

SALIVARY GLAND PROTEINS OF THE HUMAN MALARIA VECTOR, *Anopheles dirus* B (DIPTERA: CULICIDAE)

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SUMMARY

Salivary gland proteins of the human malaria vector, *Anopheles dirus* B were determined and analyzed. The amount of salivary gland proteins in mosquitoes aged between 3 - 10 days was approximately 1.08 ± 0.04 $\mu\text{g}/\text{female}$ and 0.1 ± 0.05 $\mu\text{g}/\text{male}$. The salivary glands of both sexes displayed the same morphological organization as that of other anopheline mosquitoes. In females, apyrase accumulated in the distal regions, whereas alpha-glucosidase was found in the proximal region of the lateral lobes. This differential distribution of the analyzed enzymes reflects specialization of different regions for sugar and blood feeding. SDS-PAGE analysis revealed that at least seven major proteins were found in the female salivary glands, of which each morphological region contained different major proteins. Similar electrophoretic protein profiles were detected comparing unfed and blood-fed mosquitoes, suggesting that there is no specific protein induced by blood. Two-dimensional polyacrylamide gel analysis showed the most abundant salivary gland protein, with a molecular mass of approximately 35 kilodaltons and an isoelectric point of approximately 4.0. These results provide basic information that would lead to further study on the role of salivary proteins of *An. dirus* B in disease transmission and hematophagy.

KEYWORDS: *Anopheles*; Salivary gland; Malaria; Apyrase; Alpha-glucosidase.

INTRODUCTION

Salivary glands of mosquitoes play an important role in food ingestion and digestion as well as transmission of pathogens. The saliva produced by the arthropod salivary glands is the vehicle that carries pathogens and it may also enhance or facilitate infectivity during the blood meal⁴³. Biochemically active protein molecules contained in the saliva counteract vertebrate haemostasis, allowing the arthropods to feed successfully³⁷. In addition, the saliva contains immunogens that cause human IgE and lymphocyte-mediated hypersensitivities³⁰. Analyses of salivary gland proteins of hematophagous mosquitoes have been reported in *Aedes aegypti*^{17,29,48}, *Anopheles stephensi*^{45,53}, *An. gambiae*^{1,10}, *An. darlingi*^{23,24}, *Culex pipiens*³, *Cx. quinquefasciatus*²⁵, *Ae. togoi*¹⁸ and *Armigeres subalbatus*⁴⁰. These previous analyses have mainly concentrated on the use of Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Although two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is considered to be the best method available for resolving complex mixtures of proteins^{20,28}, very little information is available on 2D-PAGE analysis of mosquito salivary gland proteins.

An. dirus complexes comprise at least seven species⁴⁴. Most of them are major vectors of malaria. Species A is widespread throughout

Thailand except in the south. Species B and C are found in sympatry in southern Thailand. Species D is commonly found in the north-western side of Thailand, and in sympatry with species A along the Thai-Myanmar border. It is also found exclusively in Myanmar and Bangladesh and the north-eastern states of India. Species E is exclusively found in western India and the Shimoga hills in Karnataka. Species F (*An. nimophilus*, Peyton and Ramalingam 1988) is found on the Thai-Malaysia border and is also reported from the monsoon forests of mountain areas in south-eastern, southern and western Thailand, and the Malaysian peninsular. *An. takasagoensis* is found exclusively in Taiwan⁴⁴.

An. dirus B, which is one of the important malaria vectors, has been studied in many areas, for example, molecular studies of insect glutathione S-transferases^{26,27,32,33,34}, genetic studies of *Anopheles* species complexes^{2,13,49,50,51}, and malaria transmission^{6,9,19,39,42}. However, little is known about the salivary gland proteins of this mosquito species. In this study, its salivary gland proteins were determined and analyzed. We report, herewith, the differential distribution of salivary components within the glands of female mosquitoes, and our initial finding on the most abundant salivary gland protein of *An. dirus* B after 2D-PAGE analysis. The information obtained from this study would be an initial step for further identification of the most abundant salivary gland

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proteins that may have a role in blood feeding and/or malaria transmission.

MATERIALS AND METHODS

Mosquito: *Anopheles dirus* B mosquitoes that originated from the Armed Forces Research Institute of Medical Sciences (AFRIMS) laboratory, Bangkok, Thailand were used in this study. The mosquito colony was routinely maintained in an insectary at the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Thailand for many years. The mosquitoes were reared and maintained in the insectary at 27 ± 2 °C with $70 \pm 10\%$ relative humidity, and a photoperiod of 12:12 (light/dark) hours. Mosquitoes aged between three and seven days after emergence were used. Adult mosquitoes were given continuous access to a 10% sucrose solution and fed on blood from immobilized mice when required.

Salivary gland dissection: Salivary glands of adult mosquitoes were dissected using fine entomological needles under a stereoscopic microscope at 4X magnification in phosphate-buffered saline [PBS; 10 mM Na₂SO₄, 145 mM NaCl (pH 7.2)] and transferred to a microcentrifuge tube with a small volume of PBS. Dissection of various regions of the female salivary glands was also performed. The medial lobes were cut at the junction of the medial and lateral lobes. The distal-lateral and proximal-lateral lobes were cut at the intermediate region separating the two lobes. The gland parts were immediately removed to separate the tubes in order to avoid possible protein contamination between the different sections of the glands. The gland parts were placed in a small volume of PBS and stored at -80 °C until use. Salivary glands of blood-fed mosquitoes were dissected within 20 min after taking a blood meal. These were either processed immediately for microscopy as described below or stored at -80 °C for later biochemical analysis.

Light microscopy: The salivary glands of adult mosquitoes were dissected in PBS and allowed to settle onto slides without drying. Photographs of the glands were taken using a digital camera (Cannon, Tokyo, Japan) attached to a light microscope.

Protein quantification: The protein content of each salivary gland pair was determined using a Micro BCA Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturer's instruction. The protein concentration was determined based on a bovine serum albumin (BSA) standard curve.

Apyrase enzymatic activity assay: Apyrase activity was determined as previously described by MARINOTTI *et al.*²¹. One μ L of salivary gland homogenate was incubated at 37 °C with 99 μ L of 50 mM Tris-HCl buffer (pH 9.0), containing 100 mM NaCl, 5 mM CaCl₂, 2 mM Adenosine S'-Diphosphate (ADP) and 20 mM β -mercaptoethanol in a flat-bottom microtiter plate. After 15 min, the reaction was interrupted by the addition of 2 μ L of reducing reagent (0.02% 1-amino-2-naphol-4-sulfonic acid, 0.12% sodium bisulfite, 0.12% sodium sulfite) and 25 μ L of 1.25 M H₂SO₄ containing 1.25% ammonium molybdate. The activity was determined colorimetrically in an ELISA reader at 630 nm. The standard curve for the inorganic phosphorous produced was determined according to FISKE & SUBARROW⁸.

Enzymatic assay for alpha-glucosidase activity detection: Alpha-glucosidase activity was determined by the glucose oxidase-peroxidase method⁷. The reactions were carried out in flat-bottom microtiter plates. Ten microliters of salivary gland homogenate were incubated for 30 min at 37 °C with 10 μ L of 100 mM phosphate buffer (pH 7.0), containing 0.1 M sucrose. After that, 150 μ L of 0.5 M Tris-HCl, pH 7.0, containing 25 U of glucose oxidase, 10 mg O-dianisidine, 0.0125% Triton X-100 dissolved in 95% ethanol and 2.5 U of peroxidase were added, and the reaction was incubated at 37 °C for one h. The activity was determined colorimetrically in an ELISA reader at 405 nm. A glucose concentration standard curve was used to determine the amount of glucose produced in the reaction.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGE was carried out according to standard techniques¹⁵. Salivary gland samples were thawed on ice and mixed in 1:2 1X SDS gel loading buffer [50 mM Tris-HCl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% Bromophenol blue, 10% glycerol]. Then, the samples were heated for five min in a boiling water bath and loaded on 12% SDS polyacrylamide gels. Molecular weight markers (Bio-rad, USA) were applied in each gel.

Two-dimensional gel electrophoresis (2D-PAGE): Two-dimensional gel electrophoresis was performed using the 2D system (Amersham Pharmacia, Sweden). Five pairs of female salivary glands were solubilized in a 125 μ L sample solubilization solution (8 M urea, 50 mM DTT, 4% CHAPS, 0.2% 3/10 Bio-lyte Ampholyte, 0.0002% Bromophenol Blue) and then loaded on an IPG strip (isoelectric point (pI) 3 - 10.7 cm, Amersham Pharmacia, Sweden) to perform the first dimension isoelectric focusing (IEF) separation. The strip was incubated in equilibration buffer [6 M urea, 2% SDS, 0.05 M Tris (pH 8.8), 20% glycerol] for 15 min. SDS-PAGE slab gels (12%) were used to separate proteins in the second dimension.

Coomassie Brilliant Blue (CBB) staining: Following the electrophoresis, gels were CBB-stained. First, the gels were fixed in 50% methanol and 10% acetic acid for 30 min, then stained with 1% CBB in 10% methanol and 5% acetic acid for two h, and finally destained in 10% methanol and 5% acetic acid until dark protein bands or spots were visible. Digital images of both SDS-PAGE and 2D CBB-stained gels were captured by scanning at 300 dpi using a color scanner.

RESULTS

The salivary glands of male and female *An. dirus* B mosquitoes are morphologically different. Those of adult females are paired organs lying in the thorax on either side of the esophagus. They have a single medial and two lateral lobes. The lateral lobe of each female salivary gland is composed of two secretory regions, proximal and distal, while the medial lobe has only one region (Fig. 1A). The salivary glands of males consist of a single small lobe (Fig. 1B).

The protein contents of whole salivary glands were quantified. The amount of salivary gland proteins in the mosquitoes aged between 3 - 10 days was approximately 1.08 ± 0.04 μ g/female (n = 30) and 0.1 ± 0.05 μ g/male (n = 30).

The biochemical analyses of the salivary glands of female *An. dirus*

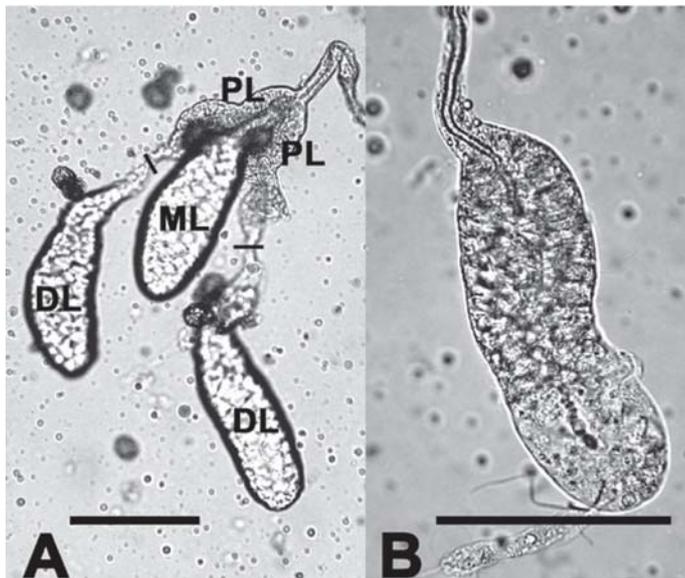


Fig. 1 - Representative adult salivary glands of the mosquito, *Anopheles dirus* B. (A) A female salivary gland. (B) A male salivary gland. PL: proximal region of the lateral lobe; DL: distal region of the lateral lobe; ML: median lobe. Bar represents 500 μ m.

B revealed the presence of apyrase and alpha-glucosidase activities (Table 1). Apyrase activity was detected by the production of inorganic phosphorous following ADP hydrolysis. The activity determined at pH 9.0 (optimum pH), was 6.5 ± 1.5 nmol of inorganic phosphorous/ minute/ salivary gland pair ($X \pm$ standard deviation, $n = 10$ samples, each sample containing three salivary gland pairs). The activity of alpha-glucosidase detected by the production of glucose from sucrose hydrolysis at pH 7.0 (optimum pH), was 0.08 ± 0.01 of glucose/ minute/ salivary gland pairs ($X \pm$ standard deviation, $n = 30$ mosquitoes). The distribution of these two enzymes within the salivary glands of female mosquitoes was determined (Table 1). The apyrase activity was mostly concentrated in the distal region of the lateral lobes. Most of the alpha-glucosidase activity was present in the proximal region of the lateral lobes.

Total proteins in whole male and female salivary glands of *An. dirus* B, as well as the various micro-dissected morphological regions of female salivary glands, were examined in CBB stained SDS-polyacrylamide gels (Fig. 2A). At least seven major and several minor protein bands were detected in the female salivary glands (Fig. 2A, lane F), some of which were labeled (P1 through P7). The molecular masses of these major protein bands were estimated at 63, 44, 43, 35, 33, 30 and 18 kilodaltons (kDa), consecutively. The male gland protein profile differed from the female one and the protein content was lower

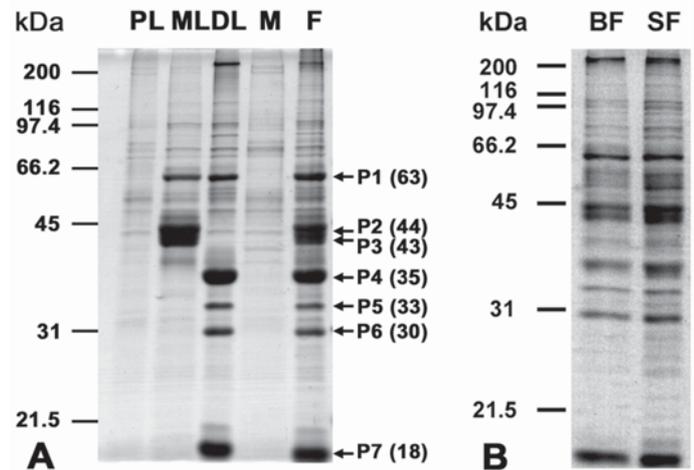


Fig. 2 - (A) Female and male salivary gland proteins of *An. dirus* B mosquitoes. Salivary proteins were separated on 12% SDS-polyacrylamide gels and stained with Coomassie Blue. Lane PL, ten proximal-lateral lobes; lane ML, ten median lobes; lane DL, ten distal-lateral lobes; lane M, fifty whole male salivary glands; lane F, ten whole female salivary glands. Molecular mass markers are indicated on the left in kDa. Labels on the right indicate protein bands found specifically in the female glands (P1 - P7) and their estimated molecular mass (in blankets). (B) Comparison of female salivary gland protein profiles between blood-fed and sugar-fed *An. dirus* B. Lane BF, ten whole glands of blood-fed mosquitoes; lane SF, ten whole glands of sugar-fed mosquitoes. Molecular mass markers are indicated on the left in kDa.

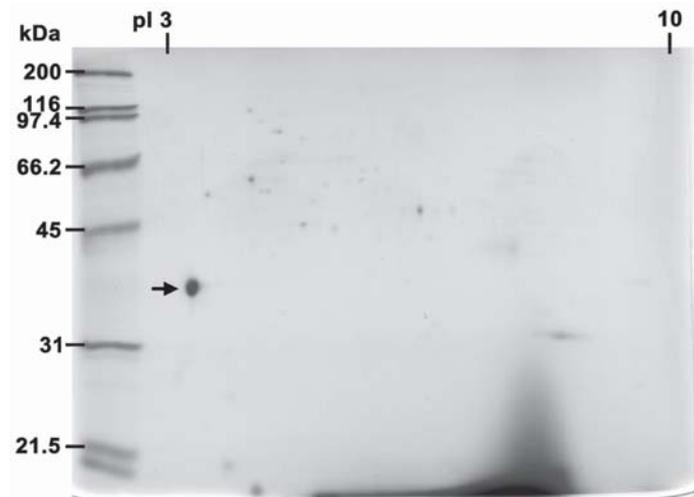


Fig. 3 - Coomassie Blue-stained 2D gel of proteins from three female salivary glands of *An. dirus* B. Arrow indicates a 35 kDa spot, the most abundant protein expressed in the salivary glands. Molecular mass markers are indicated on the left in kDa. Isoelectric points (pI) are indicated at the top.

Table 1
Distribution of apyrase and alpha-glucosidase within adult female *An. dirus* B salivary glands

	Distal region of lateral lobe	Proximal region of lateral lobe	Medial lobe
Apyrase ^a	66%	11%	23%
Alpha-glucosidase ^b	15%	78%	7%

Apyrase activity was determined by inorganic phosphate production from ADP hydrolysis as described by MARINOTTI *et al.*²¹ ($n = 20$)^a; alpha-glucosidase activity was determined by the release of glucose from sucrose by the glucose oxidase-peroxidase method⁷ ($n = 20$)^b. The total activity in the glands was set as 100%.

(lane M, fifty male glands, compared with lane F, five female glands). The different morphological regions of the female salivary glands also displayed distinct protein electrophoretic profiles. The salivary gland protein bands, P1, P4, P5, P6 and P7, appeared predominantly in the distal region of the lateral lobe (Fig. 2A, lane DL), while the female specific protein bands, P1, P2 and P3, were predominant in the medial lobe (Fig. 2A, lane ML). The protein profile of the proximal region of the lateral lobe (Fig. 2A, lane PL) appeared similar to the profile of the male salivary glands (Fig. 2A, lane M).

Figure 2B shows the salivary gland electrophoretic profiles of the blood-fed and sugar-fed mosquitoes. The protein profiles are basically similar, although there are minor differences in both after several repeats.

Separation of salivary gland proteins by 2D-PAGE yielded a protein profile with numerous individual spots, and a wide dynamic concentration range (Fig. 3). The most abundant salivary gland protein found in CBB stained 2D gels had a molecular mass of approximately 35 kDa, with a pI of approximately 4.0.

DISCUSSION

The morphology of female and male *An. dirus* B salivary glands is different. While the female glands are organized in two identical lateral lobes and a medial lobe, the salivary glands of males consist of a single small lobe. The female gland is similar to the salivary glands of *Aedes*, *Culex* and *Anopheles* species^{17,23,29,53}. Previous studies reported that the salivary glands of male *Aedes* and *Culex* mosquitoes are composed of three identical lobes, all of them formed by cells morphologically similar to that of the female proximal portion of the lateral lobes⁴³. However, the male salivary glands of *An. dirus* B follow the same morphological pattern as described for *An. darlingi*²³.

Determination of the *An. dirus* B salivary gland extracts revealed that the male glands contained 10 times less protein than the female ones. These values are consistent with the morphological differences observed between the salivary glands of males and females. These morphological and protein content differences have been observed in other mosquito species and were related with the different feeding habits of males and females^{18,21,24,25}. Females feed on both sugar and blood, whereas males feed on sugar only⁴³.

The accumulation of specific proteins in the different salivary gland regions has been described for various mosquitoes^{31,35}. Two enzymes, the apyrase and alpha-glucosidase, previously characterized in the salivary glands of *Ae. aegypti*²², *Ae. albopictus*²¹, *An. darlingi*²³ and *Cx. quinquefasciatus*²⁵, demonstrated accumulation preferentially in the distal region and proximal region of the lateral lobes, respectively. The salivary apyrase and alpha-glucosidase of *An. dirus* B females were found to have the same distribution pattern. The distal region of the lateral lobes is necessary for mosquito blood feeding. The apyrase, synthesized and accumulated in the female-specific region of the gland, has been related to inhibition of the platelet aggregation, which facilitates blood ingestion by the mosquitoes^{21,38}. This enzyme is secreted by the salivary glands only during blood feeding.

Previous work on *Ae. aegypti* and *Ae. albopictus* indicates that the

proximal regions of the lateral lobes of the mosquitoes' salivary glands synthesize and accumulate molecules that help in sugar solubilization, ingestion and digestion^{5,21}. The *Mal-I* gene expressed exclusively in the cells of the proximal region of the *Ae. aegypti* salivary glands, encodes an alpha-glucosidase that is secreted during mosquito sugar feeding. This enzyme hydrolyzes sucrose, the major natural sugar source^{16,22}.

In this study, the overall profiles of female and male salivary gland proteins of *An. dirus* B were analyzed. The protein profiles present in male and female glands are distinctly different. At least seven major proteins visualized after SDS-PAGE are female specific. One possibility is that the polypeptides not found in males are synthesized by female-specific cells and are involved in blood feeding. The predominant protein bands found in the distal region of the lateral lobes and the medial lobes of female glands were not present in the glands from non-blood sucking males that lack these regions. Specific proteins produced in different parts of the salivary glands of female *An. dirus* B are consistent with previous studies on salivary gland profiles of *An. stephensi*⁴⁵, *Ae. togoi*¹⁸ and *Ar. subalbatu*⁴⁰.

The protein profile of the salivary glands of sugar-fed female *An. dirus* B mosquitoes was compared with that of blood-fed ones. The major protein bands in the glands of both sugar-fed and blood-fed mosquitoes showed similar profiles. These results correlate with the analysis of the salivary gland proteins of sugar-fed and blood-fed *An. darlingi* mosquitoes²⁴. In other *Anopheles* species, although the total salivary gland protein content of blood-fed mosquitoes (*An. stephensi*, *An. albimanus*, *An. gambiae*, *An. freeborni*, and *An. darlingi*) is at least 10% less than that of sugar-fed controls, small differences in the amount of proteins are difficult to visualize in Coomassie Blue stained gels and/or silver stained gels¹². ORR *et al.*²⁹ observed a change in the salivary gland cells 24 hours after *Ae. aegypti* females had taken blood meals; the nucleoli of the median and lateral acini became greatly enlarged and there was a concomitant increase in RNA around the nuclei. They concluded that blood feeding may deplete the female *Ae. aegypti* salivary glands, and this depletion would lead to resynthesis of secretory products within 24 hours. Furthermore, SOLIMAN *et al.*⁴¹ reported that after *Cx. pipiens* had blood-fed, the total saliva was depleted by 64% within 24 hours, but the protein level returned to the unfed value within the next 24 - 48 hours. In *Ar. subalbatu*, however, salivary gland protein profiles between unfed and blood-fed mosquitoes were significantly different. Immediately after blood feeding, the proteins, 65 and 21 kDa, which abundantly expressed in the distal regions of the lateral lobes, were barely detectable. However, they started to appear gradually six hours later and returned to the unfed level within 48 hours⁴⁰. Results from these studies suggest that mosquitoes in a different genus may have different biochemically active molecules involving blood feeding and/or the blood digestion process.

Usually, proteomic study of the saliva and salivary glands of arthropods use SDS-PAGE to separate proteins and N-terminal sequence analysis for identification in, for example, *Ae. aegypti*⁴⁸, *An. stephensi*⁴⁷, *An. gambiae*¹⁰, *An. darlingi*⁴, *Cx. quinquefasciatus*³⁶, and *Ixodes scapularis*⁴⁶. Recent improvements in 2D-PAGE, mass spectrometry, and bioinformatics provide additional tools to characterize small amounts of protein. These methods were employed to characterize salivary proteins from *Amblyomma americanum*²⁰, *A. maculatum*²⁰, and

*Glossina morsitans morsitans*¹⁴. In this study, 2D-PAGE was also used to separate the salivary gland proteins of *An. dirus* B. The results showed that the major protein bands present on SDS-polyacrylamide gels were separated into numerous individual spots with different concentrations and pI. In addition, the most abundant protein expressed was detectable. It had a molecular mass of approximately 35 kDa and was very acidic, with a pI of approximately 4.0. Further analysis by matrix-assisted, laser desorption ionization mass spectrometry (MALDI-MS) and tandem mass spectrometry methods would provide peptide mass maps and sequences that are often sufficient in identifying proteins¹¹. The sequence information and peptide mass map can be used to query against an NCBI non-redundant database to confirm the identity of the protein. In our laboratory, identification and characterization of this major protein and analysis of *An. dirus* B salivary gland transcriptome are currently in progress. Results in this study provide information that is an initial step towards further investigations of molecular, biochemical and immunological aspects in the role of salivary proteins of *An. dirus* B in disease transmission and blood feeding.

RESUMO

Proteínas das glândulas salivares do *Anopheles dirus* B (Diptera: Culicidae), vetor da malária humana

Proteínas das glândulas salivares do *Anopheles dirus* B (Diptera: Culicidae), vetor da malária humana foram determinadas e analisadas. A quantidade de proteínas das glândulas salivares em mosquitos com três a 10 dias de idade foi de aproximadamente $1,08 \pm 0,04 \mu\text{g}$ /fêmea e de $0,1 \pm 0,05 \mu\text{g}$ /macho. As glândulas salivares de ambos os sexos mostraram organização morfológica semelhante à de outros mosquitos anofelinos. Em fêmeas, a amilase acumula-se nas regiões distais, enquanto a alfa-glucosidase foi encontrada na região proximal dos lóbulos laterais. Esta distribuição diferencial das enzimas analisadas reflete a especialização de diferentes regiões para alimentação de açúcares e sangue. Análise SDS-PAGE revelou que pelo menos sete proteínas foram encontradas nas glândulas salivares de fêmeas, das quais cada região morfológica continha diferentes proteínas principais. Perfis eletroforéticos de proteínas semelhantes foram detectados comparando-se mosquitos não alimentados e alimentados por sangue, sugerindo que não existe proteína específica induzida pelo mesmo. Análise por gel poliácridamida bi-dimensional mostrou a mais abundante proteína de glândulas salivares com aproximadamente 35 kilodaltons de massa molecular e ponto isoelétrico de aproximadamente 4,0. Estes resultados dão informações básicas que levariam a estudos adicionais sobre o papel das proteínas salivares do *An. dirus* B na transmissão da doença e hematofagia.

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