Journal of Infection and Public Health (2015) 8, 575—582

http://www.elsevier.com/locate/jiph

Proteomic analysis of immunogenic proteins from salivary glands of *Aedes aegypti*

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Received 13 January 2015; received in revised form 1 April 2015; accepted 3 April 2015

KEYWORDS
Proteomic analysis;
Immunogenic proteins;
Salivary gland;
Aedes aegypti

Summary Humans develop anti-salivary proteins after arthropod bites or exposure to insect salivary proteins. This reaction indicates that vector bites have a positive effect on the host immune response, which can be used as epidemiological markers of exposure to the vector. Our previous study identified two immunogenic proteins with molecular weights of 31 kDa and 56 kDa from salivary gland extract (SGE) of *Aedes aegypti* that cross-reacted with serum samples from Dengue Hemorrhagic Fever (DHF) patients and healthy people in an endemic area (Indonesia). Serum samples from individuals living in non-endemic area (sub-tropical country) and infants did not show the immunogenic reactions. The objective of this research was to identify two immunogenic proteins, *i.e.*, 31 and 56 kDa by using proteomic analysis. In this study, proteomic analysis resulted in identification of 13 proteins and 7 proteins from the 31 kDa- and 56 kDa-immunogenic protein bands, respectively. Among those proteins, the D7 protein (Arthropode Odorant-Binding Protein, AOBP) was the most abundant in 31-kDa band, and apyrase was the major protein of the 56-kDa band.

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http://dx.doi.org/10.1016/j.jiph.2015.04.022

Introduction

Dengue viruses, designated as DENV-1, DENV-2, DENV-3 and DENV-4, cause diseases that range from mild febrile illness and classic dengue fever (DF) to severe and potentially fatal forms of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [1,2]. Dengue has been recognized in over 100 countries, and 2.5 billion people live in areas where dengue is endemic [3,4]. Mosquito vectors transmit DENV to vertebrate hosts. *Aedes aegypti* is a main vector, and *Ae. albopictus* is a secondary vector. Infection of the female mosquito occurs during a blood feeding on a viremic human host (*i.e.*, a person infected with the dengue virus), and the virus is transmitted to a healthy person [5,6,7]. Blood feeding is needed by the mosquitoes because vertebrate host blood is important for their nutrition, egg development, and survival [8,9]. Mosquito saliva is vital for successful blood feeding because it contains numerous substances that inhibit hemostasis (*e.g.*, anti-platelet aggregation, anticoagulant, and vasoconstriction), the development of inflammation (anti-inflammatory), and the immune response. These effects of saliva also benefit the vector because it enhances the transmission of pathogens [9,10,11]. The salivary proteins of arthropods contain vasodilatory and immunomodulatory factors. The vasodilatory factors in arthropod saliva help the vector obtain a blood meal and potentially inhibit homeostasis. These proteins function as anti-coagulants and inhibit vasoconstriction. The immunomodulatory factors induce the host immune response, which manifests as an allergic response of skin itchiness and redness [9,12,13].

Some research demonstrated that the saliva or salivary gland of mosquitoes is antigenic and immunogenic. These factors induce an IgG antibody response in individuals who live in endemic areas [14,15] and travelers who are transiently exposed to vectors in tropical areas [16]. The development of a natural antibody response in people who live in an endemic area (African) is due to frequent exposure to saliva. This response is supported by the fact that IgG levels were significantly higher during the peak period, especially in persons who live in sites with higher a *Ae. aegypti* density [17,34]. This observation indicates that the vector bites have a positive effect on the host immune response. Further evidence demonstrated that hematophagous arthropod saliva contains active protein components that modify the hemostasis and cellular immune responses that are responsible for the induction of specific IgG antibodies in people living in endemic areas [13]. These antibody responses may be used as epidemiological markers of vector exposure and also support the possibility to prevent and treat allergic responses [11,34].

We identified two immunogenic proteins from the SGE of *Aedes aegypti*, 31 kDa and 56 kDa, which were recognized by serum samples from DHF patients and healthy people in an endemic area. Serum samples from individuals living in a non-endemic area (sub-tropical country), who have never traveled to tropical countries, and infants did not show immunogenic reactions with SGE. We concluded that the 31- and 56-kDa proteins from this previous study were immunogenic proteins that specifically modulate immune responses in people living in endemic areas. Therefore, the identification of these proteins using proteomic analysis (LC—MS—MS) will be a crucial step in investigating the role of these proteins in Dengue transmission.

Materials and methods

Rearing of *Ae. aegypti* (lab scale cultures) and salivary gland (SG) dissection

Mosquito larvae were collected and reared under strictly identical standard conditions at 28 °C and 60% relative humidity at Zoology Laboratory of Biology Department, Faculty of Mathematic & Natural Sciences, Jember University, Indonesia. The mosquitoes were supplied with a cotton wool pad soaked in a 10% sucrose solution. Female *Ae. aegypti* mosquitoes were selected at 7—10 days-old after their first blood feeding on rabbit blood maintained at. The SGs from female adult mosquito were dissected using a fine entomological needle under a stereomicroscope at 8× magnification. The isolated SG were pooled into a microcentrifuge tube on ice in 1x phosphate-buffered saline (PBS)/1 mM phenylmethanesulfonyl fluoride (PMSF) and stored frozen at −20 °C until needed (SG tube).

SG protein extraction and SDS-PAGE analysis

Lysis buffer containing 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 10 mM NaCl, 1% Nonidet P-40, 2 mM EDTA/NaOH was added to SG tubes (1:1). Mixtures were homogenized using a micropestle and sonicated in a water bath for 30 min. Supernatants were collected after centrifugation at 12,600 rpm for 15 min at 4 °C [6]. Protein extracts were concentrated using an eppi-membrane (10 kDa MWCO) and centrifuged at 10,000 rpm at 4 °C. Our analyses used protein extracts from SGs at a concentration

of 4 μg/μl. SG proteins were stored at −20 °C until used.

SDS-PAGE was performed according to the methods described by Laemmli with minor modification. SGE proteins were analyzed using a 12% separating gel with a 5% stacking gel. Electrophoresis was performed using a constant voltage of 25 V for the stacking gel and 80 V for the separating gel. Protein bands were detected using Coomassie Brilliant Blue (CBB) R 250 stain.

Protein identification using LC—MS/MS

Protein-containing SDS-PAGE gel pieces were subjected to in-gel digestion with trypsin as previously described [18]. Gel fragments containing 31- or 56-kDa proteins were cut from the SDS-PAGE gel. The gel plugs were dehydrated using 100 μl of 100% acetonitrile (ACN) and incubated for 15 min. ACN was discarded, and the gel was dried for 10 min using a vacuum centrifuge. Proteins were reduced in 100 μl of 100 mM NH_4CO_3/10 mM dithiothreitol (DTT) for 1 h at 56 °C. The solution was discarded, and the proteins were alkylated for 45 min at room temperature in 100 μl of 100 mM NH_4CO_3/55 mM iodoacetamide. The remaining buffer was discarded, and the gel plugs were washed with 100 μl of 100 mM NH_4CO_3 followed by 100 μl of 100% ACN. The washing step was repeated twice. The gels were dried in a vacuum centrifuge and subjected to trypsin digestion. Gels were soaked in 10 μl of 50 mM NH_4CO_3/5 mM $CaCl_2$/trypsin and incubated for 45 min on ice. The trypsin buffer was discarded, and the remaining excess of trypsin in gels was washed with 100 μl of 100 mM NH_4CO_3. This step was followed by an incubation at 37 °C overnight in 25 μl of 50 mM NH_4CO_3/5 mM $CaCl_2$. Supernatants of the gel incubations were collected into new tubes (NT1). The remaining gel was used for protein extraction by mixing the gel with 20 μl of 20 mM NH_4CO_3 in a microshaker for 20 min. The supernatant was transferred into an NT1 tube, and the remaining gel was subjected to another extraction by mixing with 20 μl of 50% ACN/5% formic acid in a microshaker for 20 min. This last step was repeated three times, and each supernatant was collected into the same NT1 tube (NT tube).

The collected supernatants were dried for 60 min using a vacuum centrifuge, and the NT tube was stored at −20 °C for a further desalting process. A 2% ACN/0.1% trifluoroacetic acid (TFA) solution was added to NT tubes and incubated for 15 min. The desalting process was performed by trapping the absorbed peptides in a silica column. This flow-through process was repeated twice. The trapped desalting peptide was dissolved in 70% ACN/0.1% TFA and dried in a vacuum centrifuge. The dried peptide samples were finally subjected to LC-MS/MS analysis for protein identification.

LC-MS/MS analysis was performed using a Q-TOF hybrid mass spectrometer (QSTAR Elite, AB SCIEX, Foster City, CA, USA) equipped with a nanoelectrospray source set in positive-ion mode (spray voltage of 1800 V). This machine was operated with Analyst v2.0 data acquisition software (AB SCIEX). The accumulated MS/MS spectra were processed using ProteinPilot software 2.0 (AB Sciex) and an *Ae. aegypti* UniProt database (version 2013_10). This software calculates a confidence percentage (the unused score) that reflects the probability that the hit was a false positive. Thresholds for the detected proteins and matched peptides were set at a sequence coverage of ≥20% and ProteinPilot unused score ≥1.31 (a confidence threshold of 95%).

Results and discussion

This research performed proteomic analyses of two immunogenic proteins with molecular weights of 31 and 56 kDa from SGs of *Ae. aegypti*. These proteins specifically modulated immune responses of people living in endemic areas, and they could also be used as reliable markers (an epidemiological indicator) of exposures to bites of *Ae. aegypti* ([19,20]). The results were consistent within individual and pooled responses (Fig. 1). We identified 13 proteins from the 31-kDa band, including the D7 protein (37-kDa salivary gland allergen Aed a2), AAEL003600-PA, the long form of D7Bclu1 salivary protein, a putative 34-kDa angiopoietin-like protein variant, a 30-kDa salivary gland allergen Aed a 3, annexin, AAEL006417-PA, putative serpin, AAEL003107-PA, AAEL007776-PA, AAEL004338-PA and malate dehydrogenase (Table 1).

Five of these proteins are secreted proteins that are involved in blood feeding, including the D7 protein (37-kDa salivary gland allergen Aed a 2), AAEL006417-PA, putative serpin, 30-kDa salivary gland allergen Aed a 3, and long-form D7Bclu1 salivary protein. Fifteen secreted proteins are responsible for the interaction between mosquitoes and vertebrate host during blood feeding. Among these proteins, the D7 protein, adenosine deaminase, apyrase, purine hydrolase and 30-kDa allergen induce an antibody response and modulate the host immune response [7]. The D7 protein (37-kDa salivary gland allergen Aed a 2) and 30-kDa salivary gland allergen Aed a 3 induce the allergic response [21]. Pyruvate dehydrogenase and malate dehydrogenase are involved in the citric acid cycle, and the expression of both proteins is increased

Table 1 Identification of immunogenic protein 31 kDa from SG of *Ae. aegypti*.

Protein name	Function	Accession number	MW	Unused score	% Coverage
37 kDa salivary gland allergen Aed a 2 OS = *Aedes aegypti* GN = D7 PE = 1 SV = 2	Blood feeding and immunoge-nicity	sp|P18153|ALL2_AEDAE	36.895	81.21	94.70
AAEL003600-PA OS = *Aedes aegypti* GN = AAEL003600 PE = 2 SV = 1	Unknown	tr|Q1HRF7|Q1HRF7_AEDAE	35.774	50.05	81.73
Long form D7Bclu1 salivary protein OS = *Aedes aegypti* PE = 2 SV = 1	Blood feeding	tr|Q95V90|Q95V90_AEDAE	38.604	31.84	81.02
Putative 34 kDa secreted protein OS = *Aedes aegypti* PE = 2 SV = 1	Unknown	tr|Q8T9V1|Q8T9V1_AEDAE	36.177	19	68.57
Angiopoietin-like protein variant (Fragment) OS = *Aedes aegypti* PE = 2 SV = 1	Unknown	tr|Q1HRV2|Q1HRV2_AEDAE	33.423	8.72	51.72
30 kDa salivary gland allergen Aed a 3 OS = *Aedes aegypti* GN = AAEL010235 PE = 1 SV = 2	Blood feeding and immunoge-nicity	sp|O01949|ALL3_AEDAE	29.155	6	21.61
Annexin OS = *Aedes aegypti* GN = AAEL011302 PE = 3 SV = 1	Calcium-dependent phospholipid binding	tr|Q16QF1|Q16QF1_AEDAE	35.756	5.99	39.81
AAEL006417-PA OS = *Aedes aegypti* GN = AAEL006417 PE = 4 SV = 1	Blood feeding	tr|Q0IF93|Q0IF93_AEDAE	38.628	3.49	75.90
Putative serpin OS = *Aedes aegypti* PE = 2 SV = 1	Blood feeding	tr|Q8T9U7|Q8T9U7_AEDAE	47.117	2.07	26.55
AAEL003107-PA OS = *Aedes aegypti* GN = AAEL003107 PE = 4 SV = 1	Unknown	tr|Q17GF0|Q17GF0_AEDAE	38.608	2.26	32.16
AAEL007776-PA (Putative 30.5 kDa secreted protein) OS = *Aedes aegypti* GN = AAEL007776 PE = 2 SV = 1	Unknown	tr|Q8T9T9|Q8T9T9_AEDAE	30.525	2.22	17.45
AAEL004338-PA OS = *Aedes aegypti* GN = AAEL004338 PE = 4 SV = 1	Pyruvate dehydrogenase	tr|Q17D51|Q17D51_AEDAE	38.508	2.02	19.77
Malate dehydrogenase OS = *Aedes aegypti* GN = AAEL007707 PE = 3 SV = 1	Cellular carbohydrate metabolic process	tr|Q171B2|Q171B2_AEDAE	36.264	2.01	31.06

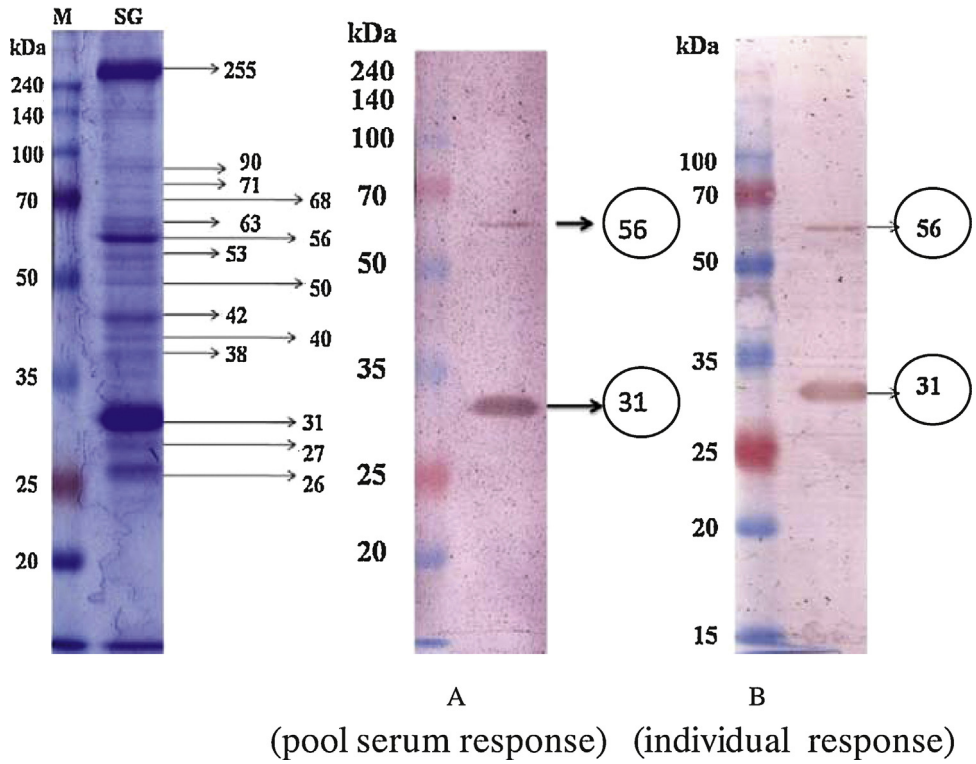

Figure 1 Immunogenic proteins (marked bands) from SG of *Ae. aegypti* (31 and 56 kDa).

in SGs that are infected dengue virus [22]. Annexins binds phospholipids in a calcium-dependent manner, and these proteins are important in various cellular and physiological processes, such as providing a membrane scaffold, which is relevant to changes in the cell shape. The functions of AAEL003600-PA, angiopoietin-like protein variant, the putative 34-kDa protein, AAEL003107-PA and AAEL007776-PA have not been reported.

The unused score (81.21) of the AOB D7 protein suggests that it is the most abundant protein in the 31-kDa band. The unused score represents the amount of peptide that is not claimed by another protein. These results indicate that people living in endemic areas have specific antibodies against the D7 protein (37 kDa salivary gland allergen Aed a2) that are not found in healthy non-exposed persons or infants. The D7 protein likely initiates a specific antibody response. The development of this natural specific antibody response in people living in endemic areas is due to the frequent exposure to *Ae. aegypti* saliva. The D7 protein is a secreted protein that is most abundant in the saliva or SGs of female blood-sucking Diptera. The D7 protein is related to the odorant-binding protein (OBP), which is adapted to bind small ligands, such as serotonin, histamine and norepinephrine. It binds host biogenic amines to antagonize vasoconstriction and platelet-aggregating, which likely aid blood feeding [23,24,25,26]. This anti-hemostatic activity protein plays an important role in the facilitation of the blood-feeding process. It also indirectly plays a significant role in the enhancing of pathogen transmission.

The D7 protein exists in two forms, including a long form (30—35 kDa) that is found exclusively in mosquitoes and sand flies. The short form (15 kDa) is found in other insects [27]. The molecular weight of the D7 protein in the SG of *Ae. aegypti* from this study supports the presence of the long form. Juhn et al. [28] reported the *in situ* hybridization patterns of 30 genes expressed in the SGs of adult female *Ae. aegypti*. Proteins involved in blood feeding, such as the D7 long form, are expressed in the distal-lateral and medial lobes of the SG. The long-form D7 protein has 10 conserved cysteine residues in its amino acid sequence, with the exception that AnSt-D7clu2 and AnAr-D7 do not have the 6th conserved cysteine, and they also lack the terminal 10th cysteine. However, all of the short-form D7 proteins have 6 conserved cysteines [23]. Molecular characterization of a D7 recombinant protein, designated rAed a2, showed that this protein was an allergen that had identical immunogenicity and biological activity as native Aed a2 [29,30]. rAed a2 induced a significant increase in IgE and IgG1 production and the development of an immediate positive skin reaction *in vivo* [21,29].

Putative serpin belongs to the serpin family of proteins, and it plays a role as an anticoagulant by inhibiting Xa in the blood coagulation process [7,25]. AAEL006417-PA and long-form D7Bclu1 belong to the odorant-binding protein (OBP) family, but they are also involved in blood feeding. A 30-kDa SG protein (Aed a2) was also indentified. This protein caused an allergic human-related immune response at the bite site. This protein is very rich in glycine and glutamic acid residues, and it was first described in *Aedes* mosquitoes, but it was also found in *An. Gambiae* [31,32]. The 30-kDa SG protein may be aegyptin, which acts as an anti-hemostatic activity protein and plays an important role in facilitating the blood-feeding process. It also indirectly plays a significant role in enhancing pathogen transmission from vector to host, and it is a proven allergen. Aegyptin binds to specific platelet glycoprotein VI (GP VI), integrin $\alpha 2\beta 1$ and VWF (von Willebrand factor) and prevents the interaction between platelets and collagen. Aegyptin acts as a specific ligand for collagen and inhibits platelet activation and thrombocyte aggregation. The matrix protein collagen plays a central role in the process of primary hemostasis and platelet activation, which triggers and stimulates thrombin formation [32].

Further analysis of the 56-kDa protein band identified 7 proteins: Apyrase, AAEL000732-PA, 5′-nucleotidase, AAEL009524, AAEL004739-PA, putative secreted protein and AAEL017349-PA (Table 2). Apyrase is a secreted protein that is involved in blood feeding. Three proteins are involved in metabolism, whereas the functions of other 3 proteins are not known. The unused score suggests that the most abundant protein in the 56-kDa band was apyrase (62.754 kDa). This result is consistent with a previous study that identified apyrase at 63 kDa [35]. However, other results also identified apyrase with molecular weights of 71 kDa and 81 kDa [7]. Transcriptomic and proteomic analyses of salivary proteins from female *Ae. Aegypti* demonstrated that apyrase is a secretory protein that is involved in the blood feeding process, and these proteins also modulate the immune response. Apyrase (68 kDa) from the salivary proteins of *Ae. Aegypti*, which was expressed in the adult female [33], was also identified as an allergen with α-glucosidase (67 kDa), D7 protein (37 kDa), and the 30-kDa protein [21]. The *Aedes albopictus* apyrase was found in the distal-lateral (80%) and medial (20%) lobes of the SG. Apyrase is a nucleoside triphosphate-diphosphohydrolase that is found in most hematophagous organisms, and it helps blood feeding by inhibiting platelet aggregation *via* the destruction of ADP or ATP. ADP is an important

Table 2 Identification of immunogenic protein 56 kDa from SG of *Ae. aegypti*.

Protein name	Function	Accession number	MW	Unused score	% Coverage
Apyrase OS = *Aedes aegypti* GN = APY PE = 1 SV = 2	Blood feeding	sp|P50635|APY_AEDAE	62.754	61.13	66.37
AAEL000732-PA OS = *Aedes aegypti* GN = AAEL000732 PE = 4 SV = 1	Unknown	tr|Q17NC2|Q17NC2_AEDAE	64.636	30.28	58.63
5′-nucleotidase OS = *Aedes aegypti* PE = 2 SV = 1	Nucleotide catabolic process	tr|Q1HQJ1|Q1HQJ1_AEDAE	61.246	13.93	39.96
AAEL009524-PA OS = *Aedes aegypti* GN = AAEL009524 PE = 4 SV = 1	Carbohydrate metabolic process	tr|Q16VL4|Q16VL4_AEDAE	66.638	6.61	25.91
AAEL004739-PA OS = *Aedes aegypti* GN = AAEL004739 PE = 3 SV = 1	Unknown	tr|Q17BX4|Q17BX4_AEDAE	69.051	6.11	28.09
Putative secreted protein OS = *Aedes aegypti* PE = 2 SV = 1	Unknown	tr|Q8T9U9|Q8T9U9_AEDAE	66.213	6.1	42.76
AAEL017349-PA OS = *Aedes aegypti* GN = AaeL_AAEL017349 PE = 2 SV = 1	ATP binding (metabolic process)	tr|Q1HR69|Q1HR69_AEDAE	72.287	2.08	25.50

inducer of the platelet aggregating process, and ADP is released by damaged cells and activated platelets [13].

Currently, only four recombinant mosquito salivary proteins from *Ae. aegypti* were identified as allergens, including rAed a 1. Recombinant apyrase (rAed1) inhibits ADP-induced platelet aggregation, which proved that the activity of recombinant apyrase is observed in platelet aggregation assays [30,33]. Three classes of apyrases were characterized at the molecular level in different blood-sucking arthropods. One class is the 5′ nucleotidase family proteins, which were cloned from *An. gambiae* and *Ae. aegypti* [26,27,33]. 5′-nucleotidases are involved in the nucleotide catabolic process, which is usually associated with SG apyrase activities in mosquitoes. The other 2 classes of identified proteins were AAEL009524-PA, which is involved the carbohydrate metabolic process, and AAEL017349-PA, which binds ATP and is involved in the stress response.

Conclusions

Thirteen proteins were characterized from the 31-kDa immunogenic proteins of the SG from *Ae. aegypti*. Five of these proteins are involved in blood feeding, 3 proteins are involved in metabolism and act as structural proteins, and the role of 5 proteins is not known. The most abundant protein in the 31-kDa band was the D7 AOB protein. The immunogenic 56-kDa band consisted of 7 proteins: one protein is involved in blood feeding, 3 proteins are involved in metabolic pathways, and 3 proteins are not known. The most abundant 56-kDa protein was apyrase.

Conflict of interest

The authors declare that there are no conflict of interest.

Ethical approval: Not required.

Acknowledgments

This study was financially supported by a Sandwich Like Program Grant 2013 and Fundamental Research Grant of Directorate General of Higher Education Indonesia.

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