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ANALYSIS OF HAEMOLYMPH PROTEINS IN THE *BRUGIA MALAYI*-SUSCEPTIBLE MOSQUITO, *AEDES TOGOI*, USING SDS-PAGE AND TWO-DIMENSIONAL GEL ELECTROPHORESIS

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Abstract- Haemolymph is the medium that transports nutrients, hormones and immune effector molecules to provide the mosquitoes with immune response against invading filarial worms. In this study, haemolymph protein profiles of adult female *Aedes togoi*, before and after intrathoracic inoculation of *Brugia malayi* microfilariae or injection with saline solution, were analyzed. SDS-PAGE revealed that haemolymph protein profiles of both mosquitoes injected and inoculated with saline solution and microfilariae, respectively, were similar. At least 5 protein bands were induced after the injection. Two-dimensional electrophoresis analysis showed that approximately 150 protein spots were resolved in the haemolymph before and after saline injection. The molecular mass of these spots varied from 10-80 kDa with a pI of 3.1-10. Through comparison and analysis, 30 protein spots were expressed differentially during the immune challenge. Seven and seventeen spots were up-regulated and down-regulated proteins, respectively. Two spots were repressed and four appeared post saline injection. Information of *Ae. togoi* haemolymph proteins obtained in this study was is an initial step for further identification of differentially expressed proteins that might help in understanding immune response in the mosquito-parasite system.
Key words- *Aedes*, *Brugia malayi*, haemolymph, protein, SDS-PAGE, 2-DE.

INTRODUCTION

Human lymphatic filariasis is a disease caused by the nematode parasites, *Wuchereria bancrofti* (bancroftian filariasis) and *Brugia malayi* (malayan filariasis). In approximately 40% of cases, the disease is manifested by lymphedema of the extremities or hydrocoel. Although human lymphatic filariasis does not increase mortality in endemic areas, morbidity causes major economic losses and often leads to psychosocial and psychosexual conditions in infected individuals. Recent efforts by the Global Program for the Elimination of Lymphatic Filariasis (GPELF) have decreased the numbers of individuals infected with, and at risk of, this parasitic disease [1].

Mosquitoes in the genus, *Aedes, Anopheles, Culex* and *Mansonia*, serve as vectors of the disease [2-5]. Mosquito control through environmental perturbation and insecticide application has been limited by environmental and human health concerns and the development of insecticide resistant mosquitoes [6]. Recently, research efforts have focused on the development of tools for the genetic alteration of mosquito vectors, with the final goal to block the parasite life cycle within these insects, making them incapable of transmitting the disease. While the success of achieving stable transformation of *Ae. aegypti* [7-9] and *An. stephensi* [10] has raised hope for the production of mosquito strains that are unable to transmit various parasites, effector molecules involved in parasite recognition, parasite development and promoters of genes active in specific tissue of mosquito vectors are required.

For this to succeed, it is important to understand the biology and molecular basis of parasite-mosquito interaction in insect organs, tissue and haemolymph, where significant interactions with parasites take place. As haemolymph is the medium that transports nutrients, hormones and immune system effector molecules to provide the mosquito with immune response against invading nematodes, and the thoracic musculature is the developmental site for *B. malayi* and *B. pahangi*, research has focused on these aspects [2, 11, 12].

In susceptible mosquitoes, microfilariae migrate from the mosquito midgut soon after being ingested and reach the thoracic muscles within 1 hour. Filarial worms develop from first-stage to third-stage (infective) larvae in the thoracic muscles and then break out of these sites. Then, they travel through the haemocoel to the head region, from which they actively emerge onto the surface of the vertebrate host's skin when the mosquito takes a blood meal [13]. In some refractory strains, the filarial worms migrate to the developmental site, but fail to develop [14]. Efforts to discover the gene(s) or gene product(s) responsible for susceptibility or refractoriness to filarial worm infection in haemolymph have not been extensive [15, 16]. In Thailand, autogenous *Ae. togoi* (Chantaburi strain) was highly susceptible to nocturnally subperiodic (NSP) *B. malayi* [17]. Therefore, as a first step towards

characterizing haemolymph molecules involved in the mosquito immune response and development of filarial worms, this study analyzed one and two-dimensional gel electrophoresis patterns of the haemolymph proteins of *B. malayi*-infected and uninfected *Ae. togoi* mosquitoes, and compared them with mosquitoes injected with saline solution.

MATERIALS AND METHODS

Mosquito: *Ae. togoi* mosquitoes (Koh Nam Sao, Chantaburi Province, Southeastern Thailand) were used in this study. This strain had been maintained in the insectary of the Department of Parasitology, Faculty of Medicine, Chiang Mai University, since 1983, and was proven to be highly susceptible to NSP *B. malayi* [17]. The method for rearing the mosquitoes followed the standard techniques [18].

Source of NSP *B. malayi* microfilariae: Microfilariae of NSP *B. malayi* were taken from an experimentally infected domestic cat maintained in the animal house of the Department of Parasitology. The filaria parasite originated from a 20-year-old woman patient in Paw District, Narathiwat province, southern Thailand, and has been used to infect domestic cats experimentally since 1982.

Isolation and inoculation of microfilariae and saline injection: *B. malayi* microfilariae were isolated from an experimentally infected cat [19]. Heparinized blood (0.5 ml) was chilled for 30 min in a wet-ice bath, then diluted with 0.5 ml of chilled distilled water and mixed thoroughly by inverting the tube and shaking it. The preparation was centrifuged for three min at 1,500 rpm at 4°C using a low speed refrigerated centrifuge with the supernatant decanted. The sediment was resuspended in 5 ml of chilled Hanks balanced salt solution (HBSS, pH 7.2–7.4). Centrifugation was repeated twice more and the final sediment resuspended in 0.5 ml of HBSS and incubated for 30 min at 37°C. The microfilariae in the suspension remained active for up to six hours. Intrathoracic inoculation of microfilariae was performed [19]. Mosquitoes were anaesthetized with ether and placed on their sides on a slide below a binocular microscope. A needle was made by drawing out a glass capillary tube in a flame until the pointed end was 80–100 μm in diameter. The injection was made into the post-spiracular area of the mesothorax, and approximately 20 microfilariae introduced by gently blowing down the attached rubber tube. Dissection of thoracic muscle of the inoculated mosquitoes 6, 12 and 24 hr after inoculation revealed that most of microfilariae remained active. Saline injection was performed using a handmade needle as described above. Approximately 0.5 μl of 0.9% NaCl was injected into the thorax of each mosquito.

Haemolymph collection: The method for haemolymph collection was modified slightly [20]. The mosquitoes were anaesthetized on ice and the tip of the proboscis was removed with microscissors. Haemolymph was expelled in a droplet at the tip of the proboscis upon pressure to the thorax. Only clear droplets were collected to avoid contamination from fat body. For one-dimensional gel electrophoresis, droplets were immediately added to a microfuge tube containing 10 μl of sodium dodecyl sulphate (SDS) gel-loading buffer [50mM Tris-HCl pH 6.8, 100mM dithiothreitol (DTT), 2% SDS, 0.1% bromphenol blue and 10% glycerol] to protect proteins from degradation while haemolymph was collected and pooled. Each 10 μl sample contained pooled haemolymph from ten mosquitoes. For two-dimentional gel electrophoresis, droplets were added to a 125 μl sample of solubilization solution (8 M urea, 50 mM DTT, 4% CHAPS, 0.2% 3/10 Bio-lyte Ampholyte, 0.002% Bromophenol Blue). Each sample of analytical gels contained pooled haemolymph from 100 mosquitoes. Samples were stored at -80°C until use.

One-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGE was performed in 12.5% or 15% polyacrylamide gel [21]. Electrophoresis was carried out under a constant current (30 mA) for 1–2 hr using Bio-rad apparatus. Samples were heated to 95°C for 5 min before loading. Molecular weight markers (Bio-Rad, USA) were applied in each gel.

Two-dimensional gel electrophoresis (2-DE): Two-dimensional gel electrophoresis was performed using the 2D system (GE Healthcare, UK). The amount of protein in each sample was determined using the Micro BCA protein assay (Pierce, CA). Samples were solubilized in a 125 μl sample of solubilization solution and then loaded on an IPG strip [isoelectric point (pI) 3–10, 7 cm, GE Healthcare, UK] to perform the first dimension isoelectric focusing (IEF) separation. Following 13 hr of rehydration, the strips were focused using Ettan™ IPGphor III (GE Healthcare, UK) according to the manufacturer's instruction. The focused IPG strips were then incubated for 15 min in 10 ml of SDS equilibration buffer (6 M urea, 2% SDS, 0.05 M Tris, pH 8.8, 30% glycerol, 0.002% Bromophenol Blue) containing 100 mg of dithiothreitol (DTT), followed by a further 15 min in 10 ml of equilibration buffer containing 250 mg of iodoacetamide. The equilibrated strips were applied to the surface of vertical 15% SDS-polyacrylamide gels and proteins and separated in the second dimension using the Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad, USA). Protein molecular weight markers (Bio-Rad, USA) were applied in each gel.

Coomassie brilliant blue staining: Following electrophoresis, the gels were stained with Coomassie brilliant blue (CBB). Initially, 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 2 hr, and finally de-stained in 10% methanol and 5% acetic acid until dark protein bands or spots were visible.

Data analysis: Gels were scanned using an ImageScaner III (GE Healthcare, UK). All gel images were

acquired at 100 µm pixel resolution under nonsaturating conditions. The 2-D PAGE images were analyzed using Ettan™ IPGphor 3 Control Software and an Image Master 2D Platinum 7 program.

RESULTS

Comparison of haemolymph protein profiles of adult female *Ae. togoi* before and after intrathoracic inoculation of *B. malayi* microfilariae and injection of saline solution by SDS-PAGE: The female mosquitoes were either injected with saline solution or inoculated with *B. malayi* microfilariae. After that, the haemolymph was collected at 6, 12, and 24 hr post injection or inoculation. These time points were chosen followed the reports of haemolymph protein profiles in *An. gambiae* changed between 6 and 24 hr after wounding or inoculating with bacteria [22]. SDS-PAGE revealed that the haemolymph protein profiles of the mosquitoes injected with saline solution or inoculated with microfilariae were similar (data not shown). Figure 1 shows that at least 5 protein bands were induced after saline injection. More differences were observed at 12 hr post injection. Therefore, analysis using two-dimensional electrophoresis was performed at 12 and 24 hr post injection.

Haemolymph characterization by two dimensional electrophoresis: The female *Ae. togoi* haemolymph was collected in 12 and 24 hr post injection. The 2-DE experiments provided evidence of many proteins in the mosquito haemolymph, with approximately 150 well-resolved spots (Fig. 2). The molecular mass of these spots varied from 10-80 kDa, with a pI range of 3.1-10. Most proteins were detected in both naïve and injected mosquitoes. The naïve gel was considered as the standard reference gel for intensity comparison of particular spots. By using the Image Master 2D Platinum program, spots were obtained that showed differences in the volume of mosquitoes injected with saline solution. Thirty protein spots were expressed differentially during the immune challenge. Seven spots were increased in intensity (up-regulated proteins), and seventeen spots were decreased in intensity (down-regulated proteins). Two spots were repressed and four appeared after saline injection. The molecular mass of these spots varied from 28-65 kDa, with a pI range of 3.1-9.3. Variation of protein expression volume is shown in Table 1.

DISCUSSION

Mosquito immunity is of special interest, due to the role these insects play as vectors of many human pathogens, including nematode worms (lymphatic filariasis), protozoa (malaria), and viruses (e.g. dengue virus). The haemolymph of mosquitoes constitutes a major barrier to infection, as a source of and fluid transporter for antibiotic peptides and components of the humoral melanization system. Insect immune responses have been studied in several insect species and their tissue. Examples include *D. melanogaster* [23-33], *An. gambiae* [20, 22], and *Ae. aegypti* [15, 34], the silkworm, *Bombyx mori* [35], and the locust, *Odaleus australis* [36].

In this study, analysis of *Ae. togoi* immune responses against saline injection and microfilariae inoculation was carried out. SDS-PAGE revealed that the haemolymph protein profiles of the mosquitoes injected with saline solution or inoculated with microfilariae were similar. At least 5 protein bands were induced after the immune challenge. Analysis by one-dimensional electrophoresis allowed detection of differentially expressed proteins by molecular mass. To obtain more details, haemolymph protein profiles were analyzed by 2-D electrophoresis. Two-dimensional gel electrophoresis has been the most widely used protein separation technology for insect immunity [20, 22, 37, 38]. With this approach, proteins are separated in the first dimension by isoelectric focusing using immobilized pH gradient strips. Then, these proteins are separated again, this time by molecular mass in standard polyacrylamide gel electrophoresis, resulting in a 2-dimensional display of proteins. By this method, it was found that after the immune challenge by saline injection, 4 groups of proteins were expressed differentially (Table 1). The results were in accordance with studies of immunity in others insects [15, 20, 22, 25, 28, 29, 32, 33, 34]. About 20% of the immune-regulated proteins in *An. gambiae* and 14% of those in *Drosophila* adult haemolymph were identified. Approximately 130 proteins described as immune-induced have been documented in larval or adult haemolymph from *D. melanogaster* by 2-D electrophoresis and protein identification methods [25, 28, 29, 32, 33]. In studies of adult haemolymph following bacterial challenge, a similar number of proteins was affect ed in *Drosophila* and *Anopheles*. For example, 50 of 350 (14%) silver-stained spots were up- or down-regulated in *Drosophila* [32, 33], while 14 of 280 (5%) silver-stained spots were upregulated in *An. gambiae* [22]. By comparison, a much larger number of spots was regulated specifically in adult fruit flies by 72 hr after fungal exposure [32, 33]. A subset comparison of 42 proteins, regulated following fungal exposure, showed that twelve of the proteins also were affected by bacterial infection. Three of the twelve were up-regulated and the other nine down-regulated.

Proteomics is a large-scale study of the gene expression at the protein level, which ultimately provides direct measurement of protein expression levels and insight into the activity state of all relevant proteins. Key elements of classical proteomics are the separation of proteins in a sample using 2-D electrophoresis and their subsequent identification by biological mass spectrometry (MS). Protein expression profiling of insect immune responses has been initiated for several insect species and their tissue, and many differentially expressed proteins have been identified by MS. Most of these studies have been carried out in *D. melanogaster* [23-33]. Other taxa, where immunity has been investigated by proteomic methods, include the mosquitoes, *An. gambiae* [20, 22] and *Ae. aegypti* [15, 34], the silkworm, *Bombyx mori* [35], and the locust, *Odaleus australis* [36].

Therefore, further identification of differentially expressed proteins using mass spectrometry and construction of a proteomic database of the *Ae. togoi* haemolymph should be performed. Identification of these proteins may have

important implications for understanding the immune response process of this mosquito species, as well as developing novel vector control strategies and understanding parasite-vector interactions.

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Table 1-List of 30 differentially expressed protein spots in the haemolymph of Ae. togoi mosquitoes.

spot number	[a]MW (kDa)	[b]pI	[c]0 hr		[d]12 hr		[e]24 hr	
			[f]NV	[g]Index	NV	Index	NV	Index
Spots with increased intensity post saline injection								
1	40	9.2	0.16	100.00	1.19	744 .13	2.59	1622.20
2	38	7.3	1.80	100.00	2.21	123.06	2.93	162.89
3	44	7.6	0.43	100.00	0.48	112.82	0.63	146.65
4	44	7.9	0.34	100.00	1.31	385.41	1.13	332.32
5	44	8.3	0.25	100.00	1.10	441.62	1.32	531.56
6	44	8.7	0.50	100.00	2.57	510.50	4.23	840.17
7	45	7.8	0.54	100.00	1.64	303.94	1.75	325.95
Spots with decreased intensity post saline injection								
8	28	3.8	1.63	100.00	0.97	59.32	0.64	39.27
9	39	8.6	0.78	100.00	0.14	18.30	0.73	93.70
10	36	7.1	0.51	100.00	0.45	88.24	0.20	39.22
11	38	7.7	0.77	100.00	0.37	48.05	0.36	46.65
12	43	7.0	1.14	100.00	0.61	54.01	0.58	50.66
13	43	7.3	1.11	100.00	0.58	52.21	0.72	62.09
14	43	9	0.58	100.00	0.54	92.18	-	-
15	43	9.3	0.68	100.00	0.07	9.58	-	-
16	47	8.5	1.42	100.00	0.38	26.57	0.49	34.44
17	51	8.6	0.39	100.00	0.11	28.82	0.10	25.35
18	50	8.0	0.46	100.00	0.35	75.88	0.11	23.16
19	51	7.3	1.54	100.00	0.78	50.63	0.50	31.90
20	64	8.8	0.31	100.00	0.19	61.92	0.18	57.84
21	65	6.2	0.50	100.00	0.17	33.87	0.22	44.43
22	65	6.4	0.63	100.00	0.13	20.48	0.32	50.07
23	65	6.6	0.50	100.00	0.07	13.49	0.18	36.23
24	65	6.8	0.33	100.00	0.07	20.87	0.11	32.75

Spots repressed post saline injection								
25	28	4.2	0.42	100.00	-	-	-	-
26	35	3.1	0.88	100.00	-	-	-	-
Spots appearing post saline injection								
27	28	4.7	-	-	0.47	100.00	0.56	119.15
28	28	5.7	-	-	0.33	100.00	0.41	42.42
29	28	9.0	-	-	0.57	100.00	0.18	31.58
30	30	6.5	-	-	0.47	100.00	0.78	165.96

[a]MW: Molecular mass
[b]pI: Isoeletric point
[c]0 hr: naïve mosquitoes
[d]12 hr: mosquitoes after 12 hr saline injection
[e]24 hr: mosquitoes after 24 hr saline injection
[f]NV: normalization volume of protein spot
[g]Index: relative volume expressed on the hour when the relative normalization volume of protein was expressed at the first hour

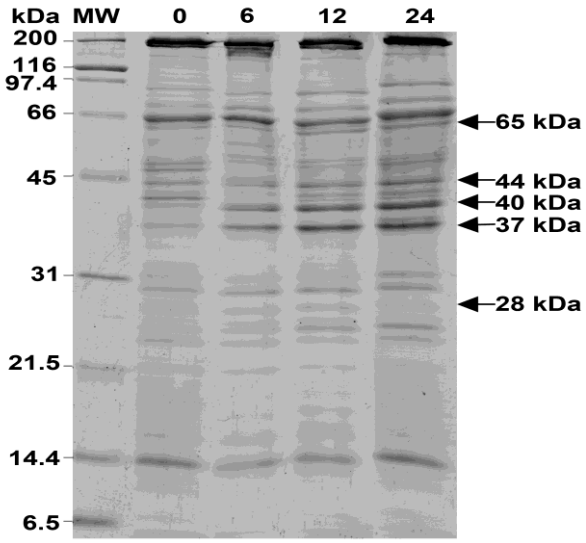

Fig. 1-SDS-PAGE of haemolymph proteins from *Ae. togoi* injected with saline solution. Haemolymph samples were collected from 5 mosquitoes at 6, 12, and 24 hr post saline injection, separated on 15% SDS-PAGE, and stained with CBB. Arrows indicate protein bands differentially expressed post saline injection. N = naïve control; 12 = 12 hr post saline injection; 24 = 24 hr post saline injection.

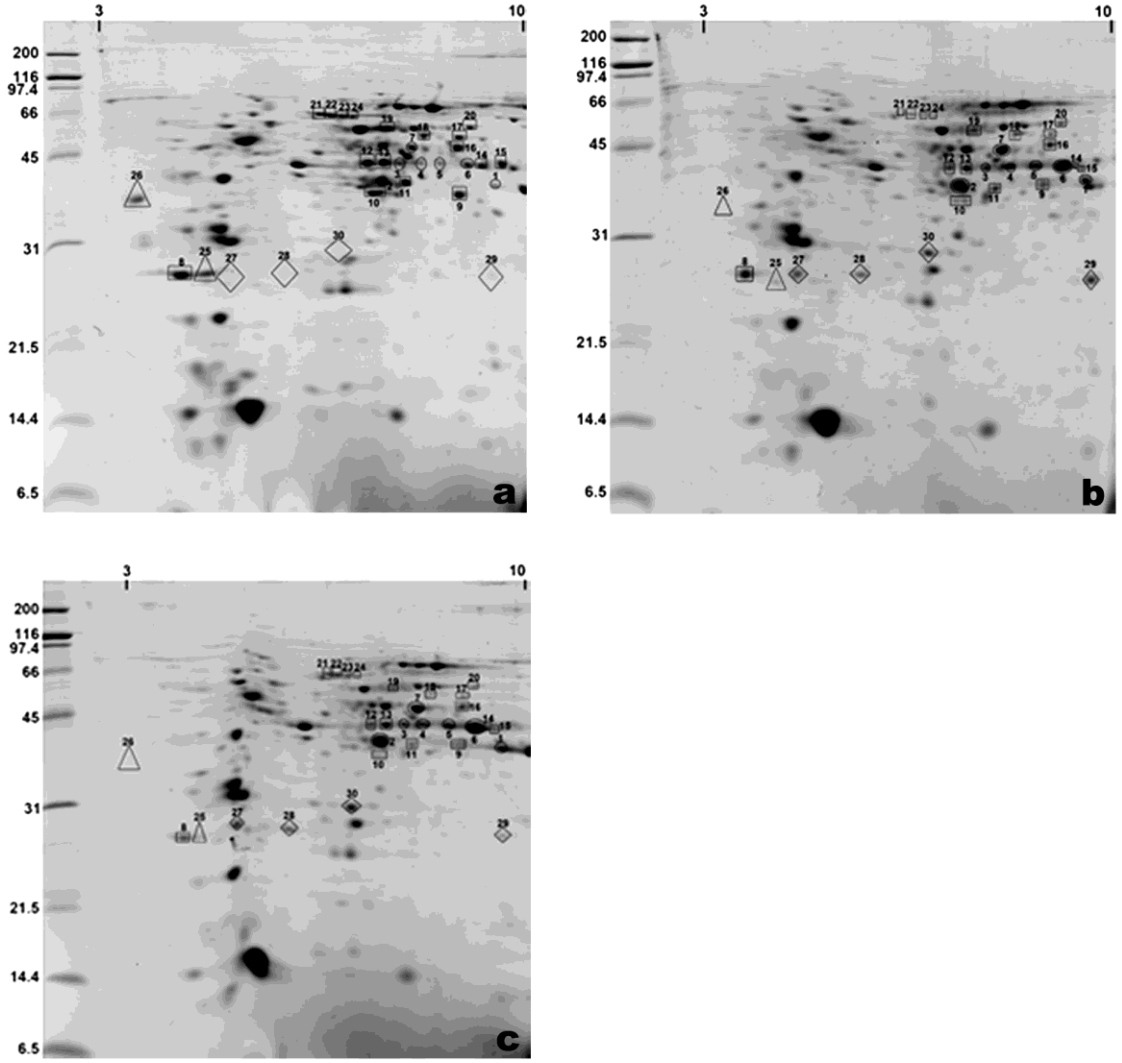

Fig. 2-(a) Representative of 2-D electrophoresis profile of haemolymph proteins from *Ae. togoi* naïve mosquitoes; (b) Representative of 2-D electrophoresis profile of haemolymph proteins from mosquitoes 12 hr post saline injection; (c) Representative of 2-D electrophoresis profile of haemolymph proteins from mosquitoes 24 hr post saline injection. Haemolymph proteins from 100 female mosquitoes were separated in the first dimension by IEF using Immobiline DryStrips of 7 cm, pH 3-10. Separation in the second dimension was performed using 15% constant gels followed by Coomassie blue staining. Molecular mass markers are indicated on the left in kDa. Isoelectric points (pI) are indicated at the top. Numbers and symbols indicate differentially expressed proteins compared among the 3 representative protein profiles. ○ = spot with increased intensity, □ = spot with decreased intensity, △ = spot absent post injection, ◇ = spot appearing post injection.