PLATELET AGGREGATION IN VITRO ANALYSIS OF 67 kDa IMMUNOGENIC PROTEIN FRACTION FROM*Aedes albopictus*SALIVARY GLAND(SKUSE) (DIPTERA: CULICIDAE)

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Abstract

Cardiovascular disease is caused by impaired heart and blood vessel function. Coronary heart disease includes acute coronary syndrome due to narrowing of the coronary arteries. Aspirin is an anti-platelet drug which is commonly used for primary and secondary prevention of coronary heart disease. The effectiveness of aspirin has limitations because 10-20% of patients who use aspirin continue to experience vascular blockage. The haematophagus arthropods salivary glands contain apyrase which can inhibit platelet aggregation and thus a potential candidate for anti-platelet drug discovery. Our studies from salivary gland protein extract of Aedes albopictus found a 67 kDa protein fraction which has a similar molecular weight range from the previously identifiedapyrase of other mosquitoes vectors. The purpose of this study was to determine the apyrase potential capacity of 67 kDa protein fraction from salivary gland of Ae. albopictus. The present study employed inhibition percentage of platelet aggregation method in determining apyrase activity. Aspirin was used as a positive control with 2 different concentrations of 0.1 mg/mL and 2 mg/mL. PBS 1mM pH 7.4 was used as a negative control treatment, while negative control without treatment only involved the addition of PRP and ADP. The inhibition percentage activity from the 67 kDa sample ranged from 3.28-37.64% whereas the total protein extract comprised of only 1%. The positive control of aspirin was 0.1 mg/mL and 2 mg/mL, resulting in inhibition percentage of 5% and 17%, respectively. The data showed that the inhibition percentage of platelet aggregation from protein 67 kDa isgenerally higher than those of total salivary gland protein extract as well as positive control. This indicated that the 67 kDa protein has a potential apyrase activity.

***Keywords*:** *Ae.albopictus, platelet aggregation, 67 kDa protein*

1. INTRODUCTION

Cardiovascular disease is caused by impaired heart and blood vessel function (Berry *et al.*, 2012). Cardiovascular diseases include coronary heart disease, heart attacks, arrhythmias, heart failure, and strokes (Ministry of Health, Republic of Indonesia, 2013). Coronary heart disease includes acute coronary syndrome due to narrowing of the coronary arteries called arteriosclerosis. Narrowing of the arteries results from the presence of saturated fat called atherosclerosis. Rupture of atherosclerotic plaque can be followed by attachment, activation of the clotting cascade, and platelet aggregation. This causes fibrin and platelets to form blood coagulation which results in inhibited blood in the blood vessels (Aziz and Yadav, 2016).

One of the anti-platelet drugs in Indonesia is aspirin. Aspirin is indicated for primary and secondary prevention of coronary heart disease. Standard anti-platelet therapy using aspirin has a thrombolytic effect. The effectiveness of aspirin has limitations because 10-20% of patients who use aspirin continue to experience vascular blockage (Yunita *et al.*, 2015).

Doucoure *et al.* (2013) statedthat *Ae. albopictus*possess immunogenic proteins ranging from 60 to 70 kDa. The apyrase detected at molecular weights between 61-68 kDa (Almeras *et al.*, 2010; Doucoure *et al.*, 2013; Fontaine *et al.*, 2011; Juhn *et al.*, 2011; Luplertlop, 2014; Oktarianti *et al.*, 2015; Peng *et al.*, 2016 and Wasinpiyamongkol *et al.*, 2010).

Apyrase is an enzyme that can be activated by calcium (or magnesium) in the plasma membrane which plays a role in the hydrolysis of ADP to produce AMP and inorganic phosphate (Smith *et al.*, 2002). Apyrase is found in the salivary glands of *Ae. albopictus* and has a role in hydrolyzing Adenosine Triphosphate (ATP) and Adenosine Diphosphate (ADP) to Adenosine Monophosphate (AMP) and Inorganic Posphate (Pi) (Hughes, 2013). ADP plays an important role as an inducer when platelet aggregation occurs (Periayah et al., 2016). In this study, the activity of the 67 kDa protein fraction of the salivary glands of *Ae.albopictus* in inhibiting platelet aggregation is indicated to have the same ability normal).

Based on the previous research, there were two immunogenic proteins from salivary glands of *Ae. aegypti* with a molecular weight of 31 kDa and 56 kDa (Oktarianti et al, 2014). The further analysis by Mass Spectrometry (LC-MS / MS) showed that the 31 kDa protein was identified as the D7 protein family and the 56 kDa protein identified as apyrase (Oktarianti et al., 2015). The salivary glands *Ae. albopictus* has immunogenic proteins with a molecular weight of 67 kDa and this protein is thought to contain apyrase (Khasanah, 2019). Therefore, the further analysis is important to carry out of thus fraction to platelet aggregation inhibition which has potencial of thrombolytic agent.

2. RESEARCH METHOD

Research Site and Setting

This research was conducted from November 2019 to July 2020, in the Biotechnology Laboratory, Faculty of Mathematics and Natural Sciences, Jember University and the Biomedical Laboratory of the Faculty of Medicine, Brawijaya University.

Isolation of Salivary Gland *Ae. albopictus*

Isolation of salivary glands in female*Ae. albopictus*was performed by microdissection technique using a dissection needle (Schmid *et al.*, 2017). Mosquitoes were placed on the glass of objects with 0.5% NaCl drop. The head and thorax were slowly separated and then a pair of clear colored salivary glands were taken. Salivary glands were collected in sterile microtubes (1:1) filled with 10µL PMSF in PBS pH 7.4 sterile and stored at -20°C prior to experiment.

Isolation and Purification of 67 kDa Protein

Salivary gland samples were analyzed for their protein profiles using the SDS-PAGE method using 12% gel seperating and 4% stacking gel. Samples were prepared by adding a buffer buffer and then heated to a thermoshaker at a temperature of 95° C for 4 minutes. SDS-PAGE analysis was performed at 150 V for 60 minutes at room temperature in an electrode buffer of pH 8.3. The SDS-PAGE gel was staining by using Coomassie Brilliant Blue staining solution for 120 minutes then destaining process three times for each 30 minutes..

The Protein fraction of 67 kDa was separated and purified by electroelution and dialysis. The protein band was accommodated in a dialysis membrane containing 1-3 ml electrode buffer solution then immersed in a pH 8.3 electrode buffer horizontally in the electrophoresis chamber. The electroelution process was carried out for 60 minutes 120 V. The resultant electrophoresis fluid was transferred into a new dialysis membrane and dialysis was carried out at 4° C for 24 hours in 500 mL PBS pH 7.4. Dialysis was performed 4 times each for 8 hours with a new PBS pH 7.4. The liquid in the membrane is removed and stored in a sterile microtube containing cold ethanol in a ratio of 1:1 for 24 hours. Then the sample was centrifuged at 10,000 rpm for 5 minutes at room temperature, the pellets were taken and dried and added with 500 µl PBS pH 7.4. The 67 kDa protein collected was placed in eppendorf and its concentration was measured with nanodrop at 0.1 mg/mL

Platelet Agragation Assay

Platelet Rich Plasma (PRP) was obtained from healthy residents of Jember taken by venipuncture and collected into an EDTA vacutainer (*Vaculab EDTA K3*, Indonesia). The sample was then swirled slowly so that

it was mixed in the EDTA vacutainer. Furthermore, after forming 2 layers, supernatant containing platelets was moved into a sterile tube. The sterile tube was then centrifuged at 3200 rpm at 4° C for 15 minutes. PRP was in the lower 1/3 and Platelet Poor Plasma (PPP) was in the upper 2/3 (Marques *et al.*, 2013).

Platelet aggregation test used the Platelet Aggregation Method combining PRP and agonist (ADP),followed by measuring the absorbance value to find out the inhibition percentage (White and Lisa, 1999; Chan *et al.*, 2018). 10 µL of 67 kDa protein fraction was incubated with 90 µL PRP for 10 minutes at room temperature in a microplate96 well. The fraction was shook at speed of 500 rpm and added 10 µL ADP 20 µM. Similar process was repeated for 10 minutes.

Furthermore, the treatment for platelet aggregation test was grouped into several treatments, namely negative control, positive control, 67 kDa protein fraction, and total extract of salivary gland of *Ae. Albopictus*. The negative control used 2 treatments namely PBS given 1mM pH 7.4 as a protein solvent and without any treatment (only PRP and ADP) while the positive control used Aspirin with a concentration of 0.1 mg/mL and 2 mg/mL (See *et al.*, 2017). Extract total protein with a concentration of 0.1 mg/mL. The absorbance value of the sample was measured at 575-650 nm using a microplate reader (Chan *et al.*, 2018).

3. RESULT AND DISCUSSION

Isolation of Salivary Gland

The results of isolation by microdisection showed that the shape of the salivary glands was intact, as there was one pair of salivary glands divided into two parts and connected with the salivary duct. Each part consisted of three lobes, namely one lobe in the middle (single medial lobes) and two lobes located on the side (lateral lobes) (Figure1). Lateral lobes are divided into proximal lobes and distal lobes(Juhn *et al.* (2011) dan Bowers *et al.* (2003)).

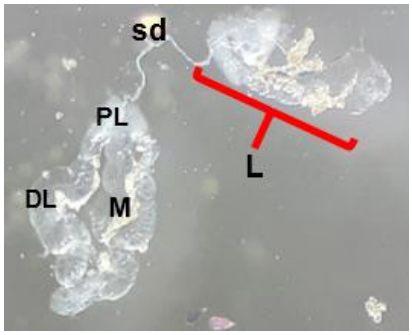

Figure 1. Salivary gland *Ae.albopictus.Salivaryduct* (sd), *Lateral lobes* (L), *Proximal lobes* (PL), *Distal lobes* (DL), *Single medial lobes* (M)(Magnifcation : 50x Microscope Nikon SMZ735. Image: Personal Documentation).

Isolation and Purification of Protein Fraction 67 kDa

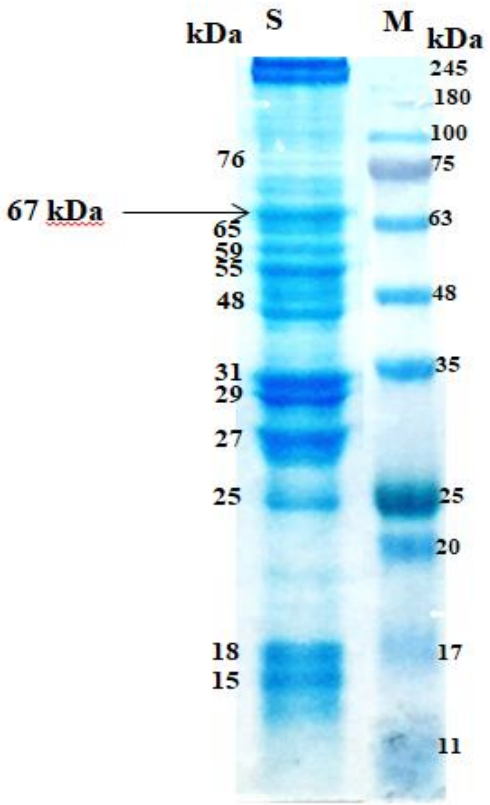

Figure 2. Salivary Gland Protein Profile of *Ae. Albopictus* M: Marker (*GangNam-STAIN™ Prestained Protein Ladder*), S: *Ae. albopictus* salivary gland (Image: Personal Documentation).

The result analysis of SDS-PAGE method showed the protein bands with molecular weights ranging from 15 kDa to 76 kDa, which can be seen in Figure 2. Protein bands with molecular weights of 31 kDa, 47 kDa, and 67 kDa are immunogenic proteins (Khasanah, 2019). According to Khasanah (2019), a protein band with a molecular weight of 67 kDa is thought to have a major component in the form of apyrase. It possesses molecular weights from 61 kDa to 68 kDa (Doucoure *et al.*, 2013; Wichit *et al.*, 2016; Dong *et al.*, 2012; Marinotti *et al.*, 1996).

The 67 kDa protein band that has been separated then purified by electroelution and dialysis methods. The purification results of the 67 kDa protein sample had a concentration of 0.1 mg/mL.

Platelet Agregation

Platelet aggregation test aimed to determine the ability of the 67 kDa protein fraction presumed to contain apyrase in inhibiting platelet aggregation. This study employed human PRP samples with the platelet aggregation method (White and Lisa, 1999). PRP samples were obtained from Jember endemic residents. Platelet aggregation testing was based the reading of absorbance values on a microplate reader. If the value of light absorbance is high, the light transmitted to the detector is low. This indicates substantial amount of platelet aggregates, so the inhibition of platelet aggregation is low. By contrast, if the absorbance value of light is low, the light transmitted to the detector is high, so that the inhibition percentage of platelet aggregation is high (Chanet al., 2018).

The negative control of this study was administering PRP and ADP as the standard for aggregate formation, whereasthe negative control treatment employed PBS 1 mM pH 7.4 as a protein solvent. Positive control used Aspirin with two different concentrations of 0.1 mg / mL and 2 mg / mL.

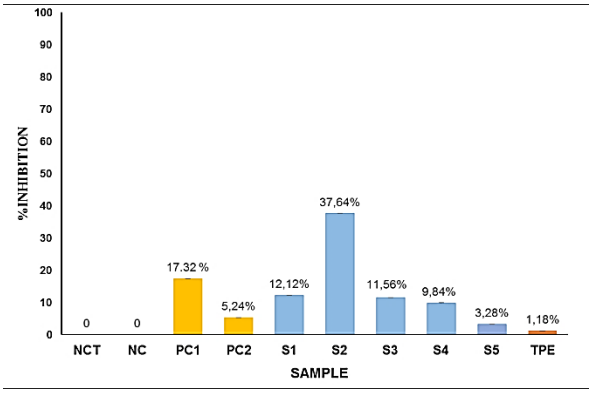

Figure. 3 Percentage inhibition of platelet aggregation. PBS 1 mM negative control pH 7.4 (NCT); negative control without treatment (NC); positive control Aspirin 2 mg/mL (PC1); positive control Aspirin 0.1 mg/mL (PC2), individual PRP + 67 kDa 0.1 mg/mL (S1-S5) and PRP + Total protein extract 0.1 mg/mL (TPE).

The inhibition of Aspirin with a concentration of 0.1 mg/L was 5.24%, while Aspirin with a concentration of 2 mg/mL was 17.32%. Then the treatment of 67 kDa protein fraction 0.1 mg/mL was tested on PRP with the addition of ADP. Figure 3 shows thatdifferent results were obtained from 5 individual PRP samples tested with a protein fraction of 67 kDa 0.1 mg/mL. The inhibition ranged from 3.28 to 37.64%. Total salivary gland protein extract (EPKS) was also tested on PRP with the addition of the same ADP. The percentage obtained was 1.18% and not higher than the 67 kDa protein sample. According to Arca *et al.* (2007) and Doucoure et al. (2013), there are many different components of protein in the salivary glands of *Ae. albopictus*. This can also occur because EPKS does not go through a protein purification process so that the ability to inhibit platelet aggregation is not specific.

ADP is an inducer or activator of platelet aggregation in the blood. ADP release granules when the receptor proteins on the platelets are active when there is vascular damage. Then the ADP binds to specific platelet membrane receptors namely P2Y1

and P2Y12. These bonds can induce changes in platelet shape which also causes attachment between platelets through Integrin (αIIbβ3) and fibrinogen receptors (Badimon *et al.*, 2012 and Oury *et al.*, 2006).

The platelet aggregation inhibition of 67 kDa protein sample is higher than positive control of Aspirin with a similar concentrations. The highest inhibition of protein sample 67 kDa was 37.64%. The same study was carried out by Erni (2019) using a different species, the *Ae. aegypti.*

The salivary glands of *Ae. aegypti*were thought contain apyrase with an inhibitory percentage 14-70% (Erni, 2019). Dong *et al.* (2012) state that apyrase can inhibit platelet aggregation by 6% in human blood. This proves that the protein fraction 67 kDa is an immunogenic protein containing apyrase (Doucoure *et al.*, 2013 and Peng *et al.*, 2001). Apyrase serves to hydrolyze ATP and ADP to AMP and inorganic phosphate from activated platelets (Hughes et al., 2013 and Williams *et al.*, 2012).

4 CONCLUSION

The protein fraction of 67 kDa from the salivary glands of *Ae. albopictus* with a concentration of 0.1 mg/mL has higher inhibition percentage of platelet aggregation than aspirin with a similar concentration is 3.28-37.64%. Thus, the protein fraction 67 kDa of *Aedes albopictus* salivary glands has a potential apyrase activity.

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