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Thrombolytic potential in bacteria isolated from fermented durian tempoyak

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Abstract. *Priskila C, Vidian V, Sanjaya A, Sugata M, Pinontoan R. 2022. Thrombolytic potential in bacteria isolated from fermented durian tempoyak. Biodiversitas 23: 5731-5737.* Cardiovascular disease is one of the leading causes of death worldwide and is primarily caused by dysregulation in the blood clotting system leading to the formation of unwanted blood clots (thrombi). Thrombolytic therapy, which aims to break down unwanted blood clots, employs various agents possessing thrombolytic activity. However, the harmful side effects of conventional thrombolytic agents necessitate the exploration of new and safer agents with high thrombolytic activities. Thrombolytic agents can be obtained from various sources, including fermented foods. Tempoyak is a condiment made from fermented durian, which is found in some regions of Indonesia, and is reported to contain beneficial microorganisms. The aim of this study was to isolate and identify thrombolytic agents from tempoyak. After several pure microbial colonies were successfully isolated from tempoyak, they were screened for proteolytic activity on skim milk agar medium. The isolated colonies with proteolytic activity were then subjected to several tests, including the whole blood clot lysis test, euglobulin clot lysis test, fibrin plate method, and zymography. Subsequently, the isolates with thrombolytic and fibrinolytic activity potential were identified in terms of their morphology and biochemistry. In this study, three bacteria with thrombolytic activity potential were successfully isolated. Fibrin zymography indicated that the molecular weights of the proteins that play roles in the fibrinolytic activity ranged from approximately 30 to 153 kDa, which were suspected to correspond to serine protease, cysteine protease, and/or metalloprotease. All three isolates were identified as belonging to the genus *Corynebacterium*. This is the first report discussing the thrombolytic and fibrinolytic activities of *Corynebacterium* sp. derived from fermented food.

Keywords: Blood clot, *Corynebacterium*, fibrinolytic activity, tempoyak, thrombolytic agent

INTRODUCTION

The human body continually aims to maintain a state of homeostasis, which describes the optimal state for carrying out its physiological functions. When blood vessels are ruptured, the body initiates an enzyme activation process to stop the bleeding. At that time, the hemostasis process begins as a result of the interactions of the complex coagulation and fibrinolytic systems in the body (Libretti and Puckett 2021). The coagulation process is aided by a protein called fibrin, which joins blood cells and other components together to form blood clots and plug the ruptured blood vessel sites (Garmo et al. 2021). After the bleeding is stopped, the coagulation process is halted and blood clots that have been formed are broken down through the fibrinolytic process, which involves the degradation or cleaving of fibrin (Bannish et al. 2017). Although the fibrinolytic process occurs naturally in the body, it can be disrupted by several factors, such as genetics, the environment, and behavior, resulting in blood clots remaining intact. Blood clots can cause blockages in blood flow, leading to various diseases such as heart disease, pulmonary embolism, and stroke (Mackman et al. 2020).

Cardiovascular disease is one of the leading causes of death worldwide and is primarily caused by dysregulation in the blood clotting system leading to the formation of unwanted blood clots (thrombi) (Alkarithi et al. 2021). Generally, thrombolysis therapy can be conducted to solve this problem. Thrombolysis is the activity of dissolving blood clots or thrombi, which can occur via fibrinolysis or hemolysis (Bannish et al. 2017). However, thrombolytic therapy has some drawbacks, including bleeding and allergies (Ali et al. 2014). One alternative to the classical thrombolytic agents are fibrinolytic enzymes, which are reportedly able to break down thrombi with very high efficacy and without significant side effects (Altaf et al. 2021).

Fibrinolytic enzymes are found widely in nature, both in food and non-food sources. Various non-food sources that have been reported to exert fibrinolytic activity include plants, algae, snake venom, and earthworms (Vijayaraghavan et al. 2019). However, fibrinolytic enzymes from these non-food sources show major limitations, such as low specificity toward fibrin and potential induction of fibrinogenesis. Furthermore, when administered orally, these proteases are difficult to absorb in the intestines and may be degraded by alimentary tract enzymes. In contrast, fibrinolytic proteases from food sources, especially fermented foods, have been reported for their health-promoting effects related to protection against heart disease. Previous studies found that this beneficial effect is closely related to the activity of microorganisms in fermented foods. The genus that is most commonly

reported for its fibrinolytic activity is *Bacillus* (Ali and Bavisetty 2020). Previously, researchers isolated *Bacillus subtilis* G8 from commercialized natto from Indonesia, which demonstrated thrombolytic and fibrinolytic activities (Lucy et al. 2019; Pinontoan et al. 2021). However, other bacteria species, such as *Staphylococcus* spp. and *Streptococcus* spp. found in fermented products have also been reported to possess fibrinolytic activity (Sharma et al. 2021). In addition to bacteria, some fungi, e.g., *Mucor* spp. and *Fusarium* spp., are also known to produce fibrinolytic enzymes (Ali and Bavisetty 2020).

Indonesia has an abundance of different types of fermented foods, one of which is tempoyak. Tempoyak is a traditional condiment made from fermented durian, originating in Indonesia. It is consumed in many provinces, such as South and West Sumatra, Bengkulu, Lampung, Jambi, West Borneo, and Aceh. Durian is typically fermented to utilize excess or poor-quality durian (Rajagukguk and Arnold 2020). There are several microorganisms found in tempoyak, including bacteria (non-lactic-acid bacteria and lactic-acid bacteria), yeasts, and molds (Hasanuddin 2010). As a fermented food containing a variety of microorganisms, tempoyak is expected to contain bacteria that may be able to produce thrombolytic agents (Sharma et al. 2021). So far, no studies have explored thrombolytic and fibrinolytic agents from tempoyak. Therefore, the aim of the present study was to isolate and characterize the thrombolytic activities of bacteria isolated from tempoyak.

MATERIALS AND METHODS

Isolation of microorganisms with proteolytic activity from tempoyak

Tempoyak, as a source of microorganisms, was bought from a local market in Palembang, Indonesia. One gram of tempoyak was dissolved in 5 mL of phosphate-buffered saline (PBS) and spread on a Glucose Yeast Extract Peptone (GYP) agar medium. The agar plate was then incubated at 37 °C for 48 hours. Single colonies were selected and purified by growing them in three-quadrant streaks on GYP agar media. The purified isolates were inoculated on a GYP broth medium and incubated at 37 °C for 24 hours. The extracellular crude enzymes were partially purified by 1:3 acetone precipitation and then used as samples for the proteolytic and thrombolytic tests (Pinontoan et al. 2021).

To analyze the protease activity of the extracellular enzymes, a protein hydrolysis test was carried out using a 2% skim milk agar medium and well diffusion method. A total of 20 µL of the partially purified extract sample was put into each well, followed by incubation at 37°C for 24 hours. The clear zones formed were then measured (Ouertani et al. 2018).

Thrombolytic activity tests: Whole blood and euglobulin clot lysis test

The whole blood clot lysis test was done using chicken blood clots. The blood clots were prepared by cutting and weighing them to about 0.16-0.18 g. Blood clots were rinsed with 0.9% NaCl (Merck) three times and weighed again. Each piece of the blood clot was put into a microtube and filled with 1 mL of partially purified extract, GYP broth (as negative control), or nattokinase 50 FU/mL (Doctor's Best, USA) prepared from a commercial supplement (as positive control). The samples were incubated at 37°C for 6 hours. After incubation, fibrinolytic activity was assessed qualitatively. The qualitative assessment was carried out by observing the color of the liquid (Lucy et al. 2019; Pinontoan et al. 2021).

To perform the euglobulin clots, chicken blood was directly collected into a tube containing ethylene diamine tetraacetic acid (EDTA). A total of 1.5 mL of chicken blood was put into a microtube and centrifuged at 2,500 rpm for 15 minutes. A total of 1 mL of the blood plasma (supernatant) formed was collected and mixed with 9 mL of cooled demineralized water (4°C) and 100 µL of 1% acetic acid (Merck). A total of 1.5 mL of the mixture was transferred to a microtube and centrifuged at 3,000 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in 20 µL of PBS and 40 µL of 25 mM $CaCl_2$ (Merck) to form a lump of euglobulin. A total of 1 mL of partially purified extract, GYP, or nattokinase were added to different microtubes containing the euglobulin clots. The samples were incubated at 37°C for 2 hours. The volume of the euglobulin clot was then observed qualitatively (Pinontoan et al. 2021).

Fibrinolytic activity tests: Fibrin plate assay and fibrin zymography

The fibrin plate assay was performed using a fibrin plate agar. The agar was made by mixing 2% agarose (Thermo Scientific) in 0.9% NaCl, 0.4% human fibrinogen (MilliporeSigma), and 80 µL 100 National Institute of Health (NIH) units/mL of bovine thrombin (MilliporeSigma). Wells were made in the agar, and 20 µL of the precipitated samples and different concentrations of nattokinase (positive control) were added to the wells. The agar was then incubated at 37°C for 8 hours (Pinontoan et al. 2021). Observations were made regarding the formation of a clear zone around the well.

The fibrin zymography was performed using 5% stacking gel and 12% resolving gel. The resolving gel was made by mixing 0.12% (w/v) fibrinogen and 50 µL thrombin into a polyacrylamide solution. A total of 10 µL of the sample was mixed with 10 µL of sample buffer. The samples and a protein marker were placed inside available wells. Electrophoresis was carried out at 50 mV for the first hour followed by 100 mV for the next two hours. The gel was soaked in a renaturation buffer and a development buffer before being stained with Coomassie Brilliant Blue R250 (Thermo Scientific) and destained (Lucy et al. 2019; Pinontoan et al. 2021). The gel was analyzed using the GelAnalyzer 19.1 application.

Identification of bacteria possessing thrombolytic activity

The bacterial isolates with thrombolytic and fibrinolytic activity were then identified morphologically using various staining and biochemical tests. Isolates were grown on

GYP agar media and the single colony formed was observed with regard to its shape, height, periphery, surface, opacity, color, and consistency. Then, three types of staining (Gram, acid-fast, and endospore) were performed. The biochemical tests carried out included starch hydrolysis, gelatin hydrolysis, catalase, indole, MR-VP (Methyl Red-Voges Proskauer), carbohydrate fermentation, and oxygen demand tests (Layly et al. 2021).

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RESULTS AND DISCUSSION

Three isolates (G1, G2, and G3) with different colony morphologies were obtained from the isolation process. The three isolates showed protease activity on skim milk agar medium, as observed by the clear zones which formed around the wells (data not shown). Subsequently, various tests were performed to investigate the thrombolytic and fibrinolytic activities of the isolates.

Whole blood and euglobulin clot lysis of partially purified isolate extracts

The whole blood clot lysis test was performed to assess a particular isolate's ability to break down blood clots (thrombosis) in the presence of whole blood components. Meanwhile, the euglobulin clot lysis test was done to evaluate the isolate's ability to break down blood clots by decreasing the volume of euglobulin clots.

The partially purified extracts of the three isolates showed the ability to break down the whole blood clots, as inferred via the increasing intensity of the red color of the liquid and the decreasing volume of the blood clots (Figure 1A). These results indicated that the thrombolytic activity of the three isolates might arise from the production of fibrinolytic agents. To confirm the fibrinolytic activity, the euglobulin clot lysis test was carried out. Euglobulin contains preserved levels of fibrinolytic factors, such as plasminogen and plasminogen activator, but low levels of anti-fibrinolytic agents, such as plasminogen activator inhibitor-1 (PAI-1) (Pinontoan et al. 2021). However, the result also showed the effect of partially purified extract of the isolate G1, G2, and G3 on the volume of the euglobulin clot (Figure 1B). After the euglobulin clots were incubated with the partially purified extracts, nattokinase, or GYP for 2 hours, there was a slight decrease in the volumes of the euglobulin clots that were incubated with G1 and a notable decrease in the volumes of the clots that were incubated with nattokinase, G2, and G3. These results suggested that the three isolates from tempoyak produced thrombolytic agents that were able to dissolve thrombi.

Fibrin plate assay and fibrin zymography of partially purified isolate extracts

A fibrin plate assay was conducted to evaluate the fibrinolytic activities of the three isolates The three isolates demonstrated the ability to degrade fibrin directly, as shown by the clear zones that formed on the fibrin plate (Figure 2). After 8 hours of incubation, the diameters of the clear zones formed by the partially purified extracts of G1, G2, and G3 were measured. The partially purified extracts' fibrinolytic activities were compared to those of the commercial nattokinase used as positive control, whose fibrinolytic activity is given in fibrinolytic units (FU). It was subsequently calculated that the partially purified extracts of G1, G2, and G3 exhibited approximate fibrinolytic activities equal to 5.9, 0.8, and 3.1 FU, respectively.

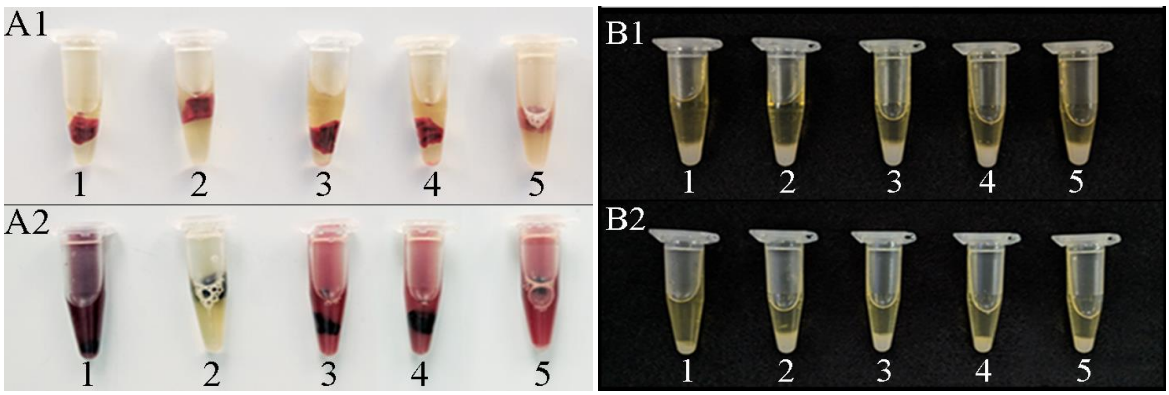

Figure 1. Partially purified extracts produced by bacteria from tempoyak showed thrombolytic activity. The whole blood clots were treated with (1) nattokinase 50 FU, (2) GYP broth, (3) a partially purified extract of G1 isolate, (4) a partially purified extract of G2 isolate, and (5) a partially purified extract of G3 isolate at (A1) 0 hour and (A2) after 6 hours of incubation at 37°C. The euglobulin clots (B) were treated with (1) nattokinase/NK 50 FU, (2) GYP broth, (3) a partially purified extract of G1 isolate, (4) a partially purified extract of G2 isolate, and (5) a partially purified extract of G3 isolate at (B1) 0 hour and (B2) after 2 hours of incubation at 37°C

Figure 2. The partially purified extracts of the bacteria isolated from tempoyak were able to directly degrade fibrin on the agar plate. Wells on the fibrin plate were filled with (A) PBS, (B) nattokinase 50 FU, (C) a partially purified extract of G1 isolate, (D) a partially purified extract of G2 isolate, (E) a partially purified extract of G3 isolate, (F) nattokinase 25 FU, (G) nattokinase 10 FU, (H) nattokinase 5 FU, and (I) nattokinase 2 FU. The plate was incubated at 37 °C for 8 hours

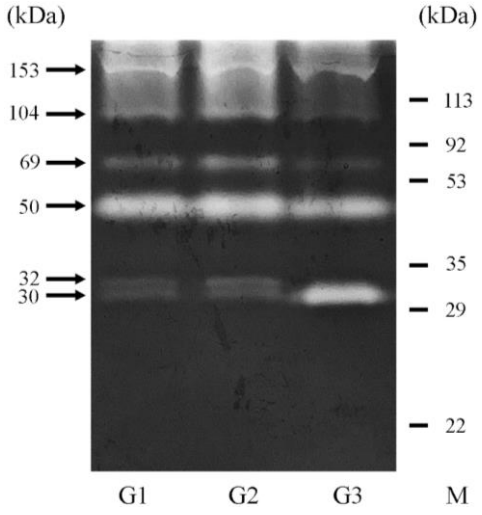

Figure 3. Fibrin zymography of the partially purified extracts of the isolates G1, G2, and G3 demonstrated several zymogram bands. The low-range protein marker (Bio-Rad; 1610305) was used as a protein molecular weight reference. The molecular weight of the protein was estimated by GelAnalyzer. The arrows indicate the estimated molecular weights of the proteins corresponding to the locations where the bands formed

Subsequently, fibrin zymography was performed to analyze the presence of a putative enzyme with fibrinolytic activity. The partially purified extracts of each G1 and G2 showed six clear bands, while the partially purified extract of G3 showed five clear bands, suggesting that several proteins may contribute to the fibrinolytic activity of the three isolates (Figure 3). Since the resultant bands were relatively thick, they were presumed to correspond to strong fibrinolytic enzymes. The clear protein bands at approximately 50 kDa were found in the partially purified extracts of the three isolates. The 50 kDa bands, which showed the strongest fibrinolytic activity, were suspected to be serrapeptidase-like metalloprotease (Salarizadeh et al. 2014) or AKD9-like serine protease (Mahmoud et al. 2021). In addition, all three partially purified isolate extracts formed a clear protein band at 30 kDa, while only two of them (G1 and G2) also showed bands at 32 kDa. These proteins with molecular weights of approximately 30 and 32 kDa are suspected to be serine proteases (Daas et al. 2018). A previous study using *B. subtilis* G8 reported that clear bands at 30.8 kDa and 26.7 kDa were assumed to correspond to subtilisin, a type of serine protease (Pinontoan et al. 2021; Dikson et al. 2022). The other bands, at approximately 69, 104, and 153 kDa, were estimated to represent Cwp22-like proteases (Zhu et al. 2019), serine proteases (Razzaq et al. 2019), or proteases that form complexes with another protein, respectively (Pan et al. 2019).

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Identification of isolated bacteria with potential thrombolytic activity

The three isolates with thrombolytic activity were then identified based on their morphology and biochemical activity (Table 1). Staining was performed to determine the cellular morphology. Three types of staining were used, namely Gram staining, endospore staining, and acid-fast staining. The three isolates were all gram-positive, non-spore-forming, and non-acid-fast *Bacilli*.

Table 1. Bacterial cell and colony morphology identification

	Isolate		
	G1	**G2**	**G3**
Cell morphology			
Gram staining	Positive	Positive	Positive
Shape	Bacilli	Bacilli	Bacilli
Endospore staining	-	-	-
Acid-fast staining	-	-	-
Colony morphology			
Shape	Circular	Irregular	Circular
Height	Flat	Raised	Convex
Periphery	Undulated	Undulated	Entire
Surface	Rough	Glistening	Rugose
Opacity	Opaque	Opaque	Opaque
Color	Yellowish white	Yellowish	Yellowish
Consistency	Buttery	Mucoid	Mucoid
Biochemical characteristic			
Starch hydrolysis	-	-	-
Gelatin hydrolysis	+	+	+
Catalase	+	+	+
Indole test	+	+	+
MR-VP	-	-	-
Carbohydrate fermentation			
Glucose	Acid and gas	Acid and gas	Acid and gas
Lactose	Acid and gas	Acid and gas	Acid and gas
Sucrose	Acid and gas	Acid and gas	Acid and gas
Oxygen demand	Aerobe	Aerobe	Aerobe

Note: (-): negative result; (+): positive result.

The morphologies of the single colonies were observed directly on the agar plate. Commonly studied characteristics of bacterial colonies included shape, height, periphery, surface, opacity, color, and consistency (Hikmawati et al. 2019). Based on our observations, the three isolates shared similar characteristics of being yellowish in color and opaque. However, other aspects of colony morphology were different among the colonies from the three isolates. The appearance of bacterial colonies can be influenced by external factors, such as the presence of other neighboring colonies (Bottery et al. 2019), which highlights the importance of further bacterial identification.

Biochemical activities were tested based on the metabolic activities of the bacteria, including starch hydrolysis, gelatin hydrolysis, catalase, indole production, MR-VP, carbohydrate fermentation, and oxygen demand. These tests were carried out according to Bergey's Manual for bacterial identification. A starch hydrolysis test was conducted to determine the ability of bacteria to produce amylase and oligo-1,6-glucosidase enzymes. These enzymes are involved in the hydrolyzation of starch. The gelatin hydrolysis test was conducted to determine the ability of the bacteria to produce gelatinase proteolytic enzymes. Gelatinase is an enzyme that can hydrolyze gelatin into polypeptides, peptides, and amino acids. The catalase test was carried out to determine the ability of the bacteria to produce catalase enzymes. Based on the results of these tests, the three isolates did not produce amylase but did produce gelatinase and catalase enzymes.

Carbohydrate fermentation tests were carried out to determine the ability of bacteria to ferment certain carbohydrates. The types of carbohydrates used in this study were glucose, lactose, and sucrose. In addition to the carbohydrates, a pH indicator (phenol red) was added to the medium to identify a decrease in pH caused by acid production. In our tests, all three isolates were able to ferment carbohydrates and produce both acid and gas.

The oxygen demand test was carried out to determine the growth characteristics of the bacteria in the presence/absence of oxygen. Based on their oxygen demand, bacteria are classified into obligate aerobes, obligate anaerobes, facultative anaerobes, and microaerophiles. Our results indicated that the three isolates consisted of obligate aerobic bacteria that generally needed oxygen to convert sugars or fats into energy.

The indole production test was conducted to determine the ability of the bacteria to break down the amino acid tryptophan into indole. The enzyme involved in breaking down tryptophan is tryptophanase. MR-VP test was conducted to determine the ability of the bacteria to produce acetyl methyl carbinol via glucose fermentation. The three isolates were able to produce indole, but they did not produce acetyl methyl carbinol.

To identify the bacteria present in the isolates G1, G2, and G3, the results of the morphological observations, staining, and biochemical tests were compared with known results in Bergey's Manual of Systematic Bacteriology. According to Bergey's Manual, the G1, G2, and G3 isolates were thought to correspond to bacteria from the genus *Corynebacterium*. A study by Permana et al. (2021) also

reported the presence of *Corynebacterium* sp. in fermented durian.

Corynebacterium is a genus of gram-positive and aerobic *Bacilli*. *Corynebacterium* spp. can be found in soil, water, plants, and foods. *Corynebacterium* spp. are often used in industrial applications to produce amino acids, nucleotides, and enzymes (Lee and Kim 2018; Sgobba et al. 2018). Although an increasing number of *Corynebacterium* spp. are recognized as probiotics (Menberu et al. 2021; Gladysheva et al. 2021; Menberu et al. 2022), there are indications that some *Corynebacterium* spp. are opportunistic, if not pathogenic, and might be involved in food spoilage as well as cause foodborne diseases (Hahne et al. 2018). Nevertheless, there have been reports of *Corynebacterium*-like microbiota in a wide variety of fermented foods, such as jeotgal from Korea, budu from Thailand and Malaysia, nam-pla from Thailand, belacan from Malaysia, ngapi from Myanmar, and shottsuru from Japan (Narzary et al. 2021). Arguably, *Corynebacterium* spp. are commonly found in fermented foods.

In conclusion, we successfully isolated three bacteria from tempoyak and characterized the thrombolytic and fibrinolytic activities of partially purified extracts produced by those bacteria in this study. The results suggest that the fibrinolytic activity is presumably mediated by various enzymes. Further research is needed to isolate and elucidate the fibrinolytic enzymes so that potent fibrinolytic agents may be developed for use in clinical applications.

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