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Protein synthesized by dengue infected *Aedes aegypti* and *Aedes albopictus*

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Abstract. The main objective of this study was to compare protein profiles of whole mosquitoes of Malaysian *Aedes aegypti* and *Aedes albopictus* after infection with virus and to investigate whether dengue virus would induce protein secretion in *Ae. aegypti* and *Ae. albopictus*. Using SDS -PAGE, it was shown that in uninfected *Ae. aegypti* and *Ae. albopictus*, the protein bands were within the range of 14 - 80 kDa with most of the bands overlapping for the two species. Comparison of the protein profile of infected and uninfected *Ae. aegypti* and *Ae. albopictus* showed five distinct molecular weight grouping at 73 - 76 kDa (Group 1), 44 - 50 kDa (Group 2), 28 - 31 kDa (Group 3), 20 – 25 kDa (Group 4) and 14 - 17 kDa (Group 5). Predominant bands for both species (infected and uninfected) were between 21 - 25 kDa and 44 - 50 kDa. Protein bands having a molecular weight of 70 kDa were only present in infected *Ae. albopictus* and those bands having molecular weight of 21 kDa were observed only in infected *Ae. aegypti*. The rate of digestion of blood meals was more rapid in *Ae. albopictus* than *Ae. aegypti*. Uninfected *Ae. albopictus* completed the blood digestion 2 days after ingestion of a blood meal whereas *Ae. aegypti* needed 3 days to complete the digestion. The rate of digestion for blood meals was slower for both mosquito species when fed with dengue virus infected blood. The digestion processes were completed 3 and 4 days after blood ingestion for *Ae. albopictus* and *Ae. aegypti*, respectively. This could be due to the presence of dengue virus in the blood, which slow down the digestion process. Appearance and disappearance of new protein bands was also observed even after the digestion has completed for both infected mosquito species. In conclusion, dengue virus was shown to induce specific proteins in both *Ae. aegypti* and *Ae. albopictus*.

INTRODUCTION

Dengue is a notorious mosquito-borne disease in the world and is transmitted in cities as well as in densely populated areas. This viral disease was said to occur more frequently compared to malaria (Scott, 1993). In Malaysia, dengue was first documented in 1902 (Skae, 1902). In 1990, the number of cases increased dramatically (Lam, 1993). Therefore, to date dengue is the most important human mosquito-borne viral pathogen.

The appearance of dengue is caused by the presence of dengue virus. In the presence of vectors, the virus can be transmitted at a faster rate. *Aedes ae*gypti and *Aedes albopitus* are mosquito vectors responsible in transmitting dengue virus infectious diseases such as dengue fever and dengue hemorrhagic fever.

A mosquito vector acquires infection by feeding on infected host. When the mosquito feeds on a vertebrate host, both humoral and cell mediated immune responses are induced by the salivary gland secretions that enter the blood stream (Ramasamy & Ramasamy, 1990). However, the main focus of attention on vector salivary glands is their role in blood feeding and transmission of disease. The saliva facilitates blood vessel location and blood ingestion, and is also the vehicle through which virus and parasite are transmitted to a vertebrate host (Ribetto, 1995).

During feeding, blood containing dengue virus is taken up through the

proboscis. Dengue virus starts to multiply in the salivary gland as well as in the midgut region. The midgut is the principal organ for digestion of the blood meal. Eventually, some kinds of protein are produced in the midgut region in order to counter the virus. Although different mosquito species may synthesis different kinds of protein, these proteins (or antibodies) are different from the proteins that are normally found elsewhere in the mosquitoes. The synthesized proteins cover the receptors or brush border region in the midgut portion to avoid membrane-pathogen interaction (Wang *et al*., 2001).

Mosquitoes have the ability to defend itself against the virus and the virus also has the potential or capability to deal with the proteins that are synthesized by the mosquito. There are a few kind of proteins make-up in the whole structure of the virus. M protein is responsible in neutralizing the synthesized protein in the mosquitoes' body (Nopporn, 1994). Therefore, to some extent, protein profiles of infected mosquitoes will be different from the normal mosquitoes.

Knowledge of physiological events taking place in the vector pathogen midgut is important in understanding vector pathogen interaction necessary for disease transmission. Identification and characterization of mosquito proteins is the first step in understanding the immunological response of host mosquitoes.

This study was conducted to characterize the proteins that could be found in blood fed mosquitoes, since these might have implication on disease transmission. Therefore the general objective of this study was to analyze proteins synthesized by *Aedes aegypt*i and *Aedes albopictus* mosquito in response to dengue virus infection.

MATERIALS AND METHODS

Mosquitoes

Four hundred 4-7 days old female *Ae. aegypti* and *Ae. albopictus* adults were obtained from the insectarium, Institute for Medical Research, Kuala Lumpur. Approximately 30 female mosquitoes were placed into each paper cup. The mosquitoes were starved overnight before blood feeding.

Dengue virus

The dengue virus (Den-2) was obtained from Department of Microbiology, University of Malaya, Kuala Lumpur.

Artificial membrane feeding

The artificial membrane feeding technique employed was modified from Graves (1980). The feeding chamber was filled in with fresh human blood. During the blood feeding, a paper cup with mosquitoes was put below the feeding chamber in contact with the artificial membrane.

Feeding and transmission procedure

A glass feeder with water jacket was covered at the bottom by wrapping a small piece of membrane, which was moistened with normal saline. Fresh normal human blood was obtained on the day of blood feeding by using venepuncture and immediately transferred into separate heparinized tubes after which the blood was placed into the feeder. One of the heparinized tubes containing 3.9ml of blood was mixed with 100µl of normal saline as a control. One hundred ul of serum of dengue type 2 virus was added to the other heparinized tubes which contained 3.9ml of normal fresh human blood each. Each heparinized tubes thus contained 4ml of mixture or solution.

The blood was presented to the mosquitoes by placing the cups containing mosquitoes below the feeder, with the surface of the nylon netting of the cup in contact with the membrane of the feeder. Water from the water bath at 38^0C was allowed to flow through the inlet and outlet of the artificial feeding system to keep the blood warm. The membrane and blood were replaced each time after feeding to prevent gradual settling of the red blood cells. Each cup of mosquitoes was allowed to feed for approximately 30

minutes to 1 hour as follows

i) *Ae. aegypti* fed with normal blood (uninfected *Ae. aegypti*)

ii) *Ae. albopictus* fed with normal blood (uninfected *Ae albopictus*).

iii) *Ae. aegypti* fed with blood and dengue virus (dengue virus - infected *Ae. aegypti*)

iv) *Ae. albopictus* fed with blood and dengue virus (dengue virus - infected *Ae. albopictus*).

After feeding, all the mosquitoes in each cup was transferred into a cage. Only fully engorged mosquitoes were collected and reared in the cages accordingly. Mosquitoes from each treatment were collected everyday for 7 days. The mosquitoes were transferred into eppendorf tubes and kept in a –70°C freezer for further use. Mosquito samples, samples of blood with dengue virus and samples of blood without dengue virus were further re-confirmed by Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR) for the presence or absence of dengue virus.

Preparation of mosquito samples for protein

The mosquitoes fed with blood and virus were used for protein profiles determination. The negative control consisted of mosquitoes fed with blood only. One mosquito from each treatment and for each species was homogenized in doubled distilled water and the extract was separated on SDS-polyacrylamide slab gel using the discontinuous system consisting of 4% acrylamide stacking gel and 12% acrylamide separating gel. Approximately 10 ul of the mosquito samples were boiled at 100 °C for five minutes before loading onto the gel. The separated protein bands were visualized by staining with Coomassie brilliant blue.

RESULTS AND DISCUSSION

Protein profile in uninfected Aedes aegypti

The protein pattern for whole mosquitoes of *Ae. aegypti* after blood meal is shown in Figure 1 and Table 1. The protein bands of each species are numbered from the top

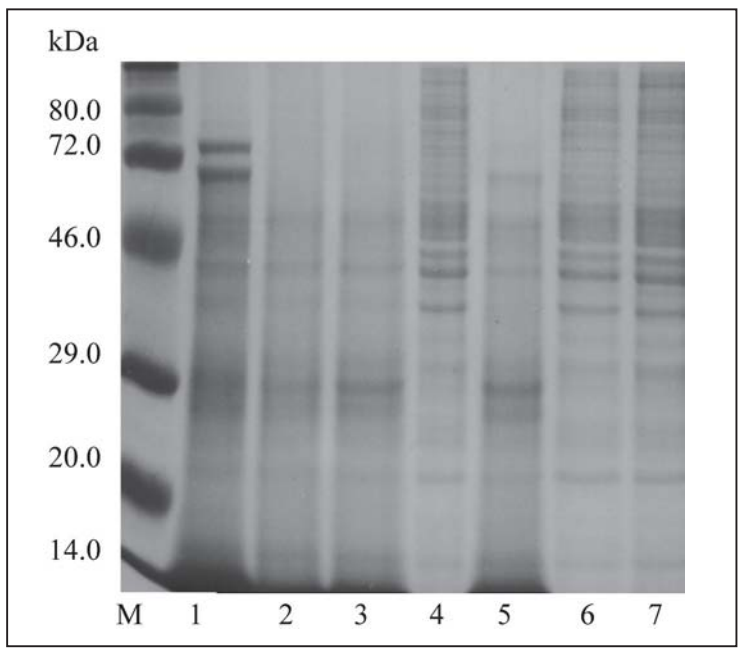

Figure 1. Protein profile of uninfected *Ae. aegypti* on acrylamide gel stained with Coomasie blue. Lane M- molecular marker, L1 to L7 *Ae. aegypti* fed with blood after day 1 to day 7.

Table 1. Molecular weight of protein profile from day 1 to day 7 in the uninfected *Aedes aegypti*

No.	Day						
	1	2	3	4	5	6	7
1.	17	17	17	17	17	17	17
2.	20	20	20,21	20,21	20,21	21	20,21
3.	28	28	28	28	28	28	28
4.	–	–	–	29	–	29	–
5.	43,44	43,44	43,44	43,44	43,44	43,44	43,44
6.	45,46	45,46	45,46	45,46	45,46	45,46	45,46
7.	–	–	–	–	–	–	–
8.	70,72	–	–	72	–	72	72
9.	–	–	–	–	–	–	–
10.	–	–	–	–	–	–	–

• only major bands have been scored

to the bottom of the gel according to their direction of migration. Protein standard markers were indicated in lane M. The protein profiles from uninfected *Ae. aegypti* revealed 11 conspicuous bands ranging from 17 to 72 kDa. At least six polypeptides can be seen with the predominant bands having molecular weight (MW) of 17, 20, 28, 43, 44, 45 and 46 kDa in all the fed mosquitoes maintained for 7 days. Expression of two proteins (70 and 72 kDa) one day after feeding and disappearance of a few proteins were observed in day 2 and day 3 (70 and 72 kDa) respectively.

Protein profile in dengue virus-infected Aedes aegypti

The polypeptide patterns of the infected *Ae. aegypti* were highly complex and showed many common proteineous components after Coomasie brilliant blue staining (Figure 2 and Table 2). SDS PAGE analysis of the infected *Ae. aegypti* showed a protein profile of bands ranging from 14 to 80 kDa. At least six polypeptides could be seen with the dominant bands having molecular weight (MW) of 14, 21, 24, 25, 45 and 46 kDa. Expression of three proteins (31, 50 and 76 kDa) were more intense in day 3 to day 7 infected *Ae. aegypti*. Two polypeptides with bands having molecular weight of 20 kDa and 72 kDa on day one infected mosquitoes and one polypeptide with the predominant band having a molecular weight of 20 kDa on day 2 were not detected three days after feeding with infected blood.

Protein profiles in uninfected Aedes albopictus

PAGE analysis of uninfected *Ae. albopictus* revealed at least 12 distinct bands ranging in size from 14 to 76 kDa (Figure 3 and Table 3). Other than the common bands (as in *Ae. aegypti*) the uninfected *Ae. albopictus* also contained a number of additional protein bands of 31 and 73 kDa on day 1 and day 2 respectively. The single molecular band of 50 kDa was not present in day 1 of the uninfected mosquitoes. The protein profiles were similar in all the uninfected mosquito from the third to the seven days after ingestion of blood meal.

Protein profile in dengue virus infected Aedes albopictus

SDS - PAGE analysis of infected *Ae. albopictus* revealed at least 15 distinct

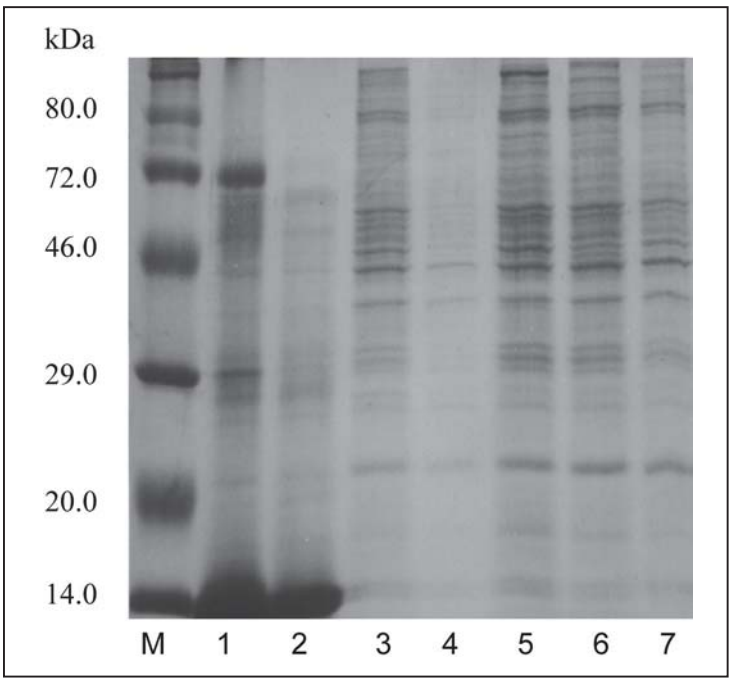

Figure 2. Protein profile of infected *Ae. aegypti* on acrylamide gel stained with Coomasie blue. Lane M- molecular marker, L1 to L7 *Ae. aegypti* fed with blood after day 1 to day 14.

Table 2. Molecular weight of protein profile from day 1 to day 7 in the infected *Aedes aegypti*

No.	Day						
	1	2	3	4	5	6	7
1.	17	14,17	17	14,17	14,17	14,17	14,17
2.	20,21	20,21	21	21	21	21	21
3.	24,25	24,25	24,25	24,25	24,25	24,25	24,25
4.	29	-	29,31	29,31	29,31	29,31	29,31
5.	45	45	45	45	45	45	45
6.	46	46	46,47	46,47	46,47	46,47	46,47
7.	–	–	50	50	50	50	50
8.	72	–	–	–	–	–	–
9.	–	–	76	–	76	76	76
10.	–	80	–	80	80	80	80·

• only major bands have been scored

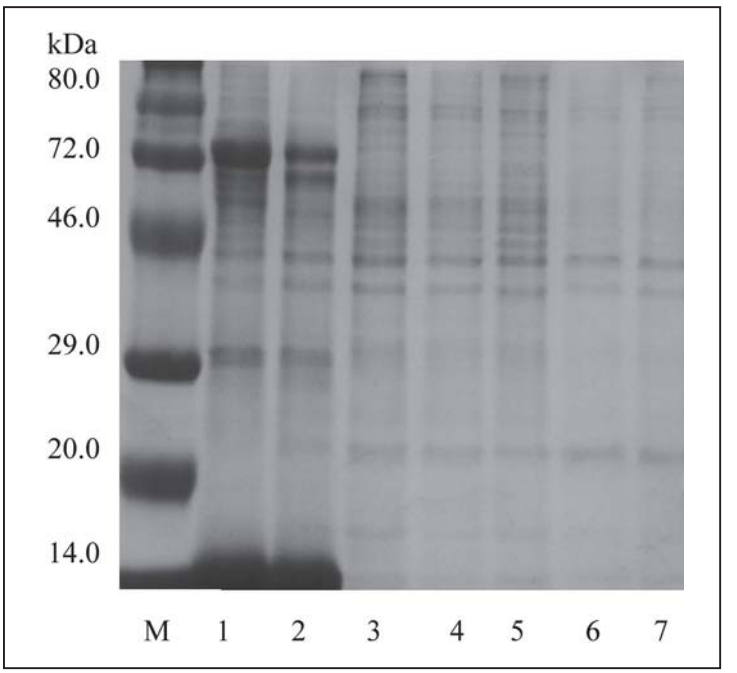

Figure 3. Protein profile of uninfected *Ae. albopictus* on acrylamide gel stained with Coomasie blue. Lane M- molecular marker, L1 to L7 *Ae. albopictus* fed with blood after day 1 to day 7.

Table 3. Molecular weight of protein profile from day 1 to day 7 in the uninfected *Aedes albopictus*

No.	Day						
	1	2	3	4	5	6	7
1.	14	14	14	14	14	14	14
2.	21	21	21	21	21	21	21
3.	25	25	25	25	25	25	25
4.	–	31	32	32	32	32	32
5.	44	44	44	44	44	44	44
6.	45	45	45	45	45	45	45
7.	70	50	48,50	48,50	48,50	48,50	48,50
8.	73	73	74	74	74	74	74
9.	76	76	76	76	76	76	76
10.	–	–	–	–	–	–	–

• only major bands have been scored

protein bands ranging in size from 14 to 80 kDa (Figure 4 and Table 4). Concentrated proteins of whole infected mosquito extract migrated in bands of 14, 24, 27, 28, 43, 44, 45, and 70 kDa in day 1 infected *Ae. albopictus*. The appearance of protein with a molecular weight of 30 kDa in day 3 fed mosquitoes was observed. There were no distinct differences in protein profile of the dengue virus-infected *Ae. albopictus* three days after blood meal. Protein bands with molecular weights of 18, 31, 76 and 80 kDa were not present on day 1 infected mosquitoes.

Protein profile in infected and uninfected Aedes aegypti

Protein profiles from uninfected and infected *Ae. aegypti* were compared and some similarities in the protein pattern were seen.

PAGE analysis of infected *Ae. aegypti* demonstrated clear differences in protein constituents when compared with the uninfected *Ae. aegypt*i. Expression of four proteins (24, 25, 31 and 76 kDa) and disappearance of a protein band 28 kDa were observed in infected *Ae. aegypti*.

Protein profile in infected and uninfected Aedes albopictus

SDS-PAGE analysis of infected *Ae. albopictus* demonstrated clear differences in protein constituents when compared with the uninfected *Ae. albopictus*. Disappearance of two proteins (21 and 50 kDa) were observed in infected *Ae. albopictus*. New proteins of molecular size 18, 27, 28 and 70 kDa were present only in infected *Ae. albopictus* from day 1 to day 7. Another new protein of molecular size of 30 kDa was only present on day 3 in infected *Ae. albopictus*. However, the predominant bands having molecular weight 14, 44 and 45 kDa were present in both the infected and uninfected *Ae. albopictus*.

Protein profile in uninfected Aedes aegypti and Aedes albopictus

Minor differences in protein profiles were noted between uninfected *Ae. albopictus*

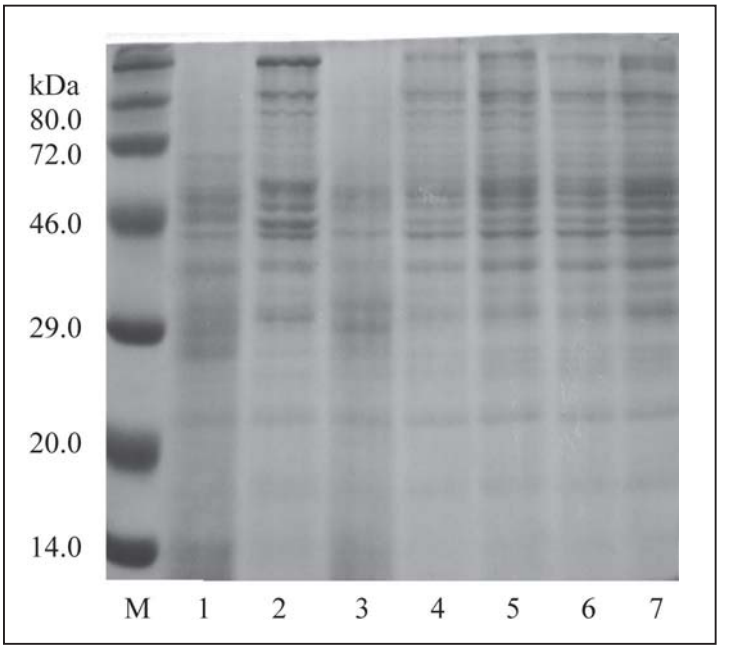

Figure 4. Protein profile of infected *Ae. albopictus* on acrylamide gel stained with Coomasie blue. Lane M- molecular marker, L1 to 7 *Ae. albopictus* fed with blood after day 1 to day 7.

Table 4. Molecular weight of protein profile from day 1 to day 7 in the infected *Aedes albopictus*

No.	Day						
	1	2	3	4	5	6	7
1.	14	14	–	14	14	14	14
2.	–	18	18	18	18	18	18
3.	24	24	24	24	24	24	24
4.	27,28	27,28	27,28	27,28	27,28	27,28	27,28
5.	–	31	30,31	31	31	31	31
6.	43,44	43,44	43,44	43,44	43,44	43,44	43,44
7.	45	45	45	45	45	45	45
8.	70	70	–	70	70	70	70
9.	–	76	76	76	76	76	76
10.	–	80	80	80	80	80	80

• only major bands have been scored

and uninfected *Ae. aegypti*. The protein profiles of both mosquitoes species form five distinct molecular weight grouping at 73 - 76 kDa (Group 1), 44 - 50 kDa (Group 2), 28 - 31 kDa (Group 3), 20 - 25 (Group 4) and 14 - 17 kDa (Group 5). Both uninfected and infected present proteins bands in the 21 - 25 kDa (Group 4) and 44 - 45 kDa (Group 2) range.

Specific bands found only in *Ae. aegypti* but not seen in *Ae. albopictus* were those between 28 - 31 kDa (Group 3). This is further evidence of the existence of species-specific band protein profile of *Ae. aegypti* and *Ae. albopictus*.

Protein profile in infected Aedes aegypti and Aedes albopictus

Comparison of the protein profile of both infected *Ae. aegypti* and *Ae. albopictus* showed that the protein profile also form five distinct molecular weight grouping. Two protein groups range between 43 - 46 kDa (Group 2) and 27 - 31 (Group 3) were present in both the mosquito species. Protein bands having a molecular weight of 70 kDa were only present in infected *Ae. albopictus* whereas protein bands having a molecular weight 21kDa were observed only in infected *Ae. aegypti*. The presence of these extra bands could be due to the proteins of the dengue virus. On the other hand, disappearance of a few bands were observed in infected *Ae. albopictus* and *Ae. aegypti* only. This could be due to the inhibition effect of dengue virus against the mosquitoes.

Effect of dengue virus against Aedes aegypti and Aedes albopictus

The present studies have identified up to 15 distinct proteins in both *Ae. aegypti* and *Ae. albopictus* mosquito. Using SDS-PAGE, it was shown that for *Ae. aegypti* and *Ae. albopictus*, the protein bands fell within the range of 14 - 80 kDa with most of the bands common for the two species. Predominant bands for both species (infected and uninfected) were between 21 - 25 kDa and 44 - 50 kDa. This confirmed previous work by Ramasamy *et al*. (1991) which showed predominant bands at 37 kDa and Lee *et al*. (1994) which showed predominant bands at 25, 35 and 67 kDa for uninfected *Ae. aegypti*. Mellink & Van Zeben (1976) identified seven fractions with 33 - 100 kDa, one low molecular weight protein at 12.5 kDa and one high molecular weight protein above 100 kDa. In addition, Moskalyk *et al*. (1996) reported

that the number of proteins in *Ae. aegypti* was in the range of 20 - 40. There were distinct differences in the protein profiles of both *Ae. aegypti* and *Ae. albopictus*, 1 - 3 days after ingestion of a blood meal. However, there was no distinct difference in the protein profile of both species 4 – 7 days after blood meals. This showed that the digestion processes were completed 3 days after ingestion of blood meal.

The rate of digestion of blood meals was more rapid in *Ae. albopictus* than *Ae. aegypti*. Uninfected *Ae. albopictus* completed the blood digestion 2 days after ingestion of a blood meal whereas *Ae. aegypti* needed 3 days to complete the digestion. The rate of digestion for blood meals was slower for both mosquito species when fed with dengue virus infected blood. The digestion processes were completed 3 and 4 days after blood ingestion for *Ae. albopictus* and *Ae. aegypti* respectively. This could be due to the presence of dengue virus in the blood, which may slow down the digestion process. Appearance and disappearance of new protein bands was also observed even after the digestion has completed for both infected mosquito species. Clements (1996) reported that rate of digestion of a blood meal in mosquitoes varies with temperature, humidity and age of the mosquitoes. Digestion of the blood meal was slower in 6-day-old virgin females of *Ae. aegypti* than in mated females of the same age. However, the injection of virgins with matrone speeded their rate of digestion, which did not differ significantly from that of mated females.

The proteins of the insect provide unique opportunity for the control of insect pest and vector borne diseases. Immunological control of blood and tissue-feeding insect by the effects of binding antibody to the protein was actively explored (Tellam *et al*., 1999). The findings reported in this study constitute an initial step in a series of investigations on the pathological, immunological and pharmacological roles of protein in mosquito in relation to man. It should now be possible by immuno-blotting techniques to identify sensitizing proteins for patients with immediate hypersensitivity reactions to mosquito bites. However, the whole body extracts are inappropriate in testing but Felix *et al.* (1991) reported that the use of whole body homogenates did not interfere with the assay of midgut trypsin which was synthesized by the midgut *Ae. aegypti* following a blood meal. Further characterization of bands should be carried out. The salivary glands and midgut should be studied separately in greater detail.

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