Identification and characterization of immune/stress function related proteins from larval forms of lepidopteran insect pests

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By

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DECLARATION

I, **Pavani. A. S. N** hereby declare that this thesis entitled "Identification and characterization of immune/stress function related proteins from larval forms of lepidopteran insect pests" submitted by me under the guidance and supervision of **Prof. Aparna Dutta Gupta** is a bonafide research work which is free from plagiarism. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

Date:

Pavani. A. S. N.
(10LAPH08)

CERTIFICATE

This is to certify that this thesis entitled "Identification and characterization of immune/ stress function related proteins from larval forms of lepidopteran insect pests" submitted by **Pavani. A. S. N**, bearing registration number **10LAPH08** in partial fulfillment of the requirements for award of Doctor of Philosophy in the School of Life Sciences is a bonafide work carried out by her under my supervision and guidance.

This thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for award of any degree or diploma.

Parts of this thesis have been:

A. Published in the following publication
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Supervisor **Head of the Department** **Dean of the School**

Dedicated to my Parents

..My every achievement, big or small, I owe it you..

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List of abbreviations

°C	degree centigrade / Celsius
20E	20-Hydroxyecdysone
AMP	Anti-microbial peptides
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
dNTPs	Deoxy ribonucleoside triphosphates
DTT	1, 4-Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
ELI	Early-last instar larvae
IPTG	Isopropyl β-D-thio galactoside
JH	Juvenile hormone
kDa	Kilodalton
LB medium	Luria-Bertani medium
LLI	Late-last instar larvae
mg	Milligram
mM	Millimolar
MOPS	3-Morpholinopropanesulfonic acid
mRNA	Messenger ribonucleic acid
NBT	Nitrotetrazolium blue
ng	Nano gram
nM	Nano molar
NP-40	Nonidet P-40 (Nonylphenyl polyethylene glycol)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenyl methyl sulfonyl fluoride
PP	Prepupae
RACE	Rapid amplification of cDNA Ends
RNA	Ribonucleic acid
RNAi	RNA interference

ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
TAE	Tris-Acetate-EDTA buffer
TCA	Trichloroacetic acid
TBS	Tris buffered saline
TE	Tris-EDTA
TEMED	N, N, N`, N`, tetramethyl ethylene diamine Tris Tris (hydroxymethyl) amino methane
v/v	Volume/volume
w/v	Weight/volume
μg	Microgram
X-Gal	5-Bromo-4-chloro-3-indolyl β-D-galactoside

General Introduction

Insects

Insects are one of the most diverse and evolutionarily successful group of animals on planet earth. It is interesting to note that even in this era of evolving parasites and pathogens, they continue to thrive successfully with their simple yet effective defense strategies. As insects are well known as agricultural and stored grain pests as well as vectors, it is important to understand their defense mechanisms and their contribution in the survival of the organism. However, the field of insect immunity gathered attention only in the past few decades and has been growing ever since. A shift of focus from basic biochemistry of insect immunity to functional genetics, using the *Drosophila* model system in the 90's led to a Nobel Prize in Physiology and Medicine in the year 2011. Also, the past decade of genomic era has immensely helped in the growth of this field at a rapid pace.

The field of insect immunity is multidisciplinary. Firstly, insects can serve as models to elucidate the fundamental aspects of the immune system conserved across the phyla and to analyze the variation in immune competence. Secondly, it helps to design novel approaches for the biological control of insect pests and vectors. Moreover, insect-pathogen interactions help in elucidating basic principles in evolutionary biology, ecology and the epidemiology of infectious diseases.

Components of insect immunity: Only a few of the countless encounters with pathogens/ parasites result in infection in insects. Insect defense system is comprised of the following components:

- Physical barriers: cuticular integument and gut serve as the first line of barriers.
- Cellular responses: coordinated responses of various types of insect blood cells commonly known as hemocytes.

- Systemic response: induced synthesis of antimicrobial peptides and other immune proteins.

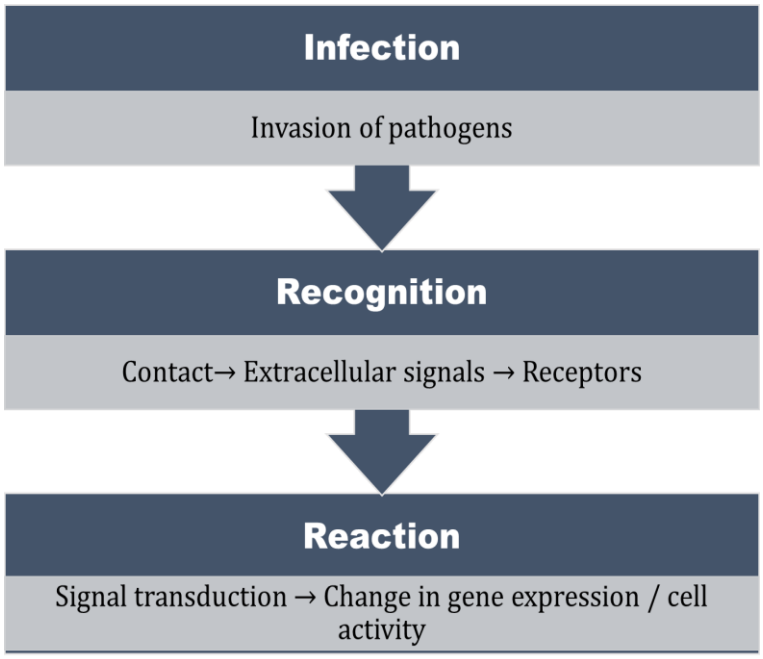

Fig. 1: A simplified scheme of insect immune response (Modified from Gillespie et al., 1997).

The aforementioned classification of insect immunity into cellular and humoral response is mainly for our convenience rather than function, since many humoral factors regulate hemocytes and hemocytes serve as important sources of humoral defense molecules.

Insect hemocyte types: Traditionally, hemocytes were classified based on morphological, functional and histochemical characteristics (Lavine and Strand, 2002). However, with the advancing technology, molecular and antigenic markers are also being used in the classification of hemocyte cell types (Lanot et al., 2001; Jung et al., 2005). Insect hemocyte nomenclature varies from species to species but a detailed understanding is primarily derived from *Drosophila*. The following are the three types of terminally differentiated hemocytes in *Drosophila* larvae:

- Plasmatocytes: They represent about 95% of the mature hemocyte population. They are the primarily phagocytes and engulf pathogens, dead cells etc.

- Crystal cells: These are non-adhesive cells and represent 5% of hemocyte population. They secrete components of phenoloxidase pathway that are responsible for melanization reaction.
- Lamellocytes: They are absent in healthy larvae but upon infection and during the metamorphosis, they rapidly develop and differentiate from prohemocytes. Their principal function is to encapsulate large pathogens, parasitoids and degenerating tissues.

In Lepidoptera, the most abundant type of hemocytes are granulocytes. They function as phagocytes and strongly adhere to foreign surface and spread symmetrically. Other class of adhesive hemocytes are the plasmatocytes. These cells play a major role in capsule formation and unlike granulocytes they spread asymmetrically on foreign surfaces. Non-adhesive hemocytes comprise of oenocytoids that contain prophenoloxidase components and spherule cells that serve as an important source of cuticular components.

Hemocyte mediated defense responses: Cellular immunity in insects mediated by hemocytes is composed of processes like phagocytosis, nodulation and encapsulation. Phagocytosis is a highly conserved defense response where the formation of a phagosome by the immune cell is induced by the binding of the target to its receptor. The receptor bound target is then engulfed via actin polymerization dependent mechanisms (Stuart and Ezekowitz, 2008). Encapsulation involves irreversible binding of multiple hemocytes to larger invaders like nematodes and parasitoids and when the target is a bacterial aggregate, the process is referred to as nodulation (Gillespie et al., 1997). The capsules formed in these processes are usually melanized and the source of phenoloxidases are the hemocytes forming the capsule (Pech and Strand, 1995; Schmidt et al., 2001).

Humoral response: Humoral response in insects can be either specific or non-specific and involves melanization, AMP synthesis and RNAi defense. It can be either a localized or systemic

response depending on the site of invasion and the type of infection. Melanization in insects plays a pivotal role in pathogen sequestration and wound healing (Gillespie et al., 1997). This process involves the formation of melanin in the site of infection which is produced by the hydroxylation and oxidation of phenols and o-phenols respectively by phenoloxidases that are activated by a cascade of specific serine proteases (Sugumaran and Kanost, 1993; Marmaras et al., 1996; Gillespie et al., 1997; Söderhäll and Cerenius, 1998; Gorman and Paskewitz, 2001; Ling and Qu, 2005). Quinoids generated during the melanization process also contribute to the generation of ROS that further assist the clearance of infection (Nappi et al., 1995). Another known function of phenoloxidases is the hardening of cuticle by sclerotization reactions that can further block the microbial invasion (Hopkins and Kramer, 1992; Kanost and Zhao, 1996).

Insect gut is one of the most common sites of invasion for bacterial pathogens where a localized response inducing ROS and AMP production serves as an initial barrier to infection (James and Xu, 2012). Peptidoglycans on the bacterial cell wall serve as immune elicitors and stimulate the synthesis of AMPs. These molecules are detected by pattern recognition receptors and trigger a systemic response thereby up-regulating the synthesis of AMPs in the fat body and their release in hemolymph (Broderick et al., 2009).

AMPs are small proteins/ peptides primarily synthesized in the fat body (Imler and Bulet, 2005) and are usually composed of less than 100 amino acid residues. They are typically cationic in nature and diverse in their structures. However, most of them fall under a limited number of families that are represented by the peptides assuming the conformation of alpha helices or disulfide-stabilized beta sheets in organic solutions. Various AMPs act on a wide spectrum of pathogens thereby suggesting different modes of action. AMPs are constitutively expressed in basal levels and upon infection genes coding AMPs are activated by multiple signaling pathways where each type of invading microbe triggers a specific signaling pathway (Bulet et al., 2005). Gram positive bacteria and the entomo-pathogenic fungi trigger the Toll

pathway whereas Gram negative bacteria activate the Imd pathway. Defense against viruses is mediated by Toll and Jak-STAT signaling pathways (Dostert et al., 2005; Zambon et al., 2005). However, inducible RNAi serves as a primary defense against viruses where small RNAs produced by the insect immune system bind to viral RNA and inactivate them (Wang et al., 2006; Zambon et al., 2006; Imler and Eleftherianos, 2009). Apart from AMPs other immune effector molecules include lysozyme, chitinases and heat shock proteins that are upregulated upon infection/ injury (Gerardo et al., 2010).

ROS in insect immunity: ROS are by-products of aerobic metabolism. Harmful and beneficial effects of ROS, antioxidant defenses that counteract ROS and the importance of redox homeostasis have been widely studied in various animal models including insects. Unlike mammals, insects lack adaptive immunity and ROS based defense responses dominate their innate immune system. Immune responses like encapsulation and melanization involve the production of ROS to kill the invading pathogen (Nappi et al., 1995; Nappi and Vass, 1998; Kumar et al., 2003). Further, autophagy in insects is activated and regulated by ROS (Huang et al., 2009; Scherz-Shouval and Elazar, 2011; Sena and Chandel, 2012). ROS also serve as key immune effector molecules in the insect gut (Ryu et al., 2010; Kuraishi et al., 2013) and ROS production to counteract bacterial/ fungal infection has been reported in several insect orders like Blattodea (Whitten and Ratcliffe, 1999), Lepidoptera (Bergin et al., 2005; Ishii et al., 2008; Mikonranta et al., 2014), Hemiptera (Garcia-Gil de Munoz et al., 2007) and Diptera (Diaz-Albiter et al., 2012). Further, in *Drosophila* gut, dual-oxidase (DUOX) dependent microbicidal ROS production serves as the first line of defense (Ha et al., 2005) and infection induced ROS acts as a signal for AMP production thereby eliciting a systemic immune response (Wu et al., 2012). Interestingly, peptidoglycan is shown to regulate DUOX expression pathway (Ha et al., 2009). High ROS levels are also required to fight off *Plasmodium* infection in mosquitoes (Kumar et al., 2003; Molina Cruz et al., 2008). Hence it could be concluded that insect immunity and

stress pathways interact with each other at several levels and together contribute to the insect's survival.

Immune and stress proteins- their role in the development of xenobiotic/ pesticide resistance: Insects develop insecticide resistance mainly by three mechanisms which are the following:

i) Behavioral: This mode of resistance development involves simple changes in the insect behavior associated with adaptation/ movement to avoid insecticide.

ii) Physiological: This includes changes in basic physiology like modification of cuticle to decrease penetration of insecticide, increased excretion and sequestration of the insecticide to reduce their availability.

iii) Biochemical/molecular: In this mechanism, insect enzymes degrade/ detoxify the insecticide before it reaches the target site, alteration of the binding/ target sites etc.

During the last decade extensive transcriptome and proteome analysis in many insect models implicate the role of many candidate genes including several immune and stress pathway components in the development of resistance (Rodriguez et al., 2008; Lei et al., 2014). Further, studies on pesticide/ biopesticide resistance development in insects identified the modification of immune and stress pathway components that led to resistance. For example, in *Helicoverpa*, coagulation of endotoxin in the gut lumen (Ma et al., 2005) and modification of MAPK pathway in diamond back moth (Guo et al., 2015) are identified to be the causative factors for Cry toxin resistance. Further, increased GST activity is known to cause Bt resistance in some dipterans (Candas et al., 2003; Boyer et al., 2012) and Bt tolerance correlates with elevated immune response in lepidopteran insect *Ephesia kuehniella* (Rahman et al., 2004). Apart from Cry toxin, several botanical and inorganic insecticides are also known to affect insect immune and stress response (George et al., 2004; Luo et al., 2005; Durmus et al., 2008; Ma et al., 2008; Buyukguzel et al., 2009; Babu and Subrahmanyam, 2010).

Rationale of the study: The emerging problem of pesticide including biopesticide resistance in the field of insect pest as well as vector control has amplified the search for new insecticides and novel control measures. However, understanding the mechanisms of resistance development is imperative in the design of better control strategies. Given the importance of immune and stress pathway components in resistance development, the current study focuses on the identification of proteins/peptides with immune/stress functions from the larvae of lepidopteran pests and evaluation of their role in insect physiology. Research in this direction yielded significant results which are presented in the following three chapters.

1. Identification and characterization of serpin2 from the rice moth, *Corcyra cephalonica* larvae.
2. Identification and characterization of Selenium Binding Protein (SeBP) from castor semilooper, *Achaea janata* larvae.
3. Differential stress responses in larval forms of castor semilooper, *Achaea janata*.

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General Materials and Methods

Chemicals and kits used for the study:

S. No.	Chemicals	Manufacturer
1	Agarose	Life Technologies (USA)
2	20-Hydroxyecdysone, Bromophenol blue, BSA, DTT, EDTA, Methoprene, Tris base, TRI reagent, TEMED, Triton X-100	Sigma Chemical Co. (USA)
3	ALP conjugated secondary antibodies, NBT/BCIP mix	BioRad Laboratories (USA)
4	Acrylamide, N-N`-methylene bisacrylamide, 2-mercaptoethanol, Glycerol, Sodium dodecyl sulphate (SDS), Glycine, Silver nitrate, Commassie brilliant blue G-250 and R-250	Sisco Research Laboratories Pvt. Ltd. (India)
5	Agar, ampicillin, Kanamycin, Luria Bertani broth, Luria Bertani Agar and Skim milk powder	HiMedia Laboratories (India)
6	SMARTer™ RACE cDNA Amplification Kit, Genomewalker™ Universal Kit	Clontech Takara Laboratories Inc., (USA)
7	RevertAid® first strand synthesis kit, InsTAclone™ PCR Cloning Kit, DNA, RNA and protein markers	Thermo Scientific (USA)
8	QIAprep® spin Miniprep Kit and QIAquick® gel Extraction Kit	Qiagen (Germany)
9	Restriction enzymes	New England Biolabs (USA)
10	Western blot Quant HRP substrate	Takara Bio Inc. (USA)
11	Nitrocellulose membrane	Amersham Biosciences (UK)
12	SYBR Green master mix and other real-time PCR reagents	Applied Biosystems (USA)

List of chemicals used in the present study

Bacterial strains used in the current study were a kind gift from Prof. Appa Rao Podile's laboratory, School of Life Sciences, University of Hyderabad. The vectors used (both cloning and expression vectors) were purchased from Clonetech Takara Laboratories, USA.

Primer designing: As the genome sequences of the model organisms are not available, for the present study, various primers (degenerate, gene specific, RACE and qRT-PCR) were designed manually. Degenerate primers were designed by aligning sequences for the gene of interest from related organisms retrieved from NCBI database and primers were designed in the conserved domains following the general primer designing guidelines. The physico-chemical properties of these designed primers like melting temperature, self and hetero dimer formation, hair pin structures etc. were analyzed using Oligo analyzer™ tool available in Integrated DNA Technologies, Inc. (IDT) web portal. All the primers were synthesized and supplied by IDT.

Primers used for analyzing genes which were used as internal control: To quantify the expression levels of target genes using qRT-PCR, it is essential to have a constitutively expressed gene as an internal control to normalize the expression levels among different samples. However, it is important that the expression levels of the endogenous control gene to not vary among the different samples. Hence, one should screen the expression levels of selected housekeeping genes in the samples and choose the one with the most stable expression as an endogenous control. For this study, a ribosomal protein, rS7 was used as this has the most stable expression levels in different conditions tested in our lepidopteran model organisms (Venkat, 2015). M13 primers are universal primers and were used to confirm the presence of insert in the vectors in cloning.

S. No.	Primer Name	Primer Sequence (5' – 3')
1	rS7 RT FP	AGC TGG ACG GTT CAC AAC TCA TCA
2	rS7 RT RP	TTC GCG GCC TGT TAG CTT CTT GTA
3	M13 FP	CGC CAG GGT TTT CCC AGT CAC
4	M13 RP	TCA CAC AGG AAA CAG CTA TGA

Table 1: List of endogenous control gene primers used for qRT-PCR and universal M13 primers

Experimental insects: For the present study two different lepidopteran insect models used were *Corcyra cephalonica* and *Achaea janata*.

i. ***Corcyra cephalonica*** **(Stainton)**

Family: Galleridae

Order: Lepidoptera

C. cephalonica, commonly known as rice moth, is a serious pest of stored grains in tropical and sub-tropical regions of the world and causes serious loss (Osman, 1984, 1986). Eggs of *C. cephalonica* were purchased from National Bureau of Agricultural Insect Resources (NBAIR), Bengaluru, India. The eggs were sterilized and placed in culture troughs containing sorghum seeds that are coarsely crushed and mixed with multi vitamin tablets powder, for hatching and the troughs were covered with muslin cloth and maintained at 26±1°C, 60±5% relative humidity (RH) and 14:10 h light: dark (L: D) photoperiod in an insect culture facility of the school. Under above conditions, the larval development is usually completed in 45-50 days and involves five instars (1^{st}, 2^{nd}, 3^{rd}, 4^{th} and the 5^{th} larval instar which includes early-last instar (ELI), mid-last instar (MLI) and late-last instar larval (LLI) stages. For all the experiments, LLI larvae were used and it is mentioned as 5^{th} instar. Pre-pupal stage extends for over 4-5 days followed by pupal stage which lasts for 7-8 days. Normally, adult moths survive for 8-10 days after emergence (Fig. 2A). Freshly emerged adults are collected and transferred to breeding cages for collection of eggs, which are used for maintenance of continuous cultures.

ii. *Achaea janata*

Family: Noctuidae

Order: Lepidoptera

A. janata, commonly known as castor semilooper, is a widespread insect pest throughout the tropical and subtropical regions of the world. Primary host of this pest is castor, which is an important non-edible oil seed crop in many countries including India and occasional hosts include economically important plants like tomato, sugar cane, rose, mustard, banana, cabbage and tea (Laksminarayana and Raoof, 2005). The larvae were obtained from Indian Institute of Oil Seeds Research (Hyderabad, India) and were maintained on castor leaves in an insect culture facility at 25±1°C with a photoperiod of 14:10 hour light: dark cycle and 70% relative humidity (Budatha et al., 2011). Under these conditions larval duration is of 16-18 days with five instars, followed by prepupal stage of 4 days and pupal stage of one week to 10 days.

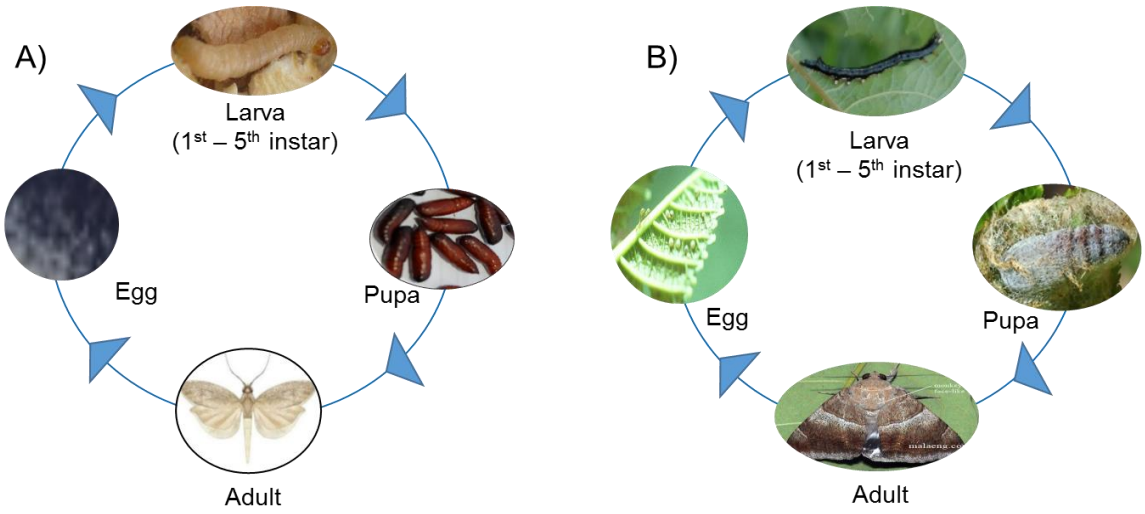


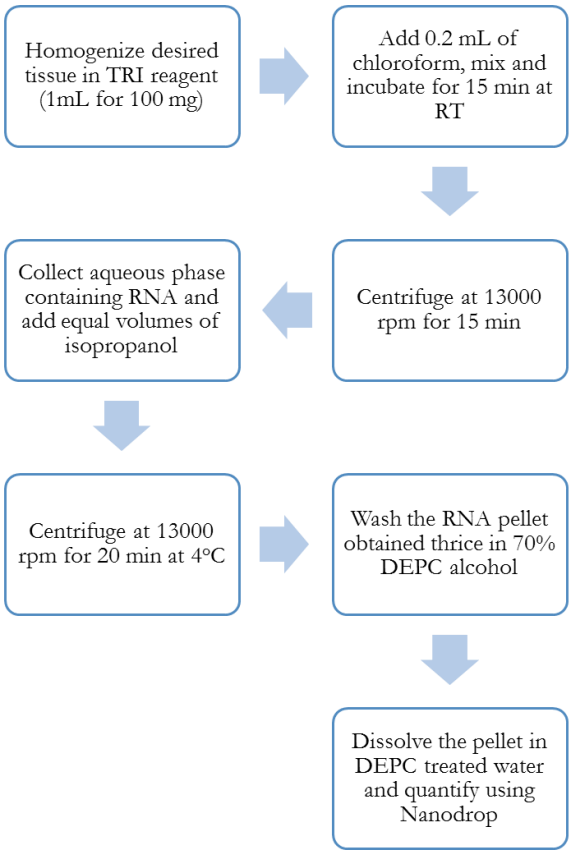
Fig. 2: Figure showing the life cycle of model organisms *C. cephalonica* (A) and *A. janata* (B). Both the insects exhibit a holometabolous life cycle with four distinct stages: Egg, larva, pupa and adult.

Tissue dissections and homogenate preparations: For dissection the insects of appropriate stage were placed on ice for narcotization. They were fixed on wax dish using insect pins. An incision was made on the middle region, desired tissues were collected in cold insect Ringer solution (130 mM NaCl, 0.5 mM KCl and 0.1 mM $CaCl_2$) from the insects by dissections. The

collected tissues were snap frozen and stored at -80°C till further use. For protein studies, the tissues were homogenized in homogenization buffer (10 mM Tris-Cl pH 7.4, 1 mM PMSF, 0.1% Triton X-100, 1 mM EDTA and 1 mM DTT) and centrifuged at 10,000 rpm for 15 min to remove debris. The supernatant was stored in aliquots at -20°C till use.

Hemolymph collection and hemocyte isolation: Hemolymph was collected from appropriate larval stages by narcotizing them on ice. Using a sharp scissors proleg was cut and the oozing hemolymph was collected in a microcentrifuge tube containing insect Ringer solution. The tubes were pre coated with 0.25% phenylthiourea to prevent melanization. Hemocytes were isolated by centrifuging the collected hemolymph at 4500 rpm for 10 min at 4°C.

Total RNA isolation:

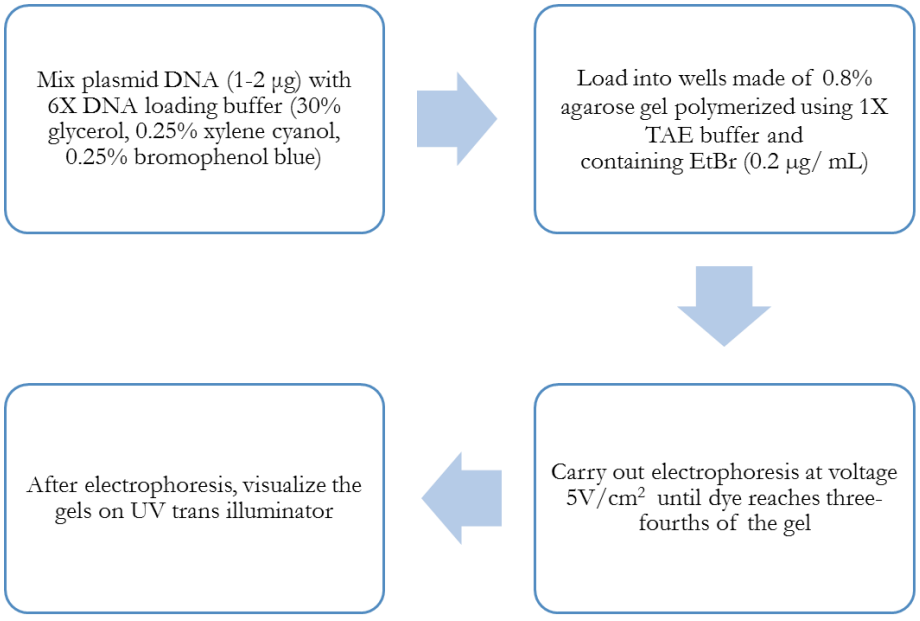


Flow chart 1: Total RNA isolation

Plasmid DNA isolation: Plasmid DNA was isolated using QIAGEN mini prep kit according to the manufacturer's protocol. A single positive colony was used to inoculate 5 mL LB broth which was allowed to grow overnight at 37°C and 180 rpm/ min. Bacterial cells were collected from this culture by centrifugation at 5000 rpm for 5 min. Cell pellet was then re suspended in 200 µL of buffer P1. To this 200 µL of P2 was added and mixed well by inverting the micro-centrifuge tube 3 to 6 times and incubated at room temperature for 5 min. 300 µL of neutralization buffer was added to this mixture, mixed well by inverting the tube and centrifuged for 15 min at 12,000 rpm. Supernatant containing plasmid DNA was collected and loaded into the column, centrifuged at 12,000 rpm for 1 min. The column was washed with wash buffer and plasmid DNA was eluted using elution buffer. The isolated plasmid DNA was quantified using NanoDrop.

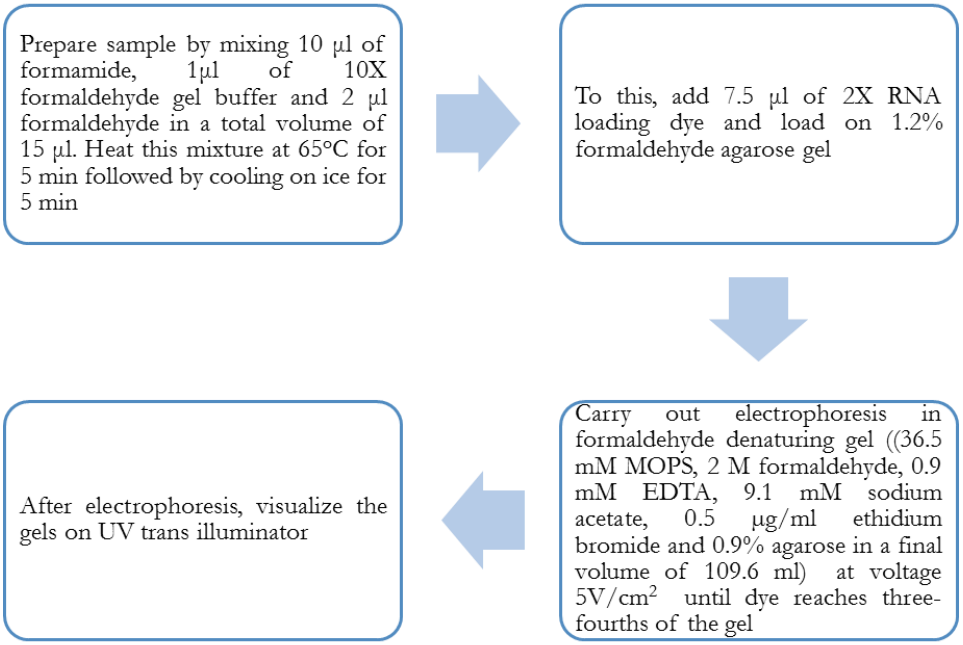
Agarose gel electrophoresis:

i) DNA electrophoresis:



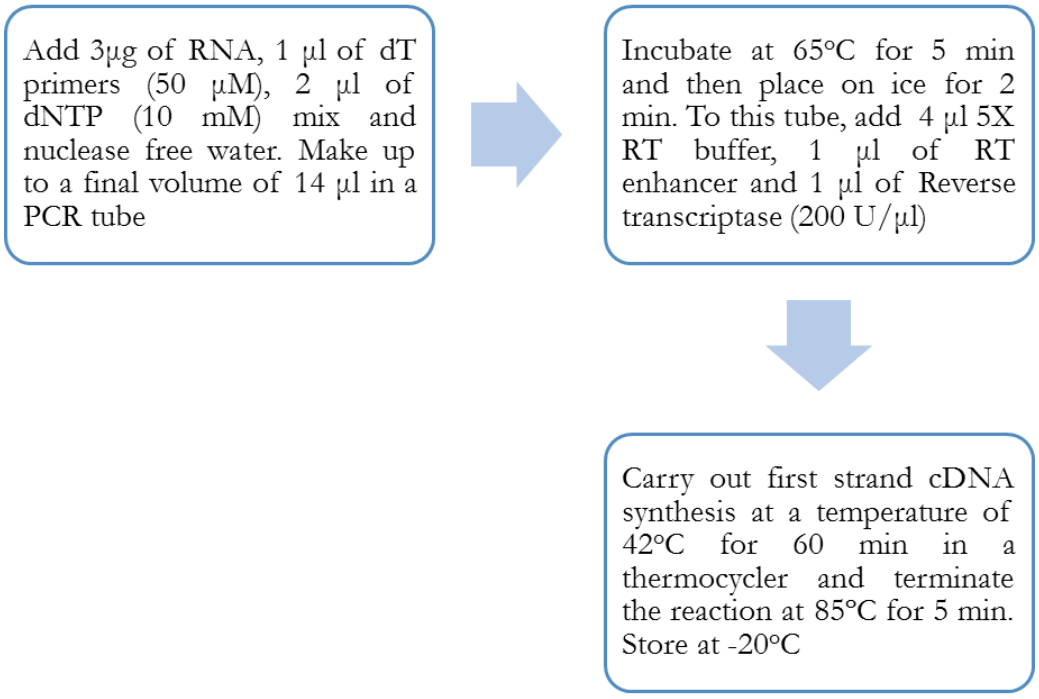
Flow chart 2: DNA electrophoresis

ii) RNA electrophoresis:



Flow chart 2: RNA electrophoresis

First strand cDNA synthesis:



Flow chart 4: cDNA synthesis

Real-time PCR analysis: 3 µg of total RNA was used for cDNA preparation using RevertAid^{TM} first strand synthesis kit (Thermo Scientific Co., USA). Gene expression was monitored using gene specific primers and SYBR green qRT-PCR kit (Applied Biosystems, CA, USA) in ABI-7500 fast real-time PCR system (Applied Biosystems, CA, USA). A 40-cycle two-step PCR was carried out in triplicates with 10 µL reaction volume containing the following components: 2.0 µL cDNA template (1:40 dilution), 1.0 µL of forward and reverse primers (10 picomoles) each, and 5.0 µL of 2X SYBR green PCR mix. Melting curve analysis was performed for each gene to check for specific amplification. 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, CA, USA). Relative quantification results were normalized with ribosomal protein S7 as endogenous control. All the results are represented as fold change in the transcript levels relative to the reference values obtained for their respective controls using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) except for tissue distribution and ontogeny profiles where relative transcript levels with respect to the endogenous control were represented.

Heterologous expression and protein purification of recombinant protein:

(i) Construction of Ccser2 and AjSeBP expression plasmids: The full length cDNA sequences of the proteins to be expressed were initially screened to identify zero cutter restriction enzyme (RE) sites present in the expression vector pET28a+. After analyzing the buffer compatibility for double digestion, specific REs were chosen (BamHI and XhoI for Ccser2; BamHI and HindIII for AjSeBP). The ORF regions were amplified using expression primers having selected RE sites at the 5' end of the forward and 3' end of the reverse primer. The amplicons thus obtained were ligated into pTZ57R/T vector and transformed into *E. coli* DH5α cells. The recombinant plasmids were isolated and digested using the above mentioned

REs to obtain the coding sequences with restriction site overhangs. These inserts were then ligated into the expression vector pET28a+ digested with the same set of REs. This ligation mixture was used to transform *E. coli* DH5α and the recombinant pET plasmids containing the expression constructs were obtained. These recombinant plasmids were then transformed into expression host *E. coli* BL21DE3.

(ii) Recombinant protein expression: Single positive colony was inoculated into 5 mL of LB broth with 50μg/mL of kanamycin, incubated for overnight at 180 rpm at 37°C. This overnight culture was used to inoculate 600 mL of LB broth with 50 μg/mL of kanamycin and allowed to grow at 37°C at 180 rpm till OD reached 0.4 at 600 nm. At this OD, culture was induced with 0.5 mM IPTG and allowed to grow for 14 hours at 18°C. This culture was centrifuged at 6000 rpm for 10 min to collect the bacterial cells. Pellet thus obtained was washed with PBS (137 mM NaCl, 10 mM Na2HPO$_4$, 1.8 mM KH_2PO_4 and 2.7 mM KCl) twice and stored at -20°C till further use.

(iii) Protein purification using Ni-NTA column: Bacterial cell pellet obtained was resuspended in equilibration buffer (50 mM Tris, 250 mM NaCl and 10 mM imidazole, pH 7.4) and cell lysis was done by sonication. After sonication, lysate was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was then applied to Ni-NTA column pre-equilibrated with equilibration buffer. After loading, the column was washed with 20 bed volumes of wash buffer (50 mM Tris, 250 mM NaCl and 40 mM imidazole, pH 7.4) and the bound protein was eluted using elution buffer (50 mM Tris, 250 mM NaCl and 250 mM imidazole, pH 7.4) in 1 mL fractions. Purity of the eluted protein fractions was checked by SDS-PAGE.

Protein estimation by Bradford method: Protein concentration in various samples was estimated using the micro-protein assay method of Bradford, 1976. Bovine serum albumin was used for preparation of standard. An aliquot of the sample was taken into a micro-centrifuge tube and the volume was adjusted to 100 μL with 10 mM TrisCl (pH 7.4). To this 1 ml of protein

reagent (1X dilution of 5X Bradford reagent, Bradford, USA) was added and mixed. After incubation for 10 min in the dark, absorbance was measured at 595 nm against blank. The protein content in the sample was calculated using a standard curve prepared using BSA.

Protein profile:

(i) Polyacrylamide gel electrophoresis: Sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the procedure described by Laemmli, (1970) using 1 cm long, 2.1 % stacking gel (pH 6.8) followed by a 7.5 cm long, 10% or 12% resolving gel (pH 8.8) depending on the size of the protein of interest. Tris-glycine (25 mM Tris and 192 mM glycine, pH 8.3) containing 0.1% SDS was used as the electrode buffer. Electrophoresis was carried out at 80 V for stacking of protein samples and 100 V for resolving until the tracking dye runs into the tank buffer. The samples were prepared by mixing an aliquot of the protein sample with sample buffer containing 0.125 M TrisCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.002% bromophenol blue followed by boiling at 100°C for 5 min.

(ii) Visualization of electrophoretically separated proteins/peptides on polyacrylamide gels:

(a) Coomassie staining: This was carried out according to the method of Wilson et al., (1983). The polyacrylamide gel containing the resolved protein samples was incubated for staining in commassie solution (0.025% Brilliant blue- R 250 in 40% methanol and 7% acetic acid) for 1 h followed by destaining with 5% methanol and 7.5% acetic acid to remove background staining and for visualization of protein bands. The incubation times for staining and destaining procedures were adjusted according to the sample needs.

(b) Silver staining: This method is relatively more sensitive when compared to the coomassie method of staining. It was carried according to the procedure of Blum et al., (1987). The gel

was incubated in a fixative solution (50% methanol, 12% acetic acid and 0.05% of formaldehyde) for 1 h to 4 h followed by three washes in 50% ethanol. Subsequently the gel was sensitized with sodium thiosulphate (20 mg/100 ml) for 30 sec to 1 min and rinsed thrice (1 min each) with distilled water. The gel was incubated in silver nitrate solution (0.2% silver nitrate with 0.076% of formaldehyde) with gentle agitation for 30 min. After incubating the gel was rinsed with distilled water thrice (1 min each) and developed with 6% sodium carbonate (w/v) containing 0.05% of formaldehyde (v/v). Once the protein bands appear on the gel, staining reaction was stopped by adding 12% acetic acid. Finally, the stained gel was thoroughly rinsed with distilled water and stored in 50% methanol.

Western blotting: The electrophoretically separated proteins were transferred to nitrocellulose membrane using Trans-Blot apparatus (Bio-Rad, USA) according to the procedure of Towbin et al., (1979). The gel and the membrane were first equilibrated in Towbin buffer (25 mM Tris, 192 mM glycine and 20% methanol) for 10 min, followed by transfer to the membrane for overnight at 25 V with 250 mA current limit. The transfer of protein to membrane was checked by reversible staining of the membrane with Ponceau S dye solution (1 mg/ ml of 5% acetic acid). After ensuring the transfer of the proteins onto the membrane, the stain was completely removed by washing the membrane with TBS (Tris buffered saline, 10 mM Tris-Cl (pH 7.4), 150 mM NaCl). For immunostaining, the protein blot was incubated with 5% skim milk powder (w/v) in TBS for 4 h at room temperature to block the non-specific binding sites followed by washing (15 min each) with TBS, TBST (TBS with 0.02% tween 20) and TBS. The blot was then incubated with the primary antibody diluted in TBS containing 5% skim milk powder (w/v) for 4 h to overnight. This was again followed by a thorough wash with TBS, TBST and TBS (15 min each). The blot was then incubated with secondary antibody (alkaline phosphatase (ALP)/ Horseradish peroxidase (HRP) conjugated anti-mouse or antirabbit IgG) diluted in 5% skim milk powder (w/v) in TBS for 2 h. The wash steps are repeated as mentioned above. The blot

was then processed with BCIP/NBT (ALP) or enhanced chemiluminiscence (ECL) substrate (HRP). The blots were imaged with Kodak Photo Imager.

Bacterial challenge: Live bacteria (*Escherichia coli* (DH5α), *Bacillus cereus,* and *Bacillus coagulans*) were grown at 37°C and 180 rpm/ min till optical density of 0.4 was reached at 600 nm. The bacterial cells were pelleted down, washed and resuspended in sterile phosphate buffered saline (PBS). 5 µl of this culture suspension was injected into the hemocoel at the penultimate abdominal segment of the fifth instar larvae using Hamilton microsyringe. The control insects received equal volume of sterile PBS. Please note that *B. cereus* were heat killed by autoclaving prior to the injection as live bacteria injection caused larval mortality in *C. cephalonica*. However, we did not observe any larval mortality in *A. janata* upon *B. cereus* injections. Hence, live bacteria were used to induce immune challenge.

Computer assisted analysis:

S. NO.	Tool	Function
1	BankIt	Submission of cloned nucleotide sequences to NCBI GenBank database
2	ClustalW	Multiple alignment analysis of DNA sequences
3	Multalin	Multiple alignment analysis of protein sequences
4	BLAST	Sequence similarity search
5	Phymol, Phylip	Construction and analysis of phylogenetic trees
6	NetPhos 2.01	Prediction of phosphorylation sites
7	NetNGlyc 3.1, NetOGlyc 1.0	Prediction of glycosylation sites
8	ExPASY	Analyze protein sequences and structures
9	VecScreen	Identification of vector contamination in nucleic acid sequences
10	SWISS MODEL	Homology modelling

List of bioinformatics tools used

Statistical analysis: Data are expressed as mean ± SEM of three independent experiments (n=3). Significance test for the means of the samples was performed using one way ANOVA (for data points with normal distribution) or Kruskal-Wallis analysis for those without normal distribution followed by Student Newman-Keul's test using Sigma Plot 12.0 software (Systat Software Inc., USA). The differences were considered significant at $P<0.05$.

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Chapter 1

Identification and characterization of Serpin2 from the rice moth, Corcyra cephalonica

1.1. Introduction

Serine Protease Inhibitors (Serpins) are one of the largest known family of protease inhibitors that were initially described as regulators of complement activation and blood coagulation in humans. However, till date more than 1500 serpins have been reported from different organisms across various phyla ranging from higher mammals to prokaryotes (Irving et al., 2002). Though majority of them function as protease inhibitors, some members serve other non-inhibitory functions like hormone transporters (Pemberton et al., 1988), molecular chaperones (Nagata, 1996) and tumor suppressors (Zhou et al., 1994).

1.1.1. Structure, function and mechanism of inhibition: A typical serpin is a single chain protein of 350 to 400 amino acid length and shows variable glycosylation. The tertiary structure constitutes a core of 7-9 α helices (denoted as hA to hI) and three β sheets (A, B and C). Majority of serpins with inhibitory functions contain a C- terminal reactive center loop (RCL) that is exposed above the body of the serpin scaffold and is responsible for interaction with target proteases, with whom they interact. Upon interaction with the target protease, a scissile bond between P1 and P10 residues of the RCL is cleaved as a result of which the target protease is ensnared to the carbonyl backbone of the P1 residue. RCL then gets inserted into the β sheet A of the serpin core and complete insertion of the RCL translocates the protease to the other side of the serpin, distorting its active site and thus rendering the protease inactive (Fig. 2) (Reichhart, 2005; Law et al., 2006).

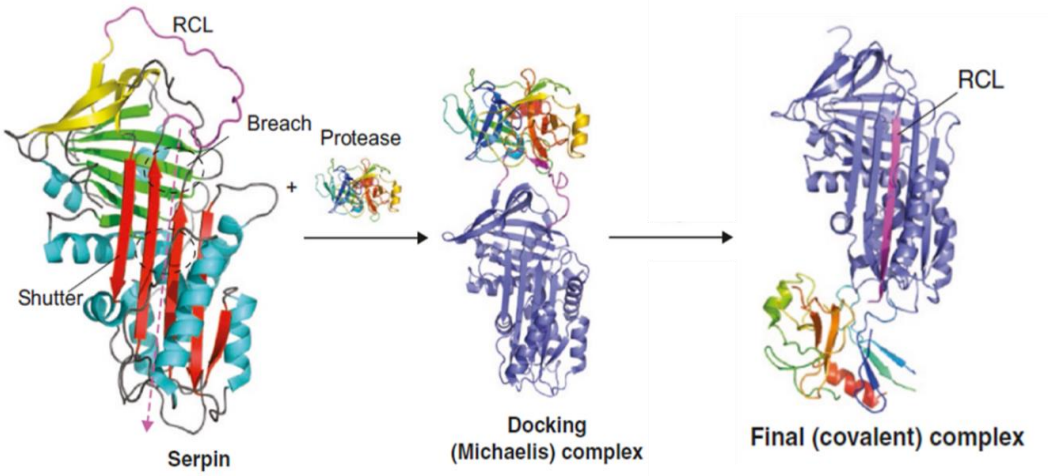

Fig. 3: Schematic presentation showing the inhibition mechanism of serpin (modified from Law et al., 2006).

1.1.2. Functional diversity of serpins in insects: Majority of the characterized arthropod serpin functions involve the control of endogenous proteases involved in processes like coagulation, prophenol oxidase activation, cytokine activation etc. (Meekins et al., 2016). All the aforementioned processes usually employ a cascade of proteolytic reactions resulting in a rapid and efficient response(s). In case these responses are not regulated and limited to be local and transient, they pose severe damage to the insect survival. Hence, these processes are always under tight regulation by serpins and this type of regulatory mechanism is an example of evolutionarily conserved strategies in the innate immune responses of animals (Potempa et al., 1994). Apart from the inhibitory function, serpins also contribute to host immunity by acting directly on the invading microbes. For example, proteinases are used by some entomo-pathogenic fungi to penetrate their host cuticle and they are also known to contribute to the virulence of bacterial pathogens (Meekins et al., 2016). Hence, some serpins have evolved to confer protection to the hosts by inactivating the proteinases released by the invading microbes. Serpin1 variants of *Manduca sexta* (Jiang and Kanost, 1997) and FP1 of *Bombyx mori* (Eguchi et al., 1993) are some of such examples. In some insects, serpins are present in the male accessory glands and during mating, these are transferred to the female suggesting their probable role in reproductive biology (Sirot et al., 2008, 2011). Further, certain members of the serpin

superfamily like Spn27A and Spn88Ea are reported to have a role in dorso-ventral axis formation during embryonic development and wing expansion respectively in *Drosophila melanogaster* (Hashimoto et al., 2003; Ligoxygakis et al., 2003). Serpins identified in the saliva of hematophagous arthropods are reported to play a vital role in blood feeding (Stark and James, 1995; Francischetti et al., 2009). In *D. melanogaster*, an intracellular serpin, Spn4a was reported to inhibit furin thus suggesting a role in the process of cellular secretion (Bruning et al., 2007).

Holometabolous insects including Lepidoptera undergo molting as well as metamorphosis, a process governed primarily by morphogenetic hormones (Gilbert et al., 2002). Controlled precision of differential titers of these hormones, mainly the juvenile hormones (JHs) and ecdysteroids, primarily 20-hydroxyecdysone (20E) and their interaction is required for normal growth and development (Gilbert *et al.*, 1996; Riddiford, 2012). Studies from our laboratory and others have reported that interplay between JH and 20E govern, not only the induction but also suppression of various genes in stage specific manner during the development (Shanavas et al., 1996; Wu et al., 2006; Chaitanya and Dutta-Gupta, 2010; Chaitanya et al., 2011; Dubrovsky and Bernard, 2014; Venkat Rao V, 2016). However, knowledge regarding the hormonal control of many genes, including serpins is limited. *In vitro* and *in vivo* experiments by Kanost et al., (1995) showed the negative regulation of *serpin 1* by 20E in *Manduca sexta*. However, to the best of our knowledge the effect of JH on serpin expression has not been evaluated yet. In the current study, we analyzed the effect of both 20E and JH on *Serpin2* transcript levels as an attempt to understand the role of these morphogenetic hormones in the regulation of insect immune function.

1.2. Materials and methods

1.2.1. Primers used:

S. No.	Primer Name	Primer Sequence (5' – 3')
Degenerate and specific primers for obtaining partial fragment		
1	Cc PI FM1	CCR YTR TCM GCW GAR WWC KTG YTR GC
2	Cc PI RM1	CGC CYK YYT CRT TSA CTT CRA TG
3	Cc PI FM2	TCA AYG CWC TYT AYT TCA ARG G
4	Cc PI RM2	TCR GTY TCR AYC TTR AAY TTS GG
5	CC A800 RP	GAA TGC CTT TTG CAC AGC
6	CC A800 FP	CAG ATG ATG ATT CCA TAC G
RACE		
7	Cc PI RACE 5P	CCT CCG CAC CCA AAT TGA AAT
8	Cc PI RACE 3P	AGA GGC TAG TAT GTT GAT CGT
9	Cc PI RACE 3N	GTA CGC GTG ACA CTT CCT AAG
10	Cc PI RACE 5N	GTC TTG CAG ATC GTA TGC ACC
Cloning complete cds		
11	CC SER FP	ATG CGG ACT ATC TAT ACG TTG ATT CC
12	CC SER RP	CTA TCC AAA GTA TGA AGT GTA AAA GAA
qRT- PCR		
13	CC SER2 RT R	GCA TCA TTC TTG ATT TCG TCT TGC
14	CC SER2 RTF	GCT TAC TTC ACT CGG GAT ACC AGA TG
Heterologous expression		
15	CC SER2 EXP FN	ATA CGG ATC CAT GGA CGC CAA CTC ATT
16	CC SER2 EXP F	ATT CGG ATC CAT GCG GAC TAT CTA TAC
17	CC SER2 EXP R	GTC ACT CGA GCT ATC CAA AGT ATG AAG

Table 2: Primers used in the identification, cloning, heterologous expression and relative expression analysis of *Serpin2* from *Corcyra cephalonica* (*CcSer2*).

1.2.2. Surgical manipulations

Thorax ligations: It is a surgical procedure used to deplete the endogenous levels of hormones (Dutta-Gupta and Ashok, 1988). Larvae to be ligated (usually late larval instar) were narcotized by placing them on ice for 10 min. A silk thread loop is slipped around the head of the narcotized larvae and thorax is ligated behind the first pair of prolegs (Ashok and Dutta-Gupta, 1991). The larval body region anterior to this ligature was cut using sterile scissors, the wound was dressed with trace of antibiotics mixture (1:1, streptomycin: penicillin) and sealed with wax (beeswax and paraffin, 0.1:10). These ligated insects were then placed in petri dishes which were covered with moist filter paper to prevent desiccation.

1.2.3. Hormone treatments: Prior to the administration of exogenous hormones, it is essential to exhaust the endogenous hormone levels. This is achieved by a surgical procedure called thorax ligation as described above. These thorax ligated late-last instar larvae (post 24 h) were used for hormone treatments. 80nM of 20-hydroxyecdysone (20E) prepared from the stock solution obtained by dissolving 1mg of hormone in 100µL of ethanol and finally diluted to 1ml with distilled water was injected into the hemocoel of thorax ligated larvae. Control insects received 0.05% ethanol (solvent for 20E). To study the effect of juvenile hormone, methoprene, a JH analog was used. 10 µl of 0.4 pg/µl of methionine was topically applied on the abdomen of thorax ligated larvae. Control insects were applied with acetone.

1.2.4. Activity studies and enzyme assays:

(i) In-gel zymography: For the visualization of inhibitor activity of recombinant CcSer2, purified recombinant CcSer2 protein was subjected to non-reducing SDS-PAGE with the resolving gel co-polymerized with 0.1% (final concentration) gelatin. After the completion of the electrophoretic run, the gel was soaked in 2.5% Triton X-100 for one hour. The gel was then washed with distilled water for several times and was incubated in enzyme solution (0.1% trypsin in 0.05 M Tris-HCl buffer, pH 8.2) at 4°C for 30 min which was followed by incubation for 90

min at 37°C. The gel was then silver stained to visualize inhibitory activity bands. Uninduced bacterial cell lysate was used as negative control.

(ii) BAPNA (Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride) assay: Purified recombinant CcSer2 of different concentrations (final volume adjusted to 500 µl with assay buffer (50 mM Tris-HCl (pH 8.2) containing 20 mM $CaCl_2$) was incubated with 500 µl trypsin (stock concentration: 20 µg/ml) for 15 minutes at 37°C. 1ml of BAPNA was added to this mixture and incubated at 37°C for 45 minutes. Reaction was stopped by addition of 200 µl of 30% acetic acid. Optical density was measured at 410nm and percentage of trypsin inhibition activity was calculated.

1.3. Results and discussion

1.3.1. Cloning of *CcSer2 from* whole body of *C. cephalonica* late-last instar (LLI) larvae:

Degenerate primers were designed based on the reported *Serpin2* sequences from other lepidopteran insects in the NCBI database (details are provided in the general materials and methods section). A partial fragment of 800 bp was obtained using first strand cDNA synthesised from the total RNA isolated from LLI larval whole body of *C. cephalonica*. The partial fragment obtained was then further confirmed using gene specific primers and the sequence identity was checked by nucleotide sequencing and BLAST analysis (Fig. 4A). This partial sequence was used to isolate complete coding sequence using RACE (rapid amplification of cDNA ends) strategy. 5' end of the partial cds was amplified using the adaptor specific forward primer and gene specific reverse primer (details in materials and methods section). Fig. 4B shows the amplification of 800 bp fragment by 5' RACE reaction. 3' end was amplified using the adaptor specific reverse primer and gene specific forward primer (details in materials and methods section). Fig. 4C shows the amplification of 750 bp fragment obtained by 3' RACE reaction. After obtaining the three partial fragments, full length cDNA from LLI larval whole body was cloned using a new set of forward and reverse primers designed based on the

sequences obtained through RACE reactions. Fig. 4D shows *C. cephalonica Serpin2* complete coding sequence obtained in the present study which was sequenced and analyzed.

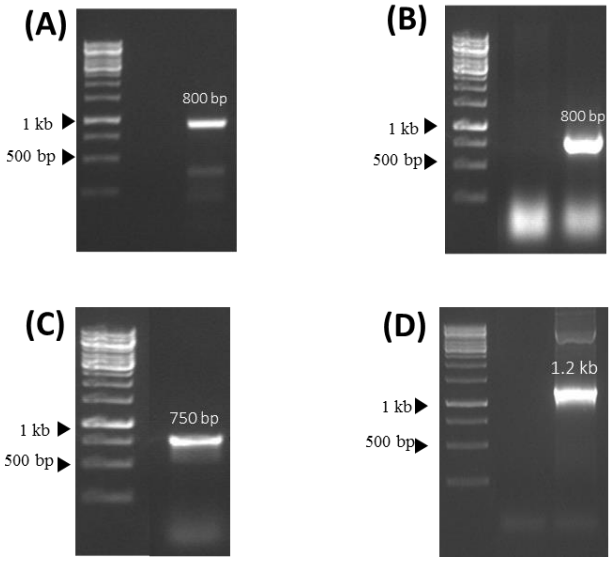

Fig. 4: Agarose gel images of amplicons obtained with (A) specific primers for *CcSer2* partial fragment (B) 5' RACE (C) 3' RACE and (D) complete coding sequence of *CcSer2*

Detailed analysis of *CcSer2* nucleotide sequence revealed that the total length of the cDNA is ~1.9 kb with an ORF of ~1.2 kb, which has ATG initiation codon and TAG stop codon. The 5' UTR region obtained is 263 bp long while 3' UTR region is 387 bp long with AATATATA polyadenylation signal (Fig. 5).

```
acatggggcaatgacattgttttgtttacaaaaacatcacaacatgtacctataaaatctaatacaacaacgtctagcaaacagttcaattaaata
cgtgaaaattttatagaggaaagataagatacgtgtacacgagataattaattttattggcaagctcggcgtagctaatcagcaaattgattagta
ttttcagcatacctccctatttaaaaaaacttttacattaatcgttgctgatttgcacttcttatctatcg
 1 atgcggactatctatacgttgattccactgtttctactgttacaagcaaaatattcttcaacggacatggacgccaactcattatcctcg
       M  R  T  I  Y  T  L  I  P  L  F  L  L  L  Q  A  K  Y  S  S  T  D  M  D  A  N  S  L  S  S
    91 gaaatactgaaattctctgcaaaattctgtaatgaacttgataaaagtgtgagtgtgatatcatcacccctctcagcagaatatttattg
       E  I  L  K  F  S  A  K  F  C  N  E  L  D  K  S  V  S  V  I  S  S  P  L  S  A  E  Y  L  L
   181 gcacttttagctctgggtaccacggatgttgcccataatgaactgcttacttcactcgggataccagatgatgattccatacgttcatct
       A  L  L  A  L  G  T  T  D  V  A  H  N  E  L  L  T  S  L  G  I  P  D  D  D  S  I  R  S  S
   271 ttcacatcagtgtcatctaaattgaaatcaatcaaaggcgtcacattgaatgttgccaatcacatttacataatggaaggtgcatacgat
       F  T  S  V  S  S  K  L  K  S  I  K  G  V  T  L  N  V  A  N  H  I  Y  I  M  E  G  A  Y  D
   361 ctgcaagacgaaatcaagaatgatgccatcaaagtttttgatgccggcattgagaagttggatttcaatttgggtgcggaggcagctcgc
       L  Q  D  E  I  K  N  D  A  I  K  V  F  D  A  G  I  E  K  L  D  F  N  L  G  A  E  A  A  R
   451 catattaacaaatgggttgaagataaaacaaaccagaaaattaaagatttgctccccctgactgcatcagtggtgataccaggcttgtt
       H  I  N  K  W  V  E  D  K  T  N  Q  K  I  K  D  L  L  P  P  D  C  I  S  G  D  T  R  L  V
   541 cttgttaatgctttgtatttcaagggaacatgggagagacaatttgagaagcatcttacaatggaaaagcctttcgatgtgaacaataat
       L  V  N  A  L  Y  F  K  G  T  W  E  R  Q  F  E  K  H  L  T  M  E  K  P  F  D  V  N  N  N
   631 actaaagtgaatatccctatgatgtttaaagaagacactttccagtacggtgaaagtgcagaattacaggcccagttattggaaatgtac
       T  K  V  N  I  P  M  M  F  K  E  D  T  F  Q  Y  G  E  S  A  E  L  Q  A  Q  L  L  E  M  Y
   721 tatgtcggtaaagaggctagtatgttgatcgtgctaccaaaggaaatcgagggattagccgatgtacttaagaaattggcggatggttat
       Y  V  G  K  E  A  S  M  L  I  V  L  P  K  E  I  E  G  L  A  D  V  L  K  K  L  A  D  G  Y
   811 gatctcatgaacgatgtaaacagaatgtattcatctaaagtacgcgtgacacttcctaagttcaaaattgagactgaaattgatttagga
       D  L  M  N  D  V  N  R  M  Y  S  S  K  V  R  V  T  L  P  K  F  K  I  E  T  E  I  D  L  G
   901 tctctattaccaaagcttggaattaaacaaatattcaaacaagaaaattcaggtttaactaaactgctaaatagccctgaaagtctttat
       S  L  L  P  K  L  G  I  K  Q  I  F  K  Q  E  N  S  G  L  T  K  L  L  N  S  P  E  S  L  Y
   991 gtatcgaaagctgtgcaaaaggcattcatcgaagtcaatgaagaaggttcggaggccgcagctgcgactgctatggtggccatgatgtgt
       V  S  K  A  V  Q  K  A  F  I  E  V  N  E  E  G  S  E  A  A  A  A  T  A  M  V  A  M  M  C
  1081 tccgccattttagatcctcctcctgtgcccgaatttactgcggaccgaccattcttggctgtaattttgattgacaacgttccattcttt
       S  A  I  L  D  P  P  P  V  P  E  F  T  A  D  R  P  F  L  A  V  I  L  I  D  N  V  P  F  F
  1171 tacacttcatactttggatag
       Y  T  S  Y  F  G  *
aattaatctttgctctcttataaattttaatatttattttgaattttgcataatattttgtaatactagtatatatgtaactggttacttgttttc
gtaatacatatacgagtattatgttgaatatataatttgttacttcttcttcttttgcatttatatttaataaatttttatatatgttgaatgtgt
atatattgtaactattgaaagtataaaaatagtaaatacaagtatattgtacaaaaaaaaaaaaaaaaaaaaaaaaagtactctgcgctgatacctc
tgctaatgatttctcgggcccgtcgactgcaaaggtagatacacatcttgtcatatatatcagagtggtattagagcttgccgtaatcgaacccca
aaa
```

Fig. 5: Full length cDNA sequence (with open reading frame (ORF) and 5' and 3' untranslated regions) and deduced amino acid sequence of CcSer2. The 5' UTR and 3'UTR regions are represented in black color, top and bottom. The coding sequence and the deduced amino acid sequence are represented in black and red colors respectively. The polyadenylation signal sequence (AATATATA) in the 3' UTR region is underlined. Conserved motifs in the protein structure that form the reactive center loop (RCL) are italicized and underlined.

In silico analysis of deduced amino acid sequence of CcSer2 shows that it has 397 amino acids and a molecular weight of 45 kDa. Further, the sequence shows the presence of conserved reactive center loop (RCL) site. The sequence also shows the presence of putative

phosphorylation and glycosylation sites with an N-terminal signal peptide of 1-21 amino acids (Fig. 6). The theoretical pI of CcSer2 is 4.91.

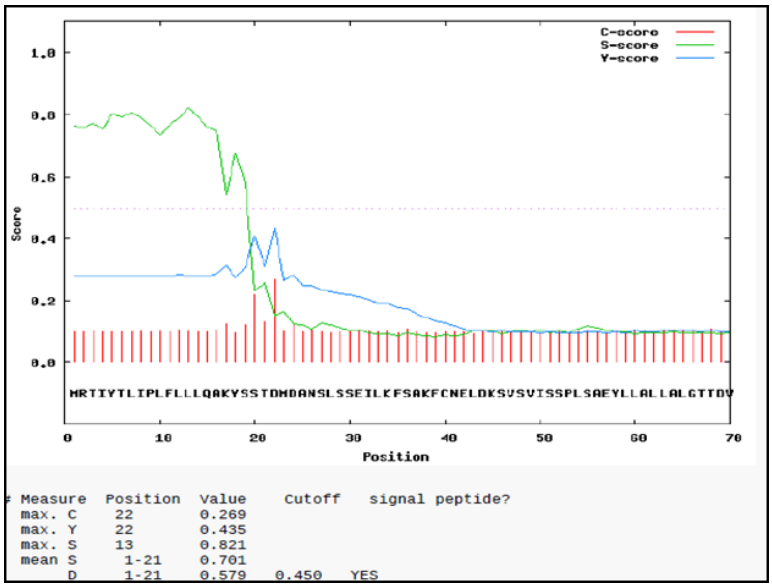

Fig. 6: Signal peptide analysis using ExPASY SignalP tool predicts the presence of putative signal peptide in CcSer2 protein sequence.

ClustalW analysis of the deduced amino acid sequence of CcSer2 cloned in the present study with other reported Serpin2 sequences show that it has more than 50% homology with Serpin2 sequence from *Bombyx mori* (GenBank Accession No. AF242200.1), *Lonomia obliqua* (GenBank Accession No. AY829815.1), *Manduca sexta* (GenBank Accession No. U79184.1), and *Spodoptera exigua* (GenBank Accession No. EF584499.1) (Fig. 7). Based on the phylogenetic tree analysis presented in figure 7, we suggest that *CcSer2* is closely related to Serpin2 from *Manduca sexta* and *Bombyx* species.

Fig. 7: Multiple alignment of CcSer2 with Serpin2 sequences from related insect species using ClustalW

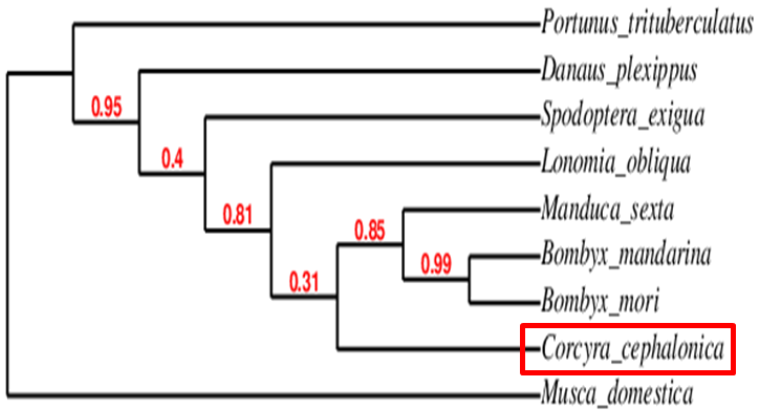

Fig. 8: Sequences were aligned using MUSCLE 3.8.31 and phylogenetic tree was constructed using PhyML software. The Serpin2 sequences used in the tree construction include *Portunus trituberculatus* (GenBank Accession no: JF412657.1), *Danaus plexippus* (GenBank Accession no: EHJ68881.1), *Spodoptera exigua* (GenBank Accession No. EF584499.1), *Lonomia obliqua* (GenBank Accession No. AY829815.1), *Manduca sexta* (GenBank Accession No. U79184.1), *Bombyx mandarina* (GenBank Accession No. KR003725.1), *Bombyx mori* (GenBank Accession No. AF242200.1), *Corcyra cephalonica* and *Musca domestica* (GenBank Accession No. KJ872511.1). CcSer2 sequence has highest homology with *Bombyx* species and *Manduca sexta.*

Homology modelling of CcSer2 yielded a valid model with maximum number of amino acids in the allowed regions of Ramachandran plot. The model also shows the presence of RCL, characteristic of inhibitory serpins (Fig. 9).

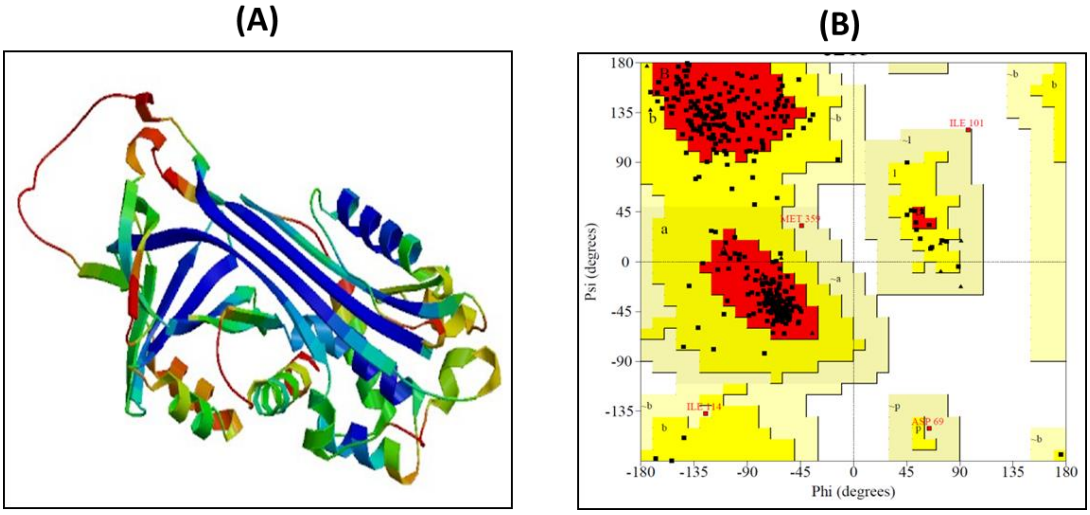

Fig. 9: Homology modeling of CcSer2. (A) Homology model of CcSer2 generated using *Anopheles gambiae* Serpin2 (PDB ID: 3PZF) shows the presence of RCL. (B) Ramachandran plot for the protein homology model generated confirms the validity with most of the amino acids in the allowed regions.

Present study clearly reveals that the Serpin2 identified from *C. cephalonica* larvae has characteristics which are not only seen in other lepidopteran insects but also from other phyla. Presence of RCL in CcSer2 once again is suggestive of its inhibitory role which might be important during the postembryonic development of *C. cephalonica*.

1.3.2. Ontogeny and tissue distribution of *CcSer2*: Serpins exhibit variation in the developmental and tissue distribution pattern among insects. Hence, developmental and tissue distribution profiles help in providing insights into the physiological roles of individual serpins. Ontogeny and tissue distribution analysis was carried out by semi-quantitative and qRT-PCR. Ontogeny results show that *CcSer2* is expressed during embryonic as well as postembryonic development (Fig. 10A). The results further show that *CcSer2* expression is fairly low in the embryo, first and second instar larvae and thereafter gradually increases upto the 5th instar, reaches its maximum during the last instar development and declines in pre-pupal and pupal

stages. These findings corroborate well with the previous reports on insect serpins (Gulley et al., 2013).

Tissue distribution profile shows that *CcSer2* is predominantly expressed in the larval gut and hemocytes (Fig. 10B). This expression pattern could be indicative of its role in digestion and immunity. However, it is different from the pattern observed in other insect models. For example, in *B. mori*, *Serpin2* is expressed in all the larval tissues (Pan et al., 2009) while in *M. sexta* its expression is confined to the hemocytes (Gan et al., 2001). The current observation reiterates that same serpin(s) in different insect models vary in their expression pattern and hence the need for individual characterization in different insect models becomes important.

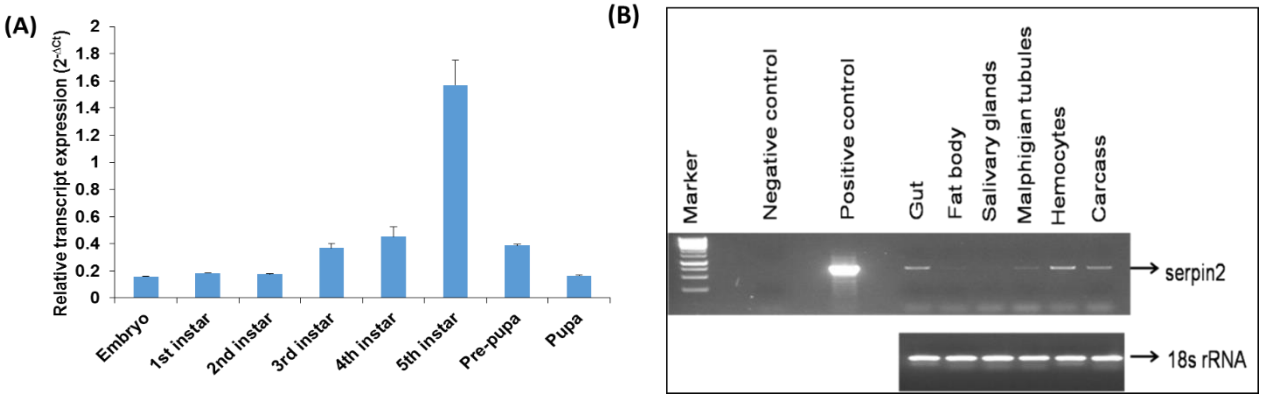

Fig. 10: Ontogeny and tissue distribution of *CcSer2*. (A) qRT-PCR analysis showing expression of *CcSer2* during various stages of larval development. (B) Semi quantitative PCR shows that *CcSer2* is predominantly expressed in the larval gut and hemocytes.

1.3.3. Heterologous expression of CcSer2, protein purification and activity studies:

Recombinant expression and purification of CcSer2 was performed according to the protocol described in the general materials and methods section. Figure 11A shows the induction of recombinant CcSer2 by IPTG. As CcSer2 specific antibodies were unavailable, the induction was confirmed by performing western blot using His-tag antibodies that detected the presence of recombinant protein both in the supernatant and pellet (Fig. 11B). Figure 11C shows pure recombinant CcSer2 obtained after affinity chromatography. Further, activity studies were carried out to confirm that the recombinant CcSer2 obtained is biologically active and

functional. Gelatin PAGE shows the presence of single band at 46 kDa corresponding to CcSer2 (Fig. 12A) and BAPNA assay further reconfirms that with increasing concentration of CcSer2 there is an increased percentage of trypsin inhibition (Fig. 12B).

Detailed analysis from other insects have also demonstrated the functional activity of recombinant Serpin2, however, there is a difference in the target protease recognition. Recombinant Serpin2 from *Manduca sexta* lacked inhibitory activity against all the tested proteinases except for human cathepsin G (Gan et al., 2001) whereas the Serpin2 characterized from *Bombyx mori* inhibits trypsin (Takagi et al., 1990). This functional variation of serpins in related insect models supports the need for individual characterization.

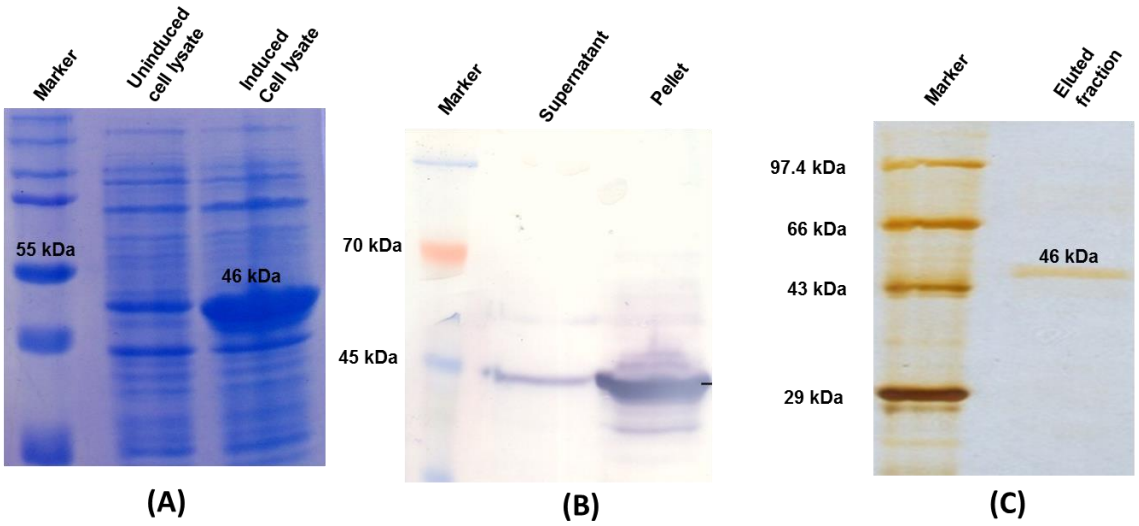

Fig. 11: Heterologous expression and purification of CcSer2. (A) SDS-PAGE profile showing the induced expression of CcSer2 at 46 kDa in the bacterial cell lysate (B) Western blot with His-tag antibodies confirming CcSer2 induction both in the pellet and supernatant (C) SDS-PAGE gel showing the pure recombinant CcSer2 obtained after affinity chromatography (Ni-NTA).

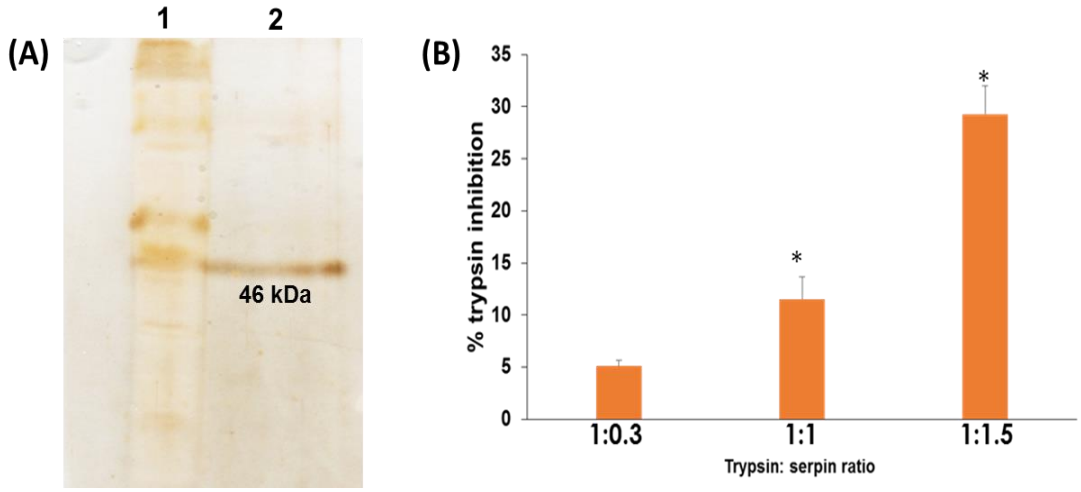

Fig. 12: Activity assays showing that purified recombinant CcSer2 is functional (A) In-gel zymogram (Gelatin PAGE) showing the presence of single band corresponding to CcSer2 at ~46 kDa [Lane 1: Bacterial supernatant, Lane 2: Purified recombinant CcSer2]. (B) BAPNA assay showing the increase in the percentage of inhibition with addition of increasing quantity of recombinant CcSer2. * indicates significant difference with P≤0.05

1.3.4. Effect of bacterial challenge: To study the effect of bacterial infection on *CcSer2* expression, mid-last instar larvae of *C. cephalonica* were injected with *E. coli* or *B. cereus* as described in the general materials and methods section. Larval gut, fat body and hemocytes were collected at different time points i.e., 1, 3, 6, 9, 12 and 24 hours post injection (hpi) and *CcSer2* transcript levels were quantified using qRT-PCR. As seen in figure 13, results show that challenge with both the bacterial species caused up-regulation of *CcSer2* levels in larval gut, fat body and hemocytes as early as 1 hpi. In larval fat body, *CcSer2* expression level increased gradually till 9 hpi and then declined and reached to basal level by 24 hpi but larval gut showed an early increase in the expression level when compared with the fat body and the level declined to a low range by 9 hpi. On the other hand, hemocytes did not show any significant alteration except for a mild upregulation observed at 6 hpi in *E. coli* challenged group.

It is interesting to note that the fold change in the expression of *CcSer2* gene varied among the tissues and fat body showed the most prominent response among the three tissues tested followed by the gut and hemocytes. However, the tissue distribution pattern seen in the

earlier section showed negligible expression of *Serpin2* in fat body under normal conditions (Fig. 10B). This differential response observed among various tissues could be primarily due to the differences in their developmental origin and functions they perform during larval stages. Insect fat body is a multifunctional metabolic tissue which is known to act as a storage organ for lipids and other macromolecules. Lipid mobilization to hemolymph under infection has been reported to play a key role in pathogen recognition and mobilized lipids are required for membrane biogenesis at the site of infection (Arrese and Soulages, 2010). Furthermore, fat body is a well-known site for the synthesis of antimicrobial peptides which play important role in innate immune function (Hoffman, 1995; Ganz, 2003). Upon bacterial challenge transcription of antimicrobial peptide genes are rapidly upregulated in fat body as well as in other tissues and within two hours large quantity of them are released into hemolymph (Shelby, 2014). The results obtained in the present study also suggest that in *C. cephalonica* larval fat body is the most responsive tissue to bacterial challenge.

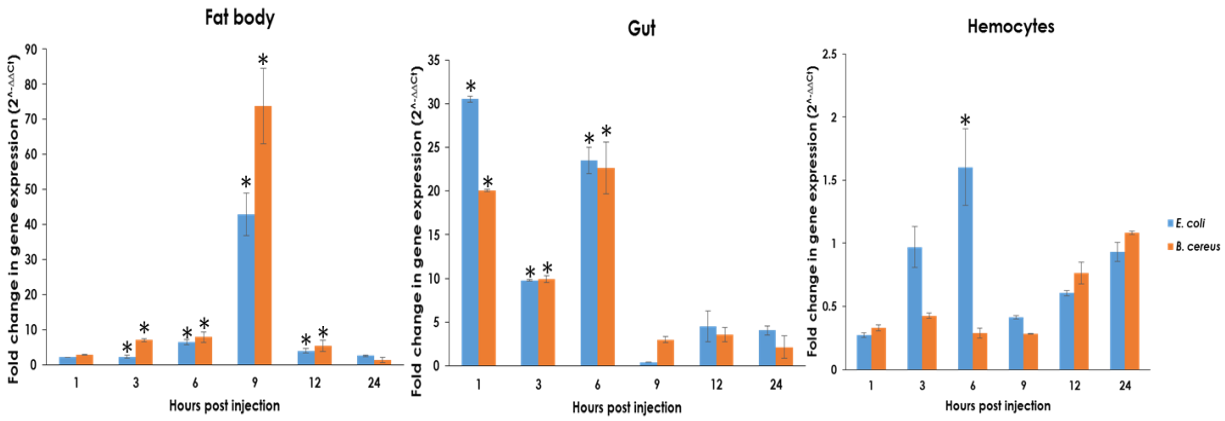

Fig. 13: Expression analysis of *CcSer2* upon bacterial challenge in the larval gut, fat body and hemocytes. *CcSer2* expression was upregulated upon bacterial challenge. The effect was more prominent in the fat body followed by gut and hemocytes. Data (n=5) were expressed as mean ± SEM (*, $P < 0.05$; ANOVA followed by Student–Newman–Keuls' post hoc test).

1.3.5. Effect of insect morphogenetic hormones: For this study, 24 h post thorax ligated LLI larvae with negligible endogenous hormone were treated with 20 hydroxy ecdysone (20E), methoprene or both (20E+ methoprene) to study the individual and combined effect(s) of these

hormones on *CcSer2* gene expression. Hormone treatments were given as described in the materials and methods section. Whole body was used to isolate total RNA and *CcSer2* expression analysis was carried out using qRT-PCR. Results of qRT-PCR (Fig. 14) clearly show that the transcription of *CcSer2* was up-regulated upon methoprene application which is a well-known and widely used juvenile hormone (JH) analogue. The increase in the expression was gradual and time dependent upto 6 h, after which it declined. Results also reveal that 20E had no direct effect on the expression of *CcSer2*. However, 20E in the presence of methoprene showed negative effect, suggesting that it probably blocks and/ or down regulates JH dependent expression.

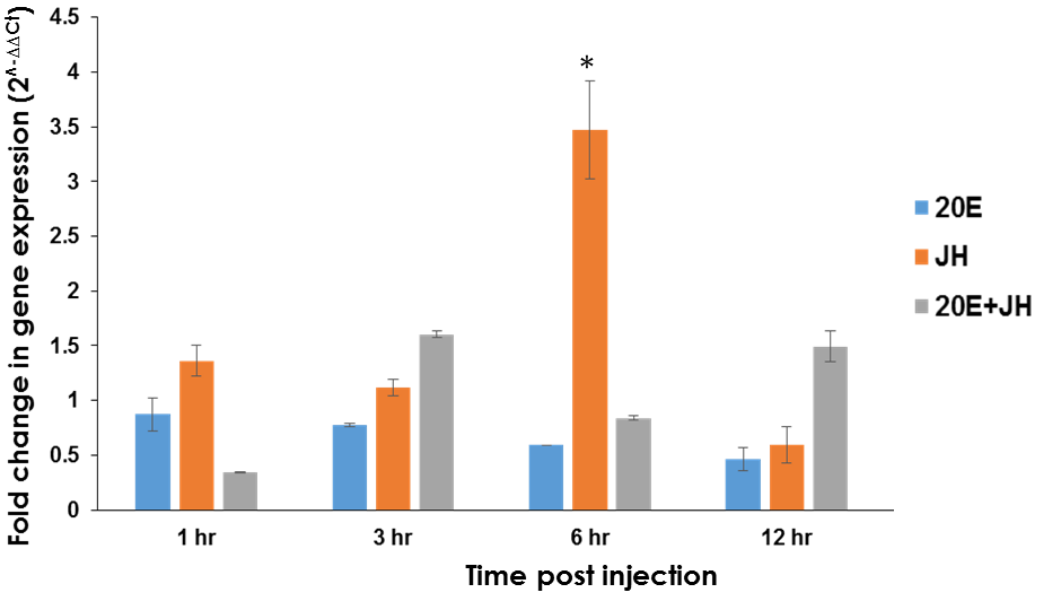

Fig. 14: Effect of insect morphogenetic hormones (20E and JH) on the expression of *CcSer2*. Expression was JH dependent with 20E having no direct effect. Data (n=5) were expressed as mean ± SEM (*, $P < 0.05$; ANOVA followed by Student–Newman–Keuls' post hoc test).

Morphogenetic hormones, JH and 20E are highly versatile in their functions and are known to coordinate various aspects of growth and development in insects. For the past three decades, several studies reported the immune modulatory actions of 20E and JH. However, it is important to note that the effects of 20E and JH are usually antagonistic (Flatt et al., 2008). The nature of the effect of these hormones on immunity (activating/inhibiting) is not universal and

changes with the nature of the immune response studied. For example, 20E application was shown to induce robust synthesis of AMPs following immune stimulation in *Drosophila* (Heyland et al., 2008) and caused an enhanced nodulation response in larvae of the flesh fly *Neobellieria bullata* (Franssens et al., 2006). A genome-wide microarray analysis revealed that 20E inhibits innate immunity while JH acts as an immune-activator in *B. mori* fat body during the postembryonic development (Tian et al., 2010). Current study also proves the antagonistic actions of these hormones where JH upregulated and 20E blocked the JH dependent transcription. Further studies have to be carried out to comprehend the basis and mechanisms of this hormonal regulation. However, present study clearly shows the *ser2* expression during postembryonic development like many other larval genes in *C. cephalonica* is not only developmentally but also hormonally regulated which is widely demonstrated by our group (Chaitanya and Dutta-Gupta, 2010; Manohar et al., 2010; Chaitanya et al., 2011; Venkat Rao et al., 2016).

1.4. Summary

In this study, we cloned the complete mRNA coding sequence of *Serpin2* from *C. cephalonica* using degenerate primer and RACE (5' and 3') strategies. Open reading frame (ORF) of *CcSer2* is ~1.2 kb which codes for a protein of molecular weight 46 kDa. *In silico* analysis revealed the presence of conserved motifs like the RCL and a putative signal peptide sequence at the N-terminal region of *CcSer2*. Expression levels of *CcSer2* varied among the larval tissues with higher levels in the gut and hemocytes when compared with fat body, which differed from the tissue distribution profile of earlier reported serpins from other lepidopteran insects. Ontogeny profile of *CcSer2* shows a developmentally regulated expression pattern which could be hormonally controlled as well. This was further confirmed by hormone treatment studies where JH dependent expression of *CcSer2* was demonstrated which was partially blocked by 20E application. Transcriptional upregulation of *CcSer2* upon immune stimulation using bacterial pathogens (both gram positive and gram negative) in three larval tissues: gut, fat body and

hemocytes clearly suggests its possible role in immunity. However, the upregulation was prominent in the fat body followed by gut and hemocytes. Recombinant CcSer2 protein was expressed using the pET28a vector system in *E. coli* BL21 host. Activity studies (in gel zymography and BAPNA assay) with recombinant CcSer2 showed that the identified Serpin2 has trypsin inhibitory activity.

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Chapter 2

Identification and characterization of Selenium Binding Protein (SeBP) from castor semilooper, A. janata

2.1. Introduction

2.1.1. Selenium (Se): Selenium is a non-metal and in nature it exists in multiple oxidation states. Se is toxic in large quantities but trace amounts of this metal are necessary for biological functions in almost all the organisms (Tapiero et al., 2003). The physiological role of Se is primarily in the antioxidant response, as a key component of the antioxidant enzymes like thioredoxin reductase and glutathione peroxidase etc. (Tinggi et al., 2008). Se supplementation increased the cellular production and activity of certain Se containing antioxidant enzymes in trophoblast cell lines (Khera et al., 2016). Other hypothesized functions include maintenance of cytochromeP450 system, DNA repair and immune system functions (Burk, 1989). Deficiency of Se in human diet causes different pathological disorders like Keshans and Kaschin-Beck disease, rheumatoid arthritis and cataract (Behne and Kyriakopoulos, 2001).

2.1.2. Selenium containing proteins: Se containing proteins identified till date can be classified into three groups. They are:

(i) Proteins that non-specifically incorporate Se in the form of selenomethionine and selenocysteine. Eg: Methionine rich proteins that occur in high concentrations.

(ii) Proteins that specifically incorporate Se in the form of genetically encoded selenocysteine (Selenoproteins). Eg: Thioredoxin reductase, glutathione peroxidase and iodothyronine deiodinase.

(iii) Proteins without selenocysteine that specifically bind Se (Se binding proteins). Eg: SeBP56 and SeBP14

2.1.3. Physiological role of Se in insects: Adequate dietary levels of Se are necessary for the survival of insects (Jensen and Trumble, 2003). High Se content in the soil can impact herbivore populations negatively by interference with molting (Jensen and Trumble, 2003), acting as feeding deterrent (Banuelos et al., 2002; Hanson et al., 2003, 2004; Vickerman et al., 2004),

causing delayed development, interfering with oviposition and reproduction (Trumble et al., 1998; Vickerman et al., 2002).

Se levels in pest insects also are likely to have a direct impact on the competence of biological control agents, such as facilitation and/or inhibition of microbial infection. Various studies indicate that the levels of Se and other micronutrients could disrupt integrated pest management strategies that depend upon biological control agents. For example, in *Trichoplusia ni*., Se supplementation boosted survival to baculovirus infection and lowered larval mortality (Popham et al., 2005). In *Heliothis virescens* dietary Se levels directly correlated with plasma Se levels that in turn correlated with baculovirus resistance (Shelby and Popham, 2007). However, the mechanisms underlying this phenomenon are unknown. The only well documented function so far for Se in animals is that it acts as a redox cofactor and gets co-translationally incorporated into selenoproteins as selenocysteine (Beck et al., 2004). Though the genome of *D. melanogaster* shows the presence of selenoproteins, enzymes that maintain redox homeostasis in insects do not depend on Se for activity as observed in mammals (Kanzok et al., 2001; Li et al., 2001; Bauer et al., 2003). Moreover, deletion of genes involved in selenoprotein synthesis and other selenoproteins yielded mixed results making the role of Se in insects debatable (Morozova et al., 2003; Hirosawa-Takamori et al., 2004).

2.1.4. Selenium binding proteins (SeBP): Earlier studies on Se metabolism focused primarily on the toxicity of this element as the amount of Se that a cell requires for its survival is very limited (Rayman, 2012). To cope with the adverse effects of Se toxicity, organisms have evolved a refined regulating mechanism to control Se levels in the cells. As mentioned above, Se is usually incorporated into proteins in the form of selenocysteine or selenomethionine. This incorporation of selenocysteine in selenoproteins requires the activity of the enzyme, selenophosphate synthetase that converts inorganic Se to activated selenophosphate, the precursor molecule that participates in biosynthetic pathways (Papp et al., 2007). As free Se is

toxic to the organism, a transport carrier is required to convert inorganic selenium to the appropriate precursor and to supply it to the biosynthetic pathways. Furthermore, the transport carrier has to be specific for Se and should be able to preserve the physical state of the metal during transport. This is carried out by a family of proteins called selenium binding proteins (SeBPs) (Sors et al., 2005).

SeBPs are ubiquitous and highly conserved proteins. In the past two decades, a number of SeBPs have been isolated, identified and characterized from different animals including mammals, amphibians, birds, fishes and molluscs (Song et al., 2006). The presence of SeBPs in these organisms has been documented by the incorporation of Selenium^{75}. They lack selenocysteine and are believed to covalently bind to Se, which is retained when they are separated using SDS-PAGE (Bansal *et al.*, 1989). The chemical form of Se in these compounds is not known. All the selenoprotein encoding genes contain a TGA codon where selenocysteine is incorporated by co-translational recoding of STOP signal (TGA) (Low and Berry, 1996). However, findings suggest that this mechanism of Se incorporation is absent in SeBPs. Moreover, there are reports that the levels of SeBPs are not dependent on dietary Se supply and therefore it could be concluded that the binding of the element is specific in these proteins (Behne et al., 2001). So far this class of proteins includes majorly two proteins of molecular mass, 14 kDa and 56 kDa, which have been detected in mouse liver (Bansal et al., 1989, 1990). In addition to these, a protein of molecular mass of about 17 kDa has been found to specifically bind to selenite both *in vitro* and *in vivo* (Sani et al., 1988). Its function is not identified yet but it has been proposed that it might play a role in the intracellular transport of Se.

The 14 kDa molecule identified from mouse liver was shown to be a fatty acid binding protein involved in growth regulation and that by modulating its function Se inhibited cell growth (Bansal et al., 1989). On the other hand the 56 kDa protein was first cloned from mouse liver cDNA and was observed to be similar to the already reported 14 kDa Se binding protein

but had different properties from the selenoproteins (Bansal et al., 1990). Later, SeBP was cloned from various other organisms including humans (Raucci et al., 2011). Being a highly conserved protein, SeBP56 has been reported to be involved in various functions including detoxification. An upregulation of this protein levels was observed in response to environmental pollutants like rPCB, 3, 3', 4, 4', 5-pentachlorobiphenyl (Ishii et al., 1996). SeBP is also suggested to be involved in the regulation of reduction- oxidation homeostasis in cases of cadmium toxicity (Jamba et al., 1997), anticarcinogenic growth regulation (Yang and Sytkowski, 1998), intra Golgi protein transport where SeBP56 along with other soluble factors plays a role in accelerating and regulating fusion of vesicular membranes (Porat et al., 2000). Further, higher expression levels of SeBP correlated with healthy cell differentiation (Li et al., 2008).

2.1.5. Status of SeBP in invertebrates and objective of the present study: Reports in invertebrate immunology reported the role of SeBP in immune response (Song et al., 2006) and oxidative stress management (Wu et al., 2010) in molluscs. Lepidopteran insect pests including stored grain pests are serious agricultural pests and cause severe damage to agricultural produce. Hence, the pests are exposed to various chemical and microbial control agents that cause severe oxidative stress in these insects. As it has been now established that Se levels play crucial role in various physiological functions of insects and can influence the efficacy of control strategies (Trumble et al., 1998; Vickerman et al., 2002; Jensen and Trumble, 2003; Hanson et al., 2003, 2004; Vickerman et al., 2004), an understanding of Se biochemistry in insects is imperative. The present study is an attempt to understand the same and focuses on the characterization of its carrier protein, SeBP in the larval forms of lepidopteran insect pest, *A. janata*.

2.2. Materials and methods

2.2.1. Primers used:

S. No.	Primer Name	Primer Sequence (5' – 3')
Degenerate and specific primers for obtaining partial fragment		
1	Aj S650 DEG FP	TAC GGV GGH SAR RAN TTY GA
2	Aj S650 DEG RP	AAR TCR SCC CAN GTC ARC TT
3	Aj S650 FP	GGA GAT TTT GTG CTC ATC GAC TCG
4	Aj S650 RP	GGA ACC TGG ACA AAG GGA GAC
RACE		
5	Aj SeBP 5P	CGA GGT GCC GTA GTG CTC CTT GTC
6	Aj SeBP 3P	CAT CAG ACG GAA CGT GGA AAG CC
7	Aj SeBP 5N	CGA GTC GAT CAG CAC AAA ATC TCC
8	Aj SeBP 3N	CAT AAC AGA TCC CCA ACA TCC CAA GC
Cloning complete cds		
9	Aj SeBP ORF FPF	ATG GGG CAT CAA GTG TAC AGC
10	Aj SeBP ORF RPF	TTA ATC TTC AGC CAA CCA GAT GTC
qRT- PCR		
11	Aj SeBP RT R	GCC GGT ATG TCG ATG ACT TTA TCG
12	Aj SeBP RTF	CTG TGC TCC CCT AGA GAT CAG
Heterologous expression		
13	Aj SeBP EXP FP	GGA TCC ATG TCT TGT AAA GGC CCC
14	Aj SeBP EXP RP	AAG CTT TTA ATC TTC AGC CAA CCA

Table 3: List of primers used in identification, cloning, heterologous expression and expression analysis of *AjSeBP*.

2.2.2. D-Galactose treatment: D-Galactose has been widely employed to generate ageing, diabetes, hyperglycemia models in rats and to induce oxidative stress (Gong and Xu, 1991). However, few studies have reported its application in insect models (Li et al., 1995; Cui et al., 2004; Gaikwad et al., 2010). For our current study, we fed the insects with D-galactose coated castor leaves to induce oxidative stress. After standardizing the dosage and the treatment period parameters for our model, the effects of D-galactose on larval growth and development were

monitored. Castor leaves of equal weights dipped in various doses of D-galactose solution (4 mg/ml and 8 mg/ml) were air-dried and fed to the neonatal larvae (3 days post hatch) till pupation. Control leaves were dipped in distilled water.

2.2.3. Collection, purification and activation of Bt crystal toxin: Bt isolate DOR1 (a local strain of *Bacillus thuringensis*) effective against *A. janata* larvae) obtained from Indian Institute of Oilseeds Research, Hyderabad was grown in Nutrient broth (Himedia) for 72 h at 180 rpm/ min at 28°C until sporulation. Bt crystal spores were harvested by centrifuging the above culture at 1100 g for 10 min at 4°C. The crystal pellet is washed with distilled water thrice and then solubilized in 50 mM sodium carbonate and bicarbonate buffer (pH 9.5) that contains 10 mM DTT. This was incubated at 37°C in water bath for 2 h for solubilization. The solubilized fraction was the dialyzed to remove DTT. For the activation of Bt toxin, the solubilized crystal proteins were digested with trypsin in a ratio of 1:50 by mass (trypsin:protoxin) at 37°C for 2 h. Proteolytic reaction was stopped by adding 1 mM PMSF.

2.2.4. Cry toxin feeding and recovery: For Cry toxin feeding and recovery experiments, 3rd instar larvae were allowed to feed on castor leaves coated with the activated toxin prepared from selected *Bt* isolate, DOR1 as mentioned above at the concentration of 140 ng/sq.cm of leaf surface, derived from LC_{50} experiments carried out in our laboratory. The insects were fed on these toxin coated leaves for either one or two days and then transferred to uncoated leaves and allowed to recover for 2 days. Samples were collected for every 24 hours in the recovery period.

2.2.5. Metal supplementation studies: To study the effect of heavy metal supplementation on *AjSeBP, CAT* and *GST* gene expression, larvae of *A. janata* were fed with castor leaves coated with various concentrations of metal salt solutions prepared in water (10, 25 and 50 ppm). For cadmium, selenium and zinc supplementation cadmium sulphate ($CdSO_4$), sodium selenite ($Na_2Se_2O_3$) and zinc sulphate ($ZnSO_4$) salts were used respectively.

2.2.6. CD spectroscopy: This study was carried out using Jasco J-810 spectropolarimeter fitted with a Peltier thermostat. The protein concentration used was 2.0 μM and measurements were carried out in the far UV region (250-200 nm). The samples were placed in a 2-mm path length rectangular quartz cell. Spectra were recorded at a scan speed of 20 nm/min with a 4 s response time and a slit width of 2 nm. To evaluate the effect of selenium ion on the secondary structure of protein, the initial recording was carried out for the native protein. Measurements were made in PBS buffer and scans recorded under the same conditions were subtracted from the protein spectra before further analysis.

2.2.7. Fluorescence spectroscopy: Binding of Selenium to recombinant AjSeBP was determined by measuring changes in the intensity of the protein fluorescence brought by metal binding. This was primarily carried out using a Spex Fluoromax 4 spectrometer. The slit widths used on the excitation and emission monochromators were 2 and 3 nm respectively. The excitation of the sample was carried out at a wavelength of 280 nm and recording of the emission spectra was done in the range of 300 and 400 nm. The protein sample of 2.0 ml with $A_{280} \leq 0.1$ in PBS was titrated against small aliquots of selenium (1 mM stock solution prepared in PBS). Once again like CD spectroscopy, the fluorescence was initially recorded for the protein alone and then after addition of each aliquot of selenium metal. For every aliquot addition an equilibration period of 2 min was given. As the volume gets altered, the recorded spectra were corrected for volume changes before final analysis of results.

2.3. Results and discussion

2.3.1. Cloning of *SeBP* from fat body of *A. janata* 5th instar larvae: Degenerate primers were designed based on *SeBP* sequences (cloned and annotated) from other invertebrates and insects (details are provided in the general materials and methods chapter). A 650 bp partial fragment was obtained using first strand cDNA prepared from total RNA isolated from 5th instar larval fat body of *A. janata*. The partial fragment obtained was further cloned using gene specific

primers (Fig. 15A) and the sequence identity was confirmed by nucleotide sequencing and BLAST analysis. This partial sequence thus obtained was used to isolate complete coding sequence using RACE (rapid amplification of cDNA ends) strategy as described in chapter 1. Figure 15B shows the amplification of 600 bp fragment by 5' RACE reaction and 1050 bp fragment obtained by 3' RACE reaction. After obtaining the three partial fragments, an attempt was made to clone full length cDNA from 5th instar larval fat body, using a new set of primers designed based on the sequences obtained in RACE reactions. Figure 15C shows *AjSeBP* complete coding sequence obtained in the present study which was sequenced.

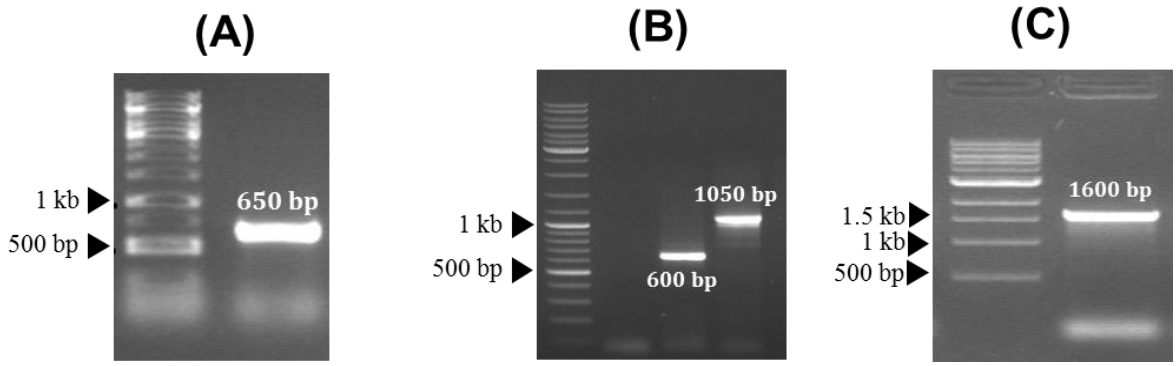

Fig. 15: Agarose gel images of amplicons obtained with (A) specific primers for *AjSeBP* partial fragment (B) Amplicons obtained by 5' RACE (600 bp) and 3' RACE (1050 bp) and (C) complete coding sequence (1.6 kb) of *AjSeBP*

Detailed analysis of *AjSeBP* sequence (Fig. 16) revealed that the total length of the cDNA is ~2.1 kb with an ORF of ~1.6 kb, which has ATG initiation codon and TAA stop codon. The 5' UTR region obtained is 170 bp long while 3' UTR region is 389 bp long. *In silico* analysis of deduced amino acid sequence of AjSeBP shows that it has 494 amino acids and a molecular weight of 56 kDa. The theoretical pI of AjSeBP is 5.97. Homology modelling of AjSeBP using SeBP from *Sulfolobus tokodaii* as template yielded a valid model showing conserved structure with maximum number of amino acids in the allowed regions of Ramachandran plot (Fig. 17A and B). Tertiary structure of AjSeBP shows that it is mainly composed of β- sheets and random coils with only a small percentage of α- helices. Phylogenetic analysis of the deduced amino acid sequence of *AjSeBP* with other reported SeBP sequences show that all the annotated

sequences of insect SeBPs branch together suggesting sequence conservation across the class (Fig. 18).

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agagacctcttatacgactcactatagggaaagcttgcatgcaggcctctgcagtcgacgggcccgggatccgattaagcagtggtatcaacgcag
agtacatggggcatcaagtgtacagctgtcagtgtagttggttgccggcatataagtgttttggagctataata
   1 atgtcttgtaaaggccccgggtacgcgtctcccctggacgccttcaataatggccccagggagaagttaatctacgtgatttgtgttcag
     M  S  C  K  G  P  G  Y  A  S  P  L  D  A  F  N  N  G  P  R  E  K  L  I  Y  V  I  C  V  Q
  91 ccggaccaaaccaaacaggactatttaggcactgtggacgtggaccctgagtctccgacatattgtcaggtcatccaccgcacttacact
     P  D  Q  T  K  Q  D  Y  L  G  T  V  D  V  D  P  E  S  P  T  Y  C  Q  V  I  H  R  T  Y  T
 181 ggaagtgttggcgacgagctccatcacagtgggtggaacgtgtgctccagctgccatcacgacagcgccctgaagaggaacctcctcata
     G  S  V  G  D  E  L  H  H  S  G  W  N  V  C  S  S  C  H  H  D  S  A  L  K  R  N  L  L  I
 271 ctgcccgcgctgagctcgagcaaagtctttgctgtagatgtcggcaccgacccacggaaaccgaagatgcataaagtaatcgacggctcc
     L  P  A  L  S  S  S  K  V  F  A  V  D  V  G  T  D  P  R  K  P  K  M  H  K  V  I  D  G  S
 361 gagatgaggtcattcaactgcagtttcccgcacacgacgcactgcctagcaaccggcgatatcatgatttcgacaatgggggataaagat
     E  M  R  S  F  N  C  S  F  P  H  T  T  H  C  L  A  T  G  D  I  M  I  S  T  M  G  D  K  D
 451 gaaaacgggaaaggagattttgtgctcatcgactcgaaaactttggaaatgaaaggaacctggacaaagggagacaaattcgctaagttt
     E  N  G  K  G  D  F  V  L  I  D  S  K  T  L  E  M  K  G  T  W  T  K  G  D  K  F  A  K  F
 541 ggttacgatttctggtatcaaccctaccataacgtaatgatctctacagaatggggtacacctaaaaggtttaagaccggtttccacgca
     G  Y  D  F  W  Y  Q  P  Y  H  N  V  M  I  S  T  E  W  G  T  P  K  R  F  K  T  G  F  H  A
 631 ggggatgtaattgacaaggagcactacggcacctcgttgaatatttataaatggtcaacgcacgagttacagcaagtcatagatttgggc
     G  D  V  I  D  K  E  H  Y  G  T  S  L  N  I  Y  K  W  S  T  H  E  L  Q  Q  V  I  D  L  G
 721 cccgacggctgtgctcccctagagatcagatttctccatgatcccaaatccgcgcagggatttgttgggtgtgcggtaaacgccaaccctt
     P  D  G  C  A  P  L  E  I  R  F  L  H  D  P  K  S  A  Q  G  F  V  G  C  A  V  N  A  N  L
 811 tacaggttttacaaaacatcagacggaacgtggaaagccgataaagtcatcgacataccggcaaaaaaagtttccaaagatggcgttgag
     Y  R  F  Y  K  T  S  D  G  T  W  K  A  D  K  V  I  D  I  P  A  K  K  V  S  K  D  G  V  E
 901 tccgagattaacggattaatgggtggcattattatatctttggacgataaatatctgtacacatcattatggatgttggcgaggtcaggc
     S  E  I  N  G  L  M  G  G  I  I  I  S  L  D  D  K  Y  L  Y  T  S  L  W  M  L  A  R  S  G
 991 aatactataacagatccccaacatcccaagctgactggccgagttcaactgggaggtgtaatcgcctcggaccccgctattaaagtgctg
     N  T  I  T  D  P  Q  H  P  K  L  T  G  R  V  Q  L  G  G  V  I  A  S  D  P  A  I  K  V  L
1081 gaagacaaagaattgaaggaacgacctcaacccattacggttaaaggaaaaacattacaaggtgcagctcaaatgatacaactttctctc
     E  D  K  E  L  K  E  R  P  Q  P  I  T  V  K  G  K  T  L  Q  G  A  A  Q  M  I  Q  L  S  L
1171 gacggaaaacgccttttcgtgtcgtcatccctgttctctccctgggacaaacagttctaccccaaaatggctgcagagggcggctggata
     D  G  K  R  L  F  V  S  S  S  L  F  S  P  W  D  K  Q  F  Y  P  K  M  A  A  E  G  G  W  I
1261 gtgaagttggacgtggacacagtgaatggaggcttgaagttggacccggacttcctcgtacacttcgggaacgaaccggacgggccagtg
     V  K  L  D  V  D  T  V  N  G  G  L  K  L  D  P  D  F  L  V  H  F  G  N  E  P  D  G  P  V
1351 ttaccacatgaaatgagatatcccggcggtgactgcacatccgacatctggttggctgaagattaa
     L  P  H  E  M  R  Y  P  G  G  D  C  T  S  D  I  W  L  A  E  D  *
aaagctaaatataactccgataaatagtgttattgcttaactaacgacattgtgccaatatttttgtatgcatgttaatatcggtaatttttgcat
agatattattacgtatactatgttttcttaaagtacattattgtaagatttgtaaggcataattatgttcaagtacatacatattaaaatataaaa
ttgtcaaaatgtattttgtataaaacgaatgtaatgcttatagatgataaatgaatatatatctatatgtgaaaaaaaaataaaaaaaaaaaaaaa
aaaaaaaaaagtactctgcgttgataccactgcttaatctagatgcattcgcgagtaccgagctcgaatacgcctctaaacaacccagcaaaatat
agggg
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Fig. 16: Full length cDNA sequence and deduced amino acid sequence of AjSeBP. The 5' UTR and 3'UTR regions are represented in black color, top and bottom. The coding sequence and the deduced amino acid sequence are represented in black and red colors respectively. Conserved motifs in the protein structure are underlined.

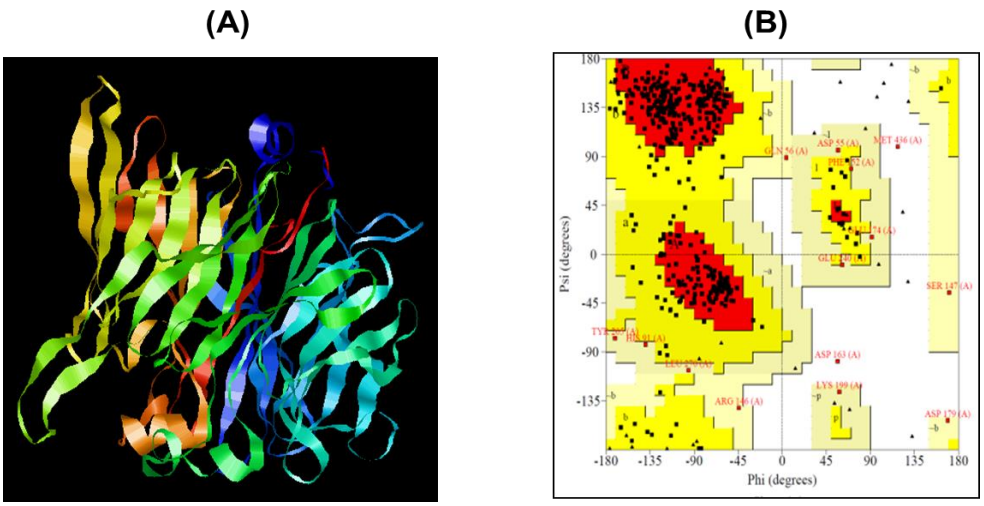


Fig. 17: Homology modeling of AjSeBP. (A) Homology model of AjSeBP generated using *Sulfolobus tokodaii* SeBP (PDB ID: 2ECE) (B) Ramachandran plot for the protein homology model generated confirms the validity with most of the amino acids in the allowed regions.

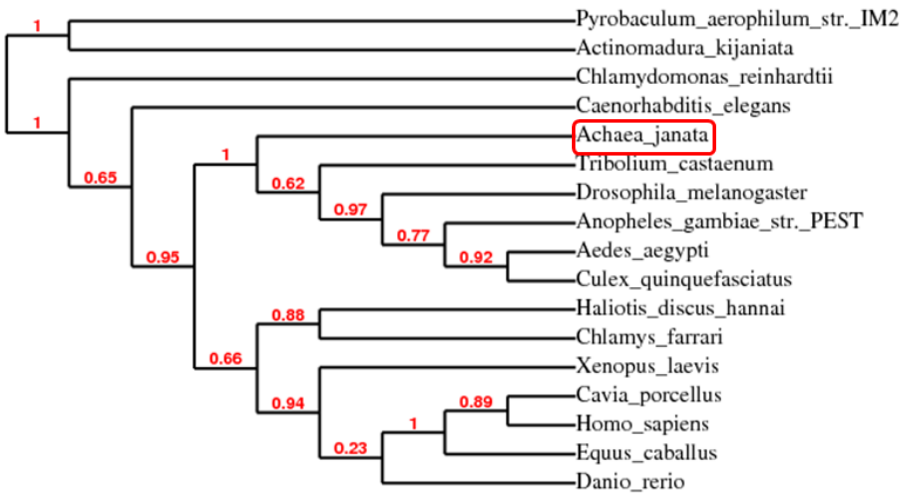


Fig. 18: Phylogenetic tree showing evolutionary relationship of AjSeBP from different organisms across the phyla. *Pyrobaculum aerophilum* (GenBank Accession no: AAL64395.1), *Actinomadura kijaniata* (GenBank Accession no: ACB46504.1), *Chlamydomonas reinhardtii* (GenBank Accession no: XP_001703358.1), *Caenorhabditis elegans* (GenBank Accession no: CAA19490.1), *Achaea janata* (sequence submitted to GenBank), *Tribolium castaenum* (GenBank Accession no: XM_965596), *Drosophila melanogaster* (GenBank Accession no: NP_650256.2), *Anopheles gambiae* (GenBank Accession no: XP_309978.4), *Aedes aegypti* (GenBank Accession no: XP_001654322.1), *Culex quinquefasciatus* (GenBank Accession no: XP_001863949.1), *Haliotis discus hannai* (GenBank Accession no: ACZ65578.1), *Chlamys farrari* (GenBank Accession no: AAX39709.1), *Xenopus laevis* (GenBank Accession no: NP_001089396.1), *Cavia porcellus* (GenBank Accession no: XP_003478942.2), *Homo sapiens* (GenBank Accession no: AAB02395.1), *Equus caballus* (GenBank Accession no: NP_001296443.1), *Danio rerio* (GenBank Accession no: AAH56590.1). Please note that AjSeBP along with SeBPs annotated from other insects fall into a single clad.

2.3.2. Ontogeny and tissue distribution of *AjSeBP*: Developmental and tissue distribution profiles often reflect the physiological role of a given protein. As this is the first report of SeBP in insects, ontogeny and tissue distribution analysis was carried out by semi-quantitative and qRT-PCR to gain a basic understanding of its function in insect physiology. Ontogeny results show that *AjSeBP* is expressed predominantly in the 5th larval instar and pre-pupal stages. (Fig. 19A). The results further show that *AjSeBP* expression is fairly low in the early larval development and increases significantly during the late- larval instar (5th instar) stage and declines thereafter during pre-pupal and pupal stages. 5th instar larva is a voracious feeder metabolically the most active larval instar in the life cycle of *A. janata* where different proteins required for various physiological processes are expressed. Higher expression of *AjSeBP* in this instar is suggestive of its involvement not only in larval development but probably in its preparation for transformation to pupa through pre-pupal stage.

Tissue distribution profile shows that *AjSeBP* is predominantly expressed in the larval gut and fat body (Fig. 19B). This expression pattern could be suggestive of its role in digestion and immunity. Insect fat body is analogous to mammalian liver and performs detoxification functions (Arrese and Soulages, 2010). Given the role of SeBP in redox homeostasis and detoxification in mammals (Jamba et al., 1997), high expression levels of *SeBP* in fat body suggests that it might be involved in similar function in insects.

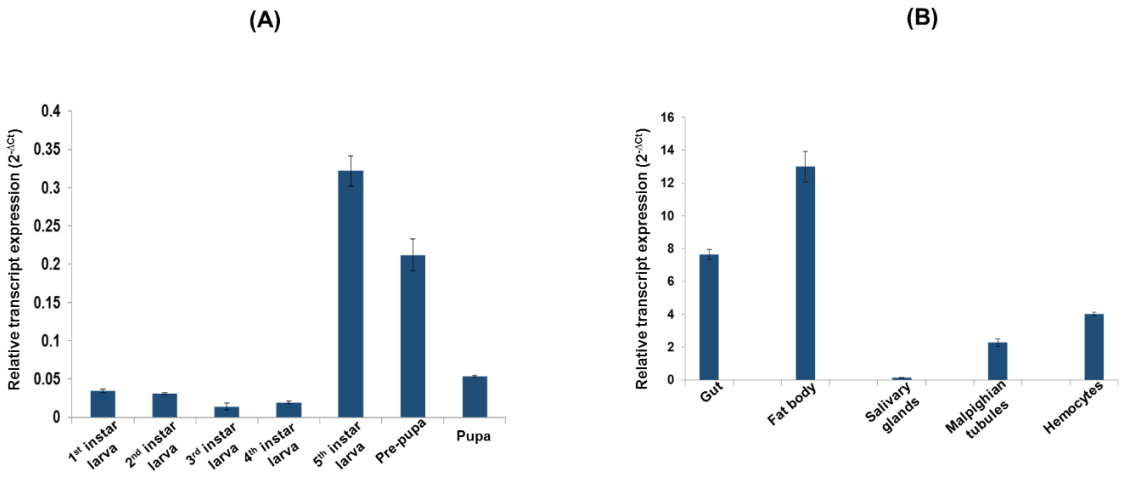


Fig. 19: Ontogeny and tissue distribution of *AjSeBP*. (A) Ontogeny- qRT-PCR analysis showing expression of *AjSeBP* during various stages of post embryonic development. (B) Tissue distribution-qRT-PCR shows that *AjSeBP* is predominantly expressed in the larval gut and fat body.

2.3.3. Heterologous expression of AjSeBP, protein purification and functional characterization: Recombinant expression and purification of AjSeBP was carried out as explained in the materials and methods. Figure 20A clearly shows the induction of recombinant AjSeBP upon IPTG addition. As AjSeBP specific antibodies were unavailable, the induction was confirmed by western blotting using His-tag antibodies that detected the presence of recombinant protein both in the inclusion bodies and cytosol (Fig. 20B). Figure 20C shows pure recombinant AjSeBP, wash and elute fractions obtained after affinity chromatography using Ni-NTA column. Further, the recombinant protein was subjected to MALDI-TOF analysis to confirm its identity. Results clearly show that the expressed recombinant protein obtained is a selenium binding protein (Fig. 21) and is highly homologous to the annotated *B. mori* SeBP.

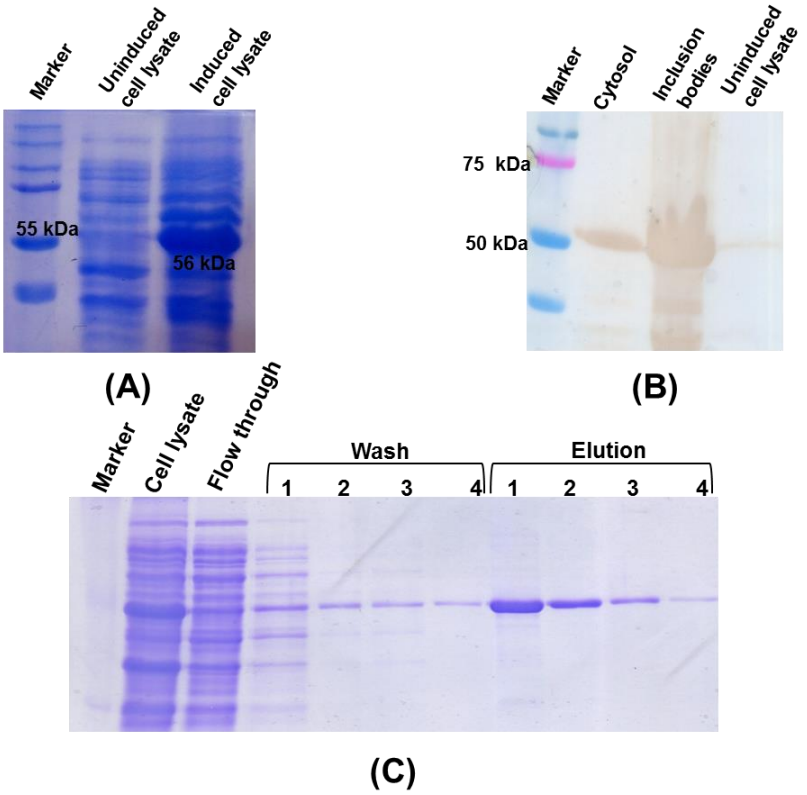


Fig. 20: Heterologous expression and purification of AjSeBP. (A) SDS-PAGE profile showing the induced expression of AjSeBP at 56 kDa in the bacterial cell lysate. (B) Western blot with His-tag antibodies confirming AjSeBP induction both in the inclusion bodies and cytosol. (C) SDS-PAGE gel showing the pure recombinant AjSeBP obtained after affinity chromatography (Ni-NTA).

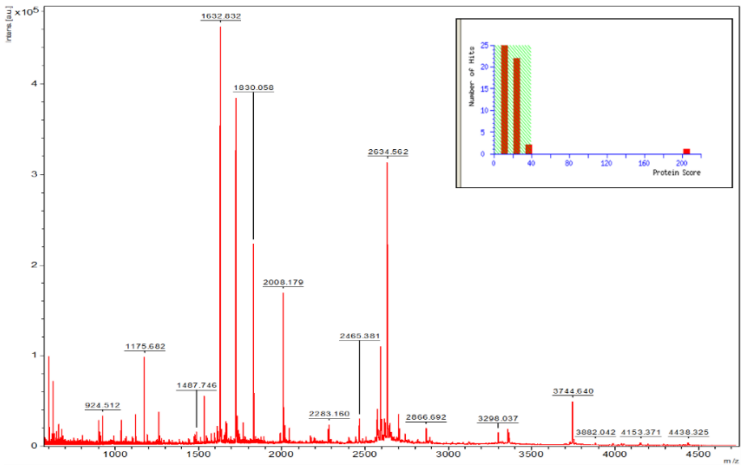


Fig. 21: MALDI-TOF spectrum of recombinant AjSeBP confirms the identity of the peptide that is homologous to predicted *Bombyx mori* Selenium binding protein with a score of 205 (Inset).

2.3.3. Effect of D-galactose feeding: Insects were fed with two different doses of D- galactose as explained in the materials and methods section. Larval gut and fat body were collected after the completion of treatment period and *AjSeBP* transcript levels were quantified using qRT-PCR. As shown in figure 22, results show that D- galactose feeding caused significant up-regulation of *AjSeBP* levels in larval gut in a dose dependent manner but no such effect was observed in the fat body. Studies in other model organisms show that SeBP expression is up-regulated under oxidative stress (Song et al., 2006) and induction of oxidative stress by D-galactose is well documented phenomena in various organisms (Gong et al., 1991; Li et al., 1995; Cui et al., 2004; Gaikwad et al., 2010). Up-regulation of SeBP in larval gut observed in the present study corroborates with the earlier findings. However, the difference in the response between the larval tissues is interesting. However to our surprise the fat body which is an important metabolic organ and is known to perform variety of functions including immune response (Hoffman, 1995; Arrese and Soulages, 2010) did not show any upregulation upon D-galactose feeding. The difference in the response between two examined tissues is interesting and the underlying mechanism needs to be investigated for getting detailed insight which allows them to respond differentially. At this juncture we can only suggest that *SeBP* genes in different tissues respond differentially even for a common stressor.

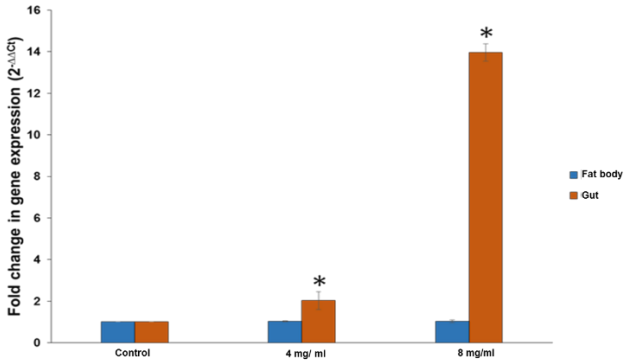


Fig. 22: Expression analysis of *AjSeBP* upon D- galactose feeding in the larval gut and fat body. *AjSeBP* expression was significantly upregulated upon D- galactose feeding in the larval gut. However, there is no effect in the fat body. Data (n=5) were expressed as mean ± SEM (*, $P < 0.05$; ANOVA followed by Student–Newman–Keuls' post hoc test).

2.3.4. Effect of bacterial challenge: To study the effect of bacterial infection on *AjSeBP* expression, 5th instar larvae of *A. janata* were injected with *E. coli, B. coagulans* and *B. cereus* as described in the materials and methods section. Larval gut and fat body were collected at different time points i.e., 3, 6, 9, and 12 hours post injection (hpi) and *AjSeBP* transcript levels were estimated using qRT-PCR. As shown in figure 23, results show that both the bacterial species, *E. coli* and *B. cereus* caused up-regulation of *AjSeBP* transcript levels in larval gut and fat body whereas *B. coagulans* did not show any significant response. In larval fat body, *AjSeBP* expression levels increased gradually from 3 hpi to 12 hpi but larval gut showed an early increase in the expression levels when compared with the fat body and the levels returned to normal range by 6 hpi in *E. coli* treated group while the higher expression levels were maintained till 12 hpi in the *B. cereus* treated group.

Bacterial challenge is known to induce oxidative stress in insects as they primarily rely on ROS based defensive responses under infection (Nappi and Christensen, 2005). The role of SeBP in oxidative stress management is well established in various organisms including humans (Tapiero et al., 2003; Tinggi, 2008). Further, studies in invertebrate models reported the up-regulation of SeBP under infection (Song et al., 2006; Wu et al., 2010). *E. coli* and *B. cereus* are known bacterial pathogens of insects (Bucher, 1960) and hence there is a significant response when *A. janata* larvae were challenged by them. On the other hand, normal expression levels observed in the *B. coagulans* treated group further justifies its non-pathogenicity in insects. The difference in the fold change in expression levels and the time points of up-regulation between the larval gut and fat body is most likely associated with the differences in their developmental origins and the physiological functions they perform during larval development. Though the up-regulation observed in the present study is in agreement with earlier reports (Song et al., 2006), the actual function of SeBP in immunity i.e., if it is a direct player in the immune response or is an indirect player that assists in scavenging the free radicals generated during the process needs to be investigated.

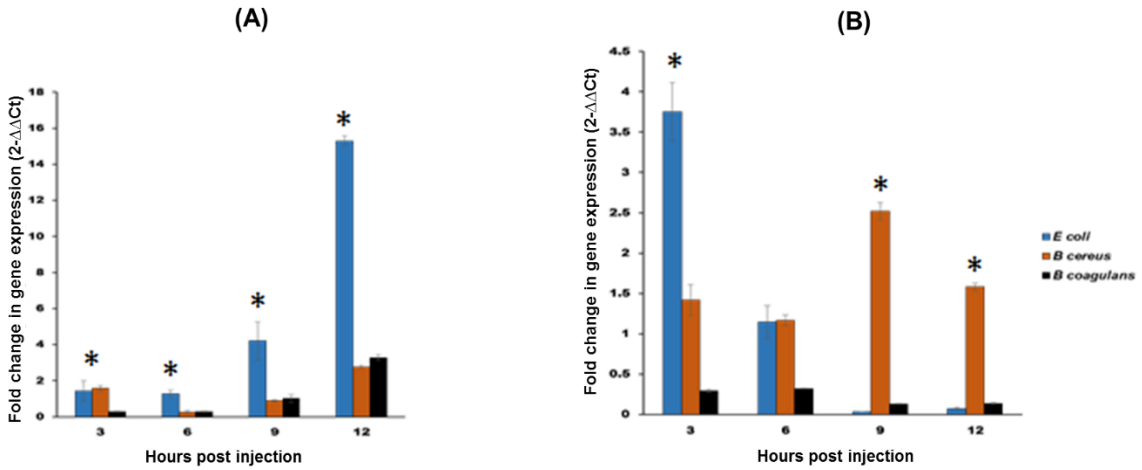


Fig. 23: Expression analysis of *AjSeBP* upon bacterial challenge in the larval fat body and gut. *AjSeBP* expression was significantly upregulated upon bacterial challenge in the larval fat body (A) and gut (B). However, the level of upregulation observed in the gut was less when compared to the fat body. Data (n=5) were expressed as mean ± SEM (*, $P < 0.05$; ANOVA followed by Student–Newman–Keuls' post hoc test).

2.3.5. Effect of Cry toxin exposure: For this study larvae were fed with Cry toxin coated leaves and allowed to recover by transferring them to normal leaves as mentioned in the materials and methods section. Larval gut was collected at specific time points and *AjSeBP* transcript levels were estimated using qRT-PCR. As shown in figure 24, results show that *SeBP* transcript levels are declined in the Cry toxin fed group and upon recovery, the expression levels show significant up-regulation. Fold change in expression is much higher in the 24 hour treated group when compared with that of the 48 hour toxin exposed group.

In lepidopteran larvae, elevated levels of selenium were reported to correlate with Cry toxin resistance (Shelby et al., 2007) and baculovirus infection (Popham et al., 2005; Shelby and Popham, 2007). However, as mentioned earlier Se is toxic to the organism and has to be bound by a carrier protein and supplied when required. As SeBP is the carrier molecule for Se, we hypothesized that SeBP expression levels might be effected under Cry toxin exposure and recovery. As hypothesized, *AjSeBP* expression levels were altered in the treated and recovery groups. The decline in the expression levels observed in the treated group can be attributed to the damage occurring at the tissue level due to Cry toxin exposure (Vinod et al., unpublished

data). Up-regulation observed in the recovery period suggests that SeBP might have a role in recovery after Cry toxin exposure that might facilitate toxin tolerance which in long term could lead to development of resistance. Further, the lower fold change in the 48h toxin fed recovery group could be a result of the excessive epithelial damage caused by Cry toxin exposure when compared with that of the 24h toxin fed group.

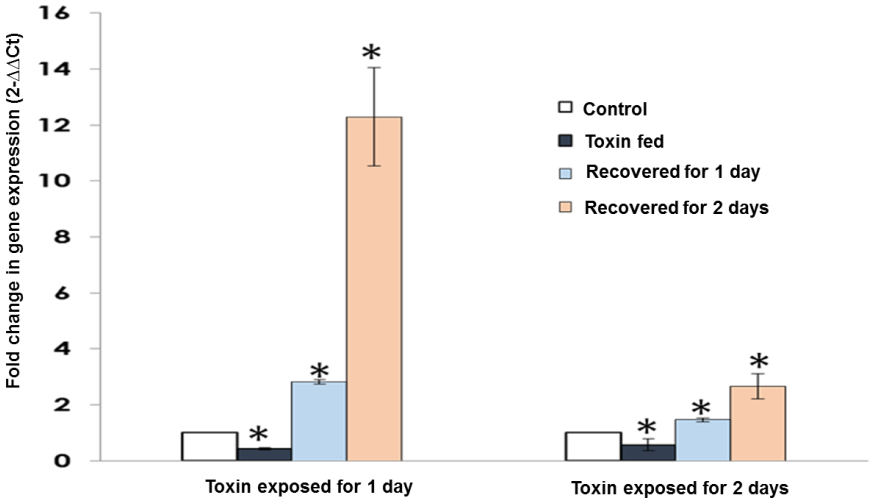


Fig. 24: Expression analysis of *AjSeBP* in the larval gut upon Cry toxin exposure and recovery. In the toxin exposed group (both 1-day and 2-day) a significant down-regulation of *AjSeBP* expression was observed when compared to the controls. But the insects in the recovery group showed elevated *AjSeBP* expression levels. However, this up regulation was more in the 1-day toxin exposed group when compared with the 2-day exposed group. Data (n=5) were expressed as mean ± SEM (*, $P < 0.05$; ANOVA followed by Student–Newman–Keuls' post hoc test).

2.3.6. Effect of Se supplementation: To gain a preliminary understanding of AjSeBP function in Se binding, Se salt supplementation was carried out and larval gut and fat body were collected as explained in the materials and methods section. As shown in figure 25, results show that Se supplementation caused significant up-regulation of *AjSeBP* transcript levels in the larval fat body of the 48 h supplemented group but not in the 24 h supplemented group. There was no effect on the expression levels in the gut either in the 24 h or in the 48h supplemented groups.

Earlier studies on Se supplementation in insects and other invertebrates have already reported an increase in the expression levels of *SeBP* (Wu et al., 2010) and other antioxidant

enzymes (Chiu et al., 2010). Our findings with fat body corroborate well with the earlier reports but it is interesting to note that *AjSeBP* levels are up-regulated only in the fat body but not in the larval gut. One of the plausible explanations for this observation could be that fat body is one of the primary tissues which performs detoxification and storage functions and is multifunctional tissue in insects (Arrese and Soulages, 2010). Further experiments on quantification of accumulated Se in the larval tissues would provide the evidence to support the above explanation.

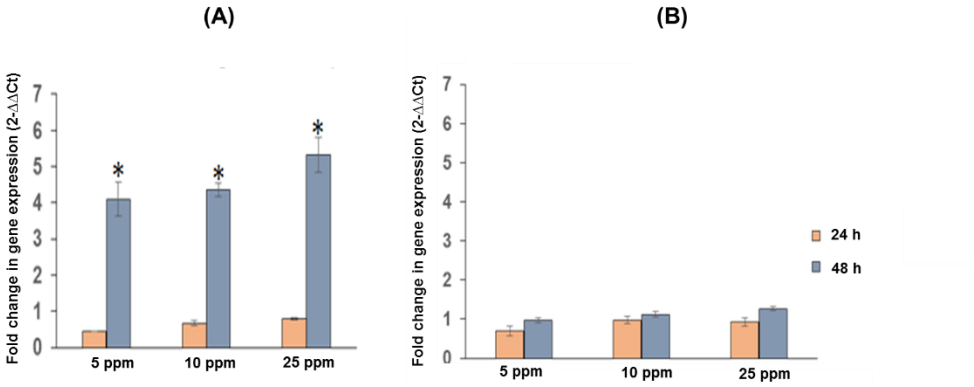


Fig. 25: Effect of Selenium supplementation on the expression of *AjSeBP* in the larval fat body (A) and gut (B). In the 24 hour fed group, no significant effect on *AjSeBP* expression was seen both in the gut and fat body. Insects supplemented with Se for 48 hours showed significant up-regulation of *AjSeBP* expression in the fat body (A). Data (n=5) were expressed as mean ± SEM (*, $P < 0.05$; ANOVA followed by Student–Newman–Keuls' post hoc test).

2.3.7. Effect of metal supplementation (Se and non Se): To investigate the specificity of up-regulation of *AjSeBP* upon Se supplementation, last instar larvae of *A. janata* were fed with leaves coated with solutions of selenium (Na_2SO_3), zinc ($ZnSO_4$) and cadmium ($CdSO_4$) salts as described in the materials and methods. As Se supplementation showed maximum response in the 48 h fed larval fat body, the tissue samples were collected from the experimental and control groups at 48h time point and *AjSeBP* transcript levels were estimated using qRT-PCR. Further, to confirm that the up-regulation of *AjSeBP* upon Se supplementation is Se specific but not a general response induced by heavy metal stress, antioxidant enzyme transcript levels (*Catalase*

and *Glutathione S- transferase*) were also estimated. As shown in figure 26, results clearly show the specific induction of *AjSeBP* upon Se supplementation. Further, up-regulation of antioxidant enzyme gene, *catalase* in all the three metal supplemented groups indicate the generation of oxidative stress caused by metal supplementation. As up-regulation of *AjSeBP* is only seen in Se supplemented group the results support the hypothesis that the response shown by the fat body is Se specific.

However, contrary to the present findings induction of *SeBP* expression by Se, iron and zinc was reported in the abalone, *Haliotis discus hannai* (Wu et al., 2010) while the current study showed up-regulation of *SeBP* only by Se but not by other metals. This is suggestive of variable response pattern in different group of organisms. Further studies have to be carried out to reach a meaningful conclusion.

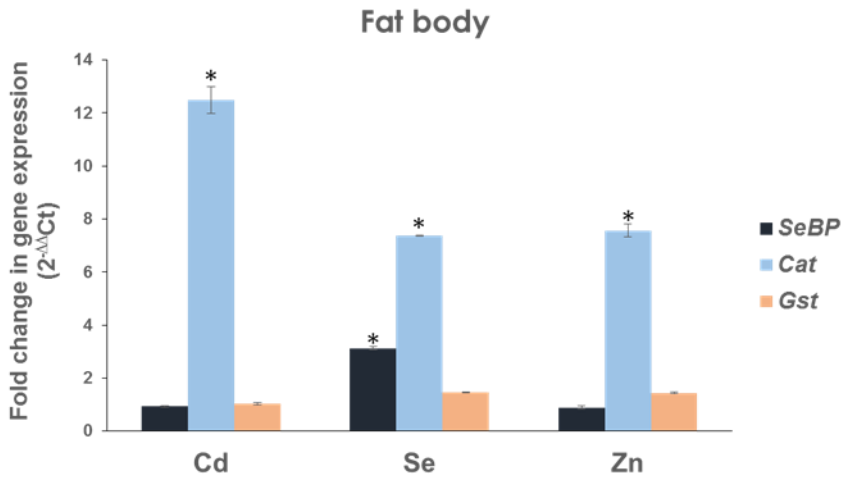


Fig. 26: Effect of Selenium (Se), Cadmium (Cd) and Zinc (Zn) supplementation on the expression of *AjSeBP* and other antioxidant enzyme genes *Catalase* (*Cat*) and *Glutathione S- transferase* (*Gst*) in the larval fat body. Significant up-regulation of *AjSeBP* expression was observed only in the Se supplemented group but not in Cd and Zn supplemented groups. Upregulation of *Cat* transcript levels were observed in all the three groups. However, no change was observed in *Gst* expression levels. Data (n=5) were expressed as mean ± SEM (*, $P < 0.05$; ANOVA followed by Student–Newman–Keuls' post hoc test).

2.3.8. Binding studies: Selenium binding ability of the recombinant AjSeBP expressed in the present study was investigated using CD as well as fluorescence spectroscopy. The details of the same are explained below:

(i) Fluorescence spectroscopy: The optimal emission spectrum obtained in the present study using recombinant AjSeBP was centered around 332 nm, while excitation was primarily carried out at 280 nm. Protein-selenium titration resulted in a 46% decrease in the emission intensity. A representative spectra of fluorescence titration of protein with selenium is presented in figure 27A where the black line corresponds to the spectrum of the protein alone and colored lines correspond to those spectra recorded in the presence of increasing concentrations of selenium. The graphical representation (Fig. 27B) shows the change in fluorescence intensity, ΔF (= F_o – F) as a function of the added metal ion concentration and provides the binding curve for the titration. Y-intercept of the plot, $F_o/\Delta F$ versus 1/ $[L]_t$, where F is the fluorescence intensity at any point during the titration, F_o is the initial fluorescence intensity and $[L]_t$ is the total concentration of the selenium gives the fluorescence intensity at saturation binding. Fluorescence titration data thus obtained was analyzed by the method described by Chipman et al., 1967 according to the following equation:

$$\log \{\Delta F/(F\text{-}F_\infty)\} = \log K_b + \log [L]_f$$

where F_∞ represents the fluorescence intensity of the protein at infinite concentration of the metal, K_b is the association constant, and $[L]_f$ is the free metal ion concentration at each point of the titration obtained from the following equation:

$$[L]_f = [L]_t - \{(\Delta F/\Delta F_\infty)\ [P]_t\}$$

where ΔF_∞ (= F_o- F_∞) is the change in fluorescence intensity at saturation binding and $[P]_t$ is the total protein concentration. The X-intercept of a double logarithmic plot of log $\{\Delta F/(F\text{-}F_\infty)\}$ *versus* log $[L]_f$ will yield the pK_b value for the association reaction. Such a plot for the binding of metal to protein is shown in figure 26C. From the X-intercept of this plot, the association

constant, K_b has been determined as $1.1 \times 10^5\ M^{-1}$. The slope of this plot is found to be ~0.86, indicating that nearly one protein molecule binds one selenium ion.

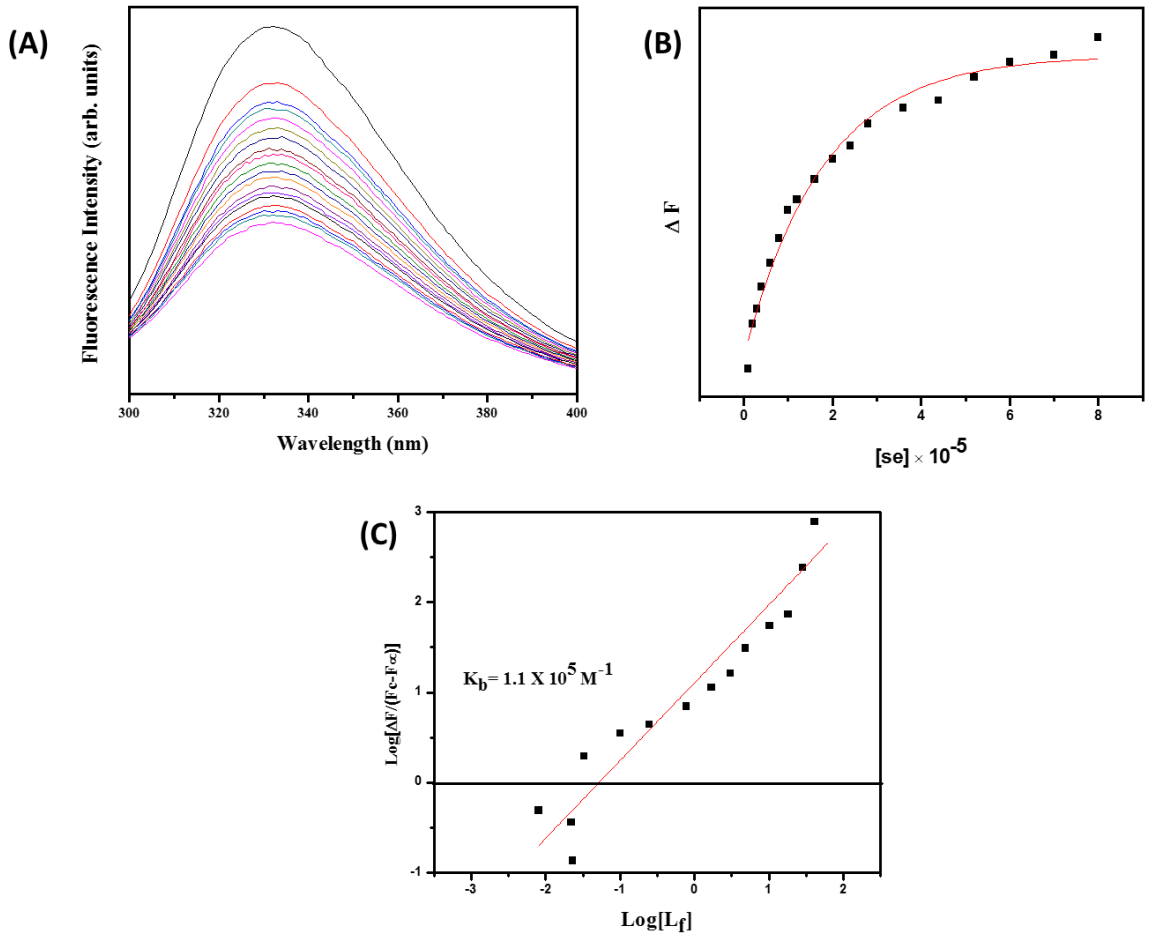


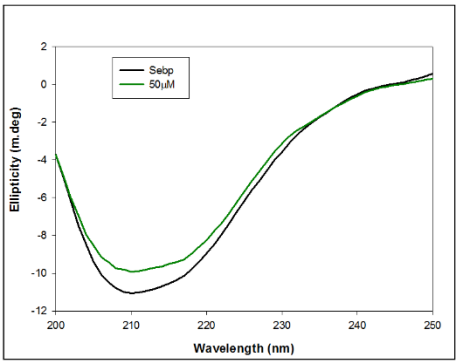
Fig. 27: Fluorescence spectroscopic measurements of Selenium binding by recombinant AjSeBP. (A) Fluorescence spectra of recombinant AjSeBP in the absence and after addition of defined aliquots from 1.0 mM selenium stock solution. Spectrum 1 corresponds to recombinant AjSeBP alone and spectra 2-19 correspond to recombinant AjSeBP in the presence of increasing concentrations of Selenium. (B) Binding curve obtained by plotting the change in fluorescence as a function of cytosine concentration. (C) Double-logarithmic plot for the binding of selenium to recombinant AjSeBP. The double logarithmic plot was obtained using Chipman analysis.

(ii) CD spectroscopy: Circular dichroic spectra of protein in native state (solid line) and in the presence of selenium ion (dashed line) are shown in figure 28A. The far UV spectrum of the native protein (Fig. 28A), characterized by a minimum around 215 nm and a small maximum around 200 nm is indicative of a predominantly β-sheet conformation. K2D Dichroweb method

was used to analyze the CD spectrum in order to gain evidence on the content of different structural elements of recombinant AjSeBP. A basis set of 43 proteins was used as a reference for fitting the experimental spectrum and the results obtained are given in figure 28B. The percentage of various secondary structures in recombinant AjSeBP as obtained from K2D Dichroweb analysis are: 15 % regular α-helix, 36 % β-sheet and 49 % unordered structures.

Overall the results obtained from analyzing the CD spectral data indicate that the secondary structure of AjSeBP is predominantly comprised of β-sheets and a relatively small α-helical content. The far UV CD spectrum of protein recorded in the presence of 50 μM selenium ion (Fig. 28A) is nearly identical to the spectrum of native protein, suggesting that the secondary structure of the protein is unaltered upon selenium binding.

(A)



(B)

α- Helices	β- Sheets	Random coils
15 %	36 %	49 %

Fig. 28: (A) Far UV (200–260 nm) circular dichroism recorded from recombinant AjSeBP (black line) and recombinant AjSeBP incubated with SeO_3^{2-}(green line) (B) Secondary structure prediction by CD spectroscopy using K2D Dichroweb.

2.4. Summary

The complete coding sequence of *AjSeBP* was ~2.1 kb with an ORF of ~1.5 kb coding for a protein of molecular weight ~56 kDa. In the larval tissues, *AjSeBP* is predominantly expressed in the larval gut and fat body and shows developmental regulation of expression. To further characterize *AjSeBP*, its mRNA expression levels were analyzed in various conditions like oxidative stress, bacterial challenge, Cry toxin recovery and metal supplementation. In oxidative stress induced by D- galactose, *AjSeBP* levels significantly increased in the larval fat body but not in the gut. Bacterial challenge with different bacteria such as *E. coli, B. cereus* and *B. coagulans* caused transcriptional upregulation of *AjSeBP* both in the larval gut and fat body. However, the effect was more prominent in the fat body when compared to gut. Among the three bacterial species used to induce infection, *E. coli* induced the maximum upregulation. To understand the role of *AjSeBP* in resistance development against Cry toxin, its expression was analyzed under sub lethal Cry toxin exposure followed by a 48 h recovery period. Though the transcript levels decreased in the toxin-exposed larval gut, it is possibly due to the tissue damage caused by Cry toxin (Vinod et al, unpublished data). However, in the recovery period the transcript levels were significantly upregulated suggesting its possible role in resistance development. Metal supplementation studies suggested that *AjSeBP* is upregulated upon selenium supplementation and this upregulation is selenium-specific. *A. janata* SeBP was recombinantly expressed using the pET28a vector system in *E. coli* BL21 host. *In vitro* metal binding studies (CD and fluorescence spectroscopy) with recombinant AjSeBP clearly show that AjSeBP binds to Selenium with a molar ration of 1:1 and has a binding constant value of 1.1 X 10^{-5} M^{-1}.

2.4. References

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Chapter 3

Differential stress responses in castor semilooper, A. janata

3.1. Introduction

Oxygen is a major essential element and oxidative metabolism is one of the most efficient ways to generate ATP (Tourmente et al., 2015). The biological processes while using oxygen, also generate reactive molecules called reactive oxygen species (ROS). Under normal physiological conditions, the organisms essentially maintain a homeostatic balance of ROS generation and degradation. This balance can be occasionally perturbed causing oxidative stress (Apel and Hirt, 2004; Finkel et al., 2007; Birben et al., 2012).

Oxidative stress is a phenomenon that occurs in all aerobic living forms and it has been implicated and associated with many diseases/ syndromes in humans and other species (Zhi-Wei, et al., 2015). Harmful effects of oxidative stress are mainly due to the volatile nature of ROS and their interaction with the biological macromolecules like proteins, lipids, DNA etc. causing damage to the cells and tissues (Stadtman, 1992, Johnson et al., 2016; Kahl and Silva, 2016, Shavva et al., 2016). Although ROS are potentially harmful to the organisms, they also play important roles in normal physiology serving as immune effector molecules and sometimes as secondary messengers in the intracellular signaling pathways (D'Autréaux et al., 2007). Hence, the organisms have evolved a complex system of antioxidant defenses to achieve homeostasis between ROS generation and their elimination thereby making the antioxidant system that maintains this balance critical for the survival of the organism (Valko et al., 2007). Detection of oxidative stress in a given tissue or cell type is extremely important, when we relate the same with various physiological and/or pathological conditions. Hence, identification of specific markers as well as designing appropriate assays become very important aspects, mere so because several of these markers are short lived and degrade over time when tissues/ samples are stored for long time. Lipid peroxidation, protein oxidation, protein nitration, ROS and antioxidants are some of the widely used markers for the determination of oxidative stress levels in tissues of animals including insects (Renault et al., 2016).

Insects have been valuable research models in the discovery of many scientific principles owing to the numerous advantages they provide. Throughout their life cycle, they thrive in pathogen rich environments, manage the harsh weathers and are exposed to a number of allochemicals. Their remarkable ability to cope up with the enormous oxidative stress generated in all these conditions, make them attractive models in this field of research. Interestingly, they also exhibit beneficial effects of ROS (De Lamirande and Gagnon, 1995; Salganik, 2001; Pham-Huy et al., 2008) and become suitable organisms to evaluate the effects. In immune pathways like melanization and respiratory burst, they employ ROS mediated microbe evasion where ROS or ROS generated intermediates sequester and kill the invading microbes (Christensen et al., 2005). Not only to the invading pathogens but ROS are equally damaging to the insect tissues as well. Hence, insects have evolved a suite of anti-oxidant enzymes that function in concert to maintain a fine redox balance and protect them from the harmful effects of ROS. Superoxide dismutase (SOD), catalase (CAT), glutathione per-oxidase (GPx), glutathione S-transferases (GSTs) are the few prominent antioxidant enzymes in insects (Ahmad, 1995; Halliwell and Gutteridge, 1999; Barbehenn, 2002; Rajarapu et al., 2011). A thorough understanding of the antioxidant defense responses in insects might help in gaining insights into their survival strategies through the course of their successful evolution and also exploit the same in designing the strategies for integrated pest management.

Majority of the existing studies on antioxidant systems in insects report the changes in the antioxidant enzyme activities in response to any given oxidative insult (Hazelton and Lang, 1983; Enayati et al., 2005; Gaikwad et al., 2010; Boyer et al., 2012). Though the activity changes seem to be enough to reach upon a conclusion, it is important to understand if the change is at the translational, post translational or it's mediated at transcriptional level. Furthermore, changes at the transcription levels are indicative of the long term responses and are therefore more useful in understanding the key phenomena like adaptations for survival and resistance development against various pesticides including biopesticides. Current study is such an attempt to monitor

the antioxidant gene changes at the transcriptional level for a given stress stimulus. Using various stress inducers like D- Galactose, immune challenge and *Bt* Cry toxin to analyze AO gene expression provides a better outlook on the importance of this AO defense system and helps identify AO gene markers, if any.

3.2. Materials and methods

3.2.1. Primers used:

S. No.	Primer Name	Primer Sequence (5' – 3')
Degenerate and specific primers for obtaining partial fragment		
1	CAT NEW DEG RP	GGW CCM GAR AAD GAG TTG GG
2	CAT NEW DEG FP	GAT GGH TAC AGR CAY ATG AAY GG
3	SOD NEW DEG RP2	CRT TDC CNG TBG TCT TRC TNA GYT C
4	SOD DEG FP	GAR TTY GGT GAY AAY ACM AAY GGN TG
5	GST NEW DEG RP	AKG TCA GCS AHK GTC A
6	SOD NEW RT RP	GCA TGG GCA GTC GTC
7	SOD DEG RP	CDC CVG CRT TDC CNG TBG TC
8	SOD RT RP	CGG ACC CAT CTG ACT TGT GAA TCA TGC
qRT-PCR		
9	CAT NEW RT R	CTT TGT GCC TGG CAT TGA GC
10	CAT NEW RT F	GGT CCA TCA CGC TGA TAG TTG G
11	GPx RT RP	CTG AAG AAA AGG GAT TAC GCA TTC TC
12	GPx RT FP	GGA ACT TCC ATA GTG GAC TGG
13	SOD FINAL RT F	CGT CTT GCT AAG TTC GTG GC
14	SOD FINAL RT R	CAT CCG TGA CGT CGG TGA TCT C
15	GST RT F	GCG AGC CAT CAG CCG ATA TCT AG
16	GST RT R	CCT CCA GCT TCT TCA GTT TGG CC

Table 4: List of primers used for identification, cloning and expression analysis of antioxidant enzyme genes

3.3. Results and discussion

3.3.1. Cloning of partial coding sequences of AO genes (*CAT, SOD, GST* and *GPx*): As the genome sequence of *A. janata* is not available, partial nucleotide sequences of the selected AO genes were obtained using degenerate primers (Fig. 29A) mentioned in the materials and methods chapter. The obtained sequences were further confirmed using specific primers (Fig. 28B) and nucleotide sequencing. The confirmed nucleotide sequences of *CAT, SOD, GST* and *GPx* (Fig. 30) were submitted to GenBank (Accession numbers: KM063183, KP939036, KM063184 and KP939037 respectively). Multiple alignment of these sequences with their counterparts from other insects using Clustal Omega (McWilliam et al., 2013) revealed the presence of the conserved domains which further confirms the identity of the cloned sequences (Fig. 31).

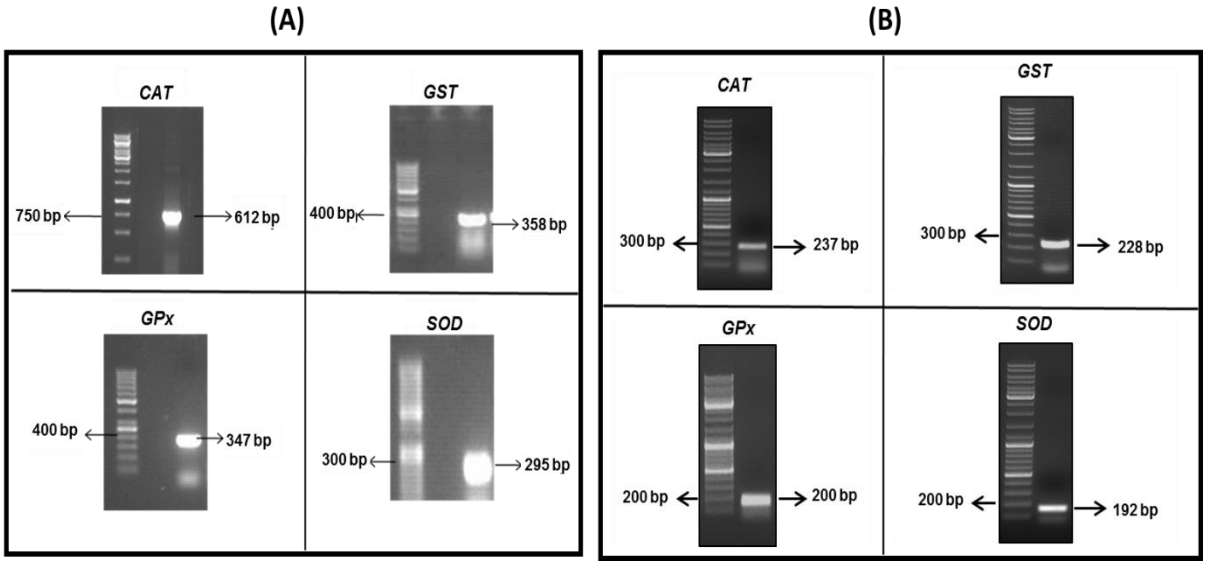

Fig. 29: Agarose gel images showing the amplicons of AO genes obtained using degenerate primers for *CAT* (612 bp), *GST* (358 bp), *GPx* (347 bp) and *SOD* (295 bp) (A) and real time primers for *CAT* (237 bp), *GST* (228 bp), *GPx* (200 bp) and *SOD* (192 bp) (B).

***CAT* mRNA, partial cds**

GATGGATACAGGCATATGAACGGTTATGGATCACACACATTCAAGCTTGTGAATGCCCAAGGTGTCGCTCACTGGGTGA
AGTTCCACTTCAAGACCAACCAGGGTATTGAGAACTTACCAGTTGACAAAGCTAATGAGTTGGCTTCCTCTGATCCTGA
TTATGCTATTAGGGATTTGTATAATGCCATTGCCAAGGGTGACTTCCCATCCTGGAATTTGCATATACAAGTCATGACT
ATGGCACAAGCTGAAAACTGCAAGTTTAATCCATTTGACTTGACAAAGGTCTGGCCGCATTCTGAATACCCCTTGATTC
CTGTGGGTAAAATGGTCTTGGACAAGAACCCTAAGAACTACTTTGCGGAAGTGGAGCAAATTGCATTCAGTCCTGCTAA
CTTTGTGCCTGGCATTGAGCCCTCGCCTGACAAGATGTTGCAAGGACGCTTGTTCTCATACAGTGATACTCACCGTCAT
CGTCTTGGAGCTAACTATTTACAAATTCCTGTGAACTGC

***SOD* mRNA, partial cds**

GACAACACAAATGGGTGCACGTCTGCCGGCGCGCACTTCAACCCCACGAAGTTGGACCACGGCGCGCCCGACGCCAGCA
TCCGTCACGTCGGAGATCTCGGCAACATTGAGTCGTATGGAGGCGTCACTAAGGTGTGCATCCAAGACTCCCAGATTTC
CCTGGCCCCTGGCACCAACAACATCATCGGCCGCACTCTCGTAGTACACGCGGACCCCGATGACTTGGGCATCGGCGGC
CACGAACTTAGCAAGACGACTCGGCAACG

***GST* mRNA, partial cds**

ATTCCACAACACACAGTTCCGACTATAGTCGACGATGGGTTCTCTCTGTGGGAGTCGCGAGCCATCAGCCGATATCTAG
TCAACAAGTATGGTGGAGACTCCACGCTGTACCCTAAAGATGCCAGAGCAAGGGCGTTGGTCGATCAGGGGCTGGACTT
CGACTTAGGAACACTATACCCGAGATTTGGGGAATATTTCTACCCTCAAATCTTTGGAGGCGCTCCCGCTGATGTGGCC
AAACTGAAGAAGCTGGAGGAAGCCCTGGTATTCCTCAACACATTCTTGGAAGGACACAAGTACGCTGCTGGAGATAAAC
TGACCTTCGCTGACAA

***GPx* mRNA, partial cds**

GTAGACGTGCAGGTGGCCGCGTATAACGGTCATGTGTGTATCATCGTGAATGTTGCTTCAATGAGCGGCCTTGAGTGCC
TCCTTGCTTGTATTGAGGAACTTATACAAAGAGTAGCTGAAAAGGGATTATCTATTCTCTCATTCCCATGCAACTTCAT
TTTTCGATCTTTACCAAAGCATACGATTTCTTCTGGATTGCCTGGATCTAGACCAAAGAGATGGTTGATTGGGAATGCA
AGAATGTGTCATCCCTTTTGGTGC

Fig. 30: Partial sequences of AO genes obtained using degenerate primers for *CAT* (612 bp), *GST* (358 bp), *GPx* (347 bp) and *SOD* (295 bp).

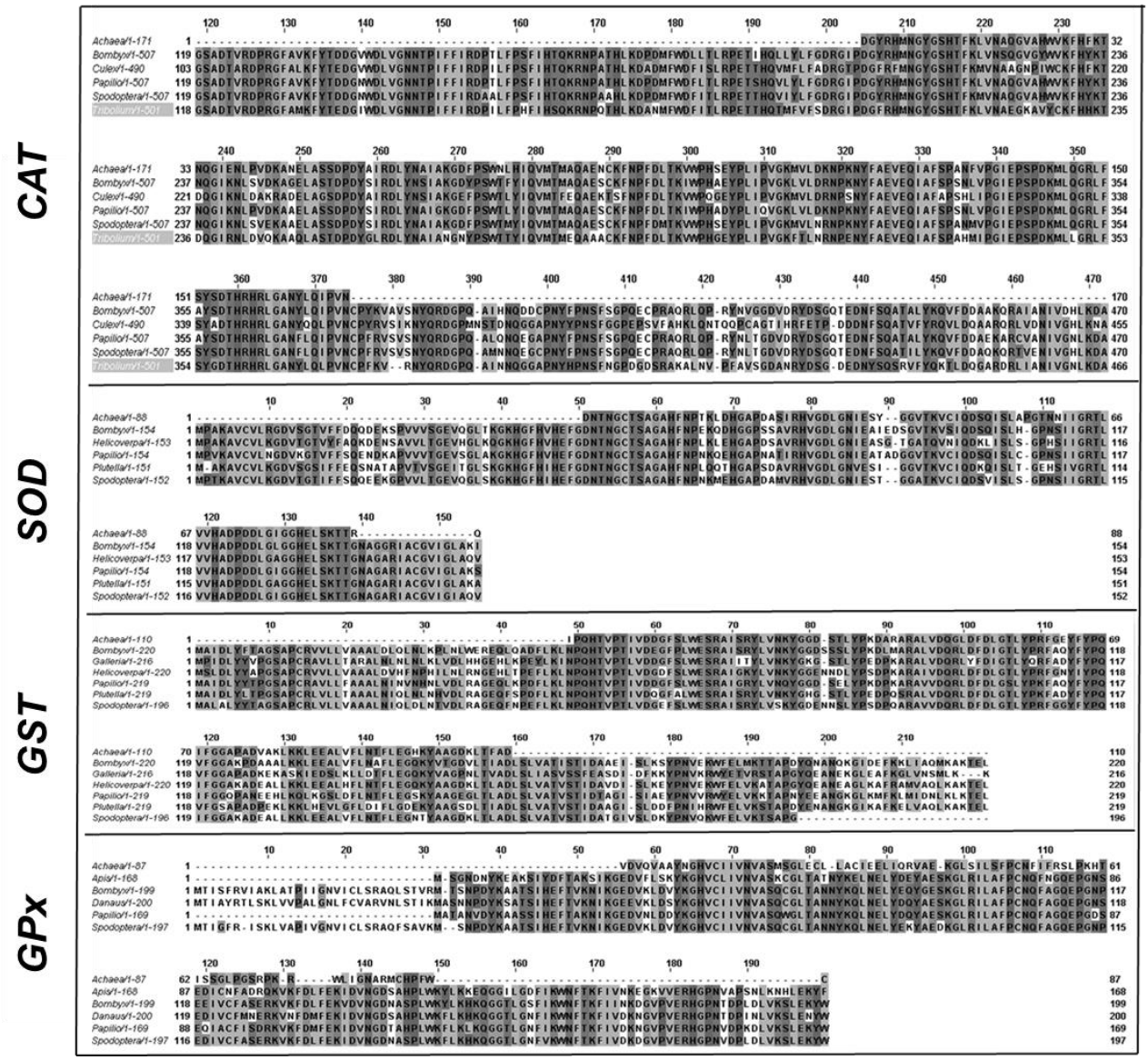

Fig. 31: Multiple alignment of AO genes from *A. janata* with other insect models. Alignment shows conserved nature of the genes among different insects.

3.3.2. Expression analysis of AO genes in larval tissues: Tissue expression studies reveal unique pattern of AO enzyme gene expression in the larval tissues (Fig. 32). *CAT* was predominantly expressed in the fat body and gut although low levels were detected in other tissues as well. *SOD* was expressed in low levels in all the tissues. *GST* was expressed primarily in the gut, fat body and Malpighian tubules while *GPx* mRNA expression was highest in the gut. Of the larval tissues examined, the lowest AO gene expression was detected in the salivary glands.

A previous study in Lepidopteran insect model correlated mitochondrial number with tissue-specific levels of AO enzymes and demonstrated maximum activity in the Malpighian tubules (Ahmad et al., 1991). In a recent study our group has also demonstrated developmentally regulated activity of mitochondrial electron transport chain enzymes in *Chilo partellus* (Venkat Rao et al., 2016). Further, tissue-specific mRNA expression analysis in other insect models have reported highest expression of AO genes in the midgut when compared with other tissues (Barbehenn, 2002; Munks et al., 2005; Krishnan and Kodrik, 2006). However, results with our model suggest otherwise. We observed prominent AO genes' expression both in the larval gut and fat body. In herbivorous insects, ROS is ingested during feeding or generated during food processing (Krishnan and Kodrik, 2006). Moreover, consumption of excess plant products serve as major source of pro-oxidants and xenobiotics (Felton and Summers, 1995). The higher expression levels in the gut of *A. janata* could be a protective response of the insect to prevent tissue damage from ROS. Further, the insect fat body, a well-known multifunctional metabolic tissue that performs versatile functions (Arrese and Soulages, 2010) might also need high expression of AO enzymes, to support its physiological activities.

The high levels of *CAT* transcript in the larval tissues of *A. janata* compared to the other three AO genes could be attributed to the fact that *CAT* is one of the primary scavengers of ROS and solely responsible for scavenging H_2O_2 in insects as they are deficient in a selenium-dependent glutathione peroxidase (Ahmad and Pardini, 1990; Sohal et al., 1990). *GST* whose primary function is to conjugate xenobiotic compounds and other peroxides to glutathione (Enayati et al., 2005) is found in low levels in the native condition which could be induced by oxidative stress.

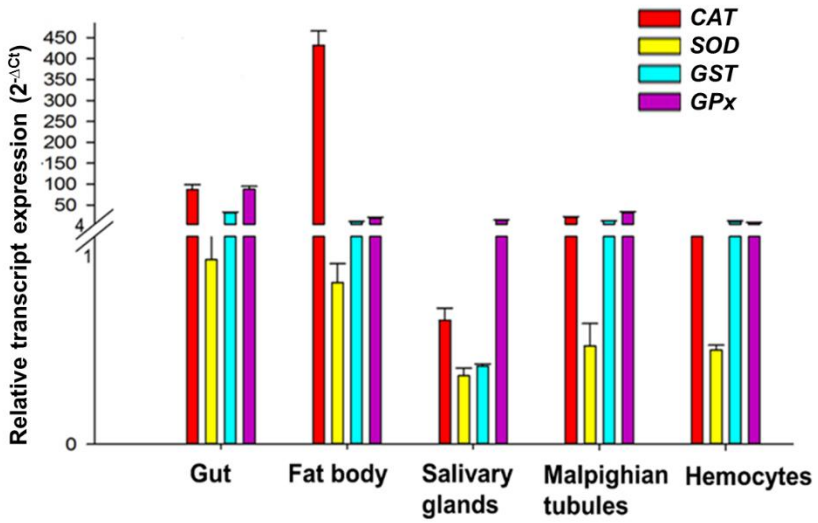

Fig. 32: Expression profile of AO genes in various larval tissues of *A. janata*. Each experiment was performed thrice and the values plotted above is the mean and standard deviation of three values.

3.3.3. Expression analysis of AO genes during the postembryonic development:

Expression of AO genes during postembryonic development is shown in figure 33. Expression of *CAT* mRNA gradually increased from 1st to 4th larval instar, reached its peak during 5th larval instar and decreased thereafter during the pre-pupal and pupal stages. *SOD* mRNA levels remain unaltered till fifth larval instar. Later, there was a two-fold and four-fold increase in the expression during the pre-pupal and pupal stages, respectively. *GST* mRNA levels remained low during the early developmental stages i.e., from 1st to 4th larval instar, rose in the 5th larval instar and reached the maximum in pre-pupal and pupal stages. Expression profile of *GPx* followed a biphasic style with low expression levels during the early larval instars, 4-fold increase during the 4th and 5th larval instars and it declined marginally during the pre-pupal and pupal stages.

So far, there has been no systematic investigation of AO enzyme mRNA expression during the postembryonic development of lepidopteran insects. However, few reports are available on the ontogenic pattern of *GST* and other AO genes in some Coleopteran and Dipteran insects where AO genes showed varied expression patterns. For example in *Agrilus planipennis*, *CAT* expression was higher in the late larval and pupal stages while *SOD* was

constitutively expressed throughout the larval development (Rajarapu et al., 2011). Moreover, isoforms of the same enzyme showed variation in their ontogenic expression pattern (Mittapalli et al., 2007). In the present study too, AO genes exhibited varied expression patterns throughout the larval instars but the significant expression levels during the pre-pupal and the pupal stages indicate the role of these antioxidants to counteract the stress generated due to the extensive metabolic activity in the insect during the larval-pupal and pupal-adult transition (Merkey et al., 2011).

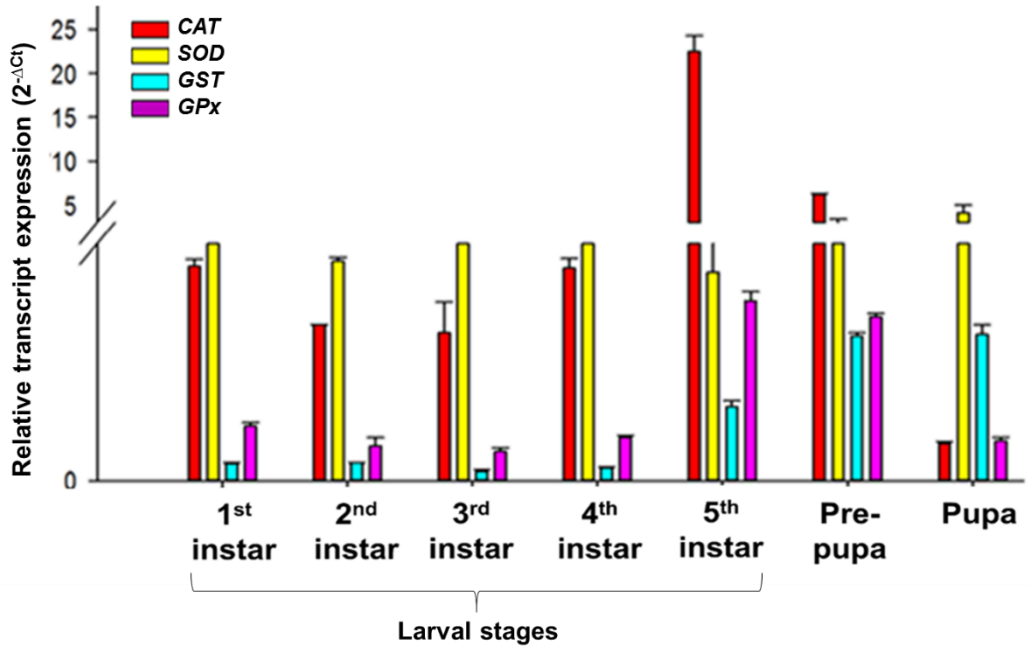

Fig. 33: Relative transcript expression of AO genes in whole body during postembryonic development of *A. janata*. Each experiment was performed thrice and the values plotted above is the mean and standard deviation of three values.

3.3.4. Expression analysis of AO genes during galactose-induced stress: As observed in figure 34, D-galactose-fed larvae exhibited stunted growth and reduced metamorphosis in a dose-dependent manner. In treated group, the larvae weighed ~50% less than the control (Figs. 34A and 34B). Also, the number of pupae formed in the treated group was significantly lower when compared with the control group (Fig. 34C). Noticeably, the pupal weight of the treated group was unaltered (Fig. 34D).

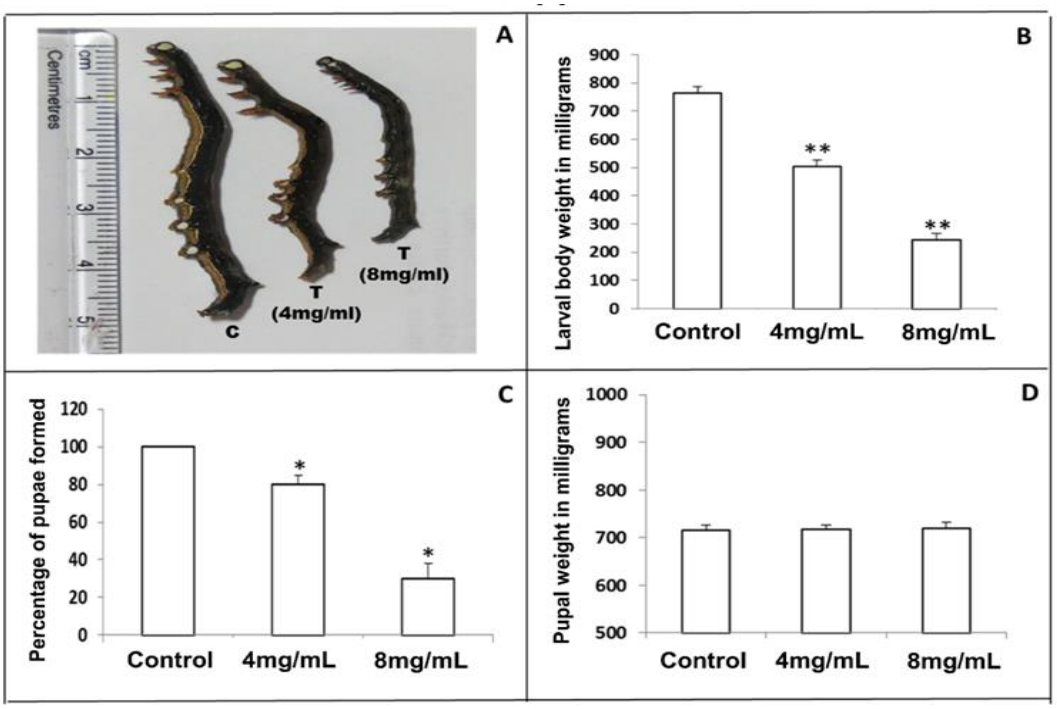

Fig. 34: Effect of D- Galactose feeding on (A) Larval growth (B) Larval body weight (C) Larval-pupal transition/ metamorphosis (D) Pupal weight. (* indicates significance P≤0.05; ** indicates significance P≤0.01).

For this study comparison was made between the control and galactose fed larvae which were age matched and the values are presented as fold change. Feeding of D-galactose modulated AO gene expression in a dose-dependent manner (Fig. 35). At lower dose (4 mg/ml), D-galactose caused significant decline in AO transcript levels both in the larval gut and fat body. At higher dose (8 mg/ml), it caused significant down regulation of mRNA levels only in the fat body while in the gut tissue, AO levels are upregulated. However, *GPx* mRNA expression showed a different pattern. At high dose, the transcript levels were upregulated in the fat body while in the gut tissue they remained similar to the low concentration group.

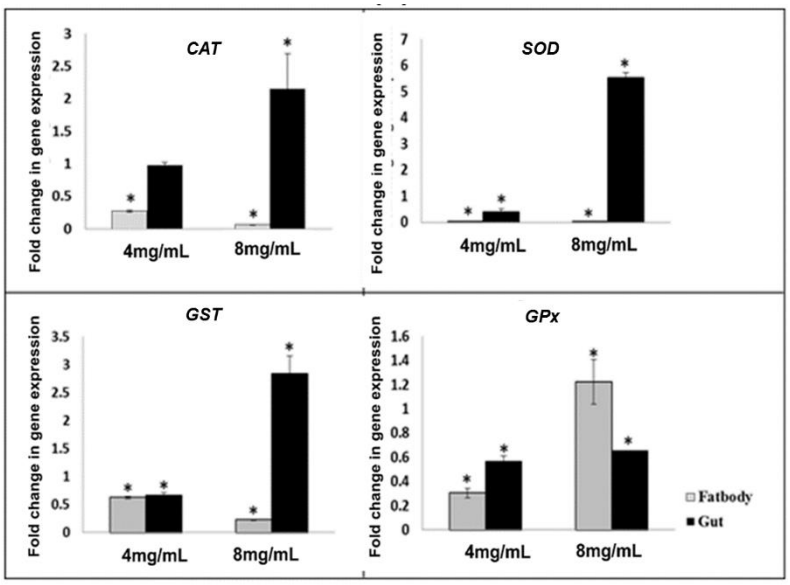

Fig. 35: Effect of D- Galactose feeding on the AO enzyme gene expression levels in the larval gut and fat body of *A. janata*. $2^{-\Delta\Delta Ct}$ is presented as fold change in gene expression.

D-Galactose has been widely employed to induce oxidative stress (Cui et al., 2004; Gaikwad et al., 2010). Similar to our result, Zhang et al., 1990; Ramana et al., 2006 also observed decreased body weight of rodents, which were subjected to D-galactose treatment. As suggested by these groups, this could be essentially due to the accumulation of polyols leading to high intracellular osmolarity followed by leaking out of cellular content (Zhang et al., 1990; Ramana et al., 2006). This reduction in the body weight of the larvae additionally affected the larval-pupal transition as it was necessary for the larvae to attain a particular weight and size to transform into pupae (Frederick, 1975). Similar pupal weights of the control and treated groups obtained in the present study further strengthen the viewpoint. However, only 50% pupae of the treated groups managed to eclose into adults (data not presented), the reason for which needs to be investigated. D-galactose treatment was shown to decrease the activity of antioxidant enzymes in rodents (Zhang et al., 1990; Ramana et al., 2006) similar to our observations that further supports the phenomenon. The decrease in the total glutathione (GSH) content upon D-galactose treatment could be attributed for the decline in *GST* and *GPx* levels in a dose-

dependent manner (Ramana et al., 2006). However, the reason for upregulation of AO genes in the gut at high dose of D-galactose needs to be investigated.

3.3.5. Expression analysis of AO genes upon bacterial challenge: Three bacterial strains were used for immune challenge: *Escherichia coli* (Gram negative), *Bacillus cereus* (Gram positive) and *Bacillus coagulans* (non-pathogen to insects). Each AO gene exhibited a difference in the pattern of induction and also in the degree of response with each microorganism tested. As seen in figure 36, the induction pattern of AO genes in the larval gut varied with the tested microorganism and the maximum upregulation observed was ~4-fold. On the other hand, larval fat body seems to show significantly high response (~70-fold) to immune challenge (Fig. 37) compared to the gut (4-fold). This difference might be due to the physiological and functional differences between these tissues in the insects (Jose and Hartenstein, 2013). Out of the three bacterial elicitors used, *E. coli* stimulated robust response with almost ~70-fold increase in the transcript levels whereas the other two *Bacillus* species down-regulated of AO enzyme transcripts. Also, of the AO enzyme mRNAs tested, *CAT* and *GPx* were expressed predominantly than the other two genes. Although *GST* showed ~25-fold upregulated mRNA levels upon *E. coli* challenge, its response was late (reached a maximum level at 12 hpi) and sustained thereafter, whilst *Cat* and *Gpx* showed an early response (at 3 hpi) with periodic bursts of expression. *Sod* transcript levels showed significant variation in the expression levels upon bacterial challenge but the fold change was much lower as compared to the other AO enzyme transcripts. This suggests that *SOD* cloned from *A. janata* in the present study might function in the management of endogenous stress rather than the induced stress from infections and other external oxidative insults.

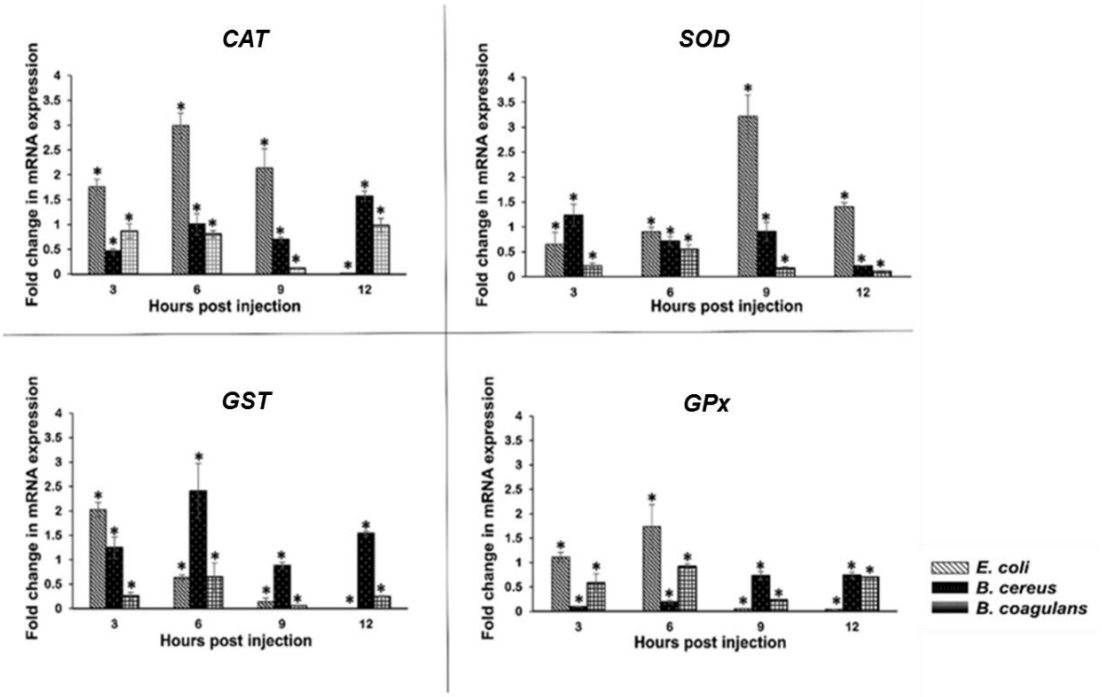

Fig. 36: Effect of bacterial challenge on AO enzyme gene expression levels in larval gut of *A. janata*. $2^{-\Delta\Delta Ct}$ is presented as fold change in gene expression. Data (n=5) were expressed as mean ± SEM (*, $P < 0.05$; ANOVA followed by Student–Newman–Keuls' post hoc test).

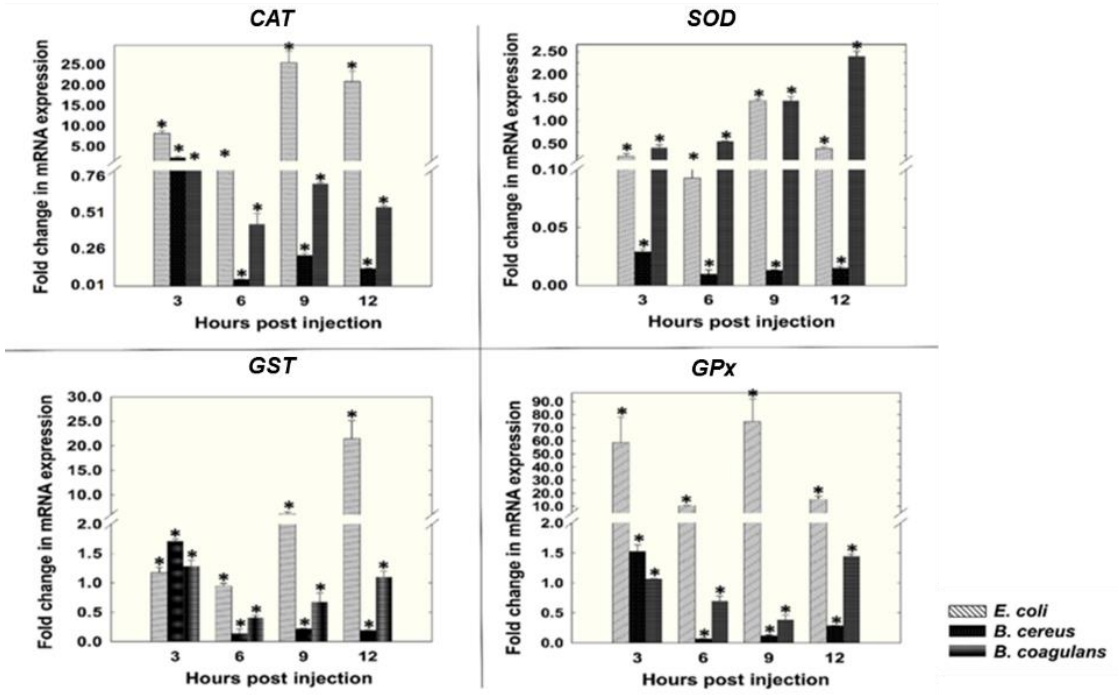

Fig. 37: Effect of bacterial challenge on AO enzyme gene expression levels in larval gut of *A. janata*. $2^{-\Delta\Delta Ct}$ is presented as fold change in gene expression. Data (n=5) were expressed as mean ± SEM (*, $P < 0.05$; ANOVA followed by Student–Newman–Keuls' post hoc test).

Insects exhibit effective immune responses despite lacking adaptive immune components. Though the immune effector components remain obscure, respiratory burst is one of the best understood mechanisms, where the enhanced generation of reactive oxygen intermediates (ROIs) kill the invading pathogen (Nappi and Vass, 1998). Apart from providing immunity to the host from infection caused by pathogens, ROS and redox systems have also been reported to affect the vector specificity in insects (Kumar et al., 2003). Moreover, ROIs are equally damaging to the insect host tissues. Hence, the dynamics between infection induced *de novo* ROI generation and efficient elimination by antioxidants is critical for host survival.

Earlier studies on the involvement of AO enzymes in invertebrate immunity suggested a differential response among the infected tissues. Each tissue exhibited a particular AO enzyme gene upregulation pattern and there was no uniformity in the expression levels of AO genes during the time-course studied (Ren et al., 2009). In insects, fat body is the principal metabolic organ and site of immune function (Arrese and Soulages, 2010). This explains the prominent response seen in the fat body as compared to the larval gut in our study. Early increase in the expression levels of *CAT* and *GPx* suggest their early response to neutralize the free radicals generated during infection. Upregulation of *GST* at 12 hpi could be a delayed response to clear the lipid peroxides generated from the damaged cell membranes. Moreover, our results further ratify the findings that Gram negative bacteria are potent inducers of immunity as observed in *Drosophila* (Lemaitre et al., 1997). Samakovlis et al., (1992) also reported the relatively strong induction of an antimicrobial peptide, cecropin *in vitro* in a *Drosophila* tumor blood cell line by bacterial lipopolysaccharide and flagellin than peptidoglycan. Altogether, it is worthwhile to point out that the AO defense is able to differentiate between different classes of bacteria.

3.3.6. Effect of *Bt* Cry toxin exposure on the expression of AO genes: Figure 38 illustrates the effect of Cry toxin exposure on the expression of AO genes. In the insects fed with Cry toxin-coated leaves, except for *GPx*, all the other AO enzyme mRNA levels were declined in the gut (both for 1-day and 2-day fed groups). Interestingly, *GPx* that seem to be a key role

player in bacterial challenge didn't respond with the same intensity during Cry toxin feeding. Once these Cry toxin-fed insects were transferred onto uncoated normal castor leaves and allowed to recover for 2 days, the AO gene expression levels significantly increased when compared with the control. The increase was more prominent in the 1-day Cry toxin exposed group. In this case, *GST* showed robust response with ~30 fold increase followed by *SOD* and *CAT*. However, fat body AO gene expression could not be monitored as Cry toxin feeding caused degeneration of the tissue.

Bacillus thuringiensis is an aerobic, gram-positive spore forming bacterium that produces a variety of insect toxins including protein crystals termed as Cry toxins during sporulation (Whitely and Schnepf, 1986). These toxins act as efficient insecticides essentially by disrupting the cell membrane of the insect midgut (Gill et al., 1992) thus disturbing the functional activity of lipid membranes and intensify lipid peroxidation (Bravo et al., 2011) forcing the insect to resort to its antioxidant defense system for survival. Induction of oxidative stress by Cry toxin has been known for a while and the implication of GSTs based insecticide resistance has also been reported in a variety of insect models (Candas et al., 2003; Boyer et al., 2012). However, insects' response to Cry toxin induced oxidative stress and the role of AO enzymes (other than GSTs) in the development of resistance has not been studied. Earlier reports on gut transcriptome analysis of *Spodoptera litura* and *Plutella xylostella* upon Cry toxin interaction showed transcriptional upregulation of oxidative stress related genes (Rodriguez et al., 2008; Lei et al., 2014) and is further seen in *A. janata* larvae (Vinod, 2016, unpublished data). Further, *Bt* exposure modulated the AO enzyme levels in rodent species too (Nadia et al., 2003). In line with these studies, our study demonstrates the modulation of AO genes upon Cry toxin feeding. The unexpected high levels of AO genes' in the recovered insect groups (1-day and 2-day) compared to the control could be one of the survival strategies which might in turn lead to the tolerance and finally in resistance development.

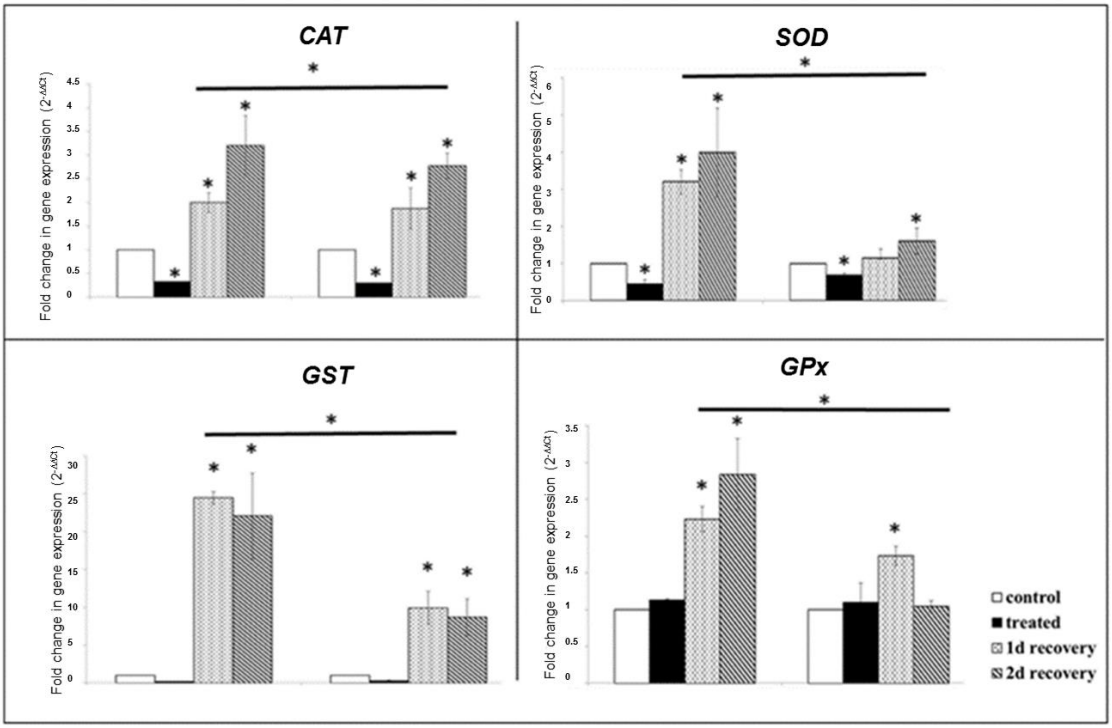

Fig 38: Effect of Cry toxin exposure (feeding and recovery) on AO enzyme gene expression levels in larval gut of *A. janata.* $2^{-\Delta\Delta Ct}$ is presented as fold change in gene expression.

3.4. Summary

Study of AO responses in the economically important polyphagous pests such as *A. janata* would help us gain a better understanding of their survival mechanisms that might further contribute in designing efficient integrated pest management strategies. This study highlights the variations in AO genes' expression in the phytophagous pest when exposed to different stress stimuli. The underlying mechanism by which the insect differentiates various stressor molecules could provide important insights into their successful survival strategies.

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Conclusion

Insects, the most diverse and evolutionarily successful group of organisms play an important role in human life as beneficial insects, agricultural pests and disease vectors. One of the primary factors that contributed to insects' success is their simple yet effective immune system. Study of insect immunity not only helps to delineate the basic principles of immunity conserved across the phyla but also in designing effective control strategies in integrated pest management.

Insects lack adaptive immunity and their innate immunity is comprised of humoral and cellular responses. Cellular response is mediated by insect immune cells primarily, hemocytes and humoral response is mediated immune proteins like AMPs, lysozyme etc. Further, earlier reports suggest ROS mediated defense mechanisms dominate insect immunity and also activate immune gene expression.

Xenobiotic/pesticide resistance is a major challenge in insect pest/ vector control and recent advances in the field of genomics and proteomics have identified several players of insect immune and stress pathway components contributing to pesticide resistance. The rationale of the current study was to identify few of the components of immunity and decipher their role if any in insecticide resistance. This was primarily carried out by identifying proteins/ peptides involved in insect immunity and characterizing their functional role in physiology of insect during post embryonic development of holometabolous insects belonging to order lepidoptera.

A serine protease inhibitor, *Ser2* was identified from the larvae of *C. cephalonica* (*CcSer2*). Using degenerate primers, a partial fragment of *CsSer2* cDNA was cloned and this partial fragment was used to obtain the complete coding sequence using 5' and 3' RACE strategies. Detailed sequence analysis show that the ORF of *CcSer2* is ~1.5 kb coding for a protein of 46 kDa with conserved motifs and a putative N-terminal signal peptide sequence. Tissue distribution profile varies from other reported insect *ser2*s, where *CcSer2* shows maximum

expression levels in the gut followed by hemocytes and the fat body. Expression of *CcSer2* is developmentally regulated suggesting hormonal control of expression which was confirmed by hormone treatment experiments where its expression was shown to be JH dependent which was partially blocked by 20E administration. Possible role of *CcSer2* in immunity has been demonstrated by immune stimulation using bacteria that induced and upregulated its transcript expression in larval gut, fat body and hemocytes. Activity studies (in gel zymography and BAPNA assay) using recombinant CcSer2 showed that the recombinant protein is functional and has trypsin inhibitory activity.

In the quest to identify novel players in insect immunity, a selenium binding protein was identified from the larvae of *A. janata* (*AjSeBP*). Using degenerate primers and RACE strategies complete coding sequence of *AjSeBP* was cloned which has an ORF of ~1.5 kb coding for a protein of molecular weight 56 kDa. *AjSeBP* is predominantly expressed in the larval gut and fat body and shows developmental regulation of expression with maximum expression in the last instar (5^{th} instar) larva. D- galactose induced oxidative stress significantly increased *AjSeBP* levels in the larval fat body. Bacterial challenge with both gram positive and gram negative bacteria induced *AjSeBP* expression with a predominant response in larval fat body when compared with gut. Possible role of *AjSeBP* in Bt toxin resistance/tolerance has been demonstrated by Cry toxin feeding and recovery experiments where significant upregulation of *AjSeBP* transcripts was observed in the recovery period. A preliminary understanding of the metal (Se) binding property of AjSeBP has been investigated by quantifying *AjSeBP* transcript levels in metal supplementations that showed the selenium-specific upregulation of *AjSeBP*. Further, *in vitro* metal binding studies (fluorescence and CD spectroscopy) using recombinant AjSeBP also demonstrated that AjSeBP binds to selenium with a molar ratio of 1:1 and has a binding constant value of 1.1×10^{-5} M^{-1}.

To understand the role of antioxidant enzyme genes in insect immunity and Bt resistance, expression analysis of four antioxidant enzyme genes *CAT, SOD, GST* and *GPx* was carried out under various stress stimuli in the larval tissues of *A. janata*. As the genome sequence of *A. janata* was not available, partial cDNA sequences for the above mentioned genes were cloned using degenerate primer strategy which were then confirmed using specific primers and nucleotide sequence analysis. Tissue distribution analysis showed the predominant expression of AO genes in larval gut and fat body. Furthermore, AO enzyme genes showed varied pattern of expression with maximum expression during the post embryonic development with maximum expression during last instar larval, pre pupal and pupal stages. D- galactose induced oxidative stress, modulated the expression of all the four AO enzyme genes in larvae of *A. janata* as a measure to counteract the stress generated. Bacterial challenge also modulated AO enzyme gene expression predominantly in the fat body when compared with the gut and the degree of response observed among the four AO enzyme genes in a given tissue is *GPx>GST>CAT>SOD*. Current study also demonstrated the modulation of AO enzyme genes upon Cry toxin feeding and during recovery. High levels of AO enzyme gene expression seen in recovered groups most likely facilitates stress management and survival of larvae. Though the current study highlights the variations in AO enzyme gene expressions, the exact underlying mechanism(s) which differentiates between different stressors needs to be elucidated.

In conclusion, present study reports the identification and characterization of candidates involved in insect immunity by facilitating melanization (*Serpin2* from *C. cephalonica*) and a novel player with possible role in immunity, stress management and Bt resistance (*SeBP* from *A. janata*). Further, AO enzymes as key players for a given stress response in *A. janata* were also identified. Observations made in the aforementioned areas clearly provide important insights into their function and could possibly serve in designing efficient strategies for the management of lepidopteran insect pests.

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Differential oxidative stress responses in castor semilooper, *Achaea janata*

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ABSTRACT

Balance between reactive oxygen species (ROS) and the antioxidant (AO) defense mechanisms is vital for organism survival. Insects serve as an ideal model to elucidate oxidative stress responses as they are prone to different kinds of stress during their life cycle. The present study demonstrates the modulation of AO enzyme gene expression in the insect pest, *Achaea janata* (castor semilooper), when subjected to different oxidative stress stimuli. Antioxidant enzymes' (*catalase* (*Cat*), *superoxide dismutase* (*Sod*), *glutathione-S-transferase* (*GST*) and *glutathione peroxidase* (*Gpx*)) partial coding sequences were cloned and characterized from larval whole body. Tissue expression studies reveal a unique pattern of AO genes in the larval tissues with maximum expression in the gut and fat body. Ontogeny profile depicts differential expression pattern through the larval developmental stages for each AO gene studied. Using quantitative RT-PCR, the expression pattern of these genes was monitored during sugar-induced (D-galactose feeding), infection-induced (Gram positive, Gram negative and non-pathogenic bacteria) and pesticide-induced oxidative stress (*Bt* Cry toxin). D-Galactose feeding differentially modulates the expression of AO genes in the larval gut and fat body. Immune challenge with *Escherichia coli* induces robust upregulation of AO genes when compared to *Bacillus coagulans* and *Bacillus cereus* in the larval fat body and gut. Cry toxin feeding predominantly induced *GST* upregulation in the gut. The current study suggests that though there are multiple ways of generation of oxidative stress in the insect, the organism tailors its response by insult- and tissue-specific recruitment of the antioxidant players and their differential regulation for each inducer.

1. Introduction

Oxygen is an essential element and plays important role in oxidative metabolism which facilitates the generation of ATP in various living organisms (Tourmente et al., 2015). The biological processes while using oxygen, also generate reactive molecules called reactive oxygen species (ROS). Under normal physiological conditions, the organisms essentially maintain a homeostatic balance of ROS generation and degradation. Occasionally, this balance can be perturbed causing oxidative stress (Finkel and Holbrook, 2000).

Oxidative stress is a universal phenomenon observed in all aerobic living forms and has been implicated and associated with many diseases/disorders in humans and other species (Ye et al., 2015). Harmful effects of oxidative stress are mainly due to the volatile nature of ROS and their interaction with the macromolecules like proteins, lipids and DNA causing damage to the cells and tissues (Stadtman, 1992). Although ROS are potentially harmful to the organisms, they also play important roles in normal physiology serving as immune effector molecules and sometimes as secondary messengers in the intracellular signaling pathways (D'Autréaux and Toledano, 2007). Hence, the organisms have evolved a complex system of antioxidant (AO) defenses to achieve homeostasis between ROS generation and their elimination thereby making this system to maintain the balance critical for the survival of the organism (Valko et al., 2007).

Insects have been valuable research models in the discovery of many scientific principles owing to the numerous advantages they provide. Throughout the life cycle, many of them thrive in pathogen-rich environments, manage harsh weathers and are exposed to a number of allochemicals. Their remarkable ability to cope up with the enormous oxidative stress generated in all these circumstances, make them attractive models in this field of research. Beneficial effects of ROS characterized in insects include immune pathways like melanization and respiratory burst where in ROS-mediated microbe evasion, ROS or ROS-derived intermediates

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sequester and kill the invading microbes (Christensen et al., 2005; Nappi et al., 2009; Nappi and Vass, 1998). Not only to the invading pathogens but ROS are equally damaging to the insect tissues as well. Hence, insects have evolved a suite of AO enzymes that function in concert to maintain a fine redox balance and protect the insects from the harmful effects of ROS. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferases (GSTs) are some of the few prominent AO enzymes in insects (Ahmad et al., 1991). A thorough understanding of the AO defense responses in insects might help in gaining insights into their survival strategies through the course of their successful evolution and also exploit this information for designing integrated pest management modules.

Majority of the existing studies on antioxidant systems in insects report the changes in the AO enzyme activities in response to any given oxidative insult. Though the activity changes seem to be enough to reach upon a conclusion, it is important to understand if the change is by *de novo* transcript synthesis or by enhanced transcript stability. Also, as the changes at the transcription levels are indicative of the long term responses, these are therefore more useful in understanding the key phenomena like adaptations for survival and resistance development. The current study is such an attempt to monitor the AO gene changes at the transcriptional level for a given stress stimulus. Using various stress inducers like D-galactose, bacteria and Cry toxin to analyze AO gene expression provides a better outlook on the importance of this AO defense system and helps to identify AO gene markers, if any.

2. Materials and methods

2.1. Insect rearing

Achaea janata belongs to the insect order Lepidoptera, which is the most destructive group of insect pests and is widespread throughout the tropical and subtropical regions of the world. This pest causes considerable yield loss of its primary host castor, which is an important non-edible oil seed crop in many countries including India. Occasional hosts include economically important plants like mustard, sugar cane, cabbage, rose, tomato, banana and tea (Lakshminarayana and Raoof, 2005).

A. janata larvae were obtained from Indian Institute of Oil Seeds Research (Hyderabad, India) and fed on castor leaves at 25 ± 1 °C and 70% relative humidity with a photoperiod of 12:12 h light: dark cycle (Budatha et al., 2011). The larvae were anesthetized on ice for 15 min and dissected in cold insect Ringer solution to isolate the tissues. The collected tissues were snap frozen using liquid nitrogen and stored at −80 °C till further use.

2.2. Cloning of AO genes from larval whole body

Total RNA was isolated from the whole body using TRI reagent™ (Sigma–Aldrich, USA). The quality of the isolated RNA was analyzed by formaldehyde–agarose gel electrophoresis and the concentration was estimated using NanoDrop-1000 spectrophotometer (Thermo Scientific Nanodrop 2000). First strand cDNA synthesis was performed using Verso cDNA synthesis kit (Thermo Scientific, Rockford, USA) according to the manufacturer's protocol. Based on the reported nucleotide sequences from Lepidoptera, degenerate primers were designed in the conserved regions of catalase (*Cat*), superoxide dismutase (*Sod*), glutathione S-transferase (*GST*) and glutathione peroxidase (*Gpx*). Amplicons obtained using degenerate primers were further confirmed using gene specific primers. Real time primers were designed for the respective AO genes to carry out further studies. All the primers used are listed in Table 1.

2.3. qRT-PCR of AO genes, in various tissues, during development and different stress conditions

For tissue expression studies, gut, fat body, salivary glands, Malpighian tubules and hemocytes were collected from 5th instar (last instar) larvae. Whole body samples of 1st to 5th instar larvae, prepupa and pupa were used for ontogeny analysis. Using the same method, larval gut and fat body samples were collected from the D-galactose fed and bacteria-challenged insect groups. As fat body was degenerated in Cry toxin exposed insects, only gut tissue was obtained. Total RNA was isolated from the respective samples and first strand cDNA was synthesized using 1 μg of total RNA as mentioned above. Gene expression was monitored using gene specific primers and SYBR green qRT-PCR kit (Applied Biosystems, CA, USA) in ABI-7500 fast real-time PCR system (Applied Biosystems, CA, USA). A 40-cycle two-step PCR was carried out in triplicates with 10 μL reaction volume containing the following components: 2.0 μL cDNA template (1:40 dilution), 1.0 μL of forward and reverse primers (10 pmol) each, and 5.0 μL of 2X SYBR green PCR mix. Melting curve analysis was performed for each gene to check for specific amplification. 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, CA, USA). Relative quantification results were normalized with ribosomal protein S7 as endogenous control. All the results were represented as fold change in the transcript levels relative to the reference values obtained for their respective controls using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) except for tissue distribution and ontogeny profiles where relative transcript levels with respect to the endogenous control were represented.

2.4. D-Galactose treatment

D-Galactose has been widely employed to generate ageing, diabetes, hyperglycemia models in rats and to induce oxidative stress (Gong and Xu, 1991 and Li et al., 1995). However, few studies have reported its application in insect models (Cui et al., 2004 and Gaikwad et al., 2010). For our current study, we fed the insects

Table 1
List of primers used for cloning and real-time analysis of *A. janata antioxidant genes:* catalase (*Cat*), superoxide dismutase (*Sod*), glutathione S-transferase (*GST*) and glutathione peroxidase (*Gpx*).

Gene	Primer	Cloning primers (5′-3′)	Real time primers (5′-3′)
Cat	Forward	GATGGATACAGGCATATGAACGG	GGTCCATCACGCTGATAGTTGG
	Reverse	CCCAACTCCTTCTCTGGTCC	CGAGTTACGGTCCGTGTTTC
Sod	Forward	GACAACACAAATGGGTGCACGTCTG	CGTCTTGCTAAGTTCGTGGC
	Reverse	GCAAGACGACTCGGCAACG	CTCTAGAGGCTGCACTGCCTAC
GST	Forward	CCACAACACACAGTTCC	GCGAGCCATCAGCCGATATCTAG
	Reverse	GGAGATAAACTGACCTTCGC	CCGGTTTGACTTCTTCGACCTCC
Gpx	Forward	GTAGACGTGCAGGTGGCCG	GGAACTTCCATAGTGGACTGG
	Reverse	GTGTCATCCCTTTTGGTGC	CTCTTACGCATTAGGGAAAAGAAGTC

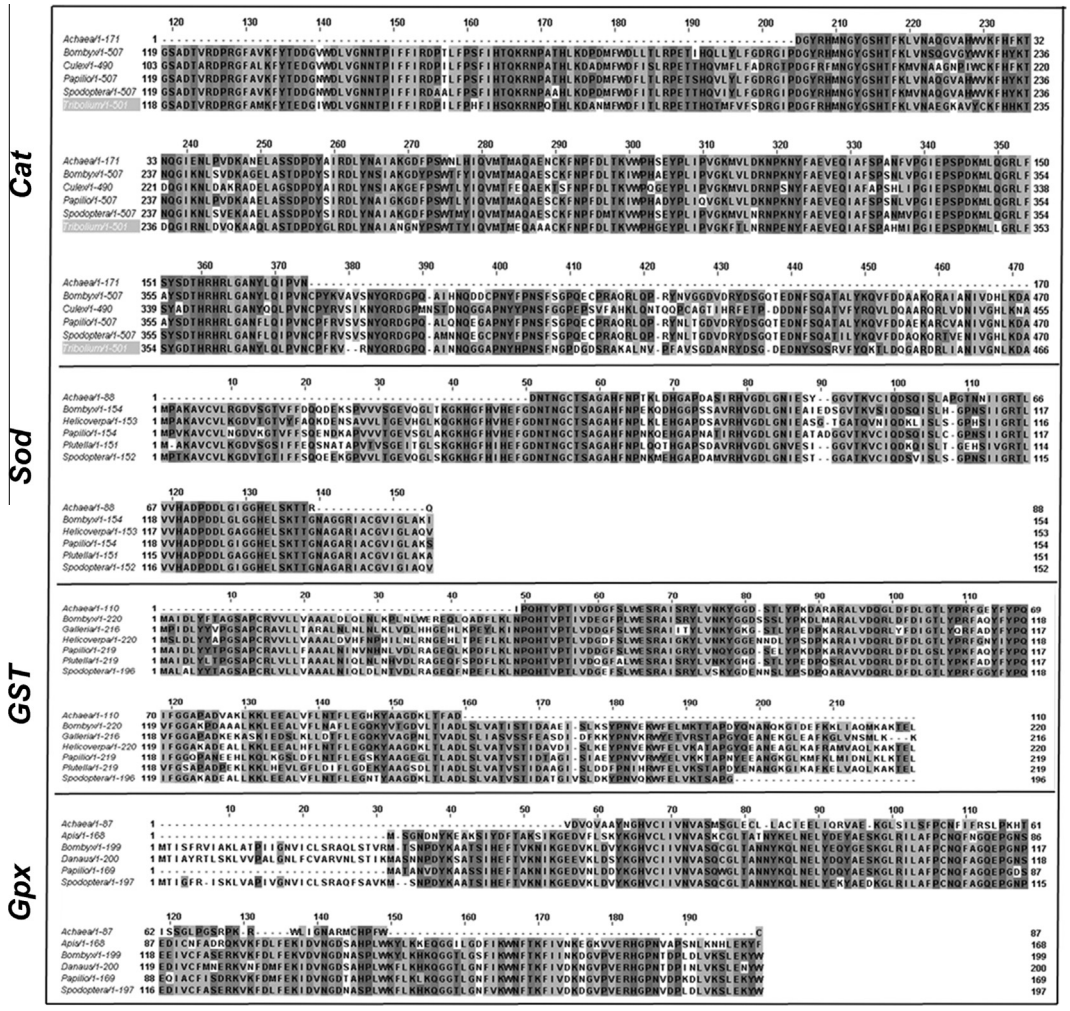

Fig. 1. Multiple alignment of the antioxidant genes: catalase (*Cat*), superoxide dismutase (*Sod*), glutathione S-transferase (*GST*) and glutathione peroxidase (*Gpx*).

with D-galactose coated castor leaves to induce oxidative stress. After standardizing the dosage and the treatment period parameters for our model (data not included), the effects of D-galactose on larval growth and development were monitored. Castor leaves of equal weights were dipped in various doses (4 mg/ml and 8 mg/ml) of D-galactose. These leaves were then air-dried and fed to the neonatal larvae (3 days post hatch) till pupation. Control leaves were dipped in distilled water.

2.5. Bacterial challenge

Live bacteria (*Escherichia coli* (DH5α), *Bacillus cereus*, and *Bacillus coagulans*) were grown till optical density of 0.4 was reached at 600 nm. The bacterial cells were pelleted down, washed and resuspended in phosphate buffered saline (PBS). 5 μl of this suspension was injected into the hemocoel at the penultimate abdominal segment of the fifth instar larvae using Hamilton micro syringe. The control insects received equal volume of PBS.

2.6. Cry toxin feeding and recovery

For Cry toxin feeding and recovery experiments, 3rd instar larvae were allowed to feed on castor leaves coated with the activated toxin prepared from selected *Bt* isolate, DOR1 from Indian Institute of Oilseeds Research, Hyderabad (Reddy et al., 2012) at the concentration of 140 ng/sq.cm of leaf surface. The insects were fed on these toxin coated leaves for either one or two days and then transferred to uncoated leaves and allowed to recover for 2 days. Samples were collected for every 24 h in the recovery period.

2.7. Statistical analysis

Data are expressed as mean ± SEM of three independent experiments ($n = 3$). Significance test for the means of the samples was performed using one way ANOVA (for data points with normal distribution) or Kruskal–Wallis analysis for those without normal distribution followed by Student Newman–Keul's test using Sigma Plot 12.0 software (Systat Software Inc., USA). The differences were considered significant at $P < 0.05$.

3. Results

3.1. Cloning of partial coding sequences of AO enzyme genes: Cat, Sod, GST and Gpx

The obtained sequences of *Cat*, *Sod*, *GST* and *Gpx* were submitted to GenBank (Accession numbers: KM063183, KP939036, KM063184, KP939037) respectively. Multiple alignment of these sequences with their counterparts from other insects using Clustal Omega (McWilliam et al., 2013) revealed the presence of the conserved domains which further confirms the identity of the cloned sequences (Fig. 1).

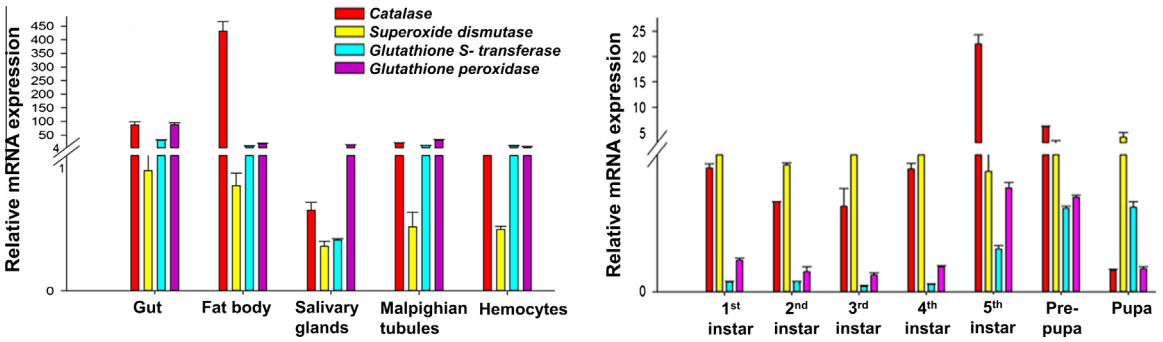

Fig. 2. Tissue distribution and developmental profile of antioxidant genes in *A. janata*.

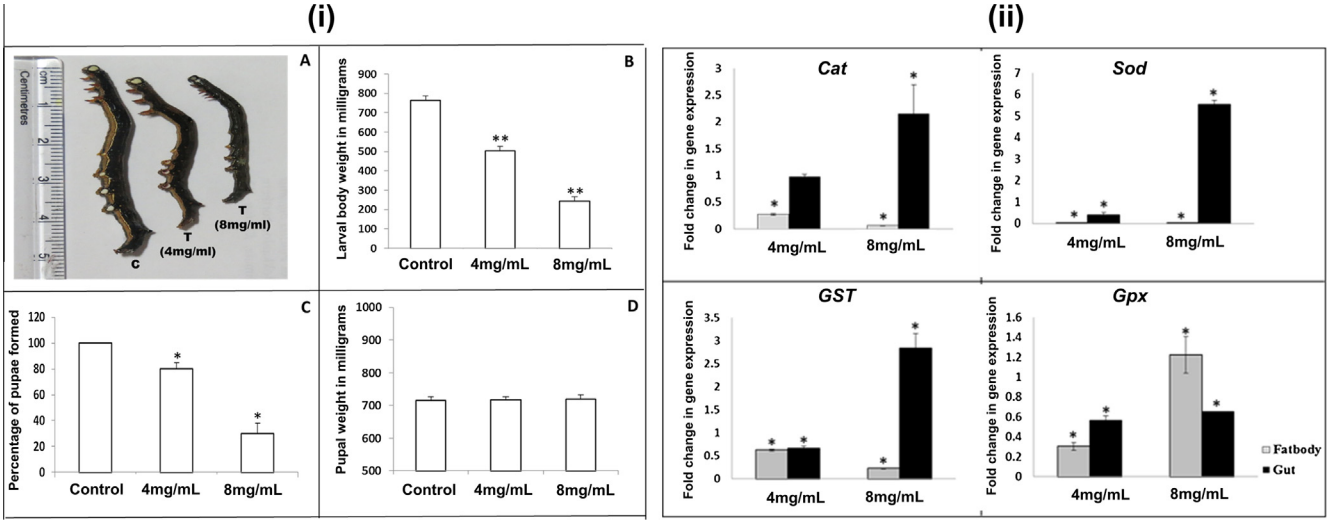

Fig. 3. Effect of oxidative stress inducer, D-galactose on (i) larval development and (ii) expression of antioxidant genes in the larval gut and fat body. * Indicates a statistically significant ($P < 0.05$) difference between the control and treated groups.

3.2. Expression analysis of AO enzyme genes in larval tissues

Tissue expression studies reveal unique pattern of AO enzyme gene expression in the larval tissues (Fig. 2). *Cat* is predominantly expressed in the fat body and gut although low levels are detected in other tissues as well. *Sod* is expressed in low levels in all the tissues. *GST* is expressed primarily in the gut, fat body and Malpighian tubules while *Gpx* mRNA expression was highest in the gut. Of the larval tissues examined, the lowest AO gene expression was detected in the salivary glands.

A previous study in Lepidopteran insect model correlated mitochondrial number with tissue-specific levels of AO enzymes and demonstrated maximum expression in the Malpighian tubules (Ahmad et al., 1991). However, results with our model suggest otherwise. We observed prominent AO genes' expression in the larval gut and fat body. The higher expression levels in the gut could be due to the presence of excess plant products that serve as the major source of pro-oxidants and xenobiotics (Felton and Summers, 1995). Moreover, insect fat body, a well-known multifunctional metabolic tissue might also need high expression of AO enzymes, to support its physiological activities.

The high levels of *Cat* in the larval tissues compared to the other three AO genes could be attributed to the fact that *Cat* is one of the primary scavengers of ROS and solely responsible for scavenging H_2O_2 in insects as they are deficient in a selenium-dependent glutathione peroxidase (Ahmad and Pardini, 1990 and Sohal et al., 1990). *GST* whose primary function is to conjugate xenobiotic compounds and other peroxides to glutathione (Enayati et al., 2005) is found in low levels in the native condition which could be induced by oxidative stress.

3.3. Expression analysis of AO genes during the postembryonic development

Expression of *Cat* mRNA gradually increased from 1st to 4th instar, reached its peak during 5th instar and decreased thereafter during the pre-pupal and pupal stages. *Sod* mRNA levels remain unaltered till fifth larval instar. Later, there was a two-fold and four-fold increase in the expression during the pre-pupal and pupal stages, respectively. *GST* mRNA levels remained low during the early developmental stages i.e., from 1st to 4th instar, rose in the 5th instar and reached the maximum in pre-pupal and pupal stages. Expression profile of *Gpx* followed a biphasic style with low expression levels during the early instars, 8-fold increase during the 4th and 5th instars and declined marginally during the pre-pupal and pupal stages.

So far, there has been no systematic investigation of AO enzyme mRNA expression during the postembryonic development of insects. However, few reports are available on the ontogenic pattern of GST where the highest enzyme activity was detected in the pupal stages (Hazelton and Lang, 1983; Kostaropoulos and Papadopoulos, 1998; Kotze and Rose, 1987). In the present study,

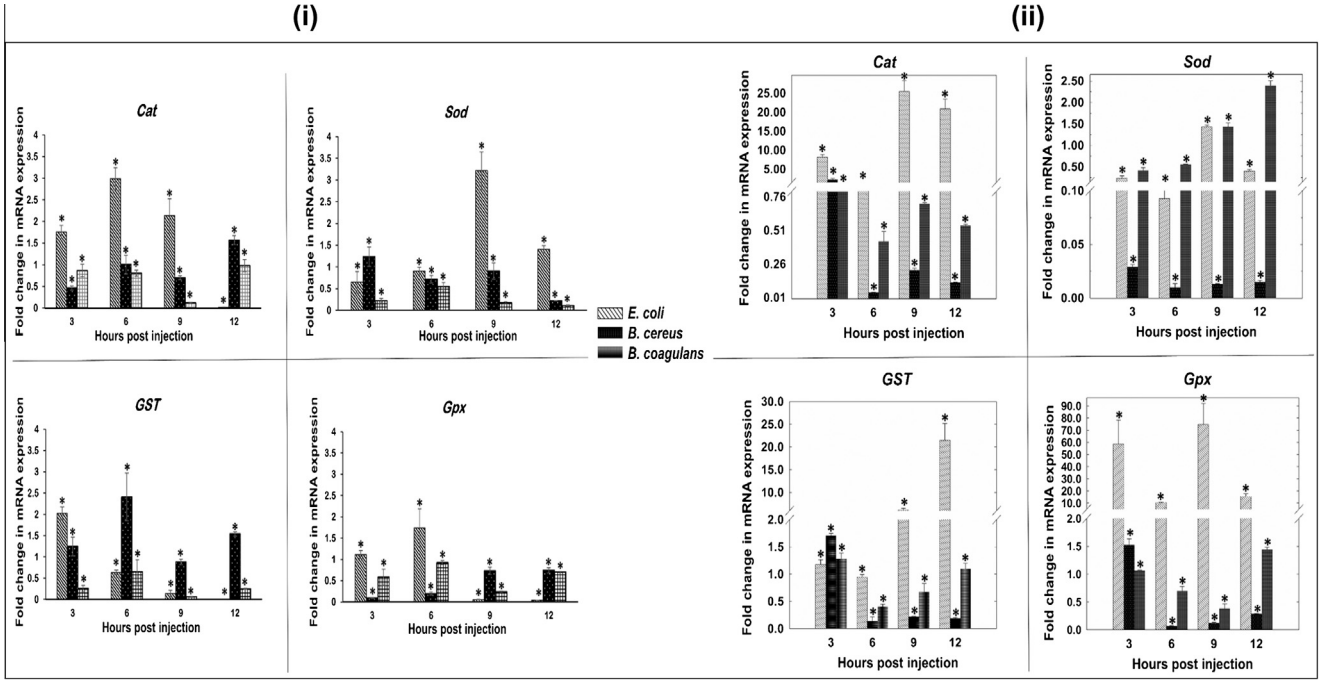

Fig. 4. Effect of infection induced oxidative stress on the expression of antioxidant genes in the larval (i) gut and (ii) fat body. * Indicates a statistically significant ($P < 0.05$) difference between the control and treated groups.

AO enzyme genes exhibited varied expression patterns throughout the larval instars but the significant expression levels during the pre-pupal and the pupal stages indicate the role of these antioxidants to counteract the stress generated due to the extensive metabolic activity in the insect during the larval-pupal and pupal-adult transition (Merkey et al., 2011).

3.4. Expression analysis of AO genes during galactose-induced stress

As observed in Fig. 3, D-galactose-fed larvae exhibited stunted growth and altered metamorphosis in a dose-dependent manner. The treated group larvae weighed ~50% less than the control (Fig. 3A and B). Also, the number of pupae formed in the treated group was significantly less when compared to the control group (Fig. 3C). Noticeably, the pupal weight of the treated group was unaltered (Fig. 3D).

Further, D-galactose modulated AO gene expression in a dose-dependent manner (Fig. 4). At low dose (4 mg/ml), D-galactose caused significant decline in AO transcript levels both in the larval gut and fat body. At high dose (8 mg/ml), it caused significant down regulation of mRNA levels only in the fat body while in the gut tissue, AO levels are upregulated. However, *Gpx* mRNA expression showed a different pattern. At high dose, the transcript levels were upregulated in the fat body while in the gut tissue they remained similar to the low concentration group.

D-Galactose has been widely employed to induce oxidative stress (Cui et al., 2004; Gaikwad et al., 2010). Similar to our result, Ramana et al., 2006; Zhang et al., 1990 also observed decreased body weight of rodents, which were subjected to D-galactose treatment. As suggested by these groups, this could be essentially due to the accumulation of polyols leading to high intracellular osmolarity followed by leaking out of cellular content. This reduction in the body weight of the larvae additionally effected the larval-pupal transition as it was necessary for the larvae to attain a particular weight and size to develop into pupae (Nijhout, 1975). Similar pupal weights of the control and treated groups observed in the present study further strengthen the viewpoint. However, only 50% pupae of the treated groups managed to eclose into adults (data not presented), the reason for which needs to be investigated.

D-galactose treatment decreased the activity of antioxidant enzymes in rodents as well (Ramana et al., 2006; Zhang et al., 1990), similar to our observations. The decrease in the total glutathione (GSH) content upon D-galactose treatment could be attributed for the decline in *GST* and *Gpx* levels in a dose-dependent manner (Ramana et al., 2006). However, the reason for upregulation of AO genes in the gut at high dose of D-galactose is unknown.

3.5. Expression analysis of AO enzyme genes upon bacterial challenge

Three bacterial strains were used for immune challenge: *E. coli* (Gram negative), *B. cereus* (Gram positive) and *B. coagulans* (non-pathogen to *A. janata*). Each AO gene exhibited a difference in the pattern of induction and also in the degree of response with each microorganism tested. As seen in Fig. 4(i), the induction pattern of AO genes in the larval gut varied with the tested microorganism and the maximum upregulation observed was 4-fold.

On the other hand, larval fat body seems to show significantly high response (70-fold) to immune challenge compared to the gut (4-fold) (Fig. 4(ii)). This difference might be due to the physiological and functional differences between these tissues in the insects (Campos-Ortega and Hartenstein, 2013). Out of the three bacterial elicitors used, *E. coli* stimulated robust response with almost 70-fold increase in the transcript levels whereas the other two *Bacillus* species induced down-regulation of AO enzyme transcripts. Also, of the AO enzyme mRNAs tested, *Cat* and *Gpx* were expressed predominantly than the other two genes. Although *GST* showed ~30-fold upregulated mRNA levels upon *E. coli* challenge, its response was late (reached a maximum level at 12 hpi) and sustained thereafter, while *Cat* and *Gpx* showed an early response (at 3 hpi) with periodic bursts of expression. Though *Sod* responded in a similar fashion as the other AO genes, the level of expression was lower.

Insects exhibit effective immune responses despite lacking adaptive immune components. Though the immune effector

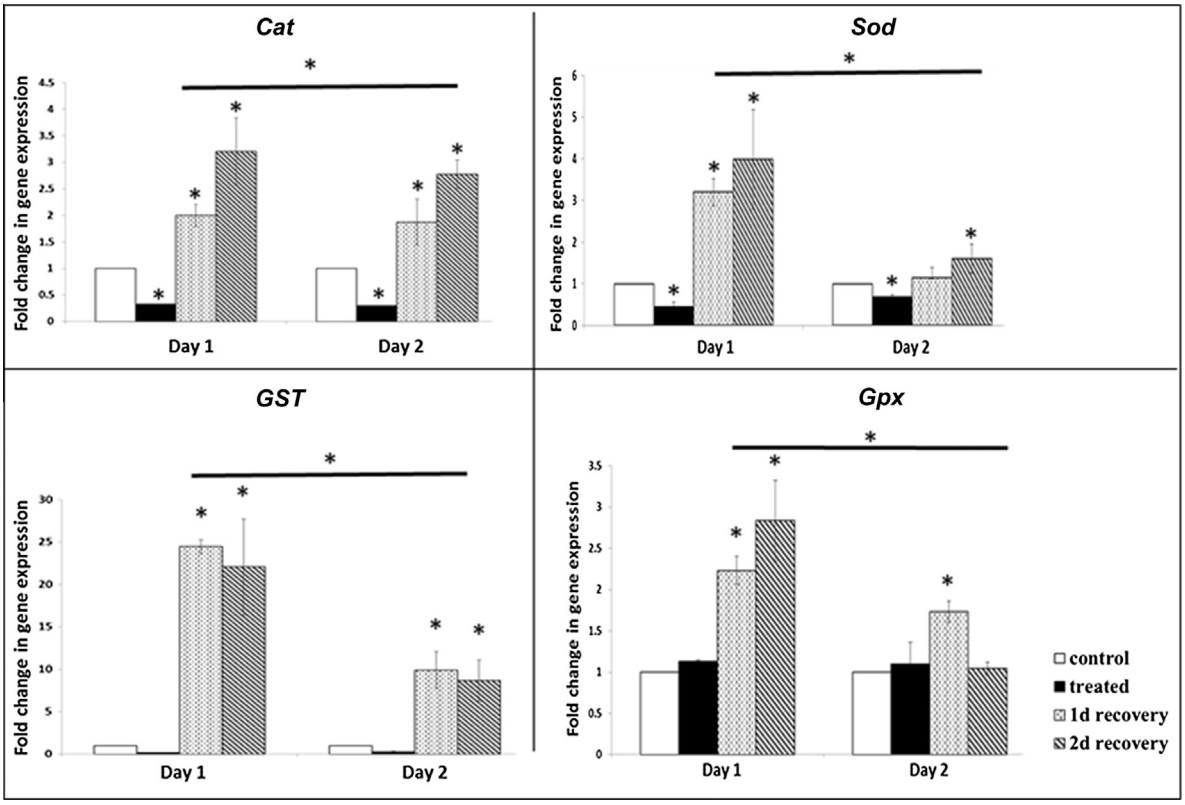

Fig. 5. Effect of Cry toxin induced oxidative stress on the expression of antioxidant genes in the larval gut. * Indicates a statistically significant ($P < 0.05$) difference between the control and treated groups.

components remain obscure, respiratory burst is one of the best understood mechanisms, where the enhanced generation of reactive oxygen intermediates (ROIs) kills the invading pathogen (Nappi and Vass, 1998). Apart from providing immunity to the host from infection caused by pathogens, ROS and redox systems have also been reported to affect the vector specificity in insects (Kumar et al., 2003). Moreover, ROIs are equally damaging to the insect host tissues. Hence, the dynamics between infection induced *de novo* ROI generation and efficient elimination by antioxidants is critical for host survival.

Involvement of AO enzyme genes in the control of microbial development has been reported in *Drosophila* where the induction of catalase upon septic injury was observed (De Gregorio et al., 2001). This is further strengthened by the discovery of an immune responsive catalase (IRC) and its role in protection from gut infection in *Drosophila* by Ha et al. (2005). Also, transcriptome studies in *Galleria melonella* have shown the inducible expression of *Gst* upon infection (Vogel et al., 2011). Our results strongly support these previous findings. However, few other studies on the involvement of AO enzymes in invertebrate immunity suggested a differential response among the infected tissues. Each tissue exhibited a particular AO enzyme gene upregulation pattern and there was no uniformity in the expression levels of AO enzyme genes during the time-course studied (Ren et al., 2009). In insects, fat body is the principal metabolic organ and site of immune function (Arrese and Soulages, 2010). This explains the prominent response in the fat body compared to the larval gut in our study. Early increase in the expression levels of *Cat* and *Gpx* suggest their early response to neutralize the free radicals generated during infection. Upregulation of *GST* at 12 hpi could be a delayed response to clear the lipid peroxides generated from the damaged cell membranes and the lower degree of response exhibited by *Sod* could probably be either due to lower transcript abundance or the presence of other physiologically active forms of the enzyme. Moreover, our results further ratify the findings that Gram negative bacteria are potent inducers of immunity as observed in *Drosophila* (Lemaitre et al., 1997). Samakovlis et al. (1992) also reported the relatively strong induction of an antimicrobial peptide, cecropin *in vitro* in a *Drosophila* tumor blood cell line by bacterial lipopolysaccharide and flagellin than peptidoglycan. Altogether, it is worthwhile to point out that the AO defense is able to differentiate between different classes of bacteria.

3.6. Effect of Cry toxin feeding on the expression of AO enzyme genes

In the insects fed with Cry toxin-coated leaves (Fig. 5), except for *Gpx*, all the other AO enzyme mRNA levels were declined in the gut (both for 1-day and 2-day treated groups) which could be due to the cell damage caused by the toxin (Chauhan, unpublished data). Once these Cry toxin-fed insects were transferred onto uncoated normal castor leaves and allowed to recover for 2 days, the AO gene levels significantly increased when compared to the control. The increase was more prominent in the 1-day Cry toxin treated group. In this case, *GST* showed robust response with ~30-fold increase followed by *Sod* and *Cat*. Interestingly, *Gpx* that seem to be a key role player in bacterial challenge didn't respond with the same intensity to Cry toxin. However, fat body AO gene expression could not be monitored as Cry toxin treatment caused degeneration of the tissue.

Bacillus thuringiensis is an aerobic, gram-positive spore forming bacterium that produces a variety of insect toxins including protein crystals termed as Cry toxins during sporulation (Whiteley and Schnepf, 1986). These toxins act as efficient insecticides essentially by disrupting the cell membrane of the insect midgut (Gill et al., 1992) thus disturbing the functional activity of lipid membranes and intensify lipid peroxidation (Alejandra et al., 2011) forcing

the insect to retort to its antioxidant defense system for survival. Induction of oxidative stress by Cry toxin has been known for a while and the implication of GSTs in insecticide resistance has also been reported in a variety of insect models (Boyer et al., 2012 and Candas et al., 2003). However, insects' response to Cry toxin induced oxidative stress and the role of AO enzymes (other than GSTs) in the development of resistance has not been studied. Earlier reports on transcriptome analysis of *Spodoptera litura* and *Plutella xylostella* guts upon Cry toxin interaction showed transcriptional upregulation of oxidative stress related genes (Rodríguez-Cabrera et al., 2008 and Lei et al., 2014). Further, *Bt* exposure modulated the AO enzyme levels in rodent species too (Shaban et al., 2003). In line with these studies, our study demonstrated the modulation of AO enzyme genes upon Cry toxin feeding. The unexpected high levels of AO genes' in the recovered insect groups (1-day and 2-day) compared to the control could be one of the survival strategies which might in turn lead to the resistance development.

4. Conclusion

This study highlights the variations in AO genes' expression in the phytophagous pest when exposed to different stress stimuli. The underlying mechanism by which the insect differentiates various stressor molecules could provide important insights into their successful survival strategies.

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