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Full Length Research Paper

Chitinase like activity of metabolites of *Pseudomonas fluorescens* Migula on immature stages of the mosquito, *Culex quinquefasciatus* (Diptera: Culicidae)

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The present study describes the chitinolytic activity of extra cellular mosquitocidal proteins of *Pseudomonas fluorescens* and its involvement during pathogenesis in insects against larvae and pupae of *Culex quinquefasciatus.* The chitinolytic activity of purified extra cellular protein was assayed by using artificial colloidal chitin polymer substrates. The effect of purified protein in the alimentary canal and gut region of the treated larvae and pupae of *C. quinquefasciatus* was studied. The extra cellular protein of *P. fluorescens* was found to exhibit chitinolytic activity and degraded the cuticular proteins in the treated larvae and pupae of *C. quinquefasciatus.* The Km and Vmax values toward pNP-$(GlcNAC)_2$ were 0.03 mM and 0.219 nmol/µg/min respectively. This result suggests that chitinolytic activity of extra cellular mosquitocidal protein of *P. fluorescens* play a critical role in chitin catabolism and was to weaken the insect's chitinous structures/ peritrophic membranes. It leads to disruption of ionic regulation and death of the larvae or pupae treated with the proteins. It is concluded that chitinolytic activity is one of the reasons for the already reported mosquitocidal effect of the proteins of *P. fluorescens*.

Key words: *Pseudomonas fluorescens*, *Culex quinquefasciatus*, chitin, mosquitocidal protein, natural chitinase, larvae, pupae.

INTRODUCTION

Mosquitoes transmit a number of important diseases in the tropical and sub-tropical countries. Control of mosquitoes using chemical pesticides has its own problems such as the resistance development in the target species and environmental pollution. Attempts have been made elsewhere to overcome these problems and the result was the isolation and development of various parasites and pathogens with mosquito control potential. Two bacterial agents such as the *Bacillus thuringiensis* and *Bacillus sphaericus* are being widely used for control of mosquitoes breeding in a variety of habitats (Geetha and Manonmani 2010).These bio-control agents are targeted against larval stages of mosquitoes as they are ingested and act as stomach poison. However, there are some recent reports indicating development of resistance in mosquitoes against microbial agents too (Mir et al., 2003). These reports have prompted many workers to look for new microorganisms and/or their metabolites with mosquito control potential.

At the Vector Control Research Centre (VCRC), Puducherry, India, we have tested culture supernatant of an indigenous strain of *Pseudomonas fluorescens* Migula against larvae and pupae of vector mosquitoes. Though it showed lethal effect on mosquito larvae, it was more pronounced on pupal stages (Padmanabhan et al., 2005). The active principles or mosquitocidal toxins present in the culture supernatant could be a cocktail of many molecules and the most abundant of them were identified, through nano LC- MS/MS, as serralysins (VCRC unpublished data). As it was very effective in

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killing the non-feeding pupal stage of mosquitoes, the effect will not be due to ingestion of toxins and the mode of action is possibly through cuticular region. In order to investigate this, in the present study, attempts were made to elucidate its detrimental effect on the cuticle of the target mosquito stages.

Chitin, composed of N-acetylglucosamine monomers connected by glycoside β-1, 4 bonds is a main component of cuticle of insects, including mosquitoes (San-lang and Wen-tsu, 1997).Chitinolytic activities may play key roles in killing insects through contact poisoning with bacterial metabolites.

The involvement of these chitinolytic activities during pathogenesis in insects has been investigated with *B. thuringiensis* subsp.*israelensis* IPS78 against larvae of the midge *Culicoides nubeculosus,* and with *B. thuringiensis* subsp. *aizawai* HD133 against caterpillars of the cotton leaf worm *Spodoptera littoralis* (Mark et al., 1998). Among the *Pseudomonades,* only *Pseudomonas aeruginosa* has been studied in detail and found to contain two different isozymes (FI and FII) of endochitinase activity in a Shrimp and Crab Shell Powder Medium. *Pseudomonas stutzeri* and *P. fuorescens* have been found to produce chitinolytic activity in batch growth cultures, but the enzyme activity has not been characterised in detail (Mette and Jan, 1999).

MATERIALS AND METHODS

Bacterial culture and separation of culture supernatant

Seed culture of the bacterium, *P. fluorescens* Migula, was obtained from the culture collection of VCRC. It 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 72 h. The culture was centrifuged at 8,000 rpm (4°C) for 30 min and the supernatant was collected for bioassay as well as isolation and purification of mosquitocidal metabolites (Prabakaran et al., 2002).

Assessment of mosquitocidal effect of the culture supernatant

In order to find out the mosquitocidal effect of the culture supernatant (CS) of the bacterium, laboratory bioassay was carried out against 4th instar larvae and pupae of *Culex quinquefasciatus* obtained from a cyclic laboratory colony maintained at the VCRC. Different concentrations of the CS, in terms of μg protein per ml, were prepared by diluting the filtrate (0.9 g protein litre^{-1}) with sterile distilled water. These were added to disposable bioassay cups containing 100 ml of chlorine free tap water and 25 larvae or pupae was introduced. A bioassay cup without the CS was served as control. Larval food containing yeast and dog biscuit (1:4 by weight) was 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). The experiment was repeated four times. Probit regression (Finney, 1971) analysis was carried out to determine LC_{50} and LC_{90} values as well as their 95% fiducial limits.

Separation and purification of the mosquitocidal active principle(s)

Proteins present in the CS of the bacterium was precipitated with 30–80% saturation of ammonium sulphate, dialyzed and fractionated by gel filtration using sephacryl S300 columns in an AKTA FPLC system (Amersham-Pharmacia,). The column was first equilibrated using a 50 mM PBS buffer (pH 7.2) containing 0.1 M sodium chloride. The loop was filled with the sample (50 µl/200 µl) and eluted at a flow rate of 1 ml per minute using 50mM PBS (pH 7.2), containing 0.1 M sodium chloride and monitored at 280 nm (Prabakaran et al., 2002; Tronsmo and Andharman, 1993). Protein fractions of 1 ml each collected was 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. The mosquitocidal protein concentration was estimated by a modified method of Lowry et al. (1951) using bovine serum albumin as standard.

Preparative non-denaturing gel electrophoresis

The concentration of mosquitocidal protein sample should be ~1 mg protein per ml in sample buffer. The mosquitocidal protein sample was diluted with an equal volume of sample Buffer. The Molecular Mass Marker Solutions and the protein sample, 68 mg total protein in 9 ml of loading buffer was loaded on top of the 10% stacking gel and electrophoresed for 20 min at 15 mA, and then for an additional 3.5 h at a constant power of 30 mA using Bio-Rad's Model 3000/300 power supply at 4°C. The gel was removed from the cassette and Stained in the staining Solution for ~1 h. The gel was destained Brilliant Blue R in destaining solution by diffusion against several changes of destaining solution. The gel was transferred into 7% (v/v) Acetic Acid Solution for a minimum of 30 min to allow the gels to uniformly swell (Totowa, 2002).

Protein profiling through SDS-PAGE and electro-elution of mosquitocidal proteins:

The ammonium sulphate precipitated crude proteins was dialyzed against PBS buffer (pH 7.0) at 4°C for 24 h. The dialysate was lyophilized and subjected to SDS-PAGE for protein profiling. The sample was boiled with solubilising buffer for 5 min and subjected to electrophoresis on a 10% (w/v) acrylamide slab gel as per the method of Ames (Ames,1974) using Tris–glycine buffer (pH 8.5). The standard marker proteins used were Myosin (205 kDa), Phosphorylase (97 kDa), Bovine serum albumin (66 kDa), Ova albumin (43 kDa), Carbonic Anhydrase (29 kDa), Soya bean Trypsin inhibitor (20 kDa), Lysozyme (14 kDa), and Aprotinin (3 kDa) (Sigma Chemical Co, USA). Gel was stained with Coomassie Brilliant Blue R-250 (Sigma Chemical Co, USA).

Proteins of similar molecular weights were eluted separately with 20 mM sodium phosphate buffer using an electro eluter (Bio rad), pooled and concentrated on Amicon Centricon concentrator (Millipore).Protein concentration was estimated by the modified method of Lowery et al. (1951) using bovine serum albumin as a standard and bio-assayed against *C. quinquefasciatus* pupae, as described earlier, to find out the mosquitocidal protein(s) (Totowa, 2002).

Assay of chitinolytic activity of mosquitocidal proteins on natural substrate

To find out the general non-mosquito specific chitinolytic activity of the mosquitocidal proteins, assay was performed using colloidal

chitin from crab shell (Sigma Chemical Co, USA) as natural substrate. The reaction mixture (100 µL) containing 25 µL (0.95 mg/ml) of the mosquitocidal protein, 0.5 mM pNP-$(GlcNAc)_2$ in 25 mM Tris, 1 mM EDTA (TE) buffer, pH 8.0, was incubated at 37°C for 24 h. The reaction was quenched by adding 0.9 mL of 0.2 M sodium borate buffer (pH 10.5). One unit of chtinolytic activity was defined as the amount of 1 nmol pNP released per min.Chitinolytic activity was measured in dark room and under the light irradiation in the absence and in the presence of colloidal chitin, respectively (Sang-Mun et al., 2008).

Comparison of chitinolytic activity with a natural chitinase

Chitinolytic assay of the mosquitocidal proteins was performed in 96 well micro titre plate using three different substrates (1 mg/ml) such as 4-nitrophenyl N-acetyl-D-β glucosaminide, 4-nitrophenyl β-D-N, N, N, triacetyl chitotriose and 4-nitrophenyl N, N-diacetyl β-D-chitobioside. *Trichoderma viride* chitinolytic enzyme (1 mg) was used as a standard for comparison and blanks were prepared with substrates alone. Mosquitocidal proteins (10 µl of 0.95 mg/ml) as well as the standard chitinolytic enzyme were diluted to a concentration of 0.2 mg/ml with 5 ml of PBS and 10 µl of (0.2 mg/ml) chitinase control enzyme was added to each of 90 µl (1 mg/ml substrate solution) of substrate. After 30 min incubation at 37°C, the reaction was arrested with 200 µl of stop solution containing 20 mM sodium carbonate. Dilute 5 µl of the 10 mM *p*-Nitro phenol solution (Sigma Chemical Co, USA) with 995 µl of Stop Solution and used as standard. Yellow colour developed in the reaction mixture due to the enzymatic hydrolysis and liberation of p-nitro phenol was measured at 405 nm.One unit of enzyme was defined as the amount of enzyme able to liberate one micromole product (as p-nitro phenol equivalent) under the assay conditions described (Yanping et al., 2008).

Chitinolytic activity of mosquitocidal protein on homogenate of larvae and pupae of *Culex quinquefasciatus*

To determine the chitinolytic activity of mosquitocidal protein, the mosquitocidal protein (1 mg/ml) was treated with 4th instars larvae and pupae of *C. quinquefasciatus* and mortality was observed after 16 h. Untreated healthy larvae and pupae were kept in freezer for 30 min and used as control larvae and pupae .The alimentary canal and gut contents were collected from the treated, untreated larvae and pupae and homogenized in 50 mM citric acid sodium phosphate buffer (pH 5.0). The homogenates were centrifuged and the supernatant was mixed with ammonium sulfate to give 70% saturation. After centrifugation, the precipitate was dissolved in the same buffer and the solution thus obtained was stored at –80°C and used in the chitinolytic assay as the enzyme solution. Chitinolytic activity was determined by measuring the reducing end group N-acetylglucosamine produced from colloidal chitin. The reaction mixture consisting of 0.5 ml standard enzyme chitinolytic (1U/ml solution and 0.5 ml of 1% (w/v) colloidal chitin (pH 7.0) was incubated at 40°C for 2 h. The reaction was stopped by adding 1ml of 96 mM 3, 5 Dinitrosalicylic acid reagent and heating in boiling water for 5 min. The reaction was then cooled to room temperature, and centrifuged at 10,000 g for 10 min. The supernatant was subjected to spectrophotometer measurement at 530 nm (Fonsfeldmann et al., 1995).

Raising of polyclonal antibodies against the mosquitocidal proteins

In order to find out the binding of the mosquitocidal protein to the midgut region of larvae and pupae of *C. quinquefasciatus*, polyclonal antibodies were raised against the mosquitocidal protein by inoculating each of 200 µg protein subcutaneously to 6-8 weeks old male BALB/c mice as per standard procedures (Harlow and Lane, 1999). Blood from the immunized animals were collected after 20 days by tail vein puncture and serum was separated. The antibody titre was assessed by ELISA and it was used for Western blot with 1:100, 1:1000 dilutions respectively (Voller et al., 1980).

Preparation of midgut homogenate of treated larvae and pupae of *C. quinquefasciatus*

The mosquitocidal protein (1 mg/ml) was exposed to the early 4th instars larvae and pupae of *C. quinquefasciatus* and mortality was observed after 16 h .The 4th instars larvae and pupae of *C. quinquefasciatus* were placed in sterile petridish- the paddles were removed from the larvae, thorax and the last abdominal segment was pulled in the opposite direction with the help of dissection needle to get the midgut.The isolated midguts from the treated untreated larvae and pupae of *C. quinquefasciatus* were washed in 0.5 ml of PBS and resuspended in 0.2 ml of PBS. The guts were homogenized in ice-cold gut extracting (GET) buffer (10 mM Tris-HCl, pH 8.0, 1 mM EGTA, 300 mM mannitol, 1 mM PMSF). Brush-border membrane Vesicles of (BBMVs) of *C. quinquefasciatus* were prepared according to the method of Wolfersberger et al. (1987) with some modifications. An equal volume of ice-cold 24 mM MgCl2 was added to the gut homogenate and the mixture was kept on ice for 15 min. After centrifugation at 2,500 rpm in 4°C for 5 min, the supernatant was transferred to another centrifuge tube. The pellet was suspended in ice-cold GET buffer with 0.5 volume of the gut homogenate. The mosquitocidal protein concentration was estimated by a modification of the Lowry et al. (1951) method using bovine serum albumin as a standard.

Immunoblot detection of mosquitocidal protein in the gut of treated mosquito larvae and pupae

The midgut proteins of untreated larvae and pupae were suspended in 4x Lammeli sample loading buffer. The samples were boiled for 5 min and separated by SDS-PAGE in Tris-glycine buffer (192 mM glycine, 25 mM Tris-HCl, pH 8.3) were electrophoretically transferred onto a nitrocellulose membrane in the MiniTrans-Blot electrophoretic transfer cell (Bio-RAD) using transferring buffer (190 mM glycine, 20% methanol and 25 mM Tris-HCl, pH 8.3) at constant voltage for overnight (Towbin et al.,1979). The membranes were washed thrice in the phosphate buffered saline containing 0.05% Tween 20 and blocked with BSA for 2 h to block the non-specific binding sites. After blocking, the proteins immobilized on the membrane were probed with 1: 500 dilution of the polyclonal antibodies developed against the mosquitocidal protein for 5 h. The nitrocellulose membrane was washed thrice with PBS-0.05% Tween 20 and the immunocomplexes were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (whole molecule) as the secondary antibody for 2 h. Binding was visualized using TMB/H_2O_2 as the substrate.

RESULTS AND DISCUSSION

Protein profile of mosquitocidal protein

In order to study the chitinolytic activity of the protein fraction of the culture supernatant of *P. fluorescens*, it was grown in GPS medium. The protein was harvested from culture supernatant collected at 72 h, precipitated

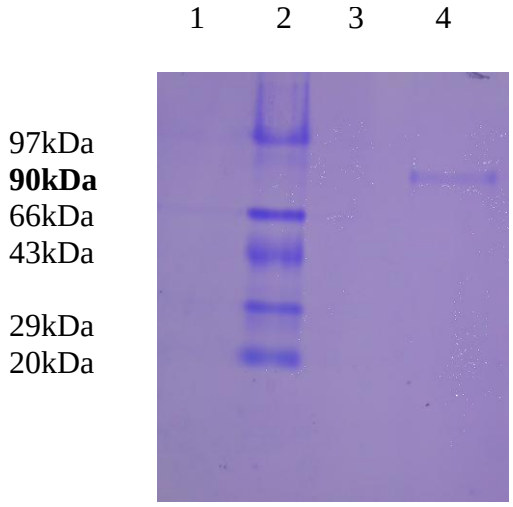

Figure 1a. Native PAGE of FPLC fractionated purified proteins of *P. fluorescens*.Lane 2: Protein Marker, Lane 4: 90 kDa Purified protein.

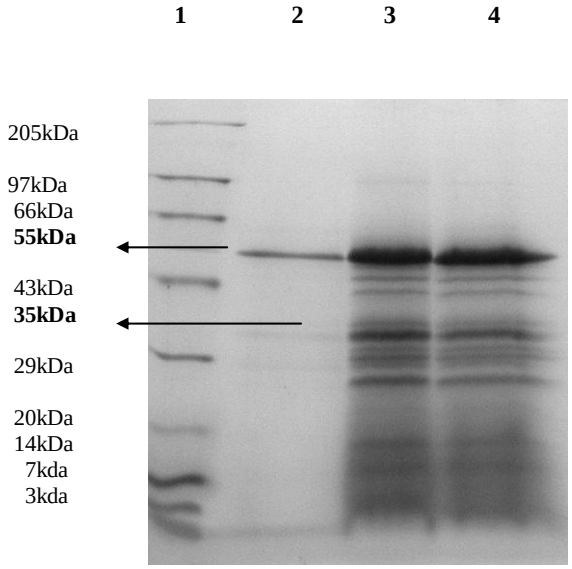

Figure 1b. SDS-PAGE of purified and crude proteins of *P. fluorescens*.Lane 1: Protein Marker, Lane 2: Purified proteins (55kDa & 35kDa) Lane 3 & 4 Crude proteins.

and dialyzed and fractionated by gel filtration using sephacryl S300 column in a FPLC system. Pure protein was subjected to 10% Native page and single band was observed with a molecular weight of 90 kDa (Figure 1a). Thus the FPLC fractionated pure protein was found to be a monomer. The LC_{50} value of pure protein was found to be 0.03 µg ml^{-1} for both pre and post treatment.

The molecular weight of mosquitocidal proteins were calculated on the basis of semi logarithmic plots of the mobility of the bands on SDS- PAGE, using a standard curve established with proteins of known molecular weight and the molecular weights of the two forms were estimated to be 55,000 and 35,000 (Figure 1b) respectively. The protein fractions of 55 and 35 kDa showed pupicidal activity on bioassay against the pupae of *C. quinquefasciatus* and hence they were considered as the mosquitocidal proteins. The protein fractions of 1 ml each collected was assayed for mosquito pupicidal and larvicidal activities against *C. quinquefasciatus* by introducing five pupae/larvae of each 1 ml fractions mixed up with 4 ml of tap water. The LC_{50} and LC_{90} values of the 4th instar larvae of *C. quinquefasciatus* was found to be 2.29, 7.79 µg ml^{-1} and The LC_{50} and LC_{90} values of the pupae of *C. quinquefasciatus* was found to be 0.33, 0.85 µg ml^{-1} (Table 1).

Chitinolytic activity of mosquitocidal proteins on natural substrate

Chitinolytic activities towards pNP-$(GlcNAc)_2$ are proportional to the function of time and also to the amount of enzyme (Figure 2a and b) The kinetic parameters (Km and Vmax) of pNP-$(GlcNAc)_2$ were calculated using Graph pad prism 5 software and were found to be pNP-$(GlcNAc)_2$, Km =0.03 mM, Vmax = 0.219 nmol/min/µg, respectively. The specific chitinolytic enzyme activity of mosquitocidal protein was shown in 0.82 ± 0.25 nmol/ug/min toward the substrate pNP-$(GlcNAc)_2$, which was obtained as an average from triplet experiments performed at RT (Figure 3). The P value of pNP-$(GlcNAc)_2$ was calculated using Graph pad Prism 5 software. Since the P value of one way (ANOVA) analysis of variance is <0.0001 at 95% confidence intervals, the results of specific activity of chitinase test is statistically significant. It is supposed to that chitin oligomers can slowly be utilized as a carbon source after hydrolyzing chitin material by mosquitocidal protein when it turned to heterotrophic condition from photosynthetic condition.

Comparison of chitinolytic activity with a natural chitinase

The *in-vitro* chitinolytic activity against various natural chitinase substrates such as Nitro phenyl N-acetyl-β-D-glucosaminide,4-Nitrophenyl N-acetyl-β-D-N,N,N, triacetylchitotriose and 4-Nitrophenyl N,N-diacetyl-β- D-chitobioside were studied (Figure 4a and b). The chitinolytic enzyme activity of mosquitocidal protein towards the Nitro phenyl N-acetyl-β-D-glucosaminide,4-Nitrophenyl N-acetyl-β-D-N,N,N,triacetylchitotriose and 4-Nitrophenyl N,N-diacetyl-β- D-chitobioside were 1.58 ± 0.15,1.12 ± 0.05, and 0.61 ± 0.03 nmol/ug/min respectively. The P value of pNP-$(GlcNAc)_2$ was calculated using Graph pad Prism 5 software. Since the average P value of one way (ANOVA) analysis of variance is <0.0001 at 95% confidence intervals, the

Table 1. Toxicity of the culture filtrates of *P. fluorescens* against 4th instar larvae and pupae of *Culex quinquefasciatus*.

Mosquito species	**Concentration of the lyophilized powder in µg/ml**					
C. quinquefasciatus	**LC_{50}**	**LCL**	**UCL**	**LC_{90}**	**LCL**	**UCL**
4th instar Larvae	2.29	1.9	2.6	7.7	5.8	10.15
Pupae	0.33	0.28	0.38	0.85	0.71	0.98

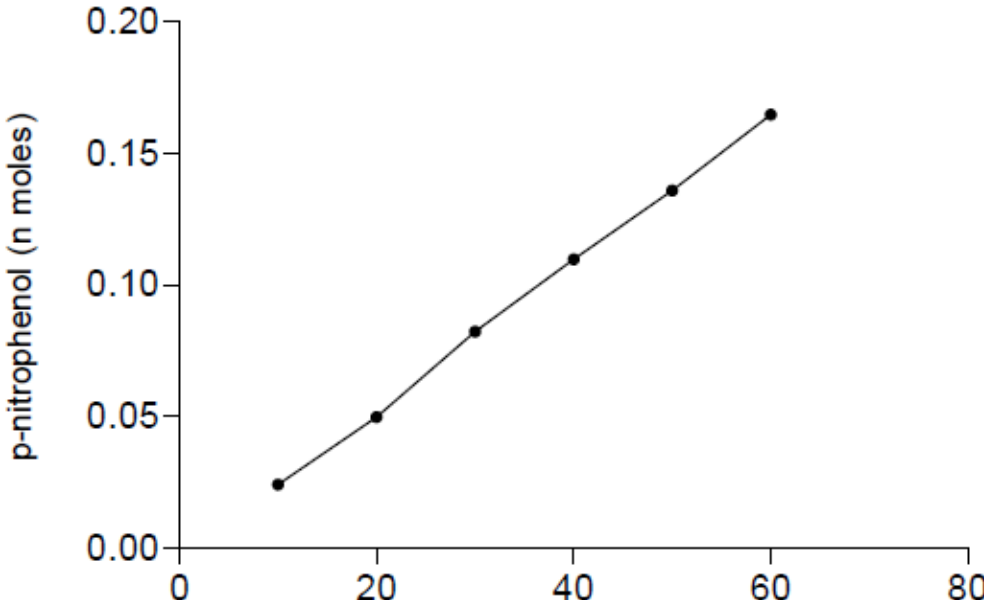

Figure 2a. Chitinase activity toward pNP-$(GlcNA)_2$ was measured in terms of time(min).

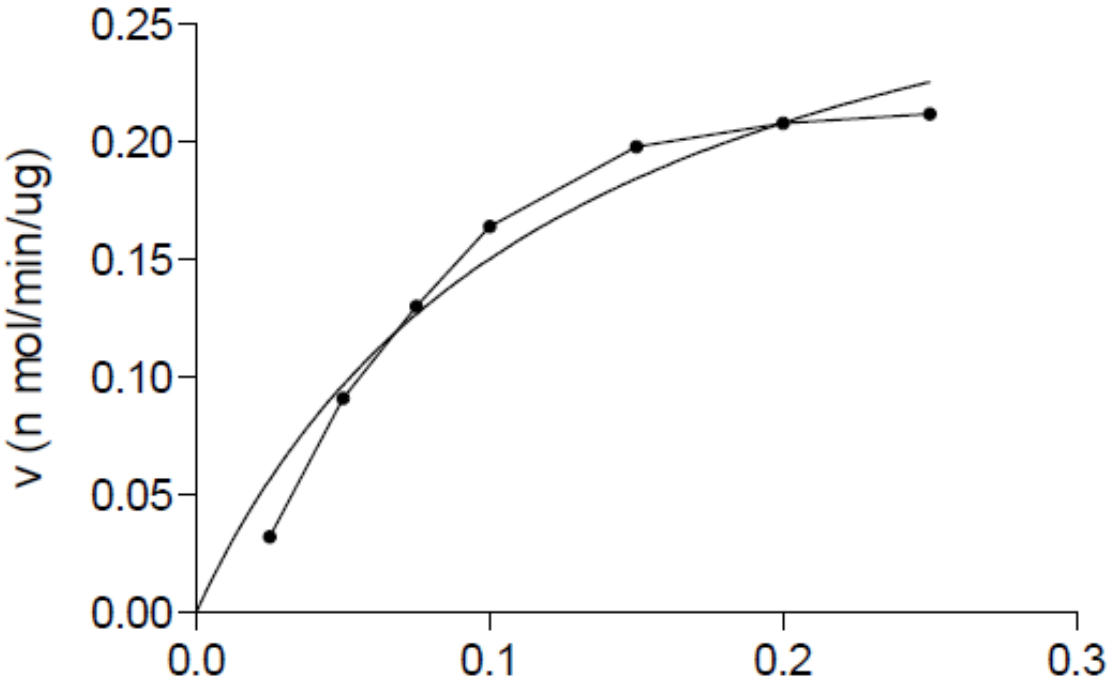

Figure 2b. Rate of p-nitro phenol formation is plotted versus pNP-$(GlcNAc)_2$ (mM substrate).

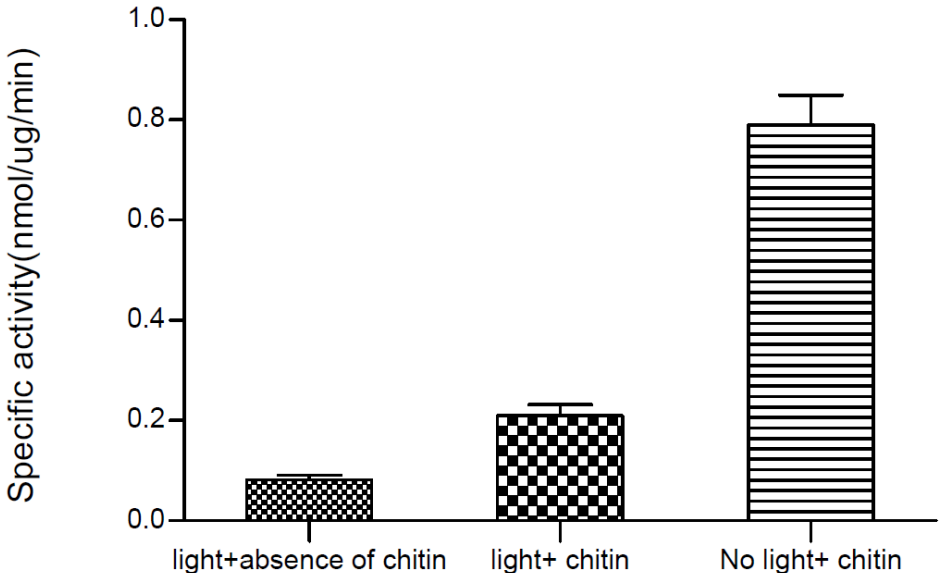

Figure 3. Specific activity of chitinase was represented as nmol/ug/min.

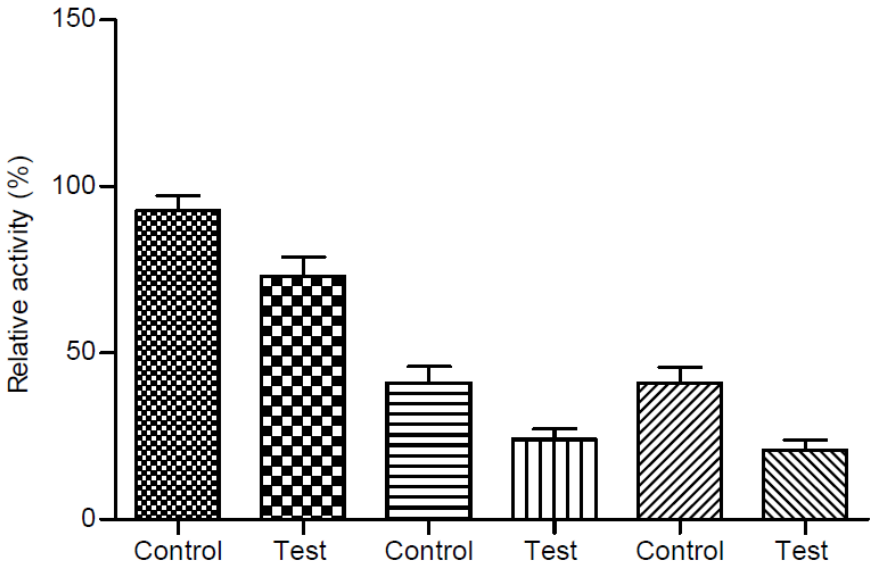

Figure 4a. *In vitro* Chitinolytic Activity of mosquitocidal protein and control chitinase on different synthetic substrates (4-Nitrophenyl N-acetyl-D-glucosaminide, 4-Nitrophenyl N-acetyl-D-N,N,N, triacetylchitotriose and 4-Nitrophenyl N,N-diacetyl- D-chitobioside) (mM).

results of in- vitro chitinolytic activity of protein on different synthetic substrate against control enzyme(mM) test is statistically significant. The highest activity was against 4-methylumbelliferyl-b-D-*N, N*-diacetylchitobioside, which suggests that the primary function of the enzyme is the removal of diacetylchitobiose from the nonreducing end of chitin or chitodextrins.

Chitinolytic activity of mosquitocidal protein on homogenate of larvae and pupae of *Cx. quinquefasciatus*

The *in vivo* chitinolytic activity was studied in the treated larvae and pupae of *Cx. quinque*fasciatus against the control larvae and pupae (Figure 5).The chitinase activity was found to be enhanced in the treated pupae than the

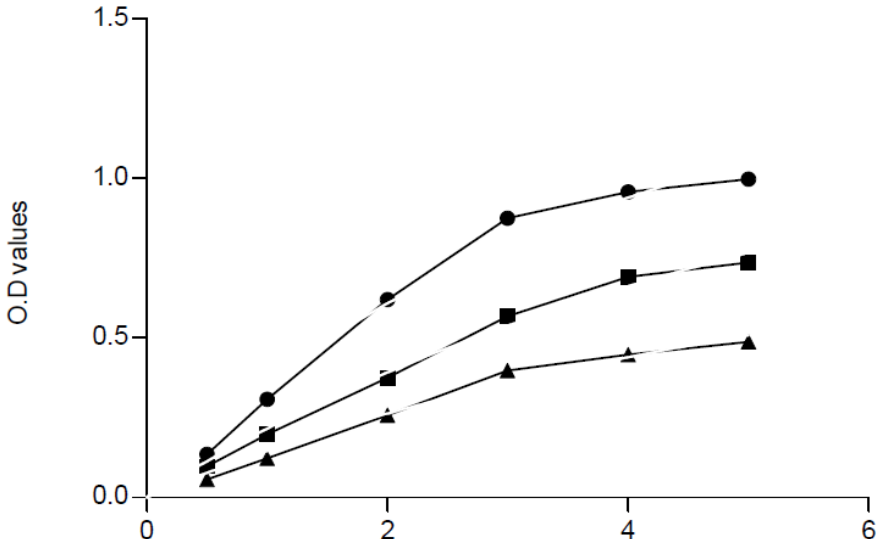

Figure 4b. Kinetic assay of Chitinolytic activity of mosquitocidal protein on different synthetic substrates (●4-Nitrophenyl N-acetyl-D-glucosaminide, ■4-Nitrophenyl N-acetyl-D-N, N, N, triacetylchitotriose and ▲4-Nitrophenyl N, N-diacetyl- D-chitobioside-mM).

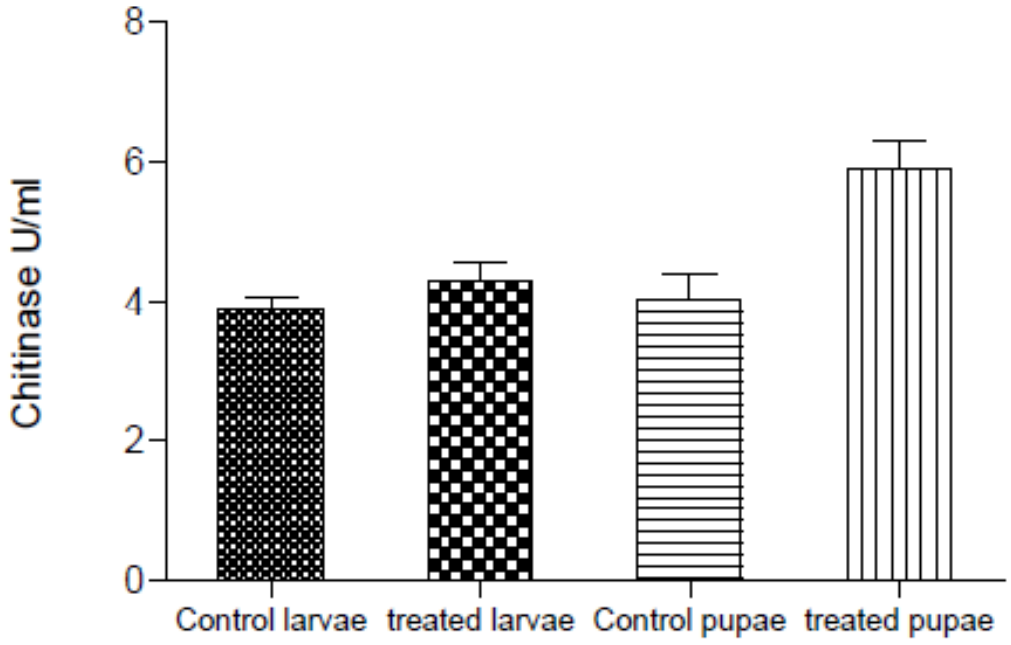

Figure 5. Chitinase assay in the larvae and pupae of *C. quinuefasciatus*.

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 as the route of mosquitocidal protein is through respiratory tract.

Immunoblot detection of mosquitocidal protein in the gut of treated mosquito larvae and pupae

The midgut protein profile of mosquitocidal protein treated larvae and pupae of *C. quinquefascitus* are shown in Figure 6. The 55KDa and 35KDa mosquitocidal proteins bands were observed in treated larvae and

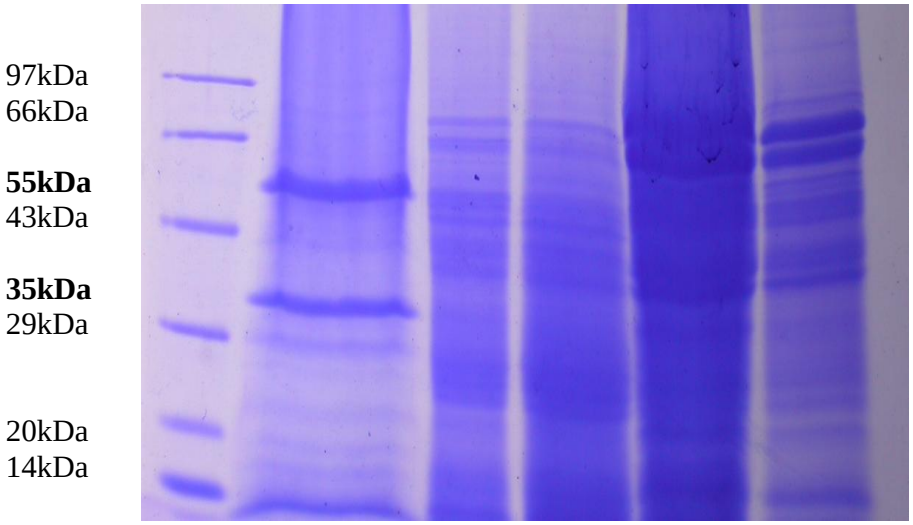

Figure 6. Protein profile of toxin treated guts of larvae and pupae .L:1 Marker, L: 2 Toxin, L: 3 Control larvae, L: 4 Treated larvae, L: 5 Control pupae, L: 6 Treated pupae.

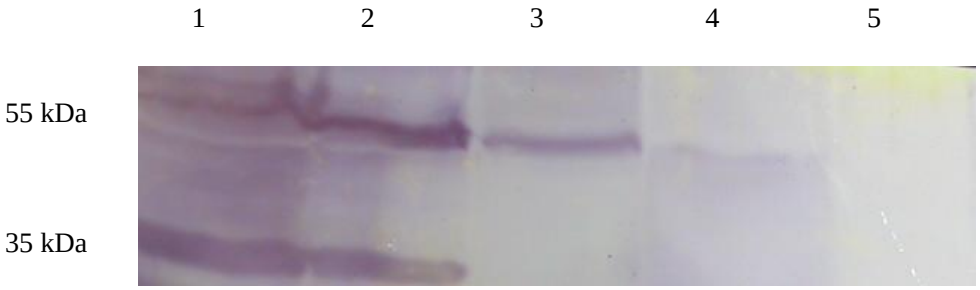

Figure 7. Western blot analysis of B 426 metabolite(s) to the gut of larvae and pupae. L 1: Toxin, L 2: Treated larvae, L 3 Treated pupae:, L 4: Control larvae, L 5: Control pupae.

pupae of *C. quinquefascitus* and it revealed that the binding of protein in midgut regions as there is no such mosquitocidal protein bands were observed in the control larvae and pupae of *C.quinquefascitus*.Immuno-staining was observed at two molecular weight levels of ~ 55 and 35 kDa in the treated larvae and pupae and this revealed that the binding of the bacterial protein in midgut regions and there was no such protein bands in the control larvae and pupae (Figure 7).

The chitinolytic activity of *P. fluorescens* mediates the complete depolymerization of the chitin present in the cuticle. It has been reported that the hyperthermophilic archaeon *Pyrococcus furiosus* contains a putative signal peptide; as well flanking a chitin-binding domain plays an important role in the degradation of chitin (Chernin et al., 1998).The results of the western blotting and immuno detection analysis revealed that the mosquitocidal toxin degrades the peritrophic membrane, which contains the chitin-binding domain and binds to the midgut epithelium of the mosquito species of *C. quinquefasciatus.* It has been reported that in insects PMs shield the midgut epithelial surfaces and can provide some level of protection from microbial invasion and infection (Gorman et al., 2000).

Insect chitin is found in the exoskeleton, respiratory tracheal system, and peritrophic matrix but is insulated from the hemolymph by epithelial layers. Exposure of chitin to the hemolymph may signal unscheduled mechanical disruption of protective barriers during injury and pathogen intrusion or extensive tissue remodelling during development. Direct contact of the hemolymph with chitin has been reported in the pupae, because of degradation of the tracheal epithelium and the larval hypodermis (Alberto et al., 2000). 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. Alberto et al. (2000) has reported that the primary components of PMs include chitin, protein and glycoprotein, but only one PM protein has been isolated and characterized thus far. The most likely action of the endogenous chitinolytic activity of *Pseudomonas fluorecens* B426 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. Thus, the present investigation confirmed that the mosquitocidal toxin efficiently hydrolysis the cuticular protein chitin as well as the peritrophic membrane which is a protective sleeve for the midgut epithelium of mosquito species and binds to the gut regions of larvae and pupae of *C. quinquefasciatus*. However, in the larvae of *C.quinquefascistus*, the mosquitocidal toxin is ingested orally and binds to the midgut epithelium causes the swelling of mitochondrial, endoplasmic reticulum and enlargement of vacuoles, followed by lysis of epithelial cells, midgut perforation and the death of larvae.

These findings together with the data here have strengthened the notion of a novel concept that the mosquitocidal toxin of *P. fluorescens* plays a significant role in the degradation of cuticular regions and peritrophic membrane and binds to the midgut epithelium of larvae and pupae of mosquito species of *C. quinquefasciatus.* Further studies on the specific recognition sites and the cleavage of peritrophic membrane will be necessary for a comprehensive understanding to explore the binding of mosquitocidal toxin to the midgut regions of larvae and pupae of mosquito species of *C. quinquefasciatus* by in vitro binding assays via immunohistochemical localization studies.

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