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# GELATINASE ACTIVITY OF METABOLITES OF *PSEUDOMONAS FLUORESCENS* MIGULA ON LARVAE AND PUPAE OF CULEX *QUINQUEFASCIATUS* (DIPTERA: CULICIDAE)

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## ABSTRACT

To determine the effect of gelatinase activity of mosquitocidal protein of *P. fluorescens* Migula strain (VCRC-B426) on larvae and pupae of *Culex quinquefasciatus* was studied. The results of Enzchek Gelatinase/Collagenase assay revealed that the mosquitocidal metabolite exhibited the gelatinase/collagenase activity and efficiently digested the fluorescein conjugate-DQ gelatin to yield highly fluorescent peptides. The arbitrary units of fluorescence emission at various concentration of substrate and various concentration of enzyme are measured. The increases in fluorescence are proportional to proteolytic activity and can be monitored with SYBER green end point assay. The $V_{max}$ and $K_m$ of Michaelis-Menten equation analysis of kinetics assay of the gelatinase activity of protein (mg/ml) at 95% confidence intervals are 18.01 and 3.335 respectively. The gelatinase activity of mosquitocidal protein was studied with increasing the DQ gelatin concentration. The $V_{max}$ and $K_m$ of Michaelis-Menten equation analysis of gelatinase assay of different concentration of DQ gelatin substrate (ug/ml) at 95% confidence intervals are 15.82 and 4.907 respectively. In this study, the gelatinase activity of mosquitocidal protein of *P. fluorescens* is likely to play important roles in the degradation of abendunt gelatine molecule in cuticular regions and peritrophic membrane of larvae and pupae of *Cx.quinquefasciatus*.

**KEY WORDS**: ***Pseudomonas fluorescens*, *Culex quinquefasciatus*, Gelatin, Gelatinase, Mosquitocidal protein, Larvae, Pupae.**

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# INTRODUCTION

Mosquitoes are important vectors of many arbo-viruses, protozoa and helminths. They can transmit many diseases, including dengue fever, malaria and yellow fever[1,2] . It is known that *Aedes albopictus* and *Culex quinquefasciatus* are the ubiquitous mosquitoes in the world. The Asian tiger mosquito, *A. albopictus*, is an invasive mosquito of considerable medical concern and has been implicated as a major vector of dengue fever[3,4] , while *C. quinquefasciatus* is a vector for Bancroftian filariasis in China [5,6,7]. The latter is also an important vector of human pathogens, such as eastern equine encephalitis, Japanese encephalitis and St.Louis encephalitis[8,9]. Management of these species, therefore, is essential for the control of these mosquito-borne diseases. The intensive use of chemical insecticides leads to the development of resistant insect populations, resulting in a reduced control and often in a negative impact on various non-target organisms and on the environment in general. Therefore, alternative control measures, including microbial control have been developed. Particularly, naturally occurring bacteria are successfully used against the larval stages of mosquitoes and black flies[10]. Biological control of mosquito larvae and pupae with naturally occurring bacteria that synthesize potent mosquitocidal toxins[11,12,13] has received much less attention, despite the fact that these bacteria have been used safely in the field for many years[14]. The mosquitocidal bacteria (eg. *Bacillus sphaericus* and *B. thuringiensis subsp. Israelensis)* among the many pathogens and parasites of vector mosquitoes. Certain strains of *B. sphaericus* and *B. thuringiensis* produce mosquitocidal protoxin crystals during sporulation. These crystals deposited inclusions alongside the spore and highly toxic to susceptible species which ingest the spores food. The protoxins solubilized in the alkaline pH of the larval midgut, where they proteolytically activated and bind to specific receptors located in the brush border epithelial cell membranes. The cells lysed by of several mechanisms, and the larva stops feeding and dies. *Bacillus sphaericus* and *B. thuringiensis* among bacteria known to produce mosquitocidal toxins, and laboratory tests of certain high toxicity strains of these bacilli against various species of *Culex, Anopheles* and *Aedes* larvae have demonstrated their safety and potential for controlling mosquitoes. *Bacillus sphaericus* has been used successfully for the control of *C.quinquefasciatus* and *Culex pipiens*[15,16] whereas *B. thuringiensis*, although useful against *Culex sp.* mosquitoes, is the strain of choice for controlling *A.aegypti*[17]. Both *B. sphaericus* and *B. thuringiensis* show toxicity to *Anopheles,* but the effect is marginal and has proved variable in the field. In general, operational with these bacilli in controlling mosquito populations has been confined mainly to temperate regions of the world where these insects merely nuisance[18]. The number and diversity of mosquitocidal toxins and the bacteria that produce them have increased gradually since their discovery, and this increase is accelerating. The toxins appear to vary in their species specificity and mode of action, making it likely that particular combinations cloned in recombinant microorganisms be chosen to enlarge the insect host range and delay prevent the development of resistance. A novel natural strain of *P.fluorescens* obtained from a soil sample collected from Pondicherry, India was used in the present study. The active principle present in the culture supernatant of *P.fluorescens* was purified and characterized already[19]. The formulation (VCRC B426) prepared from the exotoxin of *P.fluorescens* was reported to be toxic to larval as well as pupal stages of mosquitoes[20]. In this study, the gelatinase activity of mosquitocidal protein of *P. fluorescens* was studied as it has play important roles in the degradation of abundant gelatine molecule in cuticular regions and peritrophic membrane of larvae and pupae of *Cx.quinquefasciatus*.

# METHODOLOGY

### *Mosquitocidal toxin production from P.fluorescens Migula strain (VCRC-B426).*

The *P.fluorecens* Migula was grown in the GPS liquid medium containing 1.0% (wt/vol) Glucose, 1.0% (wt/vol) Peptone, 0.1M Potassium dihydrogen Phosphate, pH 7.0. 600 ml of medium was inoculated with 6 ml of seed culture and the activity of extra cellular protein production was checked in the culture supernatant at different stages from 24 hrs to 96 hrs of growth of *P.fluorecens* Migula. The 72 hrs culture was centrifuged at 8,000rpm for 30 minutes at 4°C and the supernatant was collected for the purification of the extra cellular proteins[21]. For maximum production of the mosquitocidal protein, the activities of extra cellular mosquitocidal protein production in the culture supernatant were checked at different stages of growth of *P.fluorecens* Migula. The ice-cold saturated solution of ammonium sulphate is added slowly to the protein solution, in an ice bath, and stirredcontinuously. The soluble proteins present in the supernatant were precipitated by addition of ammonium sulphate to 30 –80% of saturation. The precipitate was collected after centrifugation at 10000 rpm for 20minutes in 4°C. Each protein precipitate is dissolved individually in fresh PBS (50mM, pH 7.0) buffer and assayed for total protein. The protein concentration was estimated by a modification of the lowery et al (1951)[22] method using Bovine serum albumin as a standard.

### *Purification of Mosquitocidal protein*

The proteins present in the culture filtrate of the bacterium *P.fluorescens* Migula (VCRC B426) strain was precipitated with ammonium sulphate, dialyzed and fractionated by gel filtration using sephacryl S300 columns (Amersham-Pharmacia, Sweden) in an FPLC system. The column is first equilibrated using a 50mM PBS buffer containing 0.1M sodium chloride, pH 7.2 at a flow rate of 1 ml per minute. The loop is filled with sample (50ul/200ul) and it was eluted at a flow rate of 1 ml per minute. The protein fractions were eluted using PBS (0.05M), containing sodium chloride buffer (0.1M; pH 7.5) and monitored at 280nm[21]. The protein fractions of 1 ml each collected was assayed for mosquito pupicidal activity against *Cx. quinquefasciatus* by introducing five pupae of each 1 ml fractions, mixed with 4 ml of tap water as described above. The mosquitocidal protein concentration was estimated by a modification of the Lowry et al (1951)[22] method using bovine serum albumin as a standard.

### *Assay for gelatinase activity*

For screening of colonies, the production of gelatinase in *P.fluorescens* Migula strain (VCRC B426) was performed by using Nutrient (Himedia Laboratories) agar plates containing 3% gelatin (gelatin-TH)[23]. The clear opague zones around the *P.fluorescens culture after the overnight incubation at 37°C was considered the positive gelatinase activity.*

### *Enzyme assay on Gelatin plate*

The 1% gelatin was dissolved by heating at 56 (C and mixed with 100ml nutrient agar medium and the media was adjusted to pH 7.2. After autoclaving for 15 min, plates were poured and, after solidification, a hole was made using well cutter. 20µl of the sample solution (3 mg) was dropped into holes previously made on a gelatin agar plate using a capillary glass tube (5 mm diameter) and incubated at 37°C for 48 hours. An equal volume (3 mg) of standard gelatinase solution (1NIH unit/ml) was incubated into a hole on the gelatin agar plate as a standard protease. The gelatinolytic activity was appeared as a clear zone around the well[24].

### *Spectrophotometric assay of gelatinase activity*

Enzymatic release of azo dye from azocoll was performed to measure gelatinase activity by the method of Nakayama et al (2001)[25] with a slight modification. The different concentration of standard gelatine substrates was transferred into a 1.5ml Eppendorf tube. Tubes were incubated at 37°C for 15 min on a shaker, and standard gelatinase solution (1 NIH unit/ml) and 25µl of mosquitocidal metabolite was added to each tube containing preincubated Azocoll. The mixture was incubated for 4 hours at 37°C on a

shaker and then centrifuged at 1,500 x *g* for 5 min, followed by measurement of absorbance at 540 nm.

***Polyacrylamide-substrate gels***

Gelatin zymography was performed using a 5% polyacrylamide stacking gel and a 10% polyacrylamide resolving gel co-polymerised with 1 mg/ml gelatin (Sigma-Aldrich). Equal amounts of sample were mixed with sodium dodecyl sulphate (SDS) sample buffer [final concentration: 50 mM Tris-HCl pH 6.8, 1% (w/v) SDS, 0.025% (w/v) bromophenol blue, and 10% (v/v) glycerol] under non-reducing conditions and loaded onto the gel. After electrophoresis, the gels were washed in 50 mM Tris-HCl (pH 8.0), 5 mM CaCl2 and 2.5% (v/v) Triton X-100 over night and then incubated in 50 mM Tris-HCl (pH 7.5) and 5 mM $CaCl_2$ for 24 hours at 37°C. Gels were stained with 2.5 mg/ml Coomassie Brilliant Blue R-250 in 10% (v/v) acetic acid and 10% (v/v) isopropanol, then destained in 10% (v/v) acetic acid and 10% (v/v) isopropanol. Gelatinolytic activity appeared as a clear band on a blue background[26].

***Microplate assay for gelatinase***

Enzymatic release of azo dye from azocoll was performed to measure gelatinase activity by the method of Nakayama et al (2001)[25] with a slight modification.Azocoll (Azo dye impregnated collagen, 0.25 g, <50 mesh; Sigma Chemical Co) was washed in 50 ml of 50 mM Tris-HCl buffer (pH 7.8) containing 1 mM $CaCl_2$, kept standing for 90 min at 37°C, and then centrifuged at 1,500 x *g* for 10 min, discarding the buffer. The residue was resuspended in 50 ml of the same buffer, and 0.5-ml aliquots were transferred into a 1.5-ml Eppendorf tube. Tubes were incubated at 37°C for 15 min on a shaker, and then 25µl of mosquitocidal metabolite was added to each tube containing preincubated Azocoll. The mixture was incubated for 4 hours at 37°C on a shaker and then centrifuged at 1,500 x *g* for 5 min, followed by measurement of absorbance at 540 nm.

***Quantitative analysis using DQ gelatin as substrate***

The Enzchek Gelatinase/Collagenase Assay kit (Invitrogen molecular probes) contains DQ gelatin so heavily labeled with fluorescent that the fluorescence is quenched. This substrate gelatin is efficiently digested by most gelatinases and collagenases to yield highly fluorescent peptides. The increase in fluorescence is proportional to proteolytic activity and can be monitored with SYBER green end point assay in RT PCR (Eppendorf real plex). Collagenase purified from *Clostridium histolyticum* is provided with the Enzchek Gelatinase/Collagenase assay kit to serve as a control enzyme. Using 100ug/ml DQ gelatin and a two hours incubation period, the assay can detect the activity of this enzyme down to a final concentration of $2x10^3$ U/ml. One unit is defined as the amount of enzyme gelatinase/collagenase required to liberate 1µM of L-Leucine equivalents from collagen/gelatin at 37ºC pH 7.5.The different concentration of DQ-gelatin ranges from (12.5 to 100ug/ml) was incubated at 37ºC with the standard collagenase and the mosquitocidal metabolite of *P.fluorescens* Migula. Simillarly, the DQ- gelatin was incubated with the different concentration of inhibitor ranges from 0.1 –0.5 mM of 1, 10 phenanthroline. To this, standard collagenase and mosquitocidal metabolite was added and incubated at 37°C for two hours. Blanks were prepared with substrate and included in parallel. After incubation for two hours, the reaction rate was measured at 495 excitation and 535-emission wavelength using SYBER green end point assay in RT PCR[24,27].

***Inhibitor sensitivity***

The sensitivity to protease inhibitor was tested by determining the hydrolysis of azocasein after the preincubation with protease inhibitors.Inhibitors specific for metalloprotease orthophenonthroline (1, 10 Phe) 2mM; ethylenediaminetetra acetic acid (ETDA) 2mM, (EGTA) 2mM and Elastatinol 2mM were used. Routinely, 20µl of mosquitocidal protein was preincubated for 1 hour at room temperature with 3 µl of inhibitor in 100 µl of buffer, to give

the above concentrations of inhibitors and the enzyme activities were determined as described above with azocasein as substrate. All inhibitors were purchased from Sigma Chemicals Co[28].

***Gelatinae Assay in the larvae and pupae of Cx. quinquefasciatus***

The extra cellular protein (1mg/ml) was exposed to the 4th instars larvae and pupae of *Cx.quinquefasciatus* and mortality was observed after 16 hours. The gelatinase assay was carried out with the crude enzyme solution of treated, untreated larvae and pupae of *Cx.quinquefasciatus.* 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 the same buffer and the solution thus obtained was stored at –80° C and used in the gelatinase assay as the enzyme solution. Azocoll[29] was suspended at a final concentration of 5 mg/ml in the assay buffer (50 mM Tris [pH 7.8], 1 mM CaCl2) and incubated for 2 h at 37°C with vigorous shaking. The solution was filtered (Whatman 1MM paper), and the precipitate was resuspended immediately in the same volume of fresh buffer. Stirring was rapid enough to obtained what appeared to be a uniform suspension. Tubes (diameter, 13 mm) were prewarmed at 37°C for 15 min before the reaction was initiated by addition of enzyme. Assays were stopped by immersing tubes in an ice-water bath. Chilled tubes were then centrifuged, and the optical densities at 550 nm of supernatant fractions that were diluted 1:2 were measured. Data were obtained for every time point in triplicate[30].

## RESULTS AND DISCUSSION

The extra cellular mosquitocidal metabolite produced from the *P.fluorescens* Migula strain (VCRC B426) hydrolysis various natural substrates such as azocasein, elastin, gelatin, collagen and fibrin and showed gelatinase/collagenase, chitinase and fibrinolytic like activity (Figure 1&2). Since the average P value (<0.0001) of one way (ANOVA) analysis of varience is less than<0.05 at 95% confidence intervals, the results of activity of mosquitocidal toxin on natural substrates test is statistically significant. The $V_{max}$ and $K_m$ of Michaelis-Menten equation analysis of kinetic Assay of mosquitocidal protein activity on different natural substrate (mM) at 95% confidence intervals are tabulated (Table 1 ). The mosquitocidal protein actively hydrolysis the gelatine substrate as compared with standard gelatinase enzyme. (Figure 3). The gelatinolytic activity of mosquitocidal protein was studied on gelatin plate assay (Figure 4 a) and Zymogram assay of this protein on Polyacrylamide-substrate gel is presented in Figure 4b. The results of Enzchek Gelatinase/ Collagenase assay revealed that the mosquitocidal metabolite exhibited the gelatinase/collagenase activity and efficiently digested the fluorescein conjugate-DQ gelatin to yield highly fluorescent peptides. The arbitrary units of fluorescence emission at various concentration of substrate and various concentration of enzyme are measured. The increases in fluorescence are proportional to proteolytic activity and can be monitored with SYBER green end point assay in Eppendorf real plex (Figure 5). The kinetic assay of mosquitocidal protein against DQ gelatin is presented in Figure 6. The $V_{max}$ and $K_m$ of Michaelis-Menten equation analysis of kinetics assay of the gelatinase activity of protein(mg/ml) at 95% confidence intervals are 18.01 and 3.335 respectively.The gelatinase activity of mosquitocidal protein was studied with increasing the DQ gelatin concentration (Figure 7). The Vmax and $K_m$ of Michaelis-Menten equation analysis of gelatinase assay on different concentration of DQ gelation substrate (ug/ml) at 95% confidence intervals are 15.82 and 4.907 respectively. The inhibition of mosquitocidal metabolite by 1, 10-phenanthroline; ethylene diamine tetra acetic acid (ETDA) , (EGTA), Elastatinol (Figure 8a & 8b) and the comparison of gelatinase like assay activity of mosquitocidal protein against the Gelatinase / collaginase control enzyme are studied (Figure 9).

The Gelatinase activity was found to be enhanced in the treated pupae than the control pupae and treated larvae (Figure 10). Since the average P value(<0.0001) of one way (ANOVA) analysis of varience is less than<0.05 at 95% confidence intervals, the results of gelatinase assay on larvae and pupae of *Cx.quinquefascitus* test is statistically significant. This revealed the invasion of mosquitocidal protein through the cuticular region of pupae enhance the gelatinase activity in the treated pupae. Whereas in both treated and control larvae, there is no significant difference in the gelatinase activity as the route of mosquitocidal protein is through the respiratory tract. In this study, we focussed on the gelatinase activity in the protein fraction, since this activity would be most relevant to the role of gelatinase activity in biological control. The present study revealed that the mosquitocidal protein exhibited the broad substrate specificity towards the various natural substrates such as azocasein, elastin, gelatin, collagen and fibrin. The extra cellular protein produced from the *P.fluorescens* Migula showed gelatinase/collagenase, chitinase and fibrinolytic like activity. It has been reported that the substrate specificity of *X.nematophila* PAII indicated that it was active on a variety of natural protein substrates such as azocasein, gelatin and fibrin[31,32]. These results confirmed that the mosquitocidal metabolite(s) rapidly hydrolyzed the natural as well as synthetic peptide. Perhaps, the mosquitocidal metabolite showed a broad substrate specificity, it strengths the notion that the active principle involved in the degradation of the cuticular region of *Cx.quinquefasciatus* mosquito species. The integument of mosquitoes composed of three layers inner basement membrane, middle epidermis and the outer cuticle. The proteins of the cuticle are of three types Arthropodin, Resilin and Sclerotin. The arthropodin resembles sericin or silk like gelatin in insects, which plays a significant role to protect from invading organisms. The present study revealed that the arthropodin (gelatin) is the target substrate for the mosquitocidal toxin and it overcomes peritrophic membrane of the mosquito species and thereby supports the proposed mode of action for mosquitocidal metabolite. Thus, the present investigation confirmed that the mosquitocidal toxin efficiently hydrolysis the arthropodin (gelatin) in the cuticular region and overcome the peritrophic membrane which is a protective sleeve for the midgut epithelium mosquito species and binds to the gut regions of larvae and pupae of *Cx.quinquefasciatus*. In this study, we have investigated that the mosquitocidal metabolite of *P.fluorescens* Migula strains (VCRC B426) hydrolysis the gelatin and fibrin rapidly.

It has been reported that the endogenous metalloproteinase are inhibited by 1, 10-phenanthroline[33,34,35]. The result of this investigation confirmed that the mosquitocidal protein is metalloprotease and strongly inhibited by 1, 10-phenanthroline. This study provides the first evidence with the novel concept that the mosquitocidal metabolite hydrolysis the various substrates there by degrading the arthropodin (gelatin) in the cuticular region of dipteran mosquito species say *Cx.quinquefasciatus.* This conclusion was confirmed by the findings of degradation activity of mosquitocidal metabolite using gelatin, azocasein, elastin and fibrin. The results from the substrate analysis of this study indicated that an extra cellular metabolite of *P.fluorescens* Migula strain showed the collogenolytic/ gelatinolytic and elastolytic like activities. This study opens the way to study the mechanism action of the mosquitocidal protein against the 4$^{th}$ instar larvae and pupae of *Cx.quinquefasciatus*. These findings together with the data here have strengthened the notion of a novel concept that the mosquitocidal toxin of *P. fluorescens* Migula plays a significant role in the degradation of arthropodin (gelatin) molecule in the cuticular regions and peritrophic membrane and binds to the midgut epithelium of larvae and pupae of mosquito species of *Cx.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 *Cx.quinquefasciatus* by in vitro binding assays via immunohistochemical localization studies.

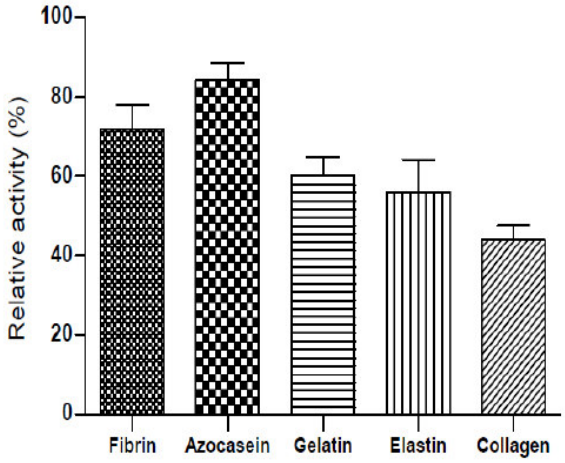


**Figure 1**
***Activity of mosquitocidal proteion of P.fluorescens Migula on natural substrates***

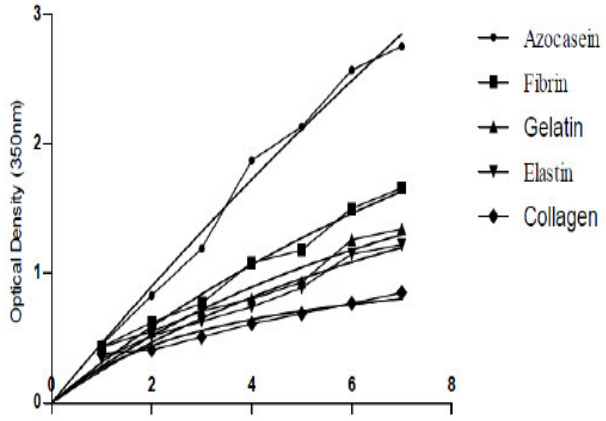


**Figure 2**
***Kinetic assay of mosquitocidal proteion activity of P.fluorescens Migula on different natural substrates(mM)***

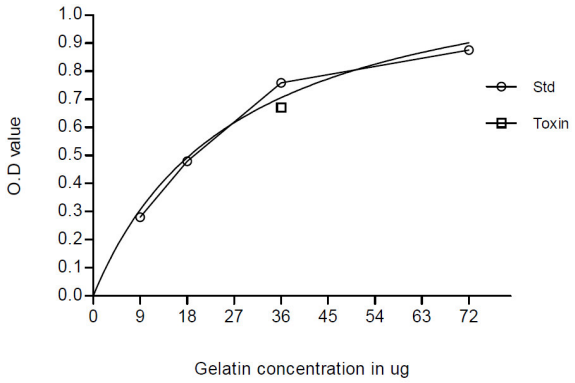


**Figure 3**
***Gelatinase activity of mosquitocidal protein of P.fluorescens Migula***

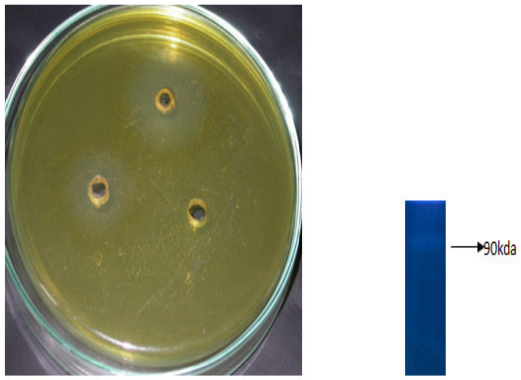


**Figure 4**

***a. Gelatinolytic activity assay on the gelatin assay plate (1%) with (a) 20µl of mosquitocidal metabolite and (b) 20 µl of standard gelatinase solution (1 NIH unit/ml) (c) 20µl sterile distilled water. Fig 4 b.Gelatin Zymograme***

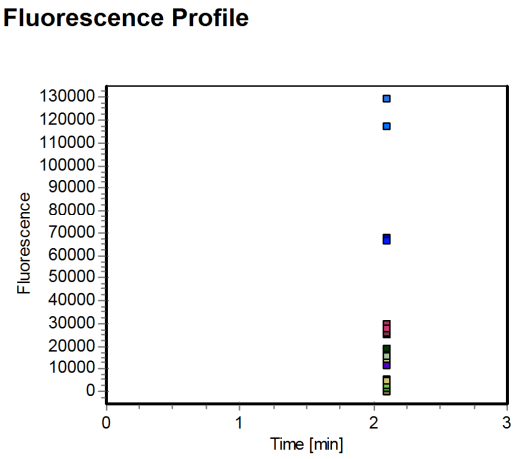


**Figure 5**

***RT-PCR SYBER green end point assay for DQ gelation substarte***

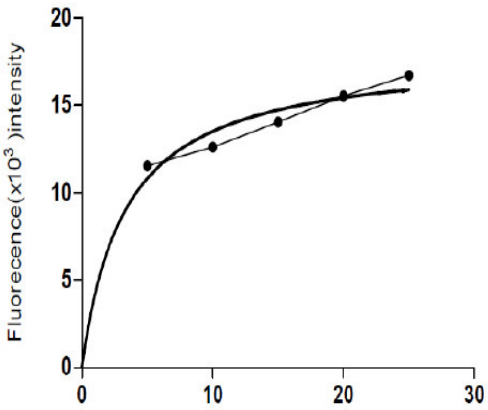


**Figure 6**

***Kinetic assay of gelatinase activity of protein of P.fluorescens Migula (mg/ml)***

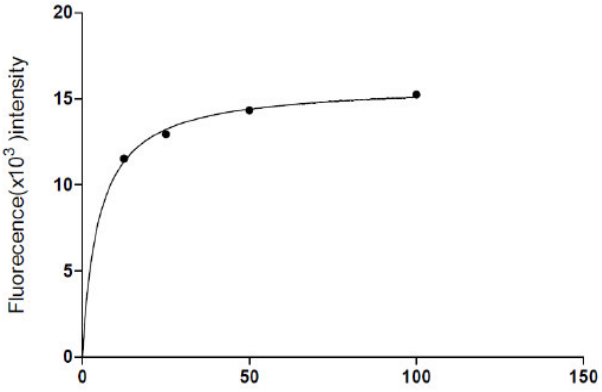


**Figure 7**
***Gelatinase assay on different concentration of DQ gelatine substrate (µg/ml)***

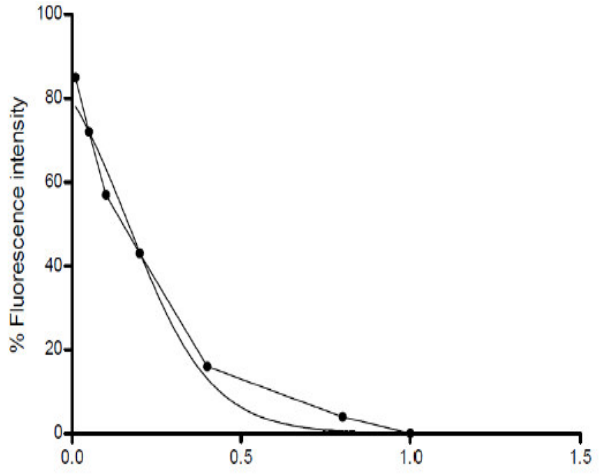


**Figure 8*a*: *Inhibition of mosquitocidal protein activity by 1,10-phenanthroline(mM)***

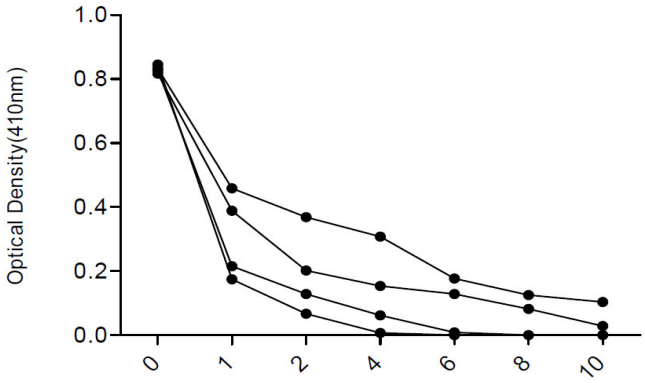


**Figure 8b: *Inhibition of mosquitocidal protein by inhibitors-1, 10- phenanthroline, EGTA, elastatinol and EDTA (mM*)**

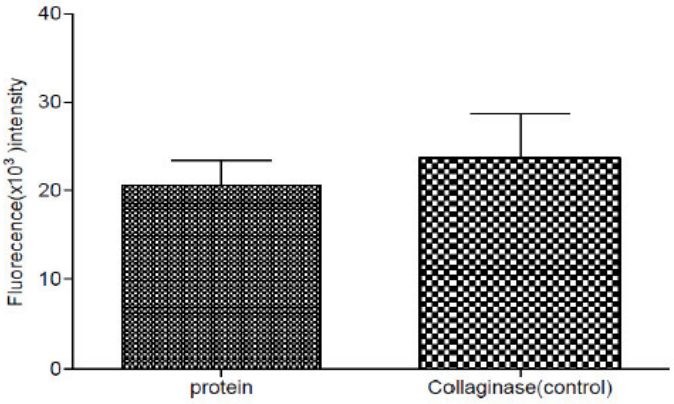


**Figure 9**
***Gelatinase activity of mosquitocidal protein against control***

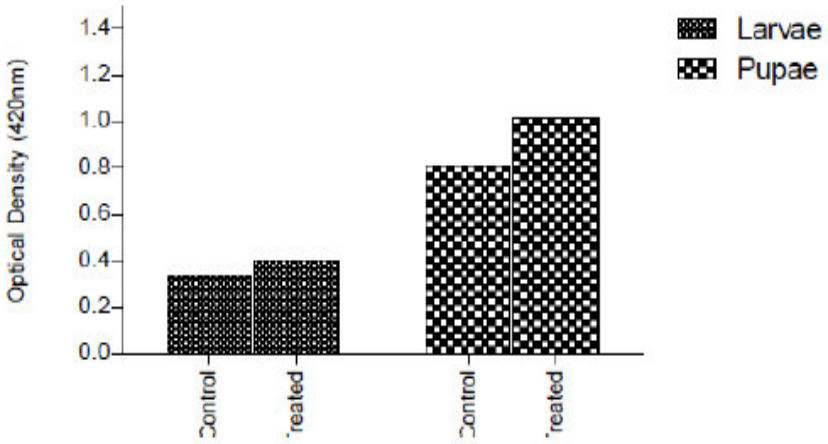


**Figure 10**
***Gelatinase assay in the larvae and pupae of Cx.quinquefasciatus***

**Table 1**
***$V_{max}$ and $K_m$ value of gelatinase activity of mosquitocidal protein on natural substarte***

| | Azocasein | Fibrin | Gelatin | Elastin | Collagen |
|---|---|---|---|---|---|
| $V_{max}$ | 21.40 | 5.644 | 3.285 | 3.232 | 1.194 |
| $K_m$ | 45.68 | 17.19 | 10.72 | 11.86 | 3.419 |

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