International Journal of Entomology Research
ISSN: 2455-4758
Impact Factor: RJIF 5.24
www.entomologyjournals.com
Volume 3; Issue 6; November 2018; Page No. 23-28

Route of entry and mode of action of protein in the larvae and pupae of mosquitoes exposed to the toxin of *Pseudomonas fluorescens* Migula

Usharani Brammacharry
Department of Genetics, Dr. A.L.M Post Graduate Institute of Basic Medical Science, University of Madras, Tamil Nadu, India

Abstract
The aim of the study is to find out the route of entry and mode of action of protein in the larvae and pupae of mosquitoes exposed to the toxins. Laboratory bioassay of the crude proteins was also carried against 4th instar larvae and pupae of *A. stephensi Liston*, *Cx. quinquefasciatus Say* and *Ae. aegypti* (L) and in vivo-binding of FITC conjugated mosquitocidal protein into the larvae and pupae of *Cx. quinquefasciatus* to study the route of entry. Among the three species of mosquitoes, the larvae and pupae of *An. stephensi* were more susceptible to the crude protein than those of *Cx. quinquefasciatus or Ae. aegypti.* Probit regression analysis was carried out to calculate the LC_{50} values for the larvae of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* of three mosquitoes species were 1.6, 2.29, and 4.9 and LC_{90} values for the larvae of three mosquitoes species were 7.0, 7.7 and 14.35 respectively. The LC_{50} and LC_{90} values for the pupae of three mosquito's species were 0.18, 0.33, 0.93 and 0.53, 0.83, 2.8 respectively. The LC_{50} and LC_{90} values for the crude and pure protein against the pupae of *Cx. quinquefasciatus* were 0.33, 0.031 and 0.85 and 0.086 respectively. The FITC conjugated mosquitocidal protein was found binding to the head, thorax and midgut regions of larvae and pupae of *Cx. quinquefasciatus.* The present study revealed that the chitin is the target substrate for the mosquitocidal toxin and leads to degradation of peritrophic membrane of the mosquito species and thereby supports the proposed mode of action for mosquitocidal metabolites. This study provides the first evidence with a novel concept that the mosquitocidal metabolite hydrolysis various substrates there by degrading the cuticular region of the mosquito.

Keywords: *Pseudomonas fluorescens, Bacillus thuringiensis, Cx. quinquefasciatus, An. stephensi, Ae. aegypti*

Introduction
Control of mosquitoes is important to prevent transmission of vector borne diseases like malaria, dengue, chikungunya, Japanese encephalitis and filarisis. Intensive use of chemical insecticides led to the development of resistant vector populations, resulting in reduced control, and often to a negative impact on various non-target organisms and on the environment in general. Therefore, alternative control measures including microbial control were developed (Charles, *et al*., 2000) [2]. Among various pathogens and parasites of mosquitoes, the bacterial agents such as *Bacillus thuringiensis* and *Bacillus sphaericus* producing mosquitocidal crystal toxins have potential to be used as biocontrol agents (Hougard, *et al*., 1993) [6]. They produce mosquitocidal protoxin crystals during sporulation and are highly toxic to susceptible species of mosquito larvae ingesting them in the aquatic environment. Protoxins get solubilized in the alkaline pH of the larval midgut and are proteolytically activated and bind to specific receptors located in the brush border epithelial cell membranes. The cells are lysed by several mechanisms, and the larva stops feeding and dies. *B. sphaericus* and *B. thuringiensis* have demonstrated to be very effective against various species of *Culex, Anopheles* and *Aedes* mosquitoes and are very safe to non-target organisms. *B. sphaericus* has been used successfully for the control of *Culex quinquefasciatus* and *Culex pipiens* (Yap, 1990; Hougard, *et al*., 1993) [18, 6] whereas *B. thuringiensis*, although useful against *Culex sp.* mosquitoes, is the strain of choice for controlling *Aedes aegypti* (Yamamoto, *et al*., 1983) [17]. Both *B. sphaericus* and *B. thuringiensis* show toxicity to *Anopheles* and their effect proved to be variable in the field (Mulla, 1990). The mode of action of *B. thuringiensis* and *B. sphaericus* on mosquito larvae is well known.

A novel natural strain of *Pseudomonas fluorescens* Migula obtained from a soil sample collected from Pichavaram Mangrove Forest at Chidambaram, India was used in the present study. The active principles present in the culture supernatant of *P. fluorescens* Migula was purified (Prabakaran, *et al*., 2002) [14] and a formulation prepared from the metabolites was reported to be toxic to larval as well as pupal stages of mosquitoes (Padmanabhan, *et al*., 2005) [13]. However, characterization of the active principles, with respect to their functional entities is yet to be done. Also, their mode of action on the target mosquito is to be studied, especially when the pupae, the non-feeding stage of mosquitoes, is susceptible to it. As the pupae are killed, the effect may not be entirely due to ingestion of toxins and the mode of action is possibly through contact poisoning. Among the *Pseudomonades*, only *Pseudomonas aeruginosa* has been studied in detail and found to produce different degradative ezymes, especially with endochitinase activity (Nielsen, *et al*., 1999) [11]. The proteases produced by this bacterium include thermolysin-like zinc-metalloproteases produced by both Gram-positive and Gram negative bacteria (Kevin, *et al*., 2004) [8]. The metalloproteases are involved in a variety of physiological processes and also have the property of

pathological tissue destruction. Degradation of host connective tissues has also been associated with zinc metalloprotease activity of *P. aeruginosa* (Olson, 1992) [12]. The present investigation aims at finding out the route of entry and mode of action of protein(s) in the larvae and pupae of mosquitoes exposed to the toxins. Knowledge on this has a significant role in the design and development of formulations based on the bacterial metabolites to control larvae and pupae of mosquitoes.

Materials and Methods

Production of mosquitocidal toxin from *P. fluorescens* Migula

Seed culture of the bacterium, *P. fluorescens* Migula, was grown in glucose peptone salt (GPS) medium containing 1.0 % (w/v) glucose and peptone, and 0.1 M potassium dihydrogen phosphate (pH 7.0). GPS medium (600 ml) in 1 litre conical flask was inoculated with 6 ml of the seed culture and incubated at 37° C and 500 rpm for 96 h. Production of extra cellular protein in the culture supernatant was checked at different stages from 24 h to 96 h of growth of the bacterium. It was done through monitoring the level of mosquitocidal activity of the proteins at 24, 48, 72 and 96 h of incubation. The 72 h culture was centrifuged at 8,000 rpm for 30 minutes at 4° C and the supernatant was collected for precipitation and purification of the extra cellular proteins (Prabakaran, *et al*., 2002; Usharani, *et al*., 2012) [12, 15].

Precipitation of the bacterial proteins

Proteins present in the culture supernatant of *P. fluorescens* Migula were precipitated with ammonium sulphate. The volume of saturated ammonium sulphate solution required was calculated at each stage to achieve a given percentage saturation of the crude extra cellular bacterial proteins. The ice-cold saturated solution of ammonium sulphate was added slowly to the protein solution to get 30 – 80% of saturation, in an ice bath, and stirred continuously. The precipitate of soluble proteins was collected after centrifugation at 10000 rpm for 20 minutes at 4°C. The precipitate was dissolved in fresh PBS (50 mM, pH 7.0) buffer and assayed for the total protein content.

The dissolved precipitate was dialyzed against PBS, pH 7.0 at 4° C for 24 h. The dialaysate sample was lyophilized and subjected to SDS-PAGE to determine the molecular weight of toxins and further subjected to chroma to focusing on DEAE-ion exchange chromatography column equilibrated with 20mM Tris buffer pH 8.0. The mosquitocidal proteins were eluted with 20 mM sodium phosphate buffer. The active mosquitocidal fractions were pooled, concentrated and washed in buffer on centricon concentrators (Amicon). The protein concentration was estimated by a modification of the Lowery, *et al*. (1951) method using Bovine serum albumin as a standard.

Purification of mosquitocidal proteins

The culture supernatant precipitated with ammonium sulphate was dialyzed and fractionated by gel filtration using sephacryl S300 columns (Amersham-Pharmacia, Sweden) in an FPLC system. The column was first equilibrated using 50 mM PBS containing 0.1 M sodium chloride, pH 7.5 at a flow rate of 1 ml per minute. The loop was filled with the sample (50 µl/200 µl) and was eluted at a flow rate of 1 ml/ minute using PBS (0.05 M), containing sodium chloride buffer (0.1 M; pH 7.5), and monitored at 280 nm (Prabakaran, *et al*., 2002) [14]. Fractions of 1 ml each collected were assayed for mosquito pupicidal activity against *Cx. quinquefasciatus* by introducing five pupae to each of 1 ml fractions mixed with 4 ml of tap water as described below. The protein concentration was estimated by a modification of the Lowry, *et al*. (1951) method using bovine serum albumin as a standard.

Assessment of the mosquitocidal effect

Laboratory bioassay of the crude proteins was carried against 4th instar larvae and pupae of *Cx. quinquefasciatus*. Different doses of the protein, in terms of µg protein per ml were prepared by diluting the supernatant (0.9 g protein litre^{-1}) with sterile distilled water. These were added to disposable cups containing 100 ml of chlorine free tap water and 25 larvae or pupae were introduced into each cup. Boassay cups without the protein served as control. Larval food containing yeast and dog biscuit (1:4 by weight) was sterilized by autoclaving at 121° C for 15 min and added in fine powder form to the bioassay cups containing larvae, but not to those containing pupae. Larval or pupal mortality was scored after 24 h exposure and corrected for control mortality, if any, using Abbott's formula (Abbott, 1995) [1]. The experiment was repeated four times. Probit regression (Finney, 1971) [4] analysis was carried out to calculate LC_{50} and LC_{90} values as well as their 95% fiducial limits. Laboratory bioassay of the crude proteins was also carried against 4th instar larvae and pupae of *A. stephensi Liston, Cx. quinquefasciatus Say* and *Ae. aegypti* (L) and Probit regression analysis was carried out to calculate LC_{50} and LC_{90} values as well as their 95% fiducial limits.

In vivo-binding of FITC conjugated mosquitocidal protein into the larvae and pupae of *Cx. quinquefasciatus*

Purified protein (10 mg) was dissolved in 1 ml of 50 mM BupH Borate buffer (pH 8.5) and transferred to a reaction tube. The FITC labelling reagent was reconstituted by puncturing the foil and 100 µl of Dimethyl formamide (DMF) was added. The appropriate amount of FITC (16.9 µl) was added to the protein containing reaction tube. The contents were mixed well and incubated at room temperature for 1 h. The sample of contents was added to the Slide -A-Lyzer MINI Dialysis unit and placed in a flotation device for dialysis. The dialysis unit was floated in a container with at least 100 ml of PBS and covered with foil to protect the FITC from light. The container was placed on a stir plate and stirred at low speed for 1 h at room temperature. The sample was removed and added to the untreated and treated larvae, pupae of *Cx. quinquefasciatus*, washed three times with PBS (pH 7.2) and mounted in D.P.X and observed under fluorescens microscope (Christine, *et al*., 1999) [3].

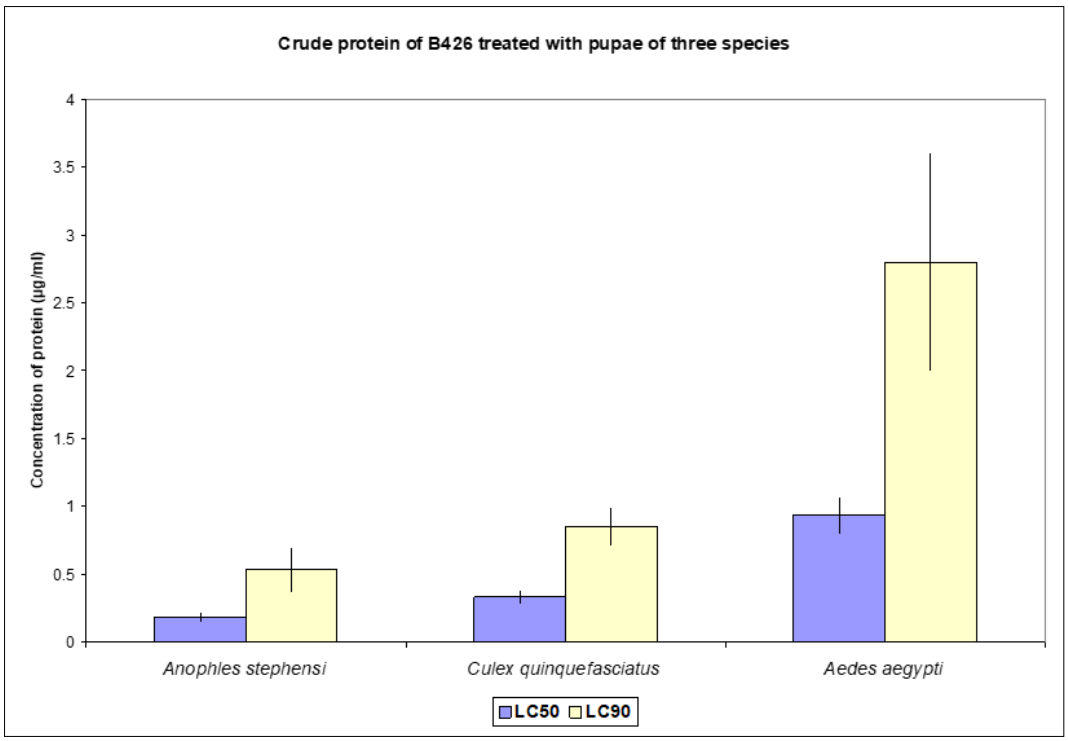

Fig 1: Bioassay of crude protein of *P. fluorescens* Migula strain treated with pupae of *A. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*.

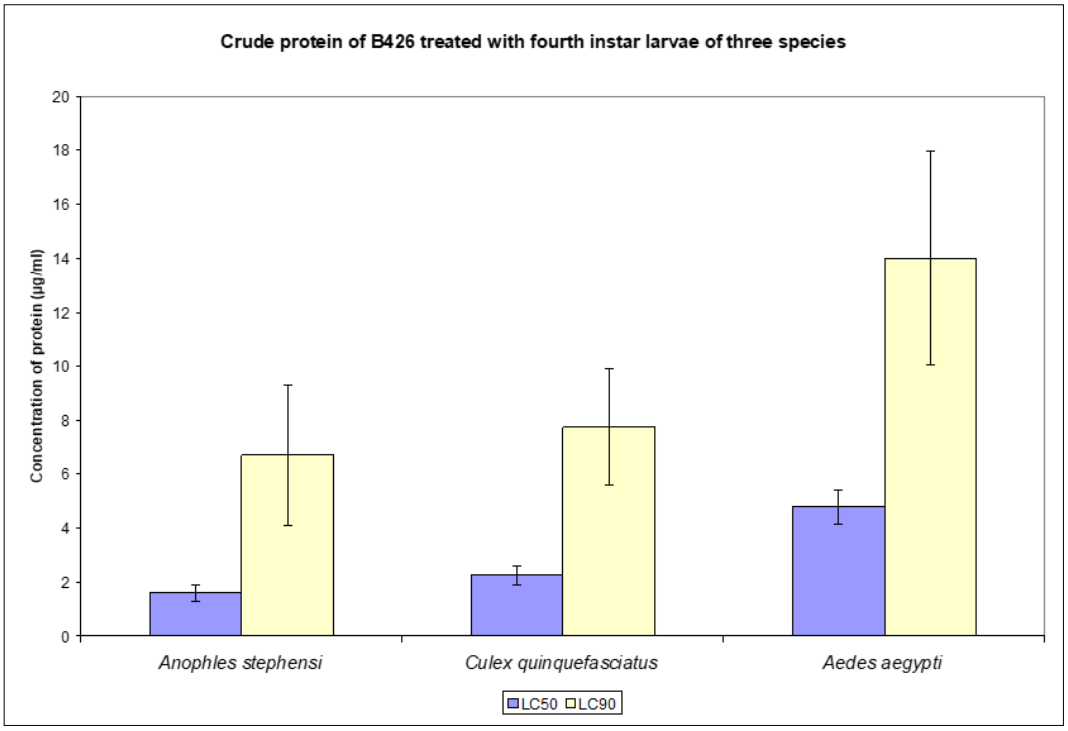

Fig 2: Bioassay of crude protein of B426 strain treated with 4th instar larvae of *A. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*.

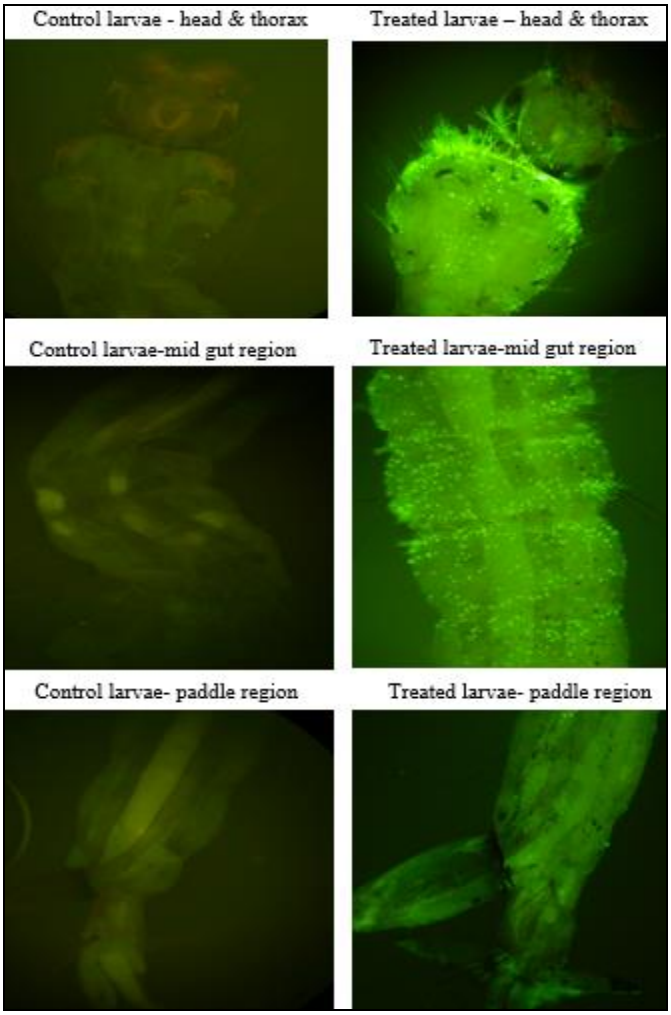

Fig 3: *In vivo*-binding of FITC conjugated mosquitocidal protein into the larvae of *Cx. quinquefasciatus*

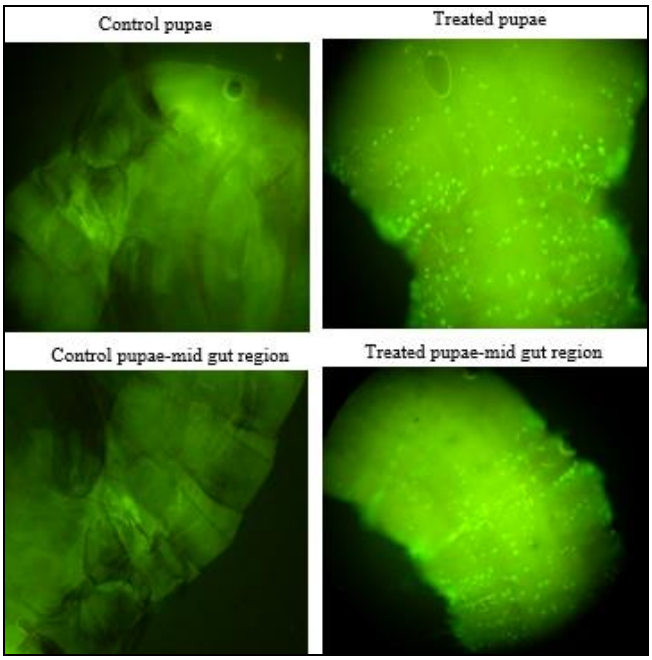

Fig 4: *In vivo*-binding of FITC conjugated mosquitocidal protein into the pupae of *Cx. quinquefasciatus*

Table 1: Toxicity of the culture supernatant of *P. fluorescens* Migula against 4th instar larvae Of *A. stephensi, Cx. quinquefasciatus* and *Ae. aegypti* mosquitoes

Mosquito species	Concentration of the lyophilized powder in µg/ml					
	LC50	LCL	UCL	LC90	LCL	UCL
An. stephensi	1.6	1.34	1.91	7.0	4.7	10.5
Cx. quinquefasciatus	2.29	1.9	2.6	7.7	5.8	10.15
Ae. aegypti	4.9	4.3	5.7	14.35	10.92	18.85

Table 2: Toxicity of the culture supernatant of *P. fluorescens* Migula against pupae of *A. stephensi, Cx. quinquefasciatus* and *Ae. aegypti* mosquitoes

Mosquito species	Concentration of the lyophilized powder in µg/ml					
	LC50	LCL	UCL	LC90	LCL	UCL
An. stephensi	0.18	0.15	0.212	0.53	0.39	0.7
Cx. quinquefasciatus	0.33	0.28	0.38	0.85	0.71	0.98
Ae. aegypti	0.93	0.81	1.07	2.8	2.1	3.7

Results and Discussion

The culture supernatant of *P. fluorescens* Migula was collected after 72 h of growth in GPS medium and the proteins were precipitated with 30 - 80% ammonium sulphate. The precipitated protein was dialyzed and fractionated by gel filtration using sephacryl S300 columns (Amersham-Pharmacia, Sweden) in an FPLC system. Laboratory bioassay of the crude protein was conducted against 4th-instar larvae and pupae of laboratory reared mosquito species such as *An. stephensi, Cx. quinquefasciatus* and *Ae. aegypti.* Among the three species of mosquitoes, the larvae and pupae of *An. stephensi* were more susceptible to the crude protein than those of *Cx. quinquefasciatus or Ae. aegypti* (Figure 1-2). Probit regression analysis was carried out to calculate the LC_{50} and LC_{90} values as well as their fiducial limits for the three species of mosquitoes (Table 1-2). In vivo bioassay was performed by introducing conjugated protein into larvae of *Cx. quinquefasciatus* to find out the route of entry of mosquitocidal proteins. The larvae and pupae were viewed under Fluorescens Microscope after the observation of mortality. The FITC conjugated mosquitocidal protein was found to be binding to the head, thorax and midgut regions of larvae and pupae of *Cx. Quinquefasciatus* (Figure 3-4).

Recently, a novel strain of *P. fluorescens* Migula obtained from a soil sample collected from Pondicherry, India was found to have mosquito larvicidal and pupicidal effects (Prabakaran, *et al*., 2002) [14]. A formulation has been developed from the metabolite(s) of this bacterium and tested against 4th instar larvae and pupae of three species of vector mosquitoes, *An. stephensi, Cx. quinquefasciatus* and *Ae. aegypti*. The formulation was found to be effective in killing both larvae and pupae of this mosquito species. It has been found to be more effective against pupal stages of the mosquitoes. However, there is no information on the identity and nature of the mosquitocidal toxins or on the mode of action on mosquitoes, especially when the pupae, the non-feeding stage of mosquitoes, are susceptible to it. Among the *Pseudomonades*, only *P. aeruginosa* has been studied in detail and found to produce different degradative ezymes, especially with endochitinase activity (Fonsfeldmann, *et al*., 1995) [5].

Proteases produced by this bacterium have been reported to be involved in a variety of pathophysiological processes like degradation of host connective tissues (Olson, 1992; Kevin, *et al*., 2004) [12, 8].

In the earlier study, the focus was on the endochitinase activity in the protein fraction, as this activity would be most relevant for the role of chitin lytic activity in biological control. Chitin lytic enzyme activities of mosquitocidal protein of *P. fluorescens* Migula was measured using either pNP-GlcNAc or pNP-(GlcNAc) $_2$ as substrates. Both have been used as typical substrates for the determination of endo- and exo-type of chitinolytic activities. This activity could be confirmed at 405 nm in spectrophotometer by measuring of p-nitrophenol released from the substrate by chitinolytic enzyme activities. Chitinolytic activities toward pNP-GlcNAc or pNP-$(GlcNAc)_2$ were proportional to the function of time. The specific chitinolytic enzyme activities of the mosquitocidal proteins was 1.82 ± 0.25 nmol/µg/min toward the substrate, pNP-$(GlcNAc)_2$, and it is in conformity with the already reported results (Usharani, *et al*., 2012**)** [16].

The substrate specificity results revealed that the mosquitocidal toxin has chitinolytic like activity and catalyse the progressive release of acetylchitobiose and N- acetyl glucosamine, and suggests that the primary function of the enzyme is the removal of diacetylchitobiose from the non-reducing end of chitin or chitodextrins. The chitinase activity was found to be enhanced in the treated pupae than in the control pupae and treated larvae. This revealed the invasion of mosquitocidal protein through the cuticular region of pupae enhance the chitinase activity in the treated pupae. Whereas in both treated and control larvae, there is no significant difference in the chitinase activity. The involvement of these chitinolytic activities during pathogenesis was investigated with *P. fluorescens* against larvae and pupae of *Cx. quinquefasciatus.* The most likely action of the endogenous chitinolytics of *P. fluorescens* is to weaken the insect's peritrophic membranes allowing more ready access of the bacterial toxins to the gut epithelia.

However, the results obtained in the earlier study clearly demonstrated that the mosquitocidal toxin of *P. fluorescens* Migula has chitinolytic activity and mediate the complete depolymerization of the chitin present in the cuticle. Insect chitin is found in the exoskeleton, respiratory tracheal system, and peritrophic matrix (PM) but is insulated from the hemolymph by epithelial layers. Chitin is of critical importance as it provides structural support for the peritrophic membrane. Therefore, temporal modulation of chitinase activity in the guts is important to maintain a functional PM (Zhicheng Shen, *et al*., 1997) [19]. The chitin can be a potential target substrate for intestinal pathogens, and it was demonstrated that degradation of chitin in the PM by a pathogen-encoded chitinolytic allowed an avian malaria parasite to overcome its mosquito vector intestinal PM barrier (Huber, *et al*., 1991) [7].

In the earlier study, the mosquitocidal protein exhibited a broad substrate activity towards various natural substrates such as azocasein, elastin, gelatine, collagen and fibrin. The proteins of *P. fluorescens* Migula showed gelatinase/collagenase, chitinase and fibrinolytic like activity (Usharani, *et al*., 2014) [15]. The integument of mosquitoes is composed of three layers, viz., the inner basement membrane, middle epidermis and the outer cuticle. The proteins of the cuticle are of three types, viz., arthropodin, resilin and sclerotin. The arthropodin resembles sericin or silk like gelatin in insects, which plays a significant role to protect from invading organisms. In the present study, attempts were made to find out whether the mosquitocidal proteins of *P. fluorescens* Migula hydrolysis the gelatine and fibrin rapidly. The mosquitocidal proteins showed fibrinolytic activity on fibrin plate (Usharani, *et al*., 2014) [15]. The most likely action of the endogenous chitinolytic activity of *P. fluorescens* Migula is to weaken the insect's peritrophic membranes allowing the more ready access of the bacterial toxins to the gut epithelia. The present study revealed that the chitin is the target substrate for the mosquitocidal toxin and leads to the degradation of peritrophic membrane of the mosquito species and thereby supports the proposed mode of action for mosquitocidal metabolites. This study provides the first evidence with the novel concept that the mosquitocidal metabolite hydrolysis various substrates there by degrading the cuticular region of the mosquito.

References

1. Abbott WS. A method of computing the effectiveness of an insecticide, J Econ Entomol. 1995; 18:265-267.
2. Charles JF, Christina NL. Mosquitocidal Bacterial Toxins: Diversity, Mode of Action and Resistance Phenomena, Mem Inst Oswaldo Cruz, Rio de Janeiro. 2000; 95:201-206.
3. Christine LW, Gina BM, Gary D, Simon N, Mark P, Alison AW. Fluorescent Labels Influence Phagocytosis of Bordetella pertussis by Human Neutrophils, Infect Immun. 1999; 67:4264.
4. Finney DJ. Probit analysis, 3rd edn, S Chand & Co Ltd, New Delhi, 1971, 50-80.
5. Fonsfeldmann Annette D, Cess W. Binding of the Cry IVD Toxin of *Bacillus thuringiensis* sub sp. *israelensis* to Larval Dipteran Midgut Protein, Appl Environ Microbiol. 1995, 2601-2605.
6. Hougard JM. Campaign against *Culex quinquefasciatus* using *Bacillus sphaericus*: results of pilot project in large urban of equatorial Africa, BuU WHO. 1993; 71:367-375.
7. Huber M, Cabib E, Miller LH. Proc Natl Acad Sci USA. 1991; 88:2807-2810.
8. Kevin SM, Efrat K, Dennis EO. Identification of residues in the *Pseudomonas aeruginosa* elastase propeptide required for chaperone and secretion activities, Microbiol. 2004; 150:3969-3977.
9. Lowry OH, Rosen rough NJ, Randall AJ. Protein measurement with the folin phenol reagent, J Biol Chem. 1951; 193:265-275.
10. Mulla M. In Bacterial Control of Mosquitoes and Black flies: Biochemistry, Genetics and Applications of *Bacillus thuringiensis* and *Bacillus sphaericus* (de Barjac, H. and Sutherland, D. eds Rutgers University Press), 1990, 134-160.
11. Nielsen TH, Christophersen C, Anthonin U, Sorensen J. Structure and production of a new bacterial cyclic depsipeptide; viscosinamide antagonistic against *Pythium ultimum* and *Rhizoctonia solani*. J. Appl. Microbiol, (in

press), 1999.
12. Olson JC, Ohman DE. Efficient production and processing of elastase and LasA by *Pseudomonas aeruginosa* require zinc and calcium ions, J Bacteriol. 1992; 174:4140-4147.
13. Padmanabhan V, Prabakaran G, Paily KP, Balaraman K. Toxicity of a mosquitocidal metabolite of *Pseudomonas fluorescens* on larvae & pupae of the house fly, *Musca domestica*, Ind J Med Res. 2005; 121:116-119.
14. Prabakaran G, Paily KP, Padmanabhan V, Hoti SL, Balaraman K. Isolation of a *Pseudomonas fluorescens* metabolite/exotoxin active against both larvae and pupae of vector mosquitoes, Pest Manag Sci. 2002; 59:21-24.
15. Usharani Brammacharry, Kummankottil Paily. Gelatinase activity of metabolites of *Pseudomonas fluorescens* Migula on larvae and pupae of Culex *quinquefasciatus* (Diptera: Culicidae), Int J Pharm Bio Sci. 2014; 5(3):234-245.
16. Usharani Brammacharry, Kummankottil Paily. Chitinase like activity of metabolites of *Pseudomonas fluorescens* Migula on immature stages of the mosquito, *Culex quinquefasciatus* (Diptera: Culicidae). Afr J Microbiol Res. 2012; 6(11):2718-2726.
17. Yamamoto T, Garcia JA, Dulmage HT. Immunological properties of the entomocidal proteins of *Bacillus thuringiensis* and its insecticidal activity. J lnvertebr Pathol. 1983; 41:122-130.
18. Yap HH. In Bacterial Control of Mosquitoes and Black flies: Biochemistry, Genetics and Applications of *Bacillus thuringiensis* and *Bacillus sphaericus* (de Barjac H. and Sutherland, D. eds), Rutgcrs University Press, 1990, 307-320.
19. Zhicheng S, Marcelo JL. Characterization of a Novel Gut-specific Chitinase Gene from the Human Malaria Vector Anopheles gambiae. J Biol Chem. 1997; 272:28895-28900.