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ARTICLE

PROTEASE FROM *Styrax paralleloneurum* LEAVES AND ITS THROMBOLYTIC ACTIVITY ASSAY

[*Protease dari Daun Styrax paralleloneurum dan Uji Aktivitas Trombolitiknya*]

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ABSTRACT

Thrombotic disease is one of the leading causes of death worldwide and enzymatic thrombolytic therapy remains the best way to achieve recanalization. Enzymatic thrombolytic agents used today have undesirable side effects, and some are expensive. Research for herbal protease enzymes was conducted because plant drugs are known to be safer. This study aimed to obtain protease enzymes from frankincense leaves and to test their thrombolytic activity. Enzyme concentration was determined by the Bradford method, and the results showed that the highest enzyme concentration was 1.6812 g/µL at 40% ammonium sulfate fraction. A quantitative assay of protease activity using Folin & Ciocalteu's phenol reagent and casein as substrate showed that 1.2609 mg of enzyme hydrolyzed 32.5 mg of casein and released 0.0980 mg of L-tyrosine. The fibrinogenolytic activity of protease against human fibrinogen tested by SDS-PAGE showed that the protease could hydrolyze fibrinogen γ-chain within 0 minutes and β-chain within 60 minutes, while α-chain could not be hydrolyzed until 720 minutes. The inhibitor effect assay showed that the protease was a serine protease. This study concluded that the protease enzyme from frankincense leaves has the potential to be used as a thrombolytic agent.

Keywords: human fibrinogen, *Styrax paralleloneurum*, thrombotic, thrombolytic agent, serine protease

ABSTRAK

Penyakit trombotik merupakan salah satu penyebab kematian terbanyak di seluruh dunia dan terapi trombolitik enzimatik tetap menjadi cara terbaik untuk mencapai rekanalisasi. Agen trombolitik enzimatik yang digunakan saat ini memiliki efek samping yang tidak diinginkan, dan beberapa di antaranya mahal. Penelitian enzim protease herbal dilakukan karena obat-obatan dari tanaman diketahui lebih aman. Penelitian ini bertujuan untuk mendapatkan enzim protease dari daun kemenyan dan menguji aktivitas trombolitiknya. Konsentrasi enzim ditentukan dengan metode Bradford, dan hasilnya menunjukkan bahwa konsentrasi enzim tertinggi adalah 1,6812 g/µL pada fraksi amonium sulfat 40%. Uji kuantitatif aktivitas protease menggunakan reagen fenol Folin & Ciocalteu dan kasein sebagai substrat menunjukkan bahwa 1,2609 mg enzim menghidrolisis 32,5 mg kasein dan melepaskan 0,0980 mg L-tirosin. Aktivitas fibrinogenolitik protease terhadap fibrinogen manusia yang diuji dengan SDS-PAGE menunjukkan bahwa protease tersebut dapat menghidrolisis rantai γ fibrinogen dalam waktu 0 menit dan rantai β dalam waktu 60 menit, sedangkan rantai α tidak dapat dihidrolisis hingga menit ke-720. Uji efek penghambatan menunjukkan bahwa protease tersebut merupakan protease serin. Penelitian ini menyimpulkan bahwa enzim protease dari daun kemenyan berpotensi untuk digunakan sebagai agen trombolitik.

Kata kunci: *Agen trombolitik, fibrinogen manusia, Styrax paralleloneurum, trombotik, protease serin*

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INTRODUCTION

Thrombolytics are drugs used to dissolve a thrombus or blood clot that forms in a blood vessel and reopen an artery or vein. Among the commercially available thrombolytic agents, uPA and tPA are generally safe but very expensive (Akhtar *et al*., 2017). All currently used thrombolytic agents have undesirable side effects, such as gastrointestinal bleeding, allergic reactions, and resistance to thrombolytic agents (Kim *et al*., 2015; Collen & Lijnen, 2005). Therefore, several studies have been conducted to obtain fibrinolytic enzymes for use as safer thrombolytic agents. The use of proteases as thrombolytic agents has been investigated in several plant species. Research on leaf and root extracts of Moringa oleifera (Moringaceae) showed that the roots of this plant have fibrinolytic and fibrinogenolytic activities (Banik *et al*., 2018). Research on the sap of Artocarpus heterophyllus (jackfruit) with isolated and purified protease also showed fibrinogenolytic activity, and it was later found that the protease belongs to the serine protease type (Siritapetawee *et al*., 2012). Protease sources that have the potential to be thrombolytic agents need to be researched further, because natural thrombolytic agents have higher fibrin specificity, longer half-life in cell plasma, and have higher ability to dissolve clots in blood vessels (Nikitin *et al*., 2021; Frias *et al*., 2021; Maqsood *et al*., 2021).

Frankincense is a plant of the Styracaceae family that has been extensively studied for its therapeutic properties. Based on several previous studies, it is known that the Styracaceae family has the potential to become therapeutic agents, such as Styrax pohlii, which has antibacterial properties, and Styrax benzoin, which has antibacterial, anti-biofilm, and anticancer properties (Jayusman, 2014; Bertanha *et al*., 2013). A family species of Styracaceae, Styrax parralleloneurum, is an endemic plant from North Sumatra, Indonesia. This plant is widely cultivated, and the local people usually harvest its resin. Meanwhile, using other plant parts, such as leaves, still needs to be researched for their medicinal potential, such as their potential as a thrombolytic agent. This finding will increase the diversity of natural sources of thrombolytic agents that can be used in the medical world. This study aims to obtain protease enzymes from frankincense leaves by performing extraction and purification, identifying and characterizing proteases, and testing thrombolytic protease activity qualitatively and quantitatively.

MATERIALS AND METHODS

Materials

The frankincense leaves used were intact, light green, 5-6 cm long, and 1.5-2.0 cm wide, from Humbang Hasundutan Regency, North Sumatra. Phosphate buffer, skimmed milk, casein, sodium chloride, acetic acid, and glycerol were purchased from CV Amor, Indonesia. Bovine serum albumin (BSA), agarose, ammonium sulfate, Folin & Ciocalteu's reagent, Bradford's reagent, Tris (hydroxymethyl) aminomethane, hydrochloric acid, human fibrinogen, trichloroacetic acid, sodium carbonate, phenyl methyl sulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), ethylene glycol bis-(2-aminoethyl)-N, N, N', N'-tetraacetic acid (EGTA), sodium dodecyl sulfate (SDS), bis-acrylamide, ammonium persulfate, tetramethylethylenediamine (TEMED), Triton X100, Coomassie Blue R-250, acetic acid, β-mercaptoethanol, bromophenol blue were purchased from Sigma Chemical.

Protease Enzyme Extraction

Forty grams of frozen frankincense leaves were mixed with 200 mL of phosphate buffer (0.5 M) at pH 7.4, pulverized using a blender, and stirred for 1 hour with a magnetic stirrer. After mixing, it was centrifuged for 15 minutes at 4°C and 8,500 rpm to obtain a supernatant containing crude protease enzyme extract. The extraction was performed in two repetitions. The protease enzyme concentration was determined by the Bradford method.

Radial Caseinolysis

Protease activity was determined qualitatively by the radial caseinolysis assay by mixing 5 mL of 1% (w/v) skimmed milk solution in 10 mL phosphate buffer (50 mM, pH 7.4) with 2.5% (w/v)

agarose solution in the same buffer (Gad *et al*., 2014). Skimmed milk consists of about 80% casein (Kailasapathy, 2016), which is needed for this assay, whereas milk still contains fat that can interfere with the reaction and observation. The homogeneous mixture was poured into a Petri dish and left until solidified. Then, a sterile paper disc was placed on the test plate. The sample solution with a concentration of 0.9124 µg/µL was dropped onto the sterile paper discs in different volumes (0.5 µL, 1.0 µL, and 1.5 µL) using a micropipette. As a control, 1.0 µL of phosphate buffer 50 mM at pH 7.4 was added to sterile paper discs. The test plate was incubated at 37°C for 18 hours. Radial caseinolysis was performed to determine the presence of proteases in the Frankincense leaves. The formation of a clear zone around the paper disc indicated the protease activity.

Partial Purification

The ammonium sulfate precipitation method partially purified the protease enzyme, followed by dialysis. The crude protease extract solution was gradually precipitated with solid ammonium sulfate $(NH4)_2SO_2$ (saturation of 20%, 40%, 60%) at 40°C and centrifuged at 8,500 rpm at 40°C for 25 minutes. The resultant pellets were enzyme precipitates that were diluted in 0.1 M Tris-HCl buffer (pH 7.0) for dialysis using a cellophane tube with a cut-off of 10 kDa. Dialysis was performed by immersing the tube containing the sample in 1 L of Tris-HCl buffer (0.001 M, pH 7.0) for 12 hours while slowly stirring with a magnetic stirrer at 40°C with periodic 1 L buffer changes every 4 hours. The concentration of the protease enzyme from the dialysis was ascertained using the Bradford technique, and the analysis was performed in two repetitions.

Fibrinolytic Assay

Ten milliliters of 3 g/mL of human fibrinogen in 20 mM Tris-HCl buffer (pH 7.4) was taken up and incubated with 20 mL of protein solution at 37°C. The reaction was stopped at different time intervals (0, 60, 120, 180, 240, 360, 480, and 720 min) by adding 30 mL of SDS-PAGE sample buffer. The results of the fibrinogenolytic assay were analyzed using 12% SDS-PAGE.

Quantitative Assay Using Follin & Ciocalteu's Phenol Reagent

Quantitative protease assay using Folin & Ciocalteu's phenol reagent was performed using casein as substrate (Folin & Ciocalteu, 1929). Five milliliters of 0.65% w/v casein solution were added and left in a water bath at 37°C for 5 minutes. Then, the sample enzymes with a concentration of 1.6812 µg/µL in different volumes (0.25, 0.5, and 0.75 mL) were added and mixed by stirring in an orbital shaker and then incubated at 37°C for 10 minutes. After incubation for 10 minutes, 5 mL of TCA reagent p.a. was added to stop the reaction. A blank solution was prepared by mixing 10 mL of 0.65% w/v casein and 5 mL of TCA reagent. The test sample and the blank solution were incubated at 37°C for 30 minutes. After 30 minutes of incubation, all of the samples were filtered through a 0.45 µm syringe filter to remove insoluble material from the sample. The resulting 2 mL filtrate was added to 5 mL of 7.5% w/v sodium carbonate and 1 mL of Folin's reagent. The solution mixture was homogenized with an orbital shaker and incubated at 37°C for 30 minutes. Two mL of the incubated mixture was added to a cuvette. The absorbance of the test blank and test samples was measured using a spectrophotometer at a wavelength of 660 nm. Measurements were performed in two repetitions.

Inhibitor effect test

An inhibitor effect test was performed to determine the type of protease in the frankincense plant. One milliliter of the enzyme was incubated with the inhibitor for 30 minutes at 37°C. The final concentration of 2 mM and 20 mM (in Tris-HCl 20 mM buffer, pH 7.4) of each inhibitor was tested in two repetitions. The inhibitors used in this test were phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), and ethylene glycol bis-(2-aminoethyl)-N, N, N0, N0 tetraacetic acid (EGTA). Protease activity was measured using casein as substrate. Five milliliters of 0.65% w/v casein solution were left in a water bath at 37°C for 5 minutes. Next, the enzyme sample incubated with the inhibitor was added and mixed by stirring in an orbital shaker, and then incubated

at 37°C for 30 minutes. After incubation, 5 mL of TCA reagent was added to stop the reaction. A blank solution was prepared by mixing 5 mL of casein at a concentration of 0.65% w/v, 1 mL of Tris-HCl buffer, and 5 mL of TCA reagent. Each test solution and blank was filtered through a 0.45 µm syringe filter to remove insoluble material from the sample. Five milliliters of sodium carbonate and 1 mL of Folin's reagent were added to the 2 mL filtrate. The solution mixture was homogenized with an orbital shaker and incubated at 37°C for 30 minutes. Two milliliters of the incubated mixture were added to a cuvette. The absorbance of the test blank solution and the test sample was measured in two repetitions using a spectrophotometer at a wavelength of 660 nm.

RESULTS

Figure 1 shows the clear zone on the plate, and Table 1 shows the diameter of the clear zone on the test media, which increased with the increasing amount of enzyme protein dripped. The protein concentration of the crude protease extract obtained in this study was 0.9124 µg/µL. For the radial caseinolytic test, a crude protease extract with volumes of 0.5 µL, 1.0 µL, and 1.5 µL was used to qualitatively observe the protease activity of the crude extract of frankincense leaves.

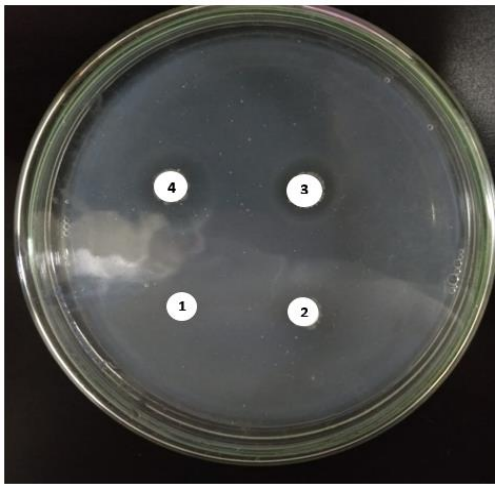

Figure 1. A clear zone is formed in each paper disc after being dropped with 5 mL (paper disc 2), 10 mL (paper disc 3), and 15 mL (paper disc 4) of 0.9124 µg/µL crude enzyme sample. Remark: There is no clear zone formed in paper disc 1, which is only dropped with buffer Tris-HCl as a negative control. *(Zona bening terbentuk pada setiap kertas cakram yang ditetesi oleh 5 mL (kertas cakram 2), 10 mL (kertas cakram 3), dan 15 mL (kertas cakram 4) sampel enzim konsentrasi 0.9124 µg/µL. Ket.: Tidak ada zona bening yang terbentuk pada kertas cakram 1, yang ditetesi oleh buffer Tris-HCl sebagai kontrol negatif).*

Table 1. Diameter of the clear zone of the protease crude extract (*Diameter zona bening dari ekstrak kasar protease*).

Enzyme volume (µL) (*Volume enzim*)	**Diameter (mm)**
0.5	7
1.0	8
1.5	12

Table 2 shows that partial purification dialysis using ammonium sulfate with a saturation level of 20% to 60% resulted in the enzyme concentration. Based on the measurement of protein concentration, the highest enzyme protein concentration was in the 40% ammonium sulfate fraction sample, which was 1.6812 µg /µL. The sample fraction with the highest concentration was then used for the quantitative protease activity and fibrinogenolytic assays.

Table 2. The concentration of the protease sample (*Konsentrasi sampel protease*).

Sample (*Sampel*)	**Substrate** (*Substrat*)
Crude extract	0.9124 ± 0.006
20% fraction	0.9433 ± 0.003
40% fraction	1.6812 ± 0.003
60% fraction	1.0449 ± 0.012

A quantitative protease activity assay was performed using casein as the substrate. The protease enzyme hydrolyzed casein and produced a product in the form of an amino acid, L-tyrosine. L-tyrosine, which contains a phenol group, can react with Folin & Ciocalteu's phenol reagent to form a blue complex, the absorbance of which is measured using a spectrophotometer. For 0.4203 mg to 1.2609 mg protease enzyme, the amount of tyrosine resulted are 0.0399 ± 0.0001 mg, 0.0751 ± 0.0004 mg, and 0.0980 ± 0.0001 mg. The more tyrosine is released from the casein, the more chromophores are produced, indicating greater protease activity (Figure 2).

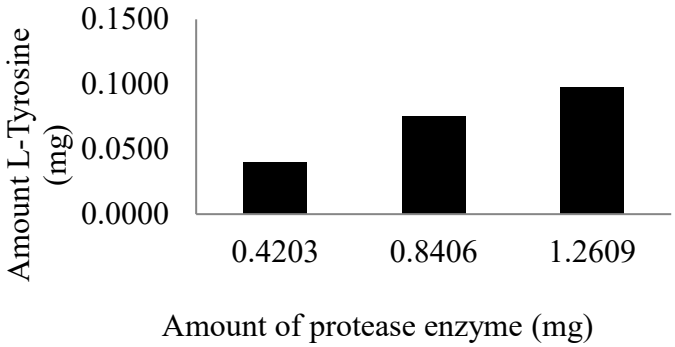

Figure 2. L-Tyrosine produced in the enzymatic reaction of frankincense leaf protease (*L-tirosin yang dihasilkan dalam reaksi enzimatik protease dari daun kemenyan*).

The fibrinogenolytic assay was performed using human fibrinogen incubated with the sample enzymes to determine the enzyme activity in degrading the fibrinogen chain using the SDS-PAGE method (Figure 3).

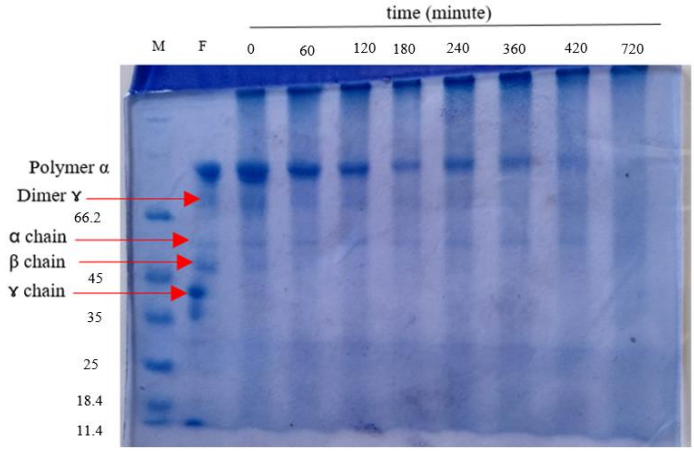

Figure 3. Electrophoresis results of human fibrinogen protein incubated with protease enzymes (*Hasil elektroforesis protein fibrinogen manusia yang diinkubasi dengan enzim protease*).

The inhibitor effect test was performed using serine protein inhibitors (PMSF) and metalloprotease inhibitors (EDTA and EGTA), as shown in Figure 4.

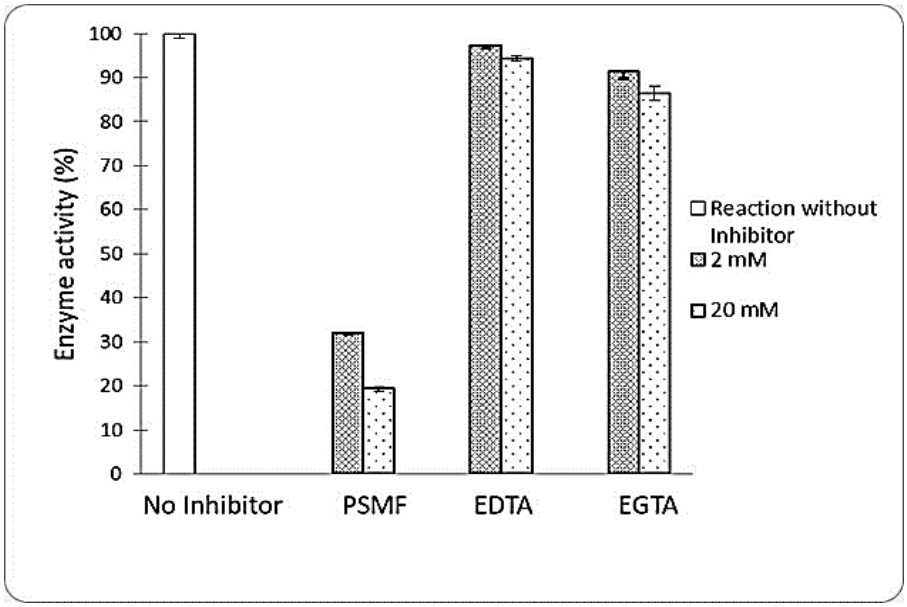

Figure 4. Effects of Inhibitors on Enzyme Activity. Note: PMSF = phenylmethylsulfonyl fluoride; EDTA = ethylenediaminetetraacetic acid, EGTA = ethylene glycol bis-(2-aminoethyl)-N,N,N0,N0 tetraacetic acid. (*Pengaruh inhibitor terhadap aktivitas enzim*).

DISCUSSION

The increasing diameter of the clear zone on the test media of the crude protease extract for the caseinolytic test indicates protease activity in the crude extract of frankincense leaves, which could hydrolyze casein in skim milk. Protease activity is one of the characteristics of thrombolytic proteases, as reported by Choi *et al*. (2014), Gogoi *et al*. (2018), and Kim *et al*. (2015), who obtained thrombolytic proteases from the leaves of *Daisies yomena*, *Leucas indica*, and *Petasites japonicas*. A quantitative protease activity of the partially purified protease in the 40% ammonium sulfate fraction was conducted, using L-tyrosine as the representative of protease activity. In a study by Uday *et al*. (2017), the protease enzyme from the sap of *Ervatamia heyneana* plant was incubated with casein substrate to observe how the enzyme activity affected changes in substrate concentration. In a study by Banik *et al*. (2018), the hydrolytic activity of proteases on casein substrates was measured based on the amount of L-tyrosine released using Folin & Ciocalteu's phenol reagents to determine the optimum pH and temperature conditions for proteases from *Moringa oleifera* leaves, marine plant proteases from *Costaria costata* (Kim *et al*., 2013), and bacterial proteases from *Serratia* sp. KG-2-1 (Taneja *et al*., 2017).

The ability of the protease to degrade the fibrinogen chain was determined by performing a qualitative fibrinogenolytic assay. Several previous studies (Kim *et al*., 2015; Choi *et al*., 2014; Siritapetawee *et al*., 2015) found that proteases degraded and cleaved the α-chain more specifically and were less able to cleave the β- and γ-chains. This activity differed from the fibrinogenolytic activity of the protease enzyme in frankincense. In this study, the frankincense protease targets the γ- and the β-chain. The γ-chain was the fastest hydrolyzed by the protease enzyme, followed by the β-chain, which was completely hydrolyzed at the 60th minute. The α-chain was the most difficult for the enzymes to break. This research indicates that hydrolysis of the α-chain occurred, but the protease could not completely hydrolyze the α-chain at the 720-minute incubation time.

Differences in cleavage patterns also occurred in the study of the fibrinogenolytic activity of herinase enzyme, a protease enzyme from the fungus, conducted by Choi *et al*. (2013). The study showed that the herinase enzyme tended to cleave the α-chain and then the γ-chain. In contrast, in previous studies on the fungus *Tricholoma saponaceum* (Kim & Kim, 2001) and the fungus *Flammulina velutipes* (Park *et al*., 2007), protease enzymes were able to degrade the α- and β-chains. However, it did not show activity against the γ-chain. Differences in cleavage patterns were also

observed for animal protease enzymes. In the fibrinogenolytic activity of the wasp venom *Polybia occidentalis,* by Czaikoski *et al*. (2010), the enzyme was shown to cleave the β-chain more specifically, followed by the γ-chain, and was unable to degrade the α-chain after 24 hours of incubation. In contrast, in another study by Izidoro *et al*. (2003) on snake venom *Bothrops pauloensis* and Pinto *et al*. (2004) on caterpillar venom *Lonomia oblique* showed that protease tended to cleave the α- and β-chains of fibrinogen. The different cleavage patterns performed by protease enzymes from different sources, such as plants, fungi, and animals, indicate that some protease enzymes may have a different cleavage pattern of the fibrinogen chain than other protease enzymes. Based on the research conducted by Bilheiro *et al*. (2013) on the sap of the *Carica candamarcensis* plant, the fibrinogenolytic ability of the protease can lead to the reduction of fibrinogen levels and inhibit the coagulation process in which the conversion of fibrinogen into fibrin and the reduced fibrin levels inhibit the formation of thrombus or blood clot. Based on this research, it can be concluded that the protease from the leaves of the frankincense plant, which was able to degrade by hydrolyzing the fibrinogen chain, showed the characteristics of a thrombolytic protein. This happened because the protease enzyme has the activity of hydrolyzing the fibrinogen chain.

This study revealed that the protease found in frankincense leaves is a serine protease, as the protease activity was almost totally suppressed by the serine protease inhibitor, 20 mM PMSF. The finding of serine protease from frankincense leaves in this research increases the diversity of serine protease sources, which are widely known for their properties in hydrolyzing thrombin and dissolving it (Hazare *et al*., 2024). Several types of fibrinogenolytic enzymes found in plants are serine proteases. Serine proteases have also been found in thrombolytic proteins isolated from the leaves of *Petasites japonicas* (Kim *et al*., 2015), the sap of *Euphorbia cf. lacteal* (Siritapetawee *et al*., 2015), *Solanum tuberosum* (Pepe *et al*., 2016), and *Crinum asiaticum* (Singh *et al*., 2011). In addition to serine proteases, metalloproteases have also been found in several types of fibrinogenolytic enzymes, such as enzymes from wasp venom *Polybia occidentalis* (Czaikoski *et al*., 2010), enzymes from snake venom *Bothrops jararaca* (Maruyama *et al*., 2002), and leaves of *Aster yomena* (kitam.) Honda (Choi *et al*., 2014). Several plants, that have been proven to have potential thrombolytic agents, have different modes of action in inhibiting clot formation in humans. Those modes of action are prolonged prothrombin time, antithrombotic and thrombolytic action, and suppressing the ability of platelet adhesion (Guguloth *et al*., 2022). Prolonging the prothrombin time will inhibit the formation of thrombin, which in turn will inhibit the process of forming fibrin from fibrinogen. Prothrombin time can be prolonged, among other things, by inhibiting the activity of clotting factors. As an antithrombotic agent, active compounds from plants can inhibit thrombin function, platelet activation, and fibrin formation. Meanwhile, as a thrombolytic agent, the active compound will convert plasminogen into plasmin, which will break down fibrin, thereby preventing the formation of blood clots. Clot formation inhibition can also occur due to suppressed platelet adhesion ability, which is mediated by von Willebrand factor (Vaseem *et al*., 2012).

CONCLUSION

In this study, the protease activity in frankincense leaves was tested qualitatively and quantitatively. Qualitatively, the protease activity was evidenced by the presence of a clear zone on the radial caseinolysis test medium. Quantitatively, the protease activity of frankincense was tested by calculating the amount of L-tyrosine produced in the protease enzymatic reaction with casein substrates, in which L-tyrosine increased as the amount of enzyme reacted increased. Partial purification of the protease enzyme at 40% ammonium sulfate saturation yielded the enzyme with the highest concentration of 1.6812 g/μL. The partially purified protease from frankincense leaves was capable of hydrolyzing the γ- and β-chains of human fibrinogen. The serine-type protease from frankincense leaves has the potential to be used as a thrombolytic agent.

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AUTHOR CONTRIBUTIONS

ERS: conducting research, collecting data; data analysis; MMM: research concept, drafting the manuscript, final revision of the manuscript; RFK: data analysis, drafting the manuscript.

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