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ISOLATION AND PURIFICATION OF A FIBRINOLYTIC ENZYME FROM BACTERIAL STRAIN ISOLATED FROM TUAK, AN INDONESIAN PALM WINE

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ABSTRACT

Palm wine, known as tuak in Indonesia, is a traditional alcoholic beverage in Tapanuli region, North Sumatera that is made by fermenting the sugary sap of sugar palm tree (*Arenga pinnata*) and the bark of tree called raru (*Vatica pauciflora* Blume). In this study, we screened 4 bacterial strains from palm wine, and found that T2 strain showed potential fibrinolytic activity. Several steps of purification were conducted including enzyme production, precipitation, dialysis, concentration, and fractionation using anion exchange chromatography. Purified enzyme was characterized for its molecular weight and fibrinolytic action using SDS-PAGE, zymography, plate fibrin, and fibrin hydrolysis assays. Purified enzyme had low molecular weight (26-28 kDa) and possessed dual direct-acting fibrinolytic and fibrinogenolytic activities. These results suggested that palm wine microbial fibrinolytic enzyme may act as plasmin-like activity for orally application in prevention and treatment of thrombosis.

KEYWORDS: **Palm wine, tuak, microbial fibrinolytic enzyme, dual actions, plasmin-like activity**

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INTRODUCTION

Healthy is very important for everyone nowadays. The cost for curing the disease is higher and higher and sometimes makes it not make sense. In 2014, World Health Statistics 2014 reported that the top three causes of the years of life lost (YLL) were ischaemic heart disease, lower respiratory infections and stroke [1]. In Indonesia, cardiovascular diseases the top noncommunicable diseases that causes about 37 % YLL. When the lining of a blood vessel is broken, collagen from endothelial cell is exposed and activates platelets which are in coagulation process form fibrin clot. The blood vessel wall can be broken down the fibrin clot by the main enzyme which is called plasmin. This enzyme is a serine protease group. The process in which a fibrin clot is broken down was called fibrinolysis. Although there are several commercial thrombolytic drugs, like tissue plasminogen activator and streptokinase, but they are expensive and have adverse effects which is bleeding complications related to systemic fibrinogenolysis of normal hemostatic plugs. Several researches have been reported about microorganisms producing fibrinolytic enzymes from various Asian fermented foods, including *Bacillus natto* from natto, a soya bean fermented food from Japan [2], Doen-jang from Korea [3], *Bacillus* sp. CK-1 from Korean fermented food Cheonggukjang [4], *Bacillus licheniformis* KJ-31 from Korean fermented fish called Jeot-gal [5], *B. amyloliquefaciens* DC-4 from douchi, a Chinese fermented food [6], and *Fusarium* sp. from tempe, an Indonesian fermented food [7]. Those enzymes have been proved for their actions as blood vessel thrombosis therapy. Interestingly, most sources of microbes producing fibrinolytic enzymes are from the *Bacillus* genus. Microbial fibrinolytic enzymes from fermented beverages have potential to be developed as drugs to cure of cardiovascular diseases, especially thrombosis. Palm wine is one of the most popular South-East Asian traditional fermented beverages made from various palm trees. In Indonesia, those beverages are known as nira and tuak and mostly produced from the sugary palm tree (*Arenga pinnata*) and coconut juice (*Cocos nucifera*). Nira is a non-alcoholic unfermented beverage that is directly tapped from the sugary sap of palm tree. Tuak is an alcoholic beverage (~3.8%) that made from nira with the addition of the tree bark called raru (*Vatica Pauciflora blume*) during 2 days fermentation [8]. Tuak contains 0.05-2% protein. Hence, microorganisms within nira and tuak may have potentials as the new source of fibrinolytic enzymes. In this study, we screened selected bacterial strains producing fibrinolytic enzymes from popular palm wine in Indonesia, such as tuak and nira. Selected strain was further purified and characterized for determining its actual fibrinolytic action.

MATERIALS AND METHODS

(i) Screening of bacterial strain

Materials used in this research were Indonesian palm wine, known as tuak (fermented beverage with the addition of tree bark called raru (*Vatica pauciflora* Blume) and nira (fermented beverage without raru), from Tapanuli region, North Sumatera province (Indonesia). A 100 µL of each sample from tuak and nira was spread on each petri dish with Luria-Agar/ LA media (tripton 1%, NaCl 0.5 %, yeast extract 0.5%, agar bacto 2%) and incubated at 37°C overnight. Some colonies which were formed from overnight incubated LA media were streaked on skim milk agar/ SMA media (agar bacto 2%, skim milk 5%, NH_4Cl 0.5%, NaCl 0.5%, K2HPO4 0.03%, MgCl2 6H2O 0.01%, and yeast extract 0.2%) and incubated at 37°C overnight. The results were shown by a clear zone on SMA media, four isolates of tuak and two isolates of nira that showing the largest zone were selected and refreshed each of the them on separate SMA media and incubated at 37°C overnight.

(ii) Crude enzyme production

The refreshed selected strains, each was mixed into SM liquid media and incubated in orbital shaker at temperature of 37°C, 120 rpm for 24 hours and for 48 hours. After 24 hours, the results were pipetted into microtiter plate for 250 µL with 4 times treatments each, then measured protein concentration of crude enzymes using ELISA microplate reader at 620 nm wavelength [9]. The remaining crudes in the media were added with Na-azida 0.2% and transfered to conical tube in order to be

centrifugated for 15 minutes at 10.000 ×g at 4°C.

(iii) Fibrin zymography [10]

The chosen strains were screened to detect fibrinolytic activity by zymography using 0.1% w/v fibrinogen substrate. Step in zymography was started by preparing separating gel with 12 % acrylamide concentration and 5% stacking gel, after a while each sample injected into the well with volume about 10-20 µL. Enzyme was dissolved into 2-mercaptoethanol buffer without heating treatment. Then the samples were loaded, running the electrophoresis with 100 Volt and 50 Ampere with the duration about 1.5-2 hours, then suspended the gel in Triton X-100 about 30 minutes, washed by distilled water then continue with digest process by suspended in 50 mM phosphate buffer (pH 8.0) at 37°C, then staining with Coomassie Brilliant Blue (CBB) R-250 about 15 minutes and destaining until getting the visualization of white proteolytic bands with blue gel background.

(iv) Purification of fibrinolytic enzymes [11]

Purification was done by separating supernatant using 12.000 ×g, 10 minutes at 40C centrifugation, then it was filtered with 0.45 µm Sarsted filter, then suspended in ammonium sulphate (80% w/v saturation) overnight at 4°C. Precipitates were resuspended in 50 mM phosphate buffer (pH 7.8) and dialyzed with the similar 20× volume buffer for 24 hours at 4°C. After dialyzed, the samples were freeze-dried and resuspended in 50 mM phosphate buffer (pH 7.8). Then samples were loaded at Hitrap DEAE FF column (GE Healthcare, Amersham) and pre-equilibrated with 50 mM phosphate buffer (pH 7.8). Elution was carried out with a linear ascending gradient of 0-1 M NaCl at a flow rate of 1 mL min^{-1}. Fractions from each column were collected and subjected into further protein quantification and fibrinolytic assay.

(v) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [12]***:***

Molecular weight of enzymes was tested by employing SDS-PAGE with 12% acyrlamide gel and low molecular weight (LMW) marker. Samples were dissolved into sample buffer contains 2-mercaptoethanol, followed by heating treatment at 95°C for 3-5 minutes. Then the samples (10 µL) were loaded and the electrophoresis was run at 100 Volt and 50 Ampere for 1.5 hours. Gel was stained with CBB R-250 and protein was indicated by the blue band with colorless background gel. Determination of molecular weight was measured according to the standard curve of LMW markers. Furthermore, fibrinolytic activity was also determined by using fibrinogen hydrolysis with SDS-PAGE analysis. A total of 2 mg of fibrinogen was mixed with 0.02 µg purified enzyme in 500 µL of 20 mM Tris-HCl (pH 8.0), then the mixture was incubated at 37°C during 0-90 minutes. At each interval of 15 minutes, 20 µL of samples was collected and then analyzed by SDS-PAGE. Hydrolysis of fibrinogen by enzymes was determined.

(vi) Fibrin plate assay [10]

To confirm the fibrinolytic activity, fibrin plate method was applied with 7 mL of 0.3% w/v fibrinogen in 50 mM phosphate buffer (pH 7.8) was mixed equal volume of 2% w/v agarose solution and 0.1 mL of thrombin solution (100 NIH) in petri dish. After the mixture was formed, samples (10 µL) were dropped on the surface and incubated at 37°C for 3 hours. Streptokinase (50 kU) was used as a reference. Fibrinolytic activity was characterized by a clear zone on fibrin plate.

RESULTS

1. Selection of bacterial strains producing fibrinolytic enzyme

There were 4 bacterial strains isolated from palm wine of tuak and nira. Crude enzymes were produced for 24 hours (N1-24, T2-24, and T3-24) and for 48 hours (N1-48, T2-48, T3-48, and T4-48), followed by protein quantification and zymographic screening for detecting fibrinolytic activity. At 24 hours, all crude enzymes had protein concentration >0.8 µg mL^{-1} (Figure 1A). Zymogram profile showed that among strains, T2 strain were positive for fibrinolytic activity (Figure 1B). Therefore, T2 strain was chosen for further enzyme production, purification, and characterization.

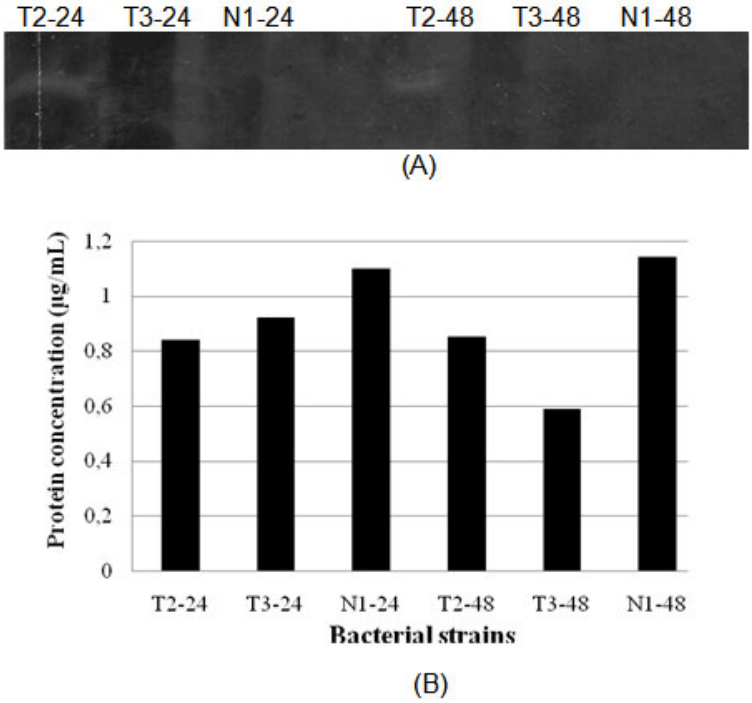

Figure 1

(A) Fibrin zymogram profile (A) and protein concentration (B) from crude enzymes of several bacterial strains isolates from palm wine (T) and nira (N). Clear zone indicated fibrinolytic activity.

2. Purification of fibrinolytic enzyme

Crude enzyme was produced from T2 strain for 24 and 48 hours incubation, followed by several steps of purification including precipitation using ammonium sulphate, dialysis, concentration using freeze drier, and fractionation using FPLC. Figure 2 showed the chromatogram profiles for enzyme purification from dialysates of 24 hours and 48 hours. All eluate fractions were quantified for protein concentration using the Bradford assay (Figure 3). Eluate 24 hours exerted 2 fractions (no 6 and 7) and eluate 48 hours had one fraction (no 4) with the highest protein levels. These selected fractions were further analyzed for their fibrinolytic activities and molecular weight.

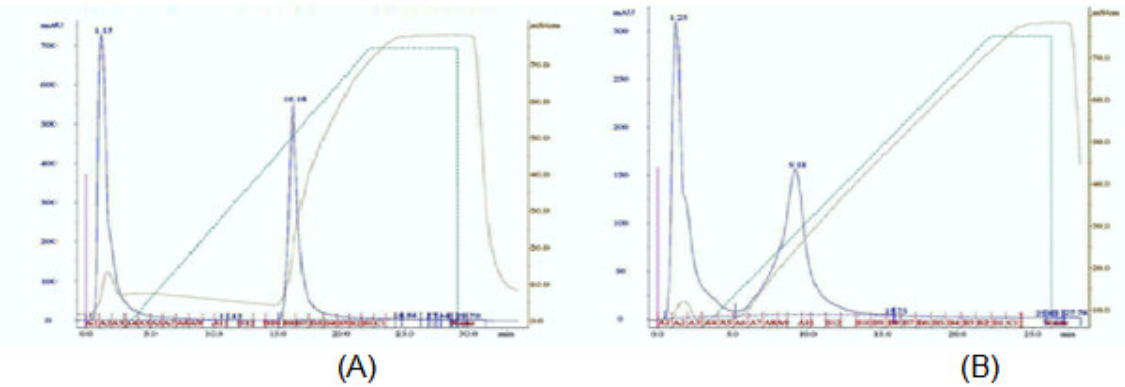

Figure 2

Chromatogram profiles of fibrinolytic enzyme from T2 strain for 24 hours (A) and 48 hours (B) through anion exchange DEAE FF column with gradient elution of NaCl (0-1 M).

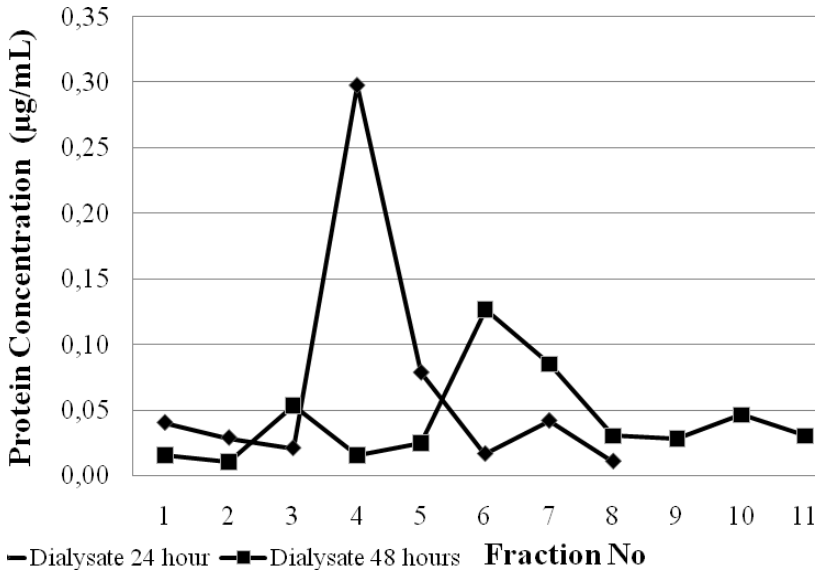

Figure 3

Protein quantification of all eluate fractions from dialysate 24 hours and 48 hours after purification with anion exchange DEAE FF column.

(3) Molecular weight of fibrinolytic enzyme

Molecular weight of fibrinolytic enzyme from selected eluate fractions was determined by SDS-PAGE analysis (Figure 4). Enzymes from both fractions had molecular weight <50 kDa. The molecular weights of enzymes were 28.1 kDa (for eluate 24 hours) and 26.9 kDa (for eluate 48 hours).

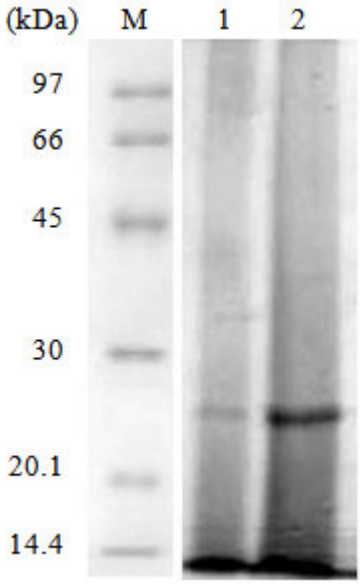

Figure 4

Molecular weight of fibrinolytic enzymes from T2 strains isolated from palm wine by SDS-PAGE analysis. M - LMW marker; lane 1 - eluate 24 hours, lane 2 - eluate 48 hours.

(4) Fibrinolytic activity

To find out whether the eluates of 24 hours and 48 hours had ability to breakdown the fibrinogen directly, SDS-PAGE analysis was applied and the results were described in Figure 5. After hydrolysisn, fibrinogen formed 3 chains, i.e. α, β, and γ, with molecular weights in range of 50-70 kDa. The fibrinogen

hydrolysis revealed that purified enzyme degraded the Aα-chain and Bβ-chains of fibrinogen in 15 minutes and degraded the γ-chain of fibrinogen more slowly. When fibrin was incubated with the enzyme, the Aα- and the Bβ-chains hydrolyzed faster than the γ-chain.

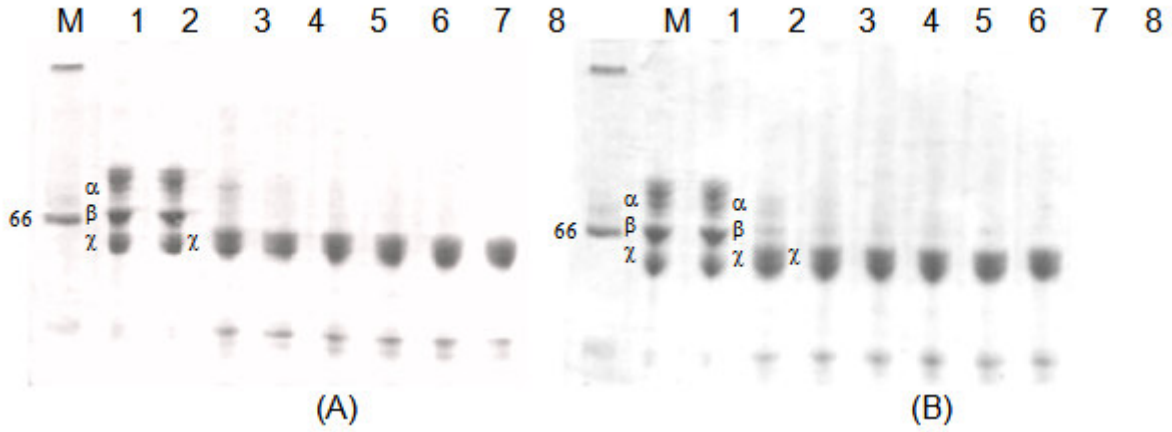

Figure 5

Fibrinolyis activity of eluate 24 hours (A) and 48 hours (B) from T2 strain isolated from palm wine by SDS-PAGE. Lane M - LMW marker, lane 1 - fibrinogen substrate as control, lane 2-8 - fibrinogen after treatment with purified enzyme for 0 minute (lane 2), 15 minutes (lane 3), 30 minutes (lane 4), 45 minutes (lane 5), 60 minutes (lane 6), 75 minutes (lane 7), and 90 minutes (lane 8).

Furthermore, fibrin plate assay was done to confirm whether the purified enzymes had fibrinolytic activity by degrading fibrin directly. Our results showed that only eluate 24 hours exert direct fibrin activity during 0-3 hours incubation at 37°C (Figure 6). Clear zone indicated fibrinolytic activity in fibrin plate, and eluate 24 hours had a clear zone diameter of 1.3 cm. Streptokinase standard did not show fibrinolytic activity for 3 hours incubation.

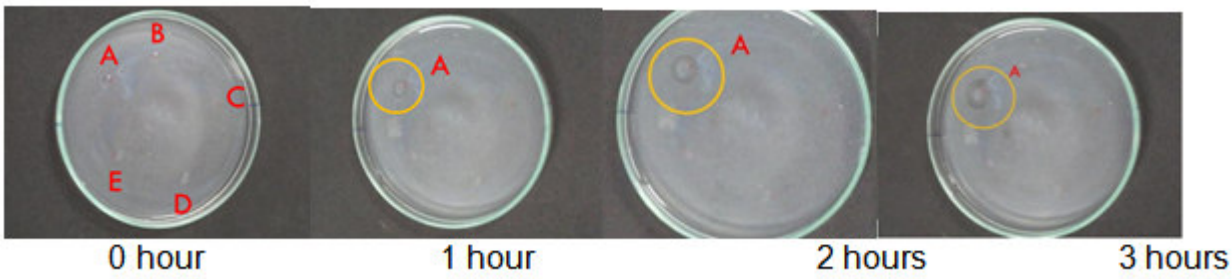

Figure 6

Fibrin plate results of crude enzyme and precipitate from T2 strains isolated from palm wine during 0-3 hours incubation at 37℃. A, eluate 24 hour; B, eluate 48 hours, C, streptokinase (standard); D, dialysate 24 hour; and E, dialysate 48 hours. Fibrinolytic activity was characterized by a clear zone on fibrin plate.

DISCUSSION

Microbial fibrinolytic enzymes, especially those from generally recognize as safe (GRAS) microorganisms, have the potential to be developed as functional food additives and drugs to prevent or cure thrombosis and other related diseases. We explored whether Indonesian traditional fermented beverages may be potential as a new microorganism source for production of fibrinolytic enzymes. Several regions in Indonesia produce fermented beverages like palm wine (tuak and nira). In this study, palm wine was taken from Tapanuli, North Sumatera province

(Indonesia) that has been known as the original region for palm wine production for decades. Our results demonstrated that among all candidate strains from palm wine, T2 strain was chosen for further enzyme production, purification, and characterization due to its fibrinolytic activity and protein content (Figure 1-3). SDS-PAGE profile showed that purified enzymes (eluates of 24 hours and 48 hours) had low molecular weights at range of 26-28 kDa (Figure 4). Other study reported that bacterial strain isolated from gembus, an Indonesian traditional fermented food had fibrinolytic enzyme with a molecular weight of 20 kDa [12]. Several reports also demonstrated that the molecular weights of fibrinolytic enzymes that were successfully isolated from various traditional fermented foods were at range of 20-100 kDa. For example, CH51 isolated from Korean fermented soy food Cheonggukjang was 27 kDa [4], KJ-31 from Korean seafood Jeot-gal was 37 kDa [5], and ATFE from Chive was 90 kDa [13].Hence, our data showed a promising indication that T2 bacterial strains isolated from Indonesian palm wine may offer a potential candidate for fibrinolytic enzyme source. Interestingly, with low molecular weight, the enzymes may be suitable for oral administration in the management of cardiovascular diseases, including thrombosis. Most therapeutic agents including enzymes are grouped in low-molecular weight (LMW) compounds which are administered systemically and exhibit non-specific biodistribution profile, short plasma circulation time, and rapid systemic elimination [14]. For oral administration, these LMW compounds are mainly considered due to the ability of compounds dissolve and reach the target. However, the use of LMW compounds were thought to be associated with side effect and low efficacy. The fibrinolytic enzymes which belong to serine protease generally are active at neutral and alkaline pH (optimum between pH 8.0 and 10) and their molecular weights are between 27.7 and 44 kDa [15]. The fibrinogen monomer contains three chains: α, β, and γ [16]. During fibrinogen hydrolysis, the purified enzymes from palm wine bacterial strain cleaved rapidly both α- and β-chains of fibrinogen to result in fibrin-digested products (FDPs) within 15 minutes, followed by the slower degradation of γ-chains (Figure 5). This result is in line with fibrinolytic enzyme KJ-31 isolated from Korean traditional Jeot-gal [5] and *Rhizopus chinensis* 12 [17]. From previous study by Hwang *et al*. [5], fibrinolytic enzyme purified from *B. licheniformis* KJ-31 also showed the degradation of fibrinogen were cleaved rapidly to make fibrin-digested products within 15 minutes. However, fibrinolytic enzyme from ATFE isolated from chive significantly degraded α-chain of fibrinogen in 10 minutes [13], indicating that fibrinolytic enzyme had relatively high substrate specificity to fibrin(ogen). Fibrinolytic action of purified enzymes from palm wine T2 strain on direct degradation of fibrin was also verified by using plate fibrin assay (Figure 6). Eluate 24 hours from T2 strain shared similar direct fibrin degrading activity with fibrinolytic enzyme isolated from Korean traditional Jeot-gal with clear zone diameter of 1.36 cm [5]. In general, there are two classes of fibrin(ogen)olytic enzymes, including the α(β)-fibrinogenase known as zinc-metalloproteinase and the β-fibrinogenase known as thermostable serine proteinase [13]. Fibrinolytic enzyme from T2 strain possesses dual direct-acting fibrinolytic and fibrinogenolytic actions, as it acts via direct cleavage of fibrin, not by plasminogen activator. The presence of plasminogen activators unnecessary for the enzymes to hydrolyze both fibrin and fibrinogen [18]. Other advantage, by direct action, the secondary effects such as platelet activation related to plasmin formation can be avoided.Furthermore, our results also indicated that streptokinase standard had no clear zone on fibrin plate during 3 hours incubation (Figure 6). It is known that streptokinase produced by *Streptococcus* sp. has no proteolytic activity by itself and activates plasminogen by an indirect mechanism [19]. Streptokinase belongs to plasminogen activators and plays role in fibrin degradation by activating plasminogen into plasmin. Therefore, it works through the indirect cleavage of fibrin and needs longer incubation time for the cleavage of fibrin.Most commercial drugs for antithrombotic therapy have been reported for their adverse effects, including hypersensitivity, haemorrhage, skin rashes, alopecia, and purpura [20]. Microbial fibrinolytic enzymes, especially those from

GRAS microorganisms, have potential to be developed as functional foods and alternative drugs to prevent or cure thrombosis and other related diseases.

CONCLUSION

Potential fibrinolytic enzyme was isolated from Indonesian palm wine T2 bacterial strains. The enzyme has been partially characterized for its moleculer weight and fibrinolytic action. Enzyme exerts dual direct-acting fibrinolytic and fibrinogenolytic actions with low molecular weight (26-28 kDa). These results suggest that palm wine microbial fibrinolytic enzyme may be potentially used for orally application for management of cardiovascular diseases particularly thrombosis. Further biochemical characteristics of fibrinolytic enzyme and molecular identification of palm wine T2 strains might be done in the future.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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