Regulatory proteins from insect haemolymph and their role in development

Thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

by

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Dec-2008
Enrolment No. 03LAPH05

Dedicated to
My Parents
and
Prof. Ch. R. K. Murthy

Contents

DECLARATION

I hereby declare that the work embodied in this thesis entitled **"Regulatory proteins from insect haemolymph and their role in development"** has been carried out by me under the supervision of Prof. Aparna Dutta Gupta and this has not been submitted for degree or diploma of any other university earlier.

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CERTIFICATE

This is to certify that Mr. G. Damodara Rao has carried out research work embodied in this thesis under my supervision and guidance for a full period prescribed under the Ph.D. ordinance of this University. We recommended his thesis **"Regulatory proteins from insect haemolymph and their role in development"** for submission for degree of Doctor of Philosophy of this University.

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Acknowledgements

I express my deep sense of gratitude and appreciation to ***Prof. Aparna Dutta Gupta*** *under whose inspiring, untiring and expert guidance the present work was carried out. I am indebted to her for the keen interest in my work, for her constant encouragement, critical suggestions and stimulatory discussions.*

I am grateful to the Dean, School Of Life Sciences Prof. A. S. Raghvendra, Former Dean Prof. T. Suryanarayana School of Life Sciences for giving me an opportunity to work at this university. I am thankful to the Head Department of Animal Sciences, Prof. S. Dayananda and Former Head of the Department Prof. Aparna Dutta Gupta and Late ***Prof. Ch. R. K. Murthy*** *for providing the necessary research facilities to carry out the present work. I also thank all the faculty members of Department of Animal Sciences and School of Life Sciences for their help and support.*

I must keep on record my special thanks to the doctoral committee members, Prof. P. Reddanna and Prof. B. Senthil Kumaran for their constructive criticism which was instrumental in designing several of the experiments discussed in this thesis. The sudden demise of Prof. Ch. R. K. Murthy is a personal loss to me in many ways.

I wish to extend my sincere thanks to Prof. Mechail Whitting, Brigham Young University, USA for allowing me to work at his laboratory and providing me with the necessary facilities and technical help during my stay. I am also grateful to Dr. Gavin Jhon Sevenson and Dr. Meghan for their help during my stay at USA.

I am grateful to Prof. Subba Reddy Palli, Kentucky University, USA for providing me the GST antibody.

I am grateful to CSIR, New Delhi for the award of Junior and Senior Research Fellowships, which provided me with the necessary financial support to pursue research. I am also thankful to the Society for systemic biology (SSB) for the financial support that helped me in carrying out part of the research work at the Brigham Young University. I also acknowledge the financial support from CSIR, DST-FIST, COSIST, UGC-SAP and UPE that was used for creating the necessary infrastructure and was used for the present study.

I take this opportunity to thank my senior Dr. Abul Arif whose initial help was instrumental in understanding the subject. I sincerely thank all my lab colleagues for their help and companionship. My special thanks to Mr. Vijaya Prasada Rao and Manohar their kindness, valuable and friendly help during the stay of my University. I am also thankful to the lab assistants Mr. Babaiah and Mr. Mallesh for their help in maintenance of insect cultures.

I thank all the non-teaching staff of School of Life Sciences especially Mr. Ankinaidu, Mr. Lallan, Mr. Nageswara Rao and Mr. Jagan for their kind help and support.

My heartful thanks to all research scholars of School of Life Sciences for their unconditional support and affection. Thanks to my friends Roy, Puli, Ramakrishna, Hari, Anand, Kumar and Srinivas sir for making the stay at university campus a memorable one.

Thanks to my parents for their ever kind blessings. I am also thankful all other family members for their unconditional support.

Abbreviations

°C : degree centigrade / celsius
20E : 20-Hydroxyecdysone
ACP : Acid phosphatase
ALP : Alkaline phosphatase
ATP : Adenosine 5` triphosphate
BCIP : 5-Bromo-4-chloro-3-indolyl phosphate
BSA : Bovine serum albumin
CcGST : *Corcyra cephalonica* GST cDNA / its deduced amino acid
CcHP19 : *Corcyra cephalonica* HP19 cDNA / its deduced amino acid
cDNA : Complementary DNA
CfGST : *Choristoneura fumiferana* GST cDNA / its deduced amino acid
CNS : Central nervous system
cpm : Count per minute
DMSO : Dimethylsulfoxide
DNA : Deoxyribonucleic acid
dNTPs : Deoxyribonucleoside triphosphates
DTT : 1, 4-Dithiothreitol
EDTA : Ethylenediaminetetraacetic acid
EGTA : Ethylene glycol-bis(2-aminoethylether)- N,N,N`,N`-tetraacetic acid
ELI : Early-last instar larvae
GST : Glutathione S-transferase
HEPES : N-(2-hydroxyethyl)piperazine-N`-(2-ethanesulfonic acid)
HGLFB : Hind gut associated lobular fat body
HP19 : Haemolymph protein of mass 19 kDa from *Corcyra cephalonica*
HP23 : Haemolymph protein of mass 23 kDa from *Corcyra cephalonica*
IgG : γ Immunoglobulin
IPTG : Isopropyl β-D-thiogalactoside
JH : Juvenile hormone
kDa : Kilodalton
LB : Luria-Bertani medium
LLI : Late-last instar larvae
MARG : Male accessory reproductive gland
mg : Milligram
MLI : Mid-last instar larvae
mM : Millimolar
MOPS : 3-Morpholinopropanesulfonic acid
mRNA : Messenger ribonucleic acid
NBT : Nitrotetrazolium blue

ng : Nanogram
nM : Nanomolar
NP-40 : Nonidet P-40 (Nonylphenyl polyethylene glycol)
PAGE : Polyacrylamide gel electrophoresis
PBS : Phosphate buffered saline
PCR : Polymerase chain reaction
PGL : Prothoracic gland
PMSF : Phenylmethylsulfonyl fluoride
PNP : Para-nitrophenol
PP : Prepupae
PTTH : Prothoracicotropic hormone
RNA : Ribonucleic acid
SDS : Sodium dodecyl sulfate
SDS-PAGE : Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SSC : Saline sodium citrate
TAE : Tris-Acetate-EDTA buffer
TBS : Tris buffered saline
TCA : Trichloroacetic acid
TE : Tris-EDTA
TEMED : N, N, N`, N`, tetramethylethylenediamine
Tris : Tris (hydroxymethyl) aminomethane
v/v : Volume/volume
w/v : Weight/volume
X-Gal : 5-Bromo-4-chloro-3-indolyl β-D-galactoside
µg : microgram
µM : micromolar

Introduction and review of literature

Insects occupy more than 70% of entire animal kingdom and are the most successful group of organism living on earth. They are usually divided into three groups *i.e.,* harmless, injurious and beneficial. A group of injurious insects referred as pests, annually destroy between 6-30% of agricultural harvest in developing countries. These losses become even more significant for stored cereal products than pre-harvest losses, because post-harvest costs are much higher than the cost of production. A wide range of lepidopteran pests cause damage and constitute a major factor that reduce the agricultural harvest globally including India. Insects on the basis of their ability to undergo metamorphosis are broadly classified into ametabolous (no metamorphosis), hemimetabolous (incomplete metamorphosis) and holometabolous (complete metamorphosis). The holometabolous group has distinct larval and pupal stages and undergoes some of the most complex transformations seen in animal kingdom (Sehnal et al., 1996; Truman and Riddiford, 1999). The present study deals with this group of insect and most of the studies are carried using rice moth, *Corcyra cephalonica*.

Insect metamorphosis-

Metamorphosis is the characteristic feature of majority of the insects, including holometabola during the postembryonic development *i.e.,* the ontogeny accomplished after hatching. Metamorphosis is marked by abrupt changes in the form and / or structure during the postembryonic development. The larval forms are the juveniles of holometabola that lack the external rudiments of wings and genitalia but possess imaginal discs (an invaginated group of undifferentiated embryonic stem cells). The larvae are voracious feeders and have different habitat and niche from the adult stage. The non-feeding pupal stages are usually hidden or somehow protected stage. The tissue degeneration and rebuilding mainly occurs at the pupal stage, which also possesses the external rudiments of wings and genitalia. The adult stage of holometabolous insect is morphologically very different from the previous stages and they are usually prolific breeders (Sehnal et al., 1996; Truman and Riddiford, 1999, 2002; Buszczak and Segraves, 2000; Tissot and Stocker, 2000).

Physiological significance of metamorphosis-

Metamorphic developments actually are the manifestation of sequential polymorphism produced by the same genome (Highnam, 1981; Nijhout and Wheeler, 1982). A group of hormones by several cascades of events control the development and decide whether a cell remains at the present stage or advances to the next one (Nijhout, 1994; Sehnal

et al., 1996; Gilbert et al., 1996; Truman and Riddiford, 2002). For example in epidermal cells, the hormones as well as their titers determine the type of cuticle produced successively in the larva, pupa and adult (Riddiford, 1982; Willis, 1996). The larval cuticular proteins are produced under high juvenile hormone (JH) titer, whereas moderate JH titer, facilitates pupal cuticular protein synthesis and in the absence of JH, the imaginal cuticular proteins are produced (Willis et al., 1982). Some authors regard the transition from larval to pupal and then to adult functional state as a developmental process during which time, it produces the imaginal cuticle (Anderson et al., 1995). During the postembryonic and adult development, each stage (larva, pupa and adult) is strictly determined and can be neither omitted nor mixed with other stages (Slama, 1975).

Postembryonic development, metamorphosis and theirs hormonal control-

Several workers have proposed a basic model of the endocrine control of postembryonic development (Nijhout and Williams, 1974; Grieneisen, 1994; Gilbert et al., 1996). According to them, the specific neurosecretory cells in the insect brain synthesize a neuropeptide, prothoracicotropic hormone (PTTH), which is transported to the corpora cardiaca that act as neurohemal site in lepidoptera (Agui et al., 1980; Smith and Gilbert, 1989; Smith and Sedlmeier, 1990). Once released into haemolymph as a result of neural, hormonal, physiological or environmental stimulus (photoperiod, temperature, humidity etc.), PTTH acts on the prothoracic glands (PGL) and stimulates ecdysteroid synthesis (Gilbert et al., 1988). Thus 3-dehydroecdysone is released into the haemolymph where it is reduced by a ketoreductase to ecdysone (Warren et al., 1988a, b; Sakurai and Williams, 1989; Sakurai and Gilbert, 1990). The prohormone ecdysone is converted to the principal molting hormone 20-hydroxyecdysone (20E) in the mitochondria and microsomes of peripheral tissues such as haemocytes, fat body, Malpighian tubules and epidermal cells (Smith et al., 1983; Smith, 1985; Zhu et al., 1991a; Riddiford et al., 2001). 20E finally exerts its effect and causes apolysis and secretion of larval, pupal or adult cuticle (Smith and Gilbert, 1989). In addition the corpora allata synthesize and secrete juvenile hormones (sesquiterpenes, JHs) into haemolymph, the second major effector hormone in insect life. In haemolymph, these hormones are bound to JH-binding proteins, which enhance the stability of JH, protect it from esterase degradation and facilitate its entry into the target cells (Riddiford, 1996; Willis, 1996; Gilbert et al., 2000).

The relative titer and interplay between JHs and ecdysteroids orchestrates the progression of one developmental stage to the next i.e., egg-larva, larva-larva, larva-pupa and pupa-adult. During postembryonic development, the ecdysteroids initiate the onset and timing of molt (the producer). The titer of JH, determines the result of the molt either by maintaining it in juvenile condition during the larval-larval molt or by allowing it to transform during larval-pupal molt. JH thus regulates the quality of molt (the director) (Sehnal and Meyer, 1968; Sehnal, 1985; Smith, 1985; Gilbert et al., 1988, 1996; Rachinsky et al., 1990; Chang, 1993; Gilbert et al., 2000; Davey, 2000). Metamorphosis, a seemingly abrupt morphological transition as viewed externally is in reality a smooth continuation of precisely regulated events (Sehnal et al., 1996). These events are controlled by the endocrine molecules that are mainly secreted by the brain, corpora cardiaca, corpora allata and PGLs. A choreographic precision of titer of mainly the morphogenetic hormones i.e., the JHs and ecdysteroids and their interaction is required for the molting and metamorphic events to occur normally (Gilbert et al., 1996). In holometabolous insects JH level is high throughout the larval instars, declines in the last instar but rises again before the pupal molt, during the prepupal stage and is absent during the pupal stage. So molting in the presence of high JH titer would result in larval-larval molt while in the presence of reduced JH titer it would result in larval-pupal molt and in the absence of JH it would result in pupal-adult molt. Ecdysteroid level rises prior to a molt and declines just before actual ecdysis. In the last larval instar, there is a small peak of ecdysteroid that occurs at a time, when JH is absent. This peak of ecdysteroid along with the following JH peak, signals that the next molt will be a pupal molt (for review see- Smith, 1985; Riddiford, 1996; De Kort et al., 1996; Davey, 2000). However, knowledge regarding the regulation of hormone dependent actions is very limited and the field remains largely unexplored with a demand for further research.

Chemistry of ecdysteroids-

Ecdysteroid is a well defined term for all compounds structurally related to ecdysone. Further it includes true ecdysteroid and ecdysteroid related compounds. The biologically active ecdysteroid refers to the molting hormone. Chemically ecdysone is the trivial name of a specific compound (22R)-2β,3β,14α,22,25-pentahydroxy-5β-cholest-7-en-6-one, a derivative of cholesterol. 20-Hydroxyecdysone (20E) is the active form, which is a result of ecdysone 20-monoxygenase catalyzed hydroxylation (Grieneisen, 1994; Rees, 1995). The two molting hormones ecdysone and 20E were originally designated as α nd β ecdysone respectively (Horn and Bergamasco, 1985). In arthropods, 20E is one of the most

ubiquitously distributed ecdysteroid utilized by the molt cycle and is also associated with various physiological events (Gilbert et al., 2002).

Juvenile hormones (JHs) and its analogues-

The JHs are a unique group of sesquiterpenoid hormones. These are synthesized and secreted by the corpora allata under the influence of allatotropins and allatostatins, which are released from brain neurosecretory cells (Schooley and Baker, 1985). The existence of JH was first reported in *Rhodnius* by Wigglesworth (1934, 1936). The term "juvenile" hormone was introduced because of its role in the retention of larval characteristics or the restraining of development towards the adult form. To date, six different JHs have been identified from various insect orders. In the lepidoptera, five JHs are produced: JH I, JH II, JH III, JH 0 and 4-methyl-JH I (Yin et al., 1994; Gilbert et al., 2000). Methyl farnesoate is the predominant JH like molecule in the crustaceans (LeBlanc, 2007). Different JH homologs have different levels of biological activity. All the JHs have a methyl ester on one end and an epoxide on the other end. Both of these structural features are required for activity.

Regulation of ecdysteroids and JHs titer-

Extensive studies have been carried out on the regulation of hormone titer as well as their synthesis (Gilbert et al., 1980a, b, 1997, 2002; Song and Gilbert, 1998). These studies orient towards the temporal, quantitative and qualitative regulations of haemolymph titers of ecdysteroids and JHs. During the larval-pupal development of insects, there is a precise temporal fluctuation in the haemolymph titer of the JHs that appears to affect changes in the haemolymph titre of ecdysteroids and vice versa (Riddiford and Truman, 1978). This sequence of interaction begins early in the last larval instar, when the JH titre is at its peak and the ecdysteroid titre at its lowest. In the presence of this high JH and low ecdysteroid titre, the prothroacicotrope, synthesize and release PTTH in the haemolymph. The PTTH then activates the PGL to synthesize ecdysone resulting in an initial subtle increase in the ecdysteroid titre, which evokes wandering behaviour and pupal commitment. This ecdysteroid surge in turn stimulates the corpora allata via the brain to synthesize JH, leading to a second increase in the JH titre, which is necessary for normal metamorphosis to the pupa (Kiguchi and Riddiford, 1978). This peak of JH titre also stimulates the PGL, indirectly contributing to the second major increase in ecdysteroid titre, which elicits the pupal moult. This model suggests that the titre and action of the ecdysteroids are of critical importance in

the governance of the physiological activities associated with growth, metamorphosis and reproduction (Doane, 1973).

Detailed studies reveal that the basal concentration of ecdysteroids in the haemolymph of various lepidopteran insects during postembryonic development ranges from 10^{-8} to 10^{-5}M, which is species specific as well as stage dependent (Calvez et al., 1976; Dutta-Gupta and Ashok, 1988). A major peak of the ecdysteroid during larval-larval and larval-pupal development is usually present during the later half of each stadium, the duration of which is once again species specific (Smith, 1985; Tissot and Stocker, 2000). During the pupal-adult development, the major peak occurs in the first half or middle of the pupal stadium (Dean et al., 1980). This pupal peak is normally 1.5 to 2.0 times greater in magnitude and duration and is attributed to the accentuated need of these compounds for extensive remodeling and differentiation of different tissues. In addition to the major peaks, there are non-molting peaks during inter-molt period which are of lower magnitude and duration. Some of the inter-molt peaks have been found to correlate temporally with metabolic activities such as synthesis of DNA, RNA, protein and other macromolecules (Truman and Riddiford, 2002). The haemolymph ecdysteroid titer at any given moment is a reflection of several metabolic processes, which include ecdysteroid biosynthesis, secretion, and transport to target tissues, tissue uptake, degradation and excretion. Thus the changing haemolymph titer of ecdysteroids in holometabolous insects is responsible for eliciting the change in commitment and is necessary for metamorphosis as well as for the critical sequence of behavioral, physiological and biochemical events termed as molting (Gilbert et al., 1980b; Nijhout, 1994; Riddiford et al., 2001).

Regulation of ecdysteroid biosynthesis-

The regulation of ecdysteroidogenesis has been studied continuously for the past several decades and recent discoveries using *Drosophila* molecular genetics have advanced our knowledge further. The availability of genome sequences, the ease of genetic manipulation and the large collection of mutants all make *Drosophila* an attractive system for understanding the mechanisms regulating steroidogenesis. Regulation of ecdysteroid synthesis is complex, and is under the control of peptide hormones as well as the JH. It has been known for some 85 years that a factor from the insect brain can stimulate the PGLs of both diptera and lepidoptera (Kope, 1922). PTTH stimulated ecdysteroid production in PGLs occurs via a cascade of events which is yet to be elucidated completely (Fig. A). Earlier

studies on *M. sexta* revealed a correlation between circulating ecdysteroid titer and adenylate cyclase activity in the PGL, suggesting a role for cAMP (Smith et al., 1985; Smith, 1993). The Ca^{+2} deprived glands failed to generate cAMP in response to PTTH indicating that cAMP production was downstream of Ca^{+2}/calmodulin sensitive adenylate cyclase (Meller et al., 1988, 1990). The group also found evidence of G-protein (guanine nucleotide binding protein) involvement in the adenylate cyclase activation. Regardless of the complicated, developmentally dynamic relationships among calcium, calmodulin, G proteins and adenylate cyclase, it is clear that PTTH causes increased cAMP formation in PGLs. Increase in intracellular cAMP levels can lead to the activation of cAMP dependent protein kinase (PKA) and subsequent phosphorylation. Earlier, Gilbert et al., (1997) showed that during the process of ecdysteroidogenesis, PTTH initiates a cascade of events, that progresses from the influx of Ca^{+2} and cAMP generation through phosphorylation of the ribosomal protein S6. This is followed by S6 dependent protein synthesis and an increase in the synthesis and export of ecdysone from the PGL. Their studies further suggested that S6 phosphorylation probably controls the steroidogenic effect of PTTH by gating the translation of selected mRNAs, whose protein products are required for increased hormone synthesis (Song and Gilbert, 1995, 1997, 1998). They have also shown that the ecdysone produced by the PGL feeds back upon the gland by increasing the expression and phosphorylation of a specific p47 USP isoform, a constituent of the functional ecdysone receptor. Thus, changes in the concentration and composition of the ecdysone receptor complex of the PGL could modulate the gland's potential for ecdysone synthesis. Recently an enzyme, ecdysteroid phosphate-phosphatase has been identified to be responsible for the dephosphorylation of 20-hydroxyecdysone 22-phosphate and ecdysone 22-phosphate for the formation of 20E apart from the *de novo* synthesis of 20E (Yamada and Sonobe, 2003).

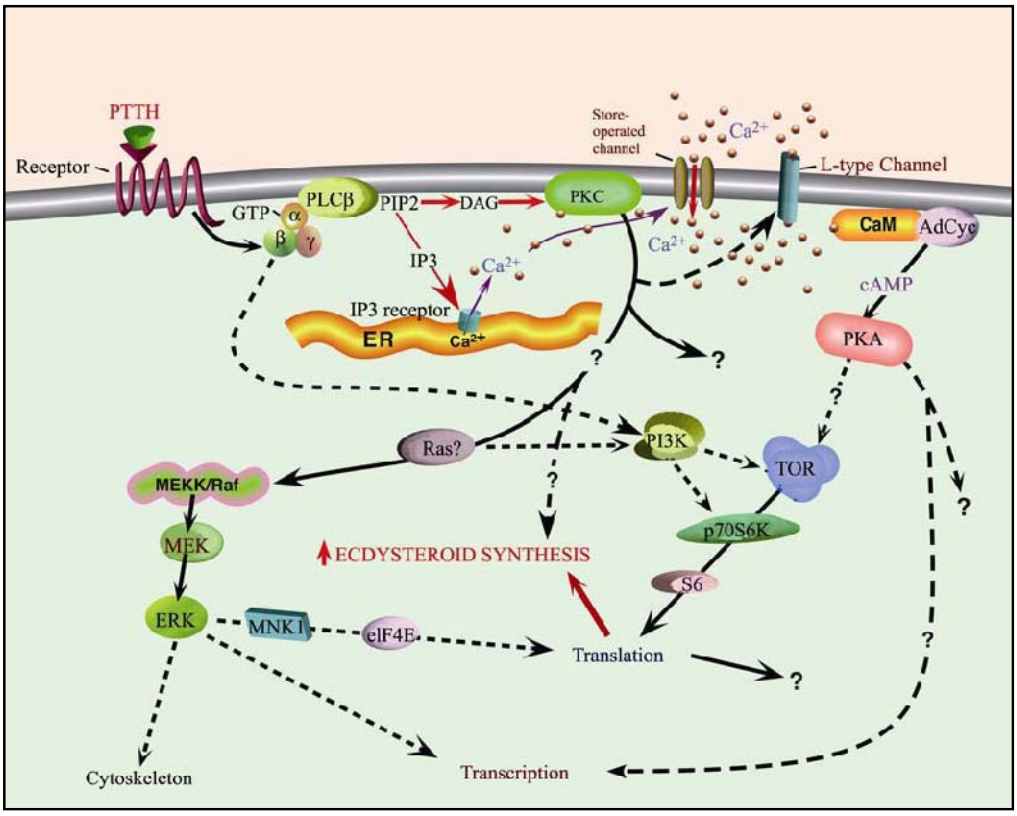


Fig. A The PTTH signal transduction cascade in prothoracic gland cells: Solid lines indicate strong interactions; dashed lines indicate hypothetical relationships. PTTH, prothoracicotropic hormone; PLCβ, phospholipase Cβ; PIP2, phosphatidylinositol-4,5-biphosphate; DAG, diacylglycerol; IP3, inositol triphosphate; GTP, guanosine triphosphate; cAMP, cyclic adenosine monophosphate; CaM, calmodulin; AdCyc, adenylyl cyclase; ER, endoplasmic reticulum; PKA, protein kinase A; PI3K, phosphoinositide 3hydroxy-dependent kinase; TOR, target of rapamycin; MEKK, MEK kinase; MEK, MAP/ERK kinase; ERK, extracellular signal-regulated kinase; S6, ribosomal protein S6; p70S6K, 70 kDa S6 kinase; MNK 1, MAP kinase-interacting kinase; Ras, a small GTP binding protein; Raf, a serine-threonine kinase; elF-4E, eukaryotic translation initiation factor 4E. (from Rybczynski, 2005).

Evidences of factors from fat body, haemolymph and other insect tissues for the regulation of ecdysteroidogenesis-

Among the secondary effectors that regulate the PGLs, JH is of particular interest since it has both stimulatory as well as inhibitory effects on the gland. Several evidences indicate an indirect stimulation of PGL by JH in post-committed last-instar *Manduca sex*ta larvae via secretion of a factor from fat body (Hiruma et al., 1978; Hiruma, 1980; Safranek et al., 1980; Gruetzmacher et al., 1984 a, b). This factor was shown to stimulate *in vitro* synthesis of ecdysone by *M. sexta* PGL and its ecdysteroidogenic effect was additive with that of PTTH. Later the factor was identified as a trypsin sensitive heat labile protein with

apparent mass of 30 kDa (Watson et al., 1985, 1987). The group hypothesized that chemical nature of the stimulatory protein facilitates the transport of sterol precursor from which ecdysone is synthesized. It was further suggested that the mode of action of this protein is distinctly different from that of PTTH. Their studies also revealed a direct correlation between the activity of the haemolymph factor and titer of JH during development of *M. sexta* (Watson et al., 1988). The ecdysteroid production may also be suppressed by another mechanism in which the PGLs themselves become refractory to PTTH stimulation during diapause (Browning, 1981; Bowen et al., 1984; Ciancio et al., 1986). Meola and Adkisson (1977) observed that release of PTTH occurs at the onset rather than at the termination, in diapausing *Heliothis zea* pupae for pharate adult development. Despite the release of this hormone, pupae remain in diapause because an unknown mechanism prevents ecdysone synthesis. Further investigation showed that when PGL from pupa which are maintained in a diapauses sustaining temperature of 19°C, when implanted in non-diapausing hosts produces ecdysone (Meola and Gray, 1984). Further, when haemolymph from non-diapausing host pupae is injected into diapausing insects kept at 19°C, ecdysone synthesis is stimulated. They concluded that the temperature sensitive mechanism controls diapause by regulating the availability of a humoral factor necessary for ecdysone synthesis. It was further reported that the fat body releases a factor into the haemolymph in response to diapause terminating temperature and that the factor is neither a free or conjugated ecdysteroid nor PTTH (Gray et al., 1987). Another interesting mechanism of regulation of ecdysteroid synthesis at the PTTH level was reported in *M. sexta*, where the PGLs synthesized 3-dehydroecdysone, which is rapidly converted to ecdysone through the mediation of a haemolymph enzyme, a 3-β forming 3-keto steroid reductase (Sakurai et al., 1989).

Biosynthesis of ecdysteroids-

Research on the biosynthesis of ecdysteroids has focused on the biosynthetic pathways for ecdysone and 20-hydroxyecdysone during larval-pupal development of insects. There has been major progress in this field over the last five years with the molecular characterization of terminal enzymatic steps. Despite of this great achievement, resulting from the utilization of molecular genetics, details of earlier steps in the biosynthetic pathway remain to be elucidated (Gilbert and Warren, 2005). Insects, which are sterol heterotrophs, obtain mainly cholesterol or phytosterols depending on their habits from dietary resorce. Phytophagous insects primarily ingest phytosterols that are first dealkylated to cholesterol, the immediate sterol precursor of ecdysteroids. In the PGL cells, cholesterol is first converted

into 7dC (7-dehydrocholesterol) by the action of the 7,8-dehydrogenase. Both dealkylation and the cholesterol to 7dC conversion are believed to involve P450-catalysed reactions by yet unidentified enzymes (Gilbert et al., 2002). However, a recent study indicates that the 7, 8-dehydrogenation may be carried out by Neverland, a Rieskedomain protein (Yoshiyama et al., 2006). The product of the 7,8-dehydrogenation, 7dC, is subjected to an unique and mysterious transformation to 5β[H]-3β, 14α-dihydroxy-cholesta-7-ene-6-one (ketodiol), the first recognizable ecdysteroid-like molecule (extensively reviewed in (Gilbert and Warren, 2005; Lafont et al., 2005). No intermediates have been characterized between 7dC and the ketodiol and even the subcellular site of this biochemical transformation remains conjectural. The nature of this so-called 'Black Box' reaction has eluded molecular and even biochemical characterization despite a great deal of investigation. Various biochemical scenarios have been proposed for this transformation that may well include P450-catalysed reactions (Gilbert and Warren, 2005). So far, the P450 enzymes involved in ecdysteroid biosynthesis (i.e. the Halloween P450s) have all been identified from 'low ecdysteroid' mutants of *Drosophila*. The only 'orphan' P450 enzyme in the Halloween family of low ecdysteroid mutants is Spook (Spo; CYP307A1). The experimental paradigm that determined the function of the other Halloween P450s has failed to assign a specific enzymatic role to Spo (Namiki et al., 2005; Ono et al., 2006). The rescue of homozygous *spo* mutants provided an artificial pulse of ecdysteroid intermediates, which indicates that Spo probably acts upstream of Phm [i.e. upstream of the ketodiol (2,22,25-trideoxyecdysone)]. However, 7dC does not rescue *spo* mutants as it does with the *woc* (Warren et al., 2001), *dnpc1a* (Huang et al., 2005) and *neverland* mutants (Yoshiyama et al., 2006), indicating that Spo is not the 7,8-dehydrogenase. Several functions have been proposed for Spo including the possibility that Spo is either involved in the Black Box reaction or synthesizes a novel signal molecule required for ecdysteroid biosynthesis (Namiki et al., 2005; Ono et al., 2006). The later steps in the biosynthetic pathway for ecdysone and 20-hydroxyecdysone are better understood and involve hydroxylations at C-2, 14, 22,and 25 for ecdysone and, in addition, at C-20 for 20-hydroxyecdysone. These hydroxylations apparently occur in a preferred sequence: C-14, C-2, C-25, and ultimately at C-22 to form ecdysone (Svoboda et al., 1975; Gilbert et al., 1977). After release from the PGL, the ecdysone is hydroxylated at C-20 in other tissues to form 20-hydroxyecdysone.

Ecdysteroids mode of action-

The isolation and purification of ecdysone and 20E by Butenandt and Karlson (1954) revolutionized the field of insect endocrinology. The widespread ramifications of this discovery later led to the present day understanding of eukaryotic gene expression. The pioneering research of Clever and Karlson (1960) and Clever (1964) revealed puffing patterns of the *Chironomous tentans* salivary gland polytene chromosome by ecdysteroid. This observation of puff regulation was later confirmed in genetic model organism, the fruit fly *Drosophila melanogaster* by several other groups (Ashburner et al., 1974; Ashburner and Richards, 1976). Based on these observations as well as through a series of detailed and elegant studies, Ashburner and group (1974, 1976) proposed a model for the regulation of gene expression by 20E. Since then this model became the basis of the knowledge of mechanism of steroid hormone action, which suggests that ecdysteroid could initiate a cascade of gene expression by directly acting on the nucleus. According to this model, the ecdysone upon binding to its specific receptor directly regulates two classes of genes, a small class of early regulatory genes and a large class of late genes. The protein products of the early genes in turn repress their own expression and induce the much larger set of late genes that play a more direct role in controlling the biological response of hormone. Extensive studies based on this model have provided insights into the molecular mechanism of 20E action (Cherbas, 1993; Antonieweski et al., 1993; Henrich and Brown, 1995; Thummel, 1996; Henrich et al., 1999; Riddiford et al., 2001). The focus of these studies have been on two major aspects- (i) Studies on the transcription factors induced by 20E and how these factors transduce and amplify the hormonal signal by coordinating the induction of secondary response genes? (ii) Discovery, cloning, characterization and expression of ecdysone receptor proteins (Segraves and Hogness, 1990; Thummel et al., 1990; DiBello et al., 1991; Palli et al., 1992; Riddiford et al., 2001; Thummel, 2002). From these studies a clear cut understanding of the mechanism by which a systemic hormonal signal is refined into stage and tissue specific developmental responses has emerged.

Hormones (peptides, amines or steroids) are chemical messengers secreted by certain endocrine tissues into the blood (haemolymph in case of insects) to regulate the activity of physiological functions of other tissues. The mechanism by which steroid hormones exert their effects is fundamentally different from other types of hormones (Truss and Beato, 1993; White and Parker, 1998; Beato and Klug, 2000). Since the steroids are too hydrophobic to

dissolve readily in the blood, they are carried along with specific carrier protein from the point of their release to the target tissues. These carrier proteins protect the hormones from enzymatic degradation which in turn extends their half-life (Hammond, 1995). In the target tissues, these hormones pass through the plasma membranes probably by simple diffusion (a process not well characterized) into the cell cytoplasm. The hormone is then mostly transported in to nucleus where it binds to specific receptor. Alternatively receptors may also be present in cytoplasm, where the hormone binds and the complex is transported to nucleus. Once hormone binds to the receptor, the receptor undergoes conformational change and dissociates itself from the heat shock proteins and becomes activated. Hence the hormone-receptor complex acts as a ligand activated transcription factor (Mcdonnell et al., 1993). The activated receptor either dimerises then binds or binds sequentially to its corresponding hormone response elements (HREs) present in the vicinity of target genes to turn on the transcription of particular DNA and then regulates the synthesis of proteins, the gene product, which in turn regulates the cellular and physiological functions (Evans, 1988; Beato, 1989). Steroid hormone receptor that acts as transcription factor in vertebrates as well as invertebrates belongs to the nuclear receptor superfamily (Beato et al., 1995; Mangelsdorf, 1995). This superfamily consists of receptors for steroids, retinoids, thyroid hormones, fatty acids, prostaglandins and orphan receptors whose ligands are unidentified. The members of this family are highly related in both (i) primary amino acid sequences and (ii) the organization of functional domains, suggesting that many aspects of their mechanism of action are conserved. In the absence of hormone, the receptor exists as an inactive oligomeric complex with a number of other proteins, including chaperons such as heat shock proteins (Hsp 90 & Hsp 70), cyclophilin 40 and p23 (Smith and Toft, 1993; Pratt and Toft, 1997). The steroid hormone receptors are structurally organized in different domains they are highly conserved DNA binding domain (DBD), nuclear localization domain and two two transactivation domains (at N-terminas and C-terminas) which have been confirmed by the results of cDNA cloning experiments (Bender et al., 1997; Riddiford et al., 2001).

Ecdysone receptor in insects-

The ecdysone receptor is a noncovalent heterodimer of two proteins the EcR protein and ultraspiracle protein (USP). These nuclear hormone receptor proteins are the insect orthologs of the mammalian farnesoid X receptor (FXR) and retinoid X receptor proteins (RXR) receptively. Indeed, based on sequence homology considerations (Hayward et al.,

1999), some researchers reserve the term USP for the EcR partner from lepidopteran and dipteran insects, and use RXR in all other instances. EcR and USP share multidomain architecture common to all nuclear hormone receptors, namely an N-terminal transcriptional activation domain (A/B domain), a DNA-binding domain (C domain, highly conserved between receptors), a linker region (D region), a ligand-binding domain (E domain, moderately conserved) and in some cases a distinct C-terminal extension (F-domain) (Koelle et al., 1991) The DNA-binding domains of EcR and USP recognise specific short sequences in DNA, and mediate the binding of the hetero dimer to these ecdysone response elements (EREs) in the promoters of ecdysone responsive genes. The ecdysteroid binding pocket is located in the ligand-binding domain of the EcR subunit, but EcR must be dimerised with a USP (or with an RXR) for high affinity ligand binding to occur. In such circumstances, the binding of an agonist ligand triggers a conformational change in the C-terminal part of the EcR ligand-binding domain that leads to transcriptional activation of genes under ECRE control (Bourguet et al., 2000). There is also a ligand-binding pocket in the corresponding domain of USP. Its natural ligand remains uncertain, and USPs appears to be locked permanently in an inactive conformation (Clayton et al., 2001).

Classification of steroid hormone action-

The central dogma for steroid hormone action has been that steroid hormones bind to their intracellular protein receptors that are ligand activated regulators of the transcription of genes. Hence the steroids trigger a genomic event that leads to transcription and protein synthesis, which in turn is responsible for the long lasting physiological response (Truss and Beato, 1993). Since the mechanism involves transcription as well as translation, there is a lag time between the binding of hormone to its receptor and to the first observable physiological effect caused by the hormone, which is sensitive to transcriptional and translational inhibitors. (Freedman, 1997). In contrast to this, these hormones may also be responsible for rapid cellular responses independent of gene transcription and / or translation (Wehling, 1997; Losel and wehling, 2003). For example the steroids may act via cell surface receptor for rapid effects through second messenger system. Therefore the steroid hormones action can be classified into two categories-

(i) **Genomic actions:** Well established classical mode of action

(ii) **Nongenomic action:** Newly emerging Rapid effects of steroids

Nongenomic actions of steroid hormones a general account-

The first report of rapid steroid effect was published way back in 1942 where intraperitoneal application of progesterone induced a prompt anaesthesia in rats (Selye, 1942). The term 'rapid actions' describes the general rate of appearance of specific biological responses that result from a steroid hormone acting through a receptor; that is, the steroid hormone forms a ligand–receptor complex that is linked by some mechanism to the production of a rapid biological response. The rapidity, of course, is system-dependent and can vary from seconds (for example, the opening of ion channels, Pietras et al., 1975) to an hour or so (for example, the inhibition of apoptosis; Kousteni et al., 2001). This contrasts with genomic responses, which generally take a few hours to days to manifest fully, and which can be blocked by inhibitors of transcription and translation. Steroid hormones can initiate biologically useful rapid responses in the absence of a functional nucleus (Meizel et al., 1997; Schwartz et al., 2001), indicating that a classic nuclear receptor need not necessarily be involved. Virtually all classes of steroid hormones have been shown to induce rapid responses. Steroids nongenomically regulate the activity of almost all the major classes of protein kinases such as PKC (Sylvia et al., 1993; Christ et al., 1995), PKA (Harrison et al., 2000), MAP kinases (Endoh et al., 1997) and tyrosine kinases (De Boland and Norman, 1998; Manegold, 1999) etc. Further the rapid nongenomic effects of steroids are characterized by at least one of the two basic features, (i) rapid physiological response which will be the first observable cellular effect in response to hormone is seen within seconds to few minutes with few exceptions where it takes little longer time and (ii) no effect of inhibitors of transcription or translation on cellular response.

Nongenomic actions of ecdysteroids-

Although the field is not as explored as in the case of steroids exchangers vertebrate but there are increasing evidences rapid nongenomic action of ecdysteroids in different insect models (Tomaschko, 1999). The favored target for the ecdysteroids is the plasma membrane and its associated protein where it interacts with Na^{+}-H^{+}channels. Thus the ecdysteroids are suggested to regulate nongenomically the ecdysteroid transport, electrolyte transport (Na^{+}, K^{+}, H^{+}, Ca^{+2}, Cl^{-}), second messenger (cAMP, Ca^{+2}level) and protein kinase activity. The uptake of ecdysteroid like other steroids across the membrane into a specific target cell is suggested to occur by diffusion. However, Spindler and his coworkers have suggested a carrier mediated transport for ecdysteroid uptake into crayfish hypodermis (Daig and Spindler 1983a, b; Spindler and Grossman, 1987). In *Sarcophaga peregrina* and *Calliphora*

vicina the ecdysteroid mediated activation of hexamerin receptor was found to be independent of transcription and protein synthesis (Ueno et al., 1983; Ueno and Natori, 1984; Chung et al., 1995; Burmester and Scheller, 1997a). The effect of ecdysteroid on selective phosphorylation of protein has also been demonstrated in *S. peregrina* (Itoh et al., 1985, 1986) and *M. brassicae* (Sass, 1988). Further, 20E also regulates the programmed cell death in salivary galnd of *Bombyx mori* via nongemonic action and it had membrane receptor for 20E (Elmogy et al., 2004). Recently it has been demonstrated that, a membrane GPCR from *Drosophila melanogaster* is able to bind to 20E and increases cAMP levels (Deepak et al., 2005). These are excellent examples of post-translational modification, independent of accompanied protein synthesis.

Regulation of ecdysteroids action-

A major area with regard to understanding the regulation of 20E action is that of tissue specificity. The diversity in function of the hormone to some extent could be due to the variation of responses among cell types that typifies the action of 20E. The 20E also shows differential effects on same tissue at different developmental stages (De Loof, 1986; Riddiford et al., 2001). Thus studies directed towards the molecular basis of differentiation during the development and the built in regulatory mechanisms at the tissue level largely aid in the understanding of 20E actions. The role of ecdysteroids, particularly 20E in eliciting the molt is no longer in question and has been established as the central dogma of the field. The role of ecdysteroids in postembryonic development of insects is well documented (Sehnal, 1989; Steele and Vafopoulou, 1989; Gilbert et al., 1996; Gu and Chow, 1996, 1997). In contrast to vertebrate systems, ecdysteroids perform a wide variety of functions in the entire insect class. Hence, it is often referred that almost the entire insect is target of ecdysteroids (Gilbert et al., 1996). It stimulates the growth and development of imaginal discs, promotes the deposition of cuticle by epidermis, regulates the growth of motor neurons, regulates defensive secretions and controls choriogenesis (Gilbert et al., 1996). The ecdysteroid also initiates the breakdown of larval structures during metamorphosis (Lockshin and Beaulton, 1974; Truman, 1996a, b). In the present work, studies have been carried out to understand the regulation of this important aspect of metamorphosis by ecdysteroids. The ecdysteroids are regulated at all levels i.e., the biosynthesis, the titer in haemolymph and the action mediated by it (Smith, 1985; Riddiford et al., 2001; Gilbert et al., 2002). As it is clear, there exists a vast amount of information regarding the regulation of ecdysteroids at the synthesis as well as

at its titer level. However, knowledge regarding the mechanism of regulation of ecdysteroid dependent actions is not very clear and the field remains largely unexplored.

In majority of holometabolous insects including lepidopterans, the 20E action is regulated to induce transition from the juvenile to adult forms. During this period there is differentiation of undifferentiated stem cells to functional adult structure. Furthermore, the differentiation of various tissues that are required during both the larval and imaginal stages as well as the ones, which are selectively imaginal, depend upon pulses of 20E (Granger and Bollenbacher, 1981; Sridevi et al., 1988a, b; Gu and Chow, 1993; Wang et al., 1995; Sehnal et al., 1996).

Extensive studies from our laboratory reveal that 20E stimulates synthesis of various proteins, in different tissues during the postembryonic development of lepidopteran insects (Ray et al., 1987a, b; Sridevi et al., 1988a, b, 1989; Ismail and Dutta-Gupta 1990a; Dutta-Gupta et al., 1996; Shanavas et al., 1996). The uptake or sequestration of storage proteins (hexamerins) by the fat body (Ismail and Dutta-Gupta, 1990b; Dutta-Gupta and Ismail, 1990, 1992; KiranKumar et al., 1997, 1998) as well as the male accessory reproductive gland (Ismail and Dutta-Gupta, 1990c, 1991; Dutta-Gupta and Ismail, 1992; Ismail et al., 1993) was also shown to be regulated by ecdysteroids. Studies from our group show revealed that the lysosomal activity in the whole body as well as in the fat body exhibits a specific pattern during postembryonic and adult development. Further the increase in the lysosomal activity is governed by the elevation of 20E levels. This increase in lysosomal activity is regulated at nongenomic level mediated by haemolymph protein (Ray et al., 1984; Sridevi et al., 1987; Ashok and Dutta-Gupta, 1988; Dutta-Gupta and Sridevi, 1991; Arif at al., 2004). Despite these studies, the mechanism of regulation of the majority of ecdysteroid dependent actions is not clear and the area requires for further research.

Peptides and proteins of haemolymph-

Haemolymph is the "blood" of insects. It is a watery fluid that fills the haemocoel. Haemolymph contains ions, molecules and cells. Often clear and color less in most insects, however some insects haemolymph may contain various pigments, making it appear yellow, blue, green, and in some rare cases of immature aquatic and endoparasitic flies, it is red due to the presence of hemoglobin All chemical exchanges between insect tissues are mediated through the haemolymph. The main difference between insect haemolymph and vertebrate blood is that haemolymph rarely contains respiratory pigments, and has a very low oxygen-

transportation capacity. Haemolymph is a reserve of water for the insect. The soft-bodied insect larvae can be 20-40% haemolymph by weight, and the adult forms usually have a bit less than 20% haemolymph. The main constituent of haemolymph is plasma. Insect plasma is characterized by high concentrations proteins, of amino acids and organic phosphates.

Larval haemolymph contains a diverse array of peptides and proteins, such as immune response proteins (Boman and Hultmark, 1987; John et al., 2001), lipophorins (Burks et al., 1992; Kanost et al., 1995; Rimoldi et al., 1996; Pennington and Wells, 2002), hormone and ion binding proteins (Huebers et al., 1988; Braun and Wyatt, 1996; Hirai et al., 1998; Maya-monteiro et al., 2000, Gudderra et al., 2002), regulatory proteins (Arif et al., 2004) and storage proteins (Telfer and kunkel 1991). The storage proteins can further be categories as like the insect storage hexamerins, iron storage proteins or ferritins, and oxygen storage proteins. While insects were thought not to have hemocyanins, a protein with potential oxygen-binding capabilities in embryonic hemolymph of the grasshopper *Schistocerca americana* was characterized (Sanchez et al., 1998). One of the major transport proteins in insects is lipophorin, which cycles between the midgut and fat body, where it picks up or delivers diacylglycerol (Canavoso et al., 2001). Also transferrins for iron transport circulate in the hemolymph. The first insect iron-binding protein was described in *Manduca sexta* (Huebers et al., 1988). Later on, insect transferrin genes were cloned from *Aedes aegypti* , *Bombyx mori, D. melanogaster*, *Drosophila sylvestris* , *Sarcophaga peregrina* and *Riptortus clavatus* (Hirai et al., 2000; Yoshiga et al., 1999; Yun et al., 1999;).

Experiments using oligonucleotide microarrays showed an additional role of transferrins in immunity (De Gregorio et al., 2001). An important portion of the hemolymph proteins consists of different enzymes and protease inhibitors. Besides glycerol playing a role in winter hardiness, insect hemolymph also contains proteins ice nucleators, which limit supercooling and induce freezing and antifreeze proteins (Duman, 2001). Furthermore, ovarian proteins, in particular vitellogenins, can reach high concentrations in the hemolymph. Other proteins circulating in the hemolymph that are heat shock proteins and pigments (seldom hemoglobin). At last, but not less important, the hemolymph is the major place of resistance during infection. Antimicrobial proteins produced by the fat body (homologue of the mammalian liver) are secreted in the hemolymph. Antimicrobial substances are recognized by pattern recognition proteins and activate the humoral response. In insects, enough information exists on the carrier proteins for JHs. These proteins appear to be present in the haemolymph of a large variety of insect orders (Goodman, 1983). However, only a few

reports are available on the existence of possible haemolymph carrier proteins for ecdysteroids. Feyereisen et al., (1977) for the first time, reported the presence of a 280 kDa high affinity ecdysteroid carrier protein in the haemolymph of *Locusta migratoria*. They further demonstrated that the majority of circulating ecdysteroid bound to this (Feyereisen 1980). Cao et al., (1983) purified a weakly acidic dimeric 270 kDa protein (monomers- 135 kDa) from the haemolymph of adult *L. migratoria* females. Despite these studies, the information regarding the transport and regulation of ecdysteroids of is not very clear and the area remains largely unexplored. Understanding the role of hemolymph proteins in development, reproduction, pathogen defense, and endocrinology is essential to our understanding insect physiology and should result in the discovery of new targets for pesticide research and development and new approaches to the management of insect pests.

Role of acid phosphatases during postembryonic and metamorphic development-

The acid phosphatases (ACPs) are a group of enzymes capable of hydrolysing esters of orthophosphoric acid in an acid medium. They are widely distributed and represent a heterologous group of enzymes with multiple isoforms and different isozymes (Egawa et al., 1995). The physiological functions of ACPs are to provide inorganic phosphate as a building block in making new cells. They are broadly classified into two types (a) the lysosomal ACPs (EC 3.1.3.2) and (b) the phosphatidic acid phosphatases or PAP (3.1.3.4). The PAPs are further classified into type 1 (PAP1) and type 2 (PAP2) PAPs. The PAP1 (38-43 kDa) is the cytosolic or membrane bound ACP and is involved in the supply of the diacylglycerols in the classical pathway of glycerolipid biosynthesis by dephosphorylating phosphatidic acid phosphate (Martin et al., 1987; Moolenaar et al., 1992). The PAP2 (35-50 kDa) is membrane bound and is involved in signal transduction that is mediated by phospholipase D (Exton, 1990; Kai et al., 1996). In this case, the phosphatidic acid cleaved from the major membrane phospholipid, phosphatidylcholine is converted by PAP to diacylglycerol, which serves as a lipid second messenger by activating protein kinase C (Nishizuka, 1984a, b; Kai et al., 1996).

In the holometabolous insects the larval structures degenerate at the beginning of metamorphosis (Schin and Clever, 1968; Radford and Misch, 1971; Lockshin and Beaulton, 1974). Lysosomal enzymes are known to play important role in histolysis of larval organs, cellular destruction, tissue remodelling and reorganisation. The metabolic fuels for these are provided primarily by the fat body. Acid phosphatase is one of the most commonly used

marker enzyme to study the lysosomal activity in insects (Verkuil, 1979, 1980). The enzyme has been identified in every organism studied to date and they exist in multiple forms and different isozymes (Konichev, 1982; Kutuzova, 1991). In *D. melanogaster*, ACP has often been used as a tool for survey of genetic polymorphism, using a major acid phosphatase gene Acph-1 (MacIntyre, 1966; Chung et al., 1996). A high increase in the activity of the lysosomal marker enzyme ACP is observed at the beginning of the wandering prepupal stages of dipteran as well as lepidopteran insects, preceding the actual metamorphosis (Verkuil et al., 1979; Ashok an Dutta-Gupta, 1988; Fialho et al., 2002). Transplantation experiments with *Calliphora erythrocephala* and thorax-ligation as well as the exogenous ecdysteroid injection studies suggest that the induction of lysosomal activity is under hormonal control possibly by ecdysteroids (Verkuil et al., 1979; Verkuil, 1980; Ashok and Dutta-Gupta, 1988). In the larval fat body of *Calpodes ethlius* a relationship could be derived between the autophagic events observed in the ultra-structural study (Locke and Collins, 1968) and variations in ACP activity in homogenates (Collins, 1975). These studies clearly suggest that the rise in ACP activity in the fat body of the insect larvae may be related to increasing autophagic activity leading to the elimination of certain cytoplasmic organelles like endoplasmic reticulum and mitochondria.

Insect Glutathione S-transferases(GSTs) and their functions-

GSTs (EC 2.5.1.18) are ubiquitously present, super-family of enzymes that play key role in detoxification of xenobiotics including insecticides and protection of cells against damage due to oxidative stress (Salinas, et al., 1999; Udomsinprasert, et al., 2005). They catalyze the conjugation of electrophilic compounds with thiol groups making the xenobiotics more water-soluble (Habig, et al., 1974). GSTs also catalyze reactions in metabolic pathways not associated with detoxification (Sheehan, et al., 2001). Most GSTs are cytosolic enzymes, present in both homo and heterodimeric forms with subunits masses of 23 to 28 kDa. Each subunit contains a specific glutathione (GSH)-binding site (G-site) in the highly conserved N-terminus adjacent to a non-specific electrophilic ligand-binding site (H-site). Insect GSTs belong to at least six distinct classes of cytosolic GSTs along with several unclassified genes (Enayati, et al., 2005). Majority of insect GSTs fall into the delta and epsilon classes. In spite of low sequence homology among GST classes they have fairly similar tertiary structures, topography of active as well as G-sites, and are induced after insecticides application (Dirr, et al., 1994; Enayati, et al., 2005). Insect GSTs are classified into two major subfamilies characterized by Ser (delta and theta classes) or Tyr (alpha, mu, pi and sigma classes), which

plays important role in lowering the pKa of the thiol group of bound GSH from 9.0 to between 6.0-6.9 to enhance the rate of nucleophilic attack of GSH towards electrophilic substrates (Caccuri et al., 1999). GSTs are known to bind non-covalently to a range of neutral or anionic lipophilic molecules. Chemicals which are not substrates for this enzymes including steroid and thyroid hormones, bile acid, bilurubin, heme, fatty acids and penicillin. Binding of these non substrate compounds is usually of moderate affinity and these values range between 10^{-8} to 10^{-5} M. Listowsky (1988) suggested that as GSTs constitute a high capacity intracellular binding pool of hormones, they might function as a binding reserve in target organs, and possibly play a buffering role to minimize the effects of transient fluxes in extracellular hormone levels. Further different GST isoenzymes may act collectively to bind GSH conjugates. Thereby help minimizing the inhibition of the specific isozyme responsible for catalyzing the formation of the particular conjugate.

Recent studies reveal that the GST levels as well as its activity regulated by metamorphic hormones during postembryonic development of Lepidopteran insects (Wu and Lu, 2008). Fat body of insects is the main metabolic centre, and performs a large number of complex functions including detoxification/degradation of xenobiotics and protection from oxidative stress (Keely, et al., 1985; Sawiciki, et al., 2003). Increasing numbers of insect GSTs are being characterized, due to their roles in insecticide detoxification, and are shown to be involved in O-dealkylation or O-dearylation of organophosphorous insecticides (Hayes, et al., 1995, 1998). However little is known about the role of GSTs of stored grain pests, which are exposed to variety of chemical formulation.

Why present study?

It is well established that metamorphosis in insects is the transition from the larval to adult stage and the events are controlled by ecdysteroids. It involves the breakdown of larval structures and the formation of new tissues that occurs either by apoptosis of individual cells or autophagy of group of cells (Thummel, 2001; Trumann and Riddiford, 2002). As a part of cell remodeling during metamorphosis, acidic autophagic vacuoles accumulate in the fat body cell and activity of several lysosomal enzymes including ACPs increases and cause the lysis of larval tissues in lepidopteran insects; which is governed by ecdysteroids. The present interest to characterize haemolymph protein (HP) in order to understand the regulation of ecdysteroid action in insects rose from the repeated interesting observation that injection of exogenous 20E stimulated the lysosomal activity in the fat body of the thorax-ligated larvae

of *C. cephalonica* (Ashok and Dutta-Gupta, 1988). However, addition of 20E to larval fat body cultures failed to stimulate the enzyme activity. Studies conducted earlier in *Manduca sexta* also revealed that the ACP activity was not induced in response to exogenous 20E alone but the reason was elusive (Caglayan, 1990). However, Ashok and Dutta-Gupta (1991) for the first time reported a significant stimulation in the fat body acid phosphatase activity, when haemolymph from final larval stage of *C. cephalonica* was added to the fat body cultures in the presence of 20E. This suggested that the hormone might require the presence of some additional factors under *in vitro* condition, which are present in the *in vivo* system to mediate the action, on lysosomal ACP activity. Later that factor was identified as haemolymph protein of 19 kDa (HP19) by Arif et al., (2004). Present study was aimed to understand the mechanism by which 20E and HP19 together induce the ACP activity larval fat body of *Corcyra*. As molecular characterization of HP19 protein revealed that its identity with GST from another lepidopteran insect *Choristoneura fumiferana,* an attempt was made to characterize the larval fat body GST from *Corcyra* and evaluate its role of GST if any in 20E induced fat body ACP activity. In addition during this study we have also identified another regulatory protein (HP23) from the larval haemolymph. which plays a role in postembryonic development of *Corcyra*.

Materials and methods

Materials-

[γ^{32}P] ATP (3000 Ci/mmol), [α^{32}P] dATP (3000 Ci/mmol) and [^{35}S] methionine (1000 Ci/mmol) were purchased from Board of Radiation Isotope and Technology (BRIT), India. Nitrocellulose membrane was from Schleicher and Schuell, Germany. The charged nylon membrane, Hybond, Sephadex G-25 & G-50 and DEAE Sephacel were procured from Amersham Biosciences. Plasmid isolation, gel elution and PCR amplification kits, Ni-NTA agarose were obtained from QIAGEN. Random primer labeling kit and restriction enzymes were from MBI Fermentas. TOPO TA cloning kit, first strand cDNA synthesis kits were and pRSET A Expression Plasmid were from Invitrogen, USA. pGEX4T-1 Expression plasmid, 2D strips and High Pure urea from GE Healthcare Life Sciences, USA. Whatman No. 1, 3 sheets were procured from Whatman, UK. Agarose, cellophane sheets, IgG purification kit and iProof™ high-fidelity DNA polymerase were purchased from Bio-Rad Laboratories, USA. BD GenomeWalker™ Universal kit was from BD Biosciences, USA. Trizol™ RNA isolation reagent, epoxy activated Sepharose and Glutathione were from Sigma Chemical Co, USA. Molecular weight cut-off membrane filters (YM-30 and YM-10) were purchased from Amicon Inc, USA. X-Omat AR X-ray film was procured from Kodak, USA. 20-Hydroxyecdysone, actinomycin D, brilliant blue G-250 & R-250, BSA, bromophenol blue, calmodulin, cycloheximide, DTT, EDTA, EGTA, Freund's complete and incomplete adjuvants, genistein, HEPES, high & low mass protein markers, leupeptin, para-nitrophenol (PNP), para-nitrophenyl phosphate bisodium salt (PNPP), phenylthiourea, PMSF, synthetic peptide substrate for kinase assay (syntide-2), streptomycin sulfate, TEMED, Triton X-100 and TC-100 insect culture medium were purchased from Sigma Chemical Co. USA. ALP conjugated secondary antibodies, NBT/BCIP mix were purchased from Bangalore Genei, India. Acrylamide, N-N`-methylene bisacrylamide, 2-mercaptoethanol, glycerol, SDS, glycine, silver nitrate, Tris base and CHAPS etc., were supplied by Sisco Research Laboratories Pvt. Ltd. India. Agar, ampicillin, MOPS, tetracycline, tryptone yeast extract etc., were purchased from HiMedia Laboratories, India. All other chemicals used were of analytical grade and were obtained from local sources in India.

Experimental insects-

Corcyra cephalonica (Stainton) - *Corcyra* commonly known as rice moth and belongs to the order Lepidoptera and family Galleridae. It is a serious pest of stored cereals, oil seeds and

legumes in the tropical and sub-tropical regions of the world (Freeman, 1976). The insects were reared in culture troughs that contained coarsely crushed sorghum seeds. Equal numbers of female and male moths were released into the troughs for egg laying. The cultures were maintained in insect culture room at 26 ± 1°C, 60 ± 5% relative humidity (RH) and 14:10 h light dark (LD) photoperiod. Under the above mentioned conditions, the larvae emerge from 4-5 days old eggs. The larval development proceeds through five instars and is completed in about 30-35 days. The final or last (=Vth) larval instar is further classified into early-last instar (ELI), mid-last instar (MLI) and late-last instar (LLI) followed by the non-feeding prepupal (PP) stage, a stage at which the larvae commits itself for metamorphosis to pupae. The prepupal stage extends over 4-5 days followed by the pupal stage, which lasts for 7-8 days. The adult moths normally survive for 8-10 days.

Stages used of C. cephalonica for experiments- For the present study mainly the early (ELI), mid (MLI), late-last instar (LLI) larvae and prepupae (PP) were used. The stages were classified based on their body weight and head capsule size. The larvae weighing 56-65 mg with head capsule size of 0.8 to 0.95 mm were categorized as ELI. Larvae weighing 66-75 mg and head capsule size of 0.96-1.03 mm were classified as MLI and those weighing 76-85 mg and head capsule size of 1.03-1.08 mm were classified as LLI. Insects collected during the wandering stage were designated as prepupae (PP). Fat body was collected from all stages and used for various studies. Haemolymph from ELI, MLI, LLI larvae and PP was collected and used for various studies.

Bombyx mori- It is commonly known as silk worm and belongs to the order Lepidoptera and family Bombycidae. The IVth instar larvae of pure Mysore strain were obtained from local breeding centre and were reared on fresh mulberry leaves under sterile conditions in insect culture room maintained at 26 ± 1°C temperature, 60 ± 5% RH and 14:10 h LD photoperiod. Staging of *Bombyx* larvae was done based on their age after the fourth ecdysis. One to two days old last instar larvae were designated as early-last instar (ELI), 5 to 6 days old as mid-last instar (MLI) and 9 to 10 days old as late-last instar (LLI) larvae. Insects collected after spinning were designated as prepupa (PP), one day old prepupa as early-prepupa (EPP), 2 days old prepupa as mid-prepupa (MPP) and 3 days old prepupa as late-prepupa (LPP). The pupal stages were designated as early-pupa (EP) 1-2 days old, mid-pupa (MP) 4-5 days old and late pupa (LP) 9-10 days old, and freshly emerged moths (< 12 h old) designated as adult were also used.

Achaea janata- It is commonly known as castor semilooper, belongs to the order Lepidoptera and family Noctuidae. The larval forms were collected from caster plantation and maintained on fresh castor leaves in insect culture room maintained at 26 ± 1°C temperature, 60 ± 5% RH and 14:10 h LD photoperiod. Fat body from late-last instar (LLI) larvae was collected and used for various studies.

Spodoptera litura- It is commonly known as tobacco caterpillar belongs to the order Lepidoptera and family Noctuidae. It is a serious polyphagous pest of agriculture crops as well as vegetables. Third instar larvae were procured from Directorate of Oil seed Research (DOR), Hyderabad, India and reared on fresh castor leaves. For the present study the last instar larvae were used.

Bacterial strains- For general transformation reactions and cloning of the cDNA in the TA vector DH-5α strain of *E. coli* was used. This is wild type bacterial stain which is sensitive to all types of antibiotics LB broth. For expression of HP23 cDNA BL21 *DE3 plys* was used as a host, which is able to grown in chloromphenicol added media.

Surgical manipulations and organ culture-

Thorax ligation- This was carried out to deplete/reduce the endogenous hormone titer (Dutta-Gupta and Ashok, 1998). Initially appropriate stage larvae to be ligated were narcotized by placing them on ice. Ligation was done by behind the 1st pair of prolegs by placing silk thread around the head of the larvae, loop was gradually tightened. Tissues anterior to the ligature were cut with sterile scissor and wound was dressed with streptomycin sulphate, finally it was sealed with mixture of paraffin and bee wax (10: 0.1). These ligated insects were kept in petriplates covered with moister filter paper.

Fat body organ culture- Fat bodies from thorax ligated insects were dissected in sterile conditions and washed with sterile insect Ringer (130 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2$ and 1 mM phenylmethylsulfonyl fluoride) and rinsed in TC-100 insect culture medium and placed in 200 µl of TC-100 medium for 1 h for preconditioning the tissue. Depending on nature of the experiment the tissue was incubated with other factors for 4hr at 25^{o}C with gentle shaking. After incubation the tissue was rinsed with cold insect Ringer and processed for homogenization.

Microinjection- Insects to be micro injected were narcotized by keeping them on ice for short period. They were injected with 10 µg of Ig G or PBS in 5 µl volume and the wound was dressed with traces of streptomycin sulphate and sealed with wax.

Tissue sample preparation-

Haemolymph collection- The larval stage of interest was narcotized on ice. The prolegs were cut with a fine sharp scissors and the oozing haemolymph was collected with the help of micro pipette into tubes precoated with 0.025% phenylthiourea in order to prevent tyrosinase activity and malanization (Whittaker, 1971). These haemolymph samples were diluted (1:20) with 10 mM Tris-HCl (pH 7.4) and were centrifuged at 1,000 g for 3 min at 4°C to sediment the haemocytes. This supernatant i.e., the cell free haemolymph samples were used immediately.

Preparation of various tissue homogenates- A 5% homogenate of desired tissue from appropriate developmental stage was prepared from the thorax ligated as well as unligated larvae of *C. cephalonica*. The tissues were rapidly dissected in cold insect Ringer and were homogenized in homogenization buffer (10 mM Tris-HCl pH 7.4, 0.1% Triton X-100, 1 mM PMSF, 1 mM EDTA and 1 mM DTT) followed by centrifugation at 1,000 g for 5 min to remove larger debris. The aliquots of the supernatant were used for various analysis such as assay of enzymes (ACP, CaM kinase II), PAGE, western analysis, ligand blotting and phosphorylation studies.

Preparation of membrane fraction- The visceral fat body from the LLI larvae was dissected in ice cold insect Ringer and homogenized on ice, in buffer A (5 mM HEPES pH 8.5 and 0.1 mM $CaCl_2$). The membrane fractions from the total fat body homogenate were prepared according to the method of Fischer et al., (1980) with slight modifications. The fat body homogenate was centrifuged at 1,000 g for 10 min at 4°C and the resultant pellet (nuclear fraction) was resuspended in buffer B [10 mM HEPES (pH 7.0), 2 mM $CaCl_2$, 10% sucrose (w/v) and 0.1% Triton X-100 (v/v)]. The supernatant was further centrifuged at 30,000 g for 30 min at 4°C and the pellet thus obtained was washed once with buffer A, followed by resuspension in small volume of buffer B. This particular fraction was used as membrane preparation and stored at -70°C.

Quantification and purification of macromolecules-

Protein- Protein content in various samples was estimated according to the micro protein assay method of Bradford (1976). BSA fraction V was used for preparation of standard curve.

Nucleic acids- The estimation of DNA or RNA was carried out by measuring absorbance at 260 nm (A_{260}) in a UV-160A Shimadzu spectrophotometer. The absorbance of the sample was also measured at 280 nm (A_{280}) to check the interference with protein (Warburg and Christian, 1941; Peterson, 1983). The purity of the sample was determined by calculating A_{260}/A_{280} ratio.

Radiolabel quantification- The proteins in homogenate were precipitated with an equal volume of 20% chilled TCA for 30 min at 4°C, followed by centrifugation at 10,000 g for 15 min. The resultant pellet was washed twice with 1 ml of 5% TCA and followed with ethanol:ether (3:1) mixture wash. The final pellet was air dried and dissolved in 100 µl of 0.1 N NaOH. An aliquot (50 µl) of sample was added to the scintillation fluid- Bray's mixture (PPO 4 g, POPOP 200 mg, naphthalene 60 g, ethylene glycol 20 ml, methanol 100 ml/L of 1,4-dioxan). The radioactivity in sample was quantified using a Packard 2100-TR Tri-Carb liquid scintillation counter. An aliquot (10 µl) of the same was used for protein estimation.

Preparation of hormones- A stock solution of 20E was prepared by dissolving 1 mg of hormone in 100 µl of ethanol, which was finally diluted to 1 ml with distilled water. This was aliquoted into small volume and stored at -20°C till use. This stock solution was further diluted as per requirement. The final concentration of ethanol in 20E never exceeded 0.05% in any of the experiments, where as JH1 was prepared by dissolving it in acetone.

Fat body organ culture studies- Visceral fat bodies from LLI larvae were dissected 24 h after ligation under sterile conditions in cold insect Ringer and transferred to 100 µl of TC-100 insect culture medium with traces of streptomycin sulfate. After rinsing, the tissue was transferred to fresh 200 µl culture containing 80 nM 20E (in 10 µl 0.05% ethanol). Control cultures were added with an equal volume of carrier solvent (10 µl 0.05% ethanol). For study the HP19 30 ng pure protein was added to the fat body culture in presence or absence of 20E or presence of inhibitor molecules (sodium fluoride, sodium potassium tartarate, LY294002, Wortmannin). Studies with GSTs were carried by adding purified fat body cytosolic GST from *C. cephalonica* to the fat body cultures in presence of hormone. These cultures were

then incubated for 4 h at 25°C with gentle shaking. At the end of incubation, the tissue was removed, rinsed in ice cold insect Ringer, homogenized as mentioned above and used for ACP assay.

Acid phosphatase (ACP) assay- The assay was carried according to the method of Henrickson and Clever (1972) with slight modification. The reaction mixture contained 200 mM sodium acetate buffer (pH 5.0) and 10 µg of protein. The enzyme sample with the buffer was incubated at 37°C for 10 min to exclude glucose-6-phosphatase activity (Beaufay et al., 1954). The reaction was initiated with the addition of 5 µmoles of substrate, p-nitrophenyl bisodium phosphate to the assay mixture followed by incubation for 1 h at 37°C. The reaction was terminated by the addition of 0.5 ml of 0.1 N NaOH. The yellow color thus developed was measured at 420 nm against a substrate blank. The p-nitrophenol (PNP) was used for the preparation of standard curve. The activity of the enzyme was expressed as nano moles of PNP released/h/µg protein.

Denaturing gel electrophoresis (SDS-PAGE)- Tris-glycine sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) with acrylamide:N,N'-bisacrylamide (30:1) was carried according to the procedure of Laemmli (1970). The protein sample was prepared by mixing an aliquot of the protein with sample buffer followed by heating at 100°C for 1 min.

2- Dimensional gel electrophoresis- The 2- dimensional electrophoresis was carried according to the method of O'Farrell (1975) with slight modifications using Bio-rad PROTEAN II xi 2-D Cell apparatus. The polymerization of the gels for IEF was done according to the manufactures protocol. The capillary tubes were polymerized using tube gel monomer solution (9.2 M urea, 3 ml acrylamide- bis-acrylamide (30:1), 0.2 ml Biolyte 5/7 ampholyte, 0.8 ml Biolyte 3/10 ampholyte, 1 ml detergent solution containing CHAPS/NP40 made up to the volume of 20 ml with double distilled water). The polymerized capillary tubes were placed in the 2-D cassette and prefocusing was done at 200V/ 1h, with 20 mM NaOH as the upper buffer and 10 mM H_3PO_4 as the lower running buffer. Approximately 10 µg of pure HP19 protein sample from rice moth was dissolved in rehydration buffer (8 M urea, CHAPS, 40 mM DTT, 0.5 % 5/7 and 3/10 ampholyte, 0.002 % bromophenol blue) and loaded on top of the prefocused gel and IEF was carried out with the following voltage, 200V/ 2 h, 500V/ 2 h and 800V/16 h. The gels from the tubes were removed according to manufacturer's protocol and were placed in equilibration buffer (6 M urea, 75 mM Tris-HCl pH 8.8, 29.3 %

glycerol, 2 % SDS and 0.002 % bromophenol blue) for 10 min. The gels were washed extensively to remove the excess buffer and were transferred on top of the SDS-PAGE. The 2-dimention was run at an initial voltage of 60 for 30 min followed by 100V till the dye front reached the bottom. The gels were removed and silver stained for visualization of protein spots.

Visualization of proteins in the gel- Staining was carried out by two methods: (i) silver staining was carried according to the procedure of Blum et al., (1987). And (ii) coomassie staining was carried according to the method of Wilson et al., (1983).

Purification of Glutathione S-transferases (GST) - 20% (w/v) larval fat body homogenate was prepared using glass homogenizer. The homogenate was centrifuged at 13,000 g for 5 min at 4°C to remove the debris. The supernatant was collected and subjected to centrifugation for 45 min at 4°C. The supernatant thus obtained was further centrifuged at 1,00,000 g for 1 h, the cytosolic fraction present in the supernatant, was collected and passed through DEAE cellulose column pre-equilibrated with 50 mM Tris-HCl (pH 7.8). The column was washed with discontinuous step gradient of NaCl. The GSTs were present in the flow through fractions as it they did not bind to the ion-exchange DEAE cellulose matrix. This is consistent with the earlier results of Habig et al., (1974). The flow though fractions that contained GSTs were pooled and applied to glutathione Sepharose affinity column, pre-equilibrated with 10 mM potassium phosphate buffer (pH 7.0), containing 1 mM EDTA and 2 mM DTT for further purification. The affinity column was first washed with washing buffer 10 mM potassium phosphate buffer (pH 7.0), containing 1 mM EDTA, 5 mM KCl and 2 mM DTT followed by elution of bound GST with elution buffer (50 mM Tris-HCl pH (8.0), 1 mM EDTA, 10 mM GSH and 2 mM DTT). The presence of protein in each fraction was checked at 280 nm and the fractions were eluted till it reached the absorbance of 0.002. At each step of purification, the presence of GST was determined by activity assay as described above and the purity was checked on 12 % SDS-PAGE.

Production of polyclonal antibodies against affinity purified GST- Polyclonal antibodies were raised against the affinity purified fat body GSTs. The protein (200 µg) was emulsified with complete Freund's adjuvant and then injected into six months old male rabbits (New Zealand variety) subcutaneously at multiple sites. For booster injections, 100 µg protein emulsified with Freund's incomplete adjuvant was used. Antiserum was collected after

administering two booster immunization. Serum from same rabbit collected prior to immunization was used as a control.

Purification of IgG- The IgG fraction containing fat body GST antibody as well as HP23 antibody was purified by affinity chromatography using protein A-agarose column (Bio-Rad) according to the manufacturer's protocol. The binding affinity of F_c regions of IgG to protein-A in the crude mixture of serum proteins was utilized for the purification. The serum protein loaded on to the protein A-agarose column pre-equilibrated with wash or binding buffer. This was followed with by the washing of the column using the same buffer until the absorbance of eluates at 280 nm reached to zero. The bound IgG fraction was eluted with 4 column volumes of elution buffer to tubes that contained 100 µl of neutralization buffer for immediate neutralization of the eluted purified IgG fraction these fractions were dialyzed against PBS, and the purity of IgG was checked on SDS-PAGE. The Ig G samples were aliqoated and stored at -20°C till further use.

Western blotting and immunostaining- The electrophoretically separated proteins were transferred (electro-blotted) to nitrocellulose membrane using miniVE blottter (Hoffer) according to the procedure of Towbin et al., (1979). The visualization of the specific reactivity was carried out using anti-rabbit IgG coupled to alkaline phosphatase followed by NBT/BCIP color reaction.

Isolation of nucleic acids- The genomic DNA from total body of LLI larvae was isolated as described in Birren et al., (1997). For RNA isolation tissue was dissected under sterile conditions in RNase free 10 mM Tris-HCl (pH 7.4). It was rinsed with the same buffer and homogenized (~20-40 mg tissue) in 1 ml of Tri-reagent (Sigma Chemical Co, USA). The homogenate was allowed to stand for 5 min at room temperature followed by addition of 200 µl chloroform and incubation on ice for 5 min. The mixture was centrifuged at 12,000 g for 15 min at 4°C. The upper aqueous layer containing nucleic acid was transferred to a fresh tube and re-extracted once again with 200 µl of chloroform. The upper aqueous phase was collected, to which 500 µl of isopropanol was added, mixed thoroughly and incubated at room temperature for 5 min. The RNA from this in the form of pellet was collected after centrifugation at 12,000 g for 20 min at 4°C. The RNA pellet was washed twice with 70% ethanol and stored at -70°C until use. During the entire preparation, care was taken to avoid

RNase contamination by treating the solutions and glassware with diethyl pyrocarbonate (DEPC) followed by sterilization.

Plasmid DNA Isolation- The bacterial strain containing the recombinant plasmid was allowed to grow in LB/amp (100 µg ampicillin per ml LB broth) for 12-14 h and the DNA was isolated using QIAGEN plasmid isolation kit.

Agarose gel electrophoresis-

Sample preparation for DNA electrophoresis- An estimated amount of plasmid DNA (1-2 µg/10 µl), undigested (circular), linearised or double digested with appropriate restriction enzymes was mixed with 2 µl of 6X DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol) and loaded into the 1% agarose gel for electrophoretic separations. The samples for genomic DNA, either digested or undigested for electrophoresis on 0.8% agarose gel were prepared as mentioned above. The digestion of DNA was carried out overnight at 37°C with the chosen restriction enzyme(s).

Sample preparation for RNA electrophoresis- The RNA sample (15-20 µg) was prepared by mixing 12.5 µl formamide, 2.5 µl 10X formaldehyde gel buffer and 4 µl formaldehyde in a total volume of 25 µl. The mix was heat denatured at 65°C for 5 min followed by snap cooling on ice for 2 min. To this, 2.5 µl of gel loading dye (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol) was added and used.

DNA electrophoresis- DNA was electrophoresed on either 1% or 0.8% (10 cm long) agarose gel. The gel was polymerized using 1X TAE. The electrophoresis was carried out using the same buffer at voltage 5V/cm^2 until the dye reached 3/4th of the length of the gel. All the ethidium bromide gels were visualized using UV-transilluminator.

RNA electrophoresis- The RNA samples were electrophoresed on 1.2% agarose-formaldehyde denaturing gel (Sambrook et al., 1989). The agarose was suspended in DEPC-treated sterile distilled water. It was boiled at 100°C and cooled till 60-65°C. To it, 1X formaldehyde gel buffer and formaldehyde (17 ml/100 ml) were added along with ethidium bromide (0.5 µg/ml). This was followed by polymerization of 10 cm long gel on horizontal gel electrophoresis system. The electrophoresis was carried using 1X formaldehyde gel buffer at voltage 5V/cm^{2}until the dye reached the end of the gel. The ethidium bromide stained gels were visualized using UV-transilluminator.

Molecular cloning and expression-

Fat body GST cDNA cloning- Degenerate primers for GST were designed from known lepidopteran cDNA sequences present in Gen bank. The primers used were (i) forward primer 5'-ATG MCC ATC GAY CTS TAC-3' and (ii) reverse primer 5'-TTR AGC ATG CTG ACR A-3'. For cDNA synthesis total fat body RNA was isolated and cDNA was synthesized using SuperScriptII First stand synthesis system for RT-PCR from Invitrogen USA. Using this cDNA as a template PCR was carried out with the help of above set of primers following conditions (initial denaturation at 94^{o}C for 2 min, later denaturation for 94^{o}C for 1 min annealing at 52^{o}C for 30 sec final extinction at 72^{o}C for 1 min for 25 cycles). The resultant PCR product was cloned in TOPO-TA vector from Invitrogen, USA.

Construction of HP19 expression plasmid- Putative HP19 clone which was available in the laboratory PCR amplified by using forward and reverse primers having *Bam HI* and *Hind III* restriction sites at 5' end of the forward and 3' end of reverse primer and ligated into pGEMT Easy vector and transformed into *E. coli* DH5α cells. Positive clones were digested with *Bam HI* at 5' end and *Hind III* at 3' end. Digested fragment was further cloned into pRSET A expression vector. The resultant HP19 clone was confirmed by PCR amplification and restriction digestion. Positive clones were used for transformation. Initially BL21DE3 *plys* cells were used as host for expression of HP19 protein. However, the purity of recombinant protein was not good enough to proceed further. Hence, HP19 was once again cloned in to pGEX4T-1 vector, having GST tag. PGEX4T-1 and HP19 clone in T-vector plasmids were double digested with *Bam HI* and *Not I* enzymes. Released HP19 insert was ligated into digested pGEX4T-1 expression vector and transformed into *E. coli* DH5α cells. Clones were confirmed by PCR and restriction digestion. After conformation recombinant plasmid was transformed into *E. coli* BL21DE3 *plys* cells.

Expression and purification of putative recombinant HP19- Single colony was inoculated into 5 ml of LB broth presence of 100 µg/ml amp incubated for over night at 200 rpm at 37^{o}C. This over night culture was inoculated into 500 ml of LB broth and allowed to grow till OD reached to 0.4 at 600 nm. At this OD culture was induced with 1mM IPTG and allowed to grow for 4 hr, after that cells were collected by centrifugation at 5000 g for 10 min. Pellet was washed with PBS for two times and resuspended in Ni-NTA binding buffer. Cell lysis was done by sonication. After sonication lysate was centrifuged at 10,000 rpm at 4^{o}C for 10

min. The supernatant was applied to Ni-NTA agarose column pre-equilibrated with Ni-NTA binding buffer. After loading column was washed with 4 volumes of wash buffer, and bound protein was eluted with elution buffer. The purity of the eluted protein was checked by SDS-PAGE. On the other hand after expression of GST tagged protein, bacterial pellet was resuspended in GST equilibration buffer and sonicated. After sonication lysate was centrifuged at 15,000 g at 4^{o}C for 10 min. The clear lysate was applied to the GSH-Sepharose 6B column pre-equilibrated with equilibration buffer, the affinity column was washed with washing buffer until OD at 280 reaches to zero. Bound protein was eluted with elution buffer containing reduced glutathione. Purity of the elutes was checked by SDS-PAGE.

Cleavage of GST tag- This was done using thrombin Cleave^{TM} kit (Sigma Chemical Co, USA). Purified recombinant protein was exchanged to thrombin cleavage buffer by dialysis, and 10 mg of recombinant protein (GST-HP19) was incubated with 1 ml of thrombin-agarose beads for 6 hr at 37^{o}C. After incubation thrombin-agarose resin was collected by centrifugation at 10,000 g for 2 min. Cleavage efficiency was checked by SDS-PAGE. The resultant cleaved recombinant protein showed functional difference with HP19 protein it is termed as haemolymph protein (HP23).

Purification of HP19 protein from haemolymph- Total haemolymph protein collected from LLI larvae was loaded on pre-equilibrated (10 mM Tris-HCl, pH 7.4) Sephadex G100-120 column (1.6 x 90 cm). Column flow rate was maintained at 1.5 ml/min. approximately 70 2ml fractions were collected, and each elute was analyzed by SDS-PAGE. Fractions which had lower quantity of protein above 50 kDa range were used for further purification. The fractions 50 kDa ranges were pooled together and were loaded to DEAE cellulose column (3 x 15 cm) flow through was collected separately and column was washed with 10 mM Tris-HCl (pH 7.4) buffer. Further bound protein was eluted with differential NaCl gradient. An aliquot of each fraction was added to the fat body cultures in the presence of 20E (80 nM) and its enhancing effect on ACP activity was analyzed. Peak fractions that stimulated the ACP activity were pooled and the purity was checked by SDS-PAGE.

Isolation of HP23 genomic clone- Total genomic DNA was isolated 200 pg of DNA template was used for Genomic PCR. The following programme used for amplification. Initial cycle at 94^{o}C for 3 min was used in the programme to activate the Taq DNA polymerase. This was followed by subsequent denaturation, annealing and elongation steps

for 30 cycles (94^{o}C -1 min, 54^{o}C - 30 sec, and 72^{o}C - 5 min). Final extension was carried out for 20 min at 72^{o}C.After PCR products were analyzed on agarose gel, cloned into TA vector and sequenced.

Genome walking of HP23 gene- Entire genome walking strategy has been outlined in Chapter 3-results section.

Nucleic acid blotting and hybridization-

Southern blotting- The total larval body genomic DNA was digested with different restriction enzymes and subjected to 0.8% agarose gel electrophoresis. After electrophoresis, the DNA was denatured by soaking the gel for 45 min in denaturing buffer with constant agitation, followed with brief washing in sterile distilled water. The gel was neutralized for 30 min in neutralization solution fallowed by transfer of DNA to nylon membrane (Hybond, Amersham Biosciences) by capillary transfer method (Sambrook et al., 1989). The membrane was soaked in 10X SSC prior to the transfer. The transfer was performed for 16-18 h using 10X SSC. After transfer, the blot was once rinsed in 6X SSC and the damped blot was UV cross-linked (between thymidine residues in the DNA and positively charged amine groups on the membrane surface) using 0.15 J/cm^{2}UV irradiation at 254 nm.

Northern blotting- RNA samples were resolved on 1.2% agarose-formaldehyde gel as described above and used for blotting on to nylon membrane by capillary transfer. Prior to transfer, the formaldehyde from the gel was removed by several changes of RNase free DEPC-treated sterile water, followed by equilibration in 20X SSC for 45 min. The nylon membrane was also presoaked in 20X SSC for 5 min. The capillary transfer was performed for 16-18 h using 10X SSC. After the transfer, the blot was once rinsed in 6X SSC and the damped blot was UV cross-linked.

Preparation of [α^{32}P] dATP labeled probe for hybridization- For this, fat body GST insert or HP23-cDNA insert corresponding to the whole open reading frame isolated by PCR using specific primers from the plasmid. Approximately 100 ng of Gel purified (using QIAquick gel extraction kit) PCR product as a template random prime labeled using HexaLabel^{TM} DNA labeling kit (MBI Fermentas) and [α^{32}P] dATP (3000 Ci/mmol, BRIT, India). For reaction 10 µl of cDNA template (~100 ng) was mixed with 10 µl random (hexamer) primer and 40 µl sterile distilled water, vortexed and spun at 10,000 g for 5 sec. The mix was

incubated in boiling water bath for 10 min followed by snap cooling on ice. The mix was spun down quickly. To this, dNTP mix (15 µM each) without dATP along with 50 µCi [α^{32}P] dATP and 1 µl Klenow fragment (5 U/µl) was added, mixed thoroughly and incubated for 20 min at 37°C. The reaction was stopped by 1 µl of 0.5 M EDTA (pH 8.0). The unincorporated dNTPs and [α^{32}P]dATP were removed by passing the mixture through Sephadex G-25 column. The probe thus prepared, had specific activity of >10^9 dpm/µg. The radiolabeled probe was stored at -20°C until further use.

Southern and northern hybridization- The blots were prehybridized in prehybridization buffer at 62°C for 3 hr followed by hybridization with radiolabeled (~3 x 10^6 dpm/µg) fat body GST or HP23 cDNA probe at 62°C for 12-16 h. The hybridized blots were washed in 1X SSC with 0.1% SDS at 37°C, followed by three washes in 0.2X SSC with 0.1% SDS at 62°C. Finally the blots were dried and exposed to Kodak X-OMAT X-ray film for detection of hybridization signal.

Quantification of Haemolymph ecdysteroids-

Ecdystreoid extraction and HPLC based quantization- Haemolymph ecdysteroids were extracted by methonolic extraction according to standard protocols. Equal volume of the haemolymph collected from control and experimental larvae was mixed thoroughly with equal volume of methanol for 1 h at 4^{o}C; mixture was centrifuged at 10,000 g for 15 min at 4^{o}C. The supernatant was collected and stored and the pellet was again resuspended in methanol and centrifuged. Both the supernatants were mixed, dried under vacuum and again dissolved in equal volume of methanol. These were used as crude ecdysteroids extracts for HPLC analysis. HPLC analysis of crude ecdysteroids were done on Agilent 1100 quaternary HPLC system equipped with ZORBAX Eclipse, XDB C_8, 4.6 mm x 150 mm, 5 µm, reverse phase column using mobile phase consisting of methanol: water (80:20) at the flow rate of 1 ml/min. Ecdysteroids in the fractions were detected at 254 nm using a UV detector.

Results- Chapter I

Background-

During the insect metamorphosis histolysis of larval organs, cellular destruction, tissue remodeling and reorganization, are mostly accomplished by the activity of lysosomal hydrolases. Stimulation of lysosomal activity by ecdysteroids is widely demonstrated in several insects including *Corcyra cephalonica* and the increase in the lysosomal activity is governed by the elevation of 20E levels. Further more extensive studies from our group suggest that the induction in lysosomal activity by ecdysteroids is governed at nongenomic level. Recently our group has demonstrated also that nongenomic regulation of acid phosphatase activity by 20E is mediated by novel regulatory haemolymph protein HP19. Molecular characterization of this protein revealed its homology (65%) with *Choristineura fumiferana* glutathione S-transferases (GSTs). Hence in the present study we purified and characterized the GSTs from *Corcyra cephalonica* upto molecular level, and made attempts to evaluate its role, if any as a regulatory protein in mediation of 20E dependent action during larval-pupal-adult development/transformation. This chapter deals with purification, biochemical characterization and molecular cloning of glutathione S-transferases from *Corcyra* fat body and its role in ability to potentiate 20E induced acid phosphatase (ACP) activity.

Determination of optimum pH and GST activity in different tissues-

For determination of pH optima enzyme assays were carried out with buffers of various pH ranging between 5 to 9. The results obtained are shown in figure 1A (black line-broad range; pink line-narrow range) clearly suggest that the optimum pH for *Corcyra* GST (CcGST) is 8.3. However, literature survey revealed that GSTs in most animal species show highest activity between pH ranges of 6.5-7.5. Hence, reconfirmation of the result obtained in present study was done by carrying out assays without addition of the enzyme in the reaction mixture. Measuring the non-enzymatic conjugation at pH 8.3 in presence of GSH and it was compared with at pH 6.5. The results do not show any significant difference in the values suggest that the highest GST activity obtained at pH 8.3 in the present insect is not due to non-enzymatic conjugation. We then assayed GST activity at pH 8.3 in different insect larval tissues and found that the activity was negligible in salivary gland and gut (Fig. 1B). However, the activity was fairly high in the visceral fat body (VFB) as well as in carcass which is associated with peripheral fat body tissue. This study indicated that most of the CcGST is present in the fat bodies of the larvae. Therefore, we have used the visceral fat bodies for the remainder of our studies.

Purification of GSTs and kinetic studies-

To further characterize the CcGST, we purified the CcGST from the visceral fat body of *C. cephalonica* larvae. The cytosolic fraction contained the major portion of GST activity, hence was used for purification by DEAE cellulose ion-exchange and GSH-Sepharose affinity chromatography. CcGST did not bind to the ion-exchange resin and entire activity was observed in the flow-thru fractions (Fig. 2A). This is consistent with the previously reported behavior of GSTs to anion-exchange resins. The flow-thru fractions were applied to reduced-GSH cross-linked with epoxy-activated Sepharose 6B affinity column to obtain the purified active GST. Purification profile of GST clearly indicated a substantial enrichment of CcGST (Table 1 & Fig. 2B). The specific activity increased nearly 5 fold in the flow-thru fractions after anion-exchange purification step and increased nearly 140 fold after affinity chromatography step. Silver staining of the SDS-PAGE of the pooled fractions of GSH-affinity eluate produced a protein with a molecular weight of 23 kDa (Fig. 2B). To further analyze the purity, affinity purified CcGST was subjected to reversed-phase HPLC, which showed presence of a single major peak (Fig. 2C). Kinetic analysis with the affinity pure CcGST with varying concentrations of CDNB and GSH at pH 8.3 to determine the V_{max} and

K_m revealed that V_{max} for CDNB is 13.76 μmoles/min/mg and K_m is 0.40 mM (Fig. 3A) while for GSH, V_{max} is 13.36 μmoles/min/mg and Km value is 0.33 mM (Fig. 3B).

Developmental regulation of GST and tissue profile-

To get further insight into GST regulation during the postembryonic development *C. cephalonica* fat body GST activity was measured at different developmental stages. The results clearly show the gradual increase of GST activity from 4th instar to LLI larval stage, followed by massive decrease in pre-pupal stage of the insect (Fig. 4A). Highest activity was seen in LLI stage of the insect. Western analysis (Fig. 4B) shows corresponding increase in amount of protein in the fat body except at pre-pupal stage. In pre-pupal stage GST protein content is equal or higher than that of LLI stage while enzymatic activity (Fig. 4A) is very low. This clearly suggests the possibility of a post-translational regulatory mechanism that regulates the activity of GST during different stages of post-embryonic development. For immunoblot analysis the proteins (20μg) from different larval tissues of *Corcyra* electrophoretically separated and transferred to nitrocellulose membrane. Results presented in figure 4 shows the proteins in the range of 20-23 kDa cross react with the anti serum generated against affinity purified fat body GSTs. Intense cross reactivity of a 23 kDa protein (Fig. 4B) was seen in lanes HGLFB (lane3), visceral fat body (lane4) and carcas (lane5). Furthermore, the content of GST protein is highest in fat body, which corresponds well with enzymatic activity presented in figure 1B. It is also interesting to note that the fat body GST antisera cross reacted faintly with a lower molecular weight protein (≈20 kDa) in gut (lane 2). However the molecular weight of the protein which immunoreacted with fat body GST antisera in the salivary gland was more are less the same as of fat body (lane 1).

Molecular characterization of Cc GST-

To further characterize the fat body CcGST, we cloned the cDNA using degenerate primers for lepidopteran specific GSTs (Fig. 5A). Cloned cDNA upon sequencing gave 701-nucleotide long sequence (Fig. 5B). The longest ORF encoded protein of 216 amino acids (Fig. 5C). The CcGST nucleotide sequence discussed here has been submitted to the GenBank with accession number DQ234273. On the basis of BLAST analysis of the deduced amino acid sequence and multiple sequence alignments with other insect GSTs using ClustalW program (Fig. 6). The newly cloned GST was identified, to be closely related to delta-class GSTs. The amino acid sequence showed 91% identity with *Galleria mellonella*,

86% identity with *Bombyx mori*, 60% identity with *Helicoverpa armigera*, 55% identity with *Anopheles dirus* and 55% identity with *Anopheles gambiae* (Fig. 6). We observed a distinct difference at the N-terminal end of the CcGST protein with the known Delta class GST from other insects, which is the presence of three serine residues (Ser-5, Ser-13 and Ser-16). Ser at 9^{th} position in AdGST and at 11^{th} position in other insect GSTs is known to play an important role in stabilizing the ionized GSH for productive binding at the G-site in all delta class GSTs (Caccuri, 1997). This Ser at 11^{th} position is replaced with Leu in this newly identified CcGST, and could be the cause for its optimal activity at higher pH. Amino acid alignment with known crystallized structure of *Anopheles dirus* AdGST revealed few distinct differences (Fig. 7). In AdGST D3, His-38, His-50, Ile-52, Glu-64, Ser-65, Arg-66 and Met-101 interact directly with glutathione by formation of hydrogen bonds and Ser-9 stabilizes the ionized GSH. Whereas in CcGST, Ile-52 is replaced with Val-52 and Met-101 is replaced with Ile-101 most importantly ionized GSH stabilizing residue in G-site Serine was replaced with leucine which does not have any group to stabilize ionized GSH.

Homology modeling of Cc GST protein-

To further confirm our hypothesis proposed in previous section we carried out homology modeling of CcGST using AdGST sequence as a template. As a result of BLAST-based template search through PDB, the crystal structure of AdGST (PDB CODE: 1JLV) was selected as the template. The homology score comparing to target protein was 55 %. A sequence alignment for sequences of the template and the target is presented in figure 7. The structurally conserved regions were determined by multiple sequence alignment, which is based on the Needleman and Wunsch Algorithm. Model was generated using InsightII. Ten different models were prepared using INSIGHTII. The RMSD values of the models were generated. The model with least RMSD value was taken for further studies. The overall secondary and tertiary structures were very similar for the two proteins. This also includes the loops adjacent to the active site. The model initially generated was energy minimized in CHARM. The final model obtained was verified with Profile-3D and Structure Alignment in InsightII. The overall self-compatibility score for this protein without the heteroatom is 87.759995, which is higher than the lower score 44.11 and close to the top score 98.030694. The root mean square deviation (RMSD) of the equivalent Cα atoms between the final model and 1JLV is 0.23 A°, indicating that the overall structures are highly identical as shown in figure 8B. the overall secondary and tertiary structures of both the proteins are more or less the same as shown in figure.8C.

The stereo chemical quality of the model was checked using the PROCHECK and ERRAT programs. Ramachandran plot of this minimized model showed that 100 % of the residues were located in the allowed regions (93.8 % most favored) and 0 % residues (no residues) outside the allowed regions (Fig. 8A). As there are no disallowed residues in the model generated, it can be used for further studies. Docking studies with GSH and CcGST model revealed that there is no serine residue in the range of hydrogen bonding distance at G-site (Fig. 9A & B). This clearly suggests that there is no stabilizer for ionized GSH in CcGST. For further validation of proposed concept the CcGST cDNA, we expressed the in *E. coli* cells. The lysate expressing the CcGST was used to measure the GST activity at pH 6.5 and 8.3 (Fig. 10). The results clearly indicate that the cloned CcGST cDNA expressed a GST enzyme that has high activity, exclusively at pH 8.3 as compared to pH 6.5. Control lysate showed negligible difference in GST activity at different pH values. This study clearly suggests that the cloned cDNA indeed encodes a GST enzyme that is uniquely active at pH 8.3. The overall folding pattern is conserved across the available 3D structures and is also recognized in the CcGST model.

Southern analysis of genomic DNA and developmental expression of CcGST by northern analysis-

Southern analysis of total genomic DNA from larval fat body digested with EcoRI, BamHI and HindIII suggests that it is s multiple copy gene (Fig. 11A). Northern hybridization with total RNA isolated from fat body of revealed the presence of two transcripts of Cc GST at all the developmental stages (Fig. 11B). However, the pattern of expression of these transcripts was fairly different at different developmental stage.

Juvenile hormone inhibits GST activity, while 20E has no effect-

To further understand CcGST regulation, we studied the effect of JH1 and 20E hormones on GST activity and protein levels at physiological concentration. Visceral fat body was cultured for different time periods in the presence or absence of hormone and was then used for GST activity assay as well as western analysis with polyclonal antibody raised against CcGST. In JH1 treated fat body cultures, there was no clear change in the CcGST protein levels (Fig. 12A). However, the activity reduced significantly in cultured fat body that were treated with JH1 for 8 and 12 h. Similar effect was observed when the JH1 was topically applied to LLI larvae (Fig. 12B). However we did not observe any effect of 20E treatment on the fat body GST activity (Fig. 13).

Fat body GST ability to potentiate 20E induced ACP activity-

As shown earlier by our group that the fat body ACP activity increases during the postembryonic development and its mediated by 20E. Furthermore a novel regulatory protein HP19 which was fairly high homology with *C. fumiferana* GST potentiated this 20E induced ACP activity in fat body cultures. To check whether the purified GST from *Corcyra* fat body can potentiate 20E induced acid phosphatase (ACP) activity in organ culture experiments, HP19 protein was substituted with purified GST in the organ culture experiment. As results show in figure 14.A clearly show that GST is unable to potentiate 20E induced ACP activity. This clearly suggests that HP19 clone which was isolated earlier (Arif et al., 2004) by immunoscreening is different from GST. Detailed amino acid alignment studies with Corcyra GST and HP19 show less than 25% similarity (Fig. 12B). Hence, the present work clearly shows that HP19 and GST two different molecules and has different functional roles in *Corcyra.*

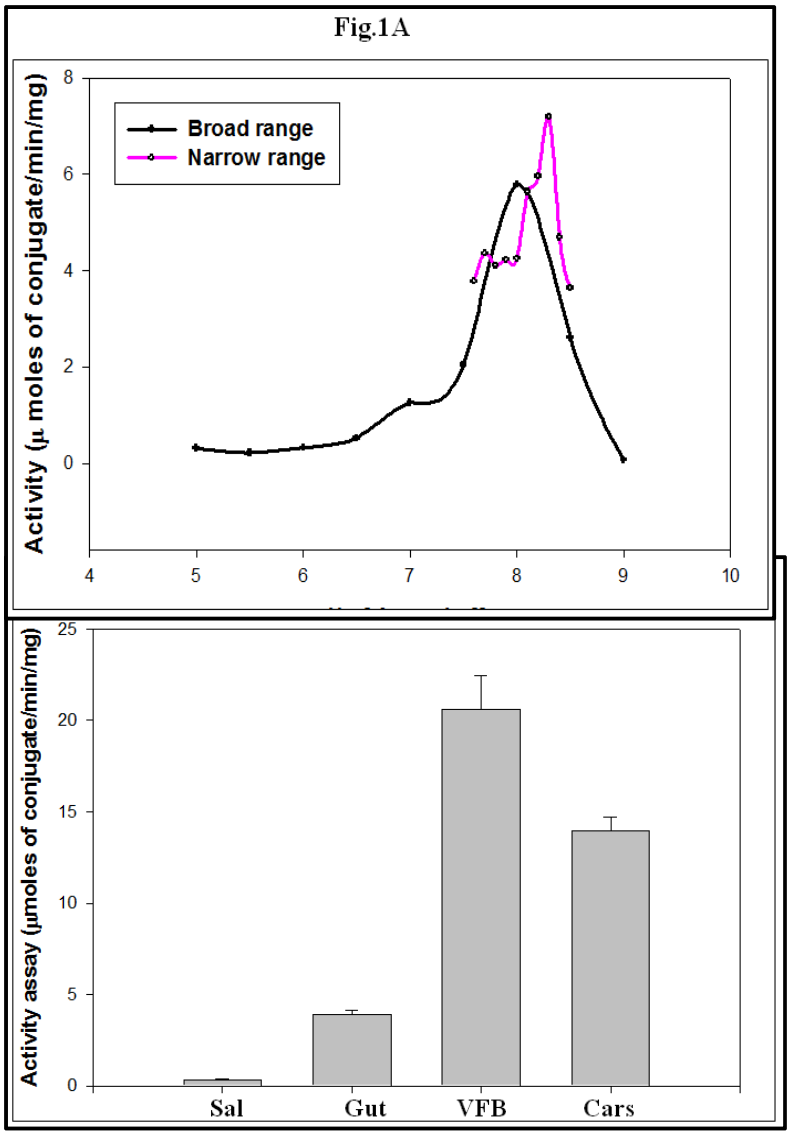
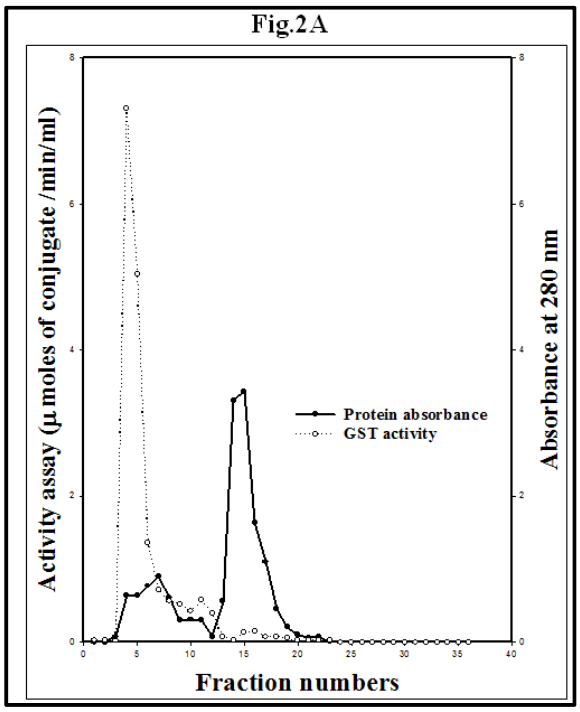


Fig. 1: Effect of pH on CDNB conjugating activity of whole body LLI larval stage CcGST. **A.** 100 mM Phosphate buffer (pH 5.0-7.0) and 100 mM Tris-HCl (pH 7.0-9.0) buffers were used for activity assay Black line indicates activity at broad range and pink line indicates activity at narrow range. **B.** Tissue profile of CcGST activity. The tissues (as shown) were isolated from LLI larvae were, homogenized and assayed for GST activity at pH 8.3 using 100 mM Tris-HCl buffer. Sal - Salivary gland, FB- fat body, Cars- carcass.



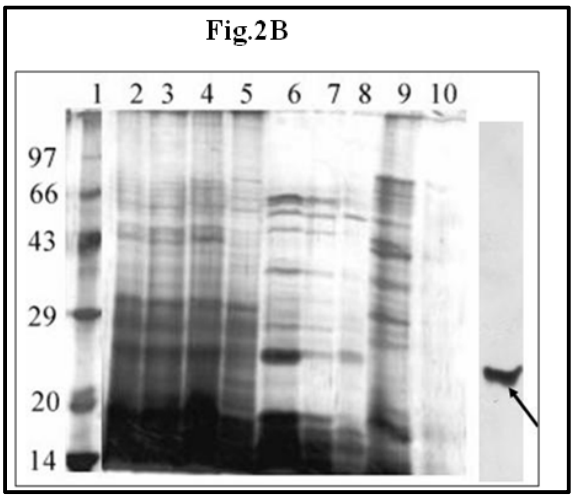


Fig. 2C

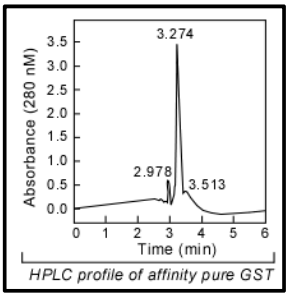


Table 1

Fraction	Total protein (mg)	Activity (µmoles/min/ml)	Total activity	Specific activity (Activity/mg)	Yield (%)	Fold purification
Crude extract	205.39	14.96	284.24	1.38	100	1.00
10 K-fraction	136.05	14.03	210.18	1.54	66.21	1.11
Cytosolic	103.22	17.04	204.16	1.98	50.25	1.28
Microsomal	23.17	1.98	4.95	0.21	11.28	0.11
DEAE eluate	42.07	12.76	229.58	5.46	20.48	2.75
GSH affinity eluate	1.08	8.39	150.81	139.62	0.52	25.57

Fig. 2: Purification and biochemical characterization of CcGST. A. DEAE Purification profile of CcGST. **B** SDS-PAGE profile of CcGST purification. Purity of CcGST was checked by silver staining at each step of purification. Affinity purified GST indicated by arrow. **C.** RP-HPLC profile of affinity pure CcGST. Affinity pure GST was passed through reversed-phase HPLC analysis to check for contaminating proteins. Protein was eluted with linear gradient of acetonitrile.

Table 1. Purification profile of fat body GST. The % yield calculated at each step on the basis of the amount of the protein recovered at that particular step with reference to the amount of protein present in the crude preparation fold purification was calculated indirectly by comparing the specific activity of the step, for which fold purification to be calculated to that of the specific activity of previous step.

Fig.3A

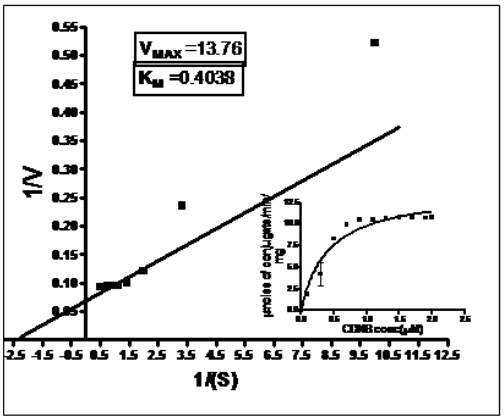


Fig.3B

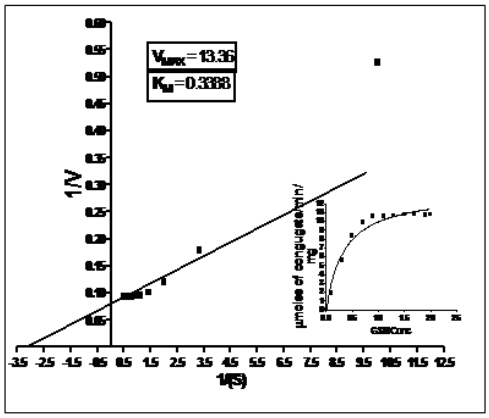
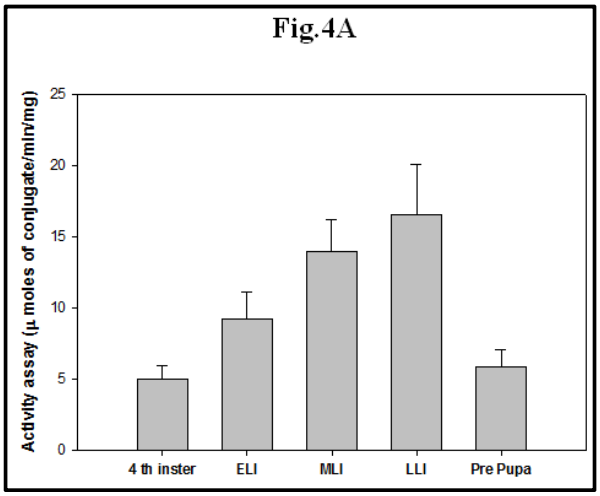


Fig. 3: Lineweaver-Burk and Michaelis-Menten plots of the CDNB conjugating acvity of fat body GST of *C. cephalonica* at pH 8.3. Initial rates were measured under standard condition for 15 min by filter paper assay. The GSH **A** and CDNB **B** concentration were varied as indicated.



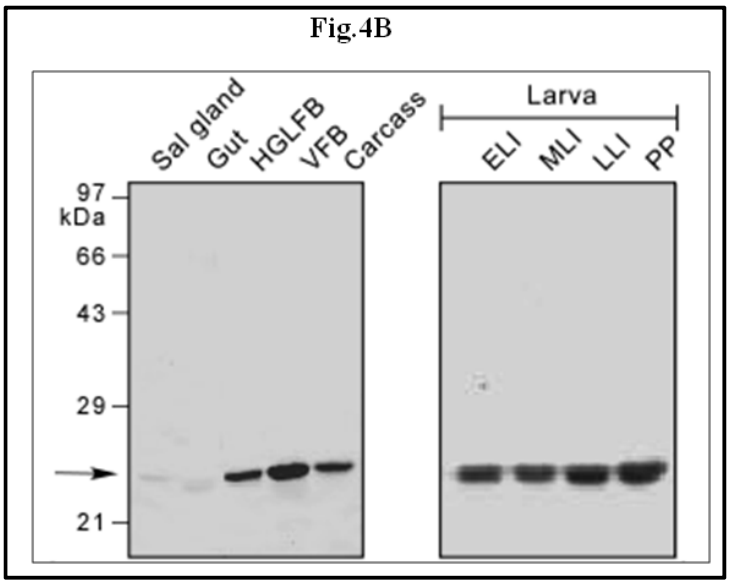


Fig. 4: A. Developmental regulation of GST activity and protein expression. GST activity profile in different larval developmental stages of *Corcyra cephalonica*. 10 µg of protein was taken for assay. Note highest activity was observed at LLI stage of the insect. **B.** Western analysis of GST protein expression in different tissues 20 µg of protein was loaded on each lane. Note high protein levels found in visceral fat body (VFB). Developmental profile of GST protein levels at different larval stages of *Corcyra*. High protein levels were observed at pre pupal stage of insect.

Fig. 5: A. PCR amplification of fat body GST cDNA at various melting temperatures. **B.** The cDNA nucleotide **C.** Deduced amino acid sequence of *C. cephalonica* GST.

Fig.6

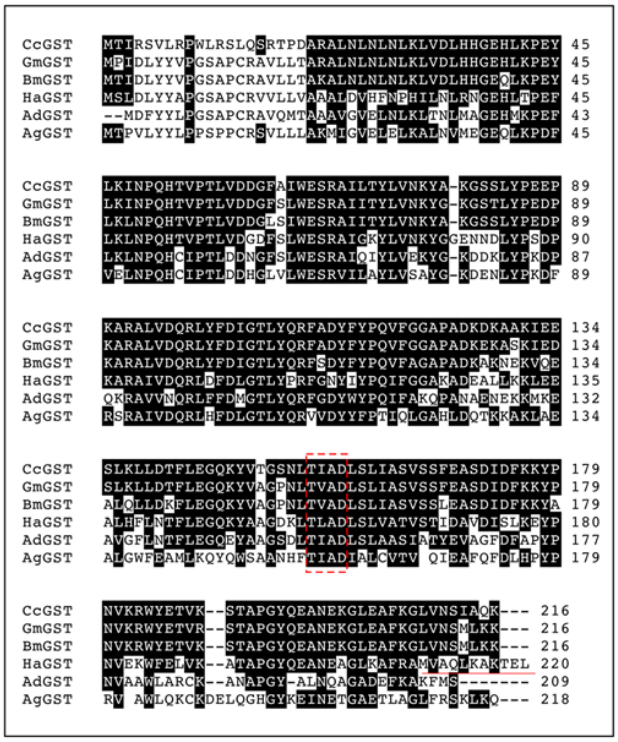


Fig.7

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CcGST      MTIRSVLRPWLRSLQSRTPDARALNLNLNLKLXDLHHGEHLKPEYLKINPQHTVPTLVDD  60
AdGST      --MDFYYLPGSAPCRAVQMTAAAVGVELNLKLTNLMAGEHMKPEFLKINPQHCIPTLVDN  58
           :     *   . ::    * *:.::***** :*  ***:***:******* :*****:
CcGST      GFAIWESRAILTYLVNKYAKGSSLYPEEPKARALVDQRLYFDIGTLYQRFADYFYPQVFG  120
AdGST      GFALWESRAICTYLAEKYGKDDKLYPKDPQKRAVVNQRLYFDMGTLYQRFADYYYPQIFA  118
           ***:****** ***.:**.*...***::*:  **:*:******:**********:***:*.
CcGST      GAPADKDKAAKIEESLKLLDTFLEGQKYVTGSNLTIADLSLIASVSSFEASDIDFKKYPN  180
AdGST      KQPANAENEKKMKDAVDFLNTFLDGHKYVAGDSLTIADLTVLATVSTYDVAGFELAKYPH  178
             **: ::  *:::::.:*:***:*:***:*..******:::*:**:::.:.::: ***:
CcGST      VKRWYETVKSTAPGYQEANEKGLEAFKGLVNXIAQ 215
AdGST      VAAWYERTRKEAPG-AAINEAGIEEFRKYFEK--- 209
           *  *** .:. ***    ** *:* *:  .:
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Fig. 6: Clust L W Alignment of CcGST with other insect GST sequences. CcGST, *Corcyra cephalonica* (DQ234273); GmGST, *Galleria mellonella* (AF336288); BmGST, *Bombyx mori* (AB176691); HaGST, *Helicoverpa armigera* (EF033109); AdGST, *Anopheles dirus* (AF273041) and AgGST, *Anopheles gambiae* (AF071161). Identical amino acids are shaded and gaps are shown as dashes. The signature sequence of delta class GST "TIAD" is underlined.

Fig. 7: Amino acid sequence alignment of CcGST and Ad GST using clustL W program showing 55% similarity.

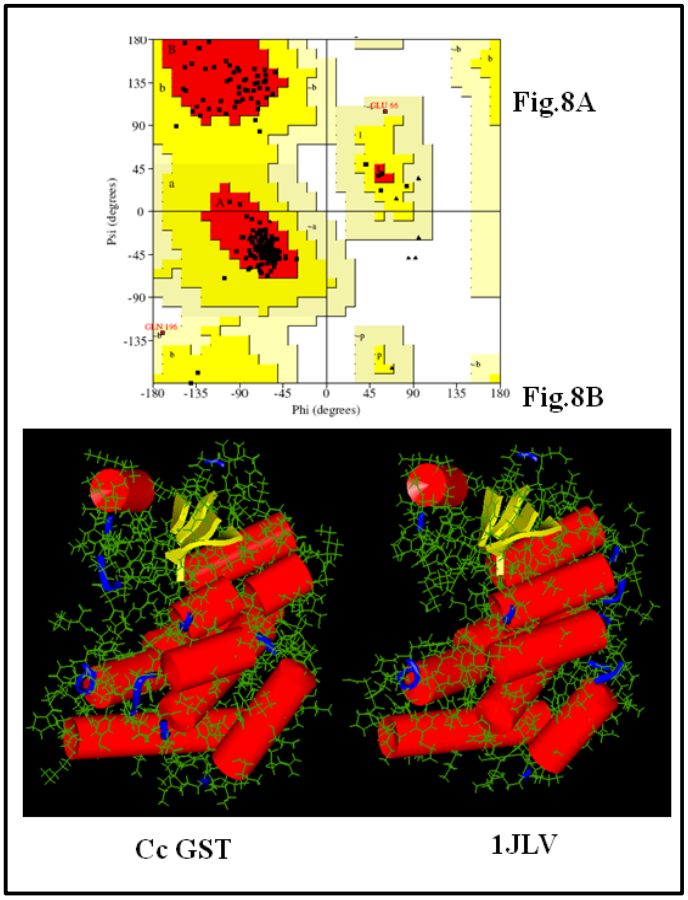


Fig. 8: A. Ramachandran plot of CcGST minimized model showed that 99.2% of the residues were located in the allowed regions (93% most favored in red color, 5.2% allowed region in yellow color and 1% generously allowed region). **B.** Secondary structures of model (Cc GST) and template (Ad GST, 1JLV).

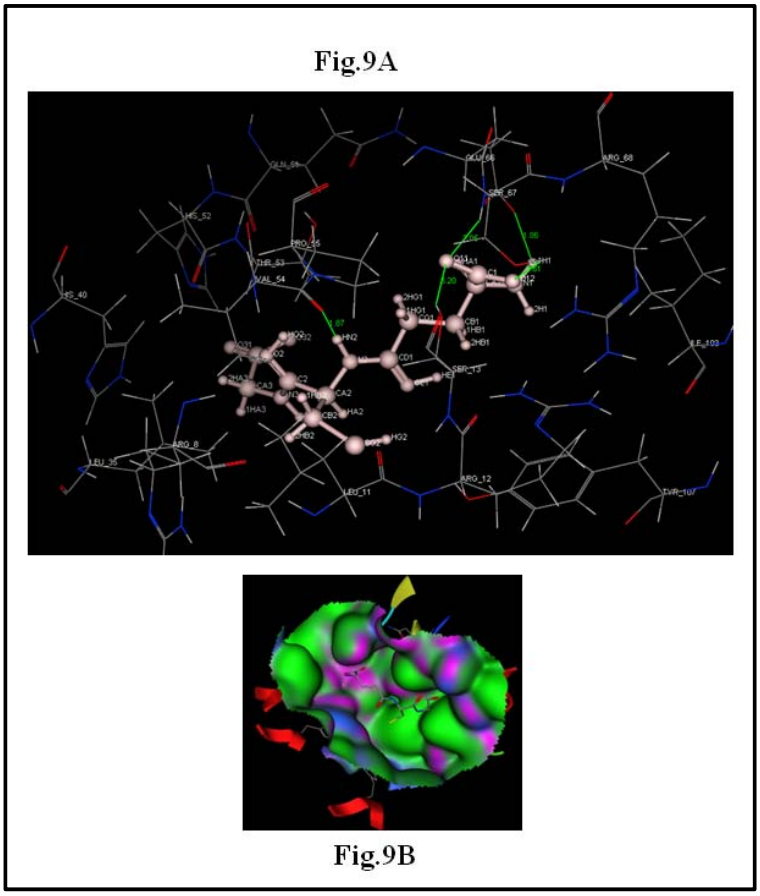


Fig. 9: A. Stereo view of hydrogen bonding interactions of reduced glutathione molecule in Cc GST active site. Hydrogen bonds were shown in blue color. **B.** GSH in active site cavity.

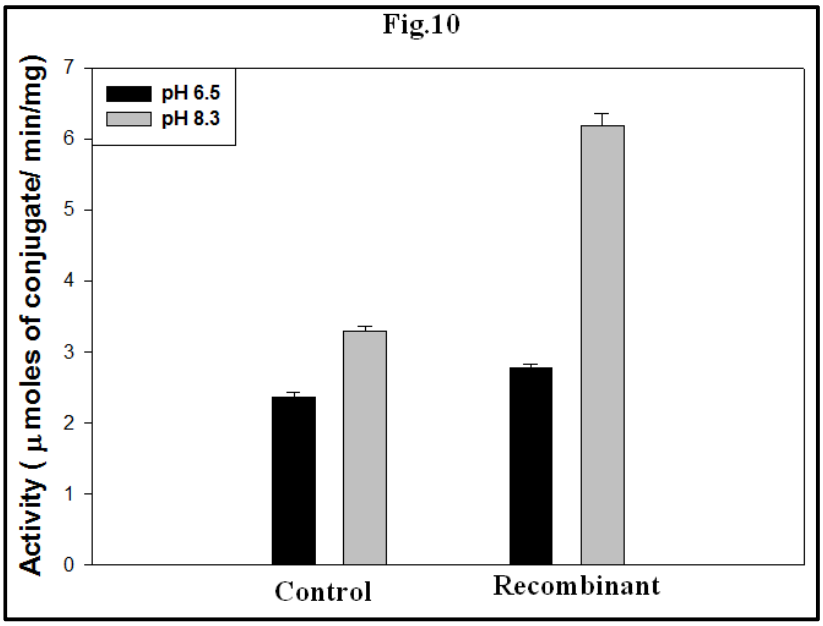


Fig. 10: Activity profile of recombinant CcGST. CcGST-cDNA was expressed in BL21-DE3 *E. coli* cells by induction with 1mM IPTG for 2h. Equal quantity of lysates (10 µg) was tested for GST activity at low (6.5) and alkaline pH (8.3). Untransformed BL21-DE3 cell lysate was used as a control.

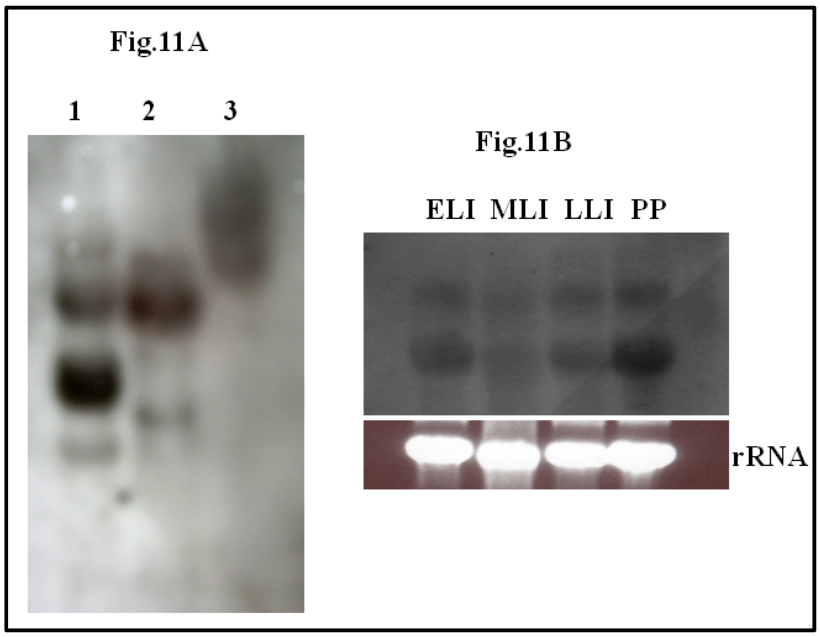
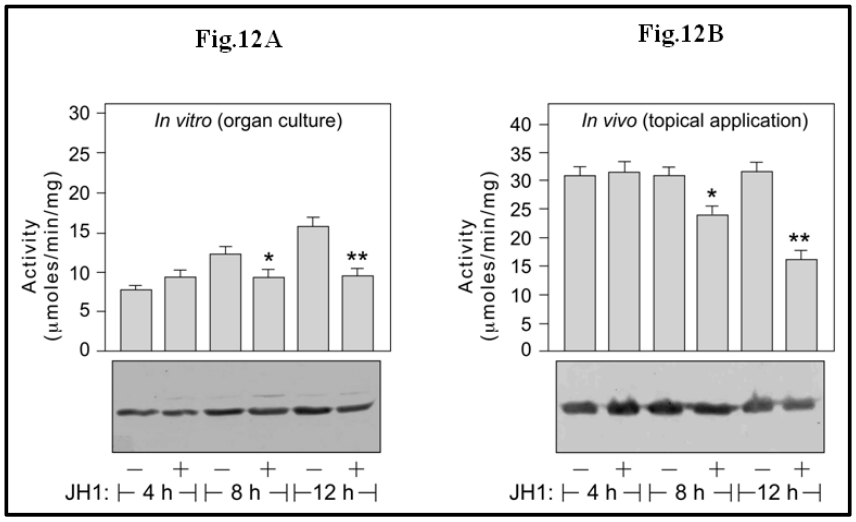


Fig. 11: Molecular characterization of GST: A. Southern blot analysis of GST gene showing multiple gene copy number. 20 µg Genomic DNA from Corcyra fat body digested with enzymes. Lane.1 BamH1, Lane.2 EcoRI, Lane.3 Hind III.
B. Developmental expression of GST transcript in *Corcyra cephalonica.* Northern blot showing different transcript levels in different developmental stages. **(**Note the presence of two different transcripts) Lane ELI-Early-last instar larvae, lane MLI- mid-last instar larvae, Lane. LLI- late-last instar larvae, lane PP- pre-pupae Total 20 µg RNA was loaded on the gel.



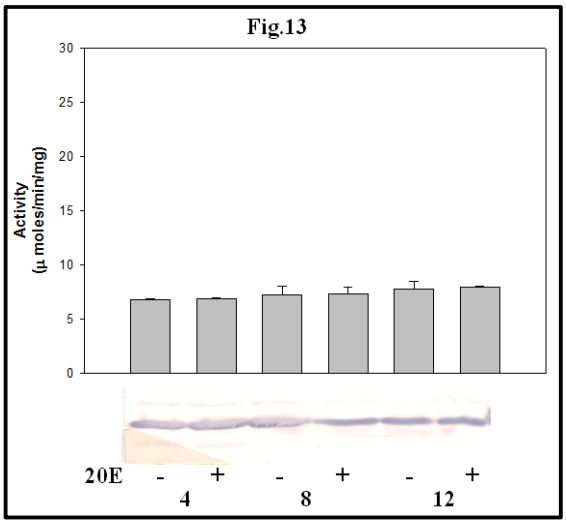


Fig. 12: Effect of JH-I on fat body CcGST.(A) *In vitro* study. LLI visceral fat bodies from LLI larvae kept in culture in TC-100 medium were incubated with or without 70 nM JH-I for indicated time. At the end of incubation, tissues were homogenized and assayed for GST activity (top) and protein expression (bottom) with anti-CcGST antibody. **(B) *In vivo* study**. 1 µl of 250 nM of JH-I was applied topically to LLI larvae and allowed to grow under natural condition for indicated time. The larvae were sacrificed and VFB homogenate was assayed for GST activity (top) and protein expression (bottom).

Fig. 13: *Effect of 20E* on fat body CcGST (*in vitro* study). LLI visceral fat bodies from LLI larvae kept in culture in TC-100 medium were incubated with or without 80 nM 20E for indicated time. At the end of incubation, tissues were homogenized and assayed for GST activity (top) and protein expression (bottom) with anti-CcGST antibody.

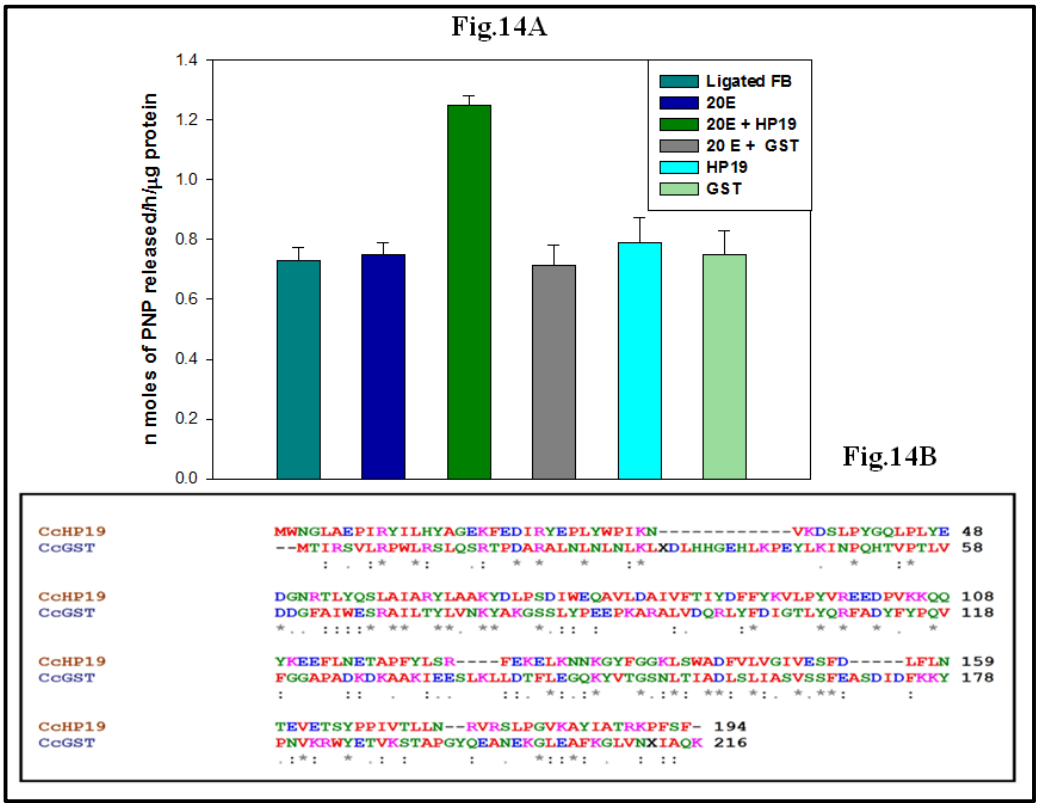


Fig. 14: A comparative study on the effects of HP19 and GST: A. Effect of affinity purified *Corcyra* fat body GST on 20E dependent fat body ACP activity. Note that the presence HP19 purified from haemolymph mediated the 20E stimulated enzyme activity, whereas presence of GST did not have any effect. The purified GST (40 ng), CcHP19 (1 µg) or purified HP19 (40 ng) were added to the fat bodies kept in culture in the presence of 80 nM 20E and incubated for 4 h at 25°C. The control contained equal volume of 0.05% ethanol. At the end of the incubation, the fat bodies were removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenized and assayed for ACP activity. Each value is the mean ± S.D. of three independent determinations. **B.** Amino acid sequence alignment of CcHP19 CcGST using clustL W program showing less than 25% similarity.

Results- Chapter II

Background-

Studies from results chapter 1 show the identification of a developmentally regulated glutathione S-transferase from *Corcyra* (CcGST) which is active at alkaline pH (pH 8.3). Molecular cloning and characterization revealed that it belongs to Delta class of GST. Clust LW analysis using deduced amino acid sequence showed that it CcGST has less than 25% similarity. Further, the CcGST does not show any 20E induced ACP potentiating activity in fat body cultures, as discussed in chapter 1 results. After confirming the HP19 and GST are two different molecules with entirely different function, HP19 cDNA was expressed and purified using bacterial expression system for further studies. This chapter deals with recombinant expression and purification of HP19 protein and evaluation of its ability to mediate 20E induced ACP activity. In addition,an attempt was also made to purification and characterize the HP19 from larval haemolymph of *Corcyra*.

Construction of HP19 expression vector and purification of recombinant protein-

PCR amplified HP19 cDNA and pRSET A expression vector were sequentially double digested with Bam H1 and Hind III restriction enzymes. Gel separated fragments (Fig. 15A) were ligated with T4 DNA ligase and *E. coli* DH5α competent cells were transformed. Recombinant clones with HP19 fragment were identified by both PCR screening and double digestion, and the results are presented in figure 15B. Positive clone was transformed into *E. coli* BL21 (DE3) *Plys* cells. Recombinant protein expression was induced with 1 mM IPTG and initially analyzed by SDS page and purified using Ni-NTA agaorse chromatography, as shown in figure 15C. The homogeneity of purified protein was satisfactory for the physiological studies. The HP19 fragment was again cloned into the GST tagged expression vector pGEX-4T1. The clone was confirmed by colony PCR and restriction digestion (Fig. 16A) and expressed in *E. coli* BL21 (DE3) *Plys* cells (Fig. 16B). Recombinant protein was purified by glutathione affinity chromatography (Fig. 16C). Purified GST tagged HP19 protein (GSTHP19) was approximately 50 kDa in size based on the mobility on SDS-PAGE it matched with the expected size of deduced amino acid sequence of HP19 cDNA. GST tag from the recombinant protein was removed by thrombin using thrombin-agarose as per the manufacturer's protocol.

Activity of recombinant HP19-

After obtaining the reasonably pure recombinant HP19, its ability for potentiation of 20E induced acid phosphatase activity was evaluated. For this recombinant protein was added to the in vitro cultured fat body and after 4 h of incubation, acid phosphatase activity in the tissue was assayed. Results presented in figure 17A clearly shows that the recombinant HP19 failed to potentiate 20E induced acid phosphatase activity, which was seen in controls native HP19 protein was added. Through literature it was known that bacterially expressed proteins lacks proper folding and post-translational modifications. We further planned to express the HP19 clone in eukaryotic expression system. Before proceeding for the same we checked cross reactivity of recombinant HP19 with antibody generated against electro eluted HP19. To our surprise the HP19 antibody failed to detect the bacterially expressed protein (Fig. 17B). However it is detected the native HP19 in the haemolymph. The experiment was repeated several times with different batches of recombinant HP19 and the results obtained were consistent, clearly suggesting that the HP19 cDNA, which was isolated by immunescreening was probably a false positive, as the isolation of HP19 clone from the

expression library was carried out using polyclonal antibody generated from electroeluted protein.

Purification of HP19 protein from LLI haemolymph-

After HP19 cDNA was found to be false positive clone. For further studies HP19 protein was purified from LLI larval haemolymph by classical chromatographic methods. For this Gel filtration (SephadexG100-120) DEAE-cellulose chromatographic methods were employed. With various modifications, the protocol was standardized for obtaining a reasonably pure protein. For this the diluted haemolymph samples were loaded on to half swelled matrix and the samples were collected at flow rate of 1.5 ml/min. At this flow rate, the samples contained only 4-6 protein below 43 kDa range from 20^{th} fraction (Fig. 18A). The fractions containing less than 43 kDa protein were pooled and loaded on to DEAE-cellulose column and flow through fractions were analyzed for 20E induced ACP potentiation activity using *in vitro* cultured fat body. The active fractions were further analyzed on SDS-PAGE to check the purity (Fig. 18B & C), and the results show pure HP19 protein in flow through fractions of DEAE-cellulose. Purified HP19 protein was further analyzed by 2D electrophoresis (Fig. 19). HP19 purified from larval haemolymph resolved into 4 spots in the PI range of 6- 7, when the nonlinear pH gradient strip was used for separation. Further attempt to separate these 4 proteins by chromatographic methods failed, which might be due to minor difference in the PI value.

HP19 possible mechanism of ACP induction-

Previous studies from our group have shown that HP19 inhibits the 20E induced phosphorylation of hexamerin receptor (Arif et al., 2002). In the present study an attempt was made to find out whether H19 is a kinase inhibitor or phosphatase activator. We have performed 20E induction of ACP potentiation assay with different phosphatase inhibitors like sodium fluoride (NaF) and sodium/potassium tartarate (Na/K-tar). These inhibitors were added to *in vitro* cultured fat bodies at various concentrations. Results presented in figure 20 A & C showed NaF and Na/K-tararate, block the 20E induced HP19 mediated ACP activity in concentration dependent manner. At 10 mM concentration NaF inhibited the 75% of ACP activity (Fig. 20B) whereas Na/K-tartarate inhibited only 50% of induced ACP activity (Fig. 20D). Earlier reports from literature suggest that the nongenomic action of 20E mediated through G-protein coupled receptor via PI3-kinase activation (Deepak et al., 2005). To further dissect, how HP19 mediated the 20E action in the present model, we have used

different PI3-kinase inhibitors. *In vitro* culture studies with fat body challenged with specific PI3-kinase inhibitors (Fig. 21A) show that 20E induced ACP activity in presence of HP19 is blocked/inhibited by PI3-Kinase inhibitors LY294002 and Wortmannin. This clearly suggests that this induction of ACP is via PI3-kinase activation. As HP19 is a protein and a macro molecule it might not cross/enter the plasma membrane, the possibility of binding proteins (receptors) at the plasma membrane was speculated. To further confirm the hypothesis, we performed ligand blot analysis using far western method. Interestingly HP19 was found to bind two binding proteins on the fat body plasma membrane in the range of 70 kDa and 67 kDa (Fig. 21B).

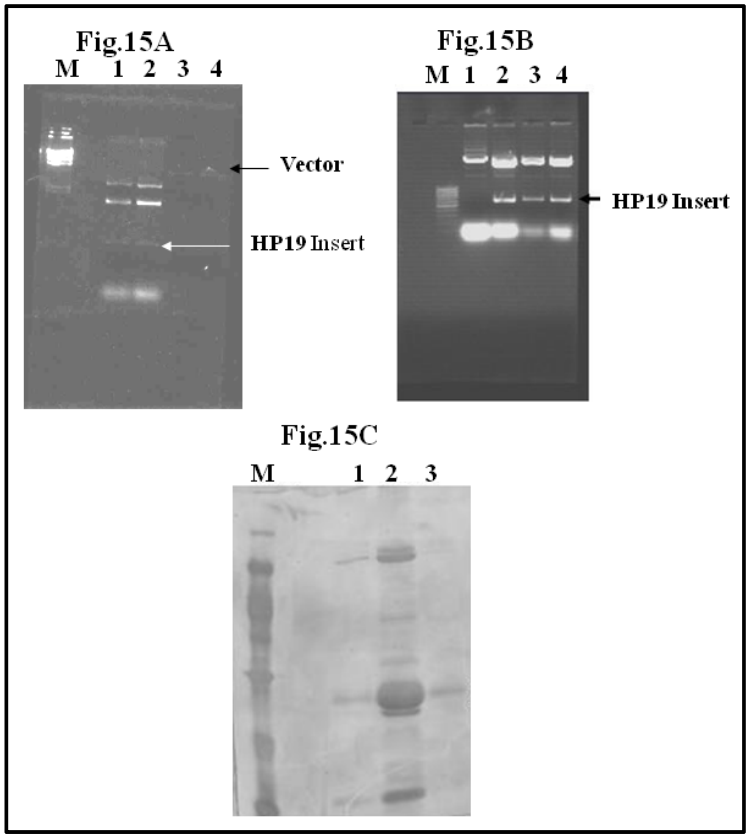


Fig. 15: HP19 Clone construction in pRSETa and purification. Restriction digestion of T vector with HP19 insert and pRSETa vector with Hind III and Bam HI enzymes. Lane M. Marker, lane 1 and 2 are Tvector-HP19 digests higher band (3.6 kb) is partially digested vector, middle band is completely digested Tvector (2.9 kb) and lower band is HP19 insert (588bp). Lane 3 and 4 are pRSETa vector (2.9 kb) digests.

B. restriction analysis of PCR +ve Recombinant pRSET a – HP19 clones. Lane M. Marker, Lane 1, 2, 3 and 4 are different clones out of which 2, 3, 4 are +ve.

C. Purification of recombinant HP19 bt Ni-NTA- agarose column. Lane M is Protein molecular marker (PMW-M), lane 1, 2 and 3 are different eluates.

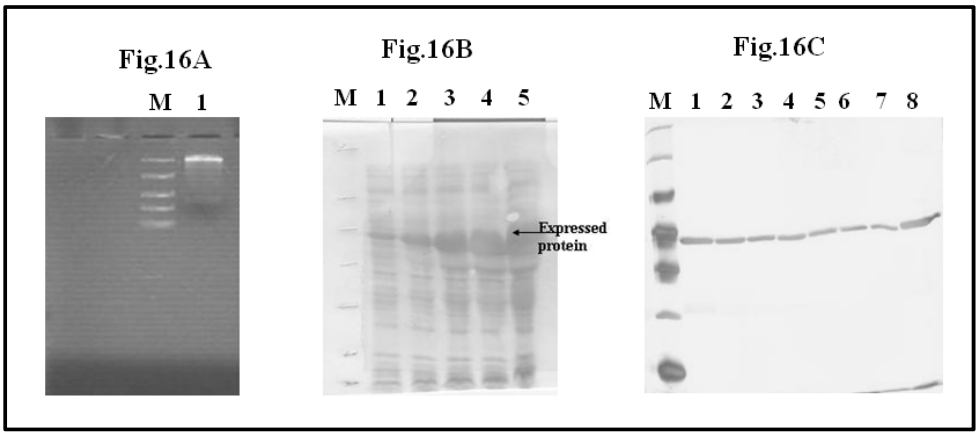


Fig. 16: HP19 clone construction in pGEX4-T1 vector and purification. A. Restriction analysis of PCR +ve recombinant clone. Lane M Marker, lane 1. Upper band is pGEX vector (5 kb) and lower band is HP19 (588 bp) insert.

B. Expression profile of GST-HP19 in *E. coli* BL21 (DE3) Plys cells at various induction times. Lane M is Protein molecular marker, lane 1 is uninduced cells, lanes 2-5 are 1-4 hour IPTG induction times respectively.

C. Purification of recombinant GSTHP19 protein by GSH-Sepharose chromatography showing different elutes.

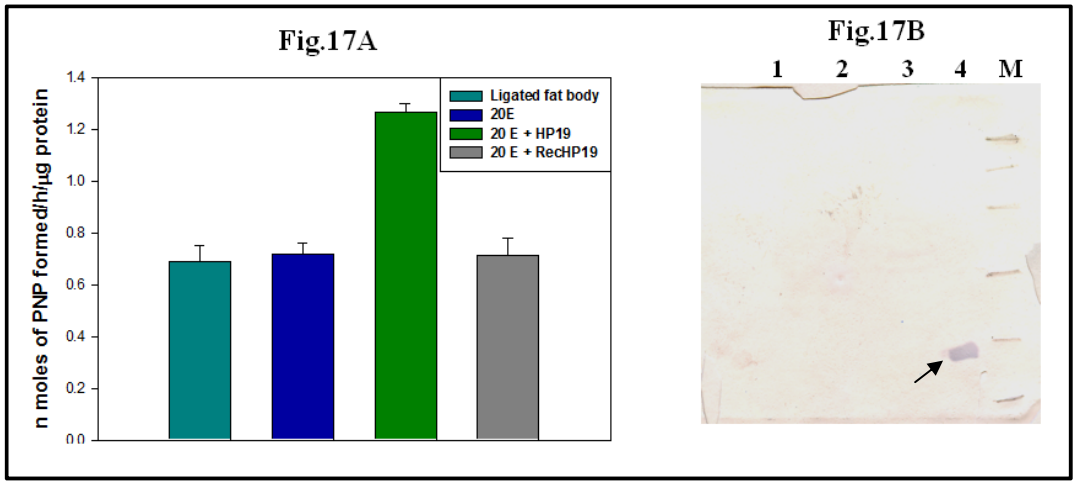
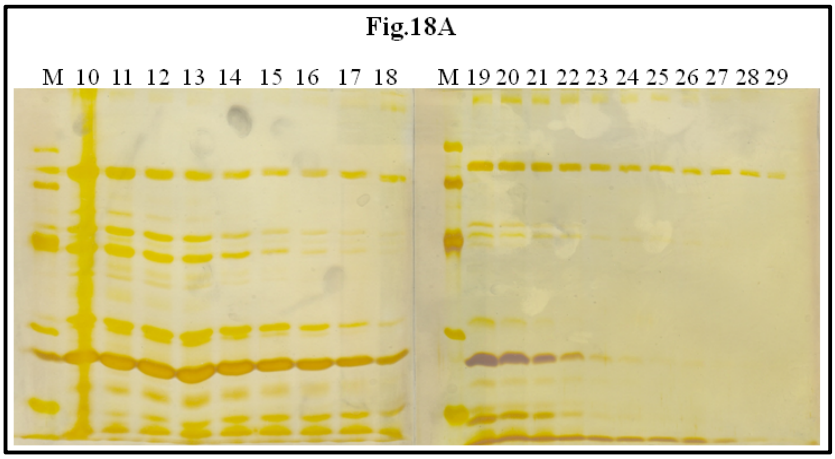


Fig. 17: Evolution of Rec.HP19 activity and cross reactivity with HP19 IgG. A. Effect of Rec.HP19 on 20E induced fat body ACP activity. Note the presence of native HP19 able to potentiate the ACP activity in *in vitro* cultured LLI larval fat bodies where as Rec.HP19 is unable to potentiate 20E induced ACP activity. The purified CcHP19 (100 ng), or purified Rec.HP19 (100 ng) were added to the fat bodies kept in culture in the presence of 80 nM 20E and incubated for 4 h at 25°C. The control contained equal volume of 0.05% ethanol. At the end of the incubation, the fat bodies were removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenized and assayed for ACP activity. Each value is the mean ± S.D. of 3 independent determinations.

B. Cross reactivity of Recombinant HP19 with native HP19 Ig G. Note native HP19 Ig G unable to detect the bacterial expressed HP19 where as it is able to detect the native HP19 from *Corcyra* haemolymph lane 4 (→).



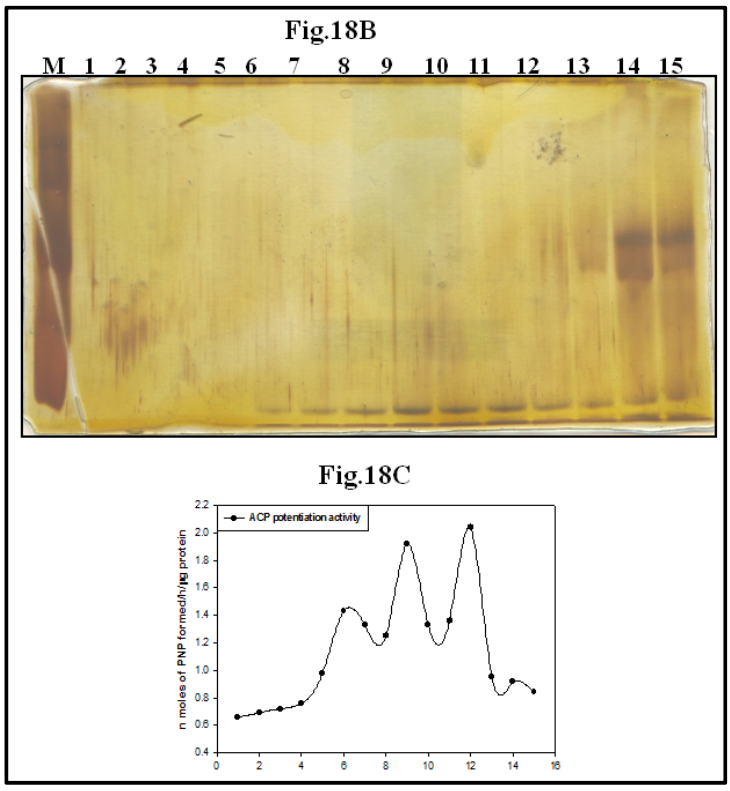


Fig. 18: Purification of HP19 from Corcyra haemolymph. A. SDS-PAGE (12%) showing the protein profile of various active haemolymph fractions from Gel exclusion chromatography (SephadexG100-120).

B. Purified active protein HP19 from DEAE- cellulose column flow through showing pure HP19 from lanes 6- 13.

C. Elution profile of haemolymph proteins on DEAE cellulose matrix in terms of their ability to mediate the ACP activity of LLI larval fat bodies kept in culture in presence of 20E. The elution was carried using 10 mM Tris-HCl.

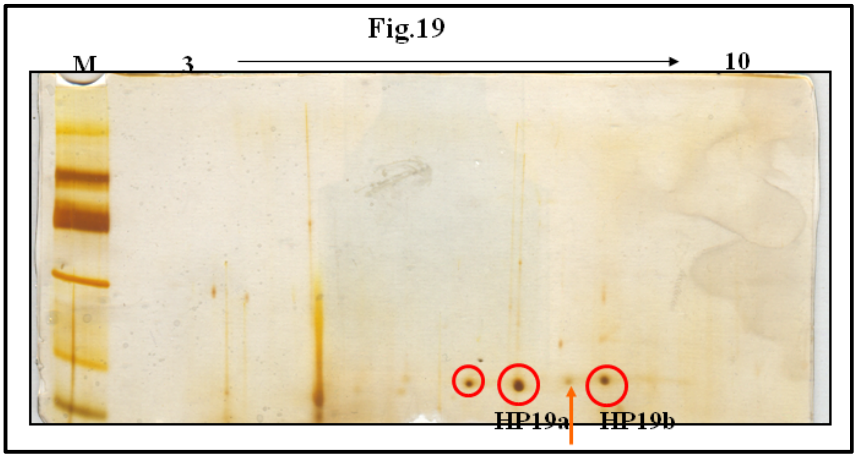


Fig. 19: 2D analysis of purified HP19 from *Corcyra* haemolymph. Purified protein was denatured by sample buffer (Iso urea solution containing 100 mg DTT, 400 mg CHAPS, 5.4 g urea 500 µl of 3/10 ampholyte in 6 ml of DDW). Denatured protein sample was loaded on to non linier 5-7 IEF strip (GE biosciences). Isoelectric focusing was carried out for 40hr (40,000 V hr) and strip was equilibrated with equilibration buffer (6 M urea, 375 mM Tris-HCl pH 8.8, 2% SDS, 20% glycerol, 2% DTT and 2% solid Acrylamide). For 5 min and loaded on to the second dimension 12% SDS-PAGE gel. Note purified HP19 protein was resolved into 4 spots at PI range of 6- 7.

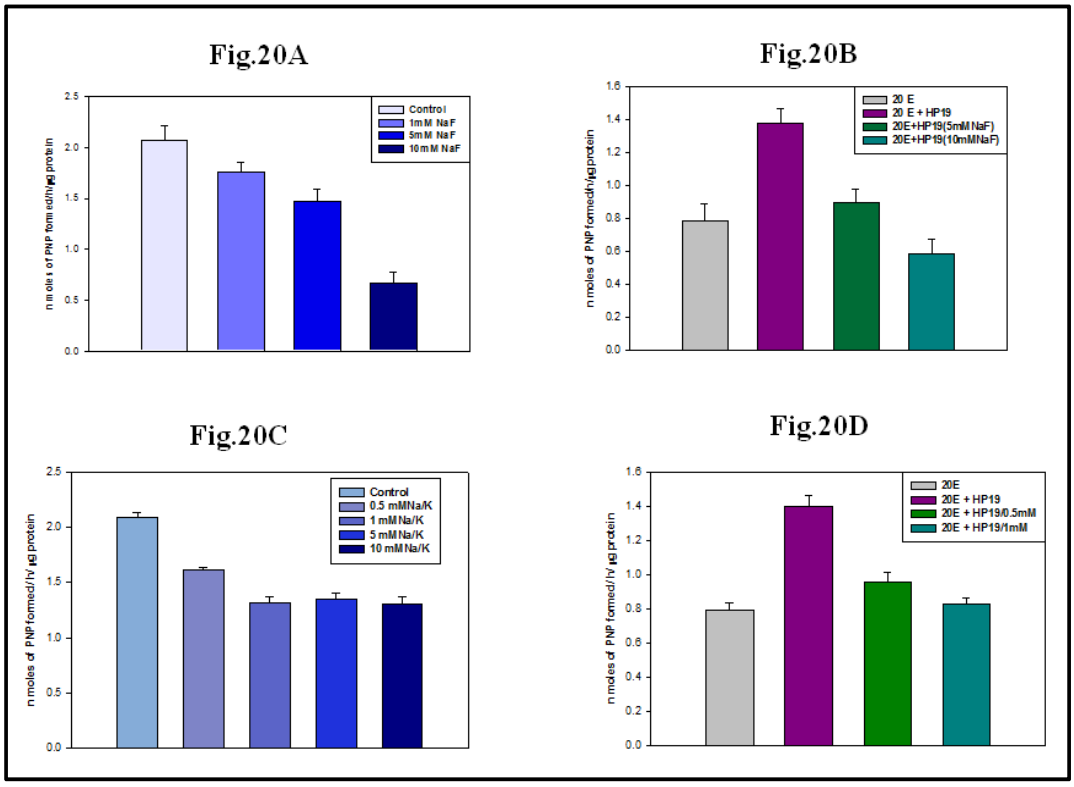


Fig. 20: Effect of phosphatase inhibitors on HP19 mediated 20E induced ACP activity in LLI fat body cultures. A&C. Effect of different concentrations of NaF and Na/K tartarate on ACP activity note at 10 mM all most 50% of ACP activity was inhibited whereas Na/K tartarate (1 mM) able to inhibit only 40% of ACP activity .

B&D. Effect of NaF and Na/K tartarate on 20E induced HP19 ACP activity in LLI fat body cultures. Note induction of ACP was reduced in concentration dependent manner in presence of both inhibitors.

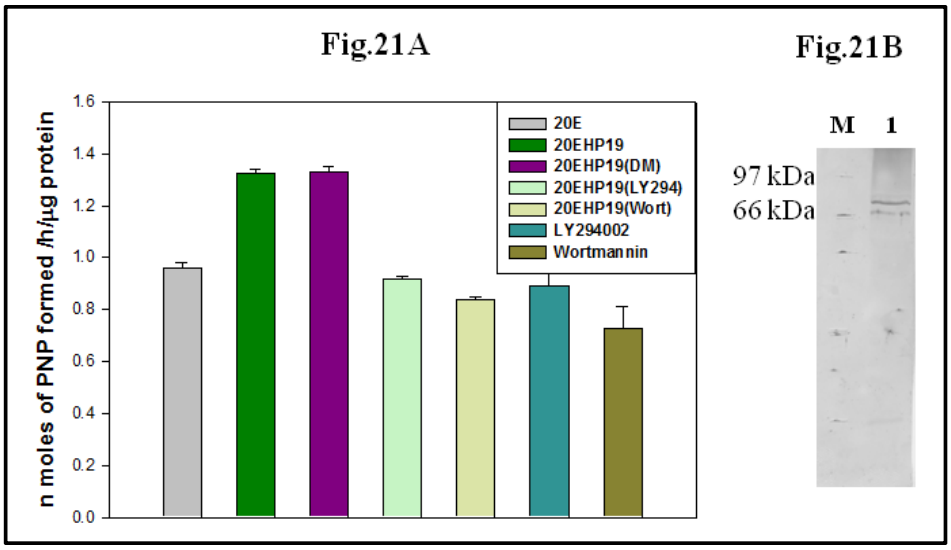


Fig. 21: A. Effect of PI-3 Kinase inhibitors on HP19 20E induced ACP activity. PI-3 kinase inhibitors LY294 and Wortmannin were able to inhibit the 20E induced ACP activity in LLI larval fat body cultures. Whereas alone they did not show any change in the ACP activity.

B. Identification of HP19 binding proteins in LLI fat body membrane. Fat body membrane fraction was separated on 12% SDS-PAGE and transferred to nitro cellulose membrane and ligand blotting was carried out in far western method using HP19 as a ligand. Note presence of two proteins at the range of 70 kDa and 68 kDa.

Results- Chapter III

Background-

Recombinant expression and purification from putative HP19 cDNA (Arif et al., 2004) produced a 23 kDa peptide, which could not potentiate 20E mediated lysosomal ACP activity. Hence in this chapter detailed study was carried out on the profile and function of this 23 kDa protein during postembryonic development of *C.cephalonica.*

Tissue specific synthesis of HP23 protein-

Putative HP19 cDNA clone isolated using immunoscreening, upon expression in bacterial system produced a 23 kDa protein which did not show HP19 activity. After clone was isolated from the larval fat body expression library it was thought worthwhile to study the profile/function of this protein in *Corcyra* development. Antibodies were raised against purified recombinant protein and antibodies were used for further studies. Western analysis of proteins from different tissues revealed the presence of HP23 protein in the visceral fat body (VFB), hind gut associated fat body (HGLFB), and haemolymph of larvae although quantity of the protein differed in different tissue as shown in figure 22A. Protein profiling studies along with the presence of two putative N-glycosylation sites clearly suggest that HP23 is expressed in the fat body and secreted in to the haemolymph during the larval development.

Developmental regulation of HP23-

Developmental profile using western analysis clearly shows that the HP23 levels of protein in the fat body during the last instar larval and prepupal stages more are less the same (Fig. 22B).While in haemolymph it was slightly high ELI larvae stage (Fig. 22C).

Developmental regulation of HP23 mRNA expression-

Northern analysis was carried out to analyse *HP23* gene transcription at various developmental stages. RNA from different developmental stages (1^{st}, 2^{nd}, 3^{rd}, 4^{th} and 5^{th} [ELI, MLI and LLI] instars) was separated on 1% formaldehyde gel and probed with radiolabeled HP23. Interestingly northern analysis revealed presence of two different size transcripts, and their sizes were 2.5 kb and 700 bp. Northern profile presented in figure 23A shows a differential pattern of *HP23* gene expression during the larval development. Both the transcripts were observed in high level in 1^{st} and 3^{rd} instar larval stages lane 1 and 3; whereas low mRNA levels were observed at other developmental stages when compared to earlier stages (Lanes 4, 5 and 6). Furthermore 700 bp fragment was not seen during the later stages of the development. After observing high HP23 transcript levels at earlier stages of larval development, we were curious to check the protein levels, during these stages of larval development. Western analysis (Fig. 23B) clearly shows that HP23 protein level found during 1^{st} to 4^{th} instar was fairly low when compared with 5^{th} instar larval stage. Even after loading 40 μg of protein per well (right panel) the detected signal was fairly faint. This clearly

suggests probably post transcriptional mechanisms regulate the HP23 translation as well as its titer during post-embryonic development.

Southern analysis of HP23 gene-

Southern analysis of larval whole body genomic DNA digested with EcoRI or HinfI, which was probed with HP23 cDNA revealed that HP23 is probably single copy gene (Fig. 23C).

Isolation of HP23 genomic clone and 5′ upstream sequences-

Results obtained from northern analysis of HP23 expression prompted us to check its genomic clone and 5′ upstream sequences as 588 bp HP23 cDNA probe detected two distinct 700bp and 2.5 kb transcript in blot. Larval genomic DNA was isolated and used as template for genomic DNA PCR. A fragment of 1390 bp was isolated along with another 500 bp fragment (Fig. 24A), which was later discarded upon sequencing as it turned out to be a nonspecific amplification. Sequencing of 1390 bp fragment has revealed the presence 4 exons (blue) and 3 introns (black) as shown in figure 25. HP23 gene has conserved intronic and exonic boundaries (GT and AG) which are shown in bold letters. 5′ upstream sequence was isolated by genome walking strategy entire strategy was outlined in figure 26. PCR with 3 digested libraries yielded 2 specific amplicons in EcoRV and Puv II, whereas no amplification was observed in Dra I library as shown in (Fig. 24B). Amplicons were cloned in TA vector and sequenced (1108 bp). *In silico* analysis of the sequence for transcription factor binding sites using fruit fly database (www.fruitfly.org/cgi-bin/seq_tools/promoter.pl) revealed many putative TF binding sites (Fig. 27). Most importantly it has binding sites for transcription factors like *hunchback* and *biocod*. Interestingly it lacks TATA box, but has a CAAT box.

Role of HP23 during postembryonic development of C. cephalonica-

After characterizing the clone up to molecular level; for gaining greater insight into the role of HP23 during the postembryonic development of *C. cephalonica*, the effect of anti-HP23 IgG fraction injection to the last instar larvae was studied. Under such circumstances due to neutralization of protein the physiological functions of the HP23 protein will be blocked/ suppressed causing which will affect the development. The mortality was significantly higher in HP23 IgG injected insects and nearly 93% of the experimental insects

died within 5 days. Whereas in controls it was only 20% mortality. In addition to this significant morphological as well as biochemical differences were observed between experimental and control insects. The larvae which were received HP23 IgG injection either died as larvae (Fig. 28Bi) or become nonviable larval pupal intermediates (Fig. 28Bii) or nonviable pupal adult intermediates (Fig. 28B iii, iv and v) on the other hand the control insects which were injected with preimmunesera/PBS underwent normal development and emerged into adults (Fig. 28A). Further analysis of various anatomical parameters revealed significant differences between HP23 IgG injected experimental insects and controls. And they are presented in table1.

Effect of HP23 Ig G injection in C. cephalonica larvae on silk secretion-

Details of larval culture after HP23 IgG and preimmunesera injection is presented in figure 29. Amount of slik accumulated in the culture plates was fairly high in HP23 IgG injected larvae (Fig. 29B and C) when compared with preimmune IgG injected larvae (Fig. 29A). This observation prompted us to analyze salivary gland in the HP23 IgG injected larvae. The results presented in figure 30 A and B clearly show that the size of the salivary gland increased in experimental insects when compared with control insects. Further the epithelial cell morphology in duct portion was fairly different in experimental insects and resembled with degenerating salivary gland. Analysis of silk protein transcript levels in the salivary gland (Fig. 30C) revealed increase in proposition of L fibroin gene transcript (lane1) in HP23 IgG injected insects. However, there is no alteration in P protein transcript level (lane3).

Various effects of neutralization of HP23 by HP23 IgG injection in last instar larvae:

(a) Effect of haemolymph from HP23 IgG insect larvae on ACP potentiation activity- In above section the results clearly show that injection of HP23 IgG blocks normal development and causes precautious metamorphic changes. Clearly suggesting that the effect at molecular level orchestrated by metamorphic hormones 20E and JH. Hence we looked into whether there was any alteration in haemolymph 20E levels of experimental insects. As our group has already demonstrated convincingly that 20E induces ACP activity in the larval fat body of *Corcyra* in *in vivo* as well as *in vitro* conditions (Ashok and Dutta-Gupta, 1988), we checked the effect of haemolymph drown from HP23 IgG injected larvae on LLI larvae fat body cultures. The larval haemolymph collected after 72 h of HP23 IgG injection larvae

significantly induced the ACP activity, whereas preimmune IgG injected larval haemolymph failed to do same (Fig. 31). This experiment further suggested that neutralization of HP23 in the haemolymph probably causes an increase in the ecdysteroid levels, hence the haemolymph from these insects could mimic the 20E effects shown in earlier work from our laboratory.

(b) Effect on fat body protein profile and phosphorylation-Comparison of the fat body protein profile from larvae, which had received HP23 IgG injection with appropriate control, indicated that after 96 h of injection hexamerin content in that fat body increased significantly when compared with control (Fig. 32A). This is probably due to sequestration of hexamerins as earlier studies from our laboratory have already demonstrated that during late larval-prepupal development of *Corcyra* which is regulated by ecdysteroids (Kiran Kumar et al., 1998; Arif et al., 2003). The above observation provides further support to our assumption that there is an increase in ecdystroid levels in HP23 neutralized larvae. For further confirmation of this *in vitro* phosphorylation of fat body proteins from HP23 neutralized insects was carried out. The degree of phosphorylation of several proteins increased significantly after HP23 IgG injection when compared with controls. Further the increase in phosphorylation was time dependent and the proteins showed increased phosphorylation were hexamerin receptor (120 kDa), CaM Kinase (59/60 kDa) and 35 kDa proteins (Fig. 32B).

(c) Effect on haemolymph ecdysteroid levels-Finally to confirm that the HP23 neutralized larvae have altered ecdysteroid levels, the ecdysteroids in haemolymph was measured, using HPLC (reff). Initially standard graph was plotted using different concentrations of 20E (Fig. 33). Equal volume heamolymph was collected at different time points after injection from both controls and HP23 IgG injected insects. Ecdystiroids were extracted and separated using HPLC. Chromatograms show significant difference between experimental and controls insects (Fig. 34), and after 72 and 96 h HP23 IgG injection on the titer of hormone in haemolymph increased significantly (Fig. 34IV and VI) when compared with respective controls.

Presence of HP23 in other lepidopteran insects-

As Hp23 was shown to play important role during development, a detailed analysis was carried to find out its presence in other Lepidopteran insects using PCR amplification. Results presented in figure. 35A clearly suggested that apart from *Corcyra, Spodoptera*

litura, Achaea janata and *Samia recina* fat body also expressed the HP23 gene. Sequencing partial cDNAs amplicons from these insects showed nearly 98% of similarity at protein level. Clust LW alignment of deduced amino acid sequences (Fig. 35B) revealed 98% sequence identity. The major difference seen between CcHP23 and Sr, Sp and Aj HP23 wasPLY (27-29) sequence at N-terminal end replaced with RSN.

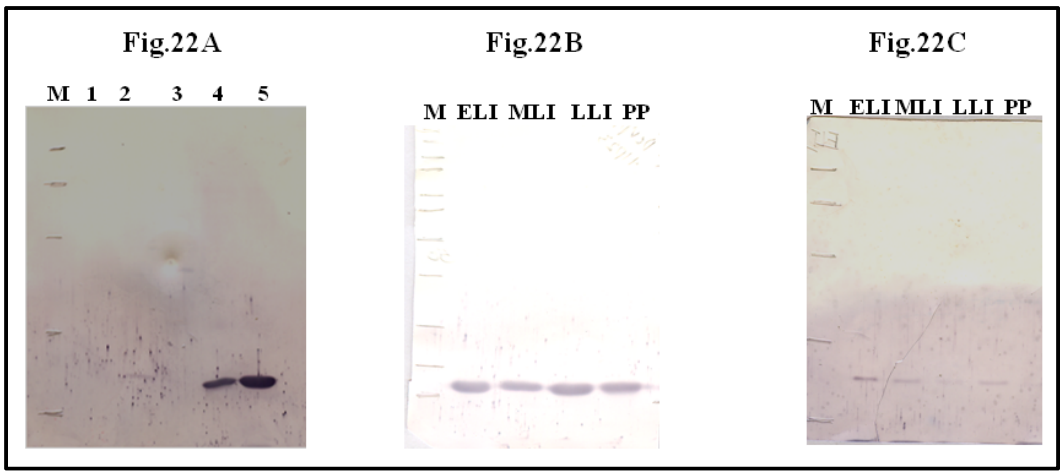


Fig. 22: A. Tissue specific expression of HP23 protein: Western analysis of HP23 protein expression in different tissues of *C. cephalonica*, 20 µg of protein was loaded on each lane. Specific cross-reactivity was found only in haemolymph (lane 2), hindgut associated lobular fat body (lane 4) and visceral fat body (lane 5). Note there is no cross-reaction in salivary gland and gut (lanes 1and 3).

B. Fat body developmental profile of HP23 protein expression (Western analysis): Visceral fat body from different developmental stages of take for this study. 20 µg of fat body homogenate was loaded on the 12% gel. HP23 Protein levels are all most similar in all the stages except in MLI stage where it is bit lower.

C. Haemolymph developmental profile of HP23 protein (Western analysis): 20 µg of haemolymph was taken for this western analysis. Amount of protein at ELI haemolymph is high when compared to other stages whereas at LLI stage protein levels are low.

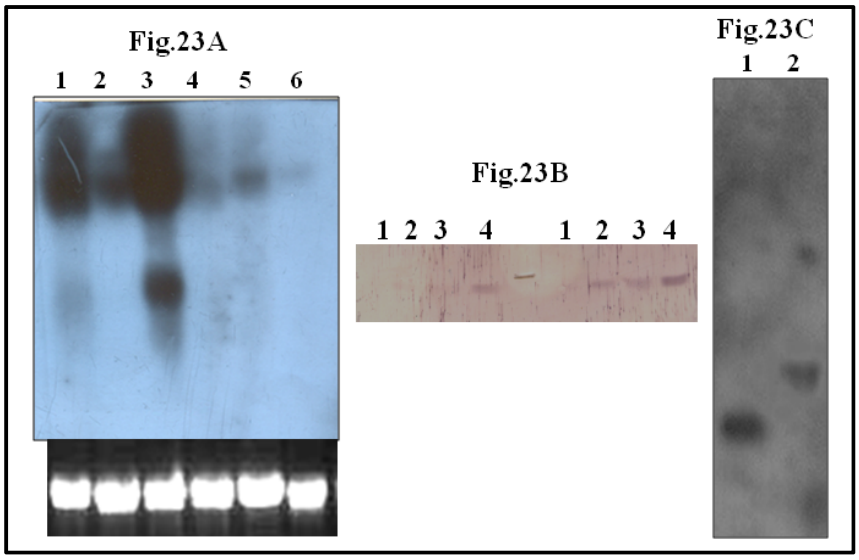


Fig. 23: Molecular characterization of HP23: A Developmental expression of HP23 transcript in *C. cephalonica*. Northern blot showing transcript levels in different developmental stages from 1st instar to 5th instar larvae. High level of transcript was observed during earlier stages of the development at 1st and 3rd instar larval stages (lanes 1 and 3) whereas it was low at later stages (4th and 5th [ELI and MLI] stages). Note the presence of second 700 bp transcript in 1st and 3rd instar larval stages.

B. HP23 protein levels at earlier stages of the development. As we have observed high transcript levels at earlier stages of the development western analysis of the whole body homogenate from 1st instar to 4th instar larval stages was carried out. For this 20 µg of protein was loaded in left panel where as 40 µg of protein was loaded in right panel. Note no protein was detected in 1st instar larvae (lane 1) whereas protein levels increased from 2nd to 4th instar larval stages (lanes 2-4).

C. Southern blot analysis showing that HP23 is a probably a single copy gene (DNA from total larval body was digested with EcoRI or Hinf I lanes 1 and 2 respectively).

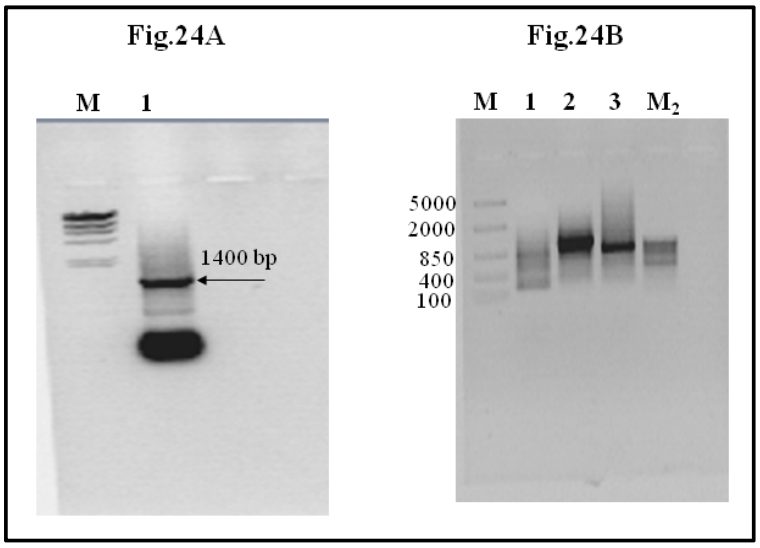


Fig. 24: A. Amplification of HP23 genomic clone: PCR amplification of HP23 gene was carried out using 20 ng of genomic DNA as a template. It's resulted in 2 bands at the range of 1400 bp and 700 bp. Later upon sequencing 700 bp amplification was found to be a nonspecific amplification.

B. PCR amplification of Genomic DNA libraries: 1% agarose gel showing amplification of 5′ upstream sequence of HP23 gene; no amplification was observed in Dra I (lane 1); amplification of ~ 900 bp fragment in EcoR V (lane 2) and ~ 800 bp yielded in Puv II (lane 3) digested library was seen.

Fig.25

CGGGATCATGTGGAACGGTTTAGCCGAACCTATTAGGTACATTTTGCATTACGCCGGAGAGAA
GTTTGAAGATATCAGATACGAACGCTCTAACTGGCCTATCAAAAATGTGAAAGATT**GT**AAGTA
GTAATTAATTATGCTACCCACTGAAAACATCTTTTTTTATTCTGTTTATCAATGCATTCATAATTT
GAACGGATTTAAGTCTAGCGGAAAAAATGTAACTTTAGAAACCTCTGTTATAACATGTTTTAAA
AATTAGCAGAACGATTGAATCTTATTTTCGATCCGATTTTGCCTTAAACTTTCGAATCTGGAGTA
AATTGAGTAGCTCAAGTAAAATCACGTAAATAAACACGTTAACAACTTGTATCATCTTTAAAGT
ATACACTTGCCTAAAATTGATGATTTTATTTTTTTGGTCAC**AG**CTTTGCCGTATGGTCAATTGCC
TCTGTACGAGGATGGAAACCGTACCTTATACCAGTCCCTGGCTATCGCTCGTTATCTTGCTGCG
AAATACGACCTCCTTCCTTCAGACATTTGGGAACAGGCTGTTTTGGATGCTATCGTTTTCACAA
TTTACGACTTCTTTTACA**GT**AAGTGGAAGTAGATGCAGCTTAAAATTCTAAATCTATTAAAATT
TTCTATTTTATTTAATTATTACAAATCTGTTTAATTAAATAGTATTTTAACATTTTCATTAGCTGTT
TCATATAACAATTGAACGTTATTTTAGATATTCAGGCATTATTAACCTTAAACAAAGTGATAATC
TCAAAGGGCAAAGTTACTCTACAACTATGCGATGGAACATGCTAAGCTGTTGTGTTTGGACTG
TATTCATTTTCTATAGAGTTCCTTCAATTGCCGAAATTCATTTAAGGAAAGTAATCCCTATAATTT
TTACAACATATTCTTATATTATTTC**AG**AGGTTTTACCATACGTCCGGGAAGAAGACTCCGTGAA
AAAGCAACAGTATAAAGAAGAATTTCTAAACGAGACTGCCCCTTTCTATTTATCTAGATTTGAA
AAGGAGCTTAAAAATAATAATGGATACTTTGGTGGGAAG**GT**GAAAAATATATATAATATTTAAT
CATAATAATAAACTAGTTTCTAACGAATGAATAATTTTAACATTGTAGTTATTACTTCAGACCATA
TCTCTCTCAGGGATAATGATTTTTGTACTATCTTTTTTATCTTAATTTATTTCTTTTACAG**TTGAGC**
TGGGCCGATTTCGTTCTCGTCGGCATCGTGGAGTCTTTCGATCTCTTCCTTAATACTGAAGTGG
AGACAAGTTATCCCACAATCGTTACCCTATTGAACAGAGTGCGGTCACTGCCGGGTGTCAAGG
CATACATCGCTACCAGAAAACCGTTTTCGTTTTAA

Fig. 25: Gene sequence of HP23 introns are shown in black color and exons are shown in blue color. Intronic and exonic boundaries are shown in bold letters.

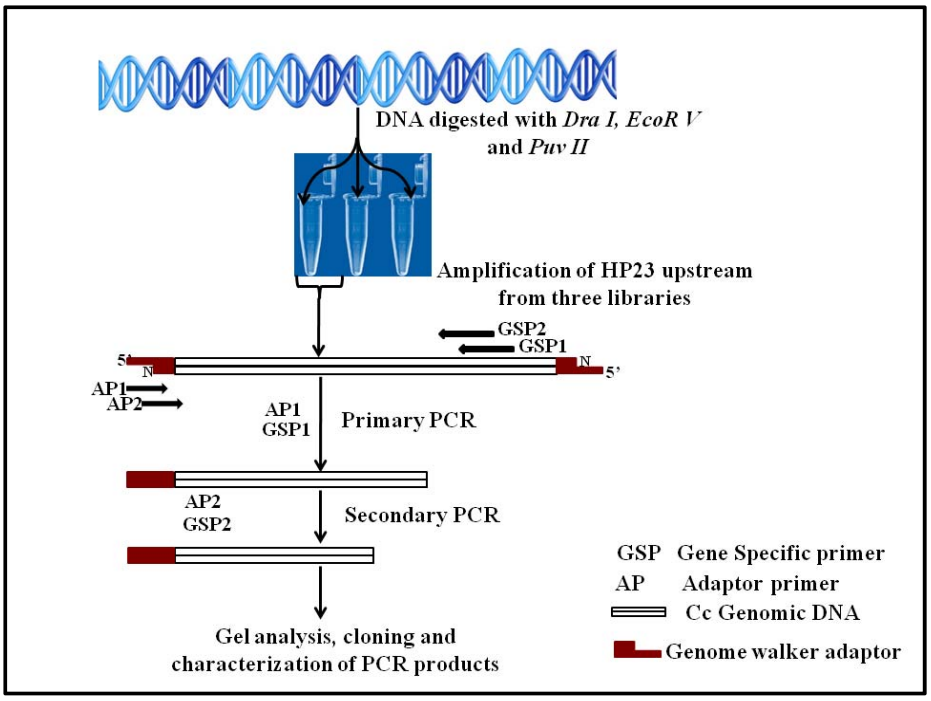


Fig. 26: Genome walking strategy for isolation of 5` upstream sequence of HP23 gene

Fig.27

TTATCCCCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGAGCAAGCTAAAGTGGCAAT
GGGCCGTCCTGGCGAGTCGGCGAAGACAGGTGGGAGAGTAAAGTTTTGGACCGGAGTGGTT
TGGATCGCATATGAGACGATAAAGCATTAGCGCATGCTCTGTTCAAGTGGACCGATTACTCTTA
ACATGCAGCCAGTTGTGTCTCGATGCGCATTGGATAAGGCTTACAGATATAACAATATGCAATC
TACAGCAGTAAGCAATCTAAAAAGCTTATTCTTCTTGTGCATGCGTTTTTTGCTTCTGTGTCAA
GTGACCCGAGATCTCTTAACATGCAGCCAGTTGGGTTCTTCAATGCGCNTTGGAATAAGGGTC
TACAAGAAATAAACAAATATGCCATTCTACAGGCAGGTAGGCTATTCTTACAAGGCTTAATTCT
TTCTTGTGGCATGCGTTTTTTTGTTCACCATTAAGGGTATAGATAAATAATTCACATTTACAGAA
ATCTATAAGGTTCATCTAATAGTTTTTAGGCTCATTAACAACTTTCGAGCTAAAGTCAGCCTCAC
CTGCATGCGGTACACCCTGCTAAATAACGGACGAGGCTCTGGCGAACAGTCGCCCATTCGC
CTGCCCAGCGTGGCGACTTATGGGCAACACTCCCCATGCATAGCGCTAGCGAAGGCCTATACC
CAGCAGTGGGTTAATATAGGCTGATAAGAATAAGTTTAATGGTTAGACAGTCATTGTAATAATC
AGCCGAGCATGTAATCCCAAATACCCACGAAGCTGGTGCTTCGTGGCCCAGAGTACACTTTCA
TCATGAATCAACAGATTCGACATGTCTGGCAATTACGATGGAGCGACTGTCGTCTGACCTCCG
CAACCATTAGTAAAGGGAAACCAGACACAATTTTGAACCATGACGGTTAGACAGTCATCTACA
ACGTCGTTATGAACAGATAACATCGTTTAGTACCTAGTTATACATCTAAAATAAGCAATGCAGTA
TACAAATGAACTTGTTGAATTTGCTATCTGCCTTGATCTACTGAATGTGGAACGGTTTAGCCGA
AGCCCTAATTCGATATAGCAATGCTTAAAA

Bicoid
Hunchback
CAAT box
Putative transcription start site (http://www.fruitfly.org/cgi-bin/seq_tools/promoter.pl)

Fig. 27: 5` upstream sequence of HP23 gene showing putative binding sites for hunchback (green), bicoid (dark saffron), CAAT box (yellow) and putative transcription site (blue) were identified by *in silico* analysis.

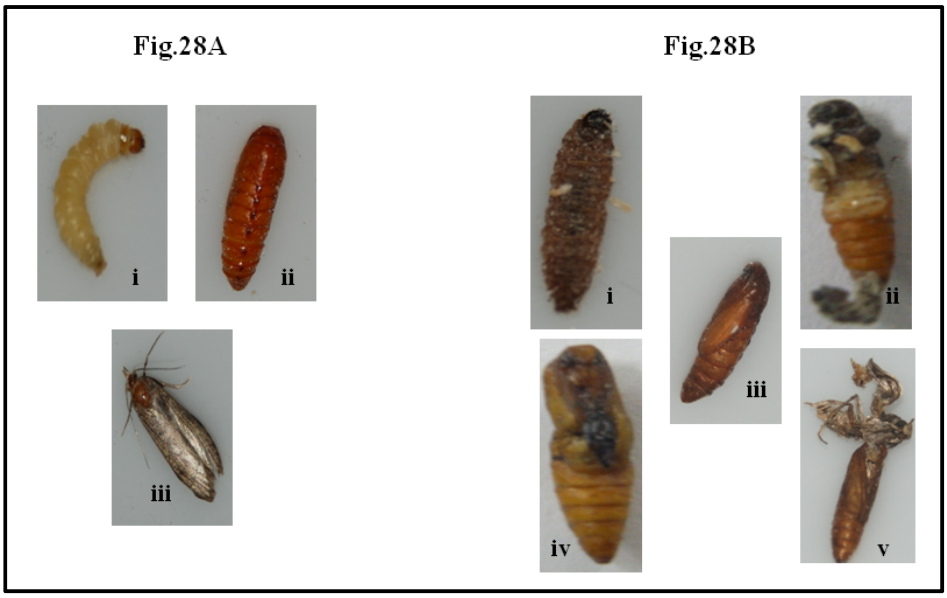


Fig. 28: Effect of HP23 IgG fraction injection to 5th instar larvae of *C. cephalonica*: The antibody injected larvae were allowed to grow in culture room along with the control groups (for details refer materials and methods chapter). Note the abnormal development of antibody injected larvae (Fig. 28B i. defective dead larvae, ii. larval pupal intermediate, iii, iv and v are pupal-audult intermediates) as compared to the control larvae (Fig. 28A)

Table 1

Controls (Pre-immune IgG fraction injected larvae/normal larvae/ PBS injected larvae)	**Experimental** (HP23 IgG injected larvae)
• 20 % mortality • Normal silk secretion • Normal salivary gland • Normal Gut and Malpighian tubule • Normal fat body • Emergence of adults in controls after 21-23 days of injection	• 93% mortality • Excessive precocious silk secretion • Enlargement of salivary gland • Purged gut and uric acid formation in Malpighian tubule suggesting metamorphic transformation • Transformation of fat body into pre-pupal fat body after 4 days of injection • Majority of them died either as larval/larval- pupal /pupal- adult intermediates no emergence of adults

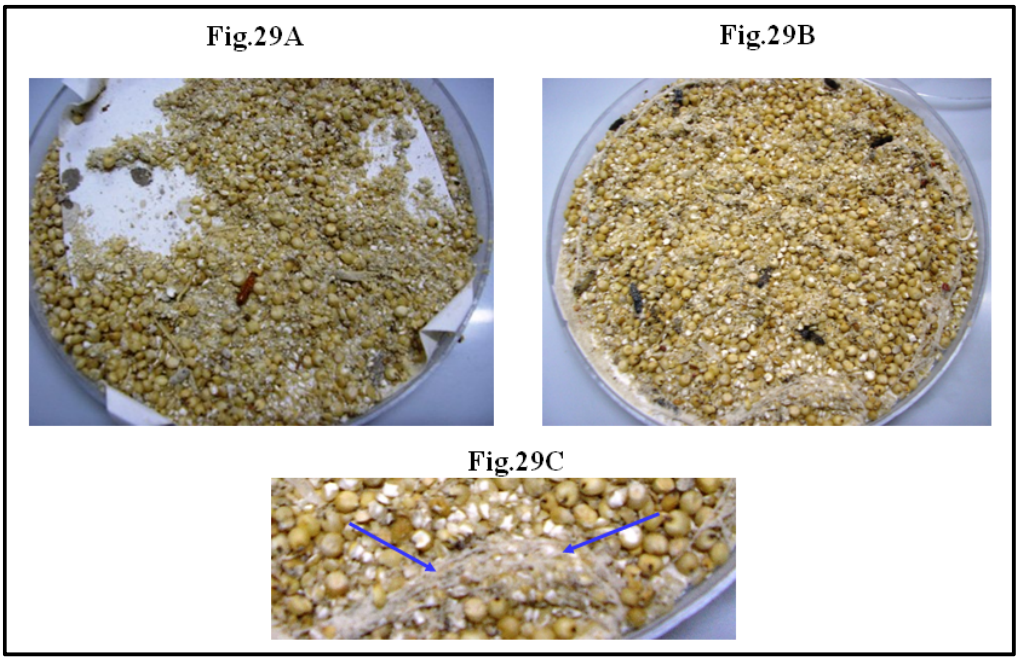


Fig. 29: Cultures of control and HP23 IgG injected larvae:
A. Control culture showing normal morphology with normal insects.

B. HP23 IgG injected culture; larvae had reduced mobility and most of the larvae died after 5 days post-injection. Note excessive silk secretion and its accumulation in (→) the experimental culture disk.

C. Close up view of silk in the plate.

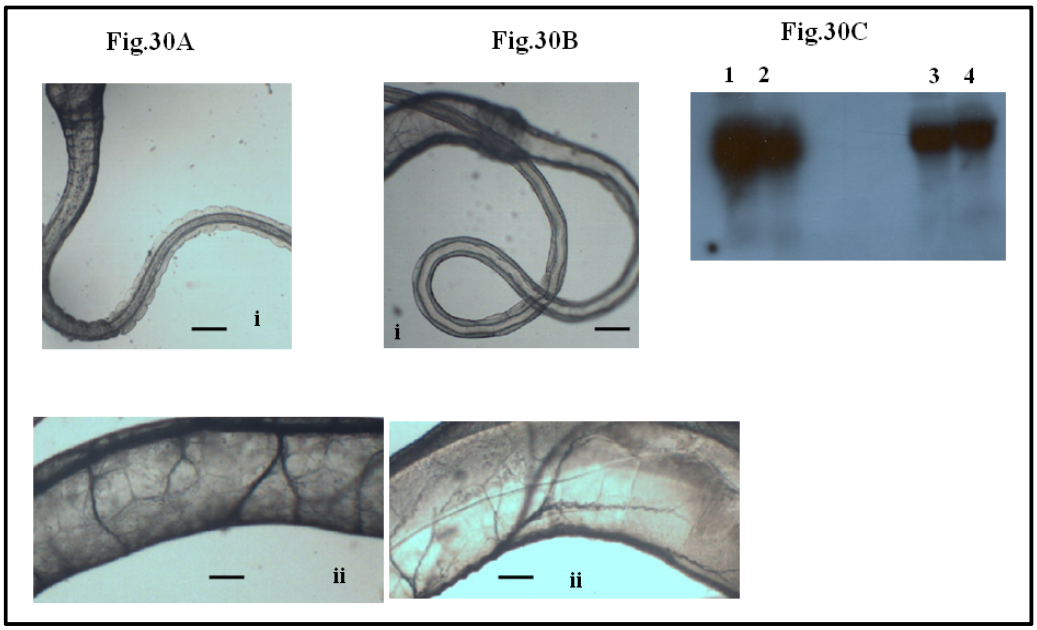


Fig. 30: Effect of HP23 IgG injection of on salivary gland morphology and silk transcript levels:

A. Micrograph of salivary gland from control insect with normal duct morphology (i) and secretory region

B. Micrograph of salivary gland from HP23 IgG injected larvae with deformed epithelial cell morphology at duct region (i) and enlarged secretory region.

C. Note the Northern analysis of fibroin L and P protein transcript levels in control and experimental insects increase in fibroin L levels in HP23 IgG injected larvae (lane1) whereas there was no change in P protein transcript levels (lane4).

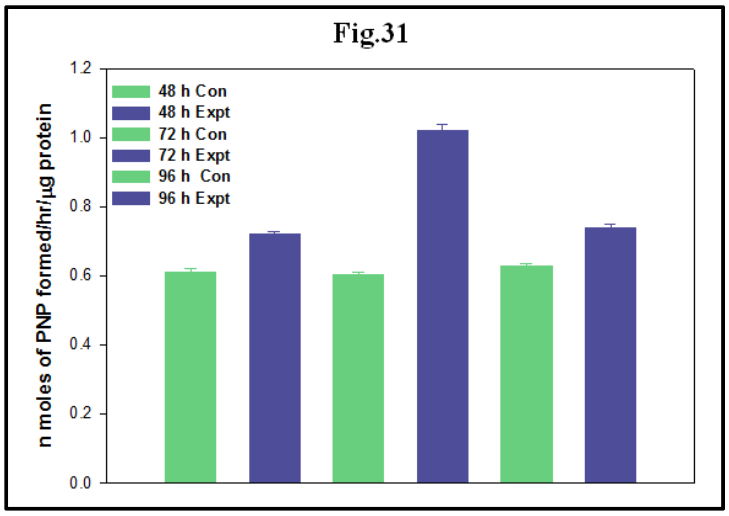


Fig. 31: Effect of HP23 IgG injection on the ability haemolymph to stimulate ACP activity in *in vitro* cultured fat body: Haemolymph was collected at different time points from control and HP23 IgG injected larvae. Equal volume of haemolymph was added in *in vitro* cultured fat bodies which were further incubated for 4 h at 28^{o}C. After incubation tissue was washed with insect Ringer, homogenized and equal quantity of protein was used for ACP assay. Note that the haemolymph from 72 h HP23 IgG injected larvae was able to simulate ACP activity in *in vitro* culture fat bodies when compared to control insect haemolymph.

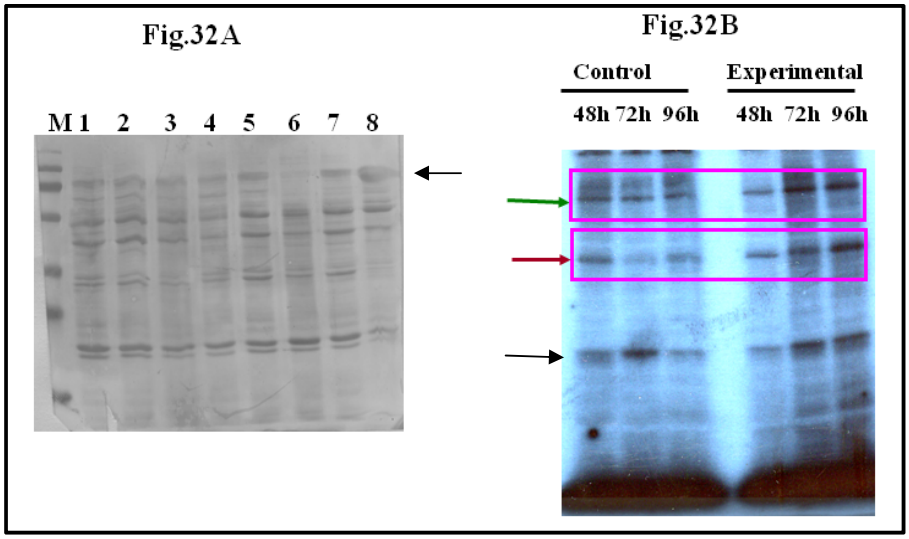


Fig. 32: Effect of HP23 IgG injection on fat body protein profile and protein phosphorylation.

A. Fat body protein profile of control and HP23 IgG injected larvae: Lanes 1, 3, 5 and 7 are fat body from 24, 48, 72 and 96 h controls respectively. Lanes 2, 4, 6, 8 are fat body from HP23 IgG injected larvae. Note the presence of higher hexamerin content in 96 h experimental insect (arrow head).

B. Phosphorylation profile of fat body from control and experimental insects at different time points (48, 72 and 96 h). Note increase in the phosporylation status of various proteins in experimental panel, specifically at 120 kDa hexamerin receptor (green arrow) 60 kDa CaM kinase II(red arrow) and 35 kDa protein.

Fig.33

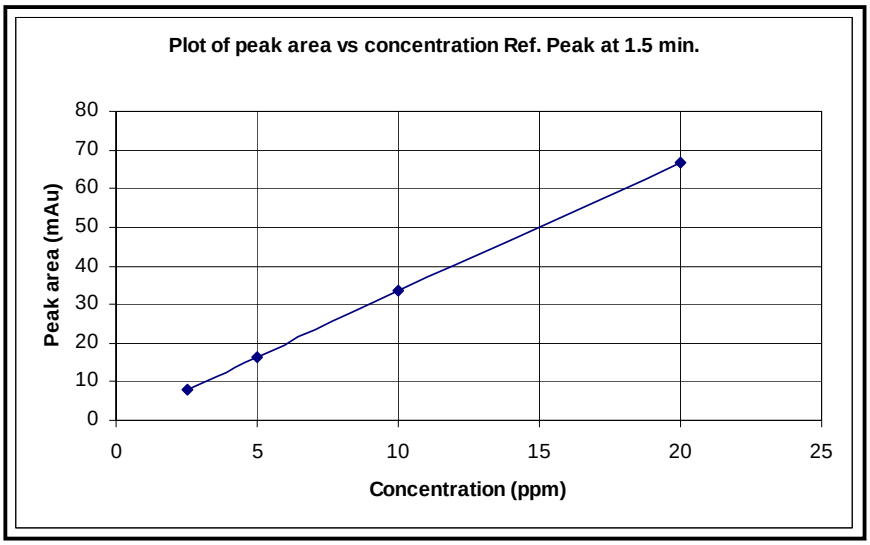


Fig. 33: Standard graph showing different 20-hydroxyecdysone concentrations vs peak area chromatogram.

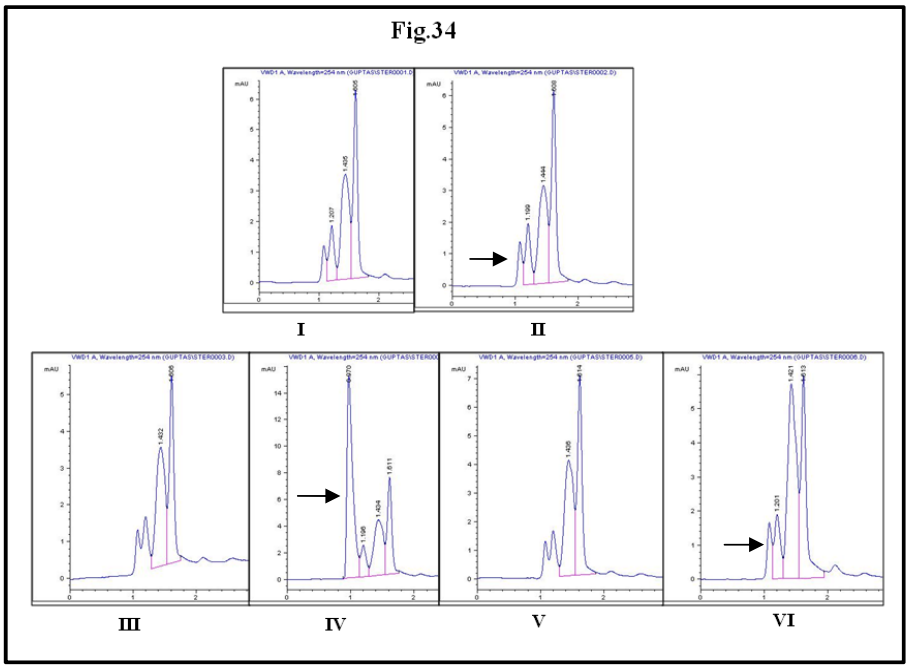
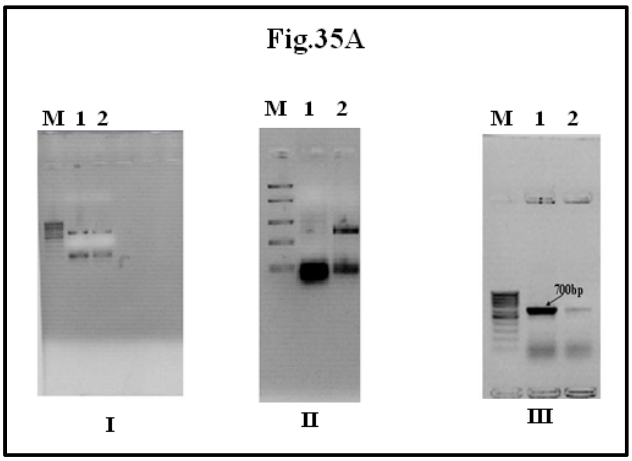


Fig. 34: Haemolymph ecdysteroid levels in HP23 IgG injected larvae: Ecdysteroids were isolated from haemolymph collected after different time points (48-96 h) and analyzed on HPLC at 254 nm. I, III, and V are chromatograms from control insects where as II, IV and VI are from HP23 IgG injected insects. Note the increase in ecdysteroid levels after 72 h (IV) and 96 h (VI) of IgG injection. (→ 20E)



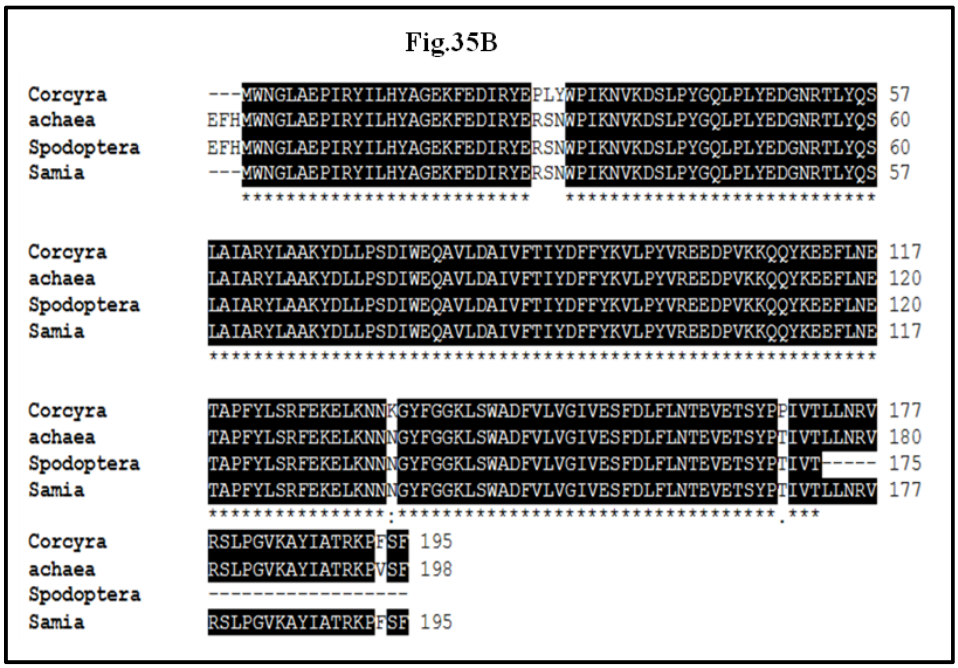


Fig. 35: A. Isolation of HP23 cDNA from other lepidopteran insects: I PCR amplification of HP23 cDNA from *Achaea janata* (lanes 1 and 2 are at different Tm.) II PCR amplification of HP23 cDNA from *Spodoptera litura* lane1 (primary amplification and lane 2 secondary amplification). III PCR amplification of HP23 cDNA from *Samia recina*.

B. Clust LW alignment of deduced amino acid sequence from *Corcyra*, *Achaea*, *Spodoptera* and *Samia*. Nearly 98% similarity was seen in all the sequences.

Discussion

The ecdysteroids are synthesized by the prothorasic gland and released into haemolymph of the insect during the postembryonic development. The changing ecdysteroid titer in haemolymph is responsible for eliciting several of the critical sequence of behavioral, physiological and biochemical events ultimately leading to the molting and metamorphosis of insects (Dean et al., 1980; Gilbert et al., 1980a, b; Nijhout, 1994; Riddiford et al., 2001).

The ecdysteroids have wide range of effects on various tissues including the fat body, the major metabolic site in the larval forms of holometabolous insects including lepidoptera (Schenkel and Scheller, 1986; Gilbert et al., 1996; Lee and Baehriecke, 2001; Thummel, 2001; Riddiford et al., 2001; Hansen et al., 2002; Roy et al., 2007). It is well known, that ecdysteroids promote remodeling/degeneration of larval structures during metamorphosis (Trumann, 1996b), either by initiating the formation of autophagic vacuoles for autophagy of group of cells seen in tissues like fat body and gut (Lockshin and Beaulton, 1974; Dean, 1978; Sass and Kovacs, 1975, 1977, 1980) or by apoptosis (the programmed death) of individual cells and salivary gland (Abrams, 1999; Iga et al., 2007). 20-Hydroxyecdysone has been shown to elicit effects on autophagic process of the fat body by stimulating the activity of lysosomal enzymes, such as acid phosphatase (Verkuil, 1979, 1980; Verkuil et al., 1979; Sass and Kovacs, 1980; Ashok and Dutta-Gupta, 1988; Sass et al., 1989). Extensive studies from our group have shown that ecdysteroids stimulate the synthesis hexamerins by the fat body cells (Ismail and Dutta-Gupta, 1990b; Dutta-Gupta and Ismail, 1990b, 1992; KiranKumar et al., 1997, 1998) and male accessory reproductive glands (MARG) (Ismail and Dutta-Gupta, 1990c, 1991; Dutta-Gupta and Ismail, 1992; Ismail et al., 1993 Manohar, 2008). Further, our group has unequivocally demonstrated that ecdysteroids regulate nongenomically not only the the activation of lysosomal acid phosphatase (ACP) in the larval fat body of *Corcyra* (Ahok and Dutta-Gupta, 1988; Arif at al., 2004) but also the uptake of hexamerin during pupal stage by activating hexamerin receptor in fat body through phosporylation (Arif et al., 2003).

Haemolymph in the insects is known to be source of several factors, which do not only regulate ecdysteroid synthesis in response to changing hormone titer but also mediate ecdysteroid dependent action in larvae and pupae (Dutkowski and Oberlander, 1974; Gray et al., 1987; Watson et al., 1987; Shiraishi and Natori, 1989; Csikos and Sass, 1997; Farkas and Sutakova, 1998; Nijhout and Grunert, 2002; Smagghe et al., 2003). These factors are synthesized

by different tissues and released into the haemolymph. Injection of 20E to *Corcyra* larvae stimulated lysosomal ACP activity. However, under *in vitro* condition it failed to stimulate the enzyme activity in fat body organ cultures (Ahok and Dutta-Gupta, 1988), similar observation was also reported in *Manduca sexta* (Caglayan, 1990). Extensive studies from our group revealed that 20E needed a haemolymph peptide (HP19) synthesized by hindgut associated lobular fat body to bring its effect (Arif et al., 2004). In *Drosophila*, *in vivo* injection of 20E to the last instar larvae caused alterations in the ultra-structure of the salivary gland finally, leading to its degeneration. However, 20E could not promote the same effect on the glands under *in vitro* conditions and lack of factors present in the haemolymph was cited as a reason for the incomplete degeneration under in vitro conditions (Farkas and Sutakova, 1998). Nijhout and Grunert (2002), demonstrated the presence of a bombyxin like heat labile protein in the haemolymph of butterfly *Precis coenia*, required for the normal growth of imaginal disks under cultured condition in presence of 20E. They also suggested that this protein acts in combination with 20E to stimulate cell division and growth of wing imaginal discs. Under *in vitro* culture studies with lepidopteran imaginal disks, a factor from fat body was shown to promote 20E dependent deposition of cuticle (Dutkowski and Oberlander, 1974). Furthermore, Smagghe et al., (2003) reported a possible interaction of a factor present in the fat body extracts from *M. sexta* with hormones like ecdysone, 20E as well as non-steroidal ecdysteroid agonist RH-2485 and their role in the regulation of the development and metamorphosis of the insect mid gut. Recently, Blackburn et al., (2004) demonstrated the stimulation of mitosis in *in vitro* cultured mid gut stem cells of *H. virescens* by the extracts from perivisceral fat body of freshly ecdysed *M. sexta* pupae. Several other studies also reported the presence of factor(s) in the haemolymph, (Stall, 1982; Meola and Gray, 1984; Watson et al., 1985, 1987, 1988; Gray et al., 1987; Shiraishi and Natori, 1989). All these observations suggested about the involvement of additional factors in the ecdysteroid mediated action as well as regulation of ecdysteriod synthesis during the postembryonic development of holometablous insects.

As a part of cell remodeling during metamorphosis, acidic autophagic vacuoles accumulate in the fat body cell and the activity of several lysosomal enzymes including ACP increase and cause the lysis of larval tissues (Sass and Kovacs, 1980; Verkuil, 1980; Thummel, 2001; Lee and Baehriecke, 2001). The fat body that fills a large fraction of the insect body cavity shows high activity of lysosomal enzymes (Hansen et al., 2002). The lysosomal enzymes play an

important role in histolysis of larval organs, cellular destruction, tissue remodeling, and reorganization. The stimulation of the lysosomal activity by ecdysteroids is well demonstrated in several insects including *C. cephalonica* and the increase in the lysosomal activity is governed by the elevation of 20E levels (Verkuil, et al., 1979; Verkuil, 1980; Ashok and Dutta-Gupta, 1988; Sass et al., 1989; Kutuzova et al., 1991). Verkuil (1979b) provided preliminary evidence for the induction of lysosomal activity by ecdysteroids and suggested that it is governed at a nongenomic level. Further more extensive studies from our laboratory demonstrated that the induction in lysosomal activity by ecdysteroids is governed at nongenomic level and mediated by haemolymph protein of 19 kDa (HP19) (Arif et al., 2004). Molecular characterization of this protein revealed its homology (65%) with *Choristineura fumiferana* fat body glutathione S-transferases (GSTs) (Arif et al., 2004). Animal GSTs with hormone regulating actions are unknown, although few studies in vertebrate GSTs speculate about their steroid binding properties or their developmental and hormonal regulation (Maruyama and Irving, 1984; Hatayama et al., 1986; Staffas et al., 1998; Sheehan et al., 2001).

In the present study we characterized the CcGST from fat body of *C. cephalonica* in order to understand its role in 20E mediated nongenomic action during the postembryonic development. As GST from this insect as well as from any other stored grain pest was not reported earlier, we carried out optimization for enzyme activity that intriguingly revealed optimal activity at pH 8.3. Although most eukaryotic GSTs are known to have optimal activity in the pH range 6.0-6.5, few reports do suggest that GSTs also have considerable activities at higher pH. A recombinant rGST M4 expressed in *E. coli* from a chemically synthesized rGST m4 gene showed optimum pH between 8.0-8.5 for the active gene product (Cheng et al., 2001). Bacterial dichloromethane dehalogenases a member of GST super family has pH optimum at 8.5 (Vuilleumier et al., 1997). In isoprene-utilizing bacterium, *Rhodococcus* sp. strain AD45 the optimal GST activity was unusually between pH 8.5-9.0 and was involved in the GSH-dependent metabolism of epoxides (van Hylckama et al., 1999). Since stored grain lepidopteran pests are known to have alkaline internal environment during development (Harrison, 2001), the GSTs with optimal pH in the alkaline range may have a functional role. Present biochemical and molecular characterization identified the CcGST as a 23 kDa protein encoded by a cDNA containing 701-nucleotide. Isolated cDNA from *Corcyra* fat body showed high homology with the GSTs of *Galleria* and *Bombyx*. Based on multiple sequence alignment and structural

alignment, the CcGST falls in the delta-class of the GST super family. The affinities for CDNB and GSH were within the range observed in delta class GSTs of other insect species (Tang and Tu, 1994; Ranson et al., 1997; Udomsinprasert et al., 2005).

There are many examples where homology modeling techniques have supported the drug discovery process especially in the target identification and/or validation, lead identification as well as lead optimization with respect to potency and selectivity. The extent of information derived from the homology model depends on the quality of the model. Since the accuracy of the homology model is related to the degree of sequence identity and similarity between the template and target, template search and sequence alignment is a crucial step in any homology modeling. For this reason extreme caution was taken into consideration, while searching for the template for CcGST and while aligning the sequences. Comparisons of numerous GST amino acid sequences as well as mutagenesis studies and crystal structures analysis led to the identification of highly conserved residues and important residues. Amino acid alignment with previously crystallized delta-class AdGST structure revealed the differences in amino acid residues involved in pocket formation for the binding of GSH in G-site (Chen et al., 2003). Interestingly homology modeling and docking studies showed that in CcGST there is no residue to stabilize the ionized GSH through H-bond formation. Further Ser-13 in the protein is far away from the H-bonding distance to stabilize ionized GSH. However, no difference was observed in H-site residues.

Western analysis with antibody generated against CcGST showed high level of CcGST in the fat body. Increased GST protein content corresponding with its high level of enzymatic activity most likely facilitates the physiological function of the fat body, which is the major metabolic center in insects and also plays important role in detoxification mechanisms as well as provides protection from oxidative stress induced damages. On the other hand, insect gut that is generally exposed to a variety of xenobiotics through food did not show the cross reactivity with antibody raised againest 23 kDa CcGST identified in fat body. However, the gut showed presence of a low molecular weight isoform with CDNB conjugating activity and it faintly cross-reacted with fat body CcGST IgG. This observation clearly suggests that the newly identified fat body CcGST might have roles other than the detoxification of xenobiotics. Though the CcGST protein level increased from ELI larval to prepupal stage, the activity profile revealed an interesting pattern. Activity rose gradually from ELI to MLI larval and was highest at LLI larval

stage, when the larvae feed actively. However, this high activity declined significantly during the non-feeding prepupal stage, where it was even lower than the ELI larval stage. This reduction in activity without any apparent change in protein level indicates possibility of post-translational modification(s), which is involved in the regulation of the GST activity at the prepupal stage without changing the protein level. To get further insight into this developmental regulation of CcGST, we analyzed the effect of hormones on GST activity. In insects, JH and 20E are known to play critical role during their metamorphosis and postembryonic development (Chang, 1993). We observed that 20E had no effect on GST activity or its protein level but JH1 application both *ex vivo* and *in vivo* showed inhibitory effect on GST activity without any alteration in protein level. In the present study, the possibility of GST exhibiting HP19 like function was evaluated by replacement of HP19, with purified fat body CcGST. However, the detailed *in vitro* fat body culture studies did not show any potentiation in 20E stimulated ACP activity, like that of HP19 purified from the haemolymph. Multiple alignment using Clust LW programme of fat body CcGST and HP19 amino acid sequences also revealed that they have less than 25% similarity. Hence the present study clearly indicates, that HP19 and GST from *Corcyra* are different molecules with different functions.

In the next part of the work putative HP19 cDNA (isolated by Arif at al., 2004) was expressed in bacterial system for to get more insight into HP19 protein. Initial expression with His tag (23 kDa) did not yield desired purity of the protein, hence the cDNA was expressed with GST-tag. After obtaining pure protein it was evaluated for its ability to induce ACP, in presence of 20E in the *in vitro* fat body cultures. However to our surprise the recombinant protein failed to mediate the 20E induced ACP activity. As eukaryotic proteins expressed in bacterial system lack proper folding and post-translational modifications (Yang, 2005) we assumed probably the recombinant HP19 lacks appropriate modification, hence it failed to mediate the 20E induction of ACP activity. Before proceeding for expression in eukaryotic expression system as a confirmational check, we checked the crossreactivity of bacterially expressed putative recombinant HP19 with HP19 IgG, (which was generated against HP19 purified from haemolymph by electroelution) and found that it could not detect recombinant protein on western blot. After knowing that cDNA which was isolated by immunoscreening was different from HP19, attempts were made to purify HP19 from LLI larval haemolymph. It was achieved successfully by two step purification protocol. 2-D analysis shows that *C.cephalonica* HP19

protein has four isoforms. Using this purified HP19 we have confirmed that HP19 mediated 20E induction of phosphatase activity is limited to lysosomes only. This was done using different inhibitors like sodium fluoride (general phosphatase) and sodium/potassium tartarate (lysosomal ACP). Further the purified protein was used to dissect, how HP19 and 20E nongenomically activate the ACP activity? Reports from the literature suggest that nongenomic action of 20E is mediated through G-protein coupled receptor via PI3-kinase activation (Deepak et al., 2005). Hence we challenged, 20E induced and HP19 mediated ACP activity with PI-3 kinase Inhibitors (LY294002 and Wortmannin), and the result confirms that the nongemnomic action of 20E in present study is mediated via PI-3 kinase activation. Later ligand blotting studies with fat body membrane, yielded presence of two binding proteins in molecular weight range of 70 kDa and 67 kDa, further substantiate our hypothesis.

In final phase of our work we carried out extensive studies on fat body secretory protein HP23. Antibodies were generated against purified recombinant protein (refer previous section) crossreacted with a protein of 23 kDa in fat body as well as haemolymph. Tissue profile of HP23 protein by western analysis clearly demonstrated that it's predominantly synthesized in the fat body of insect and released into the haemolymph during the postembryonic development. The deduced amino acid sequence from HP23 cDNA did not show a typical signal peptide necessary for transmembrane transport (Von Heijne, 1986, 1994), probably due to the lack of any hydrophobic sequence. The presence of two putative N-glycosylation sites (Asn_{51}-Arg_{52}-Thr_{53}-Leu_{54} and Asn_{116}-Glu_{117}-Thr_{118}-Ala-$_{119}$) indicates that the protein can be secreted from the synthesizing cells. Developmental profile studies reveal that it is expressed during all the stages of the larval development, with slight changes in the protein levels. Haemolymph profile at different 5^{th} instar larval and pre-pupal stages also clearly show its presence at all the stages of the development. Northern analysis of HP23 transcript revealed interesting result; HP23 cDNA probe detected two different transcripts at the range of 2.5 kb and 700 bp. This prompted us to analyze the gene and its size. Genomic PCR revealed that the gene is of 1390 bp, with 4 exons and 3 introns. Cloning and sequencing of 5` upstream sequence (1108 bp) of *HP23* gene by genome walking analysis resulted in identification of putative binding sites for hunchback and biocod transcription factors. Interestingly the upstream fragment lacks the TATA, box it show the presence of CAAT box in it. This analysis gives the answer for the having 2.5 kb fragment in northern analysis, but significance of 700 bp fragment remains elusive at this point.

Further experiments were designed to gain insight in the role of HP23 protein during the growth and development of *C. cephalonica*. The approach adopted was to block the function(s) of the protein with the help of specific antibodies. The use of antibodies to understand the role of a molecule in physiological processes has been demonstrated in several species of invertebrates including insects. Hiraoka and Hayakawa (1990) reported that a monoclonal antibody against apolipophoriin II in *Locusta migratoria* inhibited the diacylglycerol uptake in the fat body. In another study, inoculation of antibodies against β-N-acetylhexosaminidase of the bovine tick, *Boophilus microplus*, resulted in a decreased oviposition (Del Pino et al., 1998). Nijhout and Grunert (2002), showed that specific antibody against bombyxin like protein, completely removed the growth promoting activity in the haemolymph, that is required by 20E to regulate the normal growth of imaginal disks in butterfly *Precis coenia*. Recently our group has successfully demonstrated, the role of a haemolymph regulatory protein HP19 during the postembryonic development of *Corcyra* (Arif et al., 2007), in which blocking of the protein by polyclonal antibody resulted in alteration of biochemical parameters and normal developmental pattern. Keeping the above in view, the specific antibodies were generated against HP23 protein and used in the present study, basically to understand the role of HP23 in *Corcyra* postembryonic development. In the presence of antibody the protein would be immuno-complexed, hence it would not be able to perform its normal physiological function. The results obtained in the present study suggest that the injected antibodies interfered with the physiological action of the protein and caused the 95% mortality in experimental insects, remaining insects developed into either nonviable larval-pupal or pupal-adult intermediates. A detailed analysis on various parameters revealed a significant change in experimental insects when compared with the control insects. The antibody injected larvae showed increased secretion of silk, purged gut and uric acid accumulation in Malpighian tubule and transformation of fat body into pre-pupal fat body after 4 days of HP23 IgG injection. Epitheilal cells in the duct portion of the salivary gland were deformed in HP23 IgG injected insects and the fibrion L level also increased in exprimental insects. All these effects together suggest that there is a premetamorphic transformatiuon in HP23 IgG injected larvae.

As edcdysteroids are known to bring the metamophic changes during the postembryonic development of insects, in the present study we made an intial attempt to evaluate the ecdysteroids induced actions like ability of the haemolymph from HP23 blocked insects to

induce ACP activity in *in vitro* fat body cultures. Results obtained show a signeficant increase in ACP activity in the haemolymph from 72 h of antibody injected insects, further suggest that neutralization of HP23 in the haemolymph probably causes an increase in the ecdysteroid levels. Hence the haemolymph from these insects could mimic the 20E effects shown in earlier work from our laboratory (Ashok and Dutta-Gupta, 1988). Earlier studies suggested that 20E not only exerts its action through the modulation of transcription and translation but also exerts its effect at the post-translational level eg., by protein phosphorylation (Arif et al., 2003). It has been shown to stimulate *in vitro* phosphorylation of few fat body proteins in *M. brassicae* (Sass, 1988). Itoh et al., (1985, 1986), suggested that the 20E regulated phosphorylation of a 30 kDa protein was responsible for the conversion of the fat body from a synthetic to storage organ in *S. peregrina*. Casein kinase II activity in the brain of *Acheta domesticus* was shown to be inhibited by the injection of 20E (Degrelle et al., 1997). In addition our group has shown that autophosphorylation of CaM kinase II in the CNS of *Bombyx mori* (Shanavas et al., 1998) and the phosphorylation of hexamerin receptor in the fat body membranes of *C. cephalonica* (KiranKumar, 1998; Arif et al., 2003) was mediated by ecdysteroids (Arif, 2004; Arif et al., 2008). In the present study phosphorylation status of these two proteins increased significantly after 72 h and 96 in the fat body of HP23 IgG injected insects. Further, the protein profile of fat body revealed that accelerated sequestration of the hexamerins after 92 h of HP23 IgG injection. Hexamerins are high molecular weight multimeric proteins synthesized by actively feeding larval fat body cells and released into haemolymph (KiranKumar et al., 1997; Nagamanju et al., 2003), later they are taken back via a receptor mediated endocytotic process by the non-feeding pre-pupal or pupal fat body cells (Ismail and Dutta-Gupta, 1990; Kiran Kumar et al., 1997; Burmester and Scheller, 1999) which is dependent on elevation of haemolymph ecdysteroid titre. Hence, haemolymph ecdysteroid level was estimated in the HP23 IgG injected larvae using HPLC method. The level of 20E increased after 72 and 96 h of antibody injection, further supports our assumption that blocking of HP23 function at physiological level, causes an increase in the ecdysteroid levels, which in turn responsible is for precocious metamorphic transformation of insects, leading to defective development and mortality. In conclusion the present work demonstrates presence of various proteins/peptides in the larval haemolymph of lepidopteran insects, which play various regulatory role(s) during postembryonic and metamorphic development.

Summary

- *Corcyra* fat body GST has pH optima of 8.3 and tissue profile shows highest activity in visceral fat body of the insect. Purification of the protein by anion exange and GSH affinity chromatography methods yielded it is a 23 kDa protein.

- Kinetic analysis with the affinity purified CcGST with varying concentrations of CDNB and GSH at pH 8.3 to determine the V_{max} and K_m revealed that V_{max} for CDNB is 13.76 μmoles/min/mg and K_m is 0.40 mM while for GSH, V_{max} is 13.36 μmoles/min/mg and Km value is 0.33 mM.

- Western blot analysis with antibody raised against affinity purified CcGST showed high protein level of GST in fat body when compared with other tissues which corroborated with activity profile of GST from different tissues .

- Cloning and sequencing of the cDNA using degenerate primers for lepidopteran specific GSTs was carried out. Cloned cDNA was 701-nucleotide long and the longest ORF encoded protein of 216 amino acid. The CcGST nucleotide sequence discussed here has been submitted to the GenBank with accession number DQ234273.

- Identified CcGST was identified to be found to be related to delta-class GSTs. The amino acid sequence shows 91% identity with *Galleria mellonella*, 86% identity with *Bombyx mori*, 60% identity with *Helicoverpa armigera*, 55% identity with *Anopheles dirus* and 55% identity with *Anopheles gambiae*.

- Southern analysis revealed that CcGST is multiple copy gene and northern analysis shows the presence of two different transcripts with variable expression pattern during larval development.

- 20E has no effect on GST expression and activity but JH1 application, both *ex vivo* and *in vivo*, show inhibitory effect on GST activity after 72 h of application without any alteration in the expression of GST.

- The affinity purified GST is unable to mediate 20E induced ACP activity in fat body cultures which clearly suggests that HP19 and GST are different molecules. Clust LW alignment of deduced amino acid sequence of CcGST and CcHP19 shows less than 20% similarity, further confirming that the GST and HP19 are separate proteins.
- HP19 cDNA which was obtained by immune-screening of fat body expression library was cloned in two different bacterial expression vectors where GST tagged expression system yielded pure protein upon purification by affinity chromatography. However the size of the recombinant protein is 23 kDa.

- The above mentioned recombinant protein (Putative HP19) was failed to mediate 20E induced ACP activity in *in vitro* fat body cultures and did not crossreact with HP19 IgG raised against native HP19 protein. Then fresh attempts were made to purify HP19 from LLI larval haemolymph, using two step purification protocols. 2-D analysis revealed that HP19 protein has four isoforms.

- Inhibitor studies using various inhibitors show that the HP19 mediated 20E induction of phosphatase activity is limited to lysosomes only

- Further the induction of ACP activity by HP19 and 20E is mediated by PI-3 kinase activation at membrane level which is confirmed by different PI-3 kinase inhibitors. Ligand blotting studies with fat body membranes yield two binding proteins within the range of 70 kDa and 67 kDa which interact with HP19.

- Specific IgG was generated against recombinant protein (HP23) expressed in bacterial system for understating its role during postembryonic development of *Corcyra*.

- Tissue profile of HP23 protein by western analysis clearly demonstrated that it is predominantly synthesized in fat body of the insect and released into the haemolymph. Its presence is not detected in gut and salivary gland. HP23 protein is expressed during all the stages of the larval development of *Corcyra* with slight changes in the expression levels. Haemolymph profile at different stages of 5^{th} instar larvae and pre-pupal stages also clearly suggests its presences all the stages of the development.

- Northern analysis revealed presence of two different transcripts for HP23, at the with molecular size of of 2.5 kb and 700 bp. Gene cloning and sequencing revealed that it is 1390 bp in size and it has 4 exons and 3 introns. Cloning and sequencing of 5` upstream sequence (1108 bp) of *HP23* gene resulted in presences of putative binding sites for hunchback and biocoid transcription factors.

- Injection of HP23 antibody to LLI larvae suppressed the physiological action of the protein, probably by interfering with the HP23 molecule and caused 95% mortality at larval stages itself. While the remaining insects showed the defective development and gives raise larval-pupal or pupal-adult intermediates.

- HP23 antibody injection caused alteration in salivary gland morphology, excessive silk secretion which is supported by higher levels of L fibroin transcript levels.

- Haemolymph from HP23 IgG injected larvae could induce 20E mediated acid phosphatase activity in *in vitro* cultured fat body.

- Hexamerin content increased in the fatbody after 96 h of HP23 antibody injection. Further there is an increase in phosphorylation of hexamerin receptor and fat body CaM kinase II after 72 h of antibody injection. The present study further shows that HP23 IgG injection causes an elevation in haemolymph ecdysteroid titre.

- Finally the present study clearly suggests that HP19 and GST from *Corcyra* are different molecules with different function. They have significant difference at molecular level too. 20E induction of acid phosphatase activity mediated by HP19 is related to lysosomal phosphatase activation and it is via PI-3 kinase activation. We have identified another regulatory molecule HP23 from *Corcyra* fat body which is secreted into the haemolymph, and modulates ecdysteroid levels during the postembryonic development and this molecule is not unique to *Corcyra* as it is present in other lepidopterans like *Achaea janata*, *Spodoptera litura* and *Samia recina*.

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