The Apyrase Functional Properties of the 56 kDa Protein from *Aedes aegypti* Salivary Gland

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Abstract. Apyrase is an enzyme an inhibit platelet aggregation process, capable of degrading ADP in the process blood feeding and mostly found in hematophagous arthropods. While vector's blood feeding, this apyrase salivary protein is responsible for inhibiting platelet aggregation in the human host, by hydrolyzing adenosine diphosphate or adenosine triphosphate molecules that produce adenosine monophosphate thus decrease platelet aggregation. Our previous study reported that the immunogenic proteins 56 kDa from salivary gland of dengue's vector *Aedes aegypti* constituted high apyrase activity. This study wanted to analyze apyrase functional properties of this immunogenic protein. The amount of inorganic phosphate released from ADP degradation by apyrase was analyzed using by malachite green detection kit. We also further analyzed its platelet aggregation inhibition activity. The results showed that 56 kDa immunogenic protein has high apyrase activity with 33.30 nmol/well inorganic phosphate released, half of positive control activity (ATP-se) and it can inhibit platelet aggregation by in vitro was 40–50%.

Keywords: Apyrase · 56 kDa immunogenic protein · Aedes aegypti

1 Introduction

Apyrase is an enzyme mostly found in hematophagous arthropods, and is able to degrade adenosine diphosphate (ADP) and adenosine triphosphate (ATP) into adenosine monophosphate (AMP) and inorganic phosphate [1, 2]. This enzyme is important for helping hematophagous arthropods blood feeding, which interferes host blood coagulation or inhibiting platelet aggregation [2]. Apyrase activity depend on an ion-co factor, either calcium or magnesium [3]. ATP and ADP are important inducer to platelets aggregation, as they will interact with P2 receptors. The P2Y12 and P2Y1 are important receptor for ADP-induced platelet aggregation, while P2X1 is receptor for ATP [4]. They were released by injured cells during the hematophagous arthropoda blood feeding [1, 5]-[7].

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Hemostasis and inflammation could be inhibited by apyrase thus the hematophagous arthropods can blood feeding easily and enhance pathogen transmission [6]. Apyrase detected in ticks, bugs and mosquitos' saliva and the other blood-feeding arthropods. It has been identified in *Ixodes dammini* [8], and *Ornithodoros moubata* [9], *Ornithodoros savignyi* [10], *Phlebotomus papatasi* [11], and *Triatoma infestans* [12] and in mosquitoes such as *Aedes aegypti* [13], *Aedes albopictus* [14] and *Anopheles gambiae* [15].

The apyrase enzyme which is expressed by gene apyrase of adult female mosquitoes has a molecular weight about 68 kDa [16]. The distal-lateral dan medial lobes of salivary gland resul apyrase involved in blood-feeding process [17, 18]. The previous studies showed that apyrase of Aedes albopictus expressed in the distal-lateral lobes was 80% and 20% of the medial lobes [19]. *Aedes albopictus*'s apyrase has molecular weight at 61 kD [20].

The previous study apyrase abundantly was detected at 56 kDa from *Aedes aegypti* salivary gland [21]. The objective of this study is to analyzed apyrase functional properties of this immunogenic protein. The apyrase activities were measured based on the amount of inorganic phosphate released from ADP degradation using malachite green detection kit and also further analyzed its platelet aggregation inhibition.

2 Materials and Methods

2.1 Rearing and Salivary Gland Dissection

In this experiment, we used *Ae. Aegypti* which was reared on laboratory scale. Rearing of *Ae. Aegypti* was carried out on laboratory scale under controlled conditions at 28 °C with 60% relative humidity at Animal Care Unit Zoology Laboratory of Biology Department, Faculty of Mathematic and Natural Sciences, University of Jember. The cage with dimension of $1 \times 1 \times 1$ m^3 for maintaining the pupae into adult mosquitos. The male adult mosquitoes were given nutrition with 10% sucrose solution and female mosquitoes feeding by fresh blood from wistar rat. The female adult mosquitoe's salivary glands were isolated by using microdissection method. Then the salivary glands were pooled into a new microtube in PBS-PMSF (Phenyl Methyl Sulfonyl Fluoride) (Sigma-Aldrich, USA) then stored frozen at -20 °C until needed.

2.2 Sodium Dedocyl Sulphate-Polyacrilamide Gel Electrophoresis (SDS-PAGE) Analysis

Protein samples were separated based on their molecular weight using the SDS-PAGE method with 12% separating gel and 4% stacking gel. Electrophoresis was performed using a constant voltage of 120 V for ±2 h at room temperature. Protein bands were visualized using Commassie Brilliant Blue (CBB) R250 (Sigma-Aldrich, USA).

2.3 Electroelution and Dialysis

The band of 56 kDa was excised using a sharp disposable blade and then electroluted using 6–8 cm cellophane membrane that already clamped to one side. Proteins were

electro-eluted for 60 min, constant voltage 120 V at room temperature. In order to remove the chemicals that were not needed, the elution procedure was continued with a dialysis process for 24 h using fresh cold PBS in a cold chamber. The buffer was replaced with a fresh one every 8 h. The protein was precipitated and concentrated overnight in a cold chamber by adding an equal volume of cold ethanol. The supernatant was centrifuged at 12.000 rpm for 15 min at 4 °C. The pellet obtained was air-dried and resuspended in 0.05 M Tris-HCl pH 6,8.

2.4 Blood Sera Collection

Sera blood samples were taken from neonates, healthy person and Dengue Hemorrhagic Fever (DHF) patient living in Jember-East Java, Indonesia. All participants gave written informed consent to take part in the study. The collecting protocol was approved by the Ethical Committee of Dentistry Faculty, University of Jember Indonesia No: 1034 / UN25.8 / KEPK / DL / 2020.

2.5 Human Immune Response by in Vitro Analysis Using ELISA

In vitro human immune response to 56 kDa from the salivary glands of the *Aedes aegypti* was measured using indirect ELISA analysis. Indirect ELISA procedure begins with antigen coating by adding 50 μL of 56 kDa protein extract into each well and was incubated overnight at 4 °C. then washed with 250 μl of PBST (Phospate Buffer Saline Tween). The next step was coating buffer blocking by adding 200 μL of blocking buffer into each well and incubating for 1 h at 37 °C, washed again with 250 μL of PBST, then added with 50 μL of primary antibody which has been diluted in blocking buffer in a ratio of 1:100 and incubated for 1 h at 37 °C. It was washed again with 250 μL of PBST and added 50 μL of secondary antibody (anti human IgG-HRP conjugated (1:5000) (Rockland, USA) then incubated for 1 h at 37°C. Enzyme activity was detected by incubating for 10 min at room temperature with 50 μL Tetra Methyl Benzidine (TMB) (Sigma-Aldrich, USA). Enzymatic reaction was stopped using 50 μL 1M H_2SO_4 for 10 min at room temperature. Optical density (OD) at 450 nm was determined with a microplate reader.

2.6 Apyrase Activity Assay

The apyrase activity was measured based on the amount of inorganic phosphate released by ATP, using malachite green colourimetric detection kit (R&D System, MN, USA Catalog Number DY996). All incubations up to the step of alkaline phosphatase activity measurements were performed as recommended by the manufacturer instructions. The 56 kDa immunogenic protein sample (0.232 mg/ml) from *Aedes aegypti* was tested in this assay.

2.7 Platelet Aggregation

The platelet aggregation ability was measured using Hamasaki methods with minor modification (5). Platelet Rich Plasma (PRP) was prepared by centrifugation of anti-coagulated blood from healthy people at 1500 rpm for 10 min at room temperature.

Platelet aggregation was determined by measuring the change in the optical density. As much as 30 μl of 56 kDa protein (0.745 μg/μl) was added with 70 μl PRP and 10 μl ADP 20 μM (Sigma-Aldrich, USA), then incubated on a shaker for 10 min. Platelet aggregation was measured by microplate reader at a wavelength of 630 nm.

3 Result and Discussion

3.1 Apyrase Activity

The 56 kDa protein induced immune responses of people living in endemic areas. Based on western blot analysis this protein was recognized by only healthy and DHF sera sample. Proteomic analysis of these protein showed that most abundant protein from 56 kDa band was apyrase [21]. Apyrase is an enzyme that inhibits platelet aggregation process and very important in the blood feeding process in arthropods hematophagous. This enzyme was able to inhibit platelet aggregation by by breaking down the phosphodiester bonds and hydrolyzes ATP or ADP into AMP and inorganic phosphate [1, 16, 22, 23].

Determination of apyrase activity measured based on the amount of inorganic phosphate released from ATP by using the malachite green colorimetric detection kit (R&D). Malachite green is a chemical dye that has various uses, its reaction with phosphomolybdate results in an intense absorbance band at 620 nm wavelength [24]. Our results showed that 56 kDa immunogenic protein had an apyrase activity of 33.30 nmol/well inorganic phosphate released. It was higher than half of positive control activity itself (ATP-se). The apyrase activity of both positive control (ATP ase) and total extract of salivary gland were 56.97 nmol/well, 35.97 nmol/well respectively (Fig. 1).

3.2 Platelet Aggregation

The human platelet aggregation by in vitro analysis was inhibited by 56 kDa protein of salivary gland of *Aedes aegypti* up to 14–70% by destroying its ADP (Fig. 2). The percentage of inhibition in the PRP sample by apyrase showed higher results than the negative control (non-treatment) and the positive control (Aspirin 0.1 mg/ml). These results indicated that the 56 kDa protein has similar activities as apyrase, which is capable in hydrolyzing ADP to inhibit platelet aggregation in human. This study results supported our hypothesis that this protein has the same ability as aspirin which is a thrombolytic agent, and thus apyrase is potentially used as a thrombolytic agent.

Salivary gland of all arthropods hematophagous including mosquitoes, sand fly, bugs or ticks have apyrase. Apyrase is nucleoside triphosphate-diphosphohydrolase

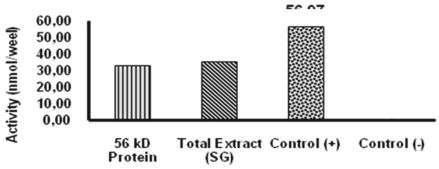

Fig. 1. Liberation of inorganic phosphate (Pi) from ATP (nmol/well) by apyrase activity.

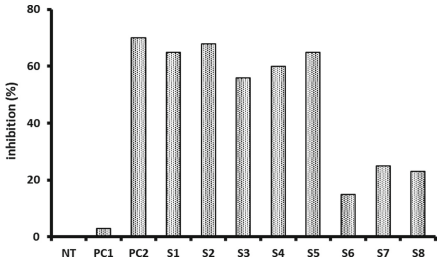

Fig. 2. Platelet aggregation inhibition. NT (negative control); PC1 PC2 (positive control Aspirin) and S1-S8 (PRP sample + 56 kD 0,1 mg/mL).

enzyme and this enzyme can hydrolyze ATP or ADP into AMP and P, so it can inhibit platelet aggregation. ADP as an inducer in the aggregation of platelet process is released from damaged cells by hematophagous arthropod bites [1, 5]. ADP is the signal for platelet aggregation, and the activities depend on the ion co-factors, both calcium, and magnesium [3].

Platelet aggregation induced by ADP can be inhibited by recombinant apyrase isolated from *Phlepotomusduboscqi* (rPduApy) by 40% without incubation and about 90% with incubation. Apyrase recombinant of *Aedes albopictus* from cloned showed a biological activity to inhibit platelet aggregation [6]. The platelet aggregation inhibition by ADP as an inducer was 8–12% [16]. Based on the ability in inhibiting platelet aggregation, apyrase was able to be developed as an anti-thrombotic protein that can be used as a potential treatment of thrombotic disease [5].

Platelet activation is induced by ADP, thrombin, and collagen molecules. Activated platelets will release mediator granules that play a role in coagulation, angiogenesis, molecular adhesion, and cytokines and chemokines. The released granules will then accelerate the activation of the platelets to form platelet aggregation [1]. This platelet activity can be inhibited by proteins produced by apyrase which is able to break down ADP thus these processes can be inhibited and facilitate mosquitoes in the blood feeding process [17].

3.3 Human Immune Response by in Vitro Analysis Using ELISA

The Elisa analysis results of cross react between 56 kDa protein salivary gland and sera human from all sample showed that the highest IgG response was detected in sera from dengue patients compared to healthy people and infants, either on individual response (Fig. 3) or pool sera response (Fig. 4).

These results indicate that people living in DHF endemic area have a spesific antibody to 56 kDa protein, and this prove that salivary proteins are immunogenic and can induce specific antibody responses [25]. They have spesific antibody due frequent exposure to saliva of mosquitoes [26]. Similiar result from another study showed that travelers in tropical country have a significant increase in antibody response to saliva mosquitoes [27].

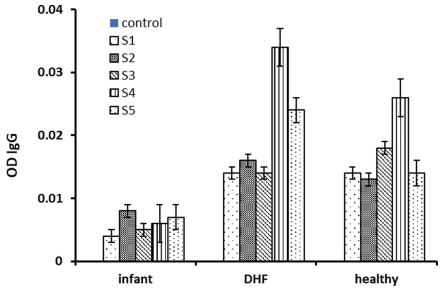

Fig. 3. Individual human immune response against protein of 56 kD.

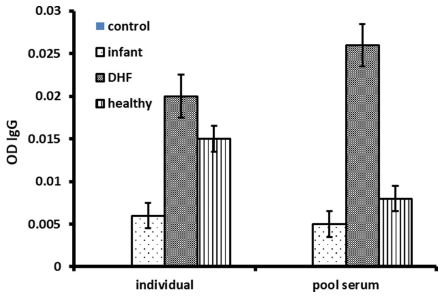

Fig. 4. Individual and pool sera human immune response against 56 kD proteins.

The concentrations of IgG as the host immune response to saliva arthropods haematophagous are correlated to the frequency of exposure [28]. Anti salivary proteins have been developed since the host exposed frequently to the mosquitoe's saliva [29]. IgG responses on DHF patient were higher than healthy people, it could be DHF patient were more exposed to *Aedes aegypti* saliva. The results of the study on malaria endemic population found that anti-saliva titers (IgG) were detected higher due to the repeated exposure to Anopheles mosquitoes [30].

A factor presence in salivary glands of *Aedes aegypti,* called 56 kDa immunogenic proteins, is believed to have apyrase activity 33.30 nmol/well, which could inhibit platelet aggregation in human plasma. The human platelet aggregation was inhibited by this immunogenic protein up to 40–50% by destroying its ADP. This protein may also elicit IgG response in humans, both from individual and pooled sera. The highest rate of IgG was detected in sera from dengue patients than those from infant and healthy people.

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Authors' Contributions. RO conceived and planned the experiments and wrote the manuscript. AS and EE carried out the experiment and contributed to the interpretation of the results. SW contributed to sample preparation and interpretation of the results. KS conceived the original idea and supervised the project. All authors read and approved the final manuscript.

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