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Proteomic Profile During Embryonic Development of Dengue Vector *Aedes albopictus* Mosquito

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Despite the potential impact of moisture on embryonation and egg eclosion of *Aedes* mosquitoes, little is known about its effect on protein synthesis during critical embryonic development as well as in the proteomic profiles. Thus, we quantify the protein concentration and proteomic profile during embryonic development of *Ae. albopictus* from far early of egg laying to egg eclosion in contact with sufficient moisture. It was observed that the concentration of protein started to decrease from the early hours (6th h) with progressing of embryonic development. There were more or less 13 bands observed in Coomassie blue staining of different embryonic stages within the range of ~58 kDa and ~7 kDa by using 12% separating gel in 1D SDS-PAGE. Among them highly expressed bands on the position of 11-13 of lower molecular weight at around 7 kDa were found in all treatments. They may have controlling effects on egg hatching. Identification of these specific proteins can give an insight direction of effective vector control way.

Key words: *Aedes*, moisture, protein profile, embryonation, egg hatching, dengue vector.

In oviparous organism, the will of embryogenesis depends largely on maternal nutrients during oogenesis (Vital *et al.,* 2010). In insects including *Aedine* mosquitoes, this latter process is the period during which, many nutrients are accumulated to further meet regulatory and metabolic needs of the developing embryo (Chippendale, 1978). Once the embryonic development is over, the pharate larva remains latent in the eggs (Novak and Shroyer, 1978), which is generally broken upon stimulation during flooding (Gjullin *et al.,* 1941). The amount of dissolved oxygen is also considered as the major factor that stimulates larval eclosion (Gillet *et al.,* 1977). This observation which clearly minimise the effect of post-flooding stimulus, strongly suggests that events prior to flooding certainly play an important role in hatch success. In general, *Aedine* females oviposit preferentially on moist sites of container habitats that result from constant evaporation and flooding events (Hill *et al.,* 2006). Upon egg deposition, it faces varying moisture conditions and it must uptake sufficient moisture to complete embryonation (Strickman, 1980). In *Aedes* mosquitoes, embryo viability has been often associated with post-oviposition moisture conditions (Gjullin *et al.,* 1950; Saifur *et al.,* 2010). The maturation of *Ae. albopictus* and *Ae. aegypti* embryos in a highly humid environment resulted

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in increased dormancy survival and hatch success (Dieng *et al.,* 2006; Harwood and Horsfall, 1959). This ability of dengue vectors embryos to remain viable after being dormant for long periods is believed to be a major problem involving dengue transmission (Vital *et al.,* 2010).

Despite the crucial role of moisture in embryogenesis, it is not the single factor that determines egg hatch success. It is well known that in insects, eggs are produced through vitellogenesis, a process during which yolk components are synthesized (Hagedorn and Judson, 1972; de Oliveira and Cruz-Landim, 2004), secreted into the hemolymph and further absorbed by the oocytes as yolk (Raikhel and Dhadialla, 1992; Seehuus *et al.,* 2006) which is mainly composed of proteins, lipids and water (Lorenz, 2003; Ziegler and Van Antwerpen, 2006; Karl *et al.,* 2007). The proteome of egg has mainly a structural function, but may additionally serve as energetic resource (Beenakkers *et al.,* 1985) during embryogenesis and predict the fitness of neonate (Diss *et al.,* 1996; van Handel, 1993). Beside these, insect egg contains proteins that can act as inhibitors or facilitators of hatching depending on their concentrations (Young *et al.,* 2000; Geiser *et al.,* 2008). Though, many studies have shown progressive hatching success with increasing submersions in *Aedes* mosquitoes (Livdhal *et al.,* 1984; Dieng *et al.,* 2006; Vitek and Livdahl, 2009), but in many cases some eggs remained un-hatched (Toma *et al.,* 2003; Nur Aida *et al.,* 2011). Despite the potential of insect egg proteins to inhibit egg hatching, no attention has been given to this phenomenon in dengue vectors.

The analysis of protein expression is a key part of proteomics, a complement to genomics, which allows for the study of the total proteome in an organism, tissue or cell. Proteomics also is used to characterize functional modifications that cannot be directly determined from DNA or mRNA (Shi and Paskewitz, 2006). Protein profiling has been proven for comparing specific proteomes variations within a given taxonomic group (Thomas and Singh, 1992) or genus (Navas *et al.,* 2002). In addition, one-dimensional-Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (1D SDS-PAGE) is a useful tool for studying protein synthesis in *Ae. aegypti* and *Ae. albopictus* in relation to dengue virus infection (Rohani *et al.,* 2005; Lee *et al.,* 2009), but these studies did not take into consideration egg of which 2.35% to 40% can be infected by vertical transmission (Lee and Rohani, 2005; Günther *et al.,* 2007). Furthermore, protein synthesis during embryogenesis of dengue vectors is poorly documented. In order to fill up this gap, while avoiding what has been already done in this topic concerning dengue vectors; here the proteomic profile were examined during embryogenesis of eggs exposed in moisture for different duration of *Ae. albopictus*, a species that is increasingly drawing importance as a public health threat.

MATERIALS AND METHODS

Colonization of mosquitoes

The *Aedes* mosquitoes used in this study were derived from wild pupae collected from outdoor containers in Gelugor, Penang Island. A colony was established in the insectarium at the School of Biological Sciences, Universiti Sains Malaysia, Penang. 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 eggs for protein extraction

Two hundred gravid females of two days old *Ae. albopictus* were kept in standard mosquito rearing cages (45 × 45 × 35 cm) provided with a 10% glucose soaked cotton. An oviposition apparatus according to Dieng *et al.,* (2010) was placed for egg laying. Mosquitoes were allowed to lay their eggs for 1 hour duration at the peak oviposition period of the day (5-7pm) (Dieng *et al.,* 2010). Then the substrates with oviposited eggs were took out from the oviposition cage and allowed to contact with water for 1, 6, 24, 48 and 72hrs respectively. After the water exposed period the substrates with eggs were dried in room temperature. The dried eggs were counted and packed with 500 eggs in each autoclaved eppendorf tube of 1.5ml within 24 hours of drying and stored in -20°C until protein extraction.

Extraction of protein from mosquito samples

Different batches of eggs were homogenized under ice in 80 ul 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 sample was centrifuged at 13,500 rpm for 15 minutes at 4°C, and collected the supernatant that contains the target protein for the measurement. Supernatant was kept at -20°C until usage.

Separation of protein

The protein was separated on SDS-polyacrylamide slab gel using the discontinuous system consisting of 5% acrylamide stacking gel and 12% acrylamide separating gel. One-dimensional SDS-polyacrylamide gel electrophoresis was performed using standard methods on the Hoefer Mighty Small II system (8 cm x 7 cm mini gels). Approximately 6 µl (3 µg) from each sample were boiled at 100 ºC for three minutes before loading onto the gel. The molecular weights of proteins in the gels were estimated by applying 2.5 µl of Kaleidoscope Prestained Standards markers (Bio-Rad, Hercules, CA) to each gel run. Electrophoresis conditions were 35 mA, 110 volts for 65 min. The separated protein bands were visualized by silver staining (ST) according to Oakley *et al.* (1980) and Coomassie blue (CB) staining (0.2% Coomassie brilliant blue in 50% MeOH in water containing 10% acetic acid for 1.30 h and de-stained overnight with the solution containing10% acetic acid and 10% methanol).

Determination of protein concentration

Protein concentrations were quantified in triplicate for each type of eggs, larvae, pupae and adults. Aliquots of the supernatants were used to determine protein quantities in triplicate using a modification of the Bradford method (Bradford, 1976) with a Bio-Rad Electroplate Reader using the kit End-point. Absorbencies were read with a 595 nm filter using a Dynatech AM60 micro-plate reader. Concentrations were expressed as optical densities. The standard curves were constructed from BSA samples diluted in the appropriate homogenization medium (dH_2O). A six-point standard curve of reduced and oxidized BSA was included with each plate. The results were averaged to obtain a single estimate of the egg protein concentration.

Data collection and analysis

To find the differences in the protein content among eggs exposed in different moisture condition, the mean protein concentrations and their standard errors were calculated. The SPSS 15.1 was used to perform the statistical analysis. Silver-stained (ST) and Coomassie blue (CB) gels were photographed with Canon camera (Canon EOS 5D Mark II, Canon, USA) and 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 effects of moisture on protein synthesis were assessed by comparing protein profiles from different stages of moisture exposed eggs. 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 comparisons were done at least 13 shared bands within the band profiles of ~200 - ~7 kDa. Discrepancies in the band pattern were considered if observed in at least two of the four replicates. The differences in darkness/lightness between identical bands were taken into account. The stronger bands were considered a higher expression level.

RESULTS

Quantitative changes of egg protein concentration during embryogenesis

The mean protein concentrations during different phases of embryonic development inside the eggs are shown in Fig. 1 and Table 1. Protein contents gradually decreased towards the progressing of embryonic development. The decreasing rate lowered significantly towards the higher development of embryo from 6 h to 72 h. The highest reduction (37.4%) observed within 24-48 h of developmental period.

Proteomes during embryonic development

The protein pattern in progressive development of embryonic events among different

Table 1. Concentration of protein during embryonic development of *Ae. albopictus.* Values with the same letter do not show a significant difference ($P < 0.05$)

During Embryogenesis	Mean Concentration ±SE µg/ml
1st hour MEG*	606.00±1.53a
6th hour MEG	594.67±5.90ab
12th hour MEG	568.00±1.73c
48th hour MEG	508.00±1.53d
72th hour MEG	457.33±1.20e

* MEG = Moisture exposed egg

Table 2. Protein band patterns during embryonic development of *Ae. albopictus* in Silver / Coomassie blue (CB) staining

Band No	MW (kDa*)	Bands of embryos in different moisture exposure periods				
		1 h	6 h	24 h	48 h	72 h
1	~ 58	+/+	+/+	+/-	-/-	0/-
2	>30	+/+	+/+	+/-	-/-	0/-
3	++/++	+/++	+/+	+/+	-/0	
4	~ 30	+/+	++/+	+/0	+/0	0/0
5	~ 25	++/++	+/++	-/+	0/+	0/-
6	++/++	++/++	+/++	+/+	+/+	
7	+/+	+/+	+/+	+/-	+/0	
8	~ 13	+/+	-/+	0/0	0/0	0/-
9	+/++	+/++	-/+	0/-	0/-	
10	++/++	++/++	++/+	+/0	-/0	
11	~ 7	-/+	+/+	+/+	++/+	++/+
12	0/0	0/0	0/0	++/+	++/++	
13	0/0	0/0	-/0	+/0	++/+	
Total Bands	11/11	11/11	11/11	10/9	6/9	

*kDa – Kilo Dalton; h – hour; (++) 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.

moisture exposed eggs is shown in Fig. 2a & 2b and Table 2. The protein profiles revealed 13 conspicuous bands ranging from ~7 to ~200 kDa in different treatments. There are five bands namely 3, 5, 6, 9 and 10 bands were highly expressed at the early stages. Among them 3 and 6 maintained moderate to low level throughout the development. Whereas, bands 5, 9 and 10 were decreased gradually and became fainted or disappeared at the last stage. Bands 1, 2, 4, 7, 8 and 11 expressed moderately in early hours and maximum of them gradually reduced or appeared very low in strength with developmental progress except bands 7 and 11. Band number 7 maintained with equal thickness until last developmental phase and 11 migrated at the same position in the PAGE with more strength throughout the progressing of embryonic development. Bands 12 and 13 initiated during 24 h and expressed highly in the subsequent phases.

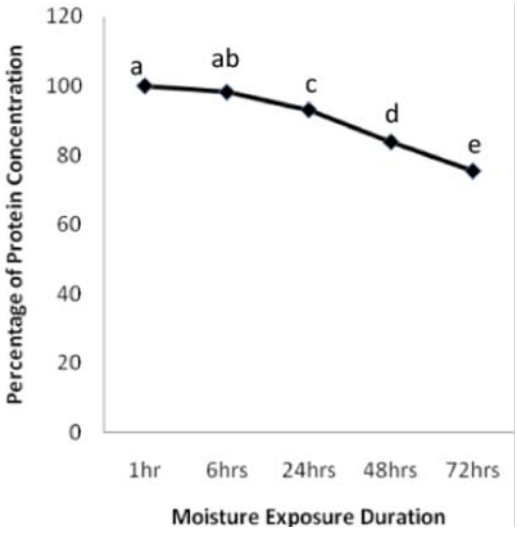

Fig. 1. Changes in protein concentration during embryonic development of *Ae. albopictus* mosquito (Values with the same letter are not significantly different) (P<0.05)

Comparisons between Silver stained (ST)/ Coomassie blue (CB) bands

Both, ST and CB stained the protein bands with equal thickness in the early hour of embryonation except bands 9 and 11. Bands 4, 7, 10, 11, 12 and 13 stained more in silver staining than CB in all stages. Bands 5, 8 and 9 followed the opposite pattern while bands 1, 2, 3 and 6 followed mixed pattern. From the latest group bands 1 and 2 gradually disappeared in ST but maintained a low frequency at the CB staining in higher developmental stages (Fig. 2a & 2b; Table 2).

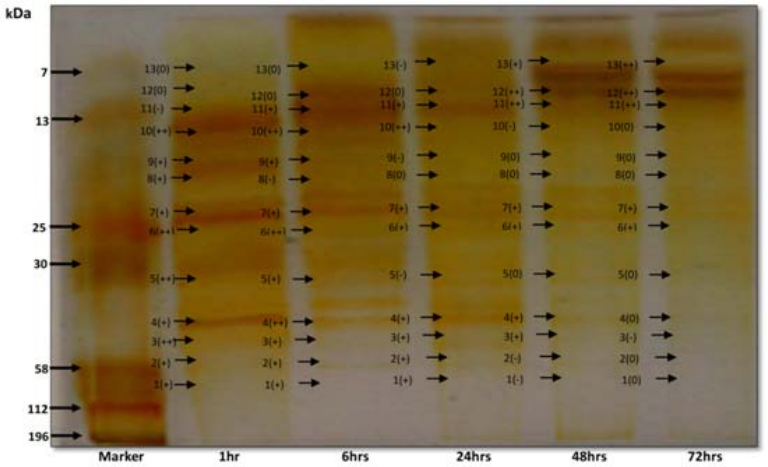

Fig. 2(a). Comparison in the protein synthesis (stained with silver nitrate) during embryonic development of *Ae. albopictus* mosquitoes; (++) indicates a very high level of synthesis; (+) indicates a high level of synthesis; (-) indicates a low level of synthesis; (0) indicates a very low level of synthesis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

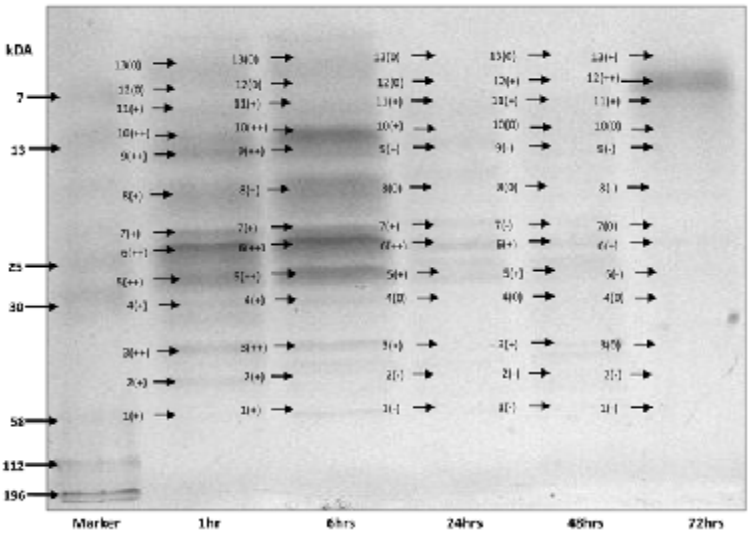

Fig. 2(b). Comparison in the protein synthesis (stained with coomassie blue) during embryonic development of *Ae. albopictus* mosquitoes; (++) indicates a very high level of synthesis; (+) indicates a high level of synthesis; (-) indicates a low level of synthesis; (0) indicates a very low level of synthesis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

DISCUSSION

Since moisture is the crucial factor for the embryonic development of *Aedine* mosquitoes, we have conducted a series of experiments to explore its' role on our test species in detail. In our previous study a correlation of embryonic development with visualized clarified embryo and spontaneous egg hatching with increasing moisture exposure of *Ae. albopictus* egg were demonstrated (Saifur *et al.*, 2010). In this work, the functional enzymes and proteins during progressive embryonic development in changing moisture condition were observed.

The yolk protein, which is the main form of food reserve in egg of many invertebrates, including insects (Chapman, 1998), are oxidised to meet the additional energy of increasing number of growing cells during embryogenesis (Srinivasan & Kesavan, 1979). Hence, the highly concentrated proteins in newly laid eggs decrease gradually. This protein declining rate indicates the variable role of

different internal and external factors including environmental conditions. Previously, it is observed that the protein concentration starts to decline after 12 h of embryonic development in principal dengue vector mosquitoes, *Ae. aegypti* (Blevins, 1973), which we notice at 6th h in our test samples. This advance change indicates faster embryonic development, which may have a great epidemiological significance. It can be explained in different ways. Firstly, faster embryonic development increases higher hatchability (Nur Aida *et al.*, 2011). Secondly, the progeny face less environmental challenges due to the short developmental period. Thirdly, the embryos face less microbial or fungal infections. Fourthly, the disease free new generation may survive longer and can complete more gonotrophic cycle (Dieng *et al.*, 2010; Saifur *et al*., 2012). All, the above situation assist to produce maximum vector population. The higher environmental temperature may trigger this faster embryonic development (Tun-Lin *et al.*, 2000) by increasing rapid enzyme activity. Our study is conducted in the temperate region, where temperature is very congenial for insects' development and growth. In this situation, identification of the activated enzymes or responsible protein can be very much effective for genetically control of this vector insect. In our proteomic profile study, we observe more or less 13 protein bands during embryonic development of the test mosquito species. There are few studies to compare this result. The embryonic proteins are most important, since they are responsible for proper embryo development and successful hatching. In this stage moisture plays the vital role to activate these important proteins or enzymes in the sense that less moistened eggs never hatch (Saifur *et al.*, 2010), may due to the lack of proper embryonic maturation or inactivity of necessary proteins and enzymes or both. To maintain a viable environment for embryo, it must maintain about 80 percent of humidity in eggs of silkworm. If it is less than 70 per cent, the hatchability becomes very low (Rahmathulla, 2012). In a suitable environment an embryo can produce sufficient amount of proteolytic enzyme, which helps to breakdown the crustacean egg shell during eclosion (De Vries & Forward, 1991).

Sometimes, some unwanted enzymes are produced during this embryonic development period, which act as a barrier of egg hatching. For example, Aprotinin, a serine protease inhibitor with a lower molecular weight, is found to interfere in embryos at blastula and gastrula stages and inhibited embryogenesis markedly in *Xenopus laevis* (Iijima *et al.*, 1999). We need to search this kind of positive or negative factors related with egg hatching to control this vector population efficiently. In this way, we see some highly expressed bands from 11-13 of lower molecular weight at around 7 kDa in all treatments with moisture. They may have controlling effects for embryonic development and egg hatching. In conclusion, embryonic development is of interest to biologists from both basic research and applied research perspectives since they are widely used for cell signalling, genetic control of early development and mechanisms of endocrine disruption (Schoenwolf, 2001). Moreover, proteomic profiling is a direct reflection of the gene expression pattern at a given stage and time which help us to understand gene regulation. Therefore, characterization of the polypeptides seen in band number 11-13 of the present study is needed to identify enzymes or proteins for finding any possible transmission blocking mechanism or to reduce the fitness of this vector mosquito.

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