Role of midgut digestive proteases upon sub-lethal exposure of Cry toxin in the larvae of castor semilooper, *Achaea janata*

Research Project submitted to Central University of Punjab

For the award of

Master of Science

In

Life Sciences (Specialization in Animal Sciences)

SURESHGOPI D

Department of Animal Sciences,

School of Basic and Applied Sciences,

Central University of Punjab, Bathinda

May, 2018

CERTIFICATE

This is to certify that the research project entitled **"Role of midgut digestive proteases upon sub-lethal exposure of Cry toxin in the larvae of castor semilooper, *Achaea janata*" submitted by Mr. SURESHGOPI D (Reg. No. 16mslsas12)** for the partial fulfillment of M.Sc. Degree in Life Sciences (specialization in Animal Sciences), has been examined by the supervisor. The supervisor finds the work done by the candidate to be satisfactory and recommend that the report be accepted.

Dr. Krishna Chaitanya Rapalli
Assistant Professor, Department of Animal Sciences

DECLARATION

I declare that the project report entitled "**Role of midgut digestive proteases upon sub-lethal exposure of Cry toxin in the larvae of castor semilooper, *Achaea janata***" has been prepared by me under the guidance of Dr Krishna Chaitanya Rapalli, Central University of Punjab, Bathinda. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institutes. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the references.

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DECLARATION

I declare that the project report entitled "**Role of midgut digestive proteases upon sub-lethal exposure of Cry toxin in the larvae of castor semilooper, *Achaea janata***" has been prepared by **Mr. SURESHGOPI D** bearing the (**Reg. No. 16mslsas12)**, under my guidance at the Department of Animal Sciences, School of Basic and Applied Sciences, Central University of Punjab.

Dr. Krishna Chaitanya Rapalli
Assistant Professor, Department of Animal Sciences

Abstract

Role of midgut digestive proteases upon sub-lethal exposure of Cry toxin in the larvae of castor semilooper, *Achaea janata*

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Keywords: *Achaea janata*, *Bacillus thuringensis*, Insecticides, Lepidoptera, Resistance, Digestive Proteases, Trypsin, Chymotrypsin

Development of synthetic insecticides to reduce the level of infestation led to deleterious effects on the environment and human health. This lead to the development of eco-friendly pest management alternatives including *Bacillus thuringensis (Bt)*. Bt produces Crystal (Cry), Cytotoxic (Cyt) and Vegetative (Vip) proteins with insecticidal activity against different orders of Lepidoptera. Of late, pest resistance against Bt is being reported in many parts of the country. The foliar spray may lead to the reduced toxicity of Bt formulation from degradation by UV light, wash-off by rain, drying, temperature, and soil acidity as well as its chemistry. Further, insects sense pesticides through odorant receptors and move away quickly. Under these conditions, there is always a possibility of a population of larvae to get exposed to sub-lethal doses of toxin which might exhibit variable effects and escape mortality and eventually generate resistance. The role played by digestive proteases in the activation of the Cry toxin and digestion when administered in sub-lethal concentration could help in the elucidation of key phenomena like adaptations for survival and resistance development. Elucidation of these digestive proteases would enhance our understanding of resistance development. The current study is an attempt to monitor the transcript levels of digestive proteases upon sub-lethal exposure of Cry toxin in the larvae of an economically important polyphagous pest castor semilooper, *Achaea janata* prevalent in the Indian subcontinent.

Mr. Sureshgopi D **Dr. R.K. Chaitanya**

Acknowledgements

I express my gratitude to supervisor ***Dr. R.K. Chaitanya,*** *Assistant Professor, Department of Animal Sciences, Central University of Punjab, Bathinda for his resourceful guidance, suggestions, constant encouragement and support.*

I thank ***Prof. Aparna Dutta Gupta,*** *Professor, School of Life Sciences, Department of Animal Biology, who has provided me with the precious insect samples, which formed the basis of this work. I also thank her for mentorship.*

I am thankful to ***Dr. Anil K Mantha,*** *Associate Professor and HOD, Department of Animal Sciences, Central University of Punjab, Bathinda for his constant encouragement.*

I also extend my thanks to all the faculty members of the Department of Animal Sciences ***Dr. Aklank Jain, Dr. Jyoti Prakash, Dr. Sunil Singh, Dr. S.R. Lone,*** *for their kind cooperation and support whenever required.*

I thank ***Dr. Vinod Kumar Chauhan*** *and* ***Mr. Narender,*** Department of Animal Biology, University of Hyderabad, *for their inputs and support*

I also thank the laboratory assistant ***Mr. Dhirendra Kumar,*** *for his help*

I thank my friends ***Kanika Singh*** *and* ***Nisha Sharma*** *who made my stay worthwhile and memorable.*

I express my profound respect and regard to ***parents*** *and* ***family members*** *for their moral support all throughout.*

SURESHGOPI D

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Abbreviations

Sr. no.	Full form	Abbreviation
1.	*Bacillus thuringenisis*	*Bt*
2.	*Achaea Janata*	*A. Janata*
3.	Trypsin	*Try*
4.	Chymotrypsin	*Chy*
5.	Alkaline phosphatase	ALP
6.	Amino peptidase-N	APN
7.	Degree Celsius	°C
8.	Base pair	Bp
9.	Crystal	Cry
10.	Cytolytic	Cyt
11	Tris acetate EDTA	TAE
12.	β –D 1 thiogalactopyranoside	IPTG
13.	Microgram	Mg
14.	Milligram	Mg
15.	Millilitre	Ml
16.	Microlitre	Ml
17.	Nanogram	Ng
18.	Nanometer	Nm
19.	Calcium chloride	$CaCl_2$
20.	Rotation per minute	Rpm
21.	Non-template control	NTC

CHAPTER 1

(Review of Literature)

1.1. Castor and Castor Semilooper, *Achaea janata*

Castor (*Riccinus communis)* is an important non-edible oilseed crop cultivated in various parts of India, Mozambique, China and Brazil, responsible for 1.7 million, 68.9, 40.0 and 37.5 thousand tons, respectively (**http://www.fao.org/faostat**). Castor oil and its derivatives have many industrial uses, mainly in paints and varnishes for surface coatings, lubricants for aviation engines as a fuel, which does not freeze at even 40 degrees of negative temperature, cosmetics, textile dyeing, nylon type synthetic polymers, resins, leather industry etc. India exports 200,000 tonnes of castor meal to Japan and other countries, which is used as biofertilizer. Its potential as a 'biofuel crop' is hindered by a number of biotic stress factors particularly, pests which degrade the crop quality and productivity (i.e., 973 kg of seeds per ha) (**Ogunniyi, 2006).**

The main defoliator of castor crop is castor semilooper, *Achaea janata*. Its occasional hosts include economically important plants like mustard, sugar cane, cabbage, rose, tomato, banana and tea (**Sujatha *et al.,* 2009**). *A. janata* L. (Noctuidae: Lepidoptera) is prevalent on castor during July–October in India. The older larvae are voracious feeders, which often totally defoliate the plants during the outbreaks and compel the farmers to abandon the fields. Its life cycle consist of 4 stages i.e. egg, larvae, pupa, and adult. The larva consists of 5 instars and each instar is of 3 days. Larva is eclosed into pupa and finally adult emerges from the pupa (**Budatha *et al.,* 2007**). This pest has developed resistance to the chemical insecticides. Hence, various genetically modified insect resistant Castor varieties are under development.

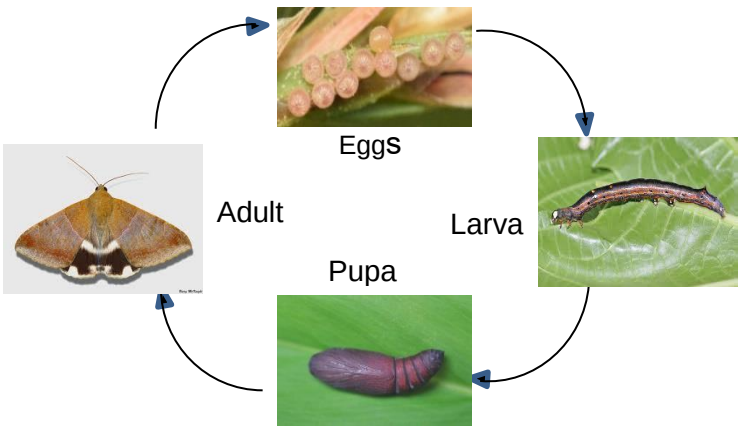

Fig.1. Life cycle of A. *janata*

1.2. Bt and its mode of action

Bacillus thuringiensis ssp*. kurstaki* is by far the most successful microbial preparation used for insect pest biocontrol. Crops such as cotton, maize, sorghum that are genetically modified with Bt have effectively countered the pests. The insecticidal activity (toxin) of Bt is contained within a very large structure called a parasporal crystal, which is synthesized during bacterial sporulation. When a target insect ingests a parasporal crystal, the Cry protoxin is activated within its gut by the combination of alkaline pH (7.5 to 8.0) and specific digestive proteases, which convert the protoxin into an active toxin with a molecular mass of approximately 60-70 kDa. The activated toxin then binds to specific receptors on the brush border membrane of the midgut epithelium columnar before inserting into the membrane. Toxin insertion creates an ion channel, which leads to an excessive loss of cellular ATP. About 15 minutes after this ion channel forms, cellular metabolism ceases; the insect stops feeding within a few hours, becomes dehydrated, and eventually dies (in about 2 to 5 days) (**Vinoth K. Chauhan *et al.,* 2017**). For Cry toxins, at least four different binding proteins/receptors have been described in different lepidopteron insects; a cadherin-like protein (CADR), a glycosylphosphatidyl-inositol (GPI)-anchored aminopeptidase-N (APN), a GPI-anchored alkaline phosphatase (ALP) and a 270 kDa glycoconjugate (**Bravo *et al.,* 2007).**

Using various criteria such as sequencing, serotyping, phage susceptibility and plasmid profiles 100 Bt subspecies have been identified and classified approximately. The Bt strains produce three types of insecticidal toxins, crystal (Cry) toxins, cytolytic (Cyt) toxins and vegetatively expressed insecticidal proteins (vip). Till 2012, a total of 229 cry toxins (Cry1Aa to Cry72Aa), 11 cyt toxins (cyt1Aa to cyt3Aa) and 102 vip toxins (vip1Aa1 to vip4Aa1) have been discovered. A total number of 342 Bt toxin genes are available for research to develop insect resistant GM crops. These toxins are biodegradable, highly insect-specific and does not affect human, plant and vertebrate populations **(Bravo *et al.,* 2015).**

1.3. Resistance to Bt

Recently, Bt resistance is being widely reported. Foliar spray of Bt formulation often results in the loss of toxicity from degradation by UV light, wash-off by rain, drying, temperature, and soil acidity as well as its chemistry. Further, insects sense pesticides through odorant receptors and move away quickly. Under these conditions, there is always a possibility of a population of larvae to get exposed to sub-lethal doses of toxin which might exhibit variable effects and escape mortality and eventually could develop resistance (**Bruce E. Tabashnik *et al.,*2015**).

One of the best-known and widely accepted resistance mechanisms to Bt is the reduction in binding of B. thuringiensis Cry toxins to their specific midgut receptors i.e. Aminopeptidases N (APN), Cadherins, Alkaline phosphatases (ALP) and ABCC transporters due to mutations in these proteins which results in cross-resistance to Cry toxins sharing recognition of the altered receptor site. Recently emerging non-receptor related resistance mechanisms involving stem cell mediated regeneration in midgut epithelium, pathogen-response (REPAT) and arylphorin genes and detoxifying proteins/enzymes pose an additional threat to Bt crops, since they could affect steps common to all Cry toxins and would result in cross-resistance to a wide range of Bt insecticidal proteins (**Liliana pardo-Lopez *et al*., 2012**). The proteinase related resistance mechanism(s) involved in reduced conversion of protoxin to activated toxin or degradation of active toxins are also reported in prominent insect pests worldwide **(Mehmet candas *et al.,*2002)**. The current study is an attempt to monitor the transcript levels of digestive proteases, particularly Trypsin and Chymotrpsin, upon sub-lethal exposure of Cry toxin in the larvae of an economically important polyphagous pest castor semilooper, *Achaea janata* prevalent in the Indian subcontinent.

1.4. Objectives of the study

- To clone digestive protease genes Trypsin (*Try*) and Chymotrypsin (*Chy*) from the midgut of *A. janata* larvae

- To monitor the expression of the digestive protease genes at transcriptional level after exposure to the sub-lethal *Bt* formulation

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CHAPTER 2

(Methodology)

2.1. Rearing and maintenance of *A. janata* larvae

A. janata were obtained from the fields with no prior exposure to any *Bt* pesticide and were reared in the laboratory conditions. The larvae were reared on sterile castor leaves and raised for three generations at 27 ± 2°C under a 14:10 h (light:dark) photoperiod and 60-70% relative humid conditions.

2.2. DOR- Bt1 treatment

DOR Bt-1 formulation consists of cry1 (cry1Aa, cry1Ab, and cry1Ac) and cry2 (cry2Aa and cry2Ab) genes with broad-spectrum potential against lepidopteran, and dipteran insects (Reddy et al., 2012). The reported IC_{50} value of the formulation is 247.52 µg/ml for a 3rd instar larvae **(Vimala Devi and Sudhakar, 2006)**. One tenth of IC_{50} value was used as a sub-lethal dose (i.e., 24.75 µg/ml of water) in the present study. Toxin-coated castor leaves were fed to the triplicate insect larvae groups (n=50 each) were maintained. Each group was continuously exposed to toxin-coated castor leaf discs (145 cm^2; 0.170 µg/cm^2) till 72 h. Control larvae were maintained on water coated leaf discs.

2.3. Midgut tissue extraction

Both the control and treated larvae were narcotized on ice and following which an incision was made with the help of sterile blade in the abdominal region of the larvae and extended through its length. The midgut was isolated, rinsed in sterile PBS and transferred immediately into Trizol reagent containing tubes.

2.4. RNA isolation from the midgut

100 µg of midgut tissue was homogenized in 1 ml of TRI Reagent (Sigma chemicals) followed by addition of 200 µl chloroform and incubation for 15 min at room temperature. The mixture was centrifuged at 12,000 g for 15 min at 4 ºC. The upper aqueous phase was collected to which 500 µl of isopropanol was added, mixed and incubated at room temperature for 10 min. The RNA pellet was collected post centrifugation at 12000 g for 20 min at 4 ºC. The pellet was washed thrice with 75%

ethanol, air dried and dissolved in 20 µl of diethyl pyro carbonate (DEPC) treated water and immediately used.

2.5. Total RNA integrity

RNA integrity was checked using Nanodrop (ND-1000) spectrophotometer. A solution with an absorbance at 260 nm of 1 contains ~ 40 µg of single stranded RNA/ml. Using this, the concentrations of various RNA samples was calculated. The absorbance of the sample was also monitored at 280 nm (A280) to check for any protein interference. The purity of the sample was determined by calculating the ratio of A260/A280.

For agarose gel electrophoresis, the RNA sample (1 µg) was mixed with 12.5 µl of formamide, 2.5 µl of10 x formaldehyde gel buffer [0.2 M MOPS, 80 mM sodium acetate and 10 mM EDTA (pH 8.0)] and 4 µl of formaldehyde in a total volume of 25 µl. The mix was denatured at 65° C for 5 min followed by snap cooling on ice for 2 min. To this mix, 2.5 µl of gel loading dye (50 % glycerol, 1 mM EDTA, 0.25 % bromophenol blue and 0.25 % xylene cyanol) was added and gel loaded. The RNA samples were electrophoresed on 1 % agarose-formaldehyde denaturing gel. The electrophoresis was carried using 1 x formaldehyde gel buffer at voltage $5V/cm^2$ until the dye reached the end of the gel. The EtBr stained gels were visualized under UV-transilluminator and analyzed using UVP-gel documentation system.

2.6. First strand cDNA synthesis

The first strand cDNA was synthesized using 1 µg of total RNA. Total RNA, 1 µl of oligo $(dT)_{20}$ (50 µM), 1 µl of dNTP (10 mM) mix and DEPC water were added to make a final volume of 10 µl in a PCR tube and incubated at 65 ºC for 5 min. To this tube, a mix of 2 µl 10 x RT buffer, 4 µl of $MgCl_2$ (25 mM), 2 µl of DTT (0.1 mM), 1 µl of RNase OUT™ (40U/µl) and 1 µl of Superscript™ III RT (200U/µl) was added to make a 20 µl reaction mixture. The mixture was incubated at 50 ºC for 50 min and terminated at 85 ºC for 5 min. To this, 1 µl of RNase H (2U/µl) was added and incubated at 37 ºC for 20 min. The cDNA synthesized was stored at -20 ºC till further use.

2.7. Cloning of partial fragments of *Try* and *Chy*.

Based on the accession numbers available on the Pubmed database specific primers were designed (using Primer BLAST) for partial amplification of Try and Chy. The list of forward and reverse primers used for the study are given below (Tab.1.). The cycling conditions for PCR amplification are initial denaturation at **95 °C** followed by denaturation at **95 °C** annealing at **60 °C** and extension at **72 °C for 35 cycles.** rS7 gene was used as internal control. No template control was set up to monitor contamination and primer-dimer formation to eliminate any false positive results. The PCR products were cloned into p-GEM-T vector.

Gene	Forward primer	Reverse primer
Try	CATAAACGCAGTGTCTTCCG	GGAATCAGCTCCTCCAACAT
Chy	GAAGACGATTTCAGTGAATTTGCCG	CTAATTGCTGGTCCGTCAGTATCAC

Tab.1. List of forward and reverse primers used for cloning of partial CDS of *Try* and *Chy*.

2.8. Agarose gel electrophoresis of DNA

The amplified DNA was electrophoresed on 0.8 % agarose gel polymerized using 1x TAE [40 mM Tris-acetate and 1 mM EDTA (pH 8.0)]. The electrophoresis was carried using the 1 x TAE buffer at voltage 5V/cm2 until the dye reached ¾th of the length of the gel.

2.9. DNA extraction from the agarose gel

The gel piece corresponding to the amplified DNA was excised and transferred to a micro centrifuge tube. To this, 3 volumes of Buffer QG was added (Buffer QG contains guanidium thiocyanate) that solubilizes the agarose gel slice and provides appropriate conditions for binding of DNA to the silica membrane of the spin column. Following binding, the column was washed with 0.75 ml of PE buffer [10 mM NaCl, 50 mM MOPS (pH 7.0) and ethanol phase] that removes any unwanted impurities

such as salts, enzymes, unincorporated nucleotides, ethidium bromide etc. The DNA was eluted with 50 µl of buffer EB [10 mM Tris-HCl (pH 8.0) with 1 mM EDTA].

2.10. Ligation of amplified DNA and the cloning vector

A reaction mixture of 6 µl of gel purified DNA (100 ng/µl), 3 µl of vector pGEM-T (55 ng/µl), 4 µl of 5 x ligation buffer, 1 µl of T4 DNA ligase (5U/µl) and 6 µl of nuclease-free water was set up. The ligation reaction was terminated after overnight incubation at 16 °C in the ligation bath. After ligation, the ligation mixture was transformed into DH5α competent cells. The transformation reaction was carried out at 42 °C for 90 sec. The mixture was plated onto LB agar plates containing ampicillin (100 µg/µl). Positive colonies were picked up based on blue-white screening.

2.12. Bacterial transformation

A single bacterial colony (DH5α) was picked from a LB agar plate and incubated for 16-20 h at 37 ºC. The colony was inoculated into 100 ml of LB broth. The culture was incubated for 3 h at 37 ºC with vigorous agitation and continuous monitoring of the growth. For efficient transformation, it is essential that the number of viable cells does not exceed 10^8cells/ml, which for most strains of E. coli is equivalent to an OD_{600} of ~ 0.4. The cells were recovered by centrifugation at 4000 rpm for 10 min at 4 ºC. The pellet obtained was resuspended by in 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution (80 mM MgCl2 and 20 mM CaCl2). The cells were recovered again by centrifugation at 4000 rpm for 10 min at 4 ºC and the pellet was resuspended freshly in 2 ml of ice-cold 0.1 M $CaCl_2$ for each 50 ml of original culture. At this point the cells were directly used for transformation. The vector with or without DNA insert were incubated with competent cells for an hour on ice and later cultured in 1 ml of LB broth for an hour at 37 ºC, centrifuged at 5000 rpm for 5 min. The pellet was resuspended in 50 µl of fresh LB broth and plated on LB Agar for blue-white screening.

2.13. Blue-white screening

The blue-white screening allows for detection of recombinant bacteria. Cells transformed with vectors containing recombinant DNA will produce white colonies and those transformed with non-recombinant plasmids (i.e. only the vector) produce blue colonies. The method is based on the principle of α-complementation of the β-galactosidase gene. The host *E. coli* strain carries the *lacZ* deletion mutant (*lacZΔM15*) which codes for the ω-peptide, while the vector harbor the *lacZα* sequence which encodes the α-peptide of β-galactosidase. Neither of the peptides is functional by itself. However, when the two peptides are expressed together i.e. when a vector containing the *lacZα* sequence is transformed into *lacZΔM15* cells, they form a functional β-galactosidase enzyme. The blue/white screening method is based on disruption of this α-complementation process. The vector carries within the *lacZα* sequence an internal multiple cloning site (MCS). This MCS within the *lacZα* sequence is where the foreign DNA is inserted thereby disrupting the gene that produces α-peptide. Consequently, in cells containing the vector with DNA insert, no functional β-galactosidase is formed. The presence of an active β-galactosidase is detected by addition of X-gal, a colorless analog of lactose that is cleaved by β-galactosidase to form 5-bromo-4-chloro-indoxyl, which then spontaneously dimerizes and oxidizes to form an insoluble blue product, 5,5'-dibromo-4,4'-dichloro-indigo. Blue colonies contain a vector with an uninterrupted *lacZα* (without insert), while white colonies indicate the presence of an insert in *lacZα*.

2.14. Plasmid isolation

The bacterial strain containing the recombinant plasmid was allowed to grow in LB/amp (100 µg ampicillin per ml LB broth) for 14-16 h and the DNA was isolated using Qiagen plasmid isolation kit. The cells were collected after centrifugation at 3,000 g for 10 min and suspended in 250 µl of P1 buffer [100 mM Tris-HCl (pH 7.5), 300 mM NaCl, 10 mM EDTA, 0.2 % (w/v) BSA and 20 mg/ml RNase A]. To the suspension, 250 µl of P2 buffer [30 % polyethylene glycol (PEG 6000) and 3 mM NaCl] was added, inverted gently 4-5 times and incubated at room temperature for 5 min. This was followed by addition of 100 µl of buffer N3 (100 mM NaCl, 100 mM

Tris-HCl pH 7.5 and 25 mM EDTA) and mixing by inversion for 4-5 times. The mix was centrifuged at 3,000 g for 10 min to separate supernatant from compact white pellet that contained DNA and was loaded on to QIAprep column. The column was then washed with 750 µl of PE buffer [10 mM NaCl, 50 mM MOPS (pH 7.0) and ethanol phase]. The plasmid DNA was eluted with 50 µl of buffer EB [10 mM Tris-HCl (pH 8.0) with 1 mM EDTA]. The recombinant plasmids containing partial DNA sequences of Try and Chy were sequences and analyzed

2.15. Quantitative PCR

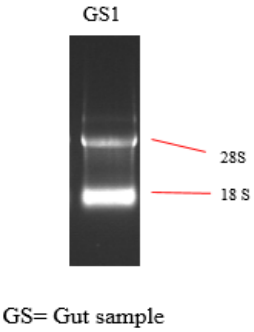
Control and Cry-toxin treated midguts collected at different time points were dissected in ice-cold TRI® reagent and the total RNA isolation was carried out immediately. All total RNA samples were treated with DNase I prior to first strand cDNA synthesis. Reverse transcription was carried out with 0.5 µg total RNA using random hexamer primers and Superscript® III reverse transcriptase according to manufacturer's protocol. Based on the partial DNA sequences of antioxidant genes, the primer sets for qRTPCR were carefully designed. Standard curve for each gene was plotted with serial dilutions of respective primers and cDNA. Gene expression was assessed by SYBR green qRT-PCR (Applied Biosystems) in ABI7500 fast real-time PCR system (Applied Biosystems). A 40-cycle two-step PCR was carried out in triplicates with 10.0 uL reaction volume containing the following components: 1.0 µL of cDNA template, 1.0 µL of forward and reverse primers each, and 5.0 µL of 2x master mix. Dissociation or melting curve analysis was performed for all the genes to check for specific amplification. The amplification efficiency was 95%-99% with slope of the curve ranging between -3.0 to -3.3. During each cycle of the PCR, fluorescence accumulation resulting from DNA amplification was analyzed and converted into cycle threshold (C_t) by the sequence detection system software (Applied Biosystems). Relative quantification results were normalized with conserved ribosomal protein S7 as endogenous control. C_t values were obtained from the exponential phase of PCR amplification. All the results are represented as change in the transcript levels relative to the reference values obtained for the control and were normalized to that of endogenous control gene (S7) C_t values using the $2^{-\Delta\Delta Ct}$ method **(Schmittgen and Livak, 2008).**

CHAPTER 3

(Results)

3.1. Quality control analysis of midgut total RNA

The total RNA from the midgut was isolated immediately after dissection as the gut tissue is rich in nucleases and proteases, which may result in RNA degradation and eventually affect the quality and the quantity. The integrity of extracted ribonucleic acid (RNA) was assessed by gel electrophoresis and subsequent analysis of the ribosomal RNA (rRNA) band. Although ribosomal RNA integrity is not an accurate measure of mRNA quality, it is useful as a readily available indicator of the general state of the purified RNA. The electrophoretic rRNA profile of insects differs significantly from the standard benchmark since the 28S rRNA of most insects contains α and β fragments, which remain hydrogen-bonded together. Upon denaturation, the masking hydrogen bonds are disrupted, releasing two similar sized fragments that both migrate closely with 18S rRNA. Usually, the ratio of 28S to 18S in intact RNA is around 2:1 simply because 28S is around double the size of 18S and incorporates more dye. The resulting rRNA profile of 28S to 18S in the ratio 1:2 thus reflects the endogenous composition of insect rRNA and not degradation **(Winneback *et al*., 2010).** The total RNA concentration obtained from 100 mg of midgut tissue was 1.3 µg/µl and the 260/280 and 260/230 ratios are > 2 indicating the purity of the RNA isolated (Fig.2.).



A.

Sample RNA Concentration	1343.9 ng/ µl
260/280	2.01
260/230	2.34

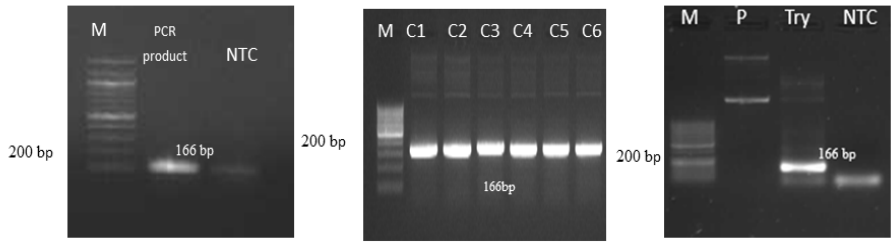
B.

Fig.2. Quality, purity and concentration of midgut total RNA. A) The image represents the qualitative estimation of isolated RNA from the gut of A. *janata*. The lighter band represents the 28s rRNA and darker band represents the 18s rRNA B) Quantitative estimation of RNA using Nanodrop spectrophotometer. (GS= gut sample).

3.2. Cloning of partial fragment of Trypsin

Based on the nucleotide sequence of *A. janata Try* gene available on PubMed (AIR09766.1), a partial fragment of 166 bp was amplified using specific primers and confirmed using Colony PCR and Plasmid PCR. The nucleotide sequence was obtained by DNA Sequencer. BlastP analysis of amino acid sequence of the putative *Try* gene showed the presence of a conserved Trypsin superfamily domain (Fig.3.)

A.



B.

Trypsin partial CDS

CATAAACGCAGTGTCTTCCGTCCTGAACGAGGTGTTCGTGCAGACGGTGAACCACACCACGTGTGAGGCGCGCTACAAGACGCTGGA
GCTTCTGACCGGACACCCTTACCCGGTCACGGACAACATGCTCTGTGCTGGACGACTCG**ATGTTGGAGGAGCTGATTCC**

Amino acid sequence:

INAVSSVLNEVFVQTVNTVHTTCEARYKTLELLTGHPYPVTDNMLCAGRLDVGGADS

C.

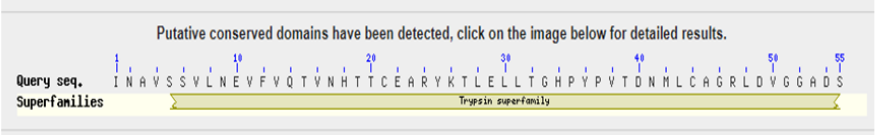


Fig.3. Cloning, confirmation and analysis of *Try*. A) Agarose gel depicting PCR amplification of *Try* using specific primers; Colony PCR for the same gene; Plasmid isolation and amplification was performed from the cultured colony of DH5α strain B) Partial CDS of *Try* and its corresponding amino acid sequence (C) BLAST P analysis of amino acid sequence of *Try*.

3.3. Cloning of partial fragment of Chymotrypsin

Based on the nucleotide sequence of *A. janata Chy* available on PubMed (AY251276.1), a partial fragment of 201 bp was amplified using specific primers and confirmed using Colony PCR and Plasmid PCR. The variations observed in the

band size during colony PCR could be due to the amplification of various *Chy* isoforms. The nucleotide sequence was obtained by DNA Sequencer. BlastP analysis of amino acid sequence of the putative *Chy* gene showed the presence of a conserved Trypsin superfamily domain (Fig.4.)

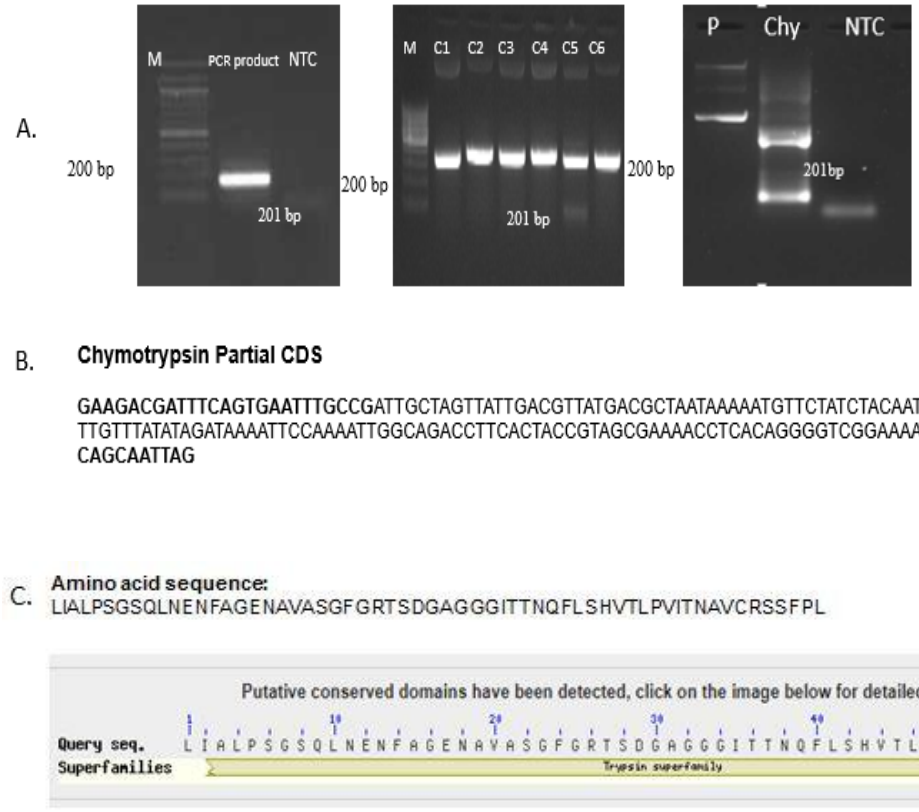


Fig.4. Cloning, confirmation and analysis of *Chy*. A) Agarose gel depicting PCR amplification of *Chy* using specific primers; Colony PCR for the same gene; Plasmid isolation and amplification was performed from the cultured colony of DH5α strain B) Partial CDS of *Chy* and its corresponding amino acid sequence (C) BLAST P analysis of amino acid sequence of *Chy*.

3.4. Relative expression of digestive proteases genes, *Try* and *Chy* upon sub-lethal Cry toxin exposure

Compared to control, the digestive protease genes, *Try* and *Chy* showed differential expression pattern upon sub-lethal Cry toxin exposure. The transcript levels of Try remains unaltered although marginal increase in expression was observed at 24, 36 and 48 h post treatment. *Chy* transcript levels were very highly elevated (> 10-15 fold) at 48 and 60 h following exposure. Overall, significant elevated levels of *Chy*

in the midgut during sub-lethal exposure of Cry toxin to the insect larvae was the most prominent observation (**Fig.5**). The C_t values obtained in triplicates for each sample are documented **(Fig.6).**

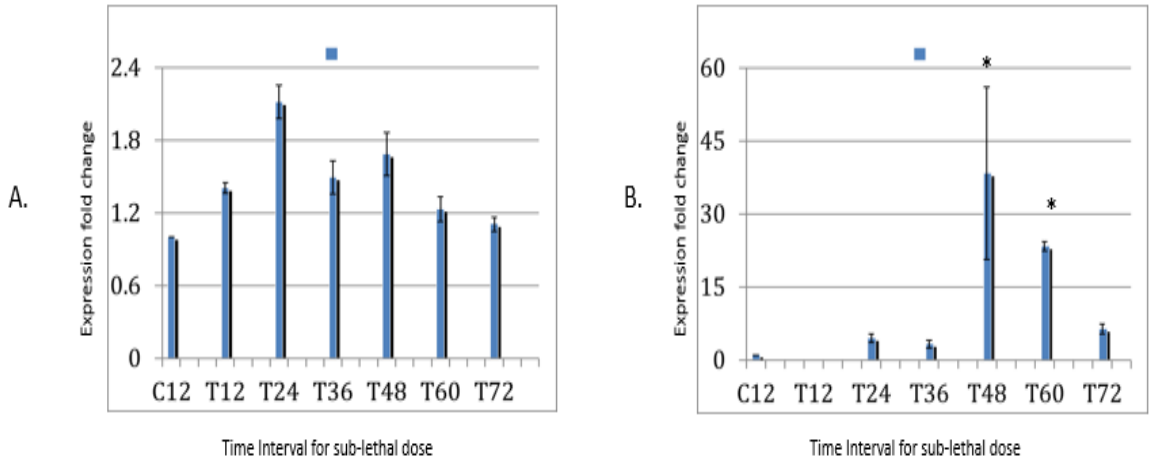


Fig.5. Relative expression of digestive proteases genes upon sub lethal Cry toxin exposure. X-axis represents time intervals C: Control, T12, 24, 36, 48, 60, 72 represent the samples collected post treatment in hours. Y-axis represents relative expression of A) *Try and* B) *Chy.* All the experiment are done in triplicates and * signifies P value < 0.5

	RS 7	Try	Chy
C12	19.93725586	25.34928703	19.82914162
	19.51820374	25.39765549	19.79645729
	19.52838898	25.1366806	19.91617012
T12	19.51820374	24.4215374	27.1229229
	19.51820374	24.40675926	26.96811295
	19.55378723	24.52906227	27.10639763
	19.22770119	23.45741463	16.6861248
T24	19.09329033	23.45741463	17.14722443
	19.13810539	23.54583359	16.90994263
	19.55378723	24.47419167	17.52131241
T36	19.51820374	24.4176712	18.20822525
	19.81393814	24.50342178	17.88139534
	19.40589142	23.91539955	13.46715355
T48	19.13093567	23.94625664	14.57966518
	19.16145515	23.83332253	13.87067032
	18.97541237	24.02122307	14.331604
T60	18.91590881	24.16956902	14.20097446
	18.89722252	23.94068718	14.30111313
	19.35211182	24.70504379	16.69535637
T72	19.47752762	24.67552376	16.46659088
	19.09048082	24.34234312	16.48207664
	19.72480801	24.54234311	16.54398330

Fig.6. C_t values in triplicates of the rs7 (internal control gene) and *Try* and *Chy*.

CHAPTER 4

(Discussion)

Discussion

Reduced Cry toxin binding to midgut receptors is widely reported resistance mechanism in most laboratory and field insect strains. However, reduced toxin binding is not always associated with resistance to Bt, and alternate mechanisms of resistance are emerging. The proteinase related resistance mechanism(s) involved in reduced conversion of protoxin to activated toxin or degradation of active toxins are also studied (**Brenda oppertet *et al*., 2015**). The lepidopteran larvae require a proteolytic enzyme complex including trypsins, chymotrypsins, aminopeptidases, and carboxypeptidases, elastases, cathepsin-B like proteases, for protein digestion. Primary protein-digestion in lepidopteron larvae depends majorly on trypsin- and chymotrypsin-like serine protease activities (**Maria Magdalena *et al.,* 2017**). The functional diversity of digestive enzymes can be correlated to insects' acquaintance to naturally occurring antagonistic biomolecules and adaptation to their host plants. Proteolytic enzymes of *Heliothis virescens* resistant to Bt subspecies *kurstaki* (HD-73) were reported to process the protoxin relatively slow and degrade the toxin faster than enzymes from a susceptible strain. In *Spodoptera littoralis larvae*, an increase in the specific activity of gut proteinases was associated with a loss of sensitivity to Cry1C, possibly due to an increase in the degradation of toxin (**Bruce E. Tabashnik *et al.,* 2015**). *Bacillus thuringiensis* (Bt)-resistant strains of the Indian meal moth, *Plodia interpunctella were found* lacking trypsin in the gut. Trypsin or trypsin-like proteases recognize highly basic residues in a substrate and these enzymes are crucial for Bt protoxin activation to enable midgut receptor binding. Chymotrypsin or chymotrypsin-like proteases are also involved in the activation of Bt protoxins. However, these enzymes recognize aromatic residues in a substrate and it was further suggested that chymotrypsin play a role in the degradation of active toxins. Many potential chymotrypsin cleavage sites were detected within the activated *Bt* toxin which could inhibit *Bt* toxin activity. The role of trypsin gene in resistance to Cry1Ac in one of the laboratory-selected resistant strains of *H. armigera* was evaluated. It was found that one of the trypsin genes, HaTryR, was reduced in the resistant strain and silencing of the gene resulted in enhance survival of susceptible larvae exposed to Cry1Ac. The present study corroborates with the earlier reports in that the chymotrypsin levels were significantly elevated in larvae exposed to sub lethal Bt toxin dose (**Cheni Liu *et al.,* 2014**). Further, studies are

warranted to check for the corroboration of trypsin and chymotrypsin activity levels with that of the obtained mRNA levels. It is also important to silence the chymotrypsin RNA and probe for toxin susceptibility of the resistant strain. Further, elucidation of the role of chymotrypsin in resistance generation can be monitored using chymotrypsin inhibitors N-tosyl-L-phenylalanylchloromethyl ketone and record the mRNA/activity levels in both resistant and susceptible strains.

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