Journal of the American Mosquito Control Association, 14(4):380–384, 1998

VARIATION IN ACID HYDROLASE ENZYME TITERS IN DIFFERENT DEVELOPMENTAL STAGES OF *AEDES TOGOI*

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ABSTRACT. Genetic mechanisms of filarial nematode susceptibility were studied in *Aedes togoi.* Acid hydrolases may play an important role in this process, including humoral or cell-mediated defenses. Levels of acid phosphatase, α-glucosidase, β-glucuronidase, and *N*-acetyl-β-glucosaminidase were determined for 1st- and 4th-instar larvae, male and female pupae, and 1- and 7-day-old adults using fluorometric and colorimetric assays. Acid phosphatase activity was highest in 1-day-old adults, moderate in larvae and pupae, and lowest in 7-day-old adults. Female 7-day-old adults had significantly higher levels than males of the same age. Moderate levels of α-glucosidase were found in larvae, with progressive increases in activity from pupae to 7-day-old adults. Levels in male pupae and 1-day-old males were higher than in females, but activity was twice as high in 7-day-old females. Activity of β-glucuronidase was greater in adults, with females showing a 2-fold higher level than males at 7 days. In contrast, *N*-acetyl-β-glucosaminidase activity was highest in 1st- and 4th-instar larvae and 1-day-old males and females. Activity also was significantly higher in male pupae, slightly greater in 1-day-old males, but twice as high in 7-day-old females when compared to males of the same age. Results showed significant changes and variation in acid hydrolase enzyme titers in the different life stages of *Ae. togoi.* These and other specific acid hydrolases could prove effective in monitoring biochemical and genetic changes in mosquito populations.

KEY WORDS *Aedes togoi,* variation, acid hydrolase enzymes, developmental stages

INTRODUCTION

Research is underway in our laboratory to obtain more information on the genetic mechanisms of filarial nematode susceptibility in the laboratory model *Aedes aegypti* (Linn.). We also are interested in whether these mechanisms occur in a natural host (Macdonald 1971, Mori et al. 1985) such as *Aedes togoi* (Theobold). A previous study found differences in the development of *Brugia malayi* (Brug) in susceptible and refractory genotypes of *Ae. aegypti* (Rodriguez et al. 1984). We also found that changes in the activities of certain acid hydrolase enzymes occur in response to challenge with this filarid (Schirf and Rodriguez, unpublished data).

However, few studies have analyzed hydrolase enzymes during the natural life cycle of these mosquitoes (Nasar-Schirf et al. 1989). Information of this nature would be useful for accurately assessing genetic changes that occur in mosquito populations after laboratory manipulations, parasitism, exposure to chemical mutagens, or environmental stress. Moreover, baseline levels and data would be established and could be applicable to other vector–host systems.

Investigators have reported increases in acid phosphatase activity in the snail host *Biomphalaria glabrata* (Say) after challenge with both *Schistosoma mansoni* (Sambon) and bacteria respectively (Cheng and Garrabrant 1977, Cheng et al. 1977). Others have described higher levels of lysosomal enzymes in refractory strains of *B. glabrata* when infected only with *S. mansoni* (Granath and Yoshino 1983). A subsequent investigation provided evidence for the destruction of *S. mansoni* sporocysts associated with elevated levels of certain lysosomal enzymes in refractory snails of the same species (Cheng and Dougherty 1989).

In mosquitoes or other insects, certain hydrolases or lysosomelike enzymes have been proposed as a humoral or cell-mediated response to immunologic challenge (Soderhall and Smith 1986, Stoffolano 1986). We have shown significant variation in the activities of 4 acid hydrolases in development stages of *Ae. aegypti* (Nasar-Schirf et al. 1989).

The present study was conducted to compare variation and patterns of 4 acid hydrolase enzyme activities in *Ae. togoi* at various stages of development. These enzymes were acid phosphatase (EC 3.1.3.2; ACP; orthophosphoric monoester phosphohydrolase), α-glucosidase (EC 3.2.1.21; α-Gic; α-D-glucoside glucohydrolase), β-glucuronidase (EC 3.2.1.31; β-GlcUr; β-D-glucuronide glucuronosohydrolase), and *N*-acetyl-β-glucosaminidase (EC 3.2.1.30; β-Glc NAc; 2-acetamido-2-deoxy-β-D-glucoside acetylaminidodeoxygluco-hydrolase). The life stages characterized included 1st- and 4th-instar larvae, male and female pupae, and 1- and 7-day-old male and female adults.

MATERIALS AND METHODS

All test mosquitoes originated from the Institute of Medical Research (IMR) strain of *Ae. togoi,* obtained from the Faculty of Medicine, National University of Singapore, Republic of Singapore. A previous experiment showed that this strain was 65% susceptible to *B. malayi* infections (Rodriguez, unpublished).

Mosquitoes were reared as described by Thompson and Rodriguez (1979), but maintained at 25°C

and 80% relative humidity under a 16-h day length in a reach-in environmental chamber (Scientific Systems, Baton Rouge, LA). First- and 4th-instar larvae, male and female pupae, and 1- and 7-day-old male and female adults were homogenized in 0.5 ml of deionized water at 4°C using a Potter-Elvejhem homogenizer. Five samples of 3 mosquitoes per each group were taken. Homogenates were centrifuged (Microcentrifuge Model 235 C, Fisher Scientific, Hampton, NH) at 14,000 rpm at 4°C for 5 min and the supernatants were collected.

Rapid and sensitive fluorometric assays were used to determine enzyme activities in supernatants. These assays were based upon enzymatic hydrolysis of nonfluorescent 4-methylumbelliferone (4-MU)-conjugated substrates to produce 4-mu, which is highly fluoroescent at alkaline pH. Purified 4-mu was used as the standard for these assays by using a Turner 112 fluorometer (Sequoia-Turner Corp., Mountain View, CA). All standards and substrates were purchased from Sigma Chemical Co. (St. Louis, MO).

Briefly, acid phosphatase activity was analyzed by the method of Robinson and Glew (1980) using 4-MU-phosphate as the substrate. α-Glucosidase activity was tested using 4-MU-α-glucosidase as the substrate according to Robinson (1956), but modified with 0.25 mM acetate buffer at pH 5.5. β-Glucuronidase was assayed based on procedures developed by Mead et al. (1955), modified with 0.25 mM acetate buffer at pH 5.0 and using 4-MU-β-Glucuronide as the substrate. Determinations for *N*-acetyl-β-glucosaminidase were adapted from Leaback and Walker (1961), using 40 mM citrate phosphate buffer at pH 4.4 and 4-MU-*N*-acetyl-β-glucosaminide as the substrate. Reactions were stopped with ammonium hydroxide–glycine buffer, pH 10.5. Again, the amount of 4-mu released was determined fluorometrically (Turner Model 112 fluorometer).

Total protein was estimated according to Bradford (1976) using a commercially available Coomassie concentrate (Bio-Rad, Richmond, CA) with bovine serum albumin as the standard. The specific activities are reported as picomoles 4-mu released per minute per microgram of protein. Appropriate artificial fluorometric substrates (purchased from Sigma Chemical) and conditions are summarized in Table 1.

Table 1. Acid hydrolase enzyme assay conditions.[1]

Enzyme	pH	Substrate concentration (mM)	Time (h)	Protein/incubation (μg)
Acid phosphatase	5.0	200	0.5	2.20
α-Glucosidase	5.5	250	1	2.20
β-Glucuronidase	5.0	250	1	2.20
N-Acetyl-β-glucosaminidase	4.4	46	1	2.20

[1] Incubation volumes were 110 μl. Reactions were stopped with 2.9 ml 0.1 M NH_4OH–glycine buffer, pH 10.5, and the amount of 4-methylumbelliforone derivative released was determined fluorometrically.

RESULTS

Figures 1 and 2 summarize mean activities of the 4 hydrolase enzymes that were determined for 5 developmental stages of *Ae. togoi.* Analyses included 1st- and 4th-instar larvae, male and female pupae, and 1- and 7-day-old male and female adults. Figure 1 specifically shows activities of the 4 hydrolases and compares the different life stages with the male adults. Figure 2 emphasizes the differences between the life stages and the 1- and 7-day-old females.

The activity of acid phosphatase was moderate in 1st- and 4th-instar larvae and male and female pupae (Figs. 1 and 2). Significantly highest activities were obtained in 1-day-old adults ($F = 118.13$; df = 4,36; $P < 0.0001$). The lowest activities were obtained for 7-day-old adults, with females having significantly higher levels than males ($t = 4.704$; df = 8; $P < 0.01$).

Levels of α-glucosidase activity were moderate in both larval stages and higher in 7-day-old females ($F = 30.22$; df = 4,36; $P < 0.0001$). When sexual differences were compared, the activity was only slightly higher in male pupae ($t = 2.46$; df = 8; $P < 0.05$). In contrast, activity for this same enzyme was twice as high in female 7-day-old adults as that than in males of the same age ($t = 4.64$, df = 8, $P < 0.01$).

β-Glucuronidase had the highest titers in 1-day-old adults and 7-day-old females, moderate levels in 4th-instar larvae and male and female pupae, and lowest levels in 1st-instar larvae ($F = 12.64$; df = 4,36; $P < 0.0001$). Levels of β-glucuronidase also were 2-fold higher in 7-day-old females than in males of the same age ($t = 4.71$; df = 8; $P < 0.01$). No significant sexual differences were found in pupae or 1-day-old adults (Figs. 1 and 2).

The levels of *N*-acetyl-β-glucosaminidase were most pronounced in 1st- and 4th-instar larvae and 1-day-old males and females ($F = 150.84$; df = 4,36; $P < 0.0001$). Lowest levels were expressed in 7-day-old males and females (Figs. 1 and 2). When sexual differences between life stages were compared, the activity of *N*-acetyl-β-glucosaminidase was significantly higher in male pupae ($t = 4.81$; df = 8; $P < 0.01$). One-day-old males also gave a higher variation in activity (Fig. 1). In contrast, activity for this same enzyme was twice as high in female 7-day-old adults as in males of the same age (Fig. 2; $t = 4.76$; df = 8; $P < 0.01$).

DISCUSSION

Hydrolytic enzymes may be involved in a variety of physiologic processes, including lysis of organ-

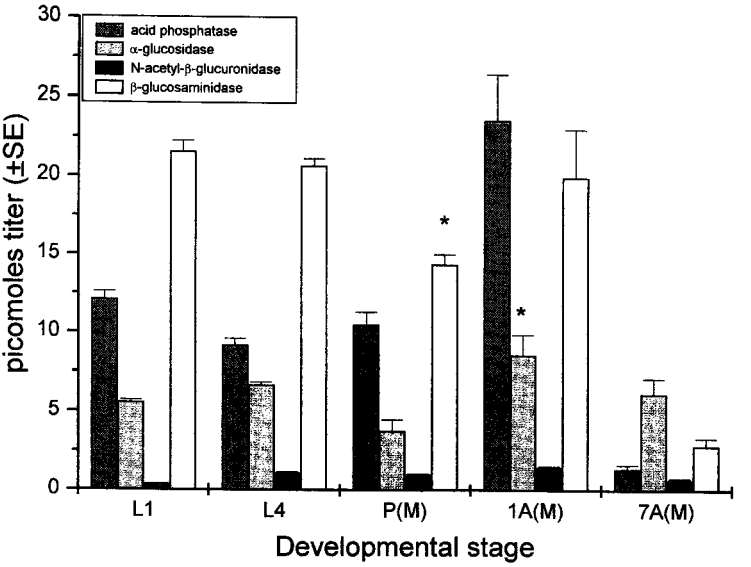

Fig. 1. Mean specific activities (± SEM) of acid phosphatase, α-glucosidase, β-glucuronidase, and *N*-acetyl-β-glucosaminidase (picomole titers) comparing larvae, pupae, and male adults of *Aedes togoi*. L1, 1st-instar larvae; L4, 4th-instar larvae; P(M), male pupae; 1A(M), 1-day-old adult males; 7A(M), 7-day-old males. $\bar{x}$ = mean of 5 sample determinations; SE, standard error of the mean. Significant *t*-test ($a < 0.05$) results are indicated by * and compare males and females within each developmental stage.

elles during metamorphosis and cellular differentiation, removal of necrotic and aged cells, and cellular immunologic defenses (Sheeler and Bianchi 1980, Johnson et al. 1986). Hydrolases or lysosomelike enzymes have been proposed as a humoral or cell-mediated response to immunologic challenge in invertebrates (Cheng et al. 1977, Soderhall and Smith 1986, Stoffolano 1986, Cheng and Dougherty 1989).

Cheng and Garrabrant (1977) showed increased acid phosphatase activity within granulocytes of *B. glabrata* challenged with *S. mansoni*. In more re-

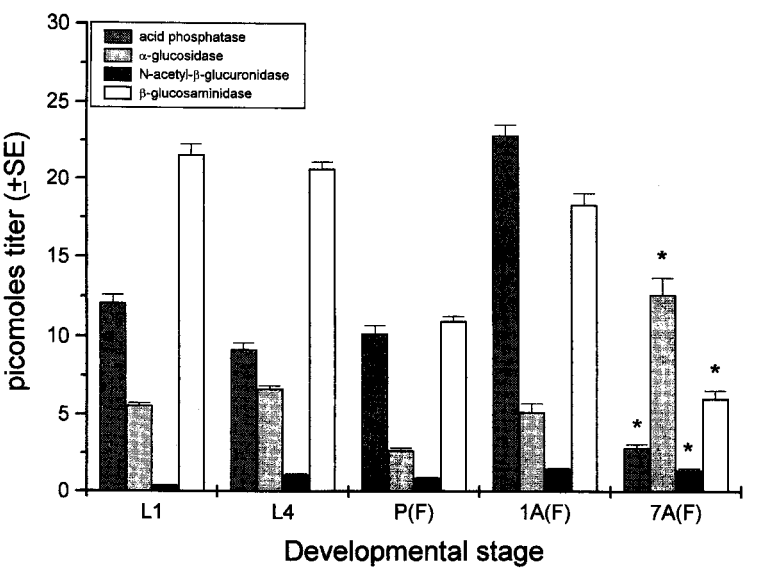

Fig. 2. Mean specific activities (± SEM) of acid phosphatase, α-glucosidase, β-glucuronidase, and *N*-acetyl-β-glucosaminidase (picomole titers) comparing larvae, pupae, and female adults of *Aedes togoi*. L1, 1st-instar larvae; L4, 4th-instar larvae; P(F), female pupae; 1A(F), 1-day-old adult females; 7A(F), 7-day-old females. $\bar{x}$ = mean of 5 sample determinations; SE, standard error of the mean. Significant *t*-test ($a < 0.05$) results are indicated by * and compare males and females within each developmental stage.

cent studies Cheng and Dougherty (1989) provided evidence for the destruction of *S. mansoni* sporocysts associated with elevated levels of acid phosphatase and aminopeptidase in refractory snails of the same species. Gilmour (1961) reported α-glucosidase activity in the gut of a number of insects, and β-glucuronidase was found in the crop liquor of the locust *Locusta migratoria* L. (Mead et al. 1955). Variable patterns of acid phosphatase, α-glucosaminidase, β-glucuronidase, and *N*-acetyl-β-glucosaminidase activities were demonstrated in different developmental stages in filarial susceptible and refractory genotypes of *Ae. aegypti* (Nasar-Schirf et al. 1989).

The present study showed significant variation in the activities of 4 acid hydrolase enzymes in the different stages of *Ae. togoi.* However, unlike in *Ae. aegypti,* the levels of acid phosphatase and β-glucuronidase were higher in either 1-day-old or 7-day-old adults, or both. *N*-Acetyl-β-glucosaminidase activity was higher in both 1st- and 4th-instar larvae, with 1-day-old adults showing similar but slightly lower levels. In *Ae. aegypti* the levels of these same enzymes (acid phosphatase, β-glucuronidase, and *N*-acetyl-β-glucosaminidase) were higher in larvae and pupae (Nasar-Schirf et al. 1989).

N-Acetyl-β-glucosaminidase can depolymerize β-1, 4-linked *N*-acetyl-β-glucosamine chains from the nonreducing terminus (Zubay 1983). Moreover, during molting, chitin is hydrolyzed and reabsorbed to form new cuticle (Gilmour 1961, Nasar-Schirf et al. 1989). Both *Ae. togoi* and *Ae. aegypti* had pronounced levels of α-glucosidase activity in 1- and 7-day-old adults. Sugar was the only food given to adults and perhaps this enzyme is important in digestion of sucrose (Chippendale 1978, Foster 1995).

Higher enzyme activities were observed in male pupae and 1-day-old male adults than in females of *Ae. togoi,* especially for α-glucosidase and *N*-acetyl-β-glucosaminidase. In *Ae. aegypti* only the latter enzyme was more prevalent in male pupae and 1-day-old adult males. Significantly higher activities of the 4 hydrolytic enzymes were observed in 7-day-old female adults of *Ae. togoi* in the present study and previously in *Ae. aegypti* than in other stages (Nasar-Schirf et al. 1989). These differences may be indicative of age, size, and metabolic differences, and could well serve as a general response or defense mechanism for parasitic infections. Sex- and age-dependent differences in enzyme activities previously were reported for β-glucuronidase (Langley et al. 1983); the higher activities of these enzymes in older females could be important for digestion of blood that females require for egg production (Engelmann 1970, Foster 1995). Recently completed experiments in our laboratory also have shown variations in the activity these same 4 acid hydrolases after challenge with *B. malayi* infections (Shirf and Rodriguez, unpublished data) and chemical mutagen exposures (Gonzalez, Brown, and Rodriguez, unpublished data). Measurement of the activities of these and other specific acid hydrolase enzymes could prove to be effective for monitoring biochemical changes in mosquito populations.

ACKNOWLEDGMENTS

We are grateful to Beng Chuan Ho, Faculty of Medicine, National University of Singapore, for providing the IMR strain of *Ae. togoi.* We also thank Lois Tews for typing the original manuscript. This study was supported in part by National Science Foundation grant DUE-9612758, Coordinating Board Grant ATP 010115-273, National Institutes of Health grant GM-08194, and the National Institute of General Medical Sciences, with cofunding through the National Institute of Allergy and Infectious Diseases.

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