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Thrombolytic protease characterization from leaves and fruit flesh of the *jernang* rattan plant (*Daemonorops draco*)

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ABSTRACT Thrombolytic agents are used for thrombolytic therapy to dissolve blood clots that form in a blood vessel. All currently used thrombolytic agents have unfavorable shortcomings, such as gastrointestinal bleeding, allergic reactions, and thrombolytic agent resistance, treatment for some of which can be quite expensive. As a result, the search for thrombolytic agents derived from plants is currently taking place. Some plants have been discovered to contain protease enzymes with thrombolytic activity; pharmaceuticals derived from plants are believed to be safer. *Jernang* rattan (*Daemonorops draco*) is a plant of the Arecaceae family and is known to produce resin. *Jernang* rattan resin is also known to have antioxidant, antiseptic, antitumor, antimicrobial, and cytotoxic activity, but very limited information on proteolytic activity of the protease from this plant. This research aims to isolate proteases from the leaves and fruit flesh of the rattan *jernang* plant (*D. draco*) and to investigate the proteolytic activity of the isolated proteases. The protease was isolated from the leaves and the fruit flesh, and then partially purified by ammonium sulfate precipitation. The radial caseinolytic assay showed that protease in a 60% ammonium sulfate fraction gave a clear zone, with diameters of 1.4 cm and 1.8 cm for the protease isolated from leaves and fruit flesh, respectively. A Folin-Ciocalteau assay showed that the enzymes isolated were able to hydrolyze casein and release L-tyrosine, with activity of 0.158 U/mL and 0.174 U/mL for the protease from the leaves and fruit flesh, respectively. A fibrinogenolytic assay showed that the protease from the fruit flesh hydrolyzed the A-α, B-β and the γ chain of human fibrinogen, while the protease from the leaves hydrolyzed the A-α and γ chain. Both proteases were inhibited by 56% by phenylmethylsulfonyl fluoride (PMSF), indicating that the enzymes are serine proteases. Based on the assay results obtained, it can be concluded that proteases isolated from the leaves and fruit flesh have potential as thrombolytic proteases.

KEYWORDS *Daemonorops draco*; Fibrinogenolytic; Protease; Serine protease; Thrombolytic

1. Introduction

Research on plants for medical purposes has been growing over time. The research is based on the specific components contained in the plants that have better therapeutic activity than synthetic drugs (Da-Yong and Ting-Ren 2019). One of the plant components that have been investigated for their therapeutic purposes is protease with thrombolytic activity. Thrombolytic activity is the ability of a compound to dissolve blood clots in blood vessels (Luzak et al. 2016; Merlyn Keziah and Subathra Devi 2018). Nowadays, many used thrombolytic agents are proteases that hydrolyze peptide bonds in protein. Drugs or therapeutic agents that have a similar mechanism to protease have drawbacks, such as side effects and contraindications that can trigger more complex problems (Rajeswari and Vidhya 2017). Therefore, the research on thrombolytic proteases derived from plants is essential since drugs obtained from plants are considered to be safe. Besides that, plant proteases have accessible availability and activity across a wide range of pH, temperature, substrates and are also useful for numerous therapeutic applications (Silva-López and Gonçalves 2019).

In this research, protease from *jernang* rattan plant *Daemonorops draco* was investigated. *Daemonorops draco* grows in Malaysia, Thailand, and Indonesia. Of the approximately 530 types of rattan plants in the world, Indonesia has 316 species spread across the islands of Sumatera and Kalimantan. The *jernang* rattan plant belongs to the Arecaceae family which is a rattan plant group (areca nut) of the *Daemonorops* genus. The resin of *jernang* rattan fruit is known by its commercial name ”Dragon’s blood” due to its deep red color. Many reports of resin utilization for medical purposes such as antimicrobial, an-

tioxidant, antitumor, and antithrombosis have been published (Wahyuni et al. 2018; Yusnelti and Muhaimin 2019; Xia et al. 2006). The resin is known to contain flavonoids (Yi et al. 2012). However, to date, no reports regarding *jernang* rattan protease. The utilization of the *jernang* rattan plant is still limited to the commercial value of its resin. High resin value can be a target of competition in economic and medical aspects. Therefore, research on the content and bioactivity of *jernang* rattan resin can serve as the basis for increasing the potential of using the other parts of the *jernang* rattan plant, such as leaves and fruit flesh. As a result, the *jernang* rattan plant's use can be expanded.

This research aims to isolate proteases from the leaves and fruit flesh of *jernang* rattan and to investigate their activities. Proteases isolated from leaves and fruit flesh of *jernang* rattan were partially purified by ammonium sulfate precipitation and assayed for their protease and fibrinogenolytic activity.

2. Materials and Methods

2.1. Preparation of enzyme extract

The enzyme extract was prepared based on the method of Sharmila et al. (2012) with modification. Leaves and fruit flesh of *jernang* rattan plant were obtained from West Aceh Regency. The leaves used were light green-colored, while the fruit flesh was taken from the old *jernang* rattan trees. The raw materials were washed to remove the impurities then cut into smaller sizes and stored in the freezer. The frozen materials were ground using a blender in 0.1 M of phosphate buffer pH 7.0. The homogenate was centrifuged at 7,500× g for 15 min at 4 °C, and the supernatant was collected as the crude extract of the enzyme. The crude enzyme was partially purified by ammonium sulfate precipitation with a gradual addition of ammonium sulfate to reach 60% saturation by stirring it at 4 °C. The mixture was incubated for 12 h at 4 °C then centrifuged at 7,500× g for 45 min at 4 °C to collect the enzyme. The precipitated enzyme was dissolved in 0.1 M Tris-HCl buffer pH 7.4 and dialyzed in a cellophane tube with 10 kDa cut off against 10 mM Tris-HCl buffer pH 7.4 for 12 h at 4 °C by slowly stirring. The buffer was replaced periodically every 4 h to maintain the buffer pH. Protein concentration was determined by Bradford assay (Bradford 1976).

2.2. Caseinolytic activity assay

The assay was performed according to the radial caseinolysis using agarose method (Saksela 1981). Briefly, 5 mL of 1% (w/v) skim milk in phosphate buffer 50 mM (pH 7.4) was mixed with 10 mL of agarose solution (2%) in the same buffer. The homogenized mixture was poured into a sterilized petri dish and incubated at room temperature until polymerized and then paper disks were placed on the top of agarose gel. As much as 10 µL enzyme extract was dripped on the paper disk, then the plate was incubated at 37 °C for 18 h.

2.3. Fibrinogenolytic activity assay

The assay was performed to investigate the fibrinogenolytic activity of isolated protease according to the method of Matsubara et al. (2000). As much as 10 µg human fibrinogen (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 200 µL of 20 mM Tris-HCl buffer pH 7.4. The assay was carried out by using a 60% ammonium sulfate fraction. The fibrinogen-buffer mixture was incubated with the protease enzyme isolated from the leaves and fruit flesh, 20 µL each. The mixture was incubated at 37 °C for 0, 60, 120, 180, and 240 min. The reaction was stopped by adding 20 µL SDS-PAGE sample buffer. The samples were analyzed by using SDS-PAGE.

2.4. Protease activity assay

Protease activity was measured based on a non-specific protease assay using Folin-Ciocalteu reagent and casein as substrate (Cupp-Enyard and Aldrich 2008). As much as 5 mL of 0.65% w/v casein solution was added into a test tube and incubated for 5 min in a 37 °C water bath. A total of 1 mL of partially purified protease was added into the casein solution and the incubation continued for 10 min. The reaction was stopped by adding 5 mL of TCA reagent (Sigma-Aldrich, St. Louis, MO, USA) into the solution. Furthermore, 5 mL of 500 mM sodium carbonate and 1 mL Folin-Ciocalteau reagent (Sigma-Aldrich, St. Louis, MO, USA) were added into the sample solution and then incubated for 30 min at 37 °C in an orbital shaker. Then, the absorbance was measured at 660 nm wavelength by using UV-Vis spectrophotometer.

2.5. Effect of inhibitors on protease activity

The effect of inhibitors was studied in 20 mM Tris-HCl buffer pH 7.4 containing 20 mM protease inhibitors i.e. phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), and ethylene glycol tetraacetic acid (EGTA) that were purchased from Sigma-Aldrich (St. Louis, MO, USA). As much as 1 mL of the enzyme sample was mixed with the inhibitor and incubated for 30 min at 37 °C. The activity of the protease was measured by Folin-Ciocalteu assay using casein as a substrate (Cupp-Enyard and Aldrich 2008).

3. Results and Discussion

3.1. Enzyme partial purification

The enzyme was partially purified from crude extract using ammonium sulfate precipitation and the protein concentration was determined using the Bradford assay (Bradford 1976). The activity of the enzyme isolated from both leaves and fruit flesh is presented in Figure 1. The protein concentration increased as the ammonium sulfate saturation level increased. The 60% ammonium sulfate fraction was used for activity measurements because it had a relatively high protein concentration.

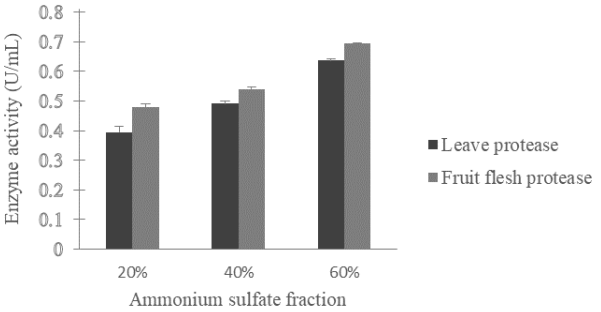

FIGURE 1 Protein concentration in ammonium sulfate fractions. Results are presented as an average of two measurements with a standard deviation.

3.2. Protease enzyme activity

3.2.1 Caseinolytic activity of protease enzyme

Caseinolysis is the ability of the protease to hydrolyze casein, a protein found in milk. Caseinolytic activity assay by radial caseinolytic method is a qualitative assay for studying protease activity. The hydrolyzing ability of the protease was demonstrated by the formation of a clear zone in the area where the protease was added (Zhang et al. 2021). The results of the caseinolytic assay are shown in Figure 2.

The results indicated that proteins isolated from leaves and fruit flesh of the *jernang* rattan plant were able to hydrolyze casein which was a characteristic of protease. The diameter of the clear zone formed on agarose-casein media increased along with the amount of enzyme added as shown in Table 1.

3.2.2 L-tyrosine release assay

The activity of protease isolated from leaves and fruit flesh was also determined by L-tyrosine release assay using Folin-Ciocalteu reagent and casein as a substrate. The results showed that enzymes isolated from leaves and fruit flesh were able to hydrolyze casein and release L-tyrosine with the activity of 0.159 U/mL and 0.174 U/mL respectively (Figure 3). Tyrosine release indicated that protease activity was present.

3.2.3 Fibrinogenolytic activity of protease enzyme

The fibrinogenolytic activity of protease was analyzed based on its ability to degrade human fibrinogen using SDS-PAGE. Fibrinogen is a dimeric plasma glycoprotein composed of three disulfide chains, namely A-α, B-β, and γ. The molecular weight of A-α, B-β, and γ chains were 63, 56, and 47 kDa, respectively. These three chains are linked by disulfide bonds with the N-terminal of the E domain (Pepe et al. 2016). For the fibrinogenolytic assay, each protease isolated from leaves and fruit flesh of *jernang* rattan was incubated with human fibrinogen, and the degradation pattern at specific time points was observed by SDS-PAGE as shown in Figure 4 and Figure 5.

From the SDS-PAGE gel, it could be seen that the protein isolated from the leaves was able to completely hy-

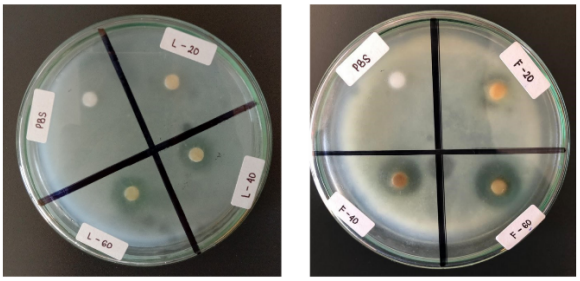

FIGURE 2 Caseinolytic activity assay of enzymes isolated from leaves (left) and fruit flesh (right) of *jernang* rattan plant. PBS = 50 mM Phosphate Buffer Saline pH 7.4, L = enzyme isolated from the leaves, F = enzyme isolated from the fruit flesh. The numbers 20, 40, and 60 represent the percentage of ammonium sulfate saturation levels.

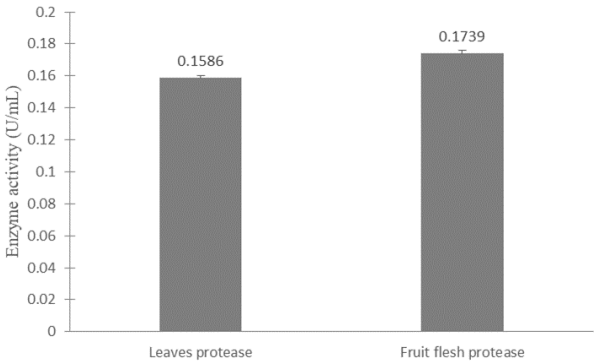

FIGURE 3 Protease activity based on the L-tyrosine released. Results are presented as an average of two measurements with a standard deviation.

TABLE 1 Radial caseinolytic assay of ammonium sulfate fractions.

Enzyme sources	Ammonium sulfate fraction	Amount of enzyme added (µg)	Clear zone diameter (cm)
Jernang Rattan Leaves	20%	0.139	-
	40%	0.382	1.2
	60%	0.587	1.4
Jernang Rattan Fruit Flesh	20%	0.474	1.4
	40%	0.657	1.6
	60%	0.672	1.8

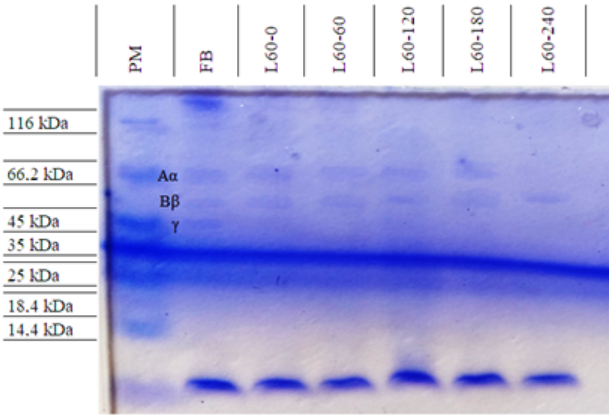

FIGURE 4 Fibrinogenolytic activity assay of protein isolated from *jernang* rattan leaves. The fibrinogen degradation profile was observed at time 0, 60, 120, 180, and 240 min of incubation. FB is human fibrinogen as a control.

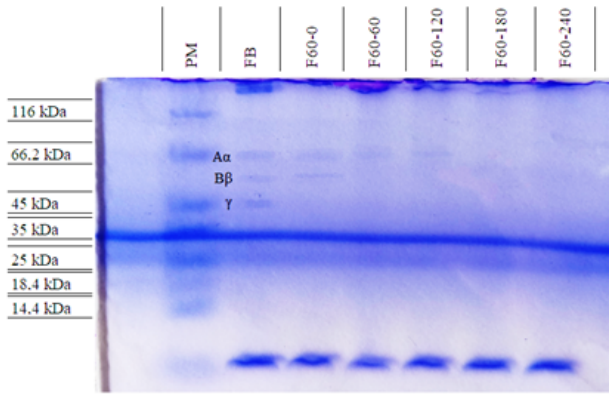

FIGURE 5 Fibrinogenolytic activity assay of protein isolated from fruit flesh of *jernang* rattan. The fibrinogen degradation profile was observed at time 0, 60,120, 180, and 240 min of incubation. FB is human fibrinogen as a control.

drolyze the A-α subunit of human fibrinogen in 240 min, while the γ subunit was hydrolyzed immediately. However, the protein could not hydrolyze the B-β subunit of human fibrinogen for incubation time applied in the experiment. Meanwhile, the enzyme isolated from the fruit flesh of *jernang* rattan hydrolyzed the subunit γ immediately, and hydrolyzed the B-β subunit and the A-α subunit completely after 60 min and 180 min incubation, respectively. Untreated human fibrinogen (FB) was used as a negative control. Hydrolysis patterns of human fibrinogen vary based on the origin of the protease that hydrolyzes it as shown in Table 2.

The presence of fibrinogen degradation products has a relationship with the ability of plasmin to bind to the E domain of fibrin and fibrinogen, which results in a competitive protein interaction (Khavkina et al. 1995). In the experiments on plasmin hydrolysis of stabilized fibrin, it was concluded that the interaction of domain E with the dimer fragment D can prevent the N-terminal groups in the α-, β-, and γ- chains from further degradation (Khavkina et al. 1995). This can be demonstrated in the mechanism of thrombin activity on fibrinogen by removing two pairs of fibrinopeptides A and B, thus opening the polymerization site of the E domain (Khavkina et al. 1995).

Several key mechanisms of proteolytic enzymes in hydrolyzing fibrinogen degradation have been explained, including defibrinogenation, inhibition of platelet aggregation, and disorders of blood coagulation cascade (Gogoi et al. 2018). It shows that the lunatrombase enzyme, a protease isolated from the Leucas indica plant, can inhibit platelet aggregation by fibrinogen due to its ability to inhibit the formation of thrombin and the blood clotting factor protein Fxa (Gogoi et al. 2018).

Based on the mechanisms described above, it can be interpreted that the ability of the *jernang* rattan proteases to hydrolyze A-α, B-β, and γ chains of fibrinogen is possibly due to the interaction of the proteases with the poly-

TABLE 2 Comparison of fibrinogenolytic activity of some plant proteases.

Plant	Part of the plant	Fibrinogenolytic activity of protease	Reference
Aster yomena	Leaves	Cleaved α, Aα and γ-γ chains of fibrin and fibrinogen.	Choi et al. (2014)
Solanum tuberosum	Leaves	Effectively hydrolyzed β subunit and partially hydrolyzed α and γ subunits of human fibrinogen.	Pepe et al. (2016)
Artocarpus heterophyllus	Latex	Effectively hydrolyzed α subunit and partially hydrolyzed β and γ subunits of human fibrinogen.	Siritapetawee et al. (2012)
Codium fragile	Whole part	Effectively hydrolyzed fibrin and fibrinogen, preferentially degrading α- and Aα chains, followed by g-g, and g-chains. Slower degradation of Bβ and β-chains.	Choi et al. (2013)
Clerodendrum colebrookianum	Leaves	Hydrolyzed -αβ chain of human fibrinogen/fibrin.	Gogoi et al. (2019)
Allium tuberosum	Whole part	Hydrolyzed only the Aα-chain of human fibrinogen.	Chung et al. (2010)
Euphorbia hirta	Latex	Hydrolyzed Aα and a-chains, followed by Bβ and β, g and g-g chains of fibrinogen.	Patel et al. (2012)
Daemonorops draco	Leaves	Hydrolyzed the A-α and γ chain of human fibrinogen.	This research
Daemonorops draco	Flesh fruit	Hydrolyzed the A-α, B-β, and γ chains of human fibrinogen.	This research

merization site of domain E on fibrinogen. Such interaction leads to the hydrolysis of the N-terminal group of α-, β-, and γ- chains by the proteases. This mechanism is a characteristic of proteases that inhibit platelet aggregation in fibrinogen. However, further studies are still needed to reveal the fibrinogenolytic mechanism of these proteases.

3.3. Effect of protease inhibitor

The effect of various protease inhibitors on enzyme activity was investigated by measuring residual enzyme activity after incubating the enzyme with each inhibitor for 30 min at 37 °C. The results are summarized in Figure 6. PMSF inhibited the activity of the enzymes isolated from leaves and fruit flesh by 56%. Meanwhile, EGTA slightly inhibited the enzyme activity while EDTA did not significantly do so.

Phenylmethylsulfonyl fluoride (PMSF) is known as a serine protease inhibitor that acts by sulfonylation reactions on the Oγ atom of the serine residue on the catalytic site of the enzyme, thereby eliminating the active site of the protease enzyme and irreversibly inhibiting the protease enzyme (Sharma and Radha Kishan 2011). Ethylenediaminetetraacetic acid (EDTA) inhibits metalloenzymes by chelating the divalent cations of enzymes thereby disrupting the stability and function of the enzymes (Fathimah and Wardani 2014). Ethylene glycol tetraacetic acid (EGTA) inhibits calcium-dependent enzymes such as trypsin and chymotrypsin because EGTA has a high binding affinity to Ca^{2+} ions (Chuang et al. 2013).

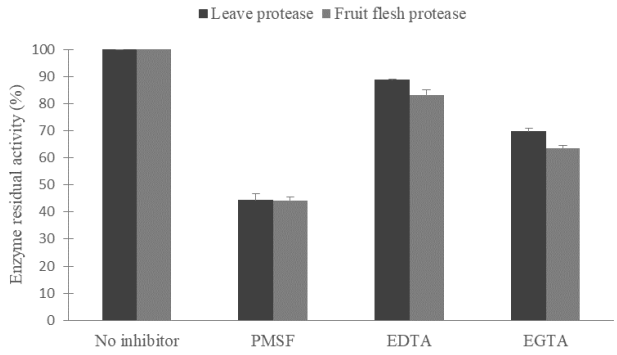

FIGURE 6 Effect of various inhibitors on enzyme activities. Results are presented as an average of two measurements with a standard deviation.

Based on the residual activity measurement, it was shown that the addition of PMSF resulted in low residual enzyme activity compared to the addition of EDTA and EGTA. Therefore, it can be concluded that the protease isolated from the leaves and fruit flesh of *jernang* rattan is likely to be a serine protease. Many studies on the effect of inhibitors on enzymes showed that most plant proteases are serine proteases (Siritapetawee et al. 2012; Patel et al. 2012; Choi et al. 2013, 2014; Pepe et al. 2016; Troncoso et al. 2022).

4. Conclusions

The results in this work suggested that the enzymes isolated from leaves and fruit flesh of *jernang* rattan plants are serine proteases that have a good potential as thrombolytic proteases. They showed the fibrinogenolytic activity by hydrolyzing the A-α, B-β, and γ chains of human fibrinogen. However, further research is still needed to investigate their ability to dissolve blood clots and to study the detailed mechanism in degrading fibrinogen/fibrin.

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Authors' contributions

RFK and MMM designed the study. UYL carried out the laboratory work. All authors analyzed the data and wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

No competing interests were disclosed.

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