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DISTINCTIONS IN PROTEOMICS AMONG DEVELOPMENTAL STAGES OF DENGUE VECTOR (*AEDES ALBOPICTUS)* MOSQUITOES

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Abstract. The embryonic proteomics that drift in the adult stage and responsible for performing visual activities including, biting and maintaining the vectorial life of dengue vector *Aedes albopictus* is not projected earlier. In a series of experiments we have indicated the concentration and extension of early proteomic cursors in the latter stages of life cycle up to the early emerging adult. We noticed about 70% gain of protein during larval developmental stages, until pupation. Newly emerged adult mosquitoes lost about 12% of protein than the pupal stage. In case of peptides, we observed 14-26 polypeptide bands during immature and early adult stages by using 12% of separating gel in ID SDS-PAGE. The bands in early larval stages up to 4th instar larvae were in the range of approximately ~58 to ~7 kDa, while in pupal and adult stages they were between ~200 and ~7 kDa. Newly emerged bands in pupal (i and ii) and adult (iii-vi) stages could be identified as stage specific peptides. The band number 10, which migrated at the same position in the PAGE with equal strength in all stages was identified as an essential peptide. Characterization of the above peptides might help in the pin pointing to possible virus transmission blocking mechanism of this vector insect.

Key words: *Aedes*, immature, proteomic profile, protein concentration, pupae, mosquito, dengue vector

INTRODUCTION

Aedes albopictus (Skuse), a species of *Aedine* mosquito, which originated in Asia, is increasingly drawing importance as a public health threat as an aggressive biter and a competent vector of at least 23 arbo-viruses including, dengue (Malavige *et al.* 2004), zika, chikungunya (Delatte *et al.* 2009), yellow fever and various types of encephalitis viruses (Rosen *et al.* 1985; Mitchell 1995a,b). It is proven to be a particularly invasive species (Hawley 1988). Its abundance and disease threat have become well established as well as increasing at an alarming rate in most countries around the world because of its ovipositional behaviour (Reiter 1998) and transovarial virus transmission character (Rosen 1987). It survives in a wide range of aquatic habitats, including

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phytolemata, dead leaves on the ground, tree holes, discarded tins, plastic containers, car parts, brick holes and rock pools (Hawley 1988, Sota *et al.* 1992, Simard *et al.* 2005).

Efforts to control dengue and dengue vector, multiple initiatives are practicing all over the dengue world. Among them, vaccine experts are trying to find a licensed dengue vaccine, but it is still in the laboratory. Until now, the most practicing weapon to reduce vector population is applying insecticides, but this strategy has proven hazardous, inadequate and ineffective (WHO 1999). Other strategies, like ovitrap, the CDC gravid trap, light trap etc. did not bring that much hope independently in this journey (Reiter 1986, Savage *et al.* 2008). Thus, integrated control approach is still the mainstay, where genetics based vector control (GVC) approaches are thinking much potential to reduce and replace the infected vector population. As a part of the latest approach, identification of the responsible protein in various biological and physiological functions is very essential.

Furthermore, during the life cycle, mosquitoes pass through different larval and pupal stages. In every stage, they must perform various physiological activities, including digestion, respiration, moulting, etc. In the adult stage, they do a lot of activities, like flying, mating, reproducing and avoiding risks. Most of these physiological activities derived from protein functions. For instance, during blood feeding when temperature rise suddenly, shock proteins help to maintain the three-dimensional integrity of enzymes and proteins and helps mosquitoes to digest ingested blood meals (Karlsson and Wickman 1990, Benoit *et al.* 2011). Besides, many mosquito proteins are involved in virus transmission and infections have been processed and identified as parts of receptors (Munoz *et al.* 1998) or co-receptors (Mendoza *et al.* 2002).

To study detail about the proteins and their expression is known as proteomics, a complement to genomics. It allows the study of the total proteome in an organism, tissue or cell and characterize their functional modifications that cannot be directly determined from DNA or mRNA (Shi and Paskewitz 2006). Protein profiling is widely known and an effective technique for taxonomic study (Onaric and Sumer 2003). Where specific proteome variation within a given taxonomic group or genus is compared by SDS-polyacrylamide gel technique (Thomas and Singh 1992, Navas *et al.* 2002). One-dimensional-sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D SDS-PAGE) is identified as useful tool for studying protein synthesis in *Ae. aegypti* and *Ae. albopictus* in relation to dengue virus infection (Lee *et al.* 1994, Rohani *et al.* 2005, Lee *et al.* 2009). Recently Al-Azab *et al.* (2013) used this technique to identify the total protein profile of different developmental stages of dengue

vector *Ae. Aegypti.* All the above studies did not take into consideration immature stages and newly emerged adult of another important dengue vector *Ae. Albopictus* mosquito. Therefore, to fill up this gap and to explore its basic proteomic composition, we conducted 1D SDS-PAGE of the developmental stages of immature and newly emerged adult (before feeding anything) of *Ae. albopictus* mosquitoes.

MATERIAL AND METHODS

Colonization of mosquitoes: The test samples were collected from a colony reared in the insectarium at the School of Biological Sciences, Universiti Sains Malaysia, Penang. Wild pupae were collected from the outdoor containers in Gelugor, Penang Island. They were identified after emergence according to the standard taxonomic key. The adult *Aedes albopictus* were separated and reared for the establishment a colony in the laboratory. The colony was maintained according to Saifur *et al.* (2010) at a room temperature of 29 ± 3ºC and a relative humidity of 75 ± 10%.

Collection of mosquito immature and adults for protein extraction: During immature rearing the early 2nd, 4th instar larvae, pupae and freshly emerged adults were collected. The adults were knocked down in a container containing ethyl alcohol soaked cotton lump. Then the samples were repeatedly washed with 1% liquid soap, 1% Clorox and deionised water for 5, 5 and 10 min, respectively. They were made batches separately in 1.5 ml Eppendorf tube with 10 individuals from each category. Three replicates were prepared for each type of sample. They were stored at –20ºC for proteomic study.

Extraction of protein from mosquito samples: The preserved samples of different batches of larvae, pupae and adults were homogenized under ice in 80 µl phosphate-buffered saline (PBS), pH 7.4, 1000 ml (NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24 g, adjust to pH 7.4 with HCl) with 0.02 mM aqueous solution of PMSF. The extracts were centrifuged at 13,500 rpm for 15 minutes at 4ºC. The supernatant that contained the target protein for the measurement were collected in new Eppendorf tubes and kept at –20ºC until usage.

Separation of protein: To perform the tests, we used one-dimensional SDS-polyacrylamide gel electrophoresis with standard methods on the Hoefer Mighty Small II system (8 cm × 7 cm mini gels). The discontinuous system consisting of 5% acrylamide stacking gel and 12% acrylamide separating gel was used to separate the proteins from the test samples. Approximately 6 µl (3 µg) from each sample of preserved supernatant were boiled at 100ºC for three minutes. Then they were loaded onto the gel. To estimate the molecular weights of the unknown

proteins, Kaleidoscope Prestained Standards markers (Bio-Rad, Hercules, CA) of 2.5 µl were used in each gel run. Electrophoresis conditions were 35 mA, 110 volts for 65 min. The separated protein bands were visualized by Coomassie blue (CB) staining (0.2% Coomassie brilliant blue in 50% MeOH in water containing 10% acetic acid for 1.30 hr and de-stained overnight with the solution containing10% acetic acid and 10% methanol).

Determination of protein concentration: Protein concentrations of all types of supernatants of larvae, pupae and adults were quantified in triplicate. Aliquots of the supernatants were used for this purpose. A modification of the Bradford method (Bradford 1976) with a Bio-Rad Electroplate Reader using the kit End-point was followed. Absorbencies were read by a Dynatech AM60 micro-plate reader with a 595 nm filter. Concentrations were expressed as optical densities. The BSA samples diluted in the appropriate homogenization medium (dH_2O) were used to construct the standard curves. A six-point standard curve of reduced and oxidized BSA was included with each plate. A single estimate of the egg protein concentration was made by doing an average of the above reading.

Data collection and analysis: The standard errors and mean protein concentrations of different test samples were calculated by using the SPSS 15.1 to find the difference in the protein contents among larval instars, pupae and adult stage. Canon camera (Canon EOS 5D Mark II, Canon, USA) was used to photograph Coomassie blue (CB) gels. After taking the photo, they were modified with Adobe Photoshop software (Adobe Systems Inc., San Jose, CA) and fixed at a contrast of 52%, lightened at of 50%. Band patterns were analyzed visually and assigned according to Prévot *et al.* (2003). The comparison of the synthesis profiles of high - low molecular weight proteins (~200 - ~7 kDa) among test samples was based on the detection of at least 13 shared bands. Discrepancies in the band pattern were considered, if observed in at least half of the total replicates. The differences in darkness/lightness between identical bands were taken into account as the dark bands are stronger or highly expressed.

RESULTS AND DISCUSSION

Quantitative changes of larval to adult protein concentration: The mean amount of protein in different developmental stages varied widely (Fig. 1, Table 1). The lowest content was in the second instar larvae which increased gradually and reached peaked at the early pupal stage then decreased steeply at the newly emerged adults. About 70% of protein gained during larval development until pupation. Newly emerged adult lost about 12% of protein than the pupal stage.

Proteomes during immature development: The progressive development of protein patterns among developmental stages of *Ae. albopictus* is shown in Fig. 2

and Table 2. The protein bands of each treatment including standard marker are numbered from the top to the bottom of the gel according to their direction of migration. The protein profiles revealed 14-26 conspicuous bands ranging from ~7 to ~200 kDa in different stages of development. Early larval stage produced a few strong bands which tremendously increased with developmental advancement. Fourth instar larvae and pupae produced a quite higher numbers of bands with higher strength. Newly emerged adults produced more faint bands

Table 1. Concentration of protein during different developmental stages of *Ae. albopictus*. Values with the same letter do not show a significant difference ($p < 0.05$)

Name of developmental stages	Mean concentration ±SE µg/ml
2nd IL*	125.00 ± 1.15[a]
4th IL	735.33 ± 2.33[b]
Pupae	792.33 ± 1.20[c]
Adult	580.33 ± 3.53[d]

*IL= Instar Larvae.

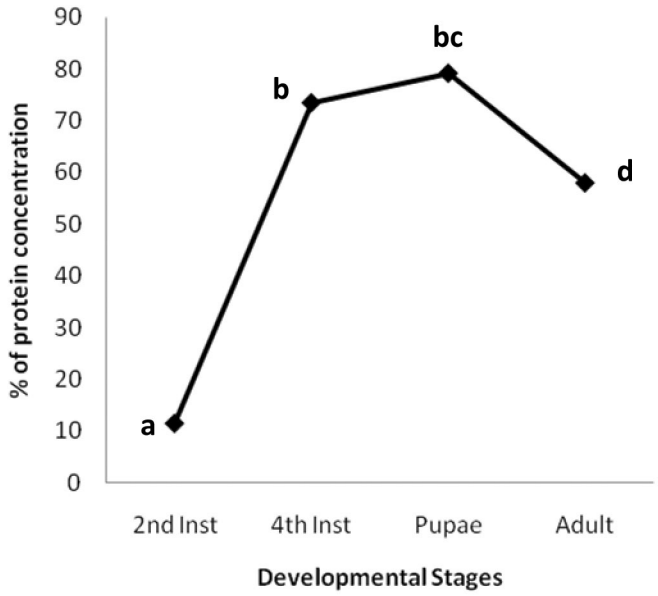

Fig. 1. Change in protein concentrations during immature development of *Ae. albopictus* mosquito. Values with the same letter are not significantly different ($p < 0.05$).

together with some new bands (iii-vi) than the previous stage. Bands i and ii were absent in the larval stages those were first appeared in the pupal stage and increased up to iii-vi in the adult stage. Bands 1-7 and 10 were more or less

present in all stages. While band no. 10 migrated at the same position in the PAGE with equal strength in all stages. Bands 8 and 11-13 were deeply stained in the fourth instars and pupal stages but appeared faint in the second instar larvae and early emerged adults. Band 9 was very much fainted or disappeared in the second instar larvae and adults.

Table 2. Protein profiles (Coomassie blue stained) during immature development of *Aedes albopictus* mosquito

Band No.	MW (kDa*)	Bands developed in different developmental stages			
		2nd Intar L*	4th Intar L	Pupae	Adults
i	~200	0	0	++	++
ii		0	0	++	++
iii	~112	0	0	0	+
iv		0	0	0	+
v	> 58	0	0	0	+
vi		0	0	0	+
1	~ 58	++	++	+	+
1a		-	+	+	+
1b		-	+	+	+
2	>30	++	++	++	+
2a		+	++	++	+
3		-	++	++	++
3a		-	++	++	++
4	~ 30	-	++	++	+
5		-	++	++	++
6	~ 25	-	++	++	++
7		++	++	++	+
7a		0	++	+	+
8		0	++	++	+
8a	~ 13	0	+	+	+
9		0	+	-	0
9a		0	+	-	0
10		++	++	++	++
11	~ 7	-	++	++	0
12		-	++	++	-
13		0	++	+	-
		14	20	22	23

*L – Larvae; (++) indicates a very high level of synthesis; (+) indicates a high level of synthesis; (-) indicates a low level of synthesis; (0) indicates a very low level of synthesis or absence of protein.

In our earlier communication, we focused on the association of moisture on the embryonic proteomic profile of *Ae. albopictus* (Saifur *et al.* 2014) followed by visualized clarified embryo and spontaneous egg hatching with increasing

moisture exposure (Saifur *et al.* 2010). In connection with the previous, this work has indicated the projection of early proteomic cursors in the latter stages of life cycle up to the emergence as an adult that perform all activities in the vectorial life of the mentioned vector mosquito species.

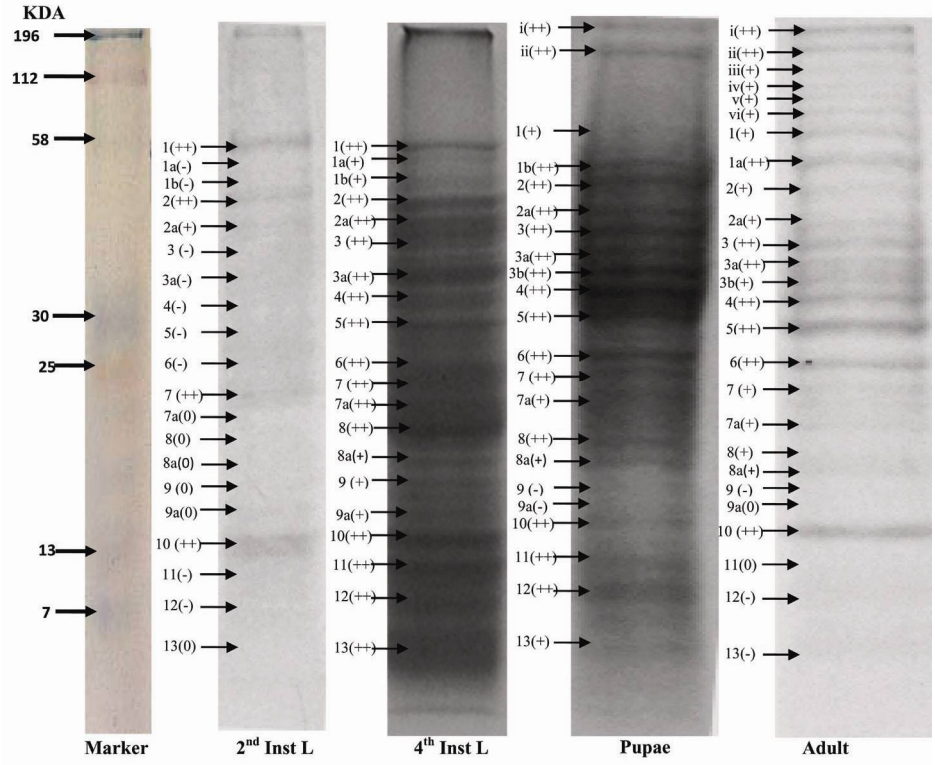

Fig. 2. Comparison in the protein synthesis during immature development of *Ae. albopictus* mosquitoes; (++) indicates a very high level of synthesis; (+) indicates a high level of synthesis; (-) indicates a low level of synthesis; (0) indicates a very low level of synthesis or absence of protein.

In general, protein is the essential element of the animal body as well as insect structure. Its higher amount in their body indicates larger body mass, which established higher reproductive success in insects (Santos *et al.* 1988, Partridge and Fowler 1993), or improved competitive ability (Warren *et al.* 2006) on life-history traits including disease vulnerability and stress resistance (Lee *et al.* 2008). Usually, the lowest protein concentration is found in the newly hatched larvae. It increases in later stages (Blevins 1973) with progressive development due to the additional proteins from external food or higher diet in their active feeding period. This additional protein is utilised for organismal growth and addition of new cells. They also perform basic cellular functions and highly specialized functions in higher stages. We observed the proof of this

continuous higher concentration of protein in higher ages of mosquito immature up to their pupal stage. In this last stage, the protein concentration was the highest due to the complex developments for formation of new organs before emergence, though previous study on *Ae. aegypti* show the opposite (Blevins 1973). Similar findings were observed in case of blowfly pupae due to the accumulation of cuticular proteins (Ring 1973), pupal size and age (Lang *et al.* 1965). Srinivasan and Kesavan (1979) correlated the increasing amount of protein concentration in larval and pupal stages of domestic flies with the synthesis of new cuticle, prior to ecdysis and the histogenesis of adult tissue prior to emergence. Chefurka (1965) correlated protein synthesis with activities of cytochrome oxidases in the developing muscles of young adult insects. However, less amount of or a non-significant increase of protein content in the pupal stage indicates its less nutrient uptake in early stages, smaller body size and the consequence activities in this phase.

Furthermore, body size in different stages of mosquitoes immature depends on many factors, including food availability and ideal environmental temperature. In the favourable temperature, the immature get sufficient food due to higher microbial growth in their aquatic habitat and their size become larger with higher amount of protein. The larger immatures produce larger adults. They can complete more gonotrophic cycles and produce more offspring (Saifur *et al.* 2012). On the contrary, the smaller larvae take comparatively longer time to become pupae and the pupae with less protein, even cannot emerge as adult. Those emerge; they feed frequently to the hosts for getting larger blood meals to fill up their insufficient body protein for running normal physiological activities followed by egg production (Xue *et al.* 1995). Normally, smaller mosquitoes feed smaller blood meals (1.6 - 2.5 ml) than larger females (2.6 - 3.5 ml) (Klowden and Lea 1978, Klowden and Lea 1979). To get sufficient blood protein for egg production and surviving in the comparatively complex outdoor environment, smaller female mosquitoes show a high frequency of biting (Sheppard *et al.* 1969, Pant and Yasuno 1973, Trpis and Hausermann 1986, Chadee and Corbet 1991). However, the present study was conducted in a place where the environmental temperature is very conducive for good health of insect immature that may have the role in the higher concentration of protein in the pupal stage.

In case of the proteomic profile in different stages of *Ae. albopictus* immature, we found more than 13 polypeptide bands by using 12% of separating gel. The number of protein bands increased with the progressive development. The number of lower kDa bands decreased and higher kDa bands increased in the latter stages. In the larval stages the highest band was MW ~60

kDa, while in pupal stage two extra bands with a higher MW~200 kDa was observed. The highest number of bands appeared in the fourth instar larvae with a lower molecular weight suggest that there were different kinds of enzyme related with feeding activities added or generated. There were some new polypeptides appeared during pupal stage, which can be explained as the non functional cells in larval stage become functional in pupal stage and secrete different kinds of enzymes and proteins to make many organs necessary for emergence as adult. Moreover, pupae covered them with a cuticular cage enriched with many proteins, including cuticle degrading proteases (Brookhart and Kramer, 1990) and chitinases enzymes (Fukamizo and Kramer 1985, Koga *et al.* 1992). They were secreted by the epidermis as an inactive precursor (Locke and Krishnan 1973, Koga *et al.* 1989) and become activated in the moulting fluid (Samuels and Reynolds 1993, St. Leger *et al.* 1986).

Regarding the basic proteomic profile of adult dengue vectors, we considered newly emerged unfed *Ae. albopictus* mosquitoes for our study to avoid unwanted or extra proteins that fed adult mosquitoes get from different hosts with their blood meals (Rohani *et al.* 2005). We found 7 more bands than the immature stage (Saifur *et al.* 2014) within the range of MW ~60 to ~200 kDa. The bands with lower MW seen in the immature stage were reduced or fainted in the higher developmental stage. In the living organisms, new proteins are made according to the direction of genes in cells to perform higher activities for survival. Adult mosquitoes do a lot of activities to fight with the environment for their survival including searching blood meals, performing oviposition activities, maintaining flight muscles. Many proteins are involved to perform these activities. The adult mosquitoes produce these necessary proteins with the help of external food i.e., blood meal by the precursor existing in the structural cells (Pan *et al.* 1969, Hagedorn and Judson 1972, Hagedorn *et al.* 1975, Benoit *et al.* 2011). However, our identified major bands in adult mosquitoes might be the key proteins to govern vectorial functions. In the same way, the stage specific proteins observed in different stages might have the controlling functions on moulting as well as emergence. Therefore, it is necessary to identify those proteomes to reduce the pupal emergence and control of vector population.

Furthermore, since proteomic profiling is the direct reflection of the gene expression and helps in understanding the gene regulation. So, their characterization or expression profile is needed for further pin pointing the possible virus transmission blocking mechanism or to modify the function of these proteins to reduce the vectorial fitness of the test species in further studies.

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