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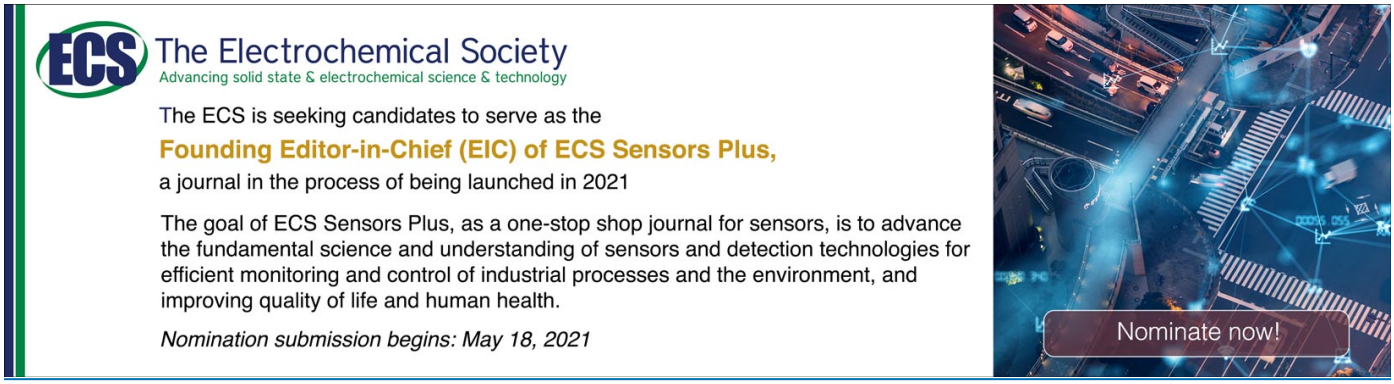

# Molecular Characterization of Secreted Proteins from Salivary Gland Immunogenic Protein of *Anopheles vagus*

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**Abstract.** Salivary mosquito's proteins have been widely acknowledged to contain factors important for pathogen transmission as well as for biomarker of Anopheles exposure. The objective of this study was to identify and characterize the secreted proteins from salivary glands and immunogenic proteins from of *Anopheles* (*An.*) *vagus*. A proteomic approach combining one-dimensional electrophoresis (1DE) was operationalized, followed by western blot analysis using human sera from healthy people living in an endemic area (Kendal, East Java - Indonesia); liquid chromatography mass spectrometry (LC-MS/MS) and bioinformatics analysis were conducted to gain direct insight into *An. vagus* salivary proteins. Three immunogenic proteins with molecular weight of 69, 75 and 232 kDa were identified. Apart from the housekeeping proteins identified by LC-MS/MS, there were also some proteins which played crucial role in the blood feeding process i.e AGA 5' nucleotidase family, for 69 & 75 kDa and SGS 4 for 232 kDa respectively. The other known proteins like vitellogenin, putative myosin class I heavy chain and heat shock protein 70 (Hsp70) were also identified. The majority of proteins were clearly characterized in Anopheles for their role in blood feeding, metabolism, cytoskeleton protein. and stress response.

## 1. Introduction

Although malaria has ever been virtually eradicated from Indonesia, currently it is recognized as a serious re-emerging threat to public health. This disease is caused by malaria parasite which is transmitted to human host by Anopheles mosquitoes as the main vector. Among the malaria vectors, *Anopheles vagus* has been confirmed as secondary malaria vector (for *Plasmodium falciparum*) in Central Java (Purworejo, Kokap) and western Timor Island (Kupang), Indonesia [1;2]. Anti-malarial drug resistances as well as vector resistance against insecticides are major public health problems which hinder the control of malaria [3]. This condition indicates that the investigation for potent therapy is of prominent importance. Therefore, the development of a vaccine can be a more efficient strategy to overcome the epidemics.

The malaria vaccine development is hindered by the sheer complexity of parasite and its life cycle, extensive antigenic variation and poor understanding of the interaction between *P. falciparum* and the human immune system [4;5;6;7]. In the last decade, a new approach in the development of vaccine for arthropod-borne diseases is using the salivary components from vectors. This approach is based on the hyphotesis that Arthropod vectors saliva contains vasomodulator and imunomodulator proteins [8;9]. The vasomodulatory factors in Arthropod saliva help the vector to obtain blood meal. There are 2 hypotheses concerning the function of imunomodulatory factor in saliva of mosquitoes. Many reports show that salivary imunomodulators can exacerbate pathogen infection [10]. (a). However, there is also evidence indicating that saliva appearsto directly protect dendritic cells from in vitro infection [11]. (b). With respect to the first case, it is likely to control pathogen transmission by vaccinating the host against the molecule(s) in saliva that potentiate the infection. This process can possibly block the

enhancing effects of salivaand thus prevent the Plasmodium from establishing infection in the host. In the second condition, it can be used directly to protect host cells from infection of transmitted pathogens. These hypotheses lead to new field of research that examines these salivary factor especially the imunomodulatory proteins to serve as target to control pathogens transmission i.e. Transmission Blocking Vaccine (TBV) or as also known as Mosquito Stage Vaccine [12]. However, specific protein as a potential target for TBV in mosquitoes i.e. Anopheles as well as *Aedes aegypti* has not yet been identified so far.

These mosquito's immunogenic proteins are also important for biomarker of Anopheles exposure. The human antibody against mosquito salivary protein generated during the blood meal can serve as immunological marker to evaluate individual exposure to Arthropod bites [13] or assess the impact of vector control interventions [14]. These strategies are of high advantage in the absence of a licenced malaria vaccine [15;16].

Here we describe an in-gel proteomic approach using SDS-PAGE 1D followed by western blot and LC-MS/MS to characterize the proteome of the salivary gland extracts (SGEs) of *An. vagus*. Our results based on mass spectrometry data analysis by using MASCOT algorithm (bioinformatic analysis) is projected to be the first preliminary step for putative functional identification of several salivary gland protein extracts (SGPE) from *An. vagus.* To our knowledge, this is the first study on proteomic identification of SGPE from *An. vagus* originating from endemic area in Indonesia.

## 2. Methods

### *2.1 Collecting of An. vagus and Salivary Glands (SG) Dissection*

The adult mosquitoes *An. vagus* were collected from Kendal, Central Java-Indonesia. *An. vagus* salivary glands were dissected using Barber and Rice's methods [17]. Fine needles were used to detach the salivary glands from thorax segment under a stereomicroscope at 4x magnification using phosphate buffer saline (PBS) and were pooled. After dissection, the tissues were immediately placed in a PBS buffer (100 µL) with protease inhibitors (PMSF) and stored at -20$^{o}$C until use.

### *2.2 Sample Analysis: 1D Gel Electrophoresis (SDS-PAGE) and Western Blotting*

SDS-PAGE was performed according to the methods described by Laemmli [18] with minor modification. SGE proteins were analyzed using 12,5% separating gel with 4,5% stacking gel. Electrophoresis was performed using a constant voltage of 120 V for ± 2 hours at room temperature. Protein bands were visualized using Commassie Brilliant Blue (CBB) R250 stain.

Proteins from SDS-PAGE analysis were transferred to a PVDF membrane under constant current (100 mA) for 1 hour by using semidry western blotting machine. The membrane was then blocked with 5% skimmed milk in 1x TBS for 1 hour at room temperature. After being washed thoroughly, the PVDF membrane was treated with the pooled sera from healthy people living in endemic area at the dilution of 1:500 and incubated overnight at 4$^{o}$C. PVDF membrane was then incubated with secondary antibodies goat-anti Human IgG alkaline phosphatase conjugated (1:5000) for 2 hours at room temperature. Colour development was done with NBT-BCIP substrate.

### *2.3 Protein Identification using LC-MS/MS Analysis*

Mass spectrometric analyses were performed by the Core Facility Proteomics at the University Medical Center Göttingen-Germany.

*2.3.1 Sample preparation.* Proteins containing SDS-PAGE gel pieces were subjected to in-gel digestion through enzymatic process. After washing, gel slices were reduced with dithiothreitol (DTT), alkylated with 2-iodoacetamide and digested with trypsin overnight. The resulting peptide mixtures were then extracted, dried in a SpeedVac, reconstituted in 2% acetonitrile/0.1% formic acid/ (v:v) and prepared for nanoLC-MS/MS as described previously [19].

*2.3.2 LC-MS/MS analysis.* For mass spectrometric analysis, samples were enriched on a self-packed reversed phase-C18 precolumn (0.15 mm ID x 20 mm, Reprosil-Pur120 C18-AQ 5 µm) and separated on an analytical reversed phase-C18 column (0.075 mm ID x 200 mm, Reprosil-Pur 120 C18-AQ, 3 µm) using a 30 min linear gradient of 5-35 % acetonitrile/0.1% formic acid (v:v) at 300 nl/ min). The eluent was analyzed on a Q Exactive hybrid quadrupole/orbitrap mass spectrometer (ThermoFisher Scientific, Dreieich, Germany) equipped with a Flexion Nanospray source operated under Excalibur

*2.4* software using a data-dependent acquisition method. Each experimental cycle was of the following form: one full MS scan across the 350-1600 *m/z* range acquired at a resolution setting of 70,000 FWHM, and AGC target of 1*10e6 and a maximum fill time of 60 ms. Up to the 12 most abundant peptide precursors of charge states 2 to 5 above a 2*10e4 intensity threshold were then sequentially isolated at 2.0 FWHM isolation width, fragmented with nitrogen at a normalized collision energy setting of 25%, and the resulting product ion spectra were recorded at a resolution setting of 17,500 FWHM, and AGC target of 2*10e5 and with a maximum fill time of 60 ms. Selected precursor *m/z* values were then excluded for the following 15 s. Two technical replicas per sample were acquired.

*2.3.3 Data processing.* Peaklists were extracted from the raw data using Raw2MSMS software v1.17 (Max Planck Institute for Biochemistry, Martinsried, Germany). Protein identification was conducted using MASCOT 2.4 software (Matrixscience, London, United Kingdom). Proteins were identified against the UniProtKB v2015.12 *Anopheles* protein entries (59919 protein entries) along with a set of 51 contaminants commonly identified in our laboratory. The investigation was performed with trypsin as enzyme and iodoacetamide as cysteine blocking agent. Up to two missed tryptic cleavages and methionine oxidation as a variable modification were granted. Search tolerances were set at10 ppm for the precursor mass and 0.05 Da for fragment masses, and ESI-QUAD-TOF specified as the instrument type. Scaffold software version 4.4.1.1 (Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they were established at greater (MISSING NOUN) than 95.0% probability by the Percolator algorithm. Protein probabilities were assigned by the Protein Prophet algorithm [20]. Protein identifications were accepted if they were established at greater (MISSING NOUN) than 99% by the Percolator algorithm and contained at least 2 identified peptides. Protein hits that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to comply with the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Proteins were annotated with GO terms from NCBI downloaded February 23, 2015 [21].

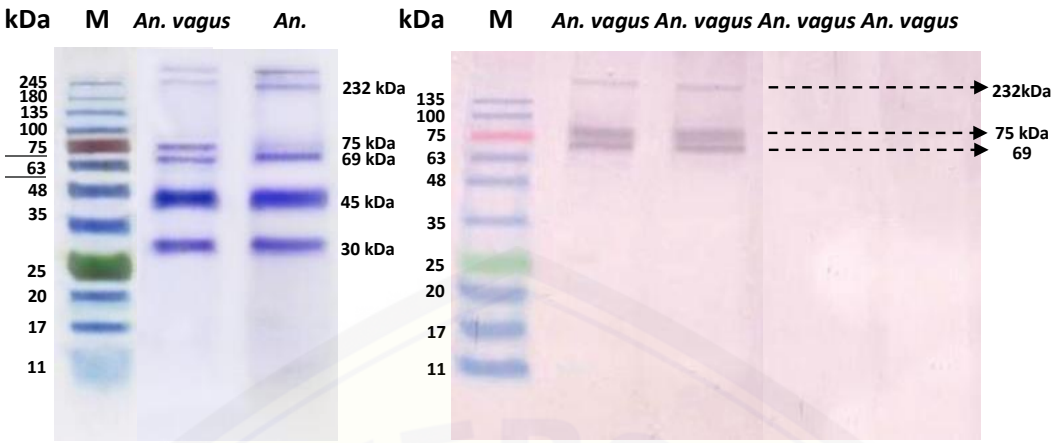
## 3. Results and Discussion

In this study, we employed a MS-based approach to categorize different putative proteins of salivary glands of *An. vagus*. Total proteins of the salivary glands homogenate were first analyzed by 1D gel electrophoresis (SDS-PAGE). There were 6 major bands of salivary glands homogenate identified after CBB staining with molecular weights ranging from 30 kDa up to 232 kDa were measured. Subsequently, there were three protein bands identified after cross-reacted with sera from healthy people living in endemic malaria (Kendal) which had molecular weights of 69, 75 and 232 kDa, meanwhile sera from healthy people living in non-endemic area (Jember) did not show an immunogenic reaction with salivary glands proteins of *An. vagus* (Figure 1).

These results indicate that healthy people living in endemic area have specific antibodies that are not found in healthy non-exposed people. Some salivary proteins are immunogenic and can initiate specific antibody responses [22]. The development of this natural specific antibodies results from frequent exposure to mosquito saliva [23].

In-gel digested peptides of *An. vagus* salivary gland were then analyzed by LC-MS/MS. Some known salivary proteins and novel proteins as well as their details, such as molecular weight, accession number, molecular function are presented in Tables 1, 2, and 3., whereas the rest of known proteins are presented as supporting information. Different proteins were assigned according to immunogenic gel bands. There were 119 proteins identified from band 69 kDa, 37 proteins from band 75 kDa and 51 proteins from band 232 kDa. These proteins with band number are shown in respective tables (Tables 1, 2 and 3), including supporting information.

Other novel proteins with feature similar to proteins in other mosquito species like *An. gambiae*, *An. sinensis* (Table 2) were also identified by MASCOT analysis. Most of these proteins were known to be involved in carbohydrate metabolism and energy pathway, whereas others were unknown. However, two novel proteins AGAP004109-PA and AGAP001424-PA were found to have signal peptide.




(a) (b)

**Figure 1.** Commassie stained SDS-PAGE gel of the salivary gland extract (a); Western blot analysis of *An. vagus* salivary glands protein (b) cross-reacted with sera from healthy people in endemic (L) and non-endemic area (R).

**Table 1.** A catalogue of known and novel proteins identified from 69 kDa band

| Protein name | Accession Number/ Vector base Accession Number | Function | MW (kDa) |
|---|---|---|---|
| AGAP011026-PA (belongs to the 5'-nucleotidase family) | tr\|Q5TVM9\|Q5TVM9_ANOGA [sp] | Blood feeding | 63,475 |
| AGAP004192-PA (belongs to the heat shock protein 70 family) | tr\|Q7PQK5\|Q7PQK5_ANOGA [sp] | Stress response | 72,743 |
| Protein disulfide-isomerase | tr\|Q5TMX9\|Q5TMX9_ANOGA [sp] | Protein disulfide-isomerase activity | 53,133 |
| AGAP002102-PA | tr\|Q7PYT9\|Q7PYT9_ANOGA [sp] | Carbohydrate metabolic process | 67,217 |
| AGAP005608-PA | tr\|Q7Q6Y1\|Q7Q6Y1_ANOGA [sp] | Unknown | 73,890 |
| ATP synthase subunit beta | tr\|E3XEC7\|E3XEC7_ANODA | Energy pathway | 53,768 |
| AGAP009310-PA | tr\|Q7PVE0\|Q7PVE0_ANOGA | Unknown | 53,249 |
| Serine hydroxymethyltransferase | tr\|A0A084VDD9\|A0A084VDD9_ANOSI | Unknown | 51,659 |
| Dper\GL12416-PA (belongs to the tubulin family) | tr\|A0A084W024\|A0A084W024_ANOSI | Cytoskeletal protein | 50,447 |

*sp: proteins with signal peptide

**Table 2.** A catalogue of known and novel proteins identified from 75 kDa band

| Protein name | Accession Number/ Vector base Accession Number | Function | MW (kDa) |
|---|---|---|---|
| AGAP004109-PA | tr\|Q7QBA6\|Q7QBA6_ANOGA [sp] | Hydrolase activity | 80,322 |

| Protein name | Accession Number/ Vector base Accession Number | Function | MW (kDa) |
|---|---|---|---|
| AGAP001424-PA | tr\|Q7PXI9\|Q7PXI9_ANOGA [sp] | ATP Binding | 91,347 |
| Uncharacterized protein | tr\|A0A084WGQ3\|A0A084WGQ3_ANOSI | Unknown | 77,357 |
| Alpha-1,4 glucan phosphorylase | tr\|Q7Q3L6\|Q7Q3L6_ANOGA | Carbohydrate metabolism | 96,839 |
| Glycerol-3-phosphate dehydrogenase | tr\|F5HLN3\|F5HLN3_ANOGA | Unknown | 81,841 |

*sp: proteins with signal peptide

**Table 3.** A catalogue of known and novel proteins identified from 232 kDa band

| Protein name | Accession Number/ Vector base Accession Number | Function | MW (kDa) |
|---|---|---|---|
| SGS4 | tr\|Q5XLG7\|Q5XLG7_ANOGA | Blood feeding | 389,184 |
| Putative myosin class I heavy chain | tr\|T1EB94\|T1EB94_ANOAQ | Cytoskeletal protein | 149,563 |
| LANB2 | tr\|M4JUU8\|M4JUU8_ANOME [sp] | Basal membrane | 179,463 |
| Vitellogenin | tr\|W8S9N9\|W8S9N9_9DIPT [sp] | Lipid transporter | 239,223 |
| AGAP006280-PA | tr\|Q7Q5P8\|Q7Q5P8_ANOGA [sp] | Unknown | 213,975 |
| AGAP006452-PA | tr\|Q7Q5G3\|Q7Q5G3_ANOGA [sp] | Unknown | 197,204 |
| AGAP007523-PA | tr\|A7URB0\|A7URB0_ANOGA | Unknown | 228,016 |

*sp: proteins with signal peptide

The role of salivary glands and their proteins is important in mosquito because parasite becomes mature and subsequently forms infectious sporozoites in salivary glands. In earlier studies, it has been reported that repeated exposures of SGE from three potential malaria vectors in Indonesia i.e. *An. aconitus*, *An. sundaicus* and *An. maculatus* are able to reduce rates of parasitemia in murine model [24]. No attempts, however, have been made to study the detailed proteome of *An. vagus* salivary glands for functional identification of such proteins. Due to the paucity of studies in this area, this study was carried out to identify total salivary gland proteins of *An. vagus* expressed by proteome analysis coupled with LC-MS/MS as an initial step towards the cataloging of the hundreds of proteins and peptides in the salivary proteome for future use in terms of blocking transmission of malaria parasites.

Among all identified proteins by LC-MS/MS, further signal peptides were also identified at the N-terminus of all identified proteins with the help of SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP), which shows the indication of secretion [25]. A sort of salivary gland proteins termed as a AGAP011026-PA (belongs to the 5'-nucleotidase family) was identified with molecular weight 63,475 kDa. Signal peptide was also identified from this protein at amino acid positions 1-23 which depicted a secreted protein (Figure 2). In *An. gambiae*, two genes are expressed in the salivary glands and annotated as apyrase and 5'-nucleotidase: both can actually be coded as proteins with apyrase activity [26;27]. Apyrases are enzymes ubiquitously found in the salivary glands of blood-feeding insects and ticks. These enzymes degrade the neutrophil-inducing substance ATP and the platelet-aggregating nucleotide ADP to AMP, presumably facilitating blood feeding [26;28].

Another protein that played an important role in stress response was identified. This was

AGAP004192-PS (belongs to the heat shock protein 70 family), with molecular weight 72,743 kDa and signal peptide at positions 1-20. As with nearly all organisms, mosquito heat shock protein (Hsp70) has been documented to increase during environmental stress [29-32]. When Hsp proteins are suppressed, mechanisms for tolerance to heat, cold, and dehydration are compromised [33]. Egg production was also reduced by 25% following knockdown of Hsp70 [34]. This provides evidence that the Hsp response is essential for successful processing of the blood meal.

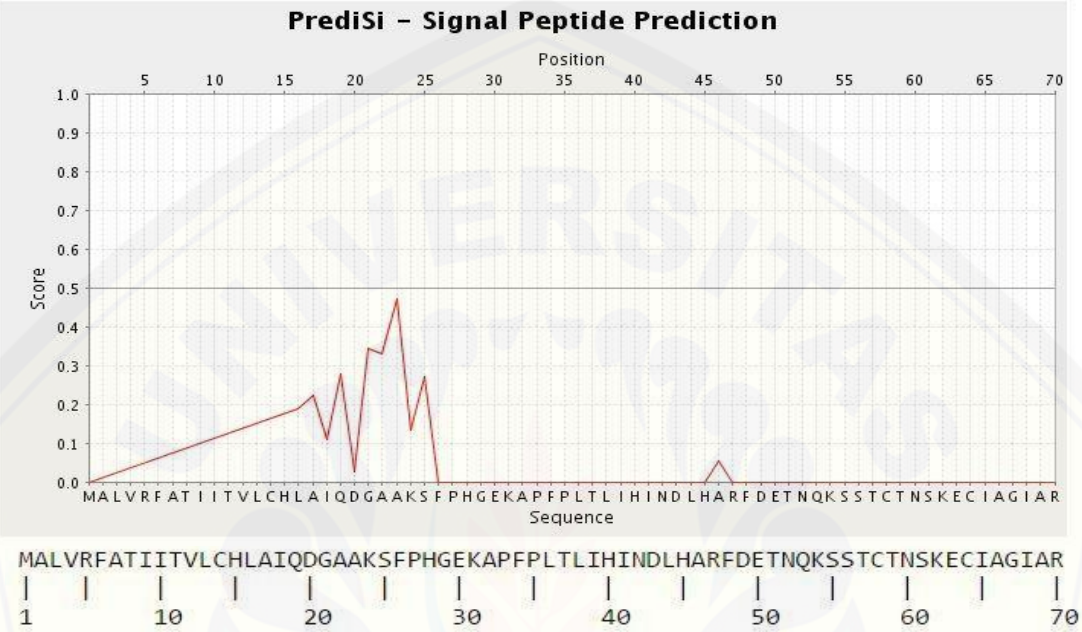



**Figure 2.** Signal peptide positions for 5'-nucleotidase protein family (the first 23 residues belong to the signal peptide of the precursor)

Further analysis of the 232-kDa protein band identified 52 proteins, some of them (7 proteins) are shown in Table 3. Among 7 proteins identified from 232 kDa band, SGS4 is associated with blood feeding behaviour [35;36]. Based on transcriptomic analysis, SGS is the only anopheline or culicine saliva protein whose mass approximates the value predicted for this ~387 kDa protein [37]. This study is consistent with our result in that bothhave identified SGS4 protein from LC-MS/MS at ~389.184 kDa. From previous study conducted, experiments involved antisera from mice exposed to mosquito bites, which were used as primary antibodies in western blots. The result was corroborated by the discovery of SGS4 as major components of mosquito saliva and also showed that it was highly immunogenic, eliciting a strong IgG response [37].

The study also discovered another protein marked for lipid transporter, such as vitellogenin. Vitellogenin genes (Vg) are known as egg yolk precursor proteins, which are used in ovary development and regulated by juvenile hormone. The mosquito is known to synthesis vitellogenin in fat body after a blood meal [38].

## 4. Conclusion

Salivary gland proteins of the Anopheles mosquitoes are considered important in the development of the plasmodium as these molecules are involved in the antihemostatics activity, which may assist blood feeding process and play a critical role in the transmission of malaria parasites. In this study, our initial studies uses proteomic approaches to identify the salivary gland of malaria vector *An. vagus*. A total of 296 known and novel proteins were analysed by LC-MS/MS from 3 immunogenic protein bands (69, 75 and 232 kDa). Some of these identified proteins are involved in blood feeding as well as

metabolism and act as structural proteins, while others remain unknown.

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