agents work through the tissue plasminogen activator (tPA) mechanism. Trypsin-like serine protease has been reported to be an anticoagulant agent because it contains sulfated polysaccharides that play a role in anticoagulant, antiangiogenic, and immunostimulating processes (7,13). The presence of thrombolytic activity is one of the potential anticoagulant (11). PT, aPTT, and PRT were used to test anticoagulant activity of crude protease. The PT, aPTT, and PRT

assays are the most commonly used tests for the examination of blood coagulation factors (14–16). Discovery of alternative anticoagulants is needed to minimize the risks and costs of drug use. The purpose of this study was to measure the anticoagulant activity of the crude protease HSFI-5 in vitro.

MATERIALS AND METHODS

A completely randomized design was used in this research. The study used three examination parameters, namely PT, aPTT, and PRT. The study was conducted at the Laboratory of Hematology of Universitas Muhammadiyah Semarang in October 2022. The controlled variables of this study included a varied volume of crude protease from *B. tequilensis* HSFI-5 (of known concentration) and length of incubation time. The number of samples used in the study was calculated using the Federer's formula. The total number of treatment in this study was 10 group, namely (1) positive control; (2) normal control; (3) direct incubation with addition of 50 µL crude protease (D 50); (4) direct incubation with addition of 100 µL crude protease (D 100); (5) 5 mins pre incubation with addition of 50 µL crude protease (5' 50); (6) 5 mins pre incubation with addition of 100 µL crude protease (5' 100); (7) 10 mins pre incubation with addition of 50 µL crude protease (10' 50); (8) 10 mins pre incubation with addition of 100 µL crude protease (10' 100); (9) 15 mins pre incubation with addition of 50 µL crude protease (15' 50); (10) 15 mins pre incubation with addition of 100 µL crude protease (15' 100). Each experiment had three replicate plants per treatment. The total study sample for each study parameter was 30. The total number of study samples was 90 samples. The blood samples used for the study were taken from a healthy volunteer who was willing to be a respondent. The research was conducted with permission from the ethics committee of the Faculty of Public Health, University of Muhammadiyah Semarang with number 377/KEPK-FKM/UNIMUS/2020.

Isolation of Crude Protease Enzyme *B. tequilensis* HSFI-5

B. tequilensis HSFI-5 isolates were subcultured on skim milk broth (SMB) medium and incubated at 37ºC for 72 hours. Bacterial cultures were centrifuged at 3000 rpm for 15 min at 4º C. The supernatant (crude protease) was harvested from the centrifugation results and could be used for in vitro anticoagulation assay (17).

In Vitro Assays

PT, aPTT, and PRT were performed to examine the anticoagulant activity of crude protease *B. tequilensis* HSFI-5.

Anticoagulant heparin was used as a positive control.

B. tequilensis HSFI-5 Crude Protease Treatment

Normal and positive controls were treated as standard procedures, while direct assay treatments with crude protease *B. tequilensis* HSFI-5 50 µL and 100 µL was carried out by means of 50 µL PPP with 50 µL/100 µL crude protease, which was analyzed for clotting that occurred as standard procedure. Test samples were prepared at 50 µL and 100 µL raw protease volumes with pre-incubation times of 5, 10, and 15 minutes.

Prothrombin Time (PT)

The PT examination used a sample of citrated platelet poor plasma (PPP). As much as 50 µL of PPP was added with 50 µL of PT reagent (TEclot PT-S, Germany) and incubated at 37ºC for 2 min before reading photometrically on a coagulometer (Coatron M1, Germany). PT results were expressed in seconds. The resulting clotting was analyzed using a Coatron M1 coagulometer according to its manufacturing method (TEclot PT-S, Germany).

Activated Partial Thromboplastin Time (aPTT)

Coagulation analysis with aPTT was performed photometrically with the TEclot aPTT-S reagent and read with a Coatron M1 device. As much as 50 µL of PPP was reacted with 50 µL of aPTT reagent and then incubated for 2 min at 37º C. The preparation of the test sample was carried out like the sample treatment for the PT test, and then the clotting time was expressed in seconds (s) following the TEclot (Germany) manufacturing procedure.

Plasma recalcification time (PRT)

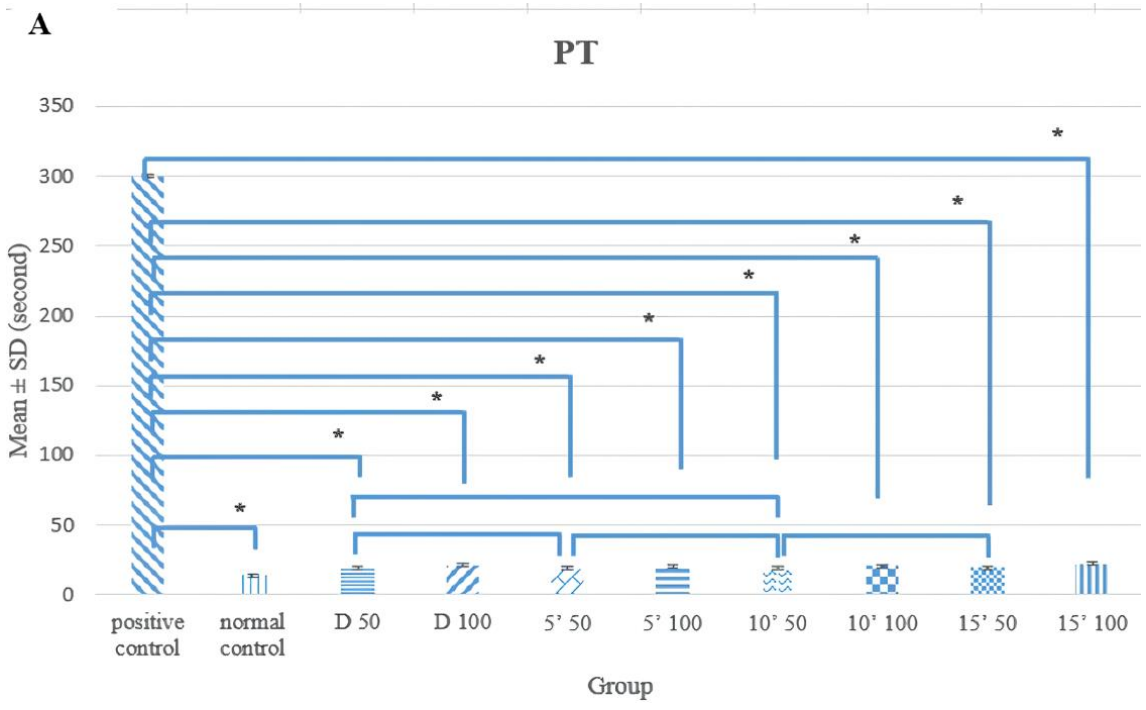
PRT examination was carried out manually with the addition of NaCl and $CaCl_2$ 0.025 M at 37ºC. The preparation of examination samples was carried out as in the treatment for PT and aPTT examinations. As much as 100 µL PPP was added to 100 µL physiological NaCl and incubated for 1 min. The mixture was then added to 100 µL $CaCl_2$ 0.025 M and incubated for 90 s. The clot that appeared every 30 seconds was observed. PRT results expressed in seconds were recorded (18).

Statistical Analysis

Data were analyzed using SPSS 25 software (IBM, USA). Kolmogorov-Smirnov test for analyzing normality of data. Normal data were followed by the one-way Anova difference test. A post hoc LSD test was performed to determine the differences between the treatment groups in the study. A significant value of less than 0.05 was expressed as the result with a significant difference.

RESULTS

The bacterial isolate of *B. tequilensis* HSFI-5 used in this study was from a previous study originally deposited in a microbank of the microbiology laboratory of Universitas Muhammadiyah Semarang (11). After subculturing in 250-mL of SMB medium, culturing and centrifugation, 35 mL of crude protease was obtained as a supernatant. This sample was then subjected to PT, aPTT, PRT and hematology assays. Initial analysis of the protein content of the crude protease yielded a nano drop value of 7,7 mg/mL. Hematology results showed that the highest mean PT examination was in the positive control group, and the lowest mean was in the normal control group. The highest and lowest aPTT test result were determined in the positive control and 15’ 50 groups, respectively. The highest and lowest mean PRT values in the positive control and 15’ 50 groups, respectively. Baseline values for the PT assay were 10-14 seconds, aPTT 22-30 seconds, and PRT 90-250 seconds. The Kolmogorov-Smirnov normality test was normal for all groups, so the one-way ANOVA test was used and performed. The p-value was 0.000 ($p<0.05$). An LSD post hoc test was used to determine differences in each group. The results of the post hoc LSD test are shown in Figure 1.



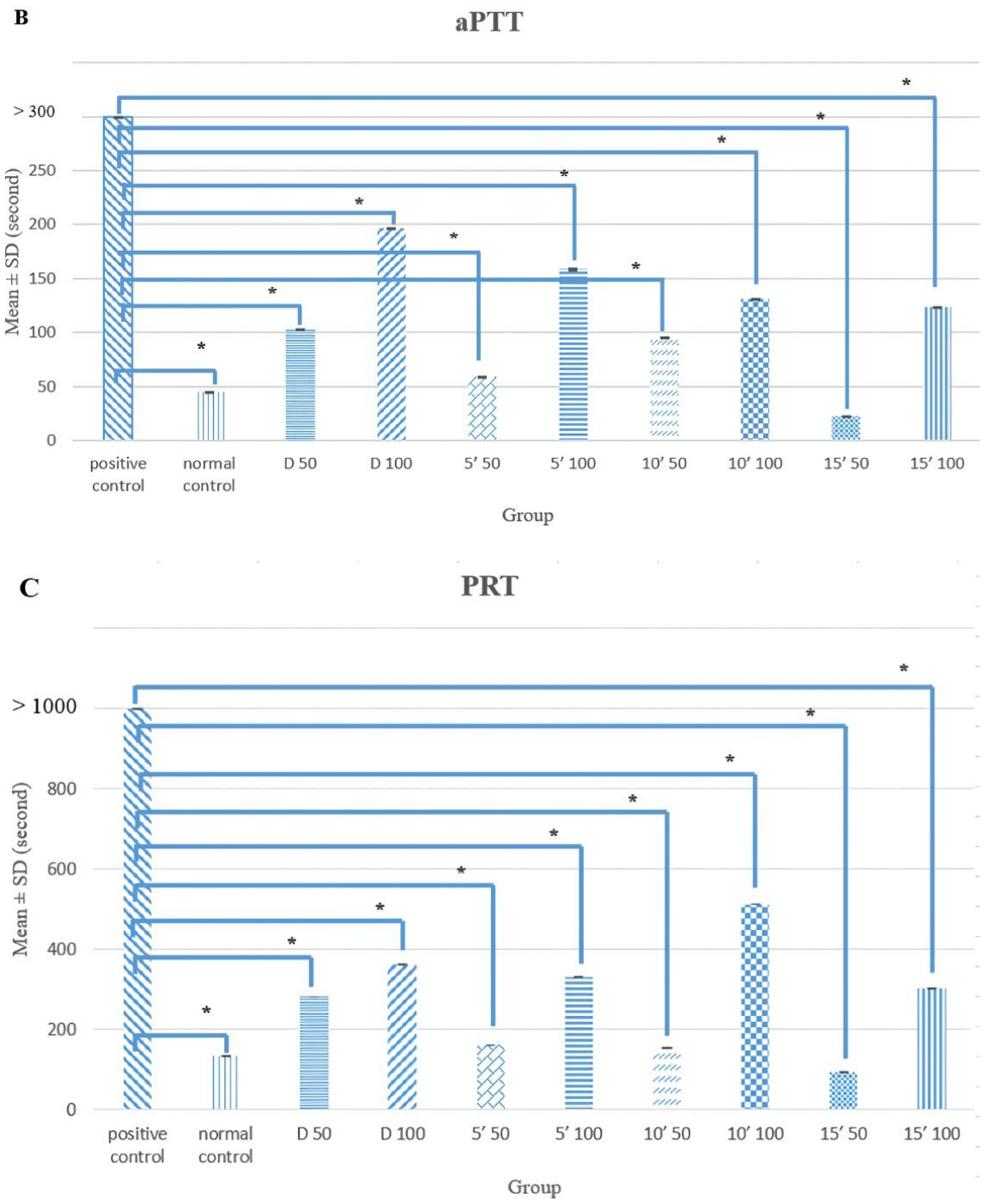


Figure 1. Post hoc LSD test results of (A) PT, (B) aPTT, (C) PRT. *significant.

DISCUSSION

The crude protease *B. tequilensis* HSFI-5 was recovered from the bacterial culture supernatant of skim milk broth medium and previous studies based on fibrin plates and gravimetric analysis reported promising fibrinolytic and clot-lytic activities respectively. Crude bacterial proteases are also expected to have anticoagulant activity, supporting their potential development as antithrombotic agents (11). In this study, we analyzed

the anticoagulant activity of bacterial proteases by performing PT, aPTT and PRT. The existence of side effects of available anticoagulants has led researchers to investigate the anticoagulant activity of the crude protease B. tequilensis HSFI-5 (19). Anticoagulant activity of crude protease B. Techylensis was analyzed by direct examination of PT, aPTT, and PRT (no delay), and by varying incubation times and crude protease volumes.

The results of the descriptive analysis of the PT studies showed a trend for the studied proteases to prolong the PT time, consistent with the increased volume of HSFI-5 crude protease. The highest yield was obtained in the group with 100 µL of crude protease *B. tequilensis* HSFI-5 added and PPP incubated for 15 min. Research data show that the longer the pre-incubation period and the more crude protease added, the longer the PT time. The most significant result of the PT examination was a 57.45% elongation compared to the normal controls. The difference in PT assay results appeared to greater with the addition of 100 µL of crude protease, suggesting that the addition of 100 µL gave optimal PT result at 37º C and 15 pre-incubation time. These results are in line with studies suggesting that fibrinolytic proteases can cause prolongation of PT (17). The PT test is a coagulation test to assess the potency of clotting factors in the extrinsic and common pathways. The presence of elongations in PT indicates that the crude protease *B. tequilensis* HSFI-5 can inhibit the coagulation processes in the extrinsic and common pathways (7).

aPTT testing is commonly used to detect coagulation factor deficiencies in intrinsic signaling pathways. The results showed that the addition of 100 µL of crude protease significantly prolonged the aPTT results for the same pre-incubation time. However, the addition of 50 µL compared to 100 µL of crude protease reduced the sample. This was probably due to the low protease concentration, which required a larger amount and longer time to inhibit clotting. Prolongation of the aPTT results compared to the normal controls resulted in up to 2.75-fold time prolongation. This indicates that the crude protease *B. tequilensis* HSFI-5 is able to inhibit the coagulation process.

All groups had extended PRT test times, but a pattern was observed in which the addition of 100 µL of crude protease significantly increased the number of samples. A 3.87-fold extension was observed in the 15 min pre-incubation group compared to controls. These results demonstrate that the optimal anticoagulant activity of crude protease is preincubated for 15 minutes in a volume of 100 µL.

Extending the PRT results, we showed that the crude protease HSFI-5 can inhibit coagulation via an intrinsic pathway. Longer incubation times and higher volumes increase anticoagulant activity because the low concentration of crude protease requires longer volumes and times to work optimally. A lower concentration of crude protease increases the amount and time to activate the anticoagulant mechanism through the serpin mechanism.

The anticoagulant mechanism of the crude protease HSFI-5 is believed to be based on the serpine mechanism. Coagulation and fibrinolysis are primarly controlled by the protease inhibitor serpine superfamily. Antithrombin (AT) is a physiological anticoagulant that targets procoagulant enzymes, particularly factor Xa and thrombin (20–23). Serpine participate in the anticoagulant mechanism by carrying out the proteolytic activity of clotting proteases in intrinsic and extrinsic pathways (24–26). Antithrombin inhibits the activity of factor Xa and thrombin (27–29).

Serpines have been identified in bacteria, archaea, eukaryotes, and viruses. Several types of serpines include subtilisin, papain, and caspase families. The PCI/AT - inhibited interaction between thrombin and thrombomodulin causes diffusion of the serpine-protease complex (30). chieving higher anticoagulant activity, smaller volumes, and minimal pre-incubation times requires the use of concentrated proteases. As a result, it can be used more effectively in the treatment of thrombosis (24,29,31).

CONCLUSIONS

The crude protease *B. tequilensis* HSFI-5 exhibits anticoagulant activity based on PT, aPTT, and PRT vacuum and may be an alternative anticoagulant in the future. The longest results for PT, aPTT, and PRT represent the positive controls, and the shortest results were obtained from the normal control for PT, and 15-min incubation with 50 µL protease for aPTT and PRT. However, *in vivo* protease anticoagulant studie are required to confirm the results of these in vitro anticoagulation studies.

AUTHOR CONTRIBUTIONS

Stalis Norma Ethica: Were involved in planning and designing the work, Dewi Seswita Zilda: Performed the enzyme preparation, Tri Joko Raharjo and Stalis Norma Ethica performed the statistical analysis. Nur Hidayati: assisted in interpreting the results and worked on the manuscript.

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CONFLICT OF INTEREST

Authors declare that no conflict of interest in this study.

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