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## Asian Pacific Journal of Tropical Biomedicine

journal homepage: [www.elsevier.com/locate/apjtb](http://www.elsevier.com/locate/apjtb)Original article <http://dx.doi.org/10.1016/j.apjtb.2016.08.012>Identification of antigenic proteins from salivary glands of female *Anopheles maculatus* by proteomic analysisYunita Armiyanti<sup>1\*</sup>, Renam Putra Arifianto<sup>2</sup>, Elisa Nurma Riana<sup>2</sup>, Kartika Senjarini<sup>2</sup>, Widodo Widodo<sup>3</sup>, Loeki Enggar Fitri<sup>4</sup>, Teguh Wahyu Sardjono<sup>4</sup><sup>1</sup>Doctoral Program in Medical Science, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia<sup>2</sup>Department of Biology, Faculty of Mathematics and Natural Sciences, University of Jember, Jember, Indonesia<sup>3</sup>Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia<sup>4</sup>Department of Parasitology, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia

## ARTICLE INFO

## Article history:

Received 4 Apr 2016

Received in revised form 17 Aug, 2nd

revised form 18 Aug 2016

Accepted 22 Aug 2016

Available online 14 Sep 2016

## Keywords:

Saliva

Immunogenic

Proteins

*Anopheles*

## ABSTRACT

**Objective:** To identify antigenic proteins from the salivary glands of female *Anopheles maculatus* using a proteomic approach to find the biomarker candidate for serological tools.

**Methods:** The identification of antigenic proteins of *Anopheles maculatus* salivary gland used these techniques: one-dimensional gel electrophoresis (sodium dodecyl sulfate polyacrylamide gel electrophoresis), western blot, and liquid chromatography–mass spectrometry.

**Results:** The proteins that have molecular weight (MW) 43 and 34 kDa were the antigenic protein. Computational bioinformatic analysis by Mascot Server revealed seven novel hypothetical proteins (MW: 43 kDa) and two novel hypothetical proteins (MW: 34 kDa). Further analysis (BLASTP, antigenicity, epitope mapping, and specificity analysis) showed that two novel proteins were identified as apolipoprotein D and cathepsin D in *Anopheles darlingi*.

**Conclusions:** The identified proteins are potential to be developed as a biomarker of mosquito bite's exposure.

## 1. Introduction

Malaria is an important health problem of the world since approximately half of the world's population have a risk of infection. Malaria is an infectious disease caused by *Plasmodium* and spreads by female *Anopheles* mosquitoes. There are 30 from 400 species that can be major vectors, including *Anopheles*

*maculatus* (*An. maculatus*) [1]. *An. maculatus* is one of major malaria vectors in Asia that widely spread in Afghanistan, Pakistan, South China, India, Indonesia, Malaysia, Thailand and the Philippines [2].

*Anopheles* mosquitoes prefer in tropic climate and humid temperature. So, *Anopheles* mosquitoes can spread easily in a tropical country, including Indonesia. As a consequence, there are many malaria-endemic areas found in the tropical countries. The society that stays in the malaria-endemic areas mostly used repellent, insecticides, and insecticide-treated nets to avoid *Anopheles* mosquitoes bites [3]. The health program to control the vector spreading is a crucial project since the vaccine was still not available yet. The malaria control programs based on ento-mological methods, such as mosquito abundance, blood feeding rates, and mortality are difficult to apply on a large scale. Another method, such as human landing catch is commonly used for evaluating individual human exposure. However, this method is limited ethically and not representative for children or under-aged [4]. Therefore, a program to control malaria vector needs a new approach to assess

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The study protocol was performed according to the Helsinki declaration and approved by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Brawijaya in statement of ethical clearance No. 251/EC/KEPK-S3/03/2014 dated 26 March 2014. Informed written consent was obtained from Health Research Ethics Committee of the Faculty of Medicine, Universitas Brawijaya.

Foundation Project: Funded by a Doctorate Research Grant of the Directorate General of Higher Education Indonesia (Grant No. 435/UN25.3.1/LT.6/2014).

Peer review under responsibility of Hainan Medical University. The journal implements double-blind peer review practiced by specially invited international editorial board members.

malaria risk by evaluating the efficacy of vector control at both population and individual levels [5].

Malaria infection mechanism begins when *Anopheles* mosquito salivary proteins inject into the host during its blood-feeding which is able to modulate the host immune response. The antibody protein, immunoglobulin G (IgG) anti-salivary protein, has been detected in malaria patients and the population of malaria-endemic areas [6]. Apyrase, *Anopheles gambiae* (*An. gambiae*) salivary gland protein 6 (gSG6), TRIO protein, *Anopheles* antiplatelet protein, glycine- and glutamate-rich salivary gland protein, and anophensin are salivary proteins that are antigenic [7]. Therefore, the salivary gland protein that is able to elicit the host immune response has been proposed as a marker of exposure to mosquito bites, even as a biomarker of malaria risk [8]. As a biomarker of malaria risk, the level of anti-gSG6 IgG antibody was also associated with malaria transmission according to strong positive association with merozoite surface protein-1 and glutamate-rich protein [6].

Recently, some studies showed gSG6 protein is a potential candidate for *An. gambiae* exposure marker [6,9]. The gSG6 is a small protein first described in *An. gambiae* with mature peptide/protein weighed 10 kDa. The human antibody response to gSG6 protein indicated a reliable indicator of human exposure to the three main malaria vectors in tropical Africa, i.e., *An. gambiae*, *Anopheles arabiensis* (*An. arabiensis*), and *Anopheles funestus* [9]. The recombinant protein of gSG6 has been studied by gSG6-P1 peptide as a biomarker of low exposure to *Anopheles* bites. The IgG response against gSG6-P1 peptide was detectable in most individuals in malaria-endemic regions in a low-rated season of mosquitoes bites exposure (September–December) in Senegal [10].

In Africa, salivary gland proteins from *An. gambiae* have been studied as an immunological marker of *Anopheles* bites exposure. But, in Asia region the study about *Anopheles* mosquitoes (*An. maculatus*) is limited. The gSG6 salivary protein has been detected in *Anopheles barbirostris* species A2 salivary glands by mass spectrometry (MS)-based proteomic analysis, but it has not been proved to be antigenic protein [11]. The first step to determine the candidate protein for biomarker of exposure to *Anopheles* mosquito bites is identification of salivary gland proteins that possess antigenic properties. The identification can be performed by the proteomic approach that provides a direct measurement of protein expression level [12]. Therefore, the study aimed to identify the antigenic proteins of *An. maculatus* salivary gland as biomarker candidate for serological tools.

## 2. Materials and methods

### 2.1. Mosquitoes collection and salivary gland dissection

The adult female *An. maculatus* were collected from Kalirejo village, Kokap District, Kulonprogo Regency in Yogyakarta by an aspirator. That mosquito species was a major vector of malaria and the dominant species in the research location. The mosquitoes were maintained under standard conditions at  $(27 \pm 2)^\circ\text{C}$  with  $(70 \pm 10)\%$  relative humidity and fed with 10% sucrose solution in the insectariums of the Entomology Laboratory of Parasitology Department, Faculty of Medicine, Gajah Mada University. The salivary glands of *An. maculatus* were dissected using fine entomological needles under a stereo microscope (4 $\times$ ) and pooled into a microcentrifuge tube containing phosphate buffer saline

with protease inhibitor (phenylmethylsulfonyl fluoride). The salivary glands were stored at  $-80^\circ\text{C}$ .

### 2.2. Salivary gland extraction and protein quantification

Dissected salivary glands (100 pairs) in phosphate buffer saline and phenylmethylsulfonyl fluoride were mixed (1:1) in lysis buffer (1.5 mmol/L  $\text{MgCl}_2$ , 10 mmol/L Tris-HCl, 10 mmol/L NaCl, 1% Nonidet P-40, and 2 mmol/L ethylenediaminetetraacetic acid, NaOH) and homogenized using a micropestle. The mixture was sonicated by a water sonicator for 30 min. Afterward, the suspension was centrifuged at 10000 r/min and  $4^\circ\text{C}$ , for 15 min. The extracted supernatant was collected and concentrated using a spin concentrator (cut-off of 10 kDa; Corning) and centrifuged (10000 r/min,  $4^\circ\text{C}$ , for 30 s). The protein concentrations of salivary gland extracts (SGEs) were determined using a nanophotometer (Implen NanoPhotometer<sup>®</sup> P 360, Germany).

### 2.3. Human serum samples

The blood was collected from 15 healthy adult residents living in Kalirejo village, Kokap District, Kulonprogo Regency in Yogyakarta. The human subject protocol for this study was approved by the Ethical Committee of Medical Research, Faculty of Medicine, Universitas Brawijaya. The level of antibodies anti-salivary gland extract IgG of *An. maculatus* in the serum samples was measured by ELISA as described by Fontaine *et al.* [13]. Five serum samples with a high level of IgG antibodies anti-salivary gland extract were used as primary antibodies in western blot [13].

### 2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

A total of 40  $\mu\text{g}$  of SGE sample was mixed in sample buffer (1:1) consisting of 0.5 mol/L Tris-HCl, 10% SDS, glycerol, distilled water, 1% bromophenol blue and  $\beta$ -mercaptoethanol. Then, the mixture was heated at  $95^\circ\text{C}$  for 5 min. Then, 20  $\mu\text{L}$  sample was loaded and separated onto 12% SDS-PAGE in V-GES (WEALTEC, USA). Molecular weight (MW) protein marker (Nacalai) was loaded on the gel stained with Coomassie Brilliant Blue.

### 2.5. Western blotting

Gels were transferred to polyvinylidene difluoride membranes (MACHEREY-NAGEL, Germany) using semidry blotting (Bio-Rad) for 1 h at 100 mA. The membranes were saturated at room temperature for 1 h with 5% w/v non-fat dried milk in blocking buffer, which consisted of Tris-buffered saline and 0.05% Tween 20 (TBS-T), then washed with TBS-T three times. Membranes were incubated with serum samples at dilution 1:20 in blocking buffer overnight at  $4^\circ\text{C}$ . Subsequently, blots were washed three times with TBS-T, then incubated with alkaline phosphatase goat anti-human IgG secondary antibody (KPL, USA) at dilution 1:2000 for 2 h at room temperature. The blot stained with nitro blue tetrazolium/bromo-4-chloro-3-indolyl phosphate and prestained broad range MW markers (9–200 kDa) (Nacalai, Japan) used to estimate the protein size.

## 2.6. In-gel protein digestion and MS analysis

The gels were digested using 10 mL trypsin digest solution (12.5 mg/mL trypsin, 25 mmol/L ammonium bicarbonate) and incubated overnight at 37 °C. The digested peptides were extracted by 10–20 mL acetonitrile containing 1% trifluoroacetic acid and incubated for 20 min. The extracts were dried by rotary evaporation and stored at –20 °C until further analysis by MS [14].

Peptides were analyzed by electrospray ionization MS using the Agilent 1260 Infinity HPLC system (Agilent) coupled to an Agilent 6540 mass spectrometer (Agilent). Tryptic peptides were loaded onto a C18 column 300 SB, 5 µm (Agilent) and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v). Spectra were analyzed to identify proteins of interest using Mascot sequence matching software (Matrix Science) with Ludwig NR database.

## 2.7. MS data analysis

The parameters were: (a) trypsin as the specific enzyme, (b) peptide mass tolerance:  $\pm 0.2$  Da, (c) fragment mass tolerance:  $\pm 0.2$  Da, (d) variable modification oxidation of methionine carbamidomethyl and database used Ludwig NR and MSPnr100. The amino acid sequence was retrieved from Mascot Server (<https://sysbio-mascot.wehi.edu.au/mascot>). For identifying the protein profile, the amino acid sequence samples were analyzed by comparing with the non-redundant protein sequence from *Anopheles* (taxid: 7164) through BLASTP (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). All identifications were manually validated based on MW, signal peptide (SignalP 4.1: <http://www.cbs.dtu.dk/services/SignalP/>) and subcellular location. Data reliability was measured by the query coverage and percent identity of each sample.

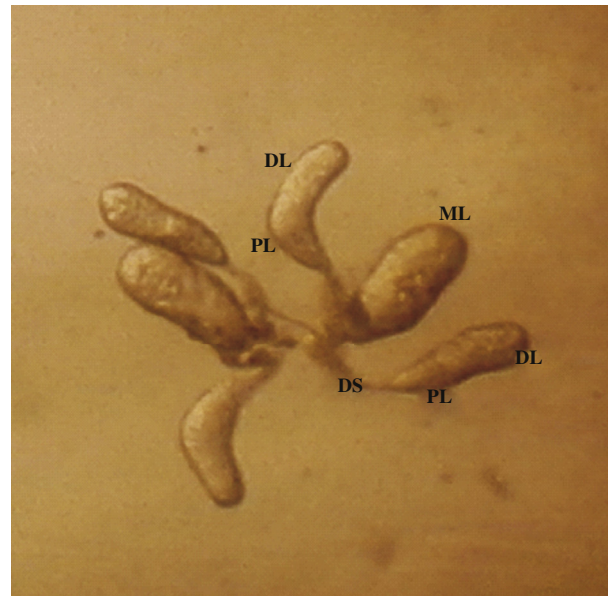
## 2.8. Analysis of antigenicity and epitope mapping

The antigenicity of proteins was investigated using Kolaskar and Tongaonkar antigenicity (<http://www.iedb.org>) and a default threshold value was 1.0 [15]. The epitope mapping was performed by using BepiPred Linear Epitope Prediction with a default threshold of 0.35. BepiPred method is a computational method to predict linear B-cell epitopes that consist a linear sequence of amino acids that can be recognized by the antibodies [16]. The similarity between all novel proteins and human's protein was also analyzed by using BLASTP (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The antigenic sites and epitopes were also visualized by 3D structure profiles using PyMol software.

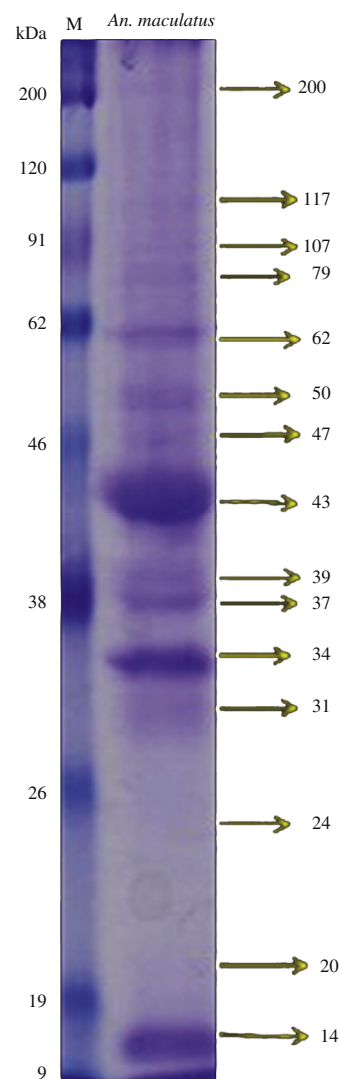
## 3. Results

### 3.1. Antigenic proteins of *An. maculatus* salivary gland

The first step to identify the candidate proteins was antigenic proteins determination by western blot. The antigen from the salivary gland of *An. maculatus* (Figure 1) and sera from five individuals from malaria-endemic areas (Kalirejo village) were used. Based on SDS-PAGE result, the proteins from salivary glands of *An. maculatus* showed numerous bands ranging from 14 kDa to 200 kDa. The MW of seven major bands was 62, 50, 43, 39, 37, 34 and 14 kDa (Figure 2). The results of western blotting showed that five bands (43, 37, 34, 20 and 14 kDa) were cross-reacted with pooled serum of five individuals from malaria-endemic areas (Kalirejo village) (Figure 3A). These

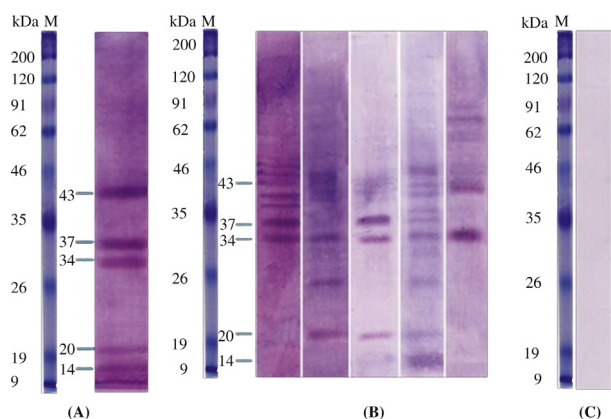


**Figure 1.** A pair of the salivary gland from adult female *An. maculatus*. DL: Distal region of lateral lobe; PL: Proximal region of lateral lobe; ML: Median lobe; DS: Ductus.



**Figure 2.** Salivary gland proteins of female *An. maculatus* mosquitoes were separated on 12% SDS-PAGE (right lane) and stained with Coomassie Blue. Lane M: Protein standard marker (kDa).

antigenic proteins were also recognized by serum as well as by pooled serum. According to the intensity of antigenic bands that showed up at the individual responses, proteins with MWs of 34 and 43 kDa showed up at all of serum samples (Figure 3B). Furthermore, the negative result appeared in the reaction between pooled sera of seven individuals living in non-malaria endemic areas with SGEs of *An. maculatus* (Figure 3C). The antigenic proteins with MWs of 34 and 43 kDa would be further identified with MS-based in gel digestion approach.



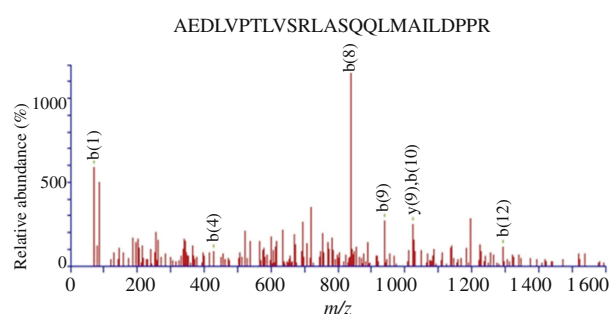
**Figure 3.** Western blotting of anti-salivary gland protein IgG antibodies in a pool of sera from 5 individuals living in malaria-endemic area as positive control (A), individual response from individuals living in malaria-endemic area (B) and a pool of sera from 7 individuals living in non-malaria endemic area as negative control (C).

### 3.2. MS analysis for antigenic proteins identification

MS data suggested that the study has identified two hypothetical proteins from 34 kDa band and seven hypothetical

proteins from 43 kDa similar to proteins of *An. gambiae*, *Anopheles sinensis* (*An. sinensis*), and *Anopheles darlingi* (*An. darlingi*) (Tables 1 and 2). The data showed the amino acid residues SREELNSGGIGEMIRPYR from 34 kDa band were matched (64%) with FMRFamide variant 1 (gi:656339519) and AGAP005518-PA (gi:118786597) proteins of *An. gambiae*.

Among seven novel hypothetical proteins from 43 kDa, three of them had the same function in carboxylic ester hydrolase activity, one of them had a function in aspartic-type endopeptidase activity, and the rest proteins had unknown function. One novel hypothetical protein that had been identified was similar to lipase of *An. darlingi* (gi:568251178) (64% similarity). Another novel hypothetical protein was identified similar to *An. darlingi* cathepsin D (aspartic proteases enzymes). This protein matched with the MS/MS spectrum that assigned the amino acid sequence AEDLVPTLVSRSLASQQLMAILDPPR (Figure 4). All identified proteins were selected based on their similarity to human's surface cell protein using BLASTP tools (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).



**Figure 4.** MS/MS spectrum of the peak at  $m/z$  911.79 corresponds to the peptide sequence, AEDLVPTLVSRSLASQQLMAILDPPR which matches a novel protein (gi:568251273) that designated as cathepsin D in *An. darlingi*.

**Table 1**

A list of novel proteins from 34 kDa antigenic band identified by LC–MS/MS using in-gel digestion approach.

No	Accession number	Protein	Query coverage (%)	Identity (%)	MW (kDa)	SignalP	Domain/function	Subcellular location
1	gi:656339519	FMRFamide variant 1 ( <i>An. gambiae</i> )	60	64	36 206	+	Neuropeptide signaling pathway	–
2	gi:118786597	AGAP005518-PA ( <i>An. gambiae</i> str. PEST)	60	64	36 266	+	Neuropeptide signaling pathway	–

**Table 2**

A list of novel proteins from 43 kDa antigenic band identified by LC–MS/MS using in-gel digestion approach.

No	Accession number	Protein	Query coverage (%)	Identity (%)	MW (kDa)	SignalP	Domain/function	Subcellular location
1	gi:568251178	Lipase ( <i>An. darlingi</i> )	91	64	40 273	+	Carboxylic ester hydrolase activity	Extracellular region/secreted
2	gi:668449414	AGAP000437-PA-like protein ( <i>An. sinensis</i> )	76	80	40 386	+	–	–
3	gi:568252110	Apolipoprotein D ( <i>An. darlingi</i> )	69	80	42 137	+	–	–
4	gi:668453186	AGAP000687-PA-like protein ( <i>An. sinensis</i> )	66	75	41 740	+	Carboxylic ester hydrolase activity	Extracellular region/secreted
5	gi:347964287	AGAP000687-PA ( <i>An. gambiae</i> )	66	75	41 152	+	Carboxylic ester hydrolase activity	Extracellular region/secreted
6	gi:668445723	AGAP002976-PA-like protein ( <i>An. sinensis</i> )	65	61	41 571	+	–	–
7	gi:568251273	Cathepsin D ( <i>An. darlingi</i> )	64	63	42 293	+	Aspartic-type endopeptidase activity/aspartyl protease	–

**Table 3**Antigenic region of selected proteins as biomarkers of *An. maculatus* bites.

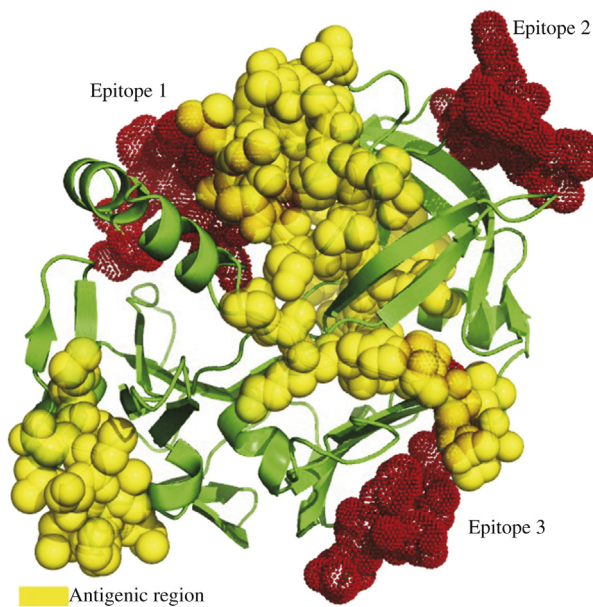
Protein identification	Accession number	Start position	End position	Peptide sequence	Peptide length
FMRFamide variant 1 ( <i>An. gambiae</i> )	gi:656339519	4	33	YLFLAIVVCESCNYFSHAEDSL	30
		54	63	VELAAAG	10
		231	241	EWSPLYPWSA	11
		286	302	EPAVALLASAH	17
AGAP000437-PA-like protein ( <i>An. sinensis</i> )	gi:668449414	4	25	FVVFAACLAVVSAVDVDYVPTS	22
		204	215	VQKHFYYHVAPE	12
		233	245	HYKIIFIKAPTVG	13
		262	271	TLVYVLVNKP	10
		281	297	DASSFVSGKPEVFFIKY	17
Apolipoprotein D ( <i>An. darlingi</i> )	gi:568252110	418	431	GVTPAYDGVAVGSA	14
		4	24	KLRCVLAVAVAVSLLCLSGT	21
		65	75	SSFLGIWYVIQ	11
		100	117	IEQVSQKAPLSLAPIKHE	18
		146	166	RPAVLVDGVASVAGSAKVVFM	21
		172	182	YAGVFSCQKIP	11
AGAP002976-PA-like protein ( <i>An. sinensis</i> )	gi:668445723	264	275	TLGCDATCCTPE	12
		282	293	GFSVDPFDLSIV	12
		6	20	KFILLMIVGQVVCRT	15
		37	52	RFLFTPESHILLTSA	16
		63	96	AGYVCIGELDLYPLPDYKYHA	34
		104	116	SSLKLGCVATYP	13
Cathepsin D ( <i>An. darlingi</i> )	gi:568251273	132	141	PPTPPPPPPPPP	10
		284	293	PAEVDQYLKD	10
				KECILRSICE	
		6	21	AATVVATLVLLALMA	16
		26	36	ADLVRVTLHKA	11
		109	118	FTNIACLLHN	10
		EPGLVFVAAK	10		
		ILGLAYSSISVDGVTPV	17		
		MVDCSLIPTLPT	12		

**Table 4**Epitope mapping of selected proteins as biomarkers of *An. maculatus* bites.

Protein identification	Accession number	Start position	End position	Peptide sequence	Peptide length
FMRFamide variant 1 ( <i>An. gambiae</i> )	gi:656339519	131	185	ELSKEYATSTEQLEPQPNVGAAGDEPAAATKR	55
		267	293	NAGLGASEEMQSTGLSESQEIK	27
AGAP000437-PA-like protein ( <i>An. sinensis</i> )	gi:668449414	20	115	RSGNGTGSRTVAEEQNATSSPAQLPEA	96
		124	193	DYVPTSYQDSVSAGADARSYGGVGGGVGGVGGG	70
		213	229	VGGGFAGGVGGVSGGVGGGFGAGAGGNVAAR	17
		243	260	SFGGAGAGGFSGAGIAASAAAGGAAYGAGY	18
		271	288	AANAGASAGAGAGGFGARFGGG	18
		299	308	SSSFGSAGSSSFG	10
		313	368	SGGSSFAGGSSFAGAGAGAGADA	56
				GAGAGASSYAKA	
				APEEPKAAAEADTLTIT	
				TVGANAGSNAYAASKTEE	55
Apolipoprotein D ( <i>An. darlingi</i> )	gi:568252110	381	435	PSQAKAGAAADASSFVSG	
				GKDAASTASA	
				IAGGASFGGAAAGADAFSSAGGSAGFDAGFGGASYD	
				AGFAGGSSFGNSGASVAGAGA	
				GGGVSAGSVGESGYNAGFNAGYTAAAAAANSGARSG	
				FGVTPAYDGVAVGSAANAG	
AGAP002976-PA-like protein ( <i>An. sinensis</i> )	gi:668445723	25	42	VQGHTYKTGECPTVEPMS	18
		45	57	QMKQSEERDGAVT	13
		295	305	QTGCPKEGEAG	11
		328	340	GEAIGDGFEEAVN	13
		349	370	YRESDYDAIVEETTNPRAER	22
Cathepsin D ( <i>An. darlingi</i> )	gi:568251273	92	158	VATYPPEPKKSPPPTPPPPPPPPPKKEEHHHHHGGE	67
		168	188	LSPAEDVQYLKDHGPTWVPPGW	21
		194	217	GKERADW	24
		226	237	PYWAAQRMDERQYQNPSSNL	12
		296	305	GIGNDGGDNFSPYQYSGWNPTAY	10
		RRKFDEDEEELE	12		
		NMLPPPGRSM			
		ELSDDYSNAMRQ			
		GSATGPVPEPLSN	13		
		DAKKSSTFEKN	11		
		FGGSDSSHYTG	11		

### 3.3. Antigenicity and epitope mapping analysis of identified proteins as potential candidate biomarker

The selected antigenic proteins of *An. maculatus* salivary gland that were not similar to human's surface cell proteins were further analyzed using bioinformatic tools to assess their antigenicity and epitope mapping. Epitope mapping using BepiPred method was performed to determine epitope of selected proteins as the binding portion of the antigen. The result of antigenicity analysis showed that selected proteins have a multi-antigenic region and polyepitope (Tables 3 and 4). However, apolipoprotein D and cathepsin D have higher antigenicity properties than other selected protein based on score value. The antigenic site and epitope region of cathepsin D protein as the best model were visualized using PyMol (Figure 5).



**Figure 5.** Predicted epitopes and antigenic region of cathepsin D (gi:568251273) as one of candidate proteins for biomarker tool of *Anopheles* mosquito bites exposure visualized by PyMol.

## 4. Discussion

The morphology of female *An. maculatus* salivary gland is similar to other *Anopheles* mosquitoes like *Anopheles stephensi* (*An. stephensi*) [17], *Anopheles dirus A* [18], and *Anopheles sundaicus* [19] that have three lobes and every lobe has a different region to maintenance function of saliva secretory. The proximal lateral lobe secretes amylase and  $\alpha$ 1-4 glucosidase that are essential for sugar feeding, while the distal lateral lobe and the medial lobe can secrete enzymes such as apyrase, anticoagulant, and vasodilator substances involved in blood-feeding [11].

The proteins of *Anopheles* salivary glands were detected as major bands with the MW, i.e., 62, 43, 39, 36–37, 33–34 kDa [18,19]. These proteins could belong to conserved proteins at the genus, subgenus or species-specific level [7]. Some proteins are ubiquitous salivary proteins that can be found in the salivary glands of many types of blood sucking insects and ticks, such as enzymes that are involved in sugar feeding (maltase), degradation of platelet aggregation (apyrase, 5'-nucleotidases), and inflammation (adenosine deaminase) [20]. Salivary proteins conserved in the genus,

subgenus, and species-specific level can be used to develop immunological marker of individual mosquito's bites exposure. However, the proteins will be an optimal candidate as biomarker depends on the purpose of serological tool utilization. Proteins that were found exclusively in anophelines, such as gSG6 can be applied as biomarker tool for a wide area with several *Anopheles* species, for example *An. gambiae*, *Anopheles funestus*, and *An. stephensi* [9]. Species-specific anopheline salivary proteins could be useful to determine the predominant mosquito populations [21].

The result of western blot showed that IgG antibodies recognized the proteins which have MW of 43, 37, 34, 20 and 14 kDa. In addition, proteins which have MW of 43 and 34 kDa were antigenic and appeared on all of the individual responses. Those proteins would act as an antigen that stimulates B-cells to produce antibodies [16]. This antigen–antibody reaction is the basic concept to develop a biomarker tool of human exposure to malaria vector. The salivary gland proteins which have a MW in the range of 35–40 kDa of *An. maculatus*, *An. gambiae*, *An. arabiensis* and *An. stephensi* showed antigenic activity towards the host. But, salivary gland proteins which have MW of 40, 35 and 11 kDa from *An. gambiae* and *An. arabiensis* exhibited high reactivity with the pooled serum [7]. The existence of cross-reactivity between some *Anopheles* species should be a consideration to determine the optimal candidate protein as biomarker tool of mosquito bites exposure.

The two novel hypothetical proteins from this study (34 kDa) are similar with proteins in other *Anopheles* mosquito species, e.g. *An. gambiae*. FMRFamide variant 1 (gi:656339519) and AGAP005518-PA (gi:118786597) proteins of *An. gambiae* contain FMRFamide domain. FMRFamide is a neuropeptide from FMRFamide-related peptide family sharing an RFamide peptide at their C-terminus and involving in myotropic activities [22]. The proteins of 43 kDa might have a function on carboxylic ester hydrolase activity, aspartic-type endopeptidase, and lipase. The member of this lipase group included secretory phospholipase A<sub>2</sub> in *Phlebotomus*, triacylglycerol lipases in the mosquito [*Culex quinquefasciatus* (*Cx. quinquefasciatus*) and *An. stephensi*], carboxylesterase in *Aedes aegypti*, and phospholipase C in *Cx. quinquefasciatus*. Phospholipase C in *Cx. quinquefasciatus* has an activity to hydrolyze platelet aggregation factor, but its molecular nature remains unknown [20]. Another novel hypothetical protein was identified similar to *An. darlingi* cathepsin D. Cathepsin D that has activity as aspartic-type endopeptidase is major catalytic classes of proteases, which are widely distributed not only in plants but also among vertebrates including insects, such as *Musca domestica*, *Stomoxys calcitrans*, and *Aedes aegypti* [23]. Cathepsins were also found in *Simulium nigrimanum* and *Culex tarsalis*, but their function has not been characterized [20].

Apolipoprotein D and cathepsin D have higher antigenicity properties than other proteins. Moreover, both proteins had epitope length ranging from 10 to 96 residues. Structural studies of B-cell epitope showed that 10–22 amino acid residues are considered to be in contact with the atoms of antibody [24]. Apolipoprotein D has five peptides with length range 11–22 residues, and cathepsin D has three peptides with length range 11–13 residues as the antigenic site. Thus, apolipoprotein D and cathepsin D proteins have the potency to be developed as candidate proteins for an immunological marker of *An. maculatus* mosquito bites exposure based on their antigenicity properties and epitope mapping. This study is the first that

revealed the candidate proteins of serological marker for human exposure to *An. maculatus* mosquito bites from antigenic proteins of the salivary gland. However, further analysis is necessary to determine the genome of *An. maculatus* salivary gland and their biological functions in blood-feeding and malaria transmission by the transcriptomic approach.

We found nine novel hypothetical proteins from 34 to 43 kDa protein bands as antigenic bands that are similar to the protein in *An. gambiae*, *An. sinensis*, and *An. darlingi*. The two expected proteins were apolipoprotein D and cathepsin D proteins that have the highest antigenicity that warrants for developing immunological marker of malaria vector bites exposure.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Acknowledgments

The authors would like to acknowledge Didik Huswo Utomo for helping the proteomics analysis. This study was funded by a Doctorate Research Grant of the Directorate General of Higher Education Indonesia (Grant No. 435/UN25.3.1/LT.6/2014).

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