CHARACTERIZATION OF A HAEMOLYMPH PROTEIN HP19 AND ITS POSSIBLE ROLE IN NONGENOMIC ACTIONS OF 20-HYDROXYECDYSONE DURING THE POSTEMBRYONIC DEVELOPMENT OF RICE MOTH, *CORCYRA CEPHALONICA*

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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Dedicated to my

Parents

DECLARATION

I hereby declare that the work embodied in this thesis entitled **"Characterization of a haemolymph protein HP19 and its possible role in nongenomic actions of 20-hydroxyecdysone during the postembryonic development of rice moth, *Corcyra cephalonica*"** has been carried out by me under the supervision of Prof. Aparna Dutta Gupta and that 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. Abul Arif** has carried out the 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 recommend his thesis **"Characterization of a haemolymph protein HP19 and its possible role in nongenomic actions of 20-hydroxyecdysone during the postembryonic development of rice moth, *Corcyra cephalonica*"** for submission for the degree of Doctor of Philosophy of this University.

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(Abul Arif)

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
CaM kinase	:	Calcium/calmodulin dependent protein kinase
CaM	:	Calmodulin
CcACP	:	*Corcyra cephalonica* ACP 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)
Hex	:	Hexamerins
HGLFB	:	Hind gut associated lobular fat body
HP19	:	Haemolymph protein of mass 19 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
MES	:	2-(N-morpholino) ethanesulfonic acid

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
PAP	:	Phosphatidic acid phosphatase
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PGL	:	Prothoracic gland
PKC	:	Calcium/phospholipid dependent protein kinase (Protein kinase C)
PMSF	:	Phenylmethylsulfonyl fluoride
PNP	:	Para-nitrophenol
POPOP	:	1, 4-bis(5-Phenyl-2-oxazolyl) benzene
PP	:	Prepupae
PPO	:	2, 5-Diphenyloxazole
PTTH	:	Prothoracicotropic hormone
RNA	:	Ribonucleic acid
RTK	:	Receptor tyrosine kinase
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

Contents for Introduction and Review of Literature

Insects-

Insects that encompass more than 70% of entire animal kingdom are the most successful group of organism living on earth. Their existence dates back to nearly 250-500 million years and are adaptable to live in air, water or land. These insects can be divided into three convenient groups *i.e.,* harmless, injurious and beneficial. The 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 (USDA-Agricultural Research Service Information Bulletin, 1995). A wide range of lepidopteran pests cause damage and constitute a major factor that reduce the agricultural harvest globally including India. To combat such losses increased emphasis on developing safer, more effective and eco-friendly methods through modern biotechnology advances are required.

Types of insects-

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.*

Molting and metamorphosis-

These are the characteristic features of all insects including holometabola during the postembryonic development *i.e.,* the ontogeny accomplished after hatching. Molting refers to the period that begins with apolysis. Apolysis is the separation of the epidermis from the old cuticle followed by a more or less pronounced morphogenesis and deposition of new cuticle and ends with ecdysis *i.e.,* the shedding of the old cuticle. Hence, molting is the shedding of the hard external covering, which is necessary to accommodate growth and changes in morphology. 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 (For review see- Wigglesworth, 1934, 1939, 1954; Safranek and Williams, 1984; Sehnal *et al.,* 1996; Buszczak and Segraves, 2000; Tissot and Stocker, 2000; Truman and Riddiford, 1999, 2002).

Metamorphosis and its physiological significance-

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 these developments and decide whether a cell remains at the present stage or advances to the next one (Wigglesworth, 1954; 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 (Piepho, 1951; 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 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).

Hormones and metamorphosis-

As mentioned above, the postembryonic development in insects involves growth, molting and metamorphosis. It is now established that 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 cues that are mainly secreted by the brain, corpora cardiaca, corpora allata and prothoracic glands (PGLs). A choreographic precision of titer of mainly the morphogenetic hormones *i.e.,* the juvenile hormones (JHs) and ecdysteroids and their interaction is required for the molting and metamorphic events to occur normally (Gilbert *et al.,* 1996). Since 1930, the role of

hormones in the regulation of insect postembryonic development has received lot of attention resulting in several studies (Wigglesworth, 1934, 1939, 1954; Safranek and Williams, 1984; Sehnal *et al.,* 1996; Henrich *et al.,* 1999; Truman and Riddiford, 1999; 2002; Tissot and Stocker, 2000; Riddiford *et al.,* 2001; Gilbert *et al.,* 2002). However, knowledge regarding the regulation of hormone dependent actions is very limited and the field remains largely unexplored with a demand for further research.

Endocrine control of metamorphosis-

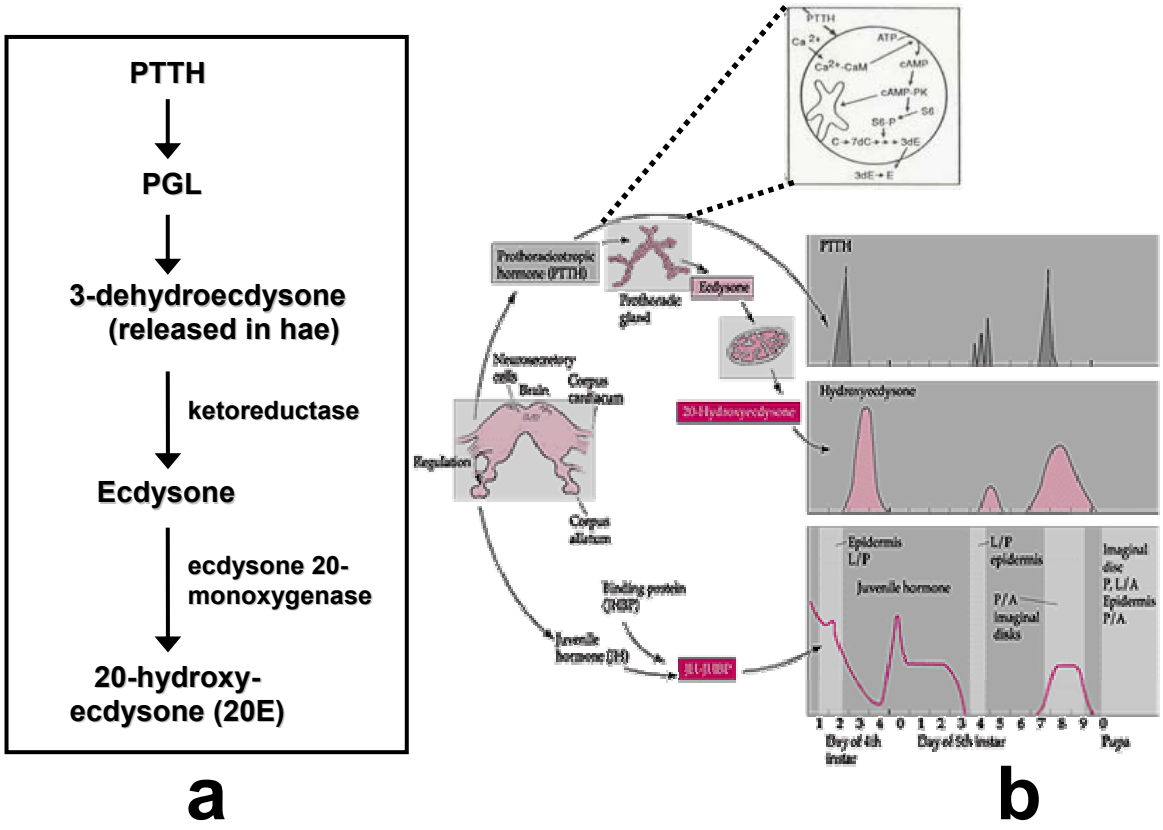

Fig. A: Simplified flow chart of 20-hydroxyecdysone biosynthesis in lepidopteran insects (a) and endocrine control of insect metamorphosis (b) (source- http://www.devbio.com/article.php?id=179&search=metamorphosis).

Molting and metamorphosis are hormonally regulated, mainly by JHs and ecdysteroids. Several workers have proposed a basic model of the endocrine control of postembryonic development (Fukuda, 1944; 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 and corpora allata that also act as neurohemal sites 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 factors (photoperiod, temperature and humidity), PTTH acts on the prothoracic glands 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 fat bodies, Malpighian tubules and epidermal cells (Smith *et al.,* 1983; Smith, 1985; Zhu *et al.,* 1991a; Riddiford *et al.,* 2001). The 20E finally exerts its effect and causes apolysis and secretion of larval, pupal or adult cuticle (Smith and Gilbert, 1989). In addition to sequestering PTTH, the corpora allata synthesize and secrete JHs into haemolymph, the second major effector hormone in insect life. In haemolymph, the hormones are bound to JH-binding proteins, which enhance the solubility 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 (sesquiterpenes) and ecdysteroids (a group of steroid hormones) 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 titers 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).

In holometabolous insects JH levels are high throughout the larval instars, declines in the last instar but rise again before the pupal molt during the prepupal stage and 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 would result in larval-pupal molt and in the absence of JH would result in pupal-adult molt. Ecdysteroid levels rise prior to a molt and decline just before actual ecdysis. In the last larval instar, there is a small peak of ecdysteroids that occurs at a time when JH is absent. This peak of ecdysteroids 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)

Choice of last instar and prepupal stages for present study-

During the postembryonic development, the cells may change their functions and these changes may or may not be related to cell divisions (Wigglesworth, 1954; Sehnal *et al.,* 1996). The extent of change is usually much greater in the last larval instar and pupa than in preceding instars making these developmental stages an ideal choice for the present study.

Chemical nature of ecdysteroids-

Ecdysteroids are well defined term for all compounds structurally related to ecdysone. It includes true ecdysteroid and ecdysteroid related compounds. The biologically active ecdysteroids refer to the molting hormone. Thus the hormonally active nonsteroidal compound such as RH5849 and its derivatives (Wing, 1988) are molting hormone agonist but not an ecdysteroid or ecdysone (Karlson, 1995). 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 molting hormone, 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 and 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 toward 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 (Williams, 1956; 1959; Yin *et al.,* 1994; Gilbert *et al.,* 2000). Methyl farnesoate is the predominant JH like molecule in the crustacean. 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.

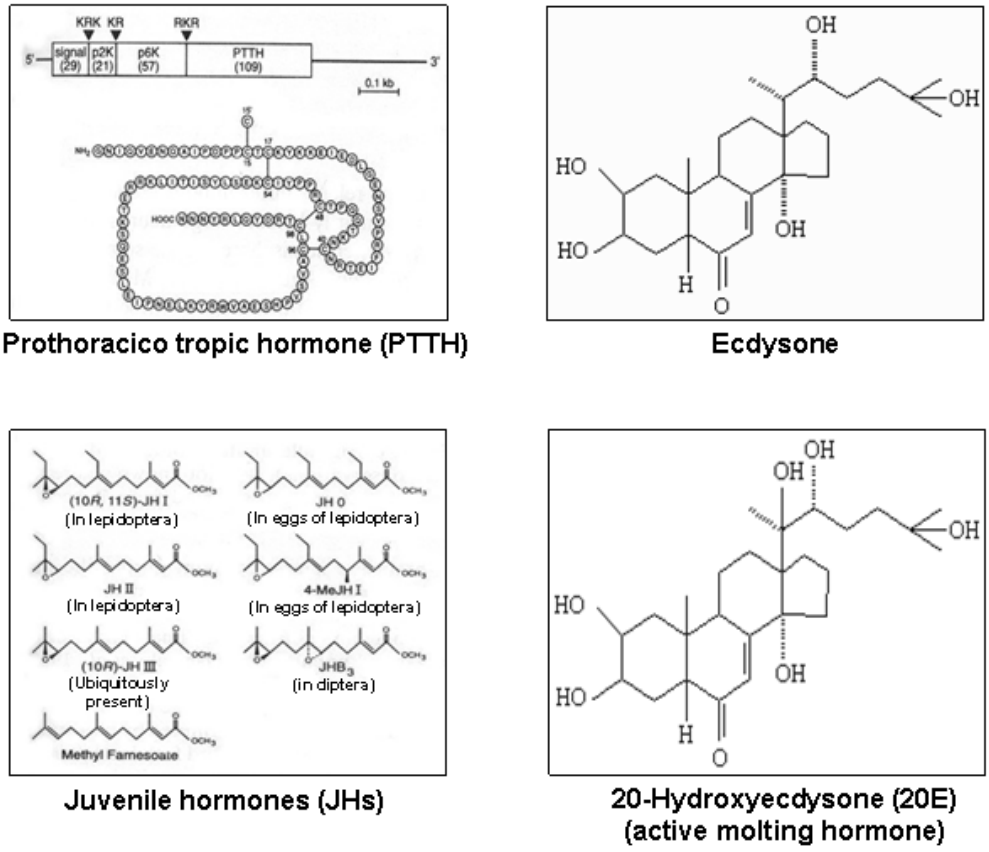

Fig. B. Major hormones of insects

Regulation of haemolymph 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; Gruetzmacher *et al.,* 1984a, b; 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 (Nijhout and Williams, 1974; Riddiford and Truman, 1978; Smith, 1985). 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, 1998). 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 titre at any given moment is therefore 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 titre 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 molting (Gilbert *et al.,* 1980b; Nijhout, 1994; Riddiford *et al.,* 2001).

Regulation of ecdysteroid biosynthesis-

The primary and secondary effectors regulate ecdysteroidogenesis. Detailed studies reveal that the cerebral neuropeptide PTTH is the primary effector in the regulation of

ecdysone biosynthesis by the PGLs (Bollenbacher and Bowen, 1983; Bollenbacher and Granger, 1985). Several evidences also suggest that secondary effectors also control the PGL activity. These include environmental signals such as temperature (Meola and Adkisson, 1977), photoperiod (Mizoguchi and Ishizaki, 1982), direct neural input (Richter and Gersch, 1983), humoral factors such as lipoproteins (Chino *et al.,* 1974) and hormones other than PTTH (Safranek *et al.,* 1980; Beylon and Lafont, 1983). These effectors either individually or in combinations precisely regulate the synthesis and release of ecdysone by PGL during postembryonic development. Thus, they control the quantitative and temporal fluctuations in the haemolymph ecdysteroid titre (Bollenbacher *et al.,* 1981; Gilbert *et al.,* 2002).

Evidences of factor (or 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 sexta* larvae via secretion of a factor from fat body (Williams, 1959; Gilbert and Schneiderman, 1959; 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 ecdysteroidogeneic 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 (Agui, 1975; 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 of 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 diapause

sustaining temperature of 19°C, implanted PGLs are able to produce ecdysone only in non-diapausing hosts (Meola and Gray, 1984). However, 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. It was also reported that the enzyme is trypsin sensitive, heat labile and has a mass between 20 to 30 kDa (Sakurai *et al.,* 1989).

Regulation of ecdysteroidogenesis by protein phosphorylation and dephosphorylation-

PTTH stimulated ecdysteroid production in PGLs occurs via a cascade of events which is yet to be elucidated completely. Earlier studies on *M. sexta* revealed a correlation between circulating ecdysteroid titers and adenylate cyclase activity in the PGL, suggesting a role for cAMP (Vadeckis *et al.,* 1976; 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 elicits increased cAMP formation in PGLs. Increase in intracellular cAMP levels can lead to the activation of cAMP dependent protein kinase (PKA) and subsequent phosphorylation (Fig. A). In recent past, Gilbert *et al.,*(1997) reported 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 suggest 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).

Regulation of enzymes of biosynthetic pathways of ecdysteroidogenesis-

In insects, ecdysone 20-monoxygenase catalyzed hydroxylation of ecdysone into the active hormone 20E has been defined as activation (Gilbert *et al.,* 1996). However, during times of decreasing hormone titers, inactivation occurs by several routes including (i) 26-hydroxylation and further oxidation to 26-oic acid, (ii) formation of various conjugates (*eg.,* phosphates) and (iii) in lepidoptera in particular, ecdysone oxidase catalysed formation of 3-dehydroecdysteroid, which is reduced to 3-epiecdysteroid, followed by phosphotransferase catalyzed formation of phosphate conjugates (Williams *et al.,* 1997). These results indicate that molting hormone stimulates at least one universal route of its own inactivation by inducing 26-hydroxylase activity, thus regulating its activity as well as titer.

Haemolymph proteins as a source of carrier for ecdysteroids-

It is generally assumed that after biosynthesis in a specific endocrine tissue, transport of the ecdysteroid hormone or prohormone occurs non-specifically. In vertebrates, almost all the steroid hormones have been known to be regulated by the presence of plasma binding globulins (Rosner *et al.,* 1991). Here the binding or carrier proteins facilitate release of hormone from the endocrine gland, constitute a circular pool of conserved hormone, buffer the effect of a sudden release of active molecules into the bloodstream and protect the hormones from enzymatic degradation that prevents the rapid depletion of hormone titer (Roe and Venkatesh, 1990). 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 of ecdysteroids is not very clear and the area remains largely unexplored.

Mode of action of ecdysteroids-

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 C*hironomous 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 (Becker, 1959; 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 suggest 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; Koelle *et al.,* 1992; Riddiford *et al.,* 2001; Thummel, 2002). From these studies a clearer understanding of the mechanism by which a

systemic hormonal signal is refined into stage and tissue specific developmental responses has emerged.

Mechanism of steroid hormone action-

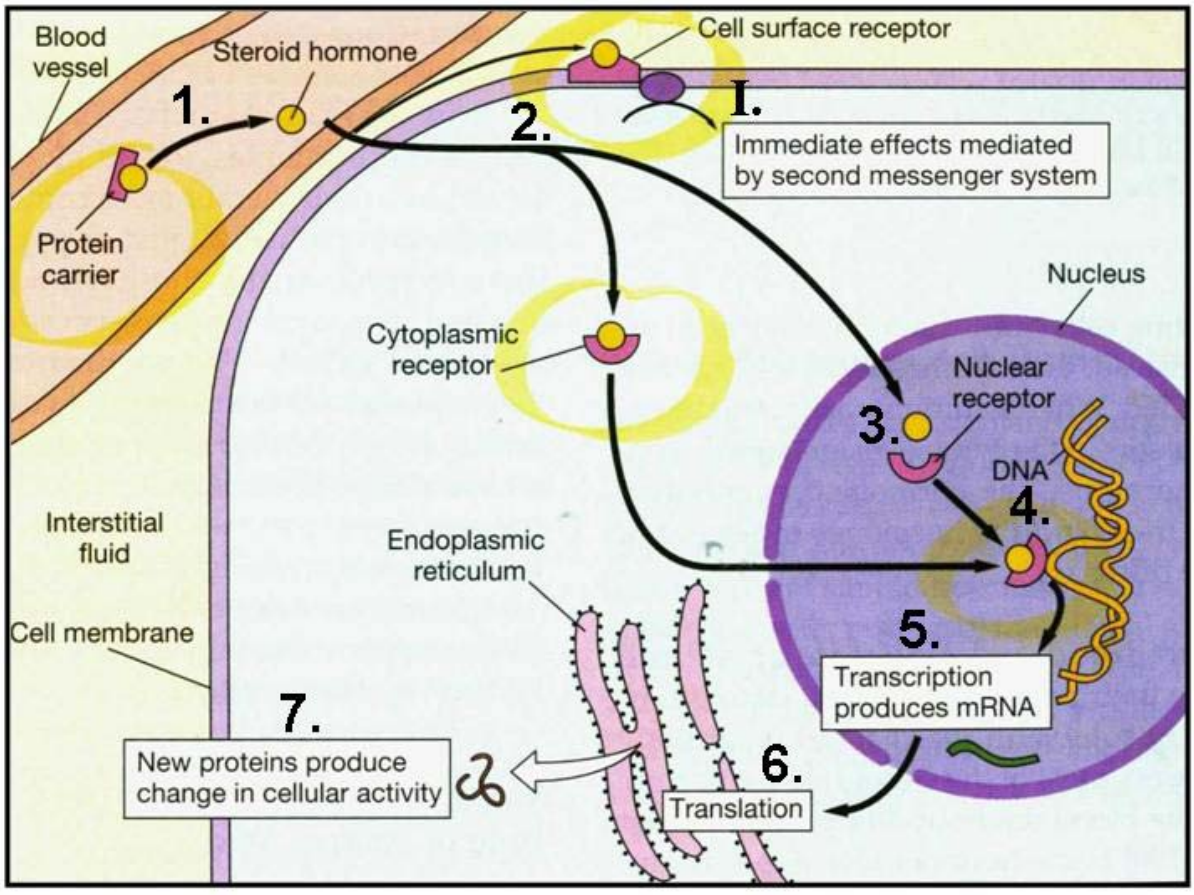

Fig. C: The generalized representation of mechanism of steroid hormone action. Steroid hormones mostly regulate the biological response in following steps- 1. Carrying of hormones to appropriate target and its dissociation from carrier protein. 2. Transport of hormone into cell cytoplasm or nucleus. 3 & 4. Binding of activated hormone receptor complex to hormone responsive elements as homo or heterodimers. 5. Gene activation and transcription. 6. Release of mRNA from nucleus and its translation into protein. 7. Changes in the cellular activity due to the regulation of newly synthesized protein. In addition to this the steroid hormones may also exert a direct rapid effect possibly through a cell surface receptor mediated by a second messenger system (I) (source- http://www.zoo.utoronto.ca/zoo344s/2003Group2/mechanism_steroid.htm).

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 and function of the other tissues. The mechanism (Fig. C) 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 on specific carrier protein from the point of their release to the target tissues. These carrier proteins protect the hormones from enzymatic degradation and extend their half-life. 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 protein and becomes activated. Hence the hormone-receptor complex acts as a ligand activated transcription factor. 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 a member of nuclear receptor super family-

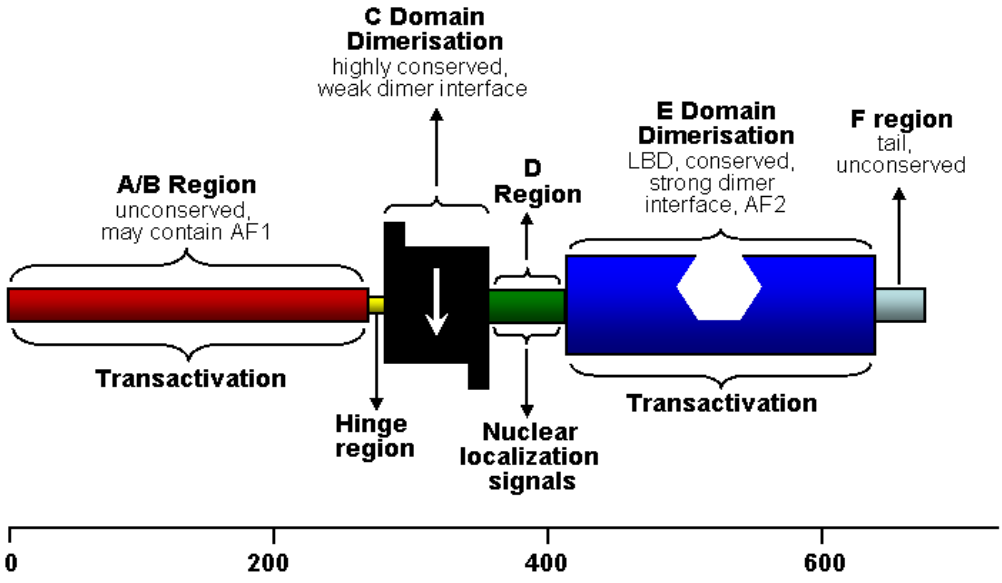

Fig. D: The generalized representation of the steroid or thyroid hormone superfamily receptor. The C domain is the highly conserved DNA binding domain (DBD) that contains cysteine rich central region compatible with the formation of two zinc fingers. The D region is responsible for nuclear localization. The E domain contains the ligand binding site and is known as the ligand binding domain (LBD). The relative position of two transactivation domains AF1 and AF2 is also shown (Modified from Bender *et al.,* 1997; Riddiford *et al.,* 2001).

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 (currently totaling 150 different proteins) consists of receptors for steroids, retinoids, thyroid hormones, fatty acids, prostaglandins and orphan receptors whose ligands are unidentified (White and Parker, 1998). The members of this family are highly related in both (i) primary amino acid sequences and (ii) the organization of functional domain, 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 (Fig. D), which have been confirmed by the results of cDNA cloning experiments (Bender *et al.,* 1997; Riddiford *et al.,* 2001).

Ecdysone receptor in insects-

Like other steroid hormones, the receptor for ecdysone is also a member of the nuclear receptor superfamily (Koelle *et al.,* 1991) that acts as a ligand dependent transcription factor (Mangelsdorf *et al.,* 1995; Freedman, 1997). Unlike the vertebrate steroid receptors which act as homodimers, the functional ecdysone receptor is a heterodimer of EcR with another member of the nuclear receptor superfamily, ultraspiracle (USP) (Yao *et al.,* 1992, 1993). The USP is the insect homolog of the vertebrate retinoid X receptor (RXR) (Oro *et al.,* 1990). The EcR-USP heterodimer binds DNA at ecdysone response elements and acts at molecular and tissue levels (For review see- Riddiford *et al.,* 2001).

Classification of steroid hormone action-

The core paradigm or 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 and 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 and are also sensitive to transcriptional or 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). For example the steroids may act via cell surface receptor for rapid effects through second messenger system (see Fig. CI). Therefore the steroid hormones action can be classified into two categories-

- **Genomic actions:** well established classical or traditional mode of action
- **Nongenomic actions:** upcoming rapid effects of steroids

Examples and characteristics of nongenomic effects of steroid hormones-

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). This was followed by several other reports on rapid effects of steroids (Klein and Henk, 1963; Edwardson and Benet, 1974; Pietras and Szego, 1977, 1999). Spach and Streeten (1964) made an excellent observation on *in vitro* effects of physiological concentrations of aldosterone on Na^+ exchange in dog erythrocytes that lacks nucleus. Their study clearly suggested that steroids can act in a pathway different from the established genomic mode of action. However, it is only recently that there has been an upsurge in the studies of rapid nongenomic actions of virtually all groups of steroids as well as thyroid hormones (Falkenstein *et al.,* 2000a; Davis *et al.,* 2002; Losel and Wehling, 2003). These non-genomic actions represent important new pathways for steroid hormone action on cellular function of varieties of cells and tissues. The important post-transcriptional or other types of rapid steroid effects include the regulations of mRNA stability in the cytoplasm (Cho and Raikhel, 2001; Nomura *et al.,* 2002), changes in membrane electrical activity (Sutter-Dub, 2002), conventional second messenger cascades such as phospholipase C (Civitelli *et al.,* 1990), phosphoinositide turnover (Morley *et al.,* 1992; Morelli *et al.,* 1993), intracellular pH (Jenis *et al.,* 1993; Wehling *et al.,* 1996), free intracellular Ca^{2+} (De Boland and Norman 1990; Wehling *et al.,* 1990), sodium transport (Christ *et al.,* 1995a; Ebata *et al.,* 1999), levels of cAMP (Christ *et al.,* 1999), cGMP (Chen and Chang, 1998), IP_3 + diacylglycerol (Christ *et al.,* 1993) and nitric oxide synthase (Wyckoff *et al.,* 2001). Steroids were also shown to regulate nongenomically the activity of almost all the major classes of protein kinases such as PKC (Sylvia *et al.,* 1993; Christ *et al.,* 1995b), PKA (Harrison *et al.,* 2000), MAP kinases (Endoh *et al.,* 1997) and tyrosine kinases (De Boland and Norman, 1998; Manegold, 1999) *etc*. All these rapid nongenomic effects of steroids are characterized by at least one of the two basic features-

- **Rapid physiological response:** 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 (Losel and Wehling, 2003).

- **No effect of the inhibitors of transcription or translation on cellular response**

Classification of non-genomic action of steroids-

To address the increasing evidence for rapid effects of steroids and the diversity of mechanisms for rapid steroid signalling, Falkenstein *et al.,* (2000a, b) proposed a Mannheim classification scheme of these rapid non-genomic effects. According to this scheme the rapid non-genomic effects can occur in following ways-

a) Through a direct effect- The steroids can directly induce the rapid effect in the absence of receptor. It involves modulation of protein function reflecting changes in membrane physico-chemical properties. The apparent steroid specificity of these effects may thus reflect variable lipophilicity and polarity. *Eg.,* interaction of high concentrations of steroids like progesterone, 17α-hydroxyprogesterone, testosterone and estradiol with membrane vesicles prepared from phosphatidylserine and from lipid extracts of human and hamster spermatozoa (Shivaji and Jagannadham, 1992; Whiting *et al.,* 2000).

b) Through a rapid non-transcriptional effect of the classical steroid receptor- *Eg.,* involvement of classical estrogen receptor in rapid stimulation of endothelial nitric oxide synthase activity in response to estrogen, which is insensitive to transcriptional inhibitor actinomycin-D, but is completely inhibited by the antagonist, tamoxifen and ICI 182,780, which bind to classical estrogen receptor (Shaul, 1997).

c) Through a distinct non-classical receptor that is possibly associated with the plasma membrane (also referred as membrane initiated steroid signalling-MISS)- This involves majority of rapid steroid effects on cellular signalling and function reported so far. The rapid responses are identified to be transmitted by membrane receptor, unrelated to classical intracellular receptors as it is unaffected by the antagonists of the classical receptors (Watson and Gametchu, 1999; Nadal, 2000; Borski, 2000; Kelly and Levin, 2001; Beyer *et al.,* 2003; Boldyreff and Wehling, 2003). *Eg.,* aldosterone effects on various ion transport mechanism and second

messenger system (Scmidt *et al.,* 2000), rapid stimulation of intestinal Ca^{2+} transport in perfused chick intestine (transcaltachia) as well as rapid stimulation of PKC and MAP kinase by $1\alpha,25(OH)_2D_3$. (Zanello and Norman, 1997; Sylvia *et al.,* 1998).

Membrane receptors for nongenomic action of steroids-

The membrane receptors have been identified for large number of steroids such as estrogen and xenoestrogen (Kelley and Levin, 2001; Nadal *et al.,* 2000), glucocorticoids (Borski, 2000), androgens (Heinlein, 2002), estradiol (Benten *et al.,* 2001), testosterone (Benten *et al.,* 1999), aldosterone (Boldyreff and Wehling, 2003, Losel *et al.,* 2002), Progesterone (Graham and Clarke, 1997) *etc*. Some authors suggest that these cell surface receptors are G protein coupled receptors (GPCR), which activate second messenger signalling mechanism (Wyckoff *et al.,* 2001). Few evidences suggest that non-genomic effect of androgens and estrogens may occur through cell surface receptor to induce MAP kinase signalling cascade and induce ERK and p38 MAP kinases stemming from G-protein activation and the resulting calcium flux (Benten *et al.,* 2001; Kelley and Levin, 2001; Heinlein, 2002). It is suggested that the membrane steroid receptors for mediation of rapid steroid effects, acts in multiple membrane localization mechanisms and receptor protein can act simultaneously or sequentially in a cell and receptor specific manner (Watson and Gametchu, 2003). Although various steroid hormones have been shown to bind to many biological membranes but the characterization of proteins to which they bind has mostly been limited to the determination of their molecular mass or their tentative identification by antibodies. Meyer *et al.,* (1996), isolated a progesterone binding protein from porcine liver membrane. Further Gerdes *et al.,* (1998) and Bernauer *et al.,* (2001) cloned and analysed two putative progesterone binding membrane proteins from human and porcine liver. However, evidence remains to be seen in terms of cellular effects by such a steroid membrane receptor (Losel and Wehling, 2003).

Recently, Zhu *et al.,* (2003b, c) unequivocally identified and characterized a progesterone membrane receptor in a fish, spotted seatrout responsible for progesterone induced meiotic maturation of oocytes. The group isolated a novel 352 amino acids protein by screening an oocyte expression library using monoclonal antibodies directed against the progestin binding oocyte membrane protein. This seatrout membrane progestin receptor (mPR) showed little sequence homology to GPCRs but contained seven putative hydrophobic transmembrane domains and was therefore considered a novel member of the heptahelical

GPCR receptor family. They further identified a family of mPR proteins from a number of different species including frog, human and mouse, some of which bound to progesterone. This mPR receptor satisfied the seven criteria for its designation as steroid membrane receptor. It had (i) a plausible structure (containing seven domains typical of GPCRs), (ii) tissue specificity (the mRNA detected only in brain and reproductive tissue), (iii) cellular distribution (present only in plasma membrane), (iv) steroid affinity (high affinity, saturable, displaceable, single binding sites for progestins), (v) role in signal transduction (activates MAP kinase and inhibits adenylyl cyclase), (vi) hormonal regulation and (vii) biological relevance. Hence, the identification of mPR gene has further redefined the non-genomic steroid mediated signalling that is often linked to GPCRs (Hammes, 2003). However, several questions such as (i) are these mPR classical GPCRs, (ii) do they bind specifically to a specific steroid or different steroids or even the non-steroids, (iii) what are the factors that direct the steroid binding to an individual target cell and (iv) how the hydrophobic steroids will specifically interact with a receptor that contains no known steroid binding domain, remain unanswered

Modulation of genomic and nongenomic actions of steroids by a cross-talk-

It is becoming increasingly clear now that steroid hormones can act at three different cellular levels *viz.,* membrane, cytosol and nucleus with interdependence on each other (Valverde and Parker, 2002; Edwards *et al.,* 2003; Losel and Wehling, 2003). The membrane target includes the non-classical steroid receptor and ligand or voltage activated ion channels. The activation of some of these receptors triggers signalling events, leading to the regulation of various kinases and phosphatases. The major targets are believed to be the classical translocating receptors, which are generally associated with signalling pathways (*eg.,* Src-PI3K. Akt and / or Src-Ras-ERK). The regulation at the membrane or cytosolic targets might ultimately determine the change in gene expression by the interaction of steroids with classical nuclear targets.

Nongenomic actions of ecdysteroids-

The classical mode of action of ecdysteroids like all other steroids is the receptor mediated regulation of gene activity influencing transcription and subsequently protein synthesis for the delayed cellular effects. There is also an increasing evidence for rapid non-genomic responses of ecdysteroids although the field is not explored as in the case of steroids

from vertebrate (Tomaschko, 1999). The favored target for the ecdysteroids is the plasma membrane and its associated protein where it interacts with Na^+-H^+ exchangers and K^+ 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). 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; Lanot *et al.,* 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; Riddiford, 1985; 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 laboratory also 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 and that an increase in the lysosomal activity is governed by the elevation of 20E levels (Rao *et al.,* 1984; Ray *et al.,* 1984; Sridevi *et al.,* 1987; Ashok and Dutta-Gupta, 1988; Dutta-Gupta and Sridevi, 1991). Despite these studies, the mechanism of regulation of the majority of ecdysteroid dependent actions are not clear and hence demands for further research.

Fat body tissue and its significance for the present study-

The fat body in insects is an important metabolic centre and biochemically most active organ in insects with multiple functions such as metabolism of proteins, carbohydrates

and lipids particularly blood sugar and haemolymph proteins such as vitellogenins and hexamerins. It is also involved in detoxification, synthesis and hormone metabolism (Keeley 1985) and is a possible source of humoral factors (Meola and Gray, 1984, Gray *et al.,* 1987). In view of the complex function performed by fat body, it is often compared to vertebrate liver and hence is a suitable tissue for the studies of the stage and tissue specific expression of genes, post-transcriptional regulation of RNA and post-translational control of proteins (Hansen *et al.,* 2002). The fat body undergoes growth and development along with the other tissues and its function changes in accordance with the developmental stage of the insect (Vanishree *et al.,* 1999). The fat body tissue is structurally organized to provide maximal exposure to the haemolymph and due to its changing metabolic role and integral position in maintaining metabolic homeostasis, it serves as an ideal model for endocrine regulated studies (Kunkel, 1981). Most of the studies with protein expression and sequestration by fat body have been carried out with the entire tissue (Locke and Collins, 1965; 1968). However, there are evidences in both diptera and lepidoptera that suggest regional differences in activity and function of fat body tissue. In case of *Helicoverpa zea,* the storage proteins are synthesized by peripheral fat body fraction but are sequestered and stored only by perivisceral fat body (Wang and Haunerland, 1994a). In the silkworm *Bombyx mori,* it has been shown that the dorsal and ventral perivisceral fat body contains the most competent cells for sequestering haemolymph protein as compared to the peripheral and hindgut associated fat body tissue (Vanishree *et al.,* 1999). Another important aspect of studies using insect fat body, as model tissue is that it functions normally under cultured condition. Nakanishi and Garen (1983), reported that ecdysteroid exerts its effect on the cultured fat body system and the expression pattern of LSP-2, P1 and G12 was similar both under *in vivo* and cultured condition.

Protein phosphorylation: a general account-

Protein phosphorylation is the major regulatory mechanism by which synthetic activities of various tissue or cell types are controlled by external physiological stimuli. It is one of the major post-translational events whose importance is established in hormone action (Cohen, 1982; Boyer *et al.,* 1983; Cochrane and Deeley, 1984). The protein phosphorylation system consists of three primary components, a protein kinase, a protein phosphatase and a substrate protein. When a protein kinase transfers the terminal (γ) phosphate from ATP to the hydroxyl group of a serine or threonine or tyrosine residue of the substrate protein, the

substrate protein is phosphorylated. These phosphate moieties can be removed from the phosphorylated protein by phosphatases. This kind of reversible protein phosphorylation is a post-translational modification well known to play a role in variety of cellular functions (Graves and Krebs, 1999; Davies *et al.,* 2000). It is a highly regulated process by which information can be shuttled from the cell surface to nucleus (Denu *et al.,* 1996). On the basis of the second messenger system required to activate specific kinase, it is classified as cAMP dependent protein kinases (PKA), cGMP dependent protein kinases, multiple types of calcium dependent protein kinase (CaM kinases) and a calcium/phospholipids dependent protein kinase (PKC). In recent years, it has become increasingly evident that many of the cellular actions of calcium in mediating the signalling are by its binding to specific kinases resulting in their activation, which thus phosphorylate a specific protein (Nestler and Greengard, 1984; Pinna and Ruzzene, 1996). Another class of kinase is tyrosine kinase which specifically catalyses the phosphorylation at tyrosine residues. These tyrosine kinases are classified into receptor and non-receptor tyrosine kinases (Hunter and Cooper, 1985).

Although extensive studies on protein phosphorylation systems have been carried in vertebrates, very little information is available on insects. However, in the last few decades, several studies have been initiated on this aspect and kinases such as CaM kinase II (Shanavas *et al.,* 1998), cyclic nucleotide dependent protein kinases like PKA (Chalaye *et al.,* 1988; Muller and Spatz, 1989; Jiang and Struhl, 1995; Lepage *et al.,* 1995; Smith *et al.,* 1996; Muller, 1997a) and cGMP-PK (Foster *et al.,* 1996; Muller, 1997b) have been well characterized from various tissues of different insect species.

Protein phosphorylation: role of 20E and other insect hormones-

Numerous report convincingly suggest that phosphorylation of specific substrates by a variety of protein kinases appears to be a general mechanism by which many hormones, neurotransmitters and other extracellular signals produce their physiological responses in specific target cells (Greengard, 1978; Graves and Krebs, 1999; Davies *et al.,* 2000; Cohen, 2002). However, only a few reports are available regarding the regulation of protein kinase activity by insect hormones. These studies suggest 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. 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). Earlier studies from our laboratory have revealed autophosphorylation of CaM kinase II in the CNS of *B. mori* (Shanavas *et al.,* 1998) and the phosphorylation of storage protein binding protein (SPBP) in the fat body membranes of *C. cephalonica* (KiranKumar, 1998). These two proteins are developmentally regulated (Shanavas *et al.,* 1998; KiranKumar, 1998) and their phosphorylation in fat body tissue was found to be 20E dependent (Vasanthi, 1999). Hence, the two proteins provided an excellent system for the present study to check if the phosphorylation of these proteins is regulated by ecdysteroid at nongenomic level and evaluate the role or function of haemolymph proteins or humoral factors in these proteins.

In addition to 20E, other insect hormones are also shown to be involved in the signal transduction mechanism. Juvenile hormone (JH) was shown to be involved in the activation of a specific Na^+-K^+ ATPase via PKC in the follicle cell membrane of *Rhodnius prolixus* (Sevala and Davey, 1989). It was also shown to stimulate protein synthesis in male accessory glands of *D. melanogaster* through the activation of PKC (Yamamoto *et al.,* 1988). PTTH has been shown to stimulate cAMP-PK activity in the prothoracic glands of *M. sexta* (Smith *et al.,* 1996). The cGMP dependent protein kinase activity in the CNS of *M. sexta* was stimulated by eclosion hormone (Morton and Truman, 1986, 1988).

Calcium dependent protein kinases: role of 20E-

Mainly two classes of calcium dependent protein kinases are known and they are calcium/calmodulin dependent protein kinases (CaM kinases) and calcium/phospholipid (phosphatidylserine) dependent protein kinases (PKC).

The CaM kinases act via the interaction of calcium with calmodulin (CaM). Calmodulin is a ubiquitous low molecular weight protein, which undergoes structural changes upon binding with Ca^{2+} and is thus activated. The Ca^{2+}/CaM complex then binds and alters the function of other cellular proteins (Cheung, 1980; Carafoli, 1987; Heizmann and Hunziker, 1990). Several studies over the last two decades established that some of the second messenger actions of calcium in a variety of tissues is due to the activation of CaM kinases and protein phosphorylation is one of the important routes by which Ca^{2+}/CaM signal transduction regulates cellular function (Hanson and Schulman, 1992a, b). Multiple types of

CaM kinases have been demonstrated and characterized in the mammalian neural and non-neural tissues. They include CaM kinase I (Nairn and Greengard, 1987), II (Kennedy and Greengard, 1981), III (Nairn *et al.,* 1985), IV (Kato *et al.,* 1992), myosin light chain kinase (Hagiwara *et al.,* 1989) and phosphorylase kinase (Cohen *et al.,* 1978). CaM kinase II is the most abundant type and has been well characterized in the neural tissue. It was first identified in rat brain as a Ca^{2+} dependent protein kinase that catalyses the phosphorylation of site 2 and 3 of synapsin 1 (Kennedy and Greengard, 1981). Rat brain CaM kinase II comprises of several related isozymes (Hanson and Schulman, 1992a, b) that consist of a catalytic domain, an autoregulatory domain containing a calmodulin binding site and a C-terminal "association domain that mediates holoenzyme formation" (Bennett and Kennedy, 1987; Lin *et al.,* 1987; Tobimatsu *et al.,* 1988). A distinct property of CaM kinase II is the autophosphorylation of a threonine residue near its calmodulin binding domain which converts the enzyme to Ca^{2+} independent form (Miller and Kennedy, 1986; Wang *et al.,* 1994). It was postulated that this autophosphorylation induce changes in subcellular distribution of enzyme in *Aplysia* and *D. melanogaster* (Saitoh and Schwartz, 1985; Willmund *et al.,* 1986) and may also be involved in prolonging the effects triggered by a transient calcium signal (Miller and Kennedy, 1986).

There are only a few reports on the characterization of CaM kinase II in insects (Cho *et al.,* 1991; Ohsako *et al.,* 1993; Shanavas *et al.,* 1998). Adult *D. melanogaster* head contains three species of CaM kinase II with molecular masses of 54/55, 58 and 60 kDa (Cho *et al.,* 1991, Ohsako *et al.,* 1993). The amino acid sequence and tissue specificity of the rat kinase are highly conserved in *D. melanogaster*. Extensive studies from our laboratory suggested that the *B. mori* CNS consists of two species of CaM kinase II with molecular mass of 59/60 kDa, which cross-react with anti-rat CaM kinase IIα monoclonal antibody and show a high degree of autophosphorylation in neural tissue (Shanavas, 1997; Shanavas *et al.,* 1998). Further, two peaks of enzyme activity occurred in the CNS of *B. mori* during the postembryonic development, the first peak at late-larval stage and the next peak at late-pupal stage (Shanavas *et al.,* 1998) that coincided with the reported parallel changes in the ecdysteroid titer in the haemolymph of *B. mori* (Calvez *et al.,* 1976). Transformed strains of *D. melanogaster*, expressing a transgene inhibitor of CaM kinase II have been shown to be deficient in an associative conditioning behavioral paradigm (Griffith *et al.,* 1993; Wang *et al.,* 1994). It was also shown to be involved in the phosphorylation of *D. melanogaster* visual phosrestin, thereby regulating photoreceptor light adaptation (Kahn and Matsumoto, 1997; Kahn *et al.,* 1998).

Another class of calcium dependent protein kinase is the calcium/phospholipid (phosphatidylserine) dependent protein kinases, also known as protein kinase C (PKC). It was originally identified as a serine/threonine kinase that was maximally active in the presence of diacylglycerol (DAG) and calcium. There are at least ten proteins of PKC family that play a critical role in regulating the cellular functions and are involved in the modulation of signal transduction (Nishizuka, 1988, 1995; Newton, 1995). A variety of hormone, growth factors and neurotransmitters are known to regulate inositol phospholipid breakdown to generate DAG and thereby mediate their actions by activating PKC (Kikkawa *et al.,* 1982; Mosior and Epand, 1993). Evidences exist for the activation of JH dependent Na^+-K^+ ATPase via PKC (Sevala and Davey, 1989). However the information with respect to 20E is not yet clear. In invertebrates, PKC regulated mechanism is suggested to play an important role in the process of neuronal plasticity (Altfelder *et al.,* 1991; Choi *et al.,* 1991; Emptage, 1993; Olds and Alkon, 1993). Recently, interest has shifted to the events downstream of PKC activation cascade, namely to the identification of physiological substrates of PKC to understand the relationship between PKC activation and cellular responses. In an invertebrate, *Hermissend crassicornis,* a 20 kDa substrate was found to exhibit GTPase activity which reduced K^+ currents. Furthermore, a change in its rate of phosphorylation was seen with learning (Nelson *et al.,* 1990). Muller (1997c) reported a 86 kDa, filament interacting protein in honeybee *Apis mellifera* as a substrate for PKC. Since the interaction of the protein with the cytoskeleton is regulated by Ca^{2+}/calmodulin and phosphorylation, this PKC substrate was suggested to be a potential site of convergency in regulation of cytoskeleton-membrane rearrangement like the members of the vertebrate myristoylated alanine rich C kinase substrate (MARCKS) family. These studies however do not throw any light on possible effects on PKC or its substrates.

Tyrosine kinases: role of 20E-

The tyrosine kinases are another very important class of protein kinases, which specifically catalyses the phosphorylation of proteins at tyrosine residues and regulate the cellular functions. These kinases are identified to be of two types, receptor tyrosine kinases (RTKs) and the non-receptor tyrosine kinases. The RTKs contain four domains- (i) an extracelluar ligand binding domain, (ii) an intracellular tyrosine kinase domain, (iii) an intracellular regulatory domain and (iv) a transmembrane domain. The amino acid sequences of the tyrosine kinase domain of RTK are highly conserved with those of PKA within the ATP binding and substrate binding regions. The RTKs are classified into at least 14 different

families based upon the structural features in their extracellular portions. Many receptors that have intrinsic tyrosine kinase activity as well as the tyrosine kinases that are associated with the cell surface receptors, contain tyrosine residues, which upon phosphorylation interact with other proteins of the signalling cascade. These other proteins contain amino acid sequence that is homologous to a domain, first identified in the c-Src proto-oncogene and was termed SH2 domain (Src homology domain 2). Another conserved protein-protein interaction domain identified is related to a third domain in c-Src and termed as SH3 domain. The interactions of SH2 domain containing protein with RTKs or receptor associated tyrosine kinase leads to tyrosine phosphorylation of the SH2 containing proteins that have enzymatic activity and caused an alteration in the activity. The non-receptor tyrosine kinases are responsible for phosphorylating variety of intracellular proteins, following activation of cellular growth and proliferation signals. These kinases distinctly belong to two different families- (a) related to Src protein and (b) Janus kinases (Jak). In both families the non-receptor tyrosine kinase couples to cellular receptor that lack enzymatic activity themselves (For review see- Hunter and Cooper, 1985; Koch *et al.,* 1991; Pazin and Williams, 1992; Fantl *et al.,* 1993; Smithgall, 1995; Gerber, 2002).

In insects, several RTKs have been identified in *D. melanogaster* (Yamamoto, 1994; Freeman, 1996; Raabe *et al.,* 1996). The *D. melanogaster* homologue of the mammalian EGF receptor has been identified as an RTK and shown to be involved in many stages of development (Doyle and Bishop, 1993; Duffy and Perrimon, 1994; Schweitzer and Shilo, 1997). Insulin receptor like tyrosine kinase activity has been reported in PGLs of *M. sexta* (Smith *et al.,* 1997). The tyrosine kinase activity was found in the cotton leaf worm *Spodoptera littoralis* (Pearce *et al.,* 1994). Phosphotyrosine containing proteins have been detected in different tissues of the mediterrenian fruit fly *Ceratitis capitata* and their role during pupation is discussed (Katsoris *et al.,* 1991). Although the role of insect hormone on tyrosine kinase signalling is not very clear. Some authors have suggested that these kinases stimulate ecdysteroid production in the mosquito *Aedes aegypti* (Riehle, 1999). In a recent study, the PTTH stimulated ecdysone secretion was shown to be dependent upon the tyrosine phosphorylation in the prothoracic glands of *M. sexta* (Smith *et al.,* 2003). Inhibition of tyrosine kinases has been suggested to impair axon extension in the nervous system of grasshopper embryo (Menon and Zinn, 1998). Studies from our laboratory suggest that phosphorylation of a 48 kDa protein in neural tissue of *B. mori* (Shanavas, 1997) and a 120 kDa storage protein binding protein (SPBP) in the fat body of *C. cephalonica* (KiranKumar,

1998) are probably mediated by tyrosine kinases. In the present study an attempt has been made to understand the physiological significance of the phosphorylation of 48 and 120 kDa proteins as the 120 kDa SPBP is involved in the uptake of hexamerins by the non-feeding prepupal and pupal fat body in *C. cephalonica*. This uptake is an essential process required for the proper growth and development of insects during pupal-adult transformation.

Hexamerins: synthesis, release and receptor mediated uptake-

As insects do not feed during prepupal and pupal stages, they depend on macromolecular supply (proteins, lipids and carbohydrates) that has previously been accumulated during the larval period and use them as building blocks for the development of imaginal tissues. In all holometabolous insects investigated so far, amino acids and energy are supplied by proteins that have been selectively taken up by the fat body from the haemolymph (Haunerland, 1996; Burmester and Scheller, 1999). Most of the sequestered proteins belong to the family of hexamerins, haemocyanin-related proteins, named according to their composition of six identical or closely related subunits (Telfer and Kunkel, 1991). Hexamerins (often called larval serum proteins, storage proteins or arylphorins due to high content of aromatic amino acids) are high molecular weight multimers, usually hexamers. Each monomer is composed of subunits in the mass range of 70-90 kDa. This characteristic feature seems to be well retained in the several orders of holometabolous insects as well as in some orders of hemimetabolous insects (Levenbook, 1985; Rahbe *et al.,* 1990; Rehn and Rolim, 1990; Telfer and Kunkel, 1991; Martinez and Wheeler, 1993; Tojo

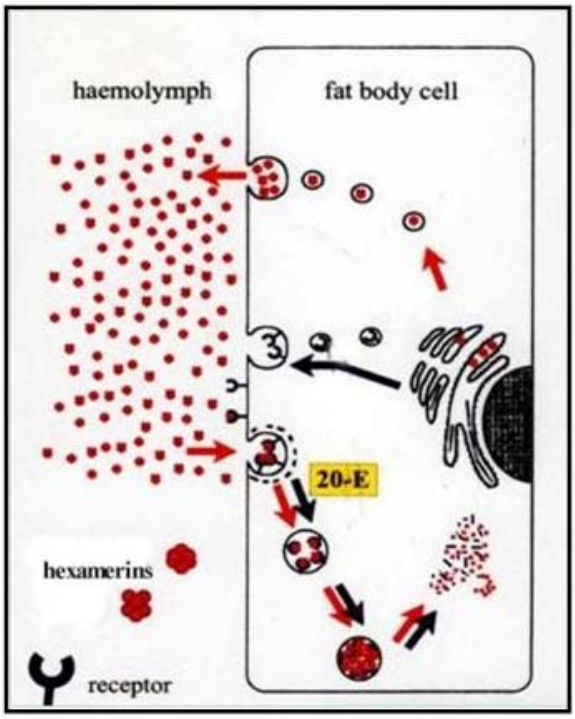

Fig. E Representation of hexamerins synthesis by the actively feeding larval fat body cells, its release into haemolymph and its uptake by the pupal fat body cells (shown in same cell) through a 20E dependent receptor mediated endocytosis. Modified from Haunerland (1996) and Burmester and Scheller (1999).

and Yoshiga, 1994; Haunerland, 1996). In *C. cephalonica*, the native protein is a hexamer (500 kDa) of three subunits with masses 86 kDa (Hex 1), 84 kDa (Hex 2) and 82 kDa (Hex 3) (Ismail, 1991; KiranKumar *et al.,* 1997; Nagamanju *et al.,* 2003). The hexamerins are synthesized predominantly by the fat body during the actively feeding larval period and are released into the haemolymph. During the last larval instar of lepidopteran insects, these proteins nearly account for 70-80% of the total soluble protein by weight (Kanost *et al.,* 1990; Telfer and Kunkel, 1991). Shortly before pupation, they are sequestered by the fat body through a receptor mediated endocytosis process and accumulate as dense protein granules to serve as a reserve pool of amino acids required for the remodeling of tissues and for deposition of cuticle during pupal-adult transformation and reproduction (Levenbook, 1985; Bean and Silhacek, 1989; Chrysanthis *et al.,* 1994; Haunerland, 1996; Burmester and Scheller, 1999; Lay *et al.,* 2004)

Endocytosis, mediated by cell-surface receptors, is an essential process in all eukaryotes and required for the uptake of various proteins (including hexamerins), hormones, nutrients and vitamins as well as for the recycling of membranes (Pierce and Robinson, 1990; Watts and Marsh, 1992; Schmid, 1995). The uptake of hexamerin from insect haemolymph by the fat body cells is a unique feature of the class "*insecta*" involving a receptor which does not belong to the low density lipoprotein (LDL) superfamily or to any other receptor known to date (Burmester and Scheller, 1997a, b, 1999).

Hexamerin receptor and role of 20E on its activation for hexamerin uptake-

The transport of hexamerins across the fat body cell membrane by the non-feeding prepupal and pupal stages requires the existence of a specific receptor. These receptors have been recognized in dipteran as well as lepidopteran insects but the sequences of the receptors are known only from the dipteran clan, notably from flesh fly *Sarcophaga peregrina* (Chung *et al.,* 1995), blow fly *Calliphora vicina* (Burmester and Scheller, 1995) and fruit fly *D. melanogaster* (Burmester *et al.,* 1999). These receptors show a significant similarity to their ligands, the hexamerins suggesting that the receptors evolved from their own ligands even before the divergence of winged insects (Burmester and Scheller, 1996; Burmester, 2002). In *S. peregrina*, a 120 kDa receptor was identified. Under the influence of 20E, the receptor acquires the ability to sequester hexamerin (Ueno and Natori, 1984; Chung *et al.,* 1995). Studies in *C. vicina* revealed that the hexamerin receptor is synthesized as a precursor (130 kDa), which is subject of a three-fold post-translational cleavage to give rise the active

receptor (Burmester and Scheller, 1997a). The onset of hexamerin uptake coincides with the third cleavage, which is initiated by ecdysteroids (Burmester and Scheller, 1997, 1999). Both in *S. peregrina* and *C. vicina,* the ecdysteroid mediated activation of the hexamerin receptor was found to be independent of transcription and protein synthesis suggesting that the receptor activation by hormone occurs at a post-translational level (Ueno and Natori, 1984; Burmester and Scheller, 1997). It has also been shown that a rise in the ecdysteroid titer at the end of larval life triggers the incorporation of hexamerins in the fat body of the fruit fly, *D. melanogaster* through fat body protein 1 (*Fbp-1*) receptor (Burmester *et al.,* 1999). Hansen *et al.,* (2002) reported the presence of an anterior fat body protein in *C. vicina*, which interacts with the hexamerin receptor and regulates hexamerin uptake by the fat body cells in the posterior part of the organ. The group further identified the binding domains of the receptor by yeast-two-hybrid system (Hansen *et al.,* 2002, 2003). These domains do not show any similarity to any functional protein domains known to date. However, the puzzling feature of the hexamerin receptor *i.e.,* the absence of a typical membrane-spanning domain, to explain how the receptor mediates endocytosis remains unclear.

In the bollworm, *Helicoverpa zea*, a single 80 kDa receptor protein was reported to mediate the uptake of VHDL and storage proteins (Wang and Haunerland, 1993; 1994a, b). Our laboratory has identified hexamerins in *C. cephalonica* and *Chilo partellus* and focused on the uptake of these proteins by the fat body and male accessory reproductive glands (MARG) (Ismail and Dutta-Gupta, 1990b, c, 1991; KiranKumar *et al.,* 1997). Using ligand binding studies, we have earlier demonstrated the presence of 120 kDa hexamerin receptor in the fat body membrane of *C. cephalonica* (KiranKumar *et al.,* 1997). The receptor was found to be present in the last larval instar and at maximal concentration in the prepupal stage. The sequestration of hexamerin in *C. cephalonica*, like in other lepidopteran insects, was not observed during the larval stage (Ismail and Dutta-Gupta, 1990b, c). However, 20E treatment induced a precocious uptake of hexamerins in the late-last instar (LLI) larval fat body (Ismail and Dutta-Gupta, 1990b). These studies suggest that ecdysteroid hormone activates the hexamerin receptor prior to hexamerin uptake.

Acid phosphatases: a general account-

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.

Autophagy and metamorphosis: regulation by ecdysteroids-

It is well established that metamorphosis in insects is the transition from the larval to adult stage and the events are controlled by ecdysteroids (Riddiford *et al.,* 2001; Truman and Riddiford, 2002; Gilbert *et al,* 2002). 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 (Lockshin and Bealuton, 1974; 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 such as ACPs increases and cause the lysis of larval tissues (Verkuil, 1980; Sass and Kovacs, 1980; Thummel, 2001; Lee and Baehriecke, 2001). The fat body that fills a large fraction of the insect body and whose function has been considered equivalent to the role of the vertebrate liver in the intermediary metabolism (Kunkel, 1981; Keeley, 1985; Vanishree *et al.,* 1999; Hansen *et al.,* 2002) shows high activity of lysosomal enzymes. The stimulation of the lysosomal activity by ecdysteroids is well demonstrated in several insects including *C. cephalonica* (Verkuil *et al.,* 1979; Verkuil, 1980; Ashok and Dutta-Gupta, 1988; Sass *et al.,* 1989; Kutuzova *et al.,* 1991). Studies also provide evidence that the induction in lysosomal activity by ecdysteroids is governed at a non-genomic level (Verkuil, 1979). However, no evidence has been provided to support the hypothesis. In the present study an attempt has been made to understand the activation of ACP by 20E in the fat body cells of the rice moth, *C. cephalonica*.

Why present study-

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 *M. 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 ACP activity, when haemolymph from 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 activity. Therefore an attempt was made in the present study to analyze the possible role of haemolymph factor(s) if any, in mediating the 20E action on selected marker proteins. Thus, a protein in the haemolymph named HP19 *i.e.,* haemolymph protein of mass 19 kDa was identified as a possible molecule in regulating few of the 20E mediated actions in some of the lepidopteran insect species.

Objectives of the present study-

Postembryonic development in insects involves growth, molting and metamorphosis. These events are controlled mainly by two morphogenetic hormones namely ecdysteroids and juvenile hormones. There is vast amount of information about the regulation of these hormones at synthesis as well as at its titer level. The information also exists about the interaction of these hormones among themselves to regulate metamorphosis. However, knowledge regarding the regulation of hormone dependent actions is limited and the field remains largely unexplored. In the present study, an attempt has been made to understand this important aspect of insect molting hormone, ecdysteroids. The study discusses the appearance of a stage and tissue specifically regulated protein HP19. This protein is identified in the present study and is found to be responsible for the regulation of few of the 20-hydroxyecdysone (20E) dependent actions.

The specific objectives of the dissertation work are given below:

1. Confirmation of the presence of factor(s) in *C. cephalonica* larval haemolymph required for 20E stimulation of acid phosphatase.
2. Understand the nature of the haemolymph factor, its isolation, characterization and developmental regulation.
3. Molecular characterization of HP19.
4. Role of HP19 in postembryonic development.
5. Effect on 20E regulated actions.
6. Mechanism of 20E regulated actions that are mediated by HP19.

Materials and Methods

Contents for Materials and Methods

Chemicals-

[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. Monoclonal anti-phosphotyrosine antibody (PY-99), polyclonal antibody against 14-3-3 (α and β subunits) and ubiquitin were procured from Santa Cruz Biotechnology, USA. The polyclonal antibody against GST of *Choristoneura fumiferana* was a generous gift of Dr. Subba Reddy Palli, University of Kentucky, USA. 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 kit were procured from QIAGEN. Bacterial strains, BM25.8 and Smart III^{TM} cDNA library construction kit were from Clontech Laboratories, USA. XL1-blue bacterial strain was from Stratagene, USA. Random primer labeling kit and restriction enzymes were from MBI Fermentas. Biotin labeling and streptavidin detection kit from Boehringer Mannheim, Germany. Whatman No. 1, 3 and cellulose phosphate P-81 sheets were procured from Whatman, UK. Agarose, cellophane sheets, IgG purification kit were purchased from Bio-Rad laboratories, USA. Tri-fast Gold RNA isolation reagent was from peqLab, Germany. Molecular weight cut-off membrane filters (YM-30 and YM-10) were 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, diolein, 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, phenylthiourea, phosphatidylserine, PMSF, PPO, POPOP, synthetic peptide substrates for kinase assay (syntide-2, MBP_{4-14}, and A-7433), streptomycin sulfate, TEMED, Triton X-100, tyrphostins *etc.,* were obtained from Sigma Chemical Co. USA. TC-100 insect culture medium was purchased from JRH Biosciences Inc. 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 *etc.,* were purchased from 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-

(A) Corcyra cephalonica (Stainton)-

It is 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).

Rearing method and life cycle of C. cephalonica-

The insects were reared in culture troughs that contained coarsely crushed sorghum seeds. Equal number of female and male moths was introduced 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 45-50 days. The final or last (Vth) larval instar is further classified into early (ELI), mid (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. The life cycles are shown in figures F and G.

Stages of C. cephalonica used 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 0.80-0.95 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 and haemolymph from these developmental stages were collected and used for various studies. (Please also refer figures F and G).

(B) 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-

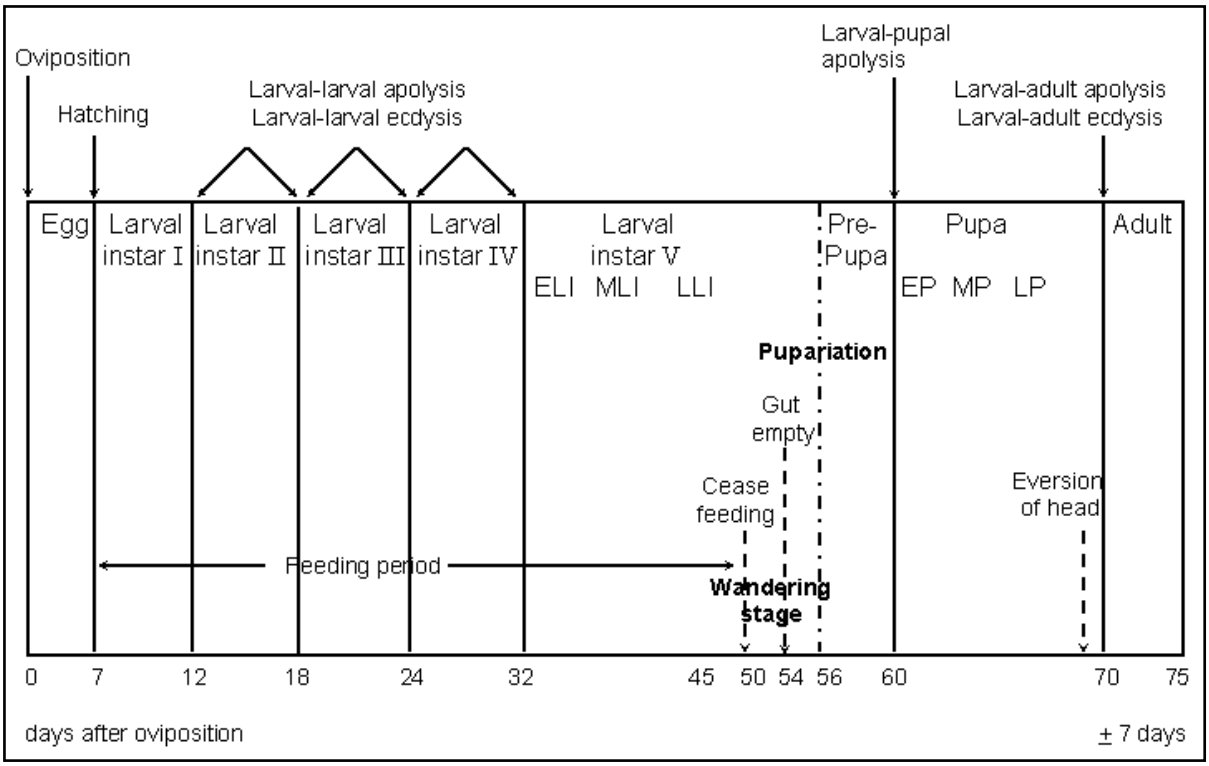

Fig. F: Schematic representation of the life cycle of rice moth, *Corcyra cephalonica*. ELI- early-last MLI- mid-last, LLI- late-last instar larvae, EP- early, MP- mid and LP- late pupae.

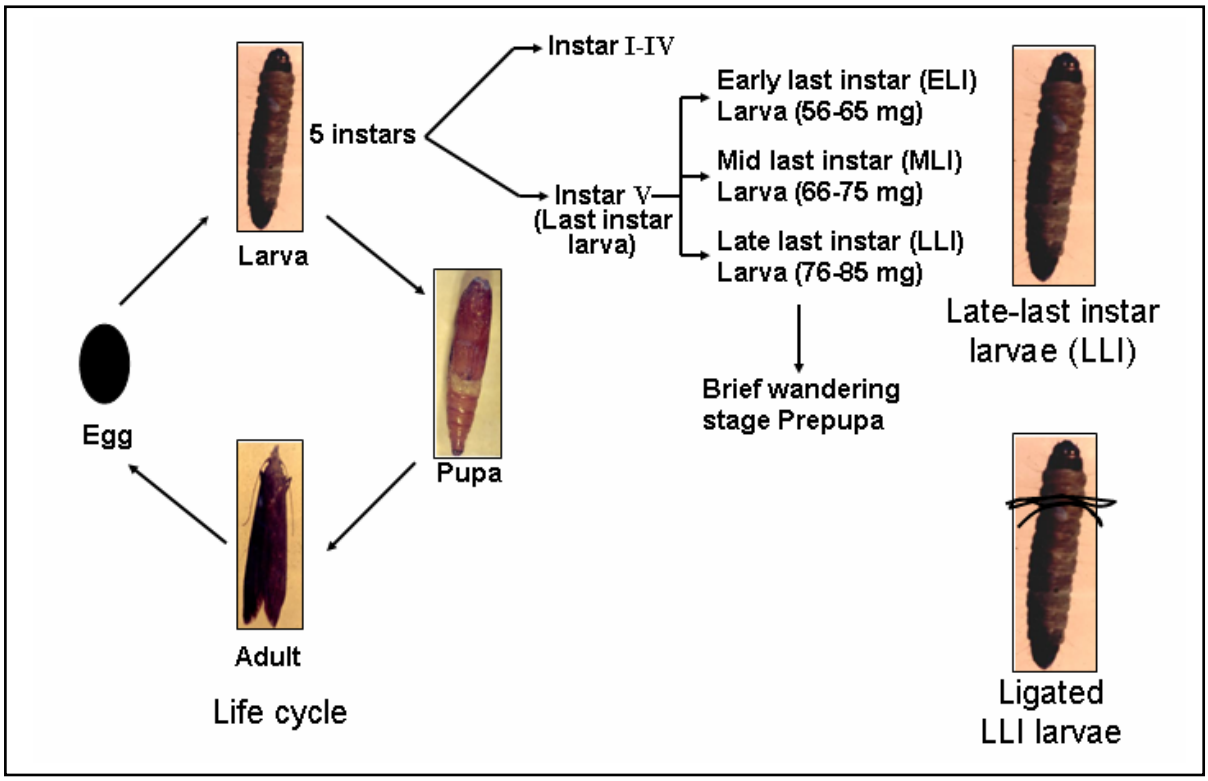

Fig. G: Simplified representation of the life cycle, classification of different developmental stages and thorax-ligation of rice moth *Corcyra cephalonica*. The LLI stage was predominantly used for most of the studies.

centre and were reared on fresh mulberry leaves under sterile conditions in insect culture room maintained at 26 ± 1°C, 60 ± 5% RH and 14:10 h LD photoperiod. Staging of *B. mori* 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). 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, late pupa (LP) 9-10 days old and freshly emerged moths (<12 h old) as adult (A). Fat body, CNS and haemolymph were collected from the required stage and used for various studies.

(C) Papilio demolius-

It is commonly known as lemon butterfly and belongs to the order Lepidoptera and family Papilionidae. The larval forms were collected from local citrus fields and maintained on fresh citrus leaves in insect culture room maintained at 26 ± 1°C, 60 ± 5% RH and 14:10 h LD period. Once again the last instar larvae were categorized as early-last instar (ELI- 1 to 2 days old), mid-last instar (MLI- 4 to 5 days old) and late-last instar (LLI- 7 to 8 days old). For certain studies prepupae (PP) were also used. Haemolymph from LLI larval stage was collected and used in the present study.

(D) Achaea janata-

It is commonly known as castor semilooper, belongs to the order Lepidoptera and family Noctuidae. The larval forms were collected from local castor field and maintained on fresh castor leaves in insect culture room at 26 ± 1°C, 60 ± 5% RH and 14:10 h LD photoperiod. Based on their age after the fourth ecdysis they were categorized as ELI (1 to 2 days old), MLI (5 to 6 days old) and LLI (8 to 9 days old). Haemolymph from LLI larval stage was used in the present study.

(E) Spodoptera litura-

It is commonly known as tobacco cut worm, belongs to the order Lepidoptera and family Noctuidae. It is a serious polyphagous pest of agricultural crops as well as vegetables. Second instar larvae were procured from Directorate of Oil seed Research (DOR),

Hyderabad, India and reared on fresh castor leaves. For the present study the haemolymph from the last instar larvae was used.

(F) Galleria mellonella-

It is commonly known as wax moth, belongs to the order Lepidoptera and family Galleridae. Studies with this insect were carried out during the visit to the laboratory of Prof. Klaus Scheller at Biocentre, Wurzburg University, Germany. The larvae were reared in constant darkness at 30°C on a semi artificial diet. The larvae within 0-12 h after ecdysis were regarded as day 1 larvae. The larvae of sixth instar preparing to ecdyse were recognized by head capsule slippage. The last instar (Vth instar) larvae were recognized by the size of the head capsule and general body pigmentation. This instar larva last approximately nine days and pupation occurs on the tenth day preceded by a short pharate pupal stage. For the present study haemolymph from the last instar instar larvae was used.

(G) Calliphora vicina-

It is commonly known as blow fly, belongs to the order Diptera and family Calliphoridae. Studies with this insect were carried during the visit to the laboratory of Prof. Klaus Scheller at Biocentre, Wurzburg University, Germany. The Robineau- Desvoidy larvae were reared on bovine meat at 23°C, 65% RH under a 16:18 h LD photoperiod. In order to synchronize development, the egg deposition was restricted to 30 min. Under these conditions, the 3rd larval instar starts 3 days after egg deposition, feeding period lasts to day 6 and is followed by the wandering stage. The pupae are formed on day 8 and adult eclosion follows after 10 additional days. For the present study the last (IIIrd) instar larval haemolymph was used.

Bacterial strains-

(A) XL1-Blue (Wood et al., 1985)-

In the present study *E. coli* bacterial strain XL1-Blue [*endA1*, *gyrA96*, *hsdR17*, *lac*$^{-}$, *recA1*, *relA1*, *supE44*, *thi-1*, (F` *lacl*^{q}*Z ΔM15*, *proAB*, Tn*10*)] was used. Tn*10* confers resistance to tetracycline. The LB/tet broth / agar (15 µg tetracycline per ml LB broth or agar) were used for library plating and screening. The LB broth (pH 7.0) contained 10 g/L bacto-tryptone, 5 g/L bacto-yeast extract and 5 g/L NaCl. For LB agar plate, 15 g/L agar was

included in the LB broth and autoclaved. The recombinants were selected by blue white (*β-galactosidase*) screening. The strain allowed regulated expression of cloned genes.

(B) BM25.8 (Palazzolo et al., 1990)-

This *E. coli* bacterial strain [*supE44*, *thi* Δ(lac-*proAB*) (F` *traD36*, $proAB^{+}$, $lac^{q}z$ *ΔM15*) *λimm434* (kan^{R}) *P1* (cam^{R}) *hsdR* (r_{k12}^{-} m_{k12}^{-}) was also used in the present study. It is lysogenic for phages λ and P1 and is used for automatic sub-cloning. The LB/kan broth or agar plates (50 μg kanamycin per ml LB broth or agar) were used for liquid culture or plating. The strain was used for *cre-lox*-mediated excision of pTriplEx2 from λTriplEx2.

Surgical manipulations and organ culture-

(A) Thorax-ligation-

This was carried using the last (Vth) instar larvae to deplete or reduce the endogenous hormone titer (Dutta-Gupta and Ashok, 1998). The appropriate larvae to be ligated were narcotized by placing them on ice. Ligation was done behind the first pair of prolegs by slipping a loop of silk thread (Ethicon, USA) around the head of the larvae (Ashok and Dutta-Gupta, 1991). The loop was adjusted behind the first pair of prolegs and gradually tightened (Fig. B). The tissues anterior to ligation were cut with sterile scissors and wound was dressed with traces of antibiotic mixture (penicillin and streptomycin) and phenylthiourea (0.025%). Finally it was sealed with wax (paraffin and beeswax, 10:0.1). These ligatures were kept in petridishes covered with moist filter paper to maintain humidity and to prevent desiccation.

(B) Microinjection-

The appropriate larvae to be microinjected were narcotized on ice and were injected with radioisotope [^{35}S] methionine or hormone using a microsyringe in a volume of 2-5 μl. The wound was dressed with traces of antibiotic mixture (penicillin and streptomycin) and phenylthiourea (0.025%). It was sealed using wax (paraffin and beeswax, 10:0.1).

(C) Organ culture-

Fat body or MARG tissues, dissected under sterile condition in ice cold insect Ringer were rinsed in 100 μl of TC-100 insect culture medium (JRH Biosciences, Inc. USA) with traces of streptomycin sulfate. This is followed by transfer to fresh 200 μl medium for 1 h as

preconditioning prior to the required experimental set up. Depending on the requirement of a specific experiment, the tissue was incubated in the culture medium along with other components for varying time periods ranging from 2-8 h at 25°C with gentle shaking. After required time incubation, tissues were rinsed in ice cold insect Ringer and processed for homogenate preparation. The choice of TC-100 insect culture medium for culture was due to the less complex composition compare to other insect media, contain no insect haemolymph (important for the present study), developed to support growth of lepidopteran cells in culture and is commonly used for insect tissue culture (Gardiner and Stockdale, 1975).

Protein sample preparation-

(A) Collection and preparation of haemolymph sample-

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 capillary tube 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.

(B) Preparation of homogenate protein sample-

A 2-5% homogenate protein sample of desired tissue from appropriate developmental stage was prepared from the ligated or unligated larvae of *C. cephalonica* as well as other insects. The tissues were rapidly dissected in cold insect Ringer (130 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2$ and 1 mM PMSF) and were homogenized in 150 µl 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 studies such as assay of enzymes (ACP, tyrosine kinase, CaM kinase II and PKC), PAGE, western & ligand blotting and phosphorylation studies.

(C) Preparation of membrane and other cellular fractions of fat body-

The fat body tissue from the LLI larvae was dissected in ice cold insect Ringer and homogenized on ice as mentioned earlier, 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 little volume of buffer B. This particular fraction was used as membrane preparation and stored at −70°C. The resultant supernatant was centrifuged further at 100,000 g for 30 min at 4°C and the pellet was washed once with buffer A and suspended in buffer B and used as microsomal fraction. The supernatant was used as cytosolic fraction. The membrane fraction of MARG tissue from late pupae or freshly emerged adults was also prepared in similar manner.

(D) Preparation of CNS protein sample-

This was prepared as described in Shanavas *et al.,* (1998). Intact CNS (brain + ventral nerve cord) were rapidly dissected from the insect of required stage, frozen in liquid nitrogen and stored at −70°C. Frozen tissue was homogenized (4 CNS/50 µl) in 50 mM HEPES buffer (pH 7.4) containing 1 mM EDTA and 1 mM DTT using all glass microhomogeniser (Kontes). Homogenates were centrifuged at 1,000 g for 5 min to remove debris. Freshly prepared homogenates were used for the phosphorylation reaction after protein estimation.

Macromolecular quantification-

(A) Protein-

Protein content in various samples was estimated according to the micro protein assay method of Bradford (1976).

Preparation of protein reagent- Ten mg of brilliant blue G-250 (Sigma) was dissolved in 5 ml of 95% ethanol. To this solution, 10 ml of 85% (w/v) orthophosphoric acid was added. The resulting solution was diluted to a final volume of 100 ml with distilled water, filtered through Whatman No. 1 filter paper and stored in an amber colored bottle at 4°C.

Procedure for protein estimation- An aliquot of the sample was taken into a tube and the volume was adjusted to 0.1 ml with 10 mM Tris-HCl (pH 7.4). To this 1 ml of protein reagent was added and mixed. After 10 min, absorbance at 595 nm was measured spectrophotometrically against a protein sample blank. The protein content in the sample was calculated using a standard curve prepared using BSA (fraction V).

(B) DNA and RNA-

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 determined 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 the ration of A_{260}/A_{280}.

(C) Preparation of sample for radiolabel quantitation-

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.

Hormone injection-

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. The LLI larvae were injected with 80 nM 20E (in 2 µl 0.05% ethanol) after the required period of thorax-ligation. Control insects received equal volume of carrier solvent (0.05% ethanol). The insects were sacrificed after the required period of hormone treatment and the visceral fat body was dissected in cold insect Ringer. Homogenates were prepared as mentioned above and were used for protein estimation and other studies.

Studies on fat bodies kept in culture-

The ribbon shaped 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

contained an equal volume of carrier solvent (10 µl 0.05% ethanol). To study the haemolymph effect, diluted (1:20) or fractionated haemolymph (~1 µg) or purified HP19 (40 ng) was added to the fat body culture in presence or absence of 20E. Studies with GSTs were carried by adding purified 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. To understand whether the factor(s) in haemolymph is proteinaceous in nature, the haemolymph samples (10 µl) were pretreated either with 1 µl of 10 N HCl or with 1 µl of 1 M NaOH or with 1 µg protease (V_8) for 20 min at room temperature or heated for 10 min at 100°C or precipitated with 100 µl of distilled ethanol and 10 µl of 5 M NaCl in ice for 15 min and then added to the fat body cultures to check its effect on enzyme activity. For ethanol precipitation, the precipitate was collected by centrifugation and dissolved in 10 mM Tris-HCl (pH 7.4). For co-culture studies the fat bodies were cultured with various tissues dissected from 24 h post-ligated LLI larvae in the above mentioned manner either in presence or absence of 20E.

Acid phosphatase (ACP) activity assay-

The assay was carried according to the method of Henrickson and Clever (1972) with slight modification. The reaction mixture contained 150 mM sodium acetate buffer (pH 5.0) and 20 µg of tissue homogenate. 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 µ moles 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 n moles of PNP released/h/µg protein.

[^{35}S] methionine incorporation studies-

In vitro incorporation of [^{35}S] methionine in fat bodies kept in culture was carried to compare the changes in the level of total protein synthesis and ACP activity stimulation in presence of 20E and HP19. The cultures were preincubated for 2 h with 10 µCi of [^{35}S] methionine (1000 Ci/mmol, BRIT, India) at 25°C, followed by incubations with 20E (80 nM) alone or HP19 (40 ng) alone or both along with actinomycin D (1 mM) or cycloheximide (1

mM) for 2 or 4 h. The incubated fat bodies were removed, homogenized as described above and equal amounts of protein were used for radiolabel quantitation after TCA precipitation.

Polyacrylamide gel electrophoresis-

(A) 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), on a 1 cm long, 2.1 % stacking gel (pH 6.8) followed by a 7.5 or 15 cm long, 12% resolving gel (pH 8.8) for resolution of HP19 and 10% (pH 8.8) for phosphorylation of fat body protein studies. Tris-glycine (25 mM Tris and 192 mM glycine, pH 8.3) with 0.1% SDS was used as the electrode buffer. Electrophoresis was carried out at 100 V until the tracking dye reached 1 cm above the base of the resolving gel. The protein sample was prepared by mixing an aliquot of the protein sample with sample buffer containing 0.125 M Tris-HCI (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.002% bromophenol blue followed by incubation at 100°C for 1 min.

(B) Non-denaturing (native) gel electrophoresis-

This was carried as described in Burmester *et al.,* (1999). For this, the haemolymph protein sample was prepared in the sample buffer containing, 120 mM Tris-citrate (pH 9), 2% sucrose and 0.05% bromophenol blue. The proteins were separated on a gel containing 12% acrylamide:N,N'-methylenebisacrylamide (30:1), 66 mM Tris-citrate, (pH 9), 0.05% ammoniumpersulfate and 0.045% N,N,N',N'-tetramethylenediamine. The electrophoresis was run in disodium tetraborate buffer (4 g/L) at 4 °C and 20 mA for 2-3 h.

(C) Silver staining of electrophoretically separated proteins on polyacrylamide gels-

This was carried according to the procedure of Blum *et al.,* (1987). The gel was incubated in fixative (50% methanol, 12% acetic acid and 50 µl of 37% formaldehyde/100 ml) for 1 h followed with 3 washes in 50% ethanol. Subsequently the gel was pretreated in sodium thiosulphate (20 mg/100 ml) for 1 min and rinsed thrice (20 sec each) with distilled water. The gel was impregnated in silver nitrate (0.2% with 187 µl of 37% formaldehyde) with gentle agitation for 30 min. The impregnated gel was rinsed with distilled water and developed with 6% sodium carbonate (w/v) and 50 µl of 37% formaldehyde (v/v). Finally, the stained gels were thoroughly rinsed with distilled water and stored in 50% methanol.

(D) Coomassie staining of polyacrylamide gels-

This was carried according to the method of Wilson *et al.,* (1983). The gel was incubated for staining in coomassie solution (0.025% brilliant blue- R250 in 40% methanol and 7% acetic acid) for 30 min. To visualize the reversible binding of stain to peptides, destaining with 5% methanol and 7.5% acetic acid was done to remove background staining.

Fractionation and purification of active haemolymph protein fraction, HP19-

(A) Fractionation of larval haemolymph proteins to determine the mass of active haemolymph fraction that mediates the 20E stimulated ACP activity in the fat bodies by gel filtration-

Total haemolymph protein (25 mg) from LLI larval stage of *C. cephalonica* was loaded on a pre-equilibrated (10 mM Tris-HCl, pH 7.4) sephadex G-50 column (1.6 x 90 cm) and eluted with the equilibration buffer. The single fractions were checked for their ability to enhance the ACP activity in LLI fat bodies kept in culture. The apparent molecular mass of the active fraction was determined by elution profile and gel electrophoresis. The calculation of approximate molecular mass range of the native 'active' haemolymph protein fraction that had the ability to mediate ACP activity from the elution profile was carried by comparing the relative elution of the different haemolymph protein fraction with that of protein markers in the MW-GF-200 kit (Sigma).

(B) Fractionation of larval haemolymph proteins active fraction for partial purification of active haemolymph protein using specific cut-off membrane fractionators-

Fractionation of total larval haemolymph protein was carried with specific molecular weight cut-off fractionators (Centricon- Amicon Inc. USA) utilizing the principle of retaining the high molecular mass protein in the retentate and allowing the low molecular mass protein pass into the filtrate portion along with the solvent under applied centrifugal force. For this, the haemolymph from appropriate larval stages, ligated or unligated (~100 larvae per fractionation) were collected into microfuge tubes precoated with 0.025% phenylthiourea. The haemolymph was diluted (1:20) with 10 mM Tris-HCl (pH 7.4) and was spun at 1,000 g for 3 min at 4°C to sediment the haemocytes and other debris. The supernatant *i.e.,* the haemolymph sample preparation (1 mg protein/ml) was first transferred into 30 kDa cut-off

fractionators (YM-30) and centrifuged at 4,000 g for 20 min at 4°C to strip the protein fractions with masses above 100 kDa. The resultant filtrate as well as the retentate were collected separately. Generally, to obtain the required molecular weight of a molecule, a filter size that is around 3 times the lower range is used (refer- Amicon technical bulletin). The filtrate with molecules <100 kDa (*i.e.,* the filtrate of 30 kDa) was again subjected to fractionation using 10 kDa cut-off fractionator (YM-10) for obtaining fraction of <30 kDa proteins. Once again, both filtrate and retentate were collected. Three fractions having masses above 100 kDa, 30-100 kDa and below 30 kDa thus obtained were used to test their effect on 20E dependent ACP activity as described above. Alternatively, in another approach to strip off the contaminating proteins from the total haemolymph protein, the samples were subjected to 60% ammonium sulfate precipitation at 4°C followed by centrifugation at 5,000 g for 20 min. The pellet thus obtained were resuspended in 10 mM Tris-HCl (pH 7.4) and transferred to 30 and 10 kDa cut-off fractionator to obtain the active haemolymph protein fraction of <30 kDa mass.

(C) Purification of active haemolymph protein fraction (HP19)-

On the basis of results on fractionation of total larval haemolymph protein from *C. cephalonica* on sephadex G-50 and specific molecular weight cut-off fractionators, the purification of active fraction was carried by salt precipitation, fractionation using specific cut-off membrane filters and gel filtration chromatography. For the purification of active fraction, haemolymph from ~500 LLI larvae was fractionated using 30 kDa cut-off fractionator as mentioned above. The <30 kDa protein fraction, which was obtained using a 10 kDa cut-off membrane fractionator during fractionation of haemolymph was available only in limited quantity and was insufficient to be used as a starting material for further purification. Hence, the filtrate from 30 kDa cut-off fractionator was used for further purification on sephadex G-50 column that was pre-equilibrated with 10 mM Tris-HCl (pH 7.4). The protein fractions were eluted with the same buffer. 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.

(D) Molecular mass determination-

The approximate molecular mass range of the native haemolymph protein fraction that had the ability to potentiate ACP activity was done by comparing the relative mobility of

the different haemolymph protein fraction eluates with that of marker proteins in the MW-GF-200 kit (Sigma) using sephadex G-50 column chromatography. The molecular masses of haemolymph protein fraction (HP19) as well as other proteins were determined by co-electrophoresing marker proteins of known masses followed by analysis using Ultra-Violet-Products (UVP) gel documentation system. The protein markers included myosin (205 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soyabean trypsin inhibitor (20.5) and lysozyme (14.3 kDa).

Production of polyclonal antibodies against HP19 and its confirmation-

(A) Electroelution of HP19-

The haemolymph from LLI larvae was collected and ~200 µg of total haemolymph protein per elution was resolved on a 15 cm long, 12% resolving SDS-PAGE with protein markers. A reference lane containing the same protein sample was coomassie stained to identify the band of interest. Prior to electrophoresis, the proteins of reference lane and the lane from which HP19 was electroeluted were treated exactly the identical way. The HP19 protein band thus separated and identified was excised from approximately sixty gels. The gel pieces containing HP19 band were placed in a dialysis tube (10 kDa cut-off) individually with 25 mM Tris, 192 mM glycine and 0.1% SDS. The horizontal electrophoretic tank was also filled with the same buffer and run for 3 h at 70 V. During the last 1 minute of the run, the flow of current was reversed. The eluted proteins along with the buffer from the dialysis tube were drained out into a microfuge tube and equilibrium dialyzed against 10 mM potassium phosphate buffer (pH 7.4) to remove SDS from the protein sample. These protein samples were lyophilized and used to raise antibody. Alternatively, the electroelution of HP19 protein was also carried using model-422 electroeluter (Bio-Rad).

(B) Production of polyclonal antibodies against HP19-

The antibody was raised against the HP19 protein that was electroeluted after slicing the protein band resolved on 12% SDS-PAGE. Three month old male rabbits (New Zealand variety) were injected with 100 µg of electroeluted HP19 (emulsified with 500 µl of complete Freund's adjuvant) by subcutaneous injections into various sites on the back. Prior to injection, the lateral ear vein was bled to collect pre-immune serum. After a fortnight, 1st booster injection was given followed by a second booster injection after seven days. For booster injections, 50 µg protein emulsified with Freund's incomplete adjuvant was used.

The blood was collected after a week of 2nd booster injection. The collected blood was left overnight at 4°C for clotting and serum was obtained by centrifugation at 5,000 g for 20 min. The serum was aliquoted and stored at −20 °C after adding 25% glycerol and 0.001% azide.

(C) Immunodiffusion-

This was performed according to the methods of Ouchterlony (1959) on a glass slide using 1% agarose in phosphate buffered saline (PBS) with 0.002% sodium azide. The antigen (20 μg purified protein) was loaded on to central well whereas the serially diluted antiserum was spread into different surrounding wells. The immunodiffusion was allowed for 36-48 h at 4°C in a humified condition until a clear precipitin line was observed.

(D) Purification of IgG-

The IgG fraction of HP19 antibody was purified by affinity chromatography using protein A-agarose column (Bio-Rad) according to the manufacturer's protocol. The binding of F_c regions of IgG to protein-A in the crude mixture of serum proteins was utilized for the purification. The serum protein was diluted (1:1) with the wash or binding buffer (20 mM sodium phosphate (pH 7.4) and 150 mM NaCl) and was loaded on to the protein A-agarose column prequilibrated with wash or binding buffer. This was followed with the washing of column using the same buffer until the absorbance of eluates at 280 nm approaches the background level. The bound IgG fraction was eluted with 4 column volumes of elution buffer (100 mM glycine-HCl, pH 3.0) to tubes that contained 100 μl of neutralization buffer (1 M Tris-HCl, pH 9.0) for immediate neutralization of the eluted purified IgG fraction. The purified IgG fraction was lyophilized and stored at −20°C till further use.

(E) Immunoprecipitation of HP19-

This was carried using protein A-agarose affinity matrix (Boehringer Mannheim) according to the manufacturer's protocol. For this, the haemolymph from LLI larvae (1:10 in 10 mM Tris-HCl, pH 7.4) was preadsorbed by incubating with homogenous protein A-agarose suspension (25 μl bed volume) for 4 h at 4°C with gentle shaking. To this, different dilution of anti-HP19 IgG was added and incubated for additional 1 h at 4°C. The mixture was further incubated with addition of 50 μl of homogenous protein A-agarose suspension for 4 h at 4°C with gentle shaking. The immuno-complex was collected by centrifugation for 30 sec at 12,000 g. The supernatant was collected separately (termed immuno-supernatant) and

was also used to check its ability to mediate the 20E dependent ACP activity. The beaded pellet was resuspended and washed thrice in 3 different buffers *i.e.,* buffer I (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 5 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF, 0.7 μg/ml pepstatin and 1 mM EDTA), high salt buffer II (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.1% NP-40, 0.05% sodium deoxycholate) and low salt buffer III (50 mM Tris-HCl pH 7.5, 0.1% NP-40, 0.05% sodium deoxycholate). Each wash was followed by incubation for 20 min at 4°C with gentle shaking and centrifugation for 30 sec at 12,000 g. After the final wash, the supernatant was removed and the beaded pellet *i.e.,* the immunoprecipitate was suspended in SDS sample buffer, incubated at 100°C for 30 sec and centrifuged at 10,000 g to sediment protein A-agarose. The supernatant was then subjected to SDS-PAGE to check the specificity of immunoprecipitation. These beaded pellets *i.e.,* the immunoprecipitates were also suspended in 10 mM Tris-HCl (pH 7.4) and used for its ability to mediate 20E dependent ACP activity.

(F) Functional assay of HP19 antibody to check its specificity against HP19-

As the HP19 antibody was raised after electroeluting the 19 kDa protein from the total haemolymph protein resolved on denaturing PAGE, couple of functional assay was performed to confirm if the antibody generated is specifically against HP19 that mediates the 20E dependent ACP activation. In one of the experiment, the fat bodies kept in culture were incubated with 80 nM 20E (in 0.05% ethanol), diluted (1:20) haemolymph from LLI larvae and different dilutions of anti-HP19 IgG fraction for 4 h at 25˚C**.** In another experiment, the HP19 present in the haemolymph of LLI larvae was first immunoprecipitated followed by addition of either the precipitate (immuno-complex) or the resulting supernatant (termed immuno-supernatant) to the fat bodies kept in culture along with 80 nM 20E for 4 h at 25˚C**.** The immunoprecipitation of HP19 from a fixed dilution (1:20) of total haemolymph protein was carried with various dilutions of anti-HP19 IgG fraction using protein A-agarose affinity beads (Boehringer Mannheim) as described in the manufacturer's protocol. The 1:10 dilution of anti-HP19 IgG fraction contained 10 μg of IgG from which the antibody was serially diluted with 10 mM Tris-HCl (pH 7.4).

Western blotting and immunostaining-

The electrophoretically separated polypeptides were transferred (electro-blotted) to nitrocellulose membrane using Trans-Blot apparatus (Bio-Rad) according to the procedure of

Towbin *et al.,* (1979). For this, the gel was first equilibrated in Towbin buffer (25 mM Tris, 192 mM glycine and 20% methanol) for 30 min followed by transfer to the membrane for 3 h at 70 V with 250 mA current limit. In case of native gel, methanol was omitted from the Towbin buffer and transfer was carried at 4°C. The transfer of protein to membrane was checked by reversible Ponceau S staining (100 mg Ponceau S in 5% acetic acid). The stain was removed by 3-4 washes with TBST *i.e.,* Tris buffered saline with Tween-20 [10 mM Tris-HCl pH 7.4, 150 mM NaCl and 0.1% Tween-20 (v/v)]. For immunostaining, the protein blot was processed with 3% BSA (w/v) in TBST for 1 h at room temperature to block the non-specific binding sites followed by washing with TBST (10 min x 5 changes). The blot was then incubated with the primary antibody diluted in TBST containing 3% BSA (w/v) for 2 h to overnight. This was again followed by a through wash in TBST (10 min x 5 changes). Thereafter, the blot was incubated with alkaline phosphatase (ALP) conjugated anti-mouse or anti-rabbit IgG for 1 h. Once again the blot was washed in TBST (10 min x 5 changes). The visualization of the specific cross-reactivity was carried with the substrates of ALP *i.e.,* NBT/BCIP (0.0033% nitroblue tetrazolium and 0.0165% 5-bromo-4-chloro-3-indolyl phosphate in 10 mM Tris-HCl pH 9.5, 5 mM $MgCl_2$ and 10 mM NaCl) for color reaction.

In situ immunodetection-

This was carried to show the localization of HP19 in the larval body of *C. cephalonica*. For this, the LLI larvae were dissected through the dorsal surface and were fixed in Carnoy's fixative (ethanol:chloroform:acetic acid- 6:3:1) for 4 h at room temperature. The tissues in the larvae were permeabilised by 3 changes of 0.1 M PBS (pH 7.4) with 0.5% Triton X-100 and 0.002% sodium azide for 24 h (Webster, 1998) followed by dehydration in a water-ethanol series. Immunostaining of these larvae were carried by treating with IgG fraction of HP19 antibodies (1:200 dilutions in blocking solution) for 24 h at 4°C with gentle shaking. The insects were then incubated with anti-rabbit IgG coupled with ALP for 2 h. Each antibody treatment was followed by three washes of TBS. Finally, the larvae were processed with the substrates of ALP (NBT/BCIP) for color reaction. Immunostained HP19 was visualized under the microscope (Wild Heerbrugg). The specificity of antibody was checked by using pre-immune rabbit serum.

Injection of antibodies for immuno-complexing HP19 in vivo-

Anti-HP19 IgG fraction (15 μg in 5 μl PBS per insect) was injected to the last (Vth) instar larvae through the dorsal surface using microsyringe. Control insect received identical

injection of pre-immune IgG fraction. These larvae along with other control such as uninjected and insect Ringer injected larvae were placed on diet (sorghum) and allowed to grow under normal insect culture room condition. Various parameters such as morphological, behavioral, and biochemical changes like ACP activity in fat body, protein profile of fat body as well as haemolymph and the sequestration pattern of hexamerins were analyzed on different days, after HP19 antibody injection. A comparison was made between the experimental and various control groups. The data presented here with reference to control insect is only shown for the pre-immune IgG fraction injected larvae, because the results obtained with other controls *i.e.,* uninjected and insect Ringer injected larvae were more or less the same as obtained with pre-immune IgG fraction injected control insect larvae.

Histological studies with fat body-

The fat body from various control as well as the HP19 antibody injected larvae were fixed in Carnoy's fixative (ethanol:chloroform:acetic acid- 6:3:1) for 4 h at room temperature. These tissues were then dehydrated in a water-ethanol series and embedded in paraffin. The sections (5 μm thick) were cut on microtome (Leica) and mounted on glass slides coated with Meyer's albumin (ovalbumin:glycerol:water- 1:1:1). The sections were deparafinized by three changes of xylene and were rehydrated in ethanol-water series just prior to the processing of slides for staining. The staining with haematoxylin and eosin was carried by first treating with nuclear stain haematoxylin (1% in 10% ethanol) for 30 min followed by a brief wash with water and differentiation under microscope. The same slide was subsequently stained with cytosolic stain eosin (1% in 10% ethanol). This was done by dehydrating the slide in water-ethanol series. After the slides were treated with 70% ethanol, it was stained with eosin for 10 min followed by quick dehydration with 90% ethanol and absolute ethanol. The slides were finally dipped in xylene and mounted with DPX mountant (A synthetic resin mixture of a polystyrene- distyrene, a plasticizer- tricresyl phosphate and xylene). The visualization of stain was done on Nikon Labophot-2 microscope.

Immunohistochemical studies-

Immunohistochemical staining of fat body sections were carried using either polyclonal antibodies against *C. cephalonica* HP19 or hexamerins. The localization of phosphotyrosine in fat body sections was carried with mouse monoclonal phosphotyrosine antibody (Santa Cruz Biotechnology). The deparafinized tissue sections were first treated with blocking solution (2% BSA and 1% non-immune goat serum in 50 mM TBS with 0.1%

Triton X-100) for 1h at 4°C. This was followed by antibody treatment (1:250 dilutions in blocking solution) for 24 h at 4°C with gentle shaking. The slides were then treated with anti-rabbit IgG coupled with ALP for 1 h. Washing after each step was done with three changes of TBS. These slides were finally processed for staining by NBT/BCIP color detection method and mounted in glycerol gel (50% glycerol, 7.5% gelatin and 0.1% azide in 0.1 M TBS) for visualization on Nikon Labophot-2 microscope (Meltzer *et al.,* 1997). The specificity of antibody was checked by processing parallel tissue sections using pre-immune serum.

Phosphorylation studies-

(A) In vitro phosphorylation of proteins-

In vitro phosphorylation of homogenate / membrane / other cellular fractions of *C. cephalonica* fat body and other tissues were carried as described in Shanavas *et al.,* (1998) with slight modification. A 40 µl reaction mixture contained 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, 10 µM ATP, 10 µM sodium orthovanadate and 20 µg of protein sample. When required EGTA (1 mM), $CaCl_2$ (1 mM), 20E (80 nM), phosphatidylserine (100 µM) and diolein (10 µM) were also added. The phosphorylation was initiated by the addition of 4 µCi of [γ^{32}P] ATP (3,000 Ci/mmol, BRIT, India) and was terminated after 1 min with 20 µl of SDS sample buffer (0.188 M Tris-HCl pH 6.8, 6% SDS, 30% glycerol, 15% 2-mercaptoethanol and 0.003% bromophenol blue) followed by immersion in boiling water for 3 min. The samples were centrifuged at 10,000 g for 5 min, the supernatant that contained phosphorylated proteins (10 µg) were subjected to 10% SDS-PAGE. The gels were silver stained, vacuum dried and exposed to Kodak X-Omat AR film at −70°C for autoradiography. To check the effect of HP19 on the phosphorylation of fat body proteins, *in vitro* phosphorylation reactions were also carried either by preincubating the fat bodies kept in culture or the homogenate in absence or presence of 20E (80 nM) with 40 ng HP19.

(B) In vitro phosphorylation of CNS proteins-

In vitro phosphorylation of CNS proteins were carried according to the method of Combest and Gilbert (1986) as per the modification of Shanavas *et al.,* (1998). A 40 µl reaction mixture contained 50 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, 10 µM ATP, 1 mM EGTA or 1 mM $CaCl_2$ and 20 µg of homogenate protein. The mixture was preincubated for 5 min at 30°C for temperature equilibration and the reaction was initiated by

the addition of 4 μCi of [γ^{32}P] ATP (3000 Ci/mmol). After 1 min of incubation, the reaction was terminated by the addition of 20 μl of 3X SDS sample buffer (0.188 M Tris-HCl pH 6.8, 6% SDS, 30% (v/v) glycerol, 15% 2-mercaptoethanol and 0.003% bromophenol blue) and immersion in boiling water for 2 min. The phosphorylated proteins (10 μg) were subjected to 10% SDS-PAGE and the gels were silver stained. For autoradiography, the stained gels were vacuum dried and exposed to Kodak X-Omat AR film at −70°C.

(C) Back phosphorylation of fat body proteins-

This was carried as described by Forn and Greengard (1978). Intact fat bodies from two 24 h post-ligated LLI larvae were dissected and rinsed thoroughly in 200 μl of TC-100 insect culture medium with traces of streptomycin sulfate. The tissue was transferred to fresh medium containing 80 nM 20E (in 10 μl 0.05% ethanol) and / or diluted or fractionated or purified HP19 (in 10 μl of 10 mM Tris-HCl, pH 7.4) and incubated for 4 h at 25°C with gentle shaking. An equal volume of carrier solvent was added to the control cultures. At the end of incubation, the fat body was removed, rinsed thoroughly with ice cold insect Ringer, homogenized and subjected to *in vitro* phosphorylation as described above.

(D) Preparation of active sodium orthovanadate solution-

Active sodium orthovanadate is a potent inhibitor of tyrosine phosphatase and was prepared according to the method of Gordon (1991) in order to have least interference in the tyrosine kinase mediated phosphorylation of hexamerin receptor. The activation refers to the depolymerization of vanadate. A 200 mM solution of sodium orthovanadate was prepared and adjusted to pH 10.0 using 1 N NaOH. At this pH, the solution is in yellow color which is boiled till it is colorless followed by cooling at room temperature. The pH was readjusted to 10 and repeat boiling and cooling was carried till the solution became colorless at this pH. The active inhibitor was aliquoted and stored at −20°C till further use.

Autoradiography-

This was employed for detection of [^{32}P] labeled polypeptides or nucleic acids. The stained gels were sandwiched between cellophane sheets and dried under vacuum at 80°C for 1 h using a Hoefer gel drier. The hybridized Southern and northern blots were wrapped in a saran wrap. These gels or blots were exposed to Kodak X-Omat X-ray film for 1-3 days

depending on the radiation counts on Geiger Muller counter using DuPont Cronex intensifying screens at −70°C and were developed and fixed as per standard procedure.

Fluorography-

This was employed for the detection of [^{35}S] methionine labeled polypeptides or proteins on SDS-PAGE and was carried according to the procedure of Bonner and Laskey (1974). The gel was immersed first in excess of DMSO for 2 h followed by second immersion in the same for 2 h. The gel was then incubated in scintillator solution (24.8 g PPO in 100 ml of DMSO) for 2 h followed with several washes in distilled water till there was no floating PPO. Finally the gel was sandwiched between cellophane sheets and was dried under vacuum as described above.

Assay of protein kinases-

(A) Assay of CaM kinase II in C. cephalonica fat body homogenates-

This was assayed by the phosphorylation of synthetic peptide substrate syntide-2 (Pro-Leu-Ala-Arg-Thr-Leu-Ser-Val-Ala-Gly-Leu-Pro-Gly-Lys-Lys) according to the method of Fukunaga *et al.,* (1989). The 50 µl reaction mixture contained 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 0.1 mM [γ^{32}P] ATP (2000-4000 cpm/pmol), 30 µM syntide-2, 2 µM calmodulin, 1 mM $CaCl_2$, and 20 µg of homogenate protein. A control reaction was carried in the presence of 1 mM EGTA. After incubation at 30°C for 1 min, the reaction was terminated by the addition of 10 µl of 0.4 M EDTA. The radioactivity was determined as described by Roskoski (1983). An aliquot (50 µl) was spotted on to 2 x 2 cm phosphocellulose strips (Whatman P-81). The strips were immersed in 75 mM phosphoric acid (10 ml per strip) and swirled gently for 2 min. The phosphoric acid was decanted and the phosphocellulose strips were washed twice (1 min each) in 75 mM phosphoric acid with gentle agitation. After drying the strips, the radioactivity was measured by liquid scintillation spectrophotocounter using toluene scintillation fluid (0.5 g POPOP, 5 g PPO/L of toluene). The assay conditions for the present study were standardized using fat body homogenates from LLI larvae.

(B) Assay of tyrosine kinase in C. cephalonica fat body homogenates-

This was done according to the methods of Casnellie *et al.,* (1982) and Pike *et al.,* (1986). The reaction mixture (25 µl) contained kinase buffer (1 mg/ml BSA, 30 mM Tris-

HCl (pH 7.5), 20 mM $MgCl_2$ and 5 mM $MnCl_2$), 80 μM ATP, 3500-4000 cpm/pmol $\gamma^{32}P$ ATP, 30 μg of homogenate protein and 40 μM substrate (synthetic peptide A-7433, Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly). The reaction was initiated by the addition of tissue homogenate. The controls used for the assay were homogenate and substrate blank. After incubation at 30°C for 2 min, the reaction was terminated by the addition of 20 μl of 10% ice cold TCA and spun for 2 min. The supernatant (35 μl) was spotted on to 2 x 2 cm phosphocellulose strips (Whatman P-81) and the radioactivity was determined as discussed in CaM kinase II assay using Packard Ultima-Gold scintillation fluid (65-70% Di-isopropylnaphtalene, 12-15% ethoxylated alkylphenol, 9-12% mono/di-phosphate ester, 1-2% sodium di-octylsulphosuccinate, 0-1% PPO and 0.1% 1,4-bis(2-methylstyryl) benzene.

(C) Assay of protein kinase C (PKC) in C. cephalonica fat body homogenates-

The PKC activity was assayed according to the procedure of Yasuda *et al.,* (1990) with slight modification. The synthetic peptide used for the assay was $pGLU^4$- myelin basic protein fragment 4-14 (MBP_{4-14}) of sequence pGLU-LYS-ARG-PRO-SER-GLN-ARG-SER-LYS-TYR-LEU. A 50 μl reaction mixture contained kinase buffer (20 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$ and 2 mM DTT), 25 μM ATP, 8,000-10,000 cpm/pmol [$\gamma^{32}P$] ATP, 0.1 mM $CaCl_2$, 10 μM diacylglycerol (diolein), 100 μM phosphatidylserine, 50 μM synthetic peptide MBP_{4-14} and 30 μg of fat body homogenate. A control reaction was carried in the presence of 1 mM EDTA with no $CaCl_2$ and lipid micelles. The reaction was initiated with the addition of [$\gamma^{32}P$] ATP after incubation at 30°C for 15 min and was terminated by the addition of stop solution (0.1 M ATP + 0.1 M EDTA). A 50 μl aliquot was spotted on to 2 x 2 cm phosphocellulose P-81 strips (Whatman) and radioactivity was determined as described in Roskoski (1983). The strips were washed 4 times in 0.4% orthophosphoric acid for 10 min each. This was followed by washing of the strips in excess of acetone and then allowed to dry. After drying the radioactivity was determined as discussed above using Packard Ultima-Gold scintillation fluid. One unit of PKC was defined as the amount of enzyme, which incorporated 1 n mol of phosphate from [$\gamma^{32}P$] ATP into MBP_{4-14} per minute under the respective conditions.

(D) Preparation of lipids for PKC assay-

The lipid micelles were prepared by the method of Takai *et al.,* (1979). Appropriate amount of phosphatidylserine (25 mg) and diacylglycerol *i.e.,* diolein (50 mg) were mixed in

little volume of chloroform. The chloroform was evaporated under the stream of nitrogen. The lipid was then suspended in 20 mM Tris-HCl (pH 7.4) and vortexed vigorously for 2 min. The suspension was sonicated until clear solution was obtained. Appropriate volume of this lipid micelle stock was used to a final concentration of 100 μM phosphatidylserine and 10 μM diolein.

Isolation of nucleic acids-

(A) 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 14-16 h and the DNA was isolated using QIAGEN plasmid isolation kit. The cells were collected after centrifugation at 3,000 g for 10 min and suspended in 250 μl of P1 buffer (100 mM Tris-HCl pH 7.5, 300 mM NaCl, 10 mM EDTA, 0.2% (w/v) BSA and 20 mg/ml RNase A). To the suspension, 250 μl of P2 buffer [30% polyethylene glycol (PEG 6000) and 3 mM NaCl) was added, inverted gently 4-5 times and incubated at room temperature for 5 min. This was followed by addition of 100 μl of buffer N3 (100 mM NaCl, 100 mM Tris-HCl pH 7.5 and 25 mM EDTA) and mixing by inversion for 4-5 times. The mix was centrifuged at 3,000 g for 10 min to separate supernatant from compact white pellet that contained DNA and was loaded on to QIAprep column. The column was then washed with 750 μl of PE buffer (10 mM NaCl, 50 mM MOPS pH 7.0 and ethanol phase). The plasmid DNA was eluted with 50 μl of buffer EB (10 mM Tris-HCl, pH 8.0 with 1 mM EDTA).

(B) Genomic DNA isolation-

The genomic DNA from total body of LLI larvae was isolated as described in Birren *et al.,* (1997). The narcotized larvae (~1.5 g) were rinsed in sterile insect Ringer, dried on Whatman-1 filter paper and were then grinded in a mortar half filled with liquid nitrogen. The pulverized tissue was transferred to a centrifuge tube and excess of liquid nitrogen were allowed to evaporate. To this, sucrose-proteinase K cell lysis buffer (27% sucrose, 1X SSC, 1 mM EDTA, 1% SDS and 200 μg/ml proteinase K) was added (1 ml/100 mg tissue) and mixed thoroughly. The lysate was incubated overnight at 37°C to allow the release of chromosomal DNA. The viscous lysate was transferred to a fresh tube to which, 10 ml of equilibrated phenol, chloroform and iso-amyl alcohol (25:24:1) mix was added followed by

centrifugation at 6,000 g for 10 min at room temperature. The upper aqueous layer was carefully removed and re-extracted with 5 ml of equilibrated phenol, chloroform and iso-amyl alcohol (25:24:1) mix. The upper aqueous phase was again collected, to which equal volume of chloroform was added, mixed thoroughly and centrifuged at 5,000 g for 5 min at room temperature to remove the residual phenol from the aqueous phase. To the aqueous phase, $1/10^{th}$ volume of 3 M sodium acetate was added and mixed thoroughly. Equal volume of iso-propanol was added to this mixture and incubated for 30 min at −20°C. The DNA was collected after centrifugation at 12,000 g for 15 min at room temperature. The DNA pellet was washed twice with 70% ethanol followed by drying under vacuum for 5 min. The genomic DNA was dissolved in small volume of TE buffer (10 mM Tris-HCl pH 8.0 with 1 mM EDTA) and stored until use.

(C) RNA isolation-

The desired tissue was dissected under sterile conditions in RNase free 10 mM Tris-HCl (pH 7.4). It was rinsed in the same buffer and homogenized (~2-4 mg tissue) in 1 ml of Tri-Fast Gold buffer (peqLab, Germany). 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.

Agarose gel electrophoresis-

(A) Sample preparation for plasmid DNA electrophoresis-

An estimated amount of plasmid DNA sample (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.

(B) Agarose gel electrophoresis for plasmid DNA samples-

The plasmid DNA was electrophoresed on 1% (10 cm long) agarose gel. The gel was polymerized using 1X TAE (40 mM Tris-acetate and 1 mM EDTA). The required quantity of agarose was suspended in appropriate volume of 1X TAE and boiled for solubilisation. The solution was allowed to cool till 60-65°C to which, ethidium bromide (0.5 µg/ml) was added. This was followed by polymerization on the horizontal gel electrophoresis system. The electrophoresis was carried using the same buffer at voltage $5V/cm^2$ until the dye reached $3/4^{th}$ of the length of the gel. All the ethidium bromide gels were visualized using UV-transilluminator and analyzed using UVP-gel documentation system.

(C) Sample preparation for genomic DNA electrophoresis-

The samples for genomic DNA, digested or undigested for electrophoresis on 0.8% agarose gel were prepared as mentioned above. The digestion of genomic DNA was carried overnight at 37°C with the chosen restriction enzymes.

(D) Agarose gel electrophoresis for genomic DNA samples-

The genomic DNA was electrophoresed on 0.8% agarose as mentioned above for plasmid DNA agarose gel electrophoresis. An agarose gel (0.8%) was polymerized on a 15 cm long horizontal gel electrophoresis system and electrophoresis was carried until the dye reached the end of the gel.

(E) 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 (0.2 M MOPS, 80 mM sodium acetate and 10 mM EDTA) 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.

(F) Agarose gel electrophoresis for RNA samples-

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/100ml) 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 and analyzed using UVP-gel documentation system.

Cloning of C. cephalonica HP19 (CcHP19) cDNA by immunoscreening of HGLFB expression library-

The strategy for cloning of HP19 is outlined in figure 22 of Chapter-II in results section.

(A) Choice of λTriplEx2 vector and SMART technology (Clontech, USA) for library construction-

The several advantages of λTriplEx2 vector made it the vector of choice for cloning. The vector offers the advantages of directional cloning, eliminates adaptor ligation and improves cloning efficiency. It provides all the advantages of directional cloning with a phagemid vector including easy conversion from phage to plasmid. In addition to this every cDNA inserted into the multiple cloning sites (MCS) of λTriplEx2 vector is expressed in all the three open reading frames (ORFs) that makes the library suitable for expression-based screening including immunoscreening methods in *E. coli*. The directional cloning with this vector provides a mean of retaining longer 5` end sequences as the vector lacks T4 adaptor ligation resulting in a loss of 1-54 bases at the 5`end.

The SMART technology is designed to maximize 5` end sequence representation in library construction using small amounts of starting material. It utilizes a modified oligo (dT) primer (CDIII primer) to begin reverse transcription and employs unique SMART III^{TM} oligonucleotide for template extension. The oligo serves as a universal PCR priming site for long-distance PCR (LD-PCR). Thus only single stranded cDNA with SMART oligo sequence at their 5` ends are amplified while amplification of incomplete cDNA is eliminated. Therefore a higher percentage of full-length double stranded cDNA is obtained.

(B) Preparation of HGLFB-cDNA expression library-

The tissue HGLFB was dissected in a drop of 10 mM Tris-HCl (pH 7.4). Total RNA was isolated using the Tri-Fast kit (peqLab, Germany). The cDNA from this RNA was

generated and amplified using the SMART III^{TM} cDNA library construction kit (Clontech, USA). The cDNA was synthesized from the HGLFB RNA using SMART^{TM} PCR cDNA synthesis kit (Clontech, USA). The cDNA was amplified by LD-PCR, cleaned by proteinase K treatment, digested with *Sfi I*, size fractionated by column chromatography and ligated into the λTriplEx2 vector arms followed by packaging in Gigapack^{R} III Gold II packaging extract (Stratagene, USA). The packaged library was titered and the titer of the unamplified library was found to be 3 x 10^6 plaques forming unit (pfu)/ml. The unamplified library was then amplified, titered (6 x 10^9 pfu/ml) and stored at −70°C in 50% DMSO until further use. The experimental procedures for library construction and amplification were carried as described in Clontech user manual PT3003-1 (www.clontech.com). Packaging of phages was done according to instructions of Gigapack^{R} III Gold II packaging extract (Stratagene, USA).

(C) Immunoscreening of expression library for HP19 cDNA clone-

For identification of cDNA clones encoding HP19, immunoscreening was carried as described by Sambrook *et al.,* (1989). A fresh overnight culture of XL-1Blue host cells was prepared in 15 ml LB/$MgSO_4$/maltose (LB broth with 10 mM $MgSO_4$ and 0.2% maltose). The bacterial cells were collected after centrifugation at 3,000 g for 5 min and were suspended in 7.5 ml of 10 mM $MgS0_4$. To obtain 3 x 10^3 recombinant phage plaques /100 mm plate or 1.2 x 10^4 recombinant phage plaques /150 mm plate, the expression library phage plaques were diluted in lambda dilution buffer (10 mM NaCl, 10 mM $MgS0_4$ and 35 mM Tris-HCl pH 7.5) and 1 µl of the diluted phages were added to 200 µl of the XL1-Blue cells in a sterile tube. The phages were allowed to adsorb to the bacteria at 37°C for 15 min. To each of this phage-bacteria mixture, 2.5 ml of melted LB/$MgS0_4$/top agarose maintained at 42°C was added, gently inverted twice and poured on to pre-warmed LB/$MgS0_4$ plates. The plates were gently swirled to evenly spread the top agarose and left at room temperature (25-30°C) for solidification. The plates were incubated at 42°C for 3-4 h for the plaques to appear. After 4 h, nitrocellulose membrane pre-soaked for 20 min in 10 mM IPTG (isopropyl β-D-thiogalactoside) was placed on the cultures avoiding any air bubbles between the membrane and plaques on top agarose. Using waterproof ink, the orientation of membranes on the plate was marked. The cultures were incubated for another 3 h at 37°C to allow the protein to adhere to the membranes. After 3 h, the filters were removed and processed for the detection of positive plaques. Till then the culture plates used for 1st round of screening of library were stored at 4°C. To process the nitrocellulose membranes, to which the recombinant plaques were transferred, the non-specific sites were blocked by immersing the

filters in TBST (20 mM Tris-HCl pH 7.4, 500 mM NaCl, 0.05% Tween 20) containing 3% BSA for 1 h with gentle shaking. This was followed by 5 washes for 10 min each in TBST and incubation for 2 h in primary antibody, anti-HP19 IgG fraction (1:250 dilutions) in TBST with 3% BSA for 1 h with gentle shaking. The membranes were again washed in TBST (5 changes x 10 min). The positive plaques bound to anti-HP19 were detected by incubation of the membranes with ALP conjugated anti-rabbit IgG for 1 h in TBST with 3% BSA. This was again followed with washes in TBST (5 changes x 10 min). To visualize the positive plaques the filters were immersed in ALP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$) containing NBT and BCIP, the substrates of ALP. The membranes were developed until the desired signal to noise ratio was clearly visible on the filters. The development was stopped by washing the membranes 2-3 times in stop solution (20 mM Tris-HCl pH 8.0 and 5 mM EDTA). The real positive plaques that developed slightly earlier and intensely than the background or the pseudo-positives were marked with a pencil. Aligning these membranes orientation marks to the original plates from which the plaque was transferred to membrane, the positive plaques were picked using sterile pipette tips. Each positive plaque was placed in a separate microfuge tube containing 500 µl of 1X lambda dilution buffer. For phage elution, the tubes were vigorously vortexed and incubated overnight at 4°C, which was subsequently used for secondary screening. The plaques picked from the second round of immunoscreening were subsequently used for tertiary screening. The 10 positive plaques obtained after third screening were processed further to convert the phage (λTriplEX2) clones to plasmid (pTriplEx2).

(D) Sub-cloning and restriction analysis of plasmid DNA for insert size determination-

The positive plaques *i.e.,* the phage λTriplEx2 clones were converted to plasmid pTriplEX2 clones by excising and circularizing the complete plasmid from recombinant phage. The conversion of phage to plasmid is a high efficiency, single step conversion via site specific recombination at *loxP* sites in *E. coli* BM25.8, a bacterial strain ideal for *cre-lox*-mediated excision of pTriplEX2 from λTriplEx2. Fresh overnight culture of BM25.8 host cells was harvested in 10 ml of LB/$MgSO_4$ broth at 31°C. The cells were allowed to grow till the absorbance at 600 nm reaches 1.1-1.4. To 10 ml of these cells, 100 µl of $MgCl_2$ (final concentration- 10 mM) was added. About 150 µl of phage elute from the positive plaques of tertiary screening was mixed with 200 µl of overnight culture and incubated for 30 min at

31°C without shaking. To these 400 µl of LB broth was added and incubated for an additional 1 h at 31°C. Finally the infected cell suspension was spread on LB/amp plate (100 µg ampicillin per ml LB broth) to obtain isolated colonies of BM25.8 containing recombinant HP19 in pTriplEx2 vector. This plasmid vector was then transformed into *E. coli* XL-1 Blue strain for better plasmid DNA yield by standard transformation protocol (Sambrook *et al.,* 1989) with chemi-competent host cells (prepared by treating XL1-Blue cells with $CaCl_2$). All the plasmid DNA isolated from the ten positive clones were subjected to restriction analysis using *EcoRI* and *NotI* enzymes. The digestion was carried out with ~1 µg of plasmid DNA in a 10 µl restriction digestion reaction at 37°C for 1 h. The reaction was terminated by 1 µl of 0.5 M EDTA and the restriction pattern was analysed on 1% agarose gel.

(E) Sequencing of HP19 cDNA clone-

All positive clones that showed good restriction pattern were sequenced using forward (5`-TCCGAGATCTGGACGAGC-3`) and reverse (5`-TAATACGACTCACTATAGGG-3`) primers provided with the SMART III^{TM} cDNA library construction kit (Clontech, USA). About 60% of the positives were found to be a glutathione S-transferase (GST) like sequences (BLAST search). One such clone was analyzed in detail. The sequencing reaction was performed with the ABI-PRISM Big-Dye terminator cycle sequencing ready reaction kit according to the manufacturer's protocol. The terminator ready reaction mix in the kit contained di-deoxynucleotide (dye terminators), dNTPs, AmpliTaqR DNA polymerase and $MgCl_2$. The plasmid DNA containing the insert (400-500 ng in 1 µl) was mixed with 2 µl of terminator ready reaction mix, 10 pM forward or reverse primers in a total volume of 20 µl. It was denatured for 2 min at 96°C. The denatured DNA was amplified in Perkin-Elmer thermocycler programmed for 25 cycle at 96°C- 30 sec, 48°C- 15 sec and 60°C- 4 min. The amplified DNA was ethanol precipitated using standard DNA precipitation protocol (Sambrook *et al.,* 1989). The DNA pellet was dissolved in 25 µl of TSR (template suppression reagent) provided with the kit and left on ice for 30 min for the DNA to dissolve completely. The sample was heat denatured for 2 min at 95°C and was finally transferred to the DNA sequencing tube for sequencing on Perkin-Elmer-310 (ABI-PRISM) sequencer.

(F) Computer assisted sequence analysis-

A deduced amino acid sequence for the analysed cDNA clone was obtained using translator tool from www.au.expasy.org/tools/dna.html server. The National Centre for

Biotechnology Information (NCBI) and search launcher (Baylor College of Medicine) server were used to compare the sequence results with other available cDNAs in the database by BLAST search (Altschul *et al.,* 1990). A multiple sequence alignment of *C. cephalonica* HP19 cDNA (CcHP19) deduced amino acids in FASTA format with best matching invertebrate GSTs was carried using Clustal-W (from www.ebi.ac.uk/clustalw) and GeneDoc 2.6 (Nicholas and Nicholas, 1997). Wherever necessary the alignment was also adjusted manually. Protean (DNASTAR, version 1.17), ProtParam tool (http://us.expasy.org/cgi-bin/protparam) was used to determine the % amino acid composition, theoretical isoelectric point (pI) and molecular mass. PROSITE tool (www.au.expasy.org/prosite) was used to ascertain the putative sites for post-translational modification in the deduced amino acid sequence of HP19-cDNA. ProtScale and bioinformatics tool from http://bioinformatics.hydroph/index.html were used to determine the hydropathic profile of CcHP19 to that of *C. fumiferana* GST (CfGST) using Kyte-Doolittle method (1982) of calculating hydrophobicity.

Cloning and sequencing of C. cephalonica ACP (CcACP) cDNA by hybridization screening of fat body expression library-

During the immunoscreening of fat body expression library for hexamerin encoding genes using hexamerin antibody, one of the false positive upon sequencing showed homology with phosphatidic acid phosphatase (PAP). In the present study this chance cloned ACP was used as the primary bait to clone ACP of *C. cephalonica*. The clone was used as RNA probe after *in vitro* transcription for expression library screening *i.e.,* hybridization screening.

(A) RNA Probe preparation for hybridization screening-

The digoxigenin labeled RNA probe was prepared by *in vitro* transcription using DIG-RNA Labeling Kit (SP6/T7) (Boehringer Mannheim). The non-radioactive DIG system uses digoxigenin, a steroid hapten to label RNA. For DIG-labeling, the cloned DNA at poly linker site of pTripEx2 vector that contains promoters for T7 RNA polymerase adjacent to the polylinker site was *in vitro* transcribed with T7 RNA polymerase in the presence of DIG-UTP. Hybridization studies were carried using DIG-labeled RNA probe and the hybrids were immunodetected with anti-digoxigenin Fab` fragments conjugated to ALP using NBT/BCIP as substrate. The cloned DNA in the vector was first linearised with *EcoRI* digestion followed by extraction with phenol, choloroform and iso-amyl alcohol mixture (25:24:1). This was followed by ethanol precipitation (Sambrook *et al.,* 1989). The linearised plasmid was then

used as template for *in vitro* transcription. The *in vitro* transcription reaction contained 1 µg of purified linearised DNA template, 2 µl of 10X NTP mix (DIG-RNA labeling mix), 2 µl of 10X transcription buffer, 1 µl of RNase inhibitor and 2 µl of T7 RNA polymerase. The reaction was carried for 2 h at 37°C and was stopped by the addition of 2 µl of 200 mM EDTA (pH 8.0). The transcribed RNA was precipitated with 0.1 volume of 4 M LiCl and 3 volumes of ethanol at −20°C for 2 h. The RNA pellet was collected after centrifugation at 12,000 g for 15 min at 4°C, washed twice in 70% ethanol. The washed DIG-labeled RNA probe was dissolved in 100 µl of RNase free sterile distilled water and stored at 4°C until use.

(B) Hybridization screening for cloning of ACP-

The recombinant phage plaque lifting from the fat body expression library on to the nitrocellulose membrane was carried by the methods of Sambrook *et al.,* (1989) as mentioned above for immunoscreening. These membranes were processed for hybridization screening with DIG-labeled RNA probe, to pick CcACP cDNA clones using ULTRAhyb^{TM} hybridization buffer (Ambion Inc. USA) and DIG nucleic acid NBT/BCIP detection kit (Boehringer Mannheim). The membrane was first prehybridized in ULTRAhyb^{TM} buffer followed by overnight hybridization in DIG-labeled RNA probe (20 µl) at room temperature. The hybridized blot was washed twice for 15 min each in wash buffer 1 (2X SSC + 0.1% SDS) and wash buffer 2 (0.1X SSC + 0.1% SDS) followed by washing in reagent DIG-1 (0.1 M maleic acid + 0.15 M NaCl at pH 7.5) for 1 min and DIG-2 (DIG-1 with 1% blocking reagent) for 30 min. The membrane was then incubated with anti-DIG conjugated with ALP for 1 h followed with 2 washes in DIG-1 and DIG-2 reagent (15 min) at room temperature. Finally, the hybridized membrane was developed using NBT [66 µl of 50 mg/ml in 70% dimethyl formamide (DMF)] and BCIP (33 µl of 50 mg/ml in DMF) substrate in DIG-3 reagent (10 mM Tris-HCl pH 9.5, 10 mM NaCl and 50 mM $MgCl_2$). The membrane was developed until the desired signal to noise ratio was clearly visible on the filters. There was one clean positive plaque that developed earlier and intensely than the background or the pseudo-positives. Aligning the membrane orientation marks to the original plates from which the plaque was transferred to membrane, the positive plaque was picked using sterile pipette tips and placed in a separate microfuge tube containing 500 µl of 1X lambda dilution buffer. The phage was eluted by vigorous vortexing and overnight incubation at 4°C which was subsequently used for secondary screening. Thus, the 2 positive plaques obtained after second screening were processed further to convert the phage (λTriplEX2) clones to plasmid

(pTriplEx2). These two clones were restriction analysed and were found to be identical. One of the clone was sequenced partially and used for studies to understand the ACP expression and changes in the mRNA transcript with 20E and HP19.

Nucleic acids blotting and hybridization-

(A) Southern blotting-

The total larval body genomic DNA was digested with different enzymes and subjected to 0.8% agarose gel electrophoresis. After electrophoresis, the DNA was denatured by soaking the gel for 45 min in several volumes of 1.5 M NaCl and 0.5 M NaOH with constant agitation, followed with brief washing in sterile distilled water. The gel was neutralized for 30 min in 1 M Tris-HCl (pH 7.4) and 1.5 M NaCl followed 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. This blot was stored at −20°C until further use. The 20X SSC used for Southern and northern blotting contained 0.3 M sodium citrate and 3M NaCl (pH 7.0).

(B) Northern blotting-

The total 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. The blot was stored at −20°C until further use.

(C) Preparation of [α^{32}P] dATP labeled probe for Southern and northern hybridization-

This was employed to determine the copy number of HP19 and ACP gene on Southern blot and their mRNA transcript on northern blot. For this, HP19-cDNA insert corresponding to the whole open reading frame and the total partial cDNA of ACP was

excised from the plasmid pTriplEx2 vector by *EcoRI* + *Not I* digestion, gel purified (using QIAquick gel extraction kit) and approximately 100 ng was random prime labeled using HexaLabel^{TM} DNA labeling kit (MBI Fermentas) and [α^{32}P] dATP (3000 Ci/mmol, BRIT, India). The reaction was carried in step wise manner. 10 µl of cDNA template (~100 ng) was mixed with 10 µl random (hexamer) primer (7.5 o.u/ml in 300 µl of 0.25 M Tris-HCl pH 8.0, 25 mM $MgCl_2$ and 5 mM DTT) 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 quick cooling on ice. The mix was again 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 (5u/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.

(D) Southern and northern hybridization-

The blots were prehybridized in 6X SSC, 5X Denhardt's reagent (0.5 g Ficoll, 0.5 g polyvinylpyrolidone and 0.5 g BSA), 0.1% SDS and 100 µg sonicated salmon sperm DNA at 68°C for 90 min followed by hybridization in same solution either with radiolabeled (~3 x 10^6 dpm/µg) HP19 cDNA or ACP cDNA probe at 68°C for 24 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 68°C. Finally the blots were dried and exposed to Kodak X-OMAT X-ray film followed by autoradiography to detect the hybridization.

Studies on hexamerins-

(A) Purification of hexamerins and generation of polyclonal antibody against purified hexamerins-

The hexamerins were purified from the total haemolymph proteins of the last instar larvae of *C. cephalonica* by a rapid protocol devised during the present study. The haemocyte free diluted haemolymph protein (1 mg/50 µl) was passed on sephadex G 50-150 (Sigma) column (1.5 x 60 cm) equilibrated with 10 mM Tris-HCl (pH 7.4) at room temperature. The protein was eluted with the same buffer at a flow rate of 1 ml/2.5 min till the absorbance of the eluates at 280 nm reached 0.002. The peak fractions that contained hexamerins were

pooled and loaded on to ion-exchange DEAE sephacel column (1.25 x 25 cm) pre-equilibrated with 10 mM Tris-HCl (pH 7.4). The bound hexamerins were eluted with a linear gradient of 0-0.5 M NaCl. The peak fractions were pooled and analyzed on 7.5% resolving SDS-PAGE for purity. The purified hexamerins thus obtained were used for production of polyclonal antibodies and for other studies. The antibody against purified hexamerins were raised in rabbits as described above for the production of polyclonal antibody against HP19 using 500 and 250 μg of pure hexamerins for primary and booster doses respectively. The specificity of hexamerin antibody was checked on western blot.

(B) Biotin labeling of hexamerins-

The hexamerins were biotinylated using biotin labeling kit (Boehringer Mannheim) utilizing the principle of formation of a stable amide bond between the free amino groups of protein to be labeled with the D-biotinoyl-ε-aminocaproic acid-N-hydroxysuccinimidester (Biotin-7-NHS). One mg of purified hexamerin was suspended in 1 ml of PBS (pH 7.4). The biotin-7-NHS was added in a molar ratio of 1:20 to the protein sample, stirred thoroughly and incubated for 2 h at room temperature with gentle stirring. The non-reacted biotin-7-NHS was removed by gel filtration using sephadex G-25 column prequilibrated with PBS (pH 7.4). The extent of biotinylation and the ratio of biotin to protein hexamerins were determined spectrophotometrically at 500 nm by avidin-HABA (4`-hydroxyazobenzene-2-carboxylic acid) assay. The absorption of avidin-HABA complex at 500 nm decreases proportionally with increased concentration of biotin as the HABA dye is displaced for avidin due to higher affinity of avidin for biotin. By using a ratio 20:1 of biotin-7-NHS and hexamerins, the level of biotinylation obtained was approximately 3-5 moles of biotin per mole of hexamerins.

(C) Ligand blotting-

This was carried according to the procedure of Kirankumar *et al.,* (1997). The membrane proteins prepared from fat body and MARG tissues were separated by SDS-PAGE and transferred to nitrocellulose membrane (Towbin *et al.,* 1979). The blotted membrane was washed (3 x 5 min) in 150 mM Tris-HCl (pH 7.4) and 150 mM NaCl followed by blocking with 2% BSA and 0.2% gelatin in the same buffer. The blot was washed (3 x 10 min) with binding buffer (5 mM MES pH 6.5, 100 mM NaCl, 8 mM $CaCl_2$, 24 mM $MgCl_2$, 0.5% Tween 20 (v/v) and 5 mg/ml BSA) followed by incubation with biotinylated hexamerins (100 μg) suspended in binding buffer for 4 h at 22°C with gentle shaking. The blot was finally washed (3 x 5 min) in 50 mM Tris-HCl (pH 7.4) and 150 mM NaCl. The protein with bound

biotinylated hexamerins were visualized with streptavidin-ALP complex using NBT/BCIP as substrate. In case of phosphorylated [γ^{32}P] fat body proteins, the probed ligand blot was subsequently processed for autoradiography.

(D) Bio-labeling of hexamerins-

The hexamerins were bio-labeled using [^{35}S] methionine. The LLI larvae were injected with 10 μCi [^{35}S] methionine (1,000 Ci/mmol, BRIT, India) and allowed to grow under normal culture condition. After 16 h, the haemolymph was collected and hexamerins were partially purified by passing it through sephadex G-25 column, in order to remove the unincorporated [^{35}S] methionine, salts and other low mass proteins. The radiolabeled proteins were resolved on SDS-PAGE and visualized by fluorography.

(E) In vitro uptake of hexamerins-

This was carried using [^{35}S] methionine labeled hexamerins and LLI fat body cultures. Intact fat bodies from two 24 h post-ligated LLI larvae were dissected under sterile conditions and rinsed thoroughly in TC-100 insect culture medium with traces of streptomycin sulfate. The tissues were preconditioned in TC-100 medium for 2 h at 25°C with gentle shaking and were then transferred to fresh medium containing 1 mM ATP and / or 80 nM 20E and 40 μM genistein or 1 mM actinomycin D or 1 mM cycloheximide and incubated for 1 h. Equal volumes of carrier solvents were added to the control cultures (without 20E / ATP). To these cultures, bio-labeled hexamerins (50,000 cpm) were added and incubated for an additional 4-6 h. The fat bodies were removed from incubation medium, rinsed thoroughly with insect Ringer and homogenized as described above. Equal quantity of protein was used for determination of the radioactivity. The uptake of hexamerin was expressed as % of labeled hexamerin in LLI larval fat body to the total amount of labeled hexamerin added to the medium *i.e.,* 50,000 cpm.

Studies on glutathione S-transferase (GST)

(A) GST activity-

The GST activity in various tissues of *C. cephalonica* was measured by the method of Habig *et al.,* (1974). Insect tissues were homogenized in buffer containing 50 mM Tris-HCl (pH.7.8), 10 mM EDTA, 15% glycerol and 0.005% phenylthiourea. The supernatant was collected carefully without disturbing the upper fatty layer and cell debris at bottom after

centrifugation at 1,000 g for 5 min. The one ml reaction mixture contained 10 µl of 100 mM 1-chloro-2,4-dinitrobenzene (CDNB), 10 µl of 100 mM reduced glutathione (GSH) and 100 mM potassium phosphate buffer (pH 6.5). The reaction was initiated by the addition of the enzyme source and the product *i.e.,* the formation of thioether conjugate was measured at 340 nm on a time scan of 0-60 sec on a UV-160A Schimadzu spectrophotometer.

(B) Purification of the cytosolic GSTs from C. cephalonica-

A 20% (w/v) homogenate from whole insect was prepared using glass homogenizer kept in ice jacket as described above in GST assay procedure. The homogenate was centrifuged at 1,000 g for 5 min at 4°C to remove the cell debris. The supernatant was subjected to centrifugation at 10,000 g for 45 min at 4°C. The obtained supernatant was further centrifuged at 100,000 g in Hitachi Ultracentrifuge for 1 h to get cytosolic fraction again in the supernatant, which was used for further purification on sephadex G-25 column to remove endogenous substrates. The elute containing the GSTs were then applied to DEAE cellulose column pre-equilibrated with 50 mM Tris-HCl (pH 7.8). The protein was eluted in discontinuous step gradient of NaCl (0-0.5 M, 0.5-1.0 M, 1-1.5 M and 1.5-3.0 M) in 50 mM Tris-HCl (pH 7.8). The GST was eluted in the flow through fractions as it did not bind to the ion-exchange DEAE cellulose column. This is consistent with the earlier results of Habig *et al.,* (1974). The flow though fractions that contained GSTs were pooled (6 mg protein) and applied to glutathione CL-agarose affinity column, prequilibrated with 10 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA and 2 mM DTT for further purification. The affinity column was first washed with washing buffer containing 10 mM potassium phosphate buffer, 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.

Statistical analysis-

The mean and standard deviation were calculated for the variables studied. The data was statistically analyzed by one way analysis of variance (ANOVA) followed by comparisons of means by Tukey or Student-Newman-Keuls multiple comparison test using Sigma Stat software (Jandel Corporation). The *$p<0.05$ was defined as the criterion for statistical significance.

Results- Chapter I

Identification, isolation and characterization of HP19 from *C. cephalonica*

Part of this work has been published in-

Arif *et al.,* (2004). The insect haemolymph protein HP19 mediates the nongenomic effect of ecdysteroids on acid phosphatase activity. ***J. Biol. Chem.*** 279, 28000-28008.

Background-

Insect metamorphosis *i.e.,* the transition from larval to adult stage is controlled by ecdysteroid hormones (Riddiford *et al.,* 2001; Trumann and Riddiford, 2002; Gilbert *et al.,* 2002). Ecdysteroids, like the steroids in vertebrates, regulate gene transcription by binding to nuclear receptors, which are ligand-activated transcription factors, converting the hormonal stimulus into a transcription response (Henrich *et al.,* 1999; Riddiford *et al.,* 2001; Scheller and Sekeris, 2003). Metamorphosis involves the breakdown of larval structures and the formation of new tissues (Trumann and Riddiford, 2002). As a part of cell remodeling during metamorphosis, acidic autophagic vacuoles accumulate in the cells of fat body and activity of several lysosomal enzymes such as ACP increases and cause lysis of larval tissues (Verkuil, 1980; Sass and Kovacs, 1980; Thummel, 2001). The fat body that fills a large fraction of the insect has been considered equivalent to vertebrate liver in intermediary metabolism (Keeley, 1985). It has been demonstrated that the stimulation of the lysosomal activity is governed by ecdysteroids (Verkuil, 1980; Sass *et al.,* 1989; Ashok and Dutta-Gupta, 1988; Kutuzova *et al.,* 1991) possibly by a nongenomic mechanism (Verkuil, 1979). Although the molecular mechanism of genomic mode of steroid action is well known, the mechanism of nongenomic steroid action remains unclear to this date (Losel and Wehling, 2003).

Earlier studies have shown that 20E stimulates ACP activity in fat bodies *in vivo* but not *in vitro* (Caglayan, 1990; Ashok and Dutta-Gupta, 1991). This result suggests that 20E, the active from of ecdysone, requires additional factor(s) to enhance ACP activity. Hence, the present study focuses on the process of ACP activation by 20E in the fat body cells of our main model insect, the rice moth *Corcyra cephalonica*. This chapter of thesis deals with the appearance of a stage and tissue specifically regulated protein, HP19, in the haemolymph of *C. cephalonica* responsible for activation of the 20E dependent stimulation of ACP activity.

Detection of factor(s) in the haemolymph of C. cephalonica and its subsequent identification as protein that mediates the 20E stimulated ACP activity in the fat bodies of thorax-ligated, hormone deprived larvae-

When insect larvae are ligated behind the first pair of prolegs, *i.e.,* behind the hormone producing glands, the posterior part of the animal is known to be relatively free from endogenous ecdysteroids (Priester *et al.,* 1979; Burmester and Scheller, 1997a; Dutta-Gupta and Ashok, 1998). Figure 1 shows the effect of thorax-ligation (Fig. 1a) and injection of exogenous 20E (Fig. 1b) on the fat body ACP activity in LLI larvae. The ACP activity

declined gradually from 6 to 72 h after ligation. As the ACP activity in the fat body was significantly lower after 24 h of ligation, this time period was used for all hormone manipulation studies. Hormone injections of 80 nM 20E, *i.e.,* the physiological concentration (Dutta-Gupta and Ashok, 1998) to 24 h post-ligated LLI larvae caused a significant increase in the ACP activity in fat bodies after 24 h, compared to the solvent treated larvae (Fig. 1b). The increase was also observed when 20E was injected to 48 h post-ligated LLI larvae for 24 h as compared to the solvent injection for 24 h to 48 h post-ligated larvae.

To study the effect of hormone on the ACP activity of fat bodies kept in culture, the tissue was dissected from 24 h post-ligated larvae and cultured for 4 h in presence of 80 nM 20E. The results show that 20E did not elicit any stimulatory effect and the activity was more or less the same as in the controls (Fig. 1c). However, addition of haemolymph from the posterior part of 24 h post-ligated or unligated LLI larvae together with 20E caused a significant increase in the ACP activity (Fig. 1d). This observation suggests that the haemolymph contains a factor (or factors) required by 20E to stimulate the ACP activity in fat body cultures. When the haemolymph was treated with alcohol, heat, acid, alkali or protease, no stimulation of the ACP activity by 20E could be observed suggesting the proteinaceous nature of the factor (Fig. 2).

Fractionation and purification of the haemolymph protein that mediates the 20E dependent ACP activity stimulation-

After loading total haemolymph protein on a sephadex G-50 column, we eluted several fractions (Fig. 3a) and checked their ability to mediate the 20E stimulated ACP activity. We found an active protein fraction with a molecular mass of approximately 22 kDa, calculated from the elution profile (Fig. 3a, inset), or 19 kDa, calculated from the mobility on a SDS-PAGE by the appearance of new protein band in the active fraction elute (Fig 3b, arrow). In another fractionation study, the total haemolymph protein was fractionated using specific molecular weight cut-off membrane filters of sizes 30 and 10 kDa (Fig. 4). Three fractions having masses above 100 kDa, 30-100 kDa and below 30 kDa were collected and the studies with them revealed that only the filtrate from 10 kDa cut off filter gave a protein fraction that mediated the 20E stimulated ACP activity of fat body cultures and an average increase from 0.7 to 1.2 n moles of PNP release/h/µg protein was consistently observed (Fig. 5a). These results clearly suggested that the active protein fraction present in the haemolymph, is a low mass protein of <30 kDa. On the basis of these results, the active

haemolymph protein fraction was purified by a strategy in which the total haemolymph protein was subjected to 60% salt precipitation, followed by fractionation using 30 and 10 kDa cut-off filters and finally the elution of active protein fraction by gel filtration chromatography (Fig. 4). The protein yield in the filtrate obtained from the 10 kDa cut off filter was insufficient to proceed for further purification. Therefore, the filtrate from the 30 kDa cut-off filters in which the HP19 is contained was used for gel filtration. The protein fraction eluted from the sephadex column that mediated the 20E dependent ACP activity (Fig. 6a), resulted in a contaminant free pure polypeptide band of 19 kDa (Fig. 6b). Hence, the active haemolymph protein was named as "HP19". Starting with 50 mg total haemolymph protein, we obtained a 98.5-fold purification with 0.05% yield (Table 1).

Electroelution of HP19 and production of polyclonal antibodies against HP19 and its confirmation-

Although a few nanogram of the protein, present in crude or partially purified fractions was found to be sufficient for ACP activity stimulation at the physiological concentration (80 nM) of 20E (Fig. 7a and b), the yield of the purified protein was very low. Other limitations in purification were the requirement of a large quantity of haemolymph of a specific developmental stage (LLI) and removal of the major contaminating protein, hexamerin that constitutes 75-80% of total haemolymph protein (Haunerland, 1996). Therefore, an antibody against HP19 was raised by electroeluting HP19. For electroelution, attempt was first made to detect the HP19 protein band on 12% SDS-PAGE on silver as well as coomassie stained gels (Fig. 8a-c). The results reveal that the protein is present in extremely low concentration and the faint HP19 protein band could be detected only when the total haemolymph protein per sample was 30 μg or more (Fig. 8a). The detection of HP19 remained difficult even when the total haemolymph protein per sample was as high as 250 μg (Fig. 8b). However, this amount of total haemolymph protein was sufficient to detect the HP19 band on coomassie stained gel also (Fig. 8c, enclosed dashed rectangle). The HP19 from approximately 60 such gels were electroeluted. Figure 8d shows the electroeluted HP19 protein that was used as antigen for the production of polyclonal antibody against HP19. The IgG fraction of this antibody was purified using protein A-agarose chromatography (Fig. 9a).

The western blots show the specificity of HP19 antibody both on denatured (Fig. 9b) and non-denatured (Fig. 9c) PAGE. The specificity of the antibody was found to be high without much non-specific cross-reaction even when the total haemolymph protein per

sample was fairly high *i.e.,* 40 and 80 µg per lane (Fig. 9b, lanes 3 & 4). A single protein band of 19 kDa detected in SDS-PAGE (Fig. 6b), denatured iimmunoblotting (Fig. 9b) and non-denatured immunoblotting (Fig. 9c) studies suggested a monomeric structure of HP19.

As the antibody was raised against the electroeluted HP19 resolved on SDS-PAGE from the total haemolymph protein, hence it was necessary to confirm whether that antibody is specifically against HP19 that mediates the 20E dependent action. For this the antibody was added in different dilutions to the fat body culture along with HP19 and 20E. The results obtained (Fig. 10) revealed that lower dilutions of antibody significantly suppressed the stimulation of 20E mediated ACP activity which is seen in presence of HP19. However, the effect (suppression) was less pronounced with higher dilutions of antibody resulting in partial stimulation of 20E dependent ACP activity. In another experiment (Fig. 11), HP19 antibody was used for the immunoprecipitation of HP19 from the total haemolymph protein and the resulting immuno-complex as well as the supernatant (termed immuno-supernatant) was added to the fat body cultures. The hormone 20E failed to stimulate the fat body ACP activity in all the cultures, which were supplemented with immuno-complex. The culture in which high dilution (insufficient to precipitate total HP19) supernatant was added 20E could stimulate the activity. The above studies suggest that the antibody is complexed with HP19, hence it was not able to mediate the 20E dependent ACP activity stimulation.

Tissue specific synthesis of HP19 by hind gut associated lobular fat body (HGLFB)-

Co-culturing of different larval tissues with fat body demonstrated that HGLFB is the only HP19 synthesizing tissue. A stimulation of the ACP activity of fat body by 20E was only observed when it was co-cultured with HGLFB (Fig. 12a). The haemolymph used in all experiments was cell-free and therefore cannot be the site of HP19 synthesis. Western analysis of proteins from different tissues also revealed the presence of HP19 only in HGLFB (Fig. 12c, lane 2) and total larval body protein (Fig. 12c, lane 5). However, the apparent mass of HP19 in this tissue was approximately 5 kDa higher than the HP19 present in haemolymph (Fig. 12c, lane 1). The faint band of HP19 in the total larval body homogenate in the western blot (Fig. 12c, lane 5) is mainly due to the lower concentration of HP19 protein present in sample. The tissue specificity of HP19 biosynthesis was further confirmed by immunohistochemical staining of different tissue sections using HP19 antibody (Fig. 13). These studies once again showed that the HP19 is localized only in HGLFB (Fig. 13b and c) and not in tissues like visceral fat body (Fig. 13d), peripheral fat body (Fig. 13e), gut (Fig.

13f), carcass (Fig. 13g), Malpighian tubule (Fig. 13h) and salivary gland (Fig. 13i). *In situ* immunodetection of HP19 in larvae again showed the presence of HP19 only in the HGLFB (Fig. 14, arrow head inside the dotted circle).

Developmental regulation of HP19-

The developmental profile studies of HP19 in *C. cephalonica* during the last (Vth) instar larval stage suggest that only the haemolymph of LLI larvae could mediate the 20E stimulated ACP activity (Fig. 15). Western analysis of proteins from haemolymph (Fig. 16b) and HGLFB (Fig. 16d and e) of different developmental stages of Vth instar larvae show that HP19 is present at maximal concentration in LLI (lane 3). HP19 is present in the haemolymph at all the developmental stages tested (Fig. 16b, lanes 1-4), but was not detectable in HGLFB of prepupae (Fig. 16d and e, lane 4). The quantity of HP19 present in the ELI and MLI was fairly low as compared to LLI. Hence, the absence of cross-reaction in ELI and MLI developmental stages in figure 16d when the total HGLFB protein extract per sample was low (8 μg/lane) compared to the appearance of cross-reaction in the figure 6e where a high concentration of total HGLFB protein extract was loaded. This study clearly suggests that HP19 is synthesized throughout the complete last larval stage by HGLFB and is released into the haemolymph. Further, the rate of synthesis is low in ELI larvae and the secretion into the haemolymph is rapid with exception of the LLI stage when the HP19 synthesis is accelerated and is paralleled by activation. These western analysis results further confirm the stage specific action of HP19 because the presence of protein was not detected in the HGLFB at prepupal (PP) stage (Fig. 16d and e, lane 4), a stage later than the active stage. The detection of HP19 protein band in haemolymph (Fig. 16b, lane 4) at PP stage is probably due to the transfer of already existing protein of LLI to PP stage.

Presence of HP19 like protein in silkworm Bombyx mori and other insect species-

We further confirmed whether a similar protein is present in other insects belonging to lepidoptera and diptera. The results presented in figure 17 suggest that apart from *C. cephalonica*, the haemolymph of other lepidopteran insects like *Bombyx mori, Spodoptera litura, Achaea janata, Galleria mellonela* and *Papilio demoleus* was able to stimulate the 20E dependent ACP activity in *C. cephalonica* fat body cultures. However, the stimulation in ACP activity was insignificant with *Calliphora vicina,* a dipteran insect as compared to the stimulation by the haemolymph of all other lepidopteran insect species. Western analysis of the haemolymph protein from these insect species with anti-HP19 IgG fraction further

indicated the presence of HP19 like protein in lepidopteran insects (Fig. 17c). However, similar protein was not detected in the haemolymph of *C. vicina* (Fig. 17c) despite of the relatively low degree of stimulation in ACP activity (Fig. 17a). The western blot also indicated that in these species, the protein is present in multiple forms or subunits as compared to the HP19 of *C. cephalonica.*

A detailed analysis of HP19 like protein in *B. mori* suggested that like *C. cephalonica*, only the haemolymph from LLI larval stage mediated the 20E dependent ACP activity stimulation (Fig. 18). Attempt on the characterization of the active haemolymph protein fraction (Fig. 19) resulted in appearance of approximately 26 kDa protein indicating that possibly this protein is responsible for the 20E dependent ACP activity stimulation (Fig. 19b, arrow head). These studies suggest that HP19 like protein is not species specific at least for the lepidopteran insect but is definitely stage specific.

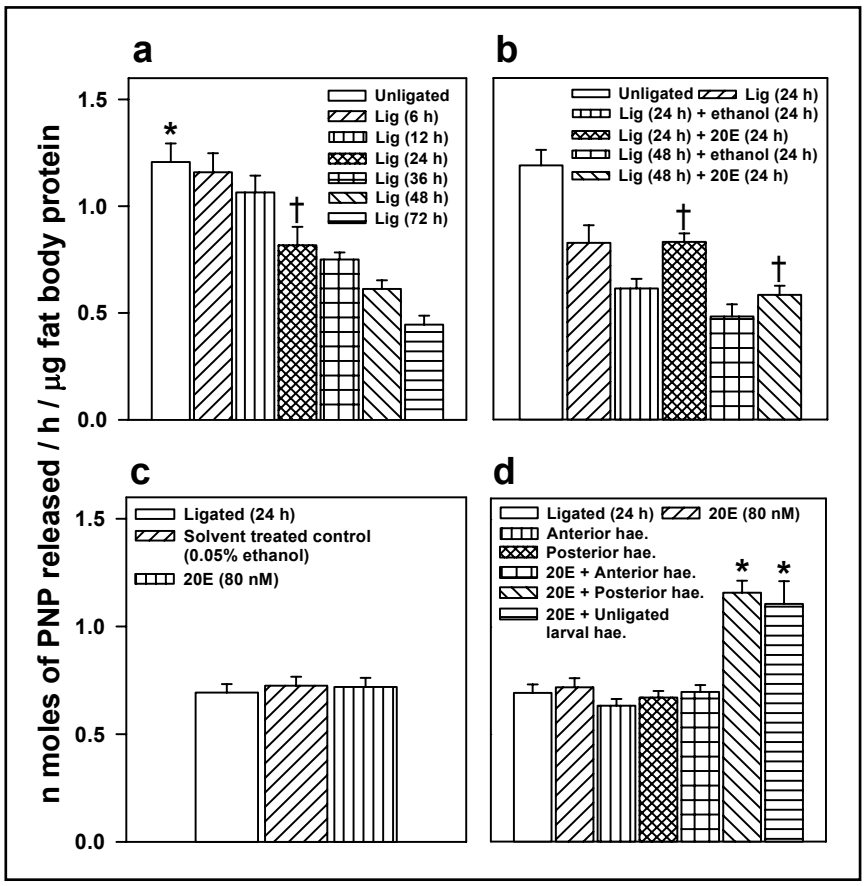

Fig. 1: Identification of a factor(s) in the haemolymph that enhances the ACP activity in fat bodies of *C. cephalonica* larvae (LLI):-

(a) Effect of thorax-ligation (Lig) on fat body ACP activity after different time periods.

(b) Effect of exogenous 20E on the fat body ACP activity in 24 h post-ligated insects. After 24 and 48 h of ligation, experimental insects were injected with 20E (80 nM in 2 µl 0.05% ethanol) while control insects received equal volume of carrier solvent (0.05% ethanol).

(c) Effect of 20E on the ACP activity of fat bodies kept in culture. The fat bodies from two 24 h post-ligated larvae were cultured as described in materials and methods with 80 nM 20E for 4 h. The solvent treated controls contained equal volume of 0.05% ethanol.

(d) Effect of 20E on the ACP activity of fat bodies kept in culture in the presence of haemolymph (hae) from *C. cephalonica* LLI larvae. The fat bodies kept in culture were incubated with 80 nM 20E and 10 µl of 1:20 diluted haemolymph for 4 h. The haemolymph was obtained from the anterior or posterior part of 24 h post-ligated and unligated LLI larvae. To the control cultures equal volume of insect Ringer was added with or without 20E.

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 four independent determinations and for each assay, fat body from 2-3 insects was pooled. For (a) * is significantly different over † and all other values ($p<0.05$), for (b) † is significantly different from previous control values ($p<0.05$) and for (d) * is significantly different from all other values.

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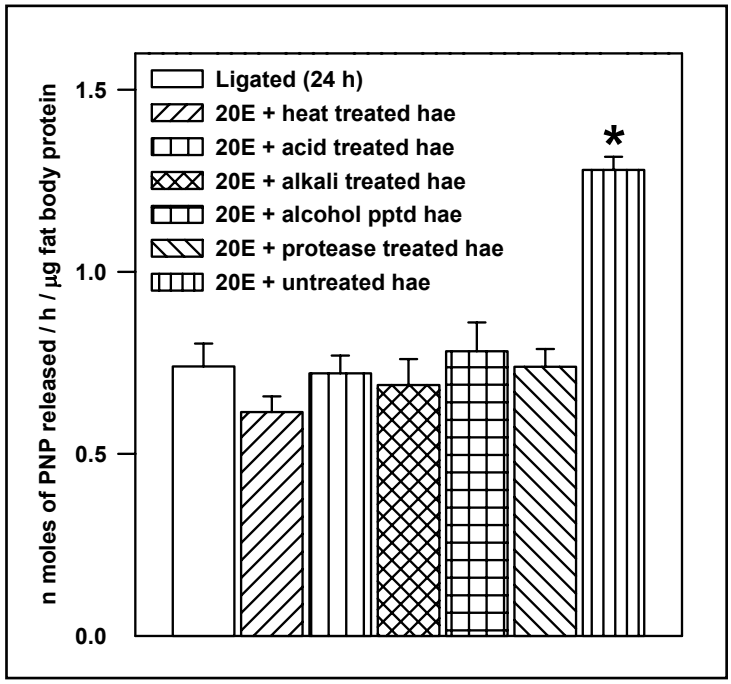


Fig. 2: Identification of the haemolymph factor(s) as protein:-

Effect of different pretreated *C. cephalonica* haemolymph (hae) in mediating the 20E dependent ACP activity of fat bodies kept in culture. The haemolymph (10 µl) was treated with heat (10 min, 100^{o}C), acid (1 µl, 10 N HCl), alkali (1 µl, 1 M NaOH), absolute ethanol (100 µl) or V_8 protease (1 µg, 15 min, 0^{o}C) prior to its addition to the fat bodies kept in culture in the presence of 80 nM 20E (in 10 µl 0.05% ethanol). 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 four independent determinations. * Significantly different over all other values ($p<0.05$).

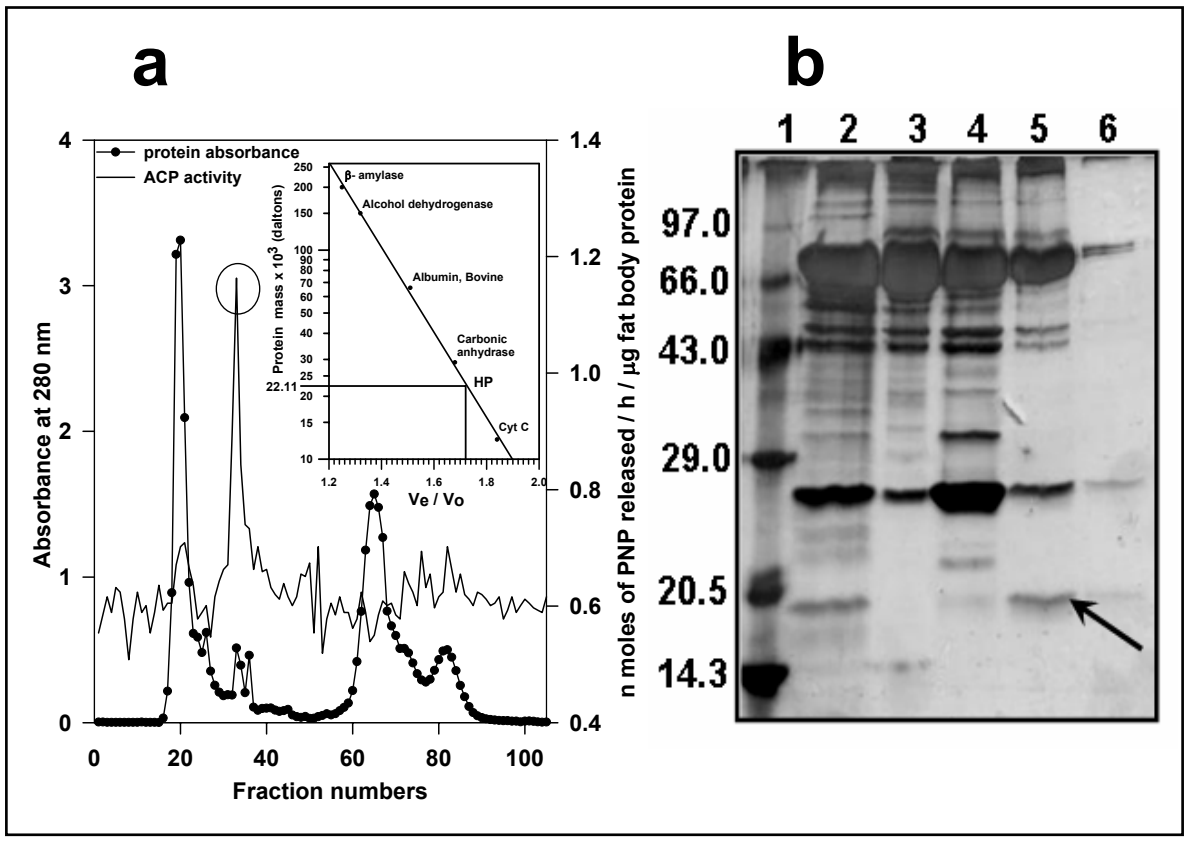


Fig. 3: Fractionation of haemolymph proteins by gel filtration chromatography and the identification of active haemolymph fraction as low mass protein:-

(a) Elution profile of haemolymph protein fractions and their ability to mediate 20E dependent ACP activity. Total haemolymph (25 mg protein) from LLI larvae was loaded on a sephadex G-50 (Pharmacia) column (1.6 x 90 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH 7.4) and eluted with the same buffer. Inset: Calculation of the approximate native molecular mass range of the active fraction using linear regression analysis on Sigma Plot and Graph Pad Prism softwares. Marker proteins: MF-GF-200 kit (Sigma). The encircled peak shows the active haemolymph fraction. Note that the calculation of active fraction (HP) from elution profile revealed a low mass protein ~22 kDa (inset).

(b) Protein profile (12% SDS-PAGE) of major haemolymph fractions along with the active fraction (lane 5) that had the ability to mediate the 20E dependent ACP activity. Lanes- protein marker in kDa (1), crude haemolymph (2), fraction 20 (3), fraction 26 (4), fraction 33- the active fraction (5) and fraction 35 (6). Note the active fraction (arrow) calculated from the mobility on SDS-PAGE revealed the appearance of a 19 kDa protein (lane 5).

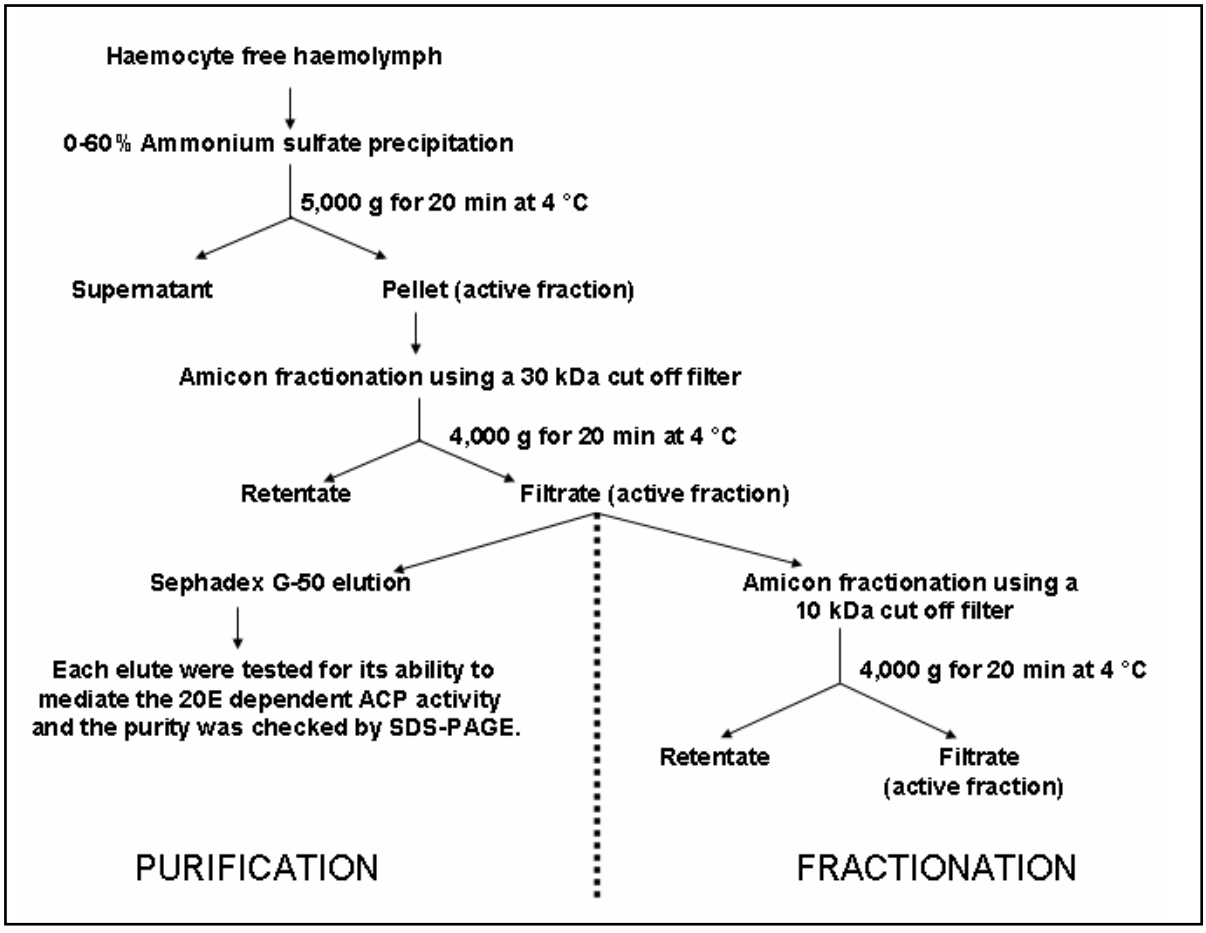


Fig. 4: Flow chart for the fractionation and purification of haemolymph protein that had the ability of mediating the 20E dependent stimulation of fat body ACP activity.

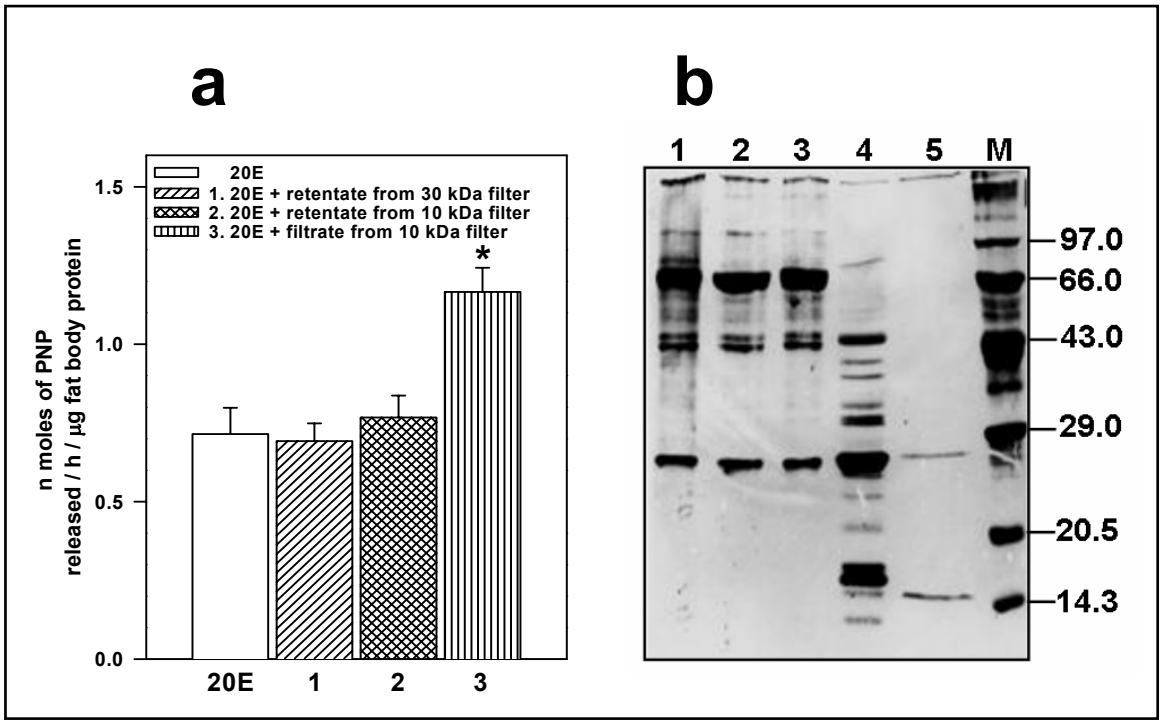


Fig. 5: Fractionation using specific molecular weight cut-off membrane fractionators for partial purification of active haemolymph fraction that had the ability to mediate 20E dependent ACP activity:-

(a) Effect of different haemolymph fractions obtained using 30 and 10 kDa fractionator on the ACP activity in fat bodies kept in culture in the presence of 20E. The fat bodies from two 24 h post-ligated LLI larvae were incubated with 80 nM 20E and 10 µl of fractionated haemolymph for 4 h. 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 4 independent determinations. *Significantly different from all other values ($p<0.05$).

(b) SDS-PAGE (12%) showing the protein profile of various haemolymph fractions. Lanes- crude haemolymph (1), proteins retained in retentate of 30 kDa filter (2), proteins in the filtrate of 30 kDa filter (3), proteins retained in retentate of 10 kDa filter (4), proteins in the filtrate of 10 kDa filter (5) and protein marker in kDa (M). The amount of protein loaded in lanes 1-4 was 10 µg and lane 5 was 3 µg.

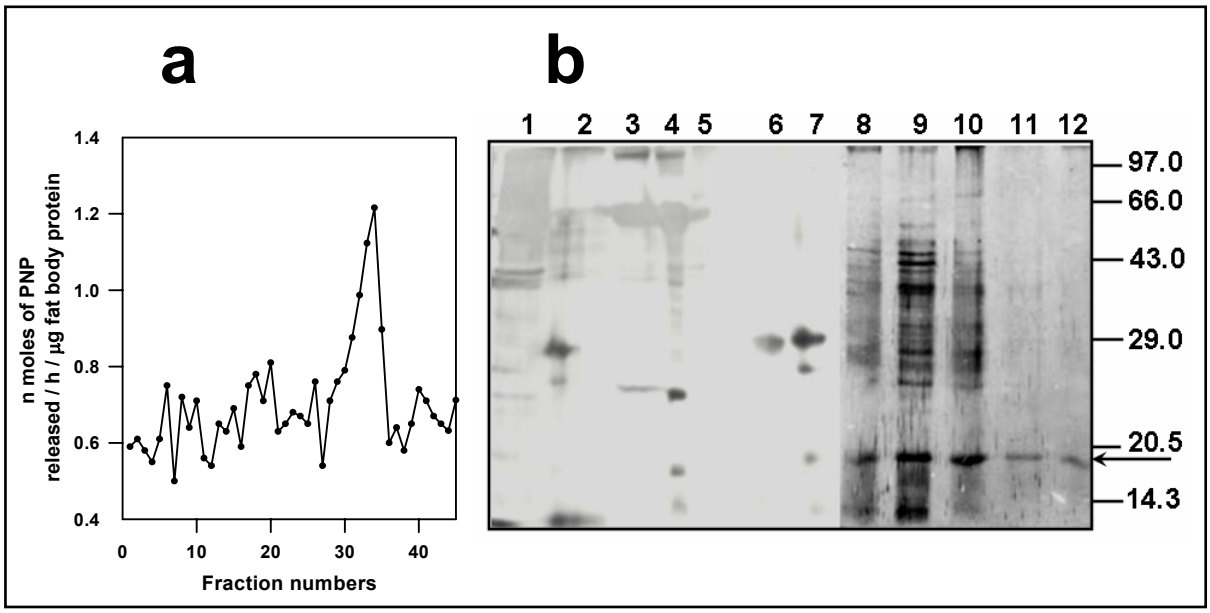


Fig. 6: Purification of a haemolymph protein enhancing the 20E dependent ACP activity in larval fat bodies kept in culture. Identification of active haemolymph fraction as 19 kDa protein hence it was termed as HP19:-

(a) Elution profile of haemolymph proteins on sephadex G 50 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 (pH 7.4).

(b) SDS-PAGE (12%) showing the purification profile of HP19. The haemolymph protein was subjected to 60% ammonium sulfate precipitation followed by fractionation using 30 and 10 kDa fractionator (Amicon). The filtrate from 30 kDa fractionator was applied on sephadex G-50 for column purification. Lanes: crude haemolymph (1), proteins in the supernatant (2) and pellet (3) after 0-60% salt precipitation, proteins from retentate (4) and filtrate (5) of 30 kDa filter, proteins from retentate (6) and filtrate (7) of 10 kDa filter and active fractions eluted from sephadex G-50 column (8-12). In lanes 1-4: 10 µg, 5-10: 5 µg and 11 & 12: total lyophilized pure protein was loaded.

Purification steps	Total protein content (mg)	% Yield	Fold purification
Haemocyte free total haemolymph protein (crude)	50	100	1
Ammonium sulfate precipitation (60% supernatant)	36.72	73.44	1.28
Amicon fractionation (30 kDa) filtrate	6.72	13.44	5.12
Amicon fractionation (10 kDa) filtrate	1.40	2.80	17.28
Sephadex G-50 eluates	**0.023**	**0.046**	**98.45**

Table 1. Purification profile of HP19:-

The % yield was calculated at each step on the basis of the amount of 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. The specific activity was calculated on the basis of the ability of pooled fractions to mediate the 20E dependent ACP activity.

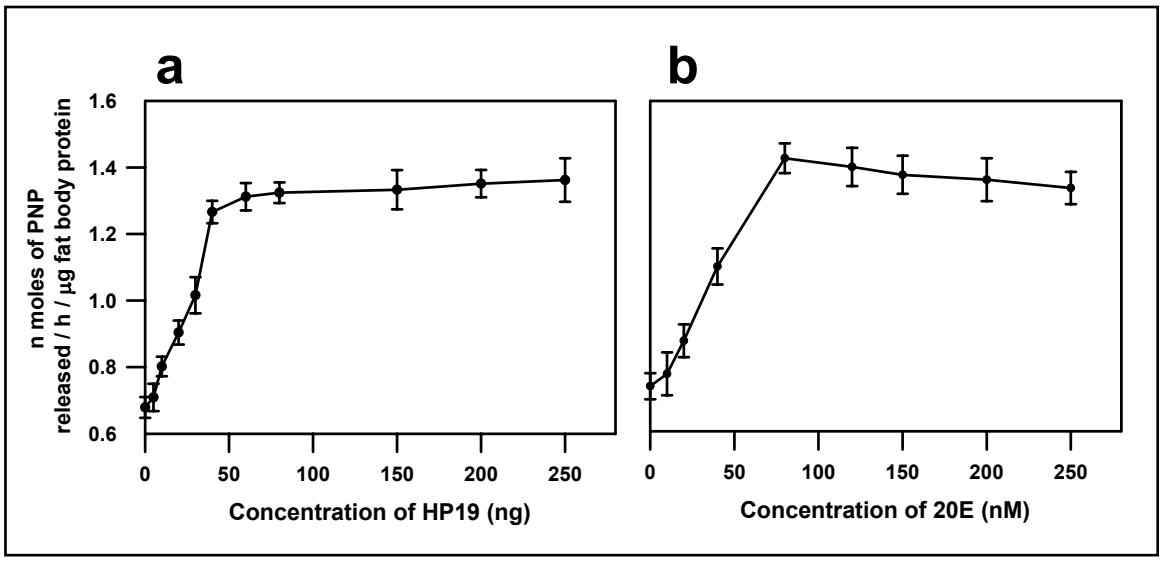


Fig. 7: Effect of different concentrations of HP19 and 20E on fat body ACP activity:-

(a) The fat bodies from two 24 h post-ligated LLI larvae were incubated with 80 nM 20E together with different concentrations of purified HP19 for 4 h at 25°C.

(b) The fat bodies from two 24 h post-ligated LLI larvae were incubated with different concentrations of 20E together with 40 ng purified HP19 for 4 h at 25°C.

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 4 independent determinations. Note that the concentration of 40 ng was effective enough to mediate the 20E dependent stimulation of fat body ACP activity and also the physiological concentration of 20E (80 nM) was sufficient and optimal for HP19 assisted stimulation.

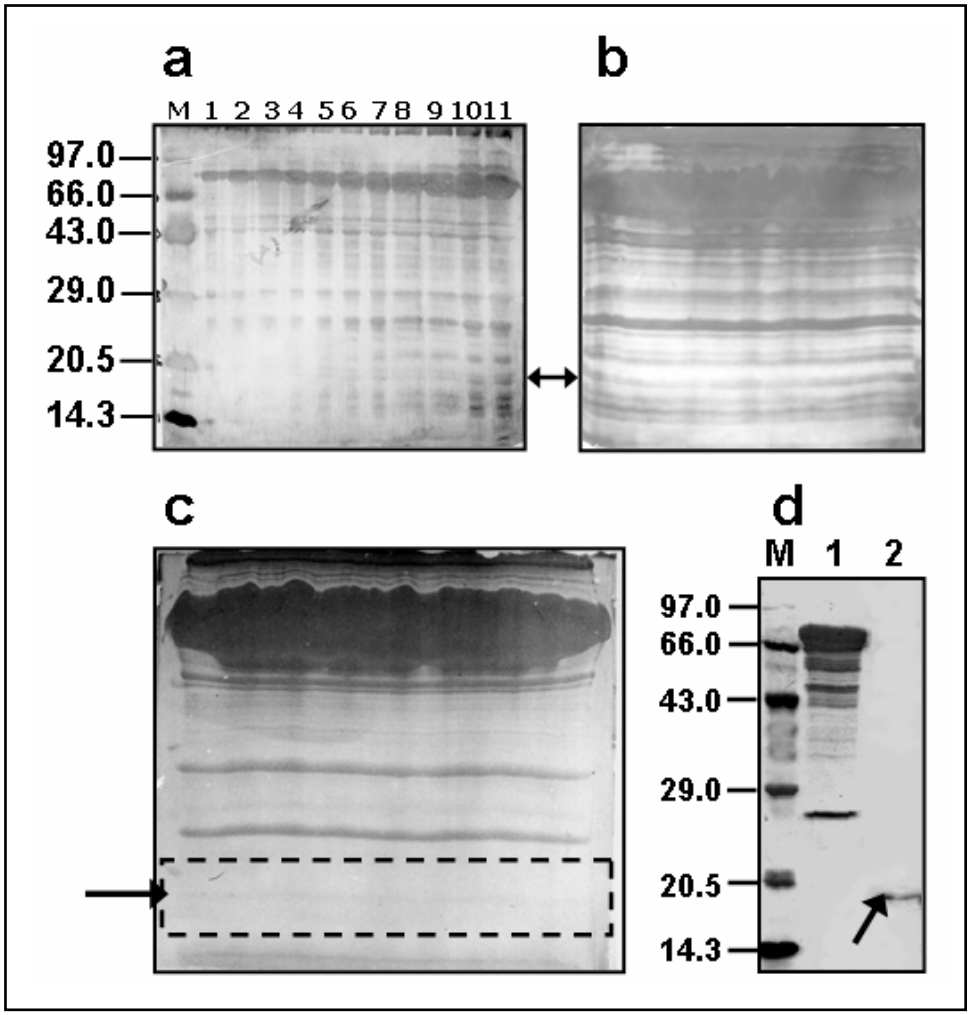


Fig. 8: Electroelution of HP19:-

(a) Profile of total haemolymph proteins from *C. cephalonica* LLI larvae to resolve HP19 on 12% (15 cm long) resolving SDS-PAGE (silver stained). Lane M- protein marker (kDa) and lanes 1 to 11 are 2, 4, 6, 8, 10, 20, 25, 30, 40 & 50 µg total haemolymph protein respectively. Note the presence of HP19 in extremely low concentration which was detected (arrow) only when the total haemolymph protein was 30 µg or more (lanes- 9 to 11).

(b) & (c) Protein profile of overloaded amount of total haemolymph proteins (250 µg) of *C. cephalonica* LLI larvae (b- silver and c- coomassie blue stained). Note that HP19 is present in extremely low concentration (arrow) and hexamerins were the major fractions seen as a thick blob around 85 kDa region. The faint HP19 band (arrow) inside the dotted rectangle of coomassie stained gel (c) was excised from 60 gels, electroeluted and injected to rabbits for the production of polyclonal antibody against HP19 as described in materials and methods.

(d) Shows the electroeluted HP19 used for antibody production. Lanes- (M) protein marker in kDa, (1) total haemolymph protein (10 µg) and (2) electroeluted HP19 (3 µg).

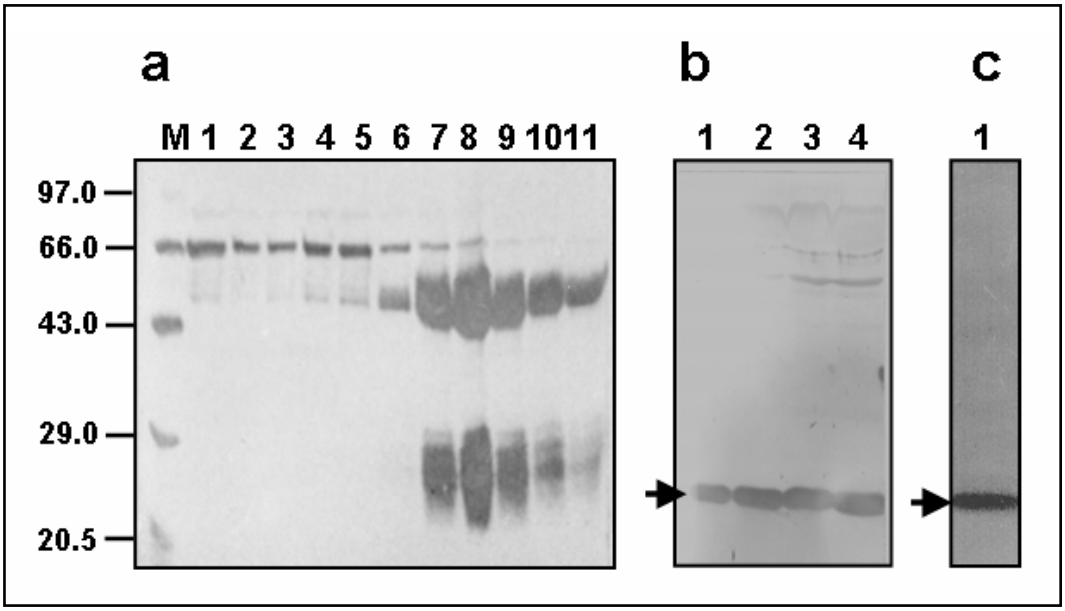


Fig. 9. Production and specificity of polyclonal antibody raised against HP19:-

(a) Shows the purification of anti-HP19 IgG fraction using affinity chromatography. The IgG fraction of HP19 antibody was purified using Protein A-agarose column (Bio-Rad) according to the manufacturer's protocol. Lanes- flow through fractions showing serum albumin (1-5) and eluted IgG fractions that bound to affinity matrix show the 50 kDa heavy chain and 25 kDa light chain (6-11).

(b) Western blot showing specific cross-reactivity of HP19 (arrow) in denatured PAGE. Lanes: 10 μg (1), 20 μg (2), 40 μg (3) and 80 μg (4) of total haemolymph proteins were loaded. Note- even high concentration of total haemolymph proteins did not show much non-specific cross-reactivity.

(c) Western blot showing the specificity for HP19 (arrow) in non-denatured PAGE. Lane 1- 20 μg of total haemolymph protein was loaded.

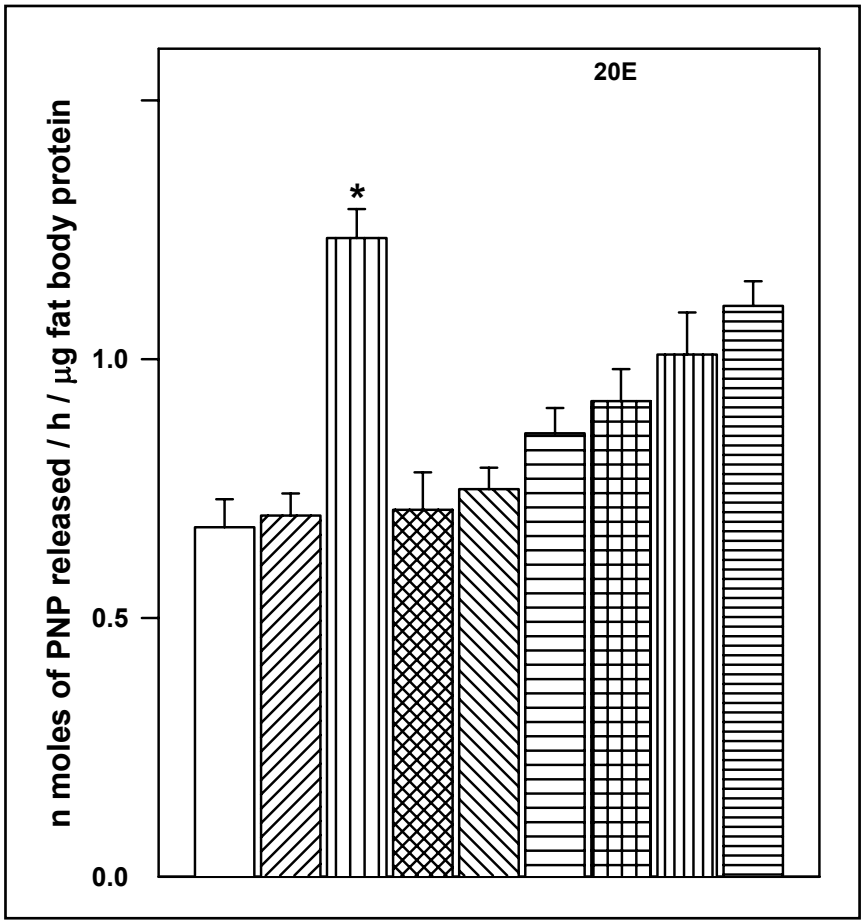


Fig. 10: Functional test of HP19 antibody to check its specificity against HP19 and to confirm it as an antibody against HP19:-

The fat bodies kept in culture were incubated in presence of 20E and fractionated haemolymph (containing HP19) that had the ability to mediate the 20E dependent ACP activity along with the serially diluted HP19 antibody for 4 h at 25 °C. Note the loss in the ability of haemolymph fraction to mediate the 20E dependent ACP activity in case of lower dilutions of antibody. The activity was gradually recovered partially as the antibody dilution increased.

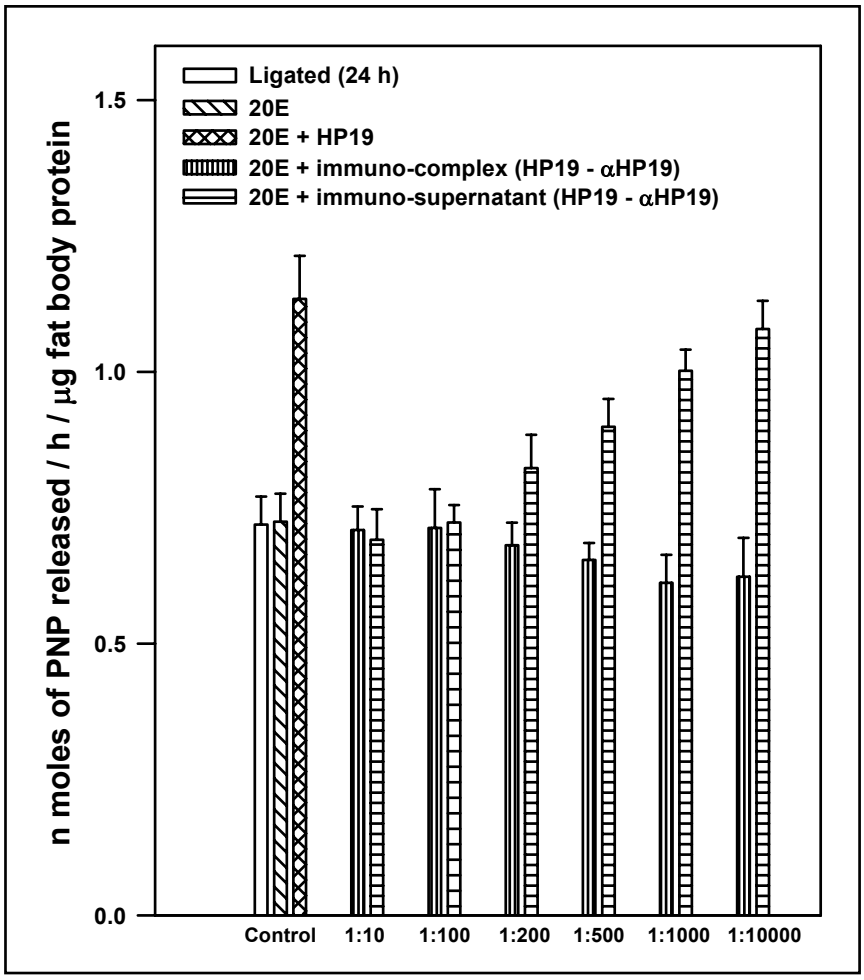


Fig. 11: Functional test of HP19 antibody (αHP19) to check its specificity against HP19 and to confirm it as an antibody against HP19:-

For this experiment, immunoprecipitated HP19 and the resultant supernatant (termed immuno-supernatant) from the total haemolymph protein was added separately to the fat bodies kept in culture in presence of 20E for 4h at 25°C. The results show that the immuno-complex failed to mediate the 20E dependent ACP activity. The immunoprecipitation was carried using serially diluted HP19 antibody with a fixed dilution of haemolymph fraction on a protein A-agarose support. The loss is also evident in the supernatant of low dilution antibody because in this case the antibody precipitated the entire protein with ACP mediating ability as compared to the supernatant obtained from the high dilution, where it was able to mediate the 20E dependent ACP activity.

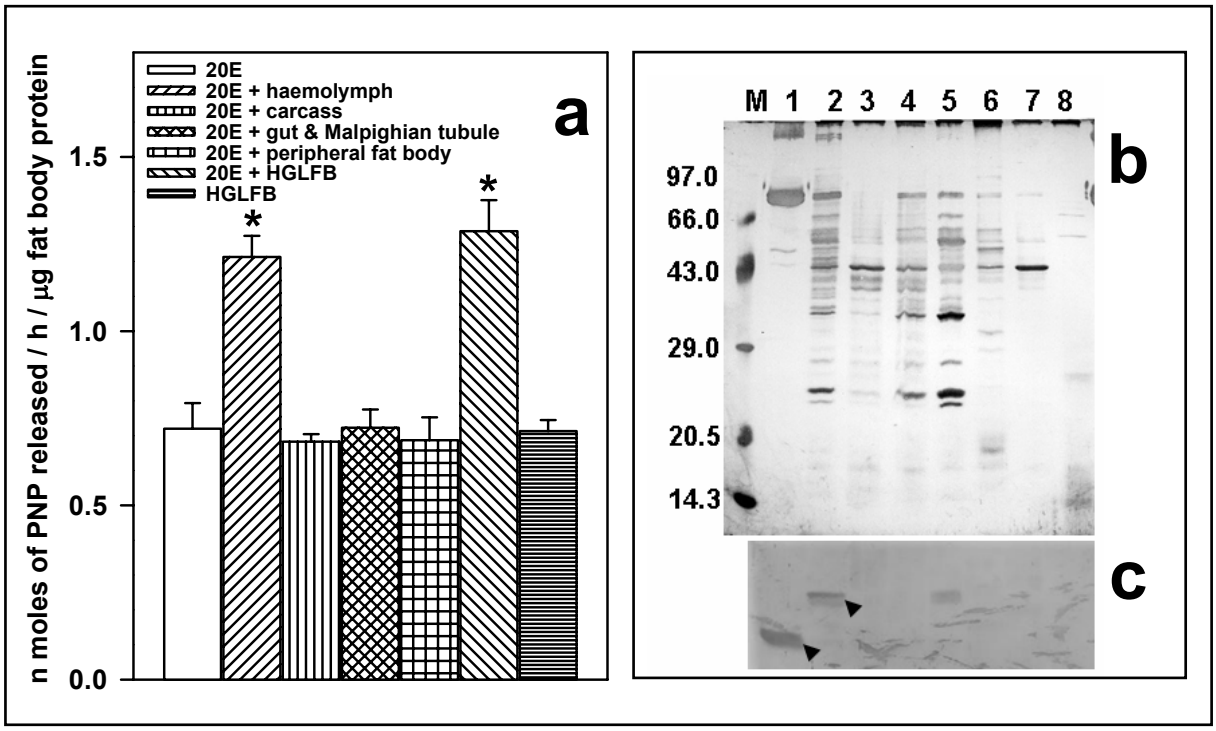


Fig. 12: Tissue-specific biosynthesis of HP19 by the hind gut associated lobular fat body (HGLFB):-

(a) ACP activity in fat bodies from 24 h post-ligated LLI larvae co-cultured with different tissues dissected from 24 h post-ligated larvae in presence of 80 nM 20E for 4 h. 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 four independent determinations. For each determination the fat bodies from two larvae were used. *Significantly different from all other values ($p < 0.05$).

(b) SDS-PAGE and (c) western blot of proteins from different tissues demonstrating the presence of HP19 only in the haemolymph and HGLFB. The protein on blot was probed with HP19 antibody. Lanes- haemolymph (1), HGLFB (2), visceral fat body (3), perivisceral fat body (4), total larval body (5), salivary gland (6), carcass (7) and gut + Malpighian tubule (8).

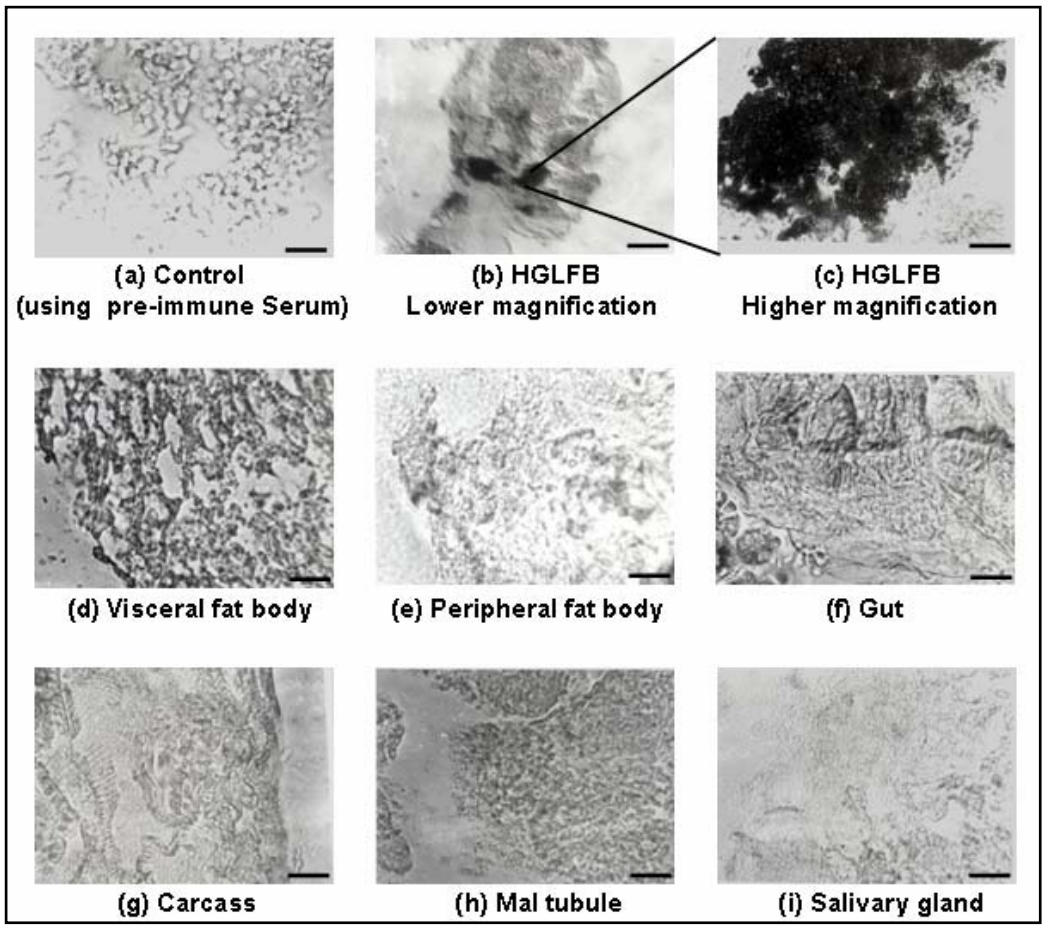


Fig. 13: Immunohistochemical localization of HP19 in HGLFB:-

The tissue sections were immunostained using anti-rabbit IgG fraction as described in materials and methods. The control slide was processed with pre-immune rabbit serum to check the specificity of antibody (a). Note the presence of HP19 in HGLFB in low (b) and high magnification (c). Other tissues like visceral fat body (d), peripheral fat body (e), gut (f), carcass (g), Malpighian tubule (h) and salivary gland (i) did not show any cross-reactivity. Scale bar for (b) 10 μm = 0.15 cm and for (a and c to i) 10 μm = 0.45 cm.

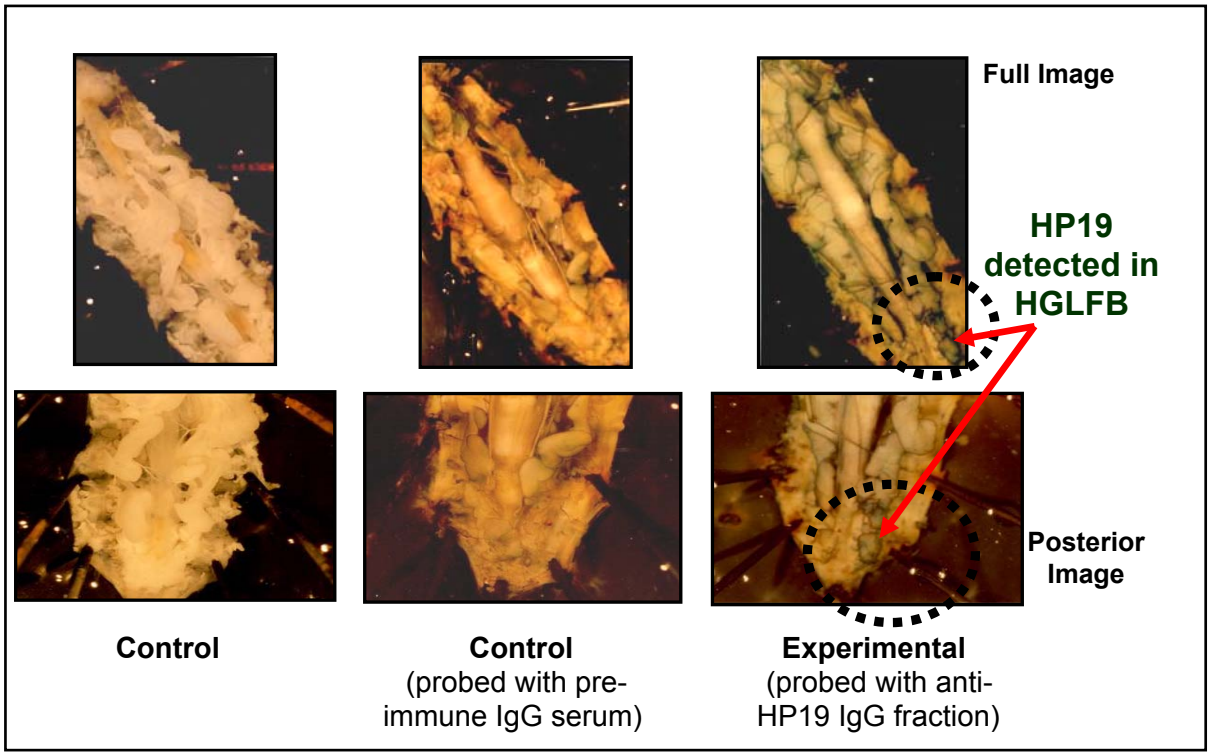


Fig. 14: *In situ* immunodetection of HP19 in *C. cephalonica* LLI larvae:-

The LLI larvae were cut open through the dorsal surface and the tissue inside the dissected larvae were fixed and processed for detection of HP19, using anti-HP19 IgG fraction after tissue permeabilisation as described in materials and methods. The immunocrossreactivity was detected in the HGLFB (arrow head inside dotted circle). One set of control larvae were processed using pre-immune serum.

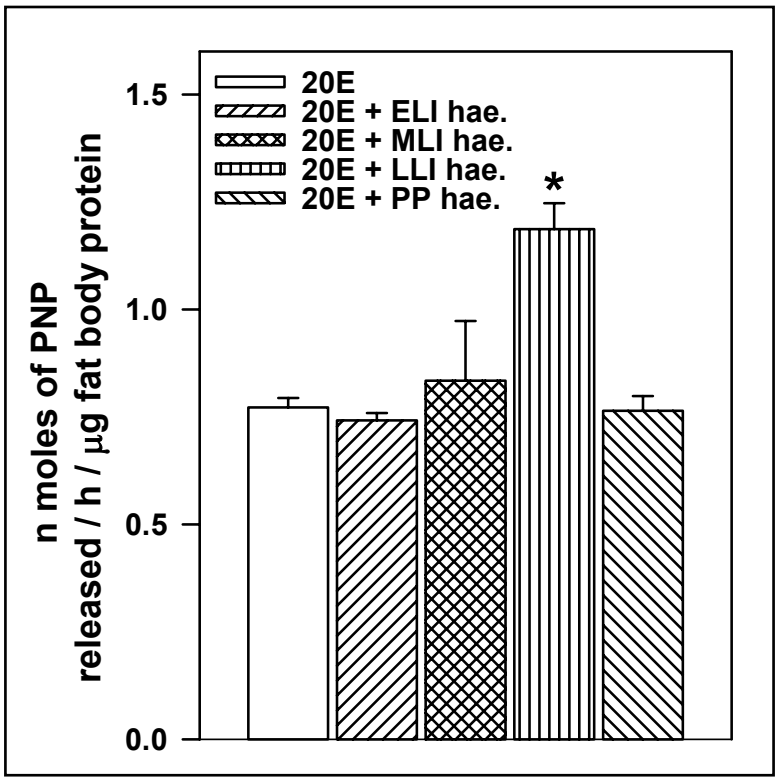


Fig. 15: Developmental regulation of HP19:-

Effect of 20E on ACP activity of fat bodies kept in culture in the presence of haemolymph from different developmental stages of *C. cephalonica.* Ten μl of fractionated haemolymph from ELI, MLI, LLI larval and prepupal (PP) stages, was added to the fat bodies kept in culture, incubated for 4 h with 80 nM 20E and assayed for ACP activity. Each value is the mean ± S.D. of four independent determinations. For each study, the fat bodies were dissected from two 24 h post-ligated LLI larvae. *Significantly different from all other values ($p < 0.05$).

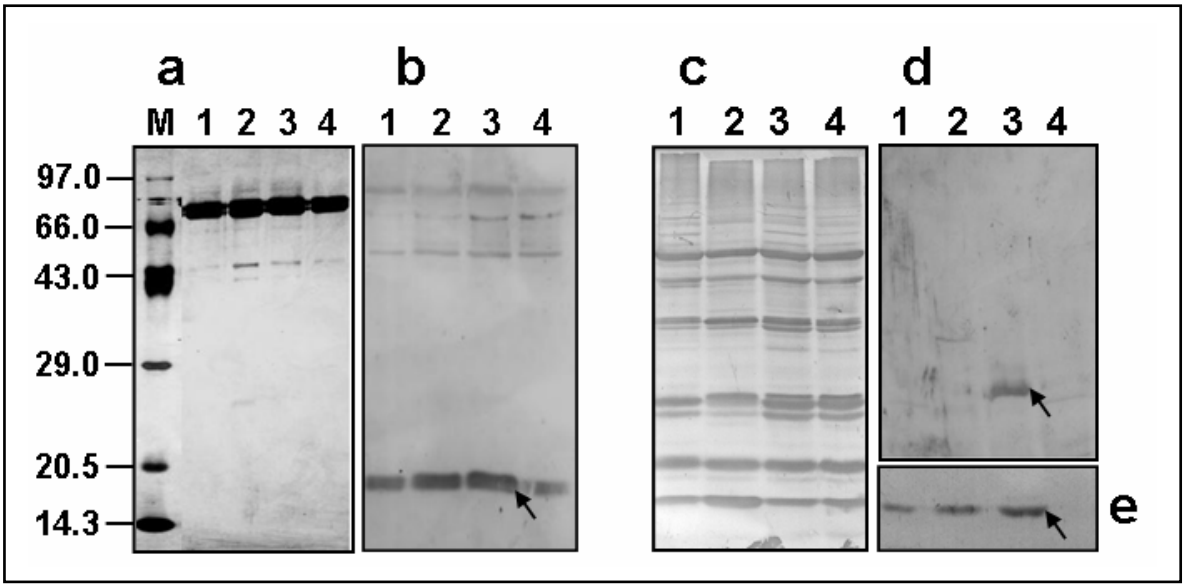


Fig. 16: Developmental regulation of HP19:-

(a) & (b) show the profile of HP19 in the haemolymph of different developmental stages of *C. cephalonica* on SDS-PAGE and western blot respectively.

(c), (d) and (e) show the profile of HP19 in the HGLFB of different developmental stages of *C. cephalonica* on SDS-PAGE (c) and western blot (d and e) respectively.

For (c) and (e), 20 µg of total protein from final (Vth) instar developmental stages *viz.,* ELI (lane 1), MLI (lane 2), LLI (lane 3) and PP (lane 4) was loaded in each lane and probed with anti-HP19 IgG fraction for detection of HP19 while in (d) 8 µg protein was loaded in each lane. Note the presence of highest concentration of HP19 in LLI stage both in haemolymph (b) as well as HGLFB (d and e) on western blots.

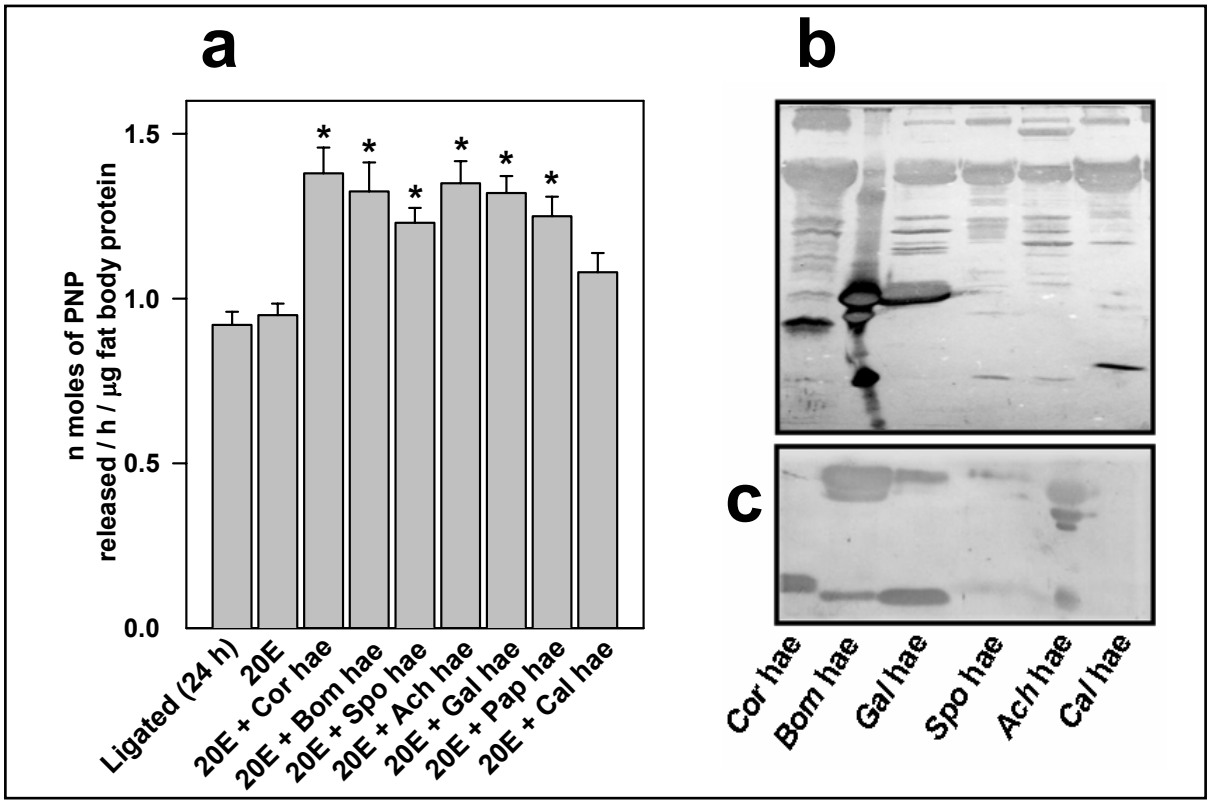


Fig. 17: Presence of HP19 like protein in other insect species:-

(a) Effect of 20E on the ACP activity of fat bodies of *C. cephalonica* kept in culture in the presence of haemolymph from different species of lepidoptera and a diptera. The fat bodies from two 24 h post-ligated *C. cephalonica* LLI larvae, kept in culture were incubated with 80 nM 20E and 10 µl of 1:20 diluted haemolymph from different species for 4 h. To the control cultures equal volume of insect Ringer was added with or without 20E. At the end of the incubation, the fat bodies were removed, rinsed, homogenized and assayed for ACP activity. Each value is the mean ± S.D. of four independent determinations. Note that haemolymph from all lepidopteran insect larvae could mediate the 20E dependent action to stimulate ACP activity. However, the degree of mediation by LLI larval haemolymph of a dipteran insect (*Calliphora vicina*) was fairly low when compared with the haemolymph of all other lepidopteran species. * Significantly different over controls and over the mediation effect by *C. vicina* haemolymph ($p<0.05$).

(b) & (c) are respectively the SDS-PAGE and western blot profile of haemolymph proteins from different species to show the presence of HP19 in these insect species. The HP19 like protein on western blot was detected using *C. cephalonica* anti-HP19 IgG fraction. Note that the haemolymph protein from all lepidopteran insects cross-reacted (multiple cross-reaction) with *C. cephalonica* HP19 antibody, however, the cross reactivity was totally absent in the diptera, *C. vicina*. Cor- *Corcyra,* Bom- *Bombyx,* Spo- *Spodoptera,* Ach- *Achaea,* Pap- *Papilio* and Cal- *Calliphora*.

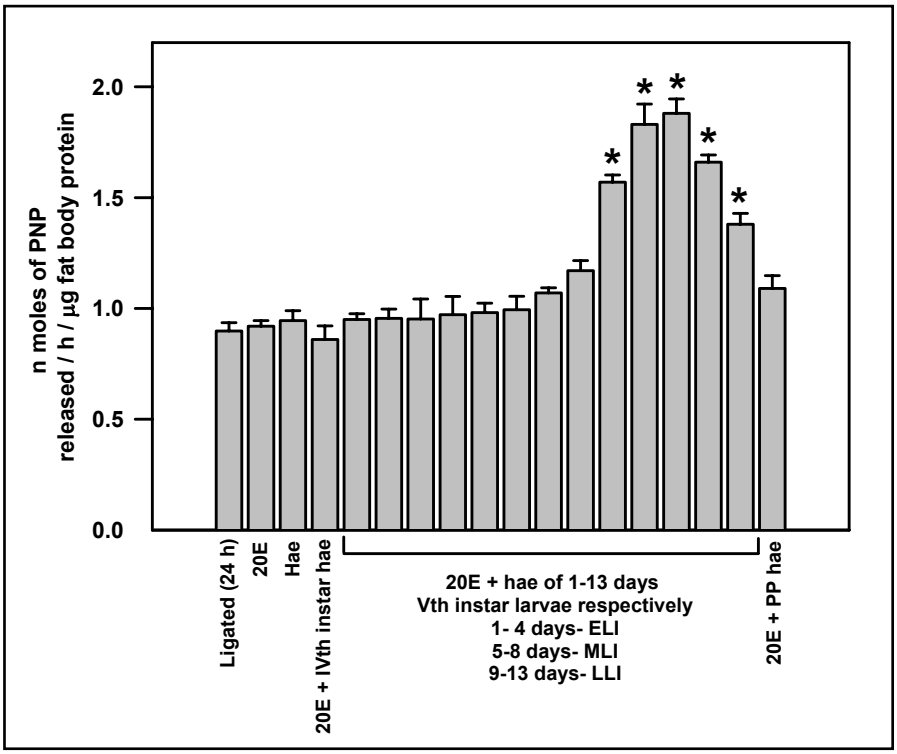


Fig. 18: Developmental regulation of HP19 like protein in silkworm, *Bombyx mori*:-

The haemolymph sample was prepared from penultimate (IVth) instar larvae, different days of final (Vth) instar larvae and prepupae (PP) and used to check its ability to mediate the 20E dependent stimulation of ACP activity of *C. cephalonica* fat bodies kept in culture. Note that only the haemolymph from LLI stage could mediate 20E dependent ACP activation. Each value is mean ± S.D. of four independent determinations. *Significantly different from all other values ($p < 0.05$).

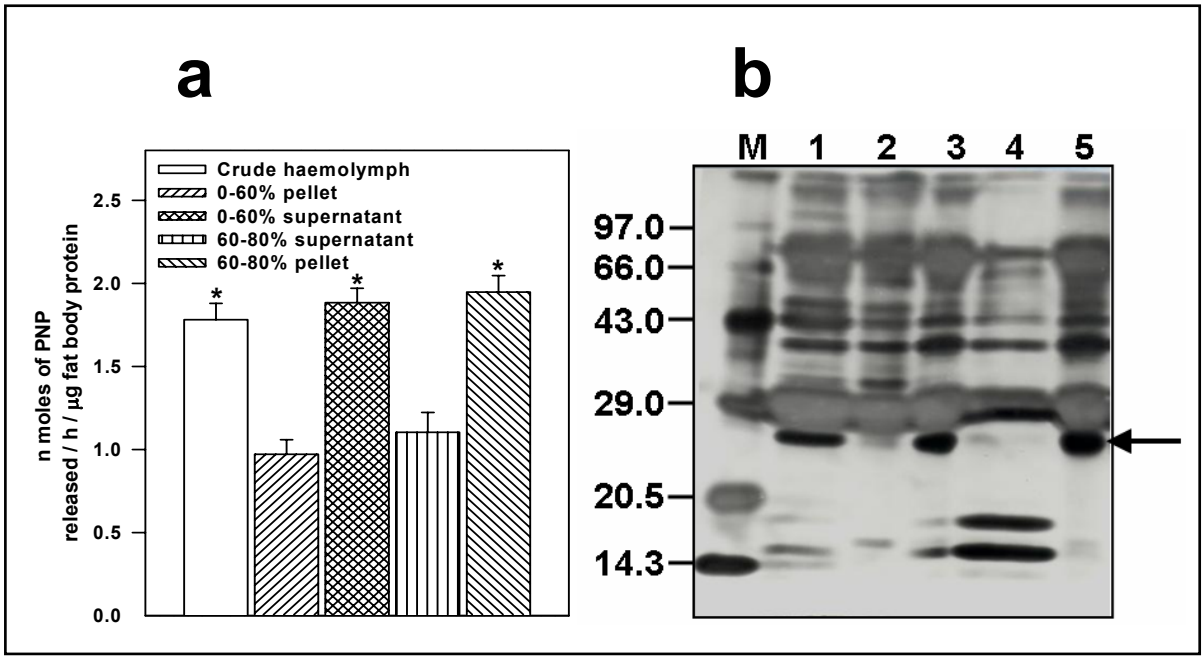


Fig. 19: Partial characterization of HP19 like protein in silkworm, *Bombyx mori*:-

The haemolymph sample from LLI stage was subjected to 60% ammonium sulfate precipitation, the supernatant obtained was subjected to 60-80% precipitation. (a) Shows the ability of different fractions to mediate the 20E dependent stimulation of ACP activity of *C. cephalonica* fat bodies kept in culture. (b) Shows the protein profile of these fractions analyzed on 12% SDS-PAGE. Note the appearance of approximately 26 kDa protein (arrow) in all the active fractions (lanes 1, 3 & 5). Lanes- protein marker in kDa (M), crude haemolymph (1), 60% pellet (2), 60% supernatant (3), 60-80% supernatant (4) and 60-80% pellet (5).

Results- Chapter II

Cloning, sequencing and molecular characterization of HP19

Part of this work has been published in-

Arif *et al.*, (2004). The insect haemolymph protein HP19 mediates the nongenomic effect of ecdysteroids on acid phosphatase activity. ***J. Biol. Chem.*** 279, 28000-28008.

Background-

Several lines of evidence suggest that the regulatory molecules present in haemolymph could be peptides or proteins (Stone and Mordue, 1980; Candy, 1981; Keeley, 1985). Present study revealed that the haemolymph factor of *C. cephalonica* that mediated the 20E dependent activation of ACP was heat labile and was also sensitive to acid and alkali treatment (Refer Chapter I, Fig. 2). In the earlier result section of the thesis, the identification, isolation and biochemical characterization of the haemolymph protein, HP19 that mediates the 20E dependent activation of ACP was presented. To gain more insight into the nature and function of HP19, a cDNA for *C. cephalonica* HP19 (CcHP19) was produced and characterized. This chapter of thesis deals with the characterization of HP19 at molecular level to understand its homology with other known regulatory proteins.

Comparison of HP19 with other regulatory conserved proteins-

Presence of HP19 like protein in the haemolymph of all the lepidopteran insects investigates so far (Fig.17) suggested that the HP19 molecule possibly is conserved in nature. Hence, in order to ascertain if this protein has any similarity with other highly conserved regulatory proteins such as 14-3-3 (Shaw, 2000; Fu *et al.,* 2002), stathmin (Sobel, 1991; Ozon, 1997) and ubiquitin (Jentsch and Pyrowolakis, 2000; Weissman, 2001), a detail study was carried out. The results revealed that HP19 has no immunological identity with these selected proteins (Figs. 20 and 21). Antibody against 14-3-3 α and β subunit showed no cross-reactivity with proteins in the mass range of HP19, in either the synthesizing tissue, HGLFB or at the release site haemolymph or any other tissues (Fig. 20a and b). Antibody against stathmin also failed to show any cross reactivity with HP19 (Fig. 21a, lane 2). Although ubiquitin antibody did show cross-reactivity with proteins in the mass range of 19 kDa in the haemolymph of *C. cephalonica* (Fig. 21b, lane 2) but further analysis by reprobing the immunoprecipitated HP19 (which was immunoprecipitated using anti-HP19 IgG fraction and shown in Fig. 21b, lane 3) on western blot using anti-ubiquitin did not show any cross-reactivity with the immunoprecipitated HP19 (Fig. 21b, lane- 4). This study clearly suggests that HP19 is different from these conserved proteins.

cDNA cloning and sequence analysis of HP19-

The novel nature and function of HP19 prompted us to look in detail the HP19 protein at molecular level. To identify the cDNA encoding the HP19 protein, a cDNA expression

library, prepared from RNA of HGLFB of LLI larvae was immunoscreened with anti-HP19 IgG fraction. The detailed cloning strategy is represented as a flow chart in figure 22. After three rounds of immunoscreening, 10 positive cDNA clones were picked for detailed examination (Fig. 23). The restriction analysis revealed 6 of the 10 clones to be of identical size (Fig. 24). Initial sequencing study with these identical size clones demonstrated significant sequence similarity among them. Furthermore, they showed homology with invertebrate glutathione S-transferases (GSTs). One of these clones was sub-cloned and totally sequenced (Fig. 25, GenBank accession AY369240). This HP19 cDNA was 634 nucleotides long, with an open reading frame of 585 bp, which encodes a protein of 195 amino acids. The calculated molecular mass of the translated unmodified protein was 22.95 kDa, which is close to the mass of HP19 detected in HGLFB, the tissue that synthesizes the protein (Fig. 12c, lane 2). The polypeptide comprises of 12.3% basic (9 arg, 1 his and 14 lys) and 13.3% acidic (10 asp and 16 glu) residues, but no cys residue. The estimated isoelectric point (pI) is 5.36. The cDNA sequence begins with the methionine start codon at position 1 and translation stop codon at 586. A 3` untranslated sequence containing a polyadenylation signal AATAAA is located at 588 nucleotide followed by a poly $(A)_{29}$ tract. The polyadenylation signal overlapped the translation stop codon by one base. Comparison of the *C. cephalonica* HP19 (CcHP19) cDNA with the sequences in the GenBank, showed 67% identity with *Choristoneura fumiferana* GST (CfGST) (Feng *et al.,* 1999). Similarity of HP19 cDNA with other invertebrate GSTs was found to be less than 38%. The comparison of the amino acid sequence of CcHP19 with the 4 best matching invertebrate GSTs is shown in figure 26. Percentage identity of CcHP19 cDNA with other GSTs is shown in figure 27. These analyses revealed that except CfGST, the identity with all other GSTs is 35% or less. Although, the CcHP19 cDNA sequence revealed 67% identity with CfGST, affinity purified GST from *C. cephalonica* had no enhancing effect on the 20E dependent ACP activity when compared with the purified or recombinant HP19 (Fig 29a). Furthermore, the haemolymph as well as the purified HP19 had negligible GST activity (Fig. 29b). Figure 28 shows the expression of recombinant CcHP19 in *E. coli* XL1 blue strain that allows regulated expression (refer Clontech protocol PT3003-1; Wood *et al.,* 1985). Analysis of ACP activity in different tissues (Fig. 30) suggests that haemolymph also has negligible ACP activity. Hence the increase in ACP activity is not due to the sequestration of the enzyme from the haemolymph but is mainly due to the stimulation. Figure 31 shows the identification of GST in various tissues from the LLI larval stage of *C. cephalonica* using antibody against the GST of *C. fumiferana* (Fig. 31a) with which the CcHP19 showed highest sequence identity *i.e.,*

67% (Feng *et al.,* 1999), and the purification of cytosolic GST by affinity chromatography (Fig. 31b, lane- 6). Although the CfGST antibody cross-reacted with a protein in haemolymph as well as HGLFB of *C. cephalonica* whose molecular range is comparable to the mass of HP19 (Fig. 31a), however, the affinity purified *C. cephalonica* cytosolic GST could not replace the function of HP19 on 20E actions on ACP activity stimulation (Fig. 29). The molecular mass of purified GST from *C. cephalonica* was found to be approximately 24.5 kDa.

Southern analysis of genomic DNA and tissue specific expression of HP19 upon northern analysis-

Southern analysis of genomic DNA of the total larval body digested with *EcoRI* or *HinfI*, and probed with HP19-cDNA revealed HP19 as a single copy gene (Fig. 32a). Northern hybridization with total RNA isolated from whole body as well as different tissues displayed the tissue specific expression of HP19 gene in HGLFB (Fig. 32b). The 0.66 kb HP19 signal obtained in northern analysis indicates a mass of about 24 kDa which corroborates with the mass of HP19 synthesized in the HGLFB detected on western blot (Fig. 12c, lane 2).

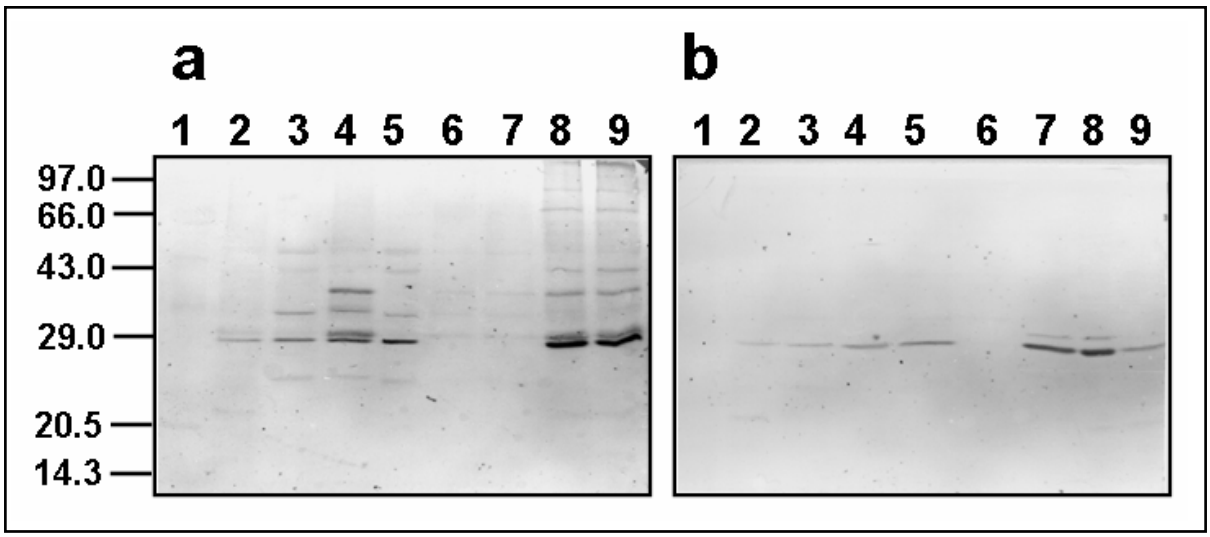


Fig. 20: Comparison of HP19 with 14-3-3 protein:-

(a) & (b) are western blot analysis of 14-3-3 α and β respectively in various tissues of LLI larvae using the respective polyclonal antibodies against these proteins (Santa Cruz Biotechnology). Note that both the antibodies failed to identify the HP19 in haemolymph (lane 1) or any other larval tissues. Lanes- haemolymph (1), total larval body (2), peripheral fat body (3), HGLFB (4), visceral fat body (5), perivisceral fat body (6), salivary gland (7), carcass (8) and gut + Malpighian tubules (9).

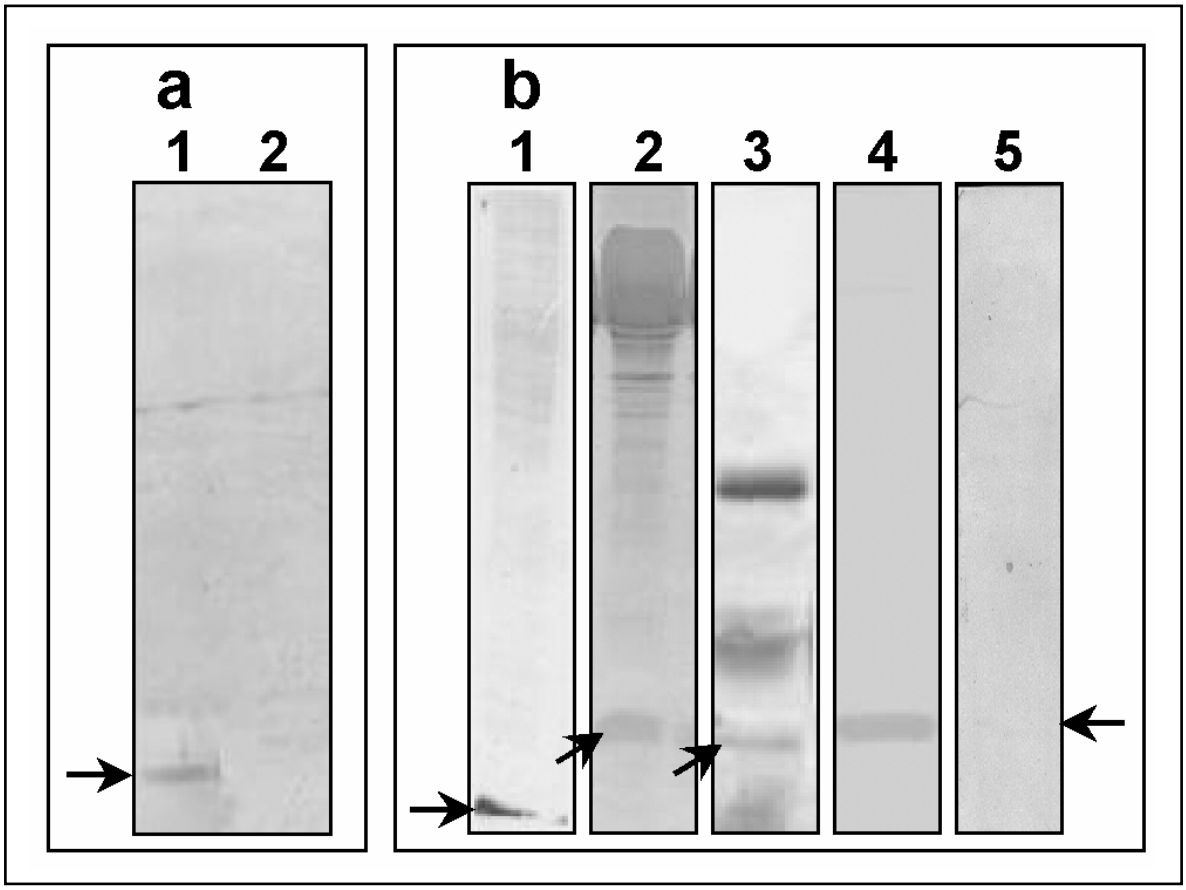


Fig. 21: Comparison of HP19 with stathmin and ubiquitin proteins:-

(a) Western blot analysis to check the similarities with stathmin using polyclonal antibody against stathmin. Note that stathmin like protein was undetected in *C. cephalonica* haemolymph. Lanes: 1- rat brain extract and 2- total haemolymph protein from LLI larvae of *C. cephalonica*.

(b) Comparison of HP19 with ubiquitin protein using polyclonal antibody against ubiquitin. Lanes (1) and (2) are the direct western blot analysis of rat brain and total haemolymph proteins from LLI larvae of *C. cephalonica,* whereas lanes 4 and 5 are the immunoprecipitated HP19 (using anti-HP19 IgG fraction), reprobed either with HP19 antibody (lane 4) or with polyclonal antibody against ubiquitin (lane 5). Lane 3- shows the immunoprecipitated HP19 (arrow) using anti-HP19 IgG fraction. The other two bands represent the heavy and light chain of IgG. Note, though ubiquitin antibody identified a 19 kDa band (arrow) in larval haemolymph in direct western blot analysis (lane 2), but fails to cross-react with immunoprecipitated HP19 (lane 5). The ubiquitin shows cross-reaction with rat brain sample (lane 1).

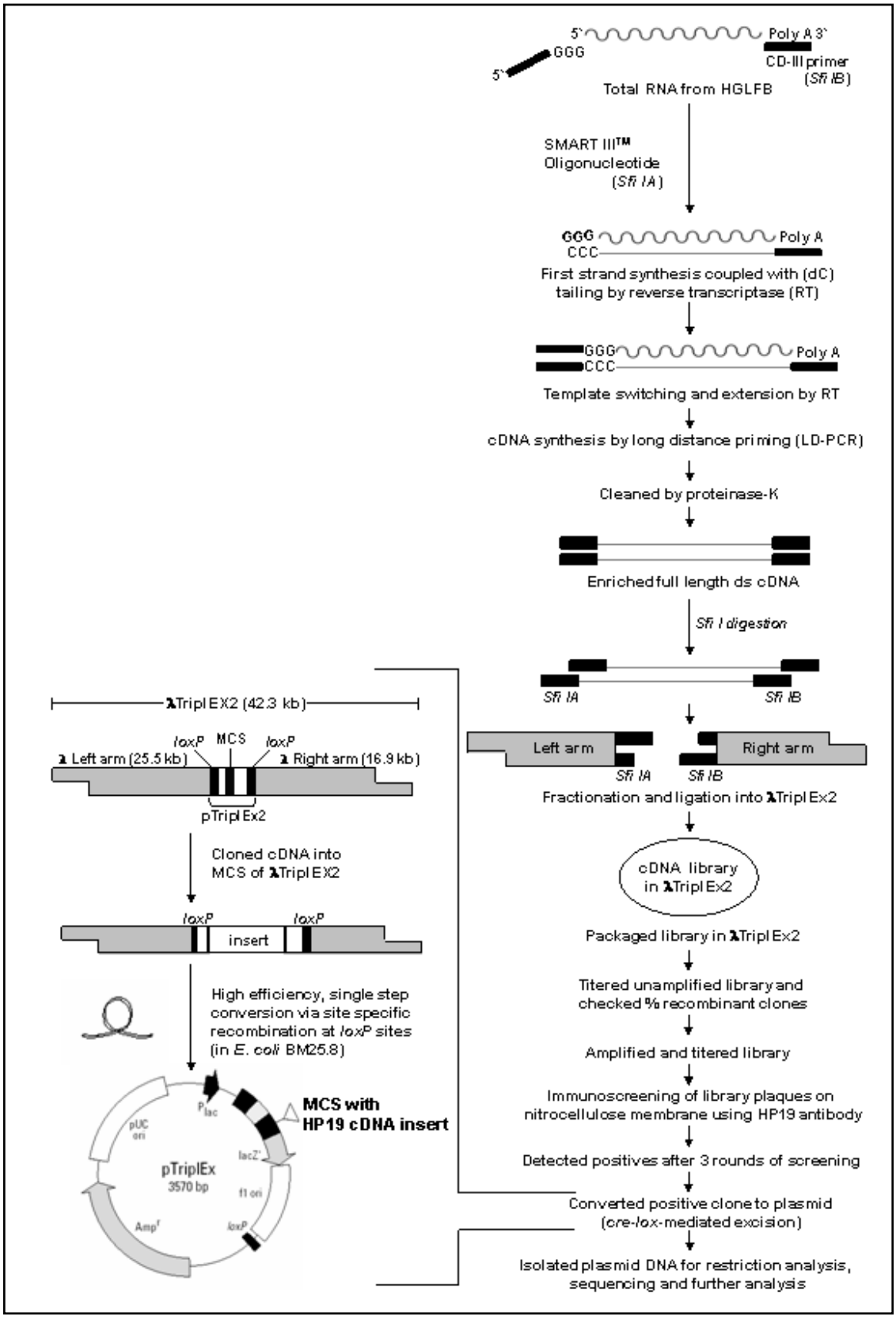


Fig. 22: Cloning strategy for cDNA cloning of HP19 gene.

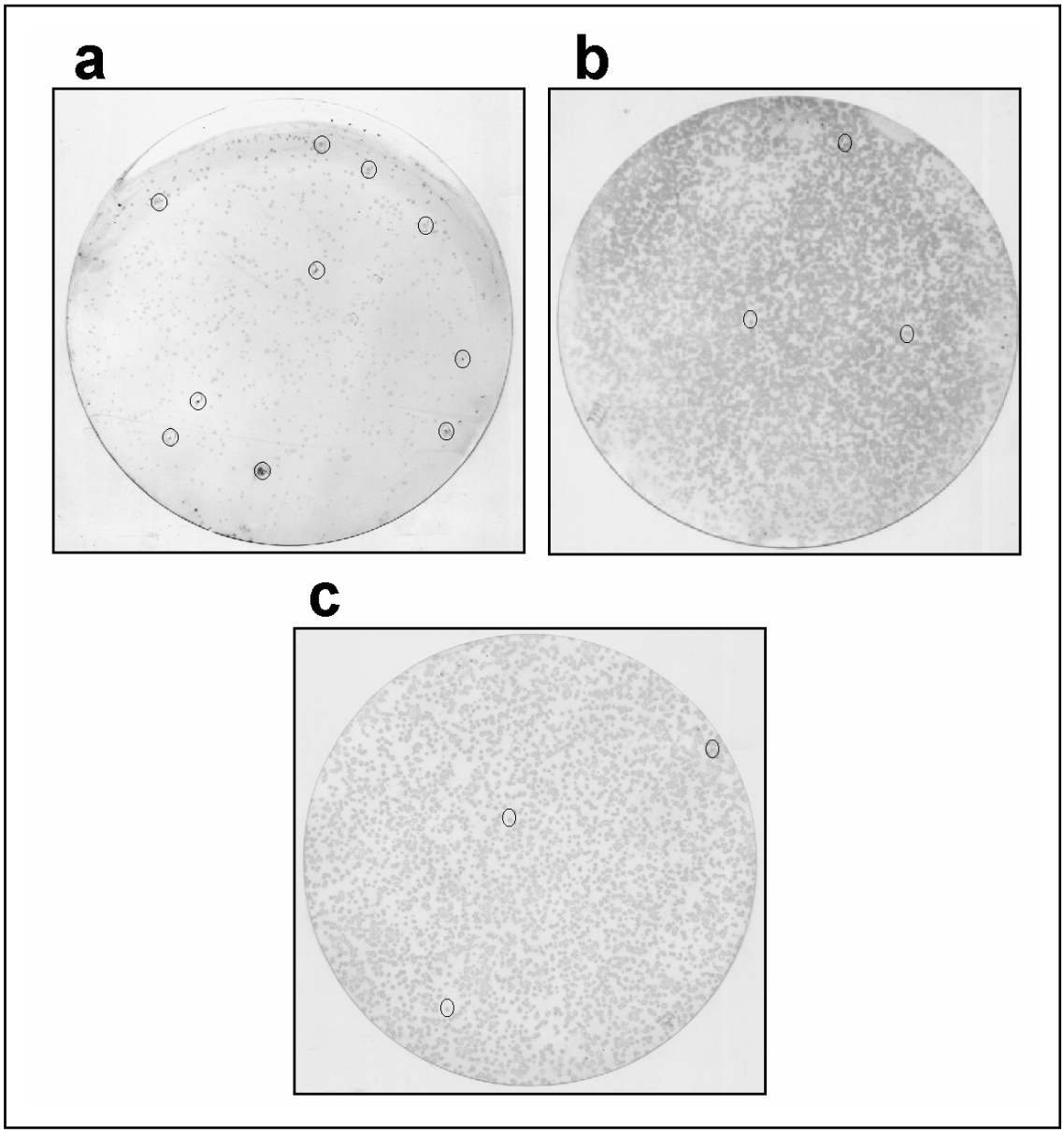


Fig. 23: Immunoscreening of HGLFB cDNA expression library using anti-HP19 IgG fraction for selection of HP19 encoding cDNA:-

(a-c) are photographs of the immunoscreening blots after 1st, 2nd and 3rd round respectively. Here only one of the blots, is shown for each round of screening. After 3 rounds, 10 positives were picked and used for further analysis.

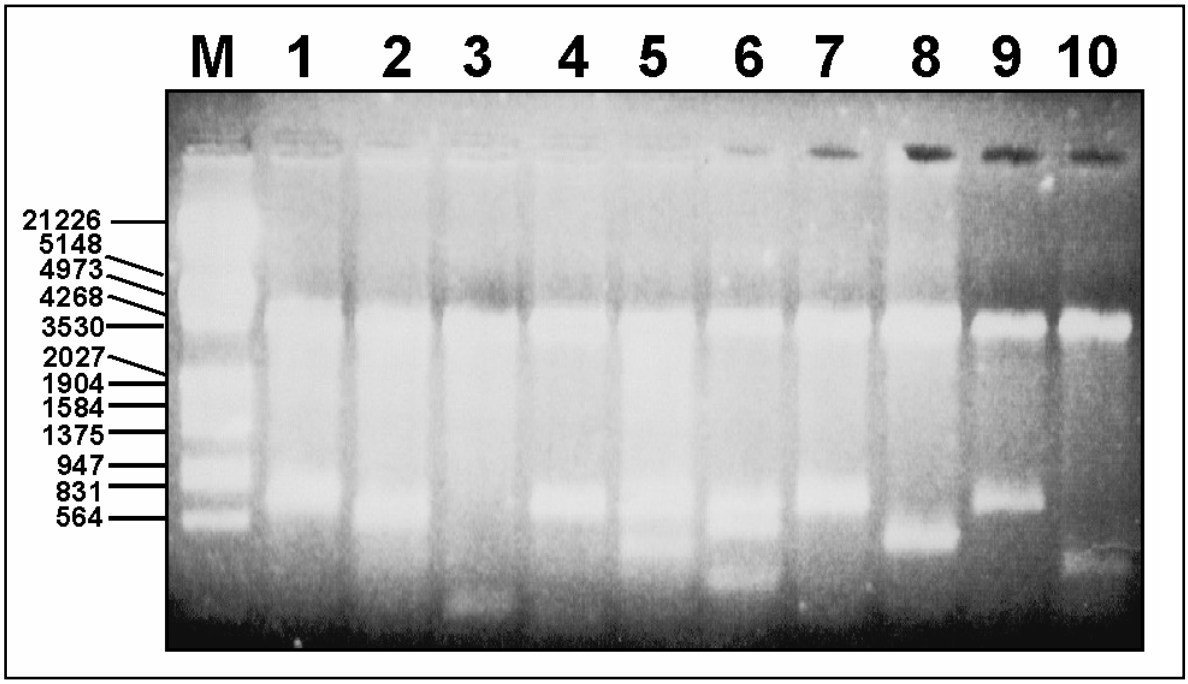


Fig. 24: Restriction analysis (double digest) of all the positives obtained after third round of immunoscreening of HGLFB-cDNA expression library:-

By immunoscreening of 6 x 10^9 recombinant phage plaques, ten positives were obtained after third round. They were *in vivo* excised, converted into plasmids and used for XL-1Blue cell transformation Approximately 1 µg of plasmid DNA was subjected to *Eco RI* + *Not I* digestion. Lane M- λDNA *Eco RI/Hind III* double digest and lanes 1 to 10 are the double digested plasmid DNA of all the ten positive clones picked during screening. The restriction analysis revealed six of these clones to be of nearly identical size (lanes 1, 2, 4, 5, 7, and 9).

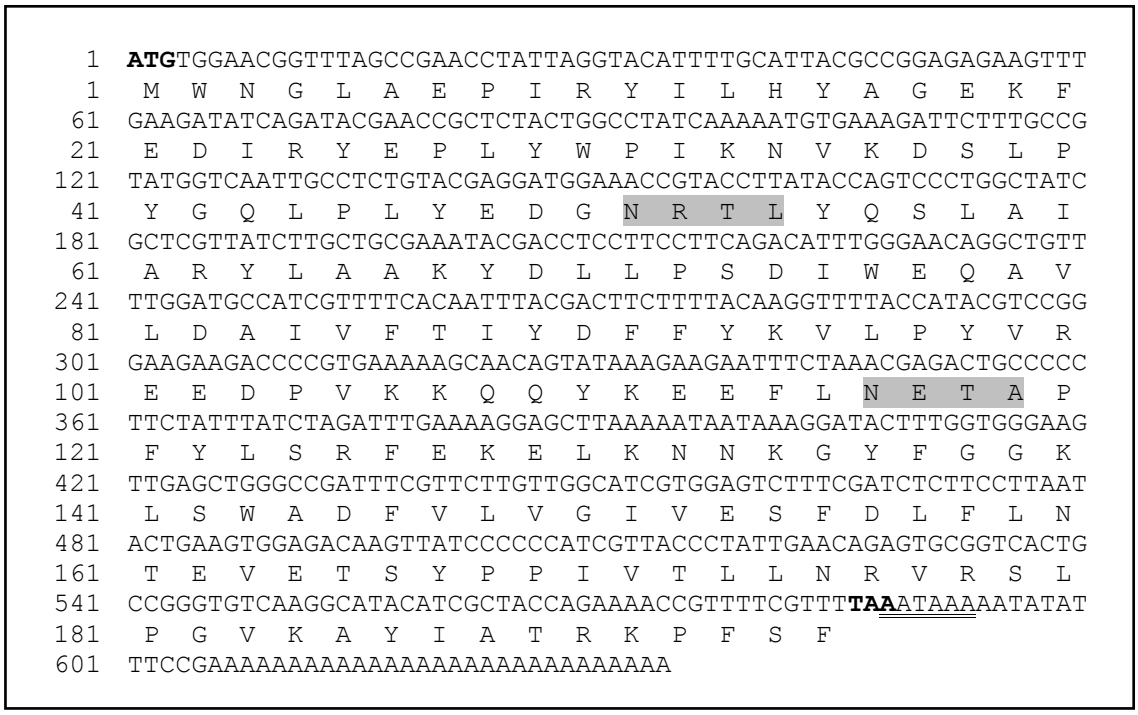


Fig. 25: The cDNA nucleotide and deduced amino acid sequence of *C. cephalonica* HP19 (CcHP19):-

The translation start signal at 1 and stop codon at 586 are in bold letters. The putative polyadenylation signal is underlined. Two putative N-glycosylation sites are shaded. The GenBank accession number for the sequence is AY369240.

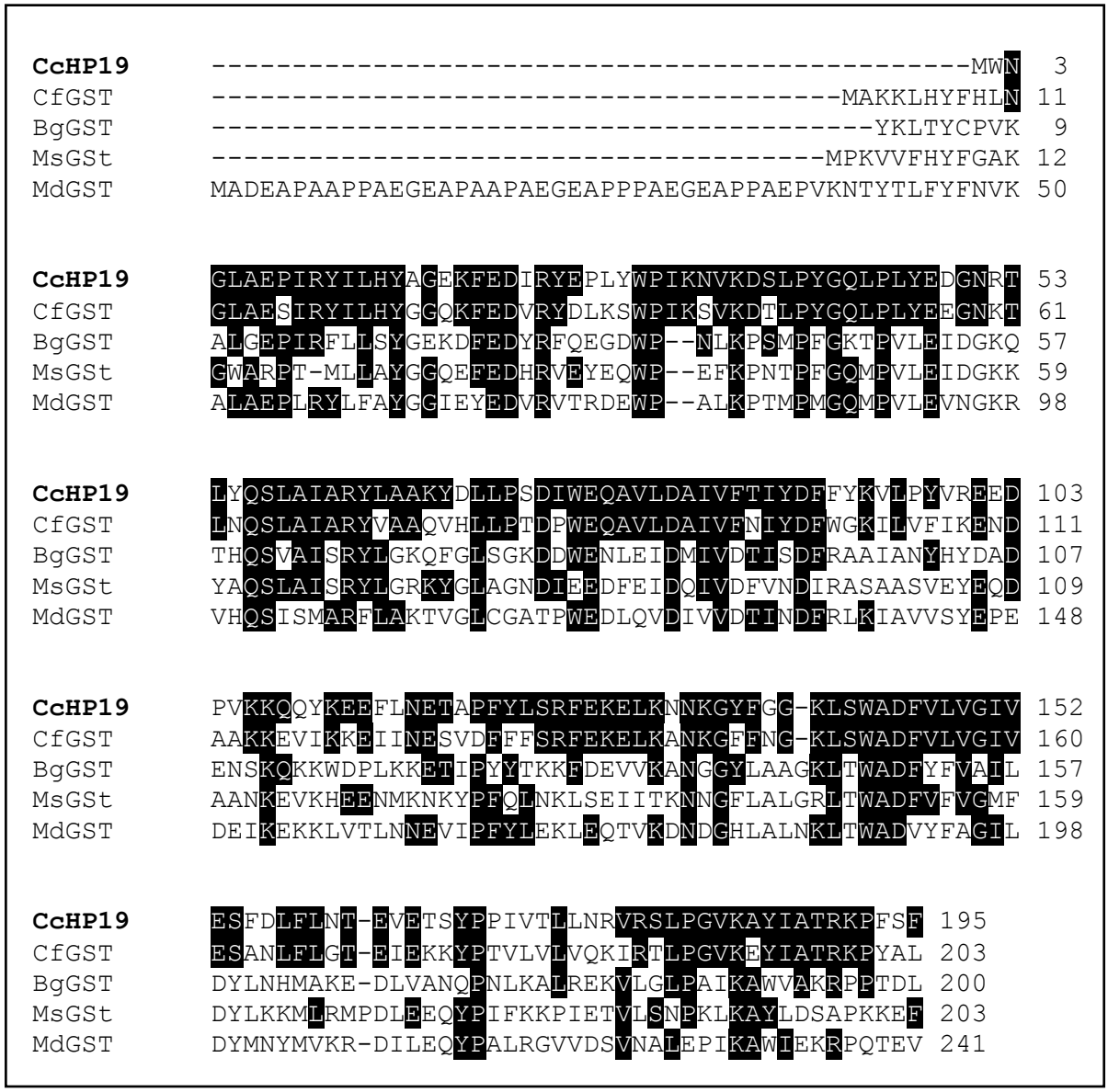


Fig. 26: Alignment of the deduced amino acid sequence of *C. cephalonica* HP19 (CcHP19) with GST sequences of other insects (BLAST search):-

Choristoneura fumiferana- CfGST (AF128867); *Blattella germanica*- BgGST (U92412); *Manduca sexta*- MsGST (L32092) and *Musca domestica*- MdGST (U02616). The identical amino acid positions are shaded and gaps are indicated by dashes. CcHP19 showed 67% identity with CfGST, 35% with BgGST, 32% with MsGST and 31% with MdGST.

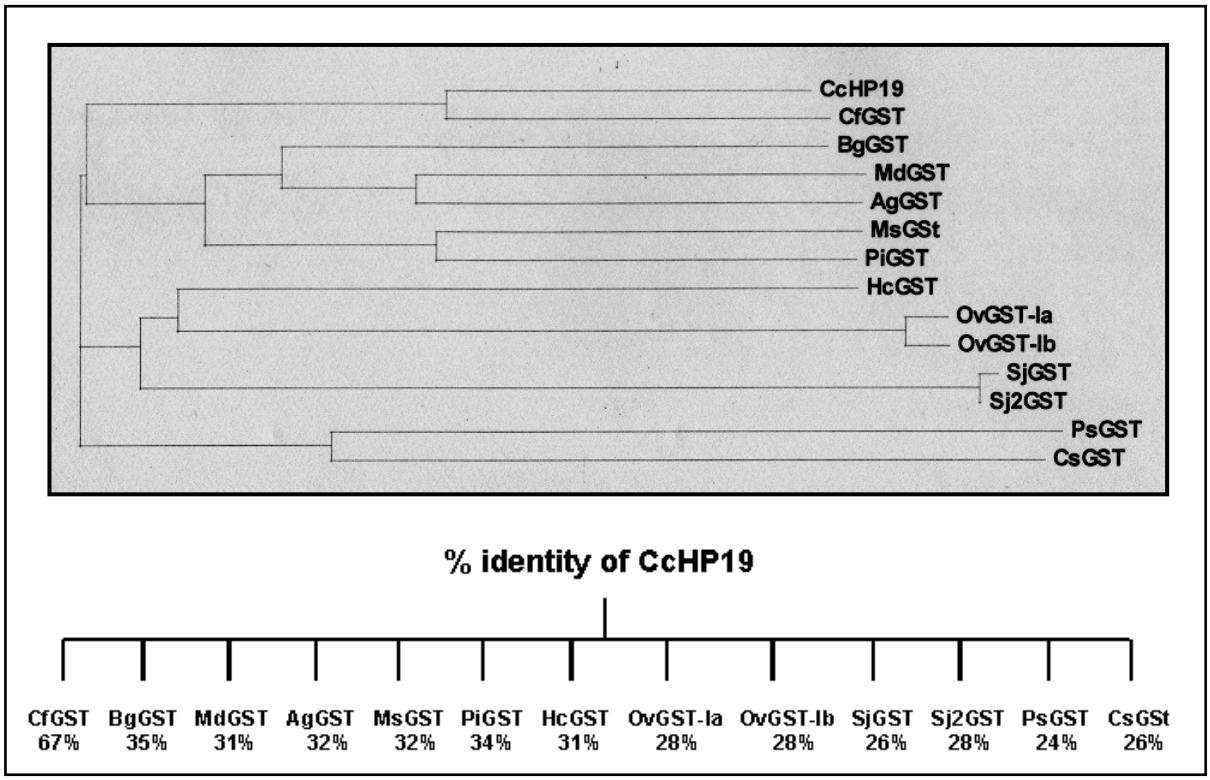


Fig. 27: Phylogenetic tree and percentage identity of *C. cephalonica* HP19 (CcHP19) with some of the invertebrate GSTs:-

Choristoneura fumiferana- CfGST (AF128867); *Blattella germanica*- BgGST (U92412); *Musca domestica*- MdGST (U02616); *Anopheles gambiae*- AgGST (L07880); *Manduca sexta*- MsGST (L32092); *Platynota idaeusalis*- PiGST (AF082570); *Haemonchus contortus*- HcGST (AF281663); *Onchocerca volvulus* Ia- OvGST-Ia (AF265556) and OvGST-Ib (AF265557); *Schistosoma japonicum*- SjGST (U58012) and Sj2GST (AF044411); *Psoroptes ovis*- PsGST (AF078684) and *Clonorchis sinensis*- CsGST (L47992).

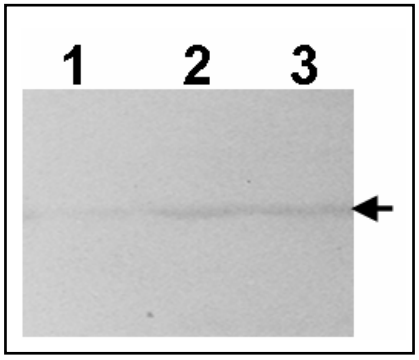


Fig. 28:- Basal level HP19 expression cloned in pTriplEx2 vector in *E. coli* XL1 Blue bacterial strain detected by anti-HP19 IgG fraction on western blot of bacterial lysates. Control (lane 1), in presence of 1 mM IPTG 1 h (lane 2) and 2 h (lane 3).

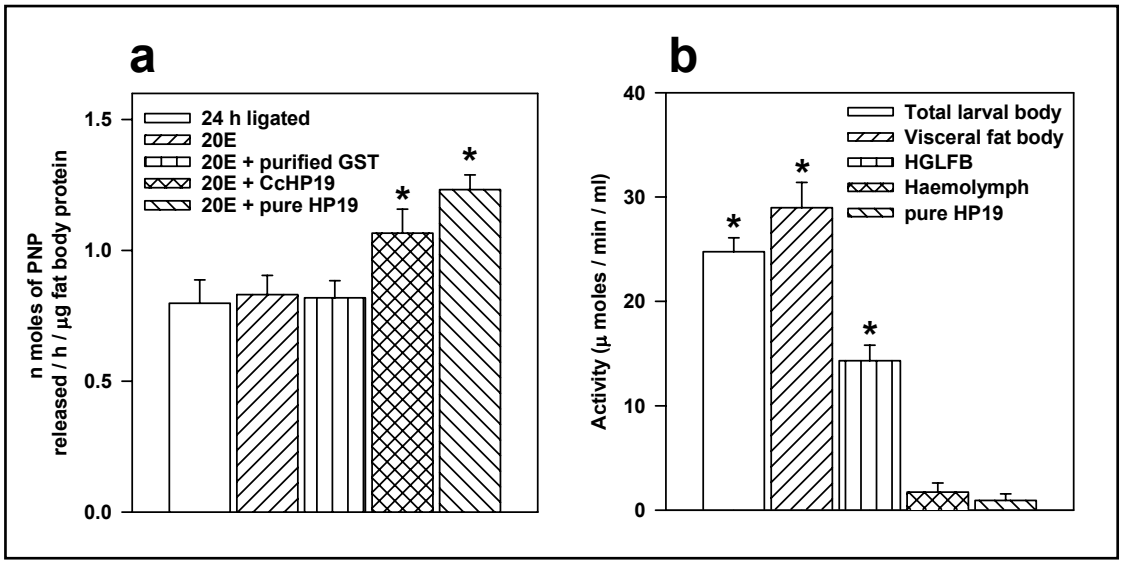


Fig. 29: A comparative study on the effects of HP19 and GST:-

(a) Effect of affinity purified cytosolic *C. cephalonica* GST on 20E dependent fat body ACP activity. Note that the presence of either recombinant HP19 or 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 four independent determinations. * Significantly different over all other values ($p<0.05$).

(b) GST activity in different larval tissues and in purified HP19. Note that both haemolymph as well as purified HP19 have negligible GST activity. * Significantly different over all other values ($p<0.05$).

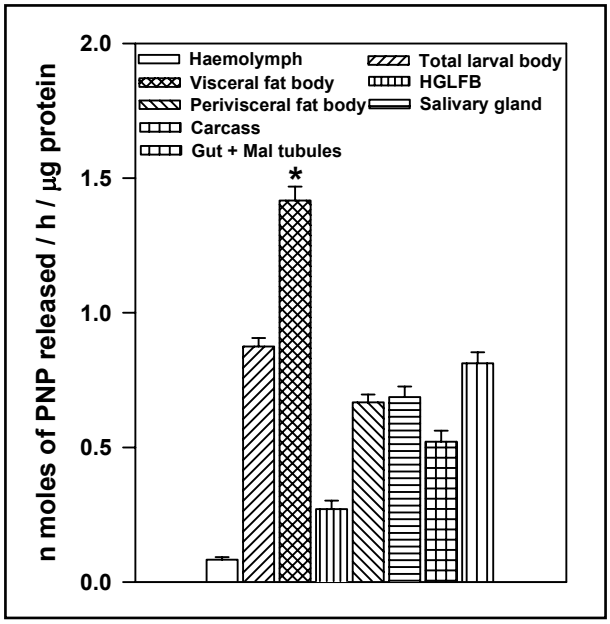


Fig. 30: ACP activity in different LLI stage larval tissues:-

Note that the activity is negligible in haemolymph and significantly low in HGLFB as compared to the activity in visceral fat body. * Significantly different over all other values ($p<0.05$).

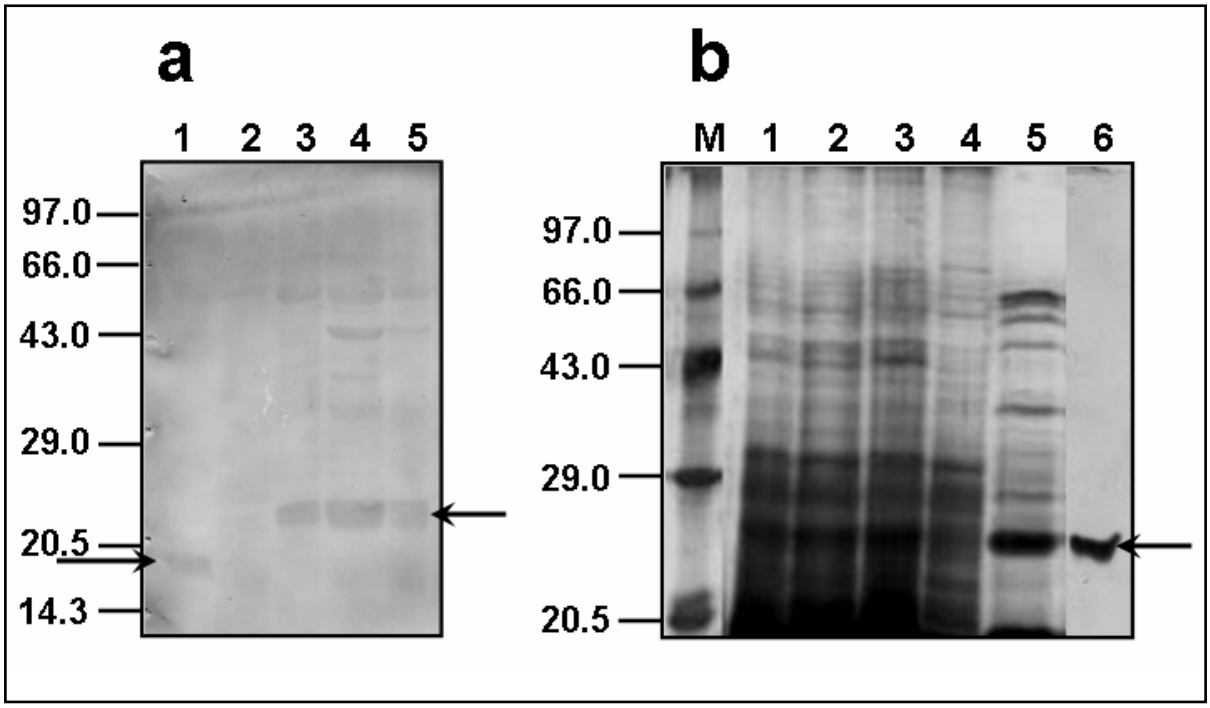


Fig. 31: Studies on glutathione S-transferase:-

(a) Western analysis of glutathione S-transferase (GST) in different larval tissues using antibody against GST of *C. fumiferana.*

(b) Purification of the cytosolic GSTs from *C. cephalonica* using glutathione CL-agarose affinity chromatography (for details refer materials and methods). Lanes- (M) protein marker in kDa, (1) crude homogenate, (2) 10,000 g 45 min supernatant, (3) 100,000 g 1 h supernatant (cytosolic fractions), (4) 100,000 g 1 h pellet (microsomal fractions), (5) DEAE eluate (active pooled fraction) and (6) affinity eluate (active fraction).

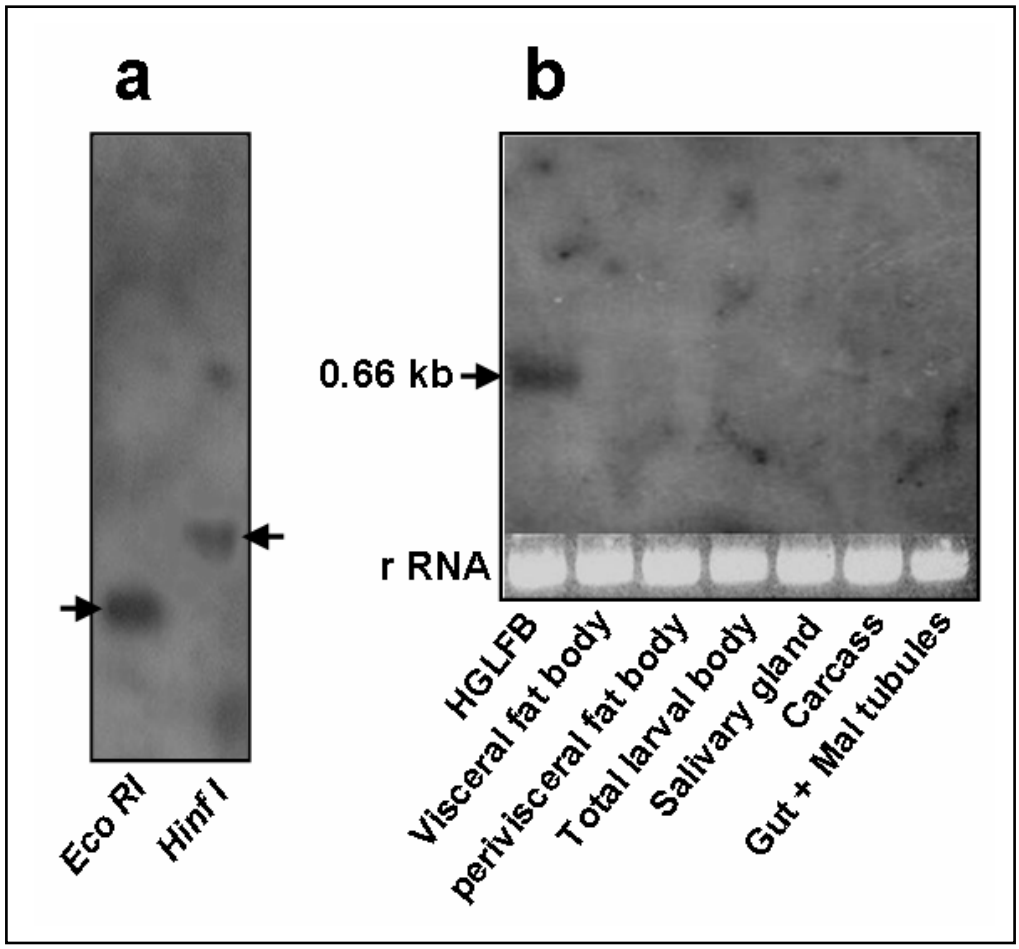


Fig. 32: Characterization of HP19 at molecular level:-

(a) Southern blot analysis showing single gene copy (arrow) of HP19. The genomic DNA (30 µg) from total larval body was digested with *EcoRI* or *Hinf I* and probed with CcHP19 cDNA as described in materials and methods.

(b) Northern blot showing the tissue specificity of the HP19 transcript in HGLFB (arrow). The ribosomal RNA shows equal loading of total RNA (20 µg). Note that HP19 is expressed only in HGLFB.

Results- Chapter III

Role of HP19 during the postembryonic development of *C. cephalonica*

Background-

The role of ecdysteroids in controlling the postembryonic development of insects is well established (Trumann and Riddiford, 2002). The hormones are known to regulate a wide variety of functions including initiation of breakdown of larval structures during metamorphosis (Gilbert *et al.,* 1996) and uptake of hexamerins (Burmester and Scheller, 1999).

Programmed cell death is crucial for the normal development and occurs mostly by apoptosis of individual cells and autophagy of cell groups. Ecdysteroid triggered regulation of autophagy is reported in *D. melanogaster* (Lee and Baehriecke, 2001; Thummel, 2001). In holometabolous insects the larval structures degenerate at the beginning of metamorphosis (Lockshin and Beaulton, 1974). Lysosomal enzymes are known to play an important role in histolysis of larval organs, tissue remodeling, cellular destruction and reorganization. The required energy and metabolic fuel are provided by the fat body. ACP is one of the commonly used marker enzyme to study the lysosomal activity in insects (Verkuil, 1979; 1980). ACPs (EC 3.1.3.2) have been found in multiple forms and different isozymes in almost all organisms investigated so far (Konichev, 1982; Kutuzova, 1991).

Earlier studies have shown that the autophagic process or the lysosomal activity in the whole animal as well as in the fat body exhibits a specific pattern during the postembryonic development. The increase in the lysosomal activity is governed by an increase of 20E titer (Verkuil, 1979; Sass and Kovacs, 1980). The administration of exogenous 20E stimulated the ACP activity in ligated larvae of *S. litura* (Sridevi *et al.,* 1987) and *C. cephalonica* (Ashok and Dutta-Gupta, 1988). However, the addition of 20E alone to the larval fat body culture of *C. cephalonica* did not alter the ACP activity (Ashok and Dutta-Gupta, 1991). Similar observations were also reported in *M. sexta*, where the ACP activity remained unchanged in fat body cultures in response to 20E (Caglayan, 1990). From these results, it could be suggested that some additional factor(s) mediate the 20E regulated stimulation of the ACP activity *in vivo*. Such a factor was identified to be present in the haemolymph of late-last instar larvae of *C. cephalonica* because only when the fat body culture was supplemented with haemolymph, a stimulation of the ACP activity by 20E could be observed (Ashok and Dutta-Gupta, 1991). In the previous chapter, the identification of and characterization of this factor as a19 kDa protein (HP19) is presented. This chapter of thesis deals with the role of HP19 in insect growth and development. The results of this study also show that ACP activity is required for the normal metamorphosis of *C. cepahlonica*. Further, it is also shown that

HP19 regulates the receptor mediated endocytosis of hexamerins that is controlled by ecdysteroid hormone.

Effect of anti-HP19 IgG fraction injection on larval growth and development-

For gaining greater insight into the role of HP19 during the postembryonic development of *C. cephalonica*, the effect of anti-HP19 IgG fraction injection to the last instar larvae was studied. Under such circumstances physiological functions of the protein will be partly blocked or suppressed, possibly resulting in altered growth and differentiation of the larvae, pupae and adults. Although the mortality rate was more or less the same in the antibody injected larvae when compared with the control larvae, we observed significant morphological and behavioral changes. Figures 33 and 34 show that the larvae which received anti-HP19 IgG fraction injections developed either in nonviable larvae (Fig. 33i and j), or in non-viable larval-pupal intermediates (Fig. 33k and l), or in non-viable pupal-adult intermediates (Fig. 33m and Fig. 34b-d) compared to the normally growing control larvae (Fig. 33b-h and Fig. 34a). Further analysis on various parameters revealed significant changes in experimental insects when compared with the controls (Table 2). Although the rate of survival was more or less the same in the experimental or control groups, the antibody injected larvae showed reduced salivary secretion, defective puparia formation, delayed reduction in body length and reduced head capsule size. Although the duration required for pupation was identical but upon antibody injection most of the larvae developed into abnormal non-viable larvae or larval-pupal intermediates and some of them could not metamorphose into adult and gave rise to non-viable pupal-adult intermediates.

Effect of anti-HP19 IgG fraction injection on fat body ACP activity-

The ACP activity profile in LLI larvae, which had received injections of anti-HP19 IgG fraction, is presented in figure 35. The results clearly show that HP19 antibody interference, which in turn is responsible for blocking the increase in the fat body ACP activity. The ACP activity did not increase and remained fairly low after 4, 7, 10 and 14 days upon antibody injection when compared with controls which showed a gradual and significant increase in ACP activity.

Effect of anti-HP19 IgG fraction injection on hexamerin uptake-

Comparison of the haemolymph and fat body protein profile from larvae, which had received anti-HP19 IgG injections with appropriate controls indicated that the injected

antibodies inhibited the hexamerin sequestration from the haemolymph by the fat body. The hexamerin concentration in the haemolymph declined in control insects after 10 days of injection and reached to a low value on 14th day (Fig. 36a, lanes 4 & 5), but in HP19 antibody injected insects, it remained more or less the same and did not decline in 10th or 14th day of post-injected insects (Fig. 36b, lanes 4 & 5). The fat body which synthesizes and releases the hexamerins at feeding larval stages and sequesters it back at the non-feeding prepupal and pupal stages, showed the presence of higher quantity of hexamerin after 14 days of post-injection (Fig. 36c, lane 5). However, this hexamerin sequestration by the fat body was found to be absent in the antibody injected larvae (Fig. 36d, lane 5).

The results obtained from morphological and histological studies also suggest that HP19 plays a role in hexamerin sequestration, which is a 20E dependent process. The whole mount preparation of fat body of controls (Fig. 37a-e) and HP19 antibody injected larvae (Fig. 37f-j) show a clear difference in the morphology which is more pronounced in 10 and 14 days post-injected larvae. Histological study reveals the presence of large number of darkly stained granules in the fat body of both control and HP19 antibody injected insects till 7 day post-injection (Fig. 37k-m and p-r). There was further decline in the number of cytoplasmic granules in both 10 days post-injected experimental and control insects. However, granules number increased significantly in controls (Fig. 37o) when compared with experimental insects after 14 day of injection (Fig. 37t) and it is mainly due to the sequestration of hexamerins from haemolymph. Immunohistochemical study using hexamerin antibody (Fig. 38, lane 2) further substantiate the histological findings, where intense immunostaining is seen only in controls after 10 and 14 days post-injection (Fig. 37x and y) and which is more or less absent in HP19 antibody injected insect fat body (Fig. 37c` and d`). Figure 38 shows the specific antibody raised against the purified hexamerins of *C. cephalonica*. The hexamerins were purified (Fig. 38a) by passing the total haemolymph proteins of *C. cephalonica* using gel filtration column sephadex G50-150 (Fig. 38a, lane 2) and ion exchange column, DEAE sephacel (Fig. 38a, lane 3). The purified hexamerins were used for antibody production. Western analysis (Fig. 38c) revealed that the antibody was highly specific for hexamerins and it identified the hexamerin both in haemolymph (lane 1) and in fat body (lane 2).

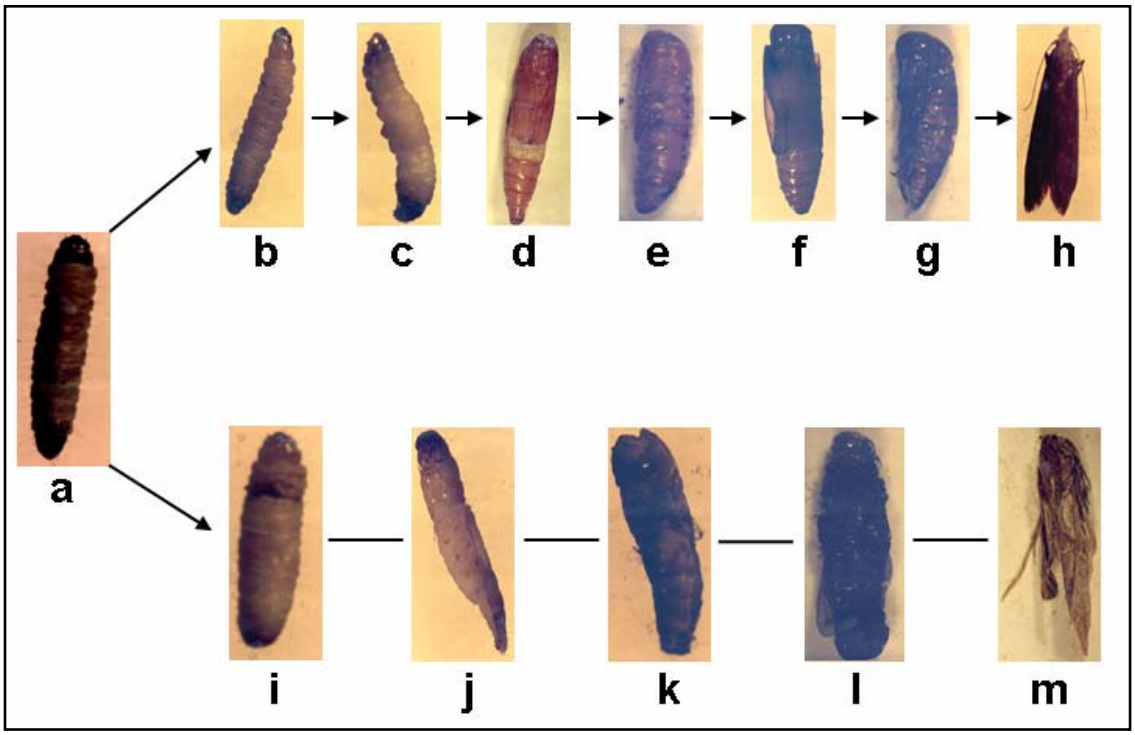


Fig. 33: Effect of anti-HP19 IgG fraction injection to final (Vth) instar larvae (a) of *C. cephalonica*:-

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 (i-m) as compared to the control larvae (b-h). Each arrow head in the control set indicate the gradual and normal development of the last (Vth) instar larvae into a healthy adult.

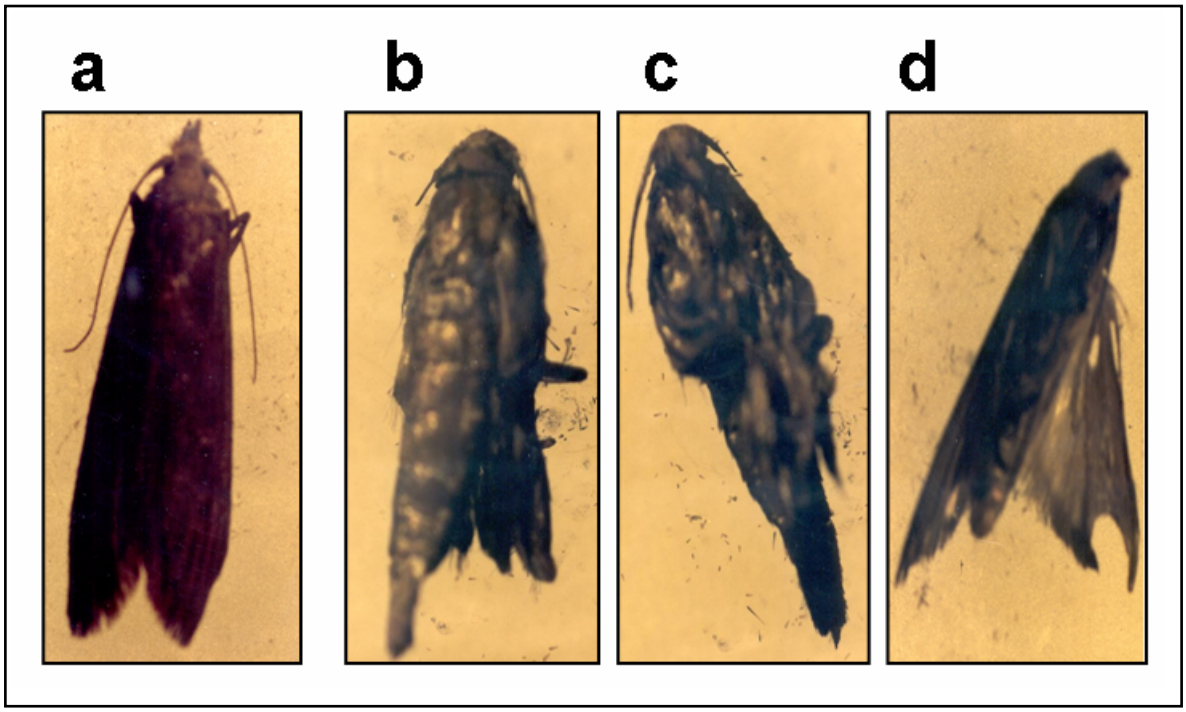


Fig. 34. Effect of anti-HP19 IgG fraction injection to final instar larvae (See Fig. 33a) of *C. cephalonica*:-

Development of non-viable pupal-adult intermediates upon injection of antibody (b-d) as compared to the control insect (a)

Controls (Normal *i.e.,* uninjected, wounded and pre-immune serum injected larvae)	**Experimental (HP 19 antibody injected larvae)**
■ 10 % mortality. ■ Normal silk secretion ■ Reduction in body and head capsule size from 8 days onward in all controls. ■ Pupation after 13-15 days, except in insect Ringer control where the pupation was little delayed, pupa however was well developed. ■ Emergence of well developed adults in all types of controls after 21-23	■ 15% mortality. ■ Reduced silk secretion ■ Delayed reduction in body and head capsule size, seen after 11 days of injection. ■ Pupation normally seen after 13-15 days but abnormally developed *i.e* non viable larval-pupal intermediate. ■ Delayed metamorphosis and almost all adults abnormally developed, *i.e* nonviable pupal-adult intermediate.

Table 2. Morphological and behavioral changes upon anti-HP19 IgG fraction injection to final (Vth) instar *C. cephalonica* larvae.

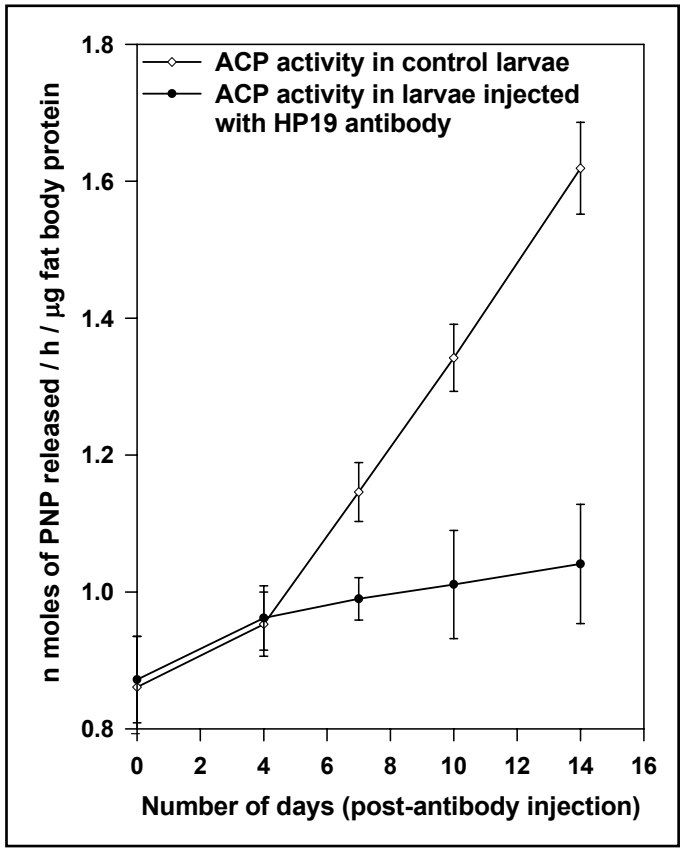


Fig. 35. Changes in the ACP activity in *C. cephalonica* larvae after different days of anti-HP19 IgG fraction injection:-

Note that the increase in ACP activity was marginal in antibody injected insects when compared with control insects, where a gradual increase is seen. Each value is mean $\pm$ S.D. of four independent experiments and for each assay fat body from 2-3 insects was pooled.

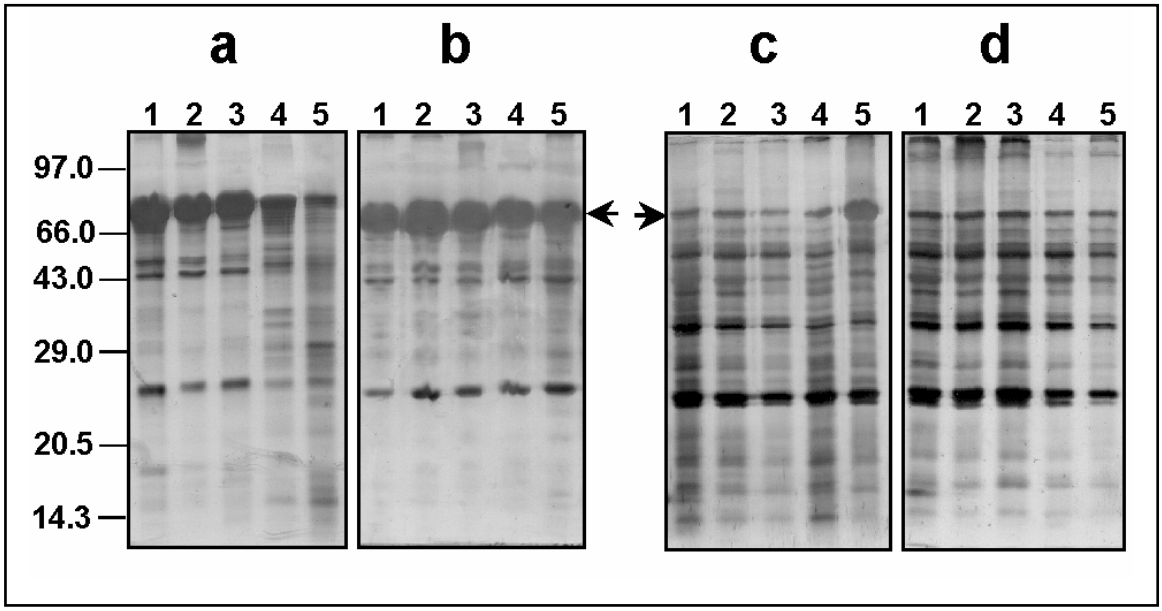


Fig. 36: SDS-PAGE profile of haemolymph and fat body proteins from anti-HP19 IgG fraction injected insects (b & d) and control insects (a & c):-

Note the decline in hexamerin concentration (arrow) in haemolymph (a, lane 5) and appearance of it in the fat body protein (c, lane 5) in control after 14 days of post-injection. These changes however were absent both in haemolymph (b) and fat body (d) of the antibody injected larvae. Lanes (1) 0 day *i.e.,* the day of injection and (2-5) 4, 7 10 and 14 days post-injected.

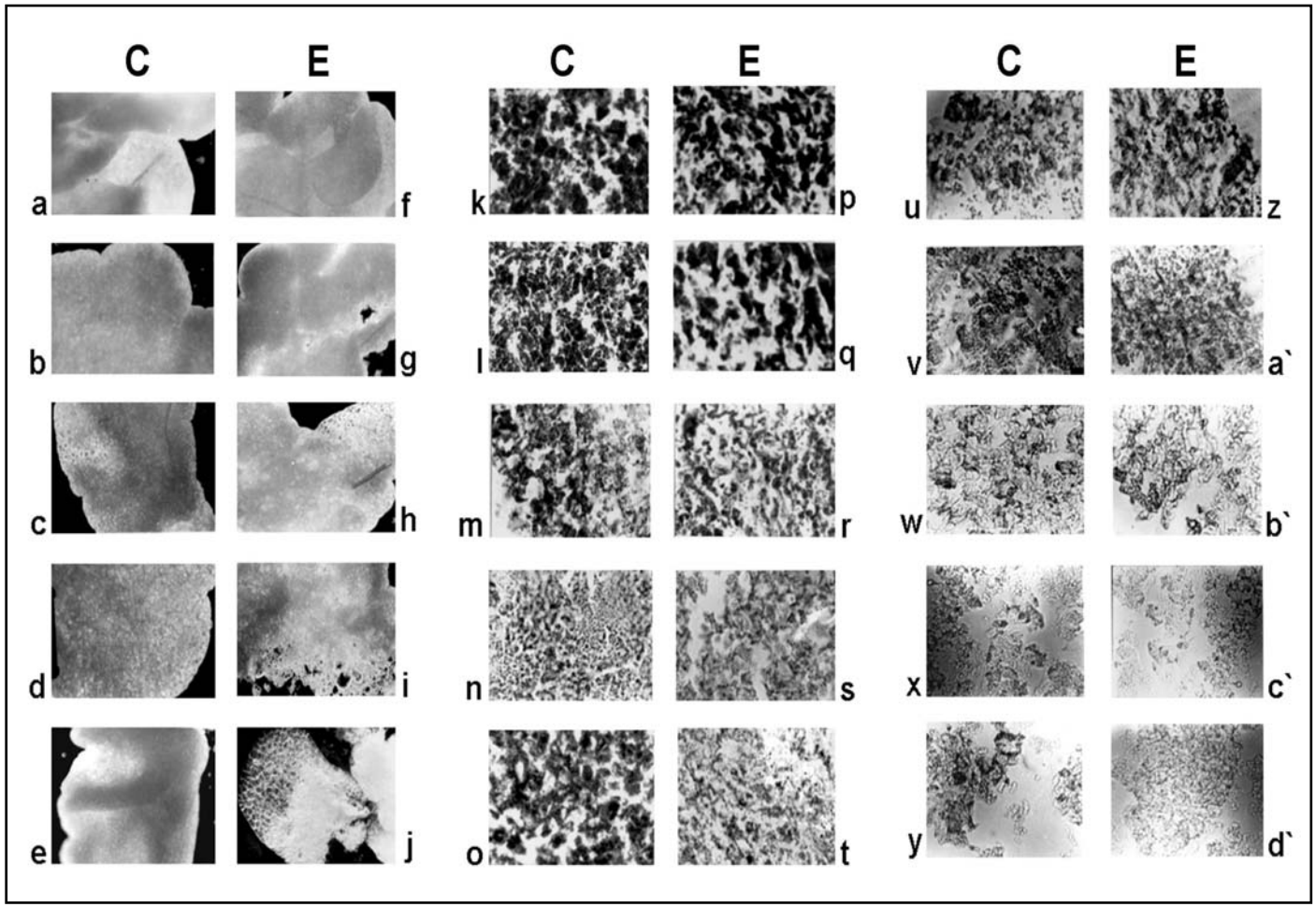


Fig. 37. Shows the morphological (a-j), histological (k-t) and immunohistochemical (u-d`) changes in control (C) as well as in the HP19 antibody injected larvae (E):-

The changes are quite evident with respect to hexamerin sequestration and the HP19 antibody injected larvae showed more or less no sequestration and very little immuno-staining (carried out using hexamerin antibody) was detected in these insects (d`) when compared with controls (y).

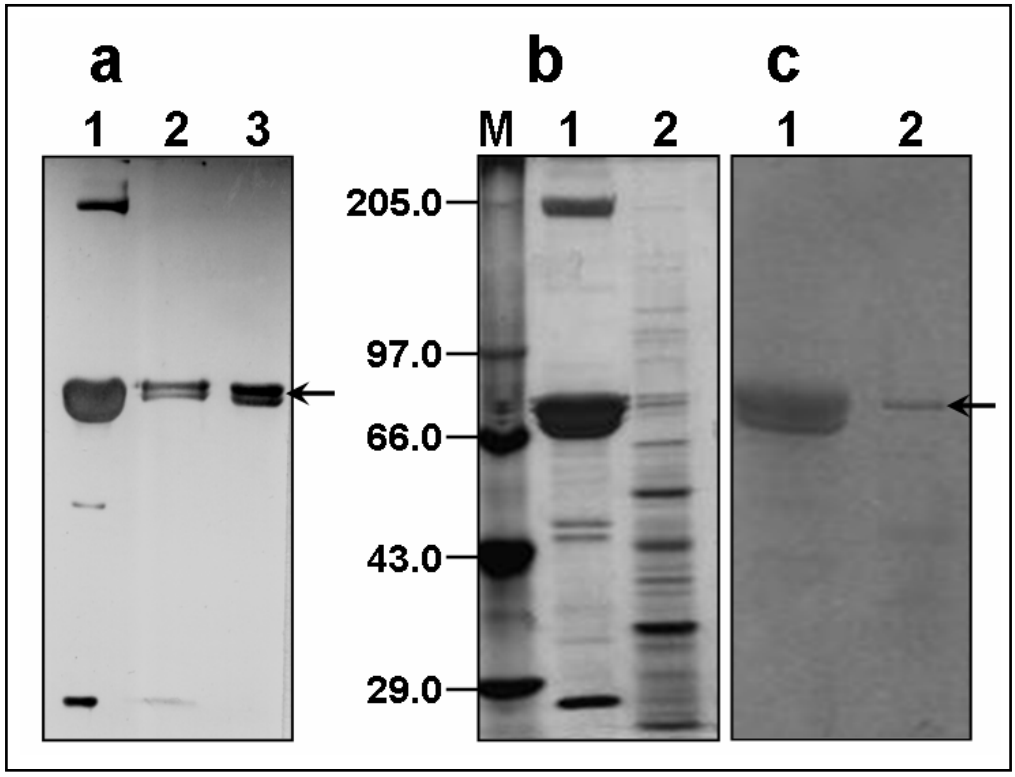


Fig. 38: Purification of hexamerins and production of polyclonal antibody against hexamerins:-

(a) SDS-PAGE showing a comparative profile of proteins at various steps of purification from *C. cephalonica* larval haemolymph.

(b & c) are respectively the SDS-PAGE profile and corresponding western blot of total haemolymph and fat body protein of *C. cephalonica*. The western blot was probed with antibody raised in rabbit against the purified hexamerins shown in (a- lane 3) as described in materials and methods chapter.

Lanes- protein marker in kDa (M), crude haemolymph (1), sephadex G-50 eluate (2) and pooled peak fraction eluate from DEAE sephacel column (3).

Results- Chapter IV

Effect of HP19 on 20-hydroxyecdysone regulated actions

Part of this work has been published in-

Arif *et al.,* (2003). Tyrosine kinase mediated phosphorylation of the hexamerin receptor in the rice moth *Corcyra cephalonica* by ecdysteroids. ***Insect Biochem. Mol. Biol.*** 33:921-928.

Arif *et al.,* (2002). Juvenile hormone stimulated tyrosine kinase mediated protein phosphorylation in the CNS of the silk worm, *Bombyx mori*. ***Arch. Insect Biochem. Physiol.*** 50:139-146.

Arif *et al.,* (2001). A rapid & reproducible protocol for the purification of insect storage protein. ***Entomon*** 26: 1-11.

Background-

Extensive studies from other as well as our laboratory have revealed that in addition to the increase in lysosomal activity in the whole body as well as in the fat body, 20E also regulates a wide variety of functions in insects. It is reported to stimulate the synthesis of various proteins in different tissues during the postembryonic development of the lepidopteran insects (Ray *et al.,* 1987a, b; Sridevi *et al.,* 1988a, 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 (KiranKumar *et al.,* 1997) as well as the male accessory reproductive glands (Ismail and Dutta-Gupta, 1990c, 1991; Dutta-Gupta and Ismail, 1992; Ismail *et al.,* 1993) was also shown to be regulated by ecdysteroids.

Hexamerins are multimeric proteins that are synthesized stage specifically by the fat body of actively feeding larval stage and released into the haemolymph. The fat body again sequesters these proteins during the non-feeding prepupal and pupal stages to meet its energy requirement (Haunerland, 1996). This uptake occurs through a unique receptor-mediated endocytosis process and the receptor does not belong to the low-density lipoprotein receptor super family (Burmester and Scheller, 1999). Studies from different groups suggest a post-translational processing mechanism of hexamerin receptor activation for hexamerin uptake, which is regulated by ecdysteroids (Burmester and Scheller, 1999). Studies on receptor activation in different insects were shown to be independent of gene activation and suggested to occur at a post-translational level (Ueno and Natori, 1984; Burmester and Scheller, 1997a). Previous studies from our laboratory suggested that hexamerin receptor undergoes protein phosphorylation that is regulated by 20E (KiranKumar, 1998; Vasanthi, 1999).

The post-translational modifications of proteins by reversible protein phosphorylation are well known to regulate several cellular actions (Graves and Krebs, 1999; Davies *et al.,* 2000). These phosphorylation events are often suggested to be regulated by hormones. The ecdysteroids are also known to stimulate phosphorylation of few fat body proteins with their significance being discussed (Itoh *et al.,* 1985; Sass, 1988). In the present study an attempt is made to understand the role of 20E on the phosphorylation of few fat body proteins of *C. cephalonica* and effect of HP19 on them. In this endeavor three protein, namely hexamerin receptor, tyrosine kinase and CaM kinase II were identified as potential markers. This section of thesis deals with the extensive study carried on these proteins.

Phosphorylation of 120 and 60 kDa protein induced by 20E-

Results of the i*n vitro* phosphorylation fat body proteins in *C. cephalonica* under different phosphorylating conditions revealed that only a few proteins of masses 32, 48, 60 and 120 kDa were phosphorylated (Fig. 39b). Of these 60 and 120 kDa were the major bands labeled with [γ^{32}P] in presence of 20E (lane 2). The 120 kDa protein was earlier identified as the hexamerin receptor by ligand blotting (KiranKumar *et al.,* 1997). The phosphorylation of the 120 kDa protein was found to be independent of calcium and was significantly enhanced by 20E (lanes 2 & 4). Furthermore, the addition of protein kinase C activators (lane 5) did not have any effect on the phosphorylation of the 120 kDa protein. The 60 kDa protein was earlier identified as CaM kinase II in the CNS of *B. mori* (Shanavas *et al.,* 1998). In the present study the calcium dependence (Fig. 39b, lanes 2 & 4) for the phosphorylation of 60 kDa protein supports the tenet that this might be similar to CaM kinase II of *B. mori* reported by Shanavas *et al.,* (1998). Furthermore, the 60 kDa protein was also found to be phosphorylated in the fat body of *B. mori* and was identified as CaM kinase II (Vasanthi, 1999). The results presented in figure 40 suggest that for the *in vitro* phosphorylation reaction, 1 min time incubation at room temperature was optimal and a significant level of phosphorylation was seen in 120, 60 and 48 kDa proteins.

Phosphorylation of 48 kDa protein in silk worm Bombyx mori-

The phosphorylation of fat body proteins of *C. cephalonica* revealed phosphorylation of 4 proteins of masses 32, 48, 60 and 120 kDa (Fig. 39b). Of these, 48 kDa protein has been earlier shown to undergo phosphorylation under the influence of juvenile hormones I (JHI) in *B. mori* CNS (Shanavas *et al.,* 1998). Protein phosphorylation studies were extended to *B. mori*, basically to find if the phosphorylation profile of proteins in different tissues is comparable with *C. cephalonica*. It was interesting to note that the same protein was phosphorylated in the fat body of *C. cephalonica* (Fig. 39b, lane 3, arrow). In the present study, the phosphorylation of 48 kDa protein was found to be dependent on a tyrosine kinase as the monoclonal antibody against phosphotyrosine identified the phosphorylated 48 kDa protein (Fig. 41a, lane 1). Further genistein, a broad-spectrum tyrosine kinase inhibitor, inhibited the phosphorylation (Fig. 41b, lane 2) as compared to control (Fig. 41b, lane 1). This 48 kDa protein phosphorylation was found to occur in all the insect tissue investigated (Fig. 41c) with significant phosphorylation in CNS (lane 1) and salivary gland (2). Because the phosphorylation of this protein was shown to be regulated by JHI (Shanavas, 1997) and

not by 20E, hence, it was not considered worthwhile to extend the study further on 48 kDa protein as the present study is focused on the regulation of ecdysteroid dependent actions.

Phosphorylation of 32 kDa protein in protein kinase C (PKC) dependent manner and studies on the endogenous fat body PKC activity-

The results in figure 39 reveal phosphorylation of a 32 kDa protein in the presence of PKC activator phosphatidylserine and diolein (Fig. 39b, lane 5). However, presence of 20E did not show any significant change in the phosphorylation status of this protein (Fig. 39b). The study was extended to understand if the endogenous fat body PKC activity is regulated by 20E. For this, we used synthetic peptide $pGLU^4$- myelin basic protein fragment 4-14 (MBP_{4-14}) as substrate. The rate of phosphorylation of this substrate by PKC revealed normal Michaelis-Menten kinetics with respect to the concentration of ATP and substrate (Fig. 42a and b). In the presence of saturating concentration of substrate the K_m value for ATP was 19.41 μM. The synthetic peptide proved to be an effective substrate for *C. cephalonica* fat body PKC with an apparent K_m of 15.24 μM and a V_{max} of 0.83 pmol/min/mg of fat body homogenate protein. The fat body PKC activity is developmentally regulated with activity gradually decreasing from ELI larvae to prepupal stage (Fig. 43a). Thorax-ligation of LLI larvae for different time periods showed gradual decline in the activity (Fig. 43b), indicating about the hormonal dependence. The 24 h post-ligation provided a fat body tissue that can be manipulated for hormonal regulation studies. However, the hormone 20E could not alter the enzyme activity when injected for 24 h to 24 or 48 h post-ligated larvae (Fig. 43c). These results and the pattern of developmental regulation of endogenous PKC activity suggest that probably the PKC activity and the protein phosphorylation due to this is regulated by JHs and not by 20E. As 20E was found to be ineffective to regulate the PKC activity and the associated phosphorylation, the study on this phosphorylation was not carried further.

Regulation of calcium/calmodulin dependent protein kinase II (CaM kinase II) activity and autophosphorylation in the fat body of C. cephalonica-

The results from the i*n vitro* phosphorylation of fat body proteins in *C. cephalonica* revealed the phosphorylation of a 60 kDa protein (Fig. 39b). This was earlier identified as an autophosphorylation of CaM kinase II in the fat body by Vasanthi (1999) and in CNS by Shanavas *et al.,* (1998). The calcium dependence for the phosphorylation of 60 kDa protein (Fig. 39b, lanes 2 & 4) supports the tenet that this might be similar to CaM kinase II of *B.*

mori reported by Shanavas *et al.,* (1998) and Vasanthi (1999). The results also suggest that the autophosphorylation is induced by the presence of ecdysteroid, 20E (Fig. 39b, lane 2). The study was further extended to find out the endogenous fat body CaM kinase II activity and effect of 20E on it (Fig. 44). The results suggest that fat body CaM kinase II activity is developmentally regulated with highest activity at LLI larval stage (Fig. 44a). Thorax-ligation for different time periods showed gradual decline in the enzyme activity (Fig. 44b), and 24 h of ligation provided a fat body tissue, ideal for hormone manipulation studies. The hormone 20E induced the enzyme activity sharply, when injected for 6 or 12 h to 24 h post-ligated ELI or LLI larvae. This induction in activity was also observed when 20E was added to the fat bodies kept in culture for 4 h (Fig. 44d). These studies clearly suggest that 20E induces the activity and autophosphorylation of 60 kDa CaM kinase II.

Effect of thorax-ligation and dependence on 20E phosphorylation of 120 kDa protein-

In the present study, extensive studies were carried on the phosphorylation of 120 kDa protein of the fat bodies of *C. cephalonica*. As evident from the *in vitro* phosphorylation of fat body proteins (Fig. 39b), the 120 kDa protein phosphorylation is independent of calcium and was significantly enhanced by 20E (lanes 2 & 4). The study was further extended to confirm if this phosphorylation is 20E dependent. Thorax-ligation for different time periods showed a gradual decline in the degree of phosphorylation of the 120 kDa protein (Fig. 45a, lanes 2-5 & corresponding densitogram) as compared to the unligated larvae (Fig. 45a, lane 1). Thorax-ligation inhibits the release of ecdysteroids into the abdominal part of the larvae. When different concentrations of 20E were added to homogenate prepared from 24 h post-ligated larvae, a dose dependent increase in phosphorylation of 120 kDa protein was observed (Fig. 45b, lanes 3-5 & corresponding densitogram) as compared to the control (Fig. 45b, lane 2 & corresponding densitogram). Furthermore, the degree of phosphorylation detected with 40 nM 20E (Fig 45b, lane 3) and 80 nM 20E (Fig. 45b, lane 4) was fairly high and comparable with homogenate of unligated larval fat body (Fig 45b, lane 1). Hence, the concentration of 80 nM 20E was used for all the subsequent phosphorylation studies.

Identification of the phosphorylated 120 kDa protein as the membrane receptor for hexamerins-

Earlier studies from our laboratory reported that a 120 kDa polypeptide- (i) is located in the cell membrane of fat bodies, (ii) binds to *C. cephalonica* hexamerin and (iii) is

responsible for the hexamerin sequestration. Additional experiments were carried out to unambiguously identify the phosphorylated 120 kDa as the hexamerin receptor. The fat body proteins were first phosphorylated either in absence or in presence of 20E, transferred to nitrocellulose membrane followed by incubation with biotinylated hexamerin. The results revealed that the biotinylated hexamerin selectively bound to 120 kDa protein (Fig. 46b, lanes 1 & 2), hence it could be considered as the hexamerin receptor. This ligand blot, when autoradiographed, showed that the same 120 kDa protein was highly phosphorylated in presence of 20E (Fig. 46c, lane 2).

Independence of tissue integrity for the phosphorylation of 120 kDa hexamerins-

The 120 kDa hexamerin receptor was hyperphosphorylated in the membrane fractions of the fat body as compared to the phosphorylation in fat body homogenate (Fig. 47a) suggest that phosphorylation has significance in the physiological function. The low degree of phosphorylation of the same protein in other fractions is probably due to the contamination of these fractions during preparations. *In vitro* phosphorylation studies with the fat body homogenate and membrane fractions in absence or presence of 20E revealed that 20E induced the phosphorylation in both (Fig. 47b and c). Back phosphorylation experiments were carried to identify whether the 120 kDa receptor was also phosphorylated under the influence of 20E in intact fat body. The rationale of the study was to check that if this protein is already phosphorylated in intact tissue in response to 20E treatment, then these sites would be occupied by endogenous unlabeled phosphate, hence they would be less available or unavailable for accepting labeled phosphate during the *in vitro* reaction. The pattern of phosphorylation obtained with homogenates prepared from 20E treated fat body showed considerably low incorporation of [γ^{32}P] into the 120 kDa protein when compared with a solvent treated control *i.e.,* no 20E (Fig. 47c). These studies in combination suggest that tissue integrity is not essential for the receptor phosphorylation as it occurs in homogenate or membrane preparations as well as in intact tissue. This also indicates about the possible physiological role of phosphorylation in activation of hexamerin receptor at nongenomic level for regulating stage dependent hexamerin uptake.

Tyrosine kinase dependence of the 20E-induced phosphorylation of 120 kDa hexamerin receptor –

The 20E induced phosphorylation of the 120 kDa protein was found to be calcium (Fig. 39b, lane 4) as well as protein kinase C independent (Fig. 39b, lane 5). Hence, the

possibility of tyrosine kinase mediation was checked. Tyrosine phosphorylation was studied by using anti-phosphotyrosine antibodies (Donaldson and Cohen, 1992). Immunoblot analysis of the phosphorylated protein with anti-phosphotyrosine antibody showed cross reactivity of the antibody with the 120 kDa receptor. As compared to 20E untreated (Fig. 48a, lane 1), the 20E treated phosphorylated 120 kDa protein showed a higher cross-reactivity (Fig. 48a, lane 2) which confirmed the above assumption, *i.e.,* tyrosine kinase mediates the 120 kDa protein phosphorylation, and is induced by 20E. Genistein, an inhibitor of tyrosine kinase, partly inhibited the 20E induced phosphorylation of the 120 kDa protein (Fig. 48b, lane 3) when compared with the 20E treated sample (Fig. 48b, lane 2).

Hexamerin uptake and identification of receptor phosphorylation in other hexamerin sequestering tissue-

Earlier studies from our laboratory have demonstrated that hexamerins are actively sequestered into male accessory reproductive glands (MARG) of several insects including *C. cephalonica* and the process is steroid dependent (Ismail and Dutta-Gupta, 1990c, 1991; Ismail *et al.,* 1993). In the present work, studies were carried out to determine the role of phosphorylation in other hexamerin sequestering tissue *i.e.,* MARG and its physiological significance in hexamerin uptake. The results in figure 49a revealed that MARG tissue like the fat body of *C. cephalonica* is capable of sequestering hexamerins. Ligand binding studies show that the 120 kDa receptor responsible for hexamerin uptake is also present in MARG tissue (Fig. 49c, lane 2) and like the hexamerin receptor present in fat body (Fig 49c, lane 1), it undergoes phosphorylation (Fig. 49d, lane 2). This phosphorylation is however absent in the non-hexamerin sequestering insect tissues (Fig. 50. lanes 2-4). The weak signal seen in the epidermis and central nervous system protein extract is due to the presence of contaminating fat body protein present in the extract (Fig. 50, lane 2). These studies clearly suggest that the phosphorylation is an important post-translational modification for 120 kDa hexamerin receptor and has a possible role in hexamerin sequestration in fat body as well as MARG tissues.

20E induced phosphorylation of 120 kDa hexamerin receptor stimulates hexamerin uptake-

The results presented in figure 51 show, that the hexamerins are synthesized by the fat body cells (Fig. 51a) and released into the haemolymph (Fig. 51b) where it accumulates. The rate of hexamerin synthesis by the fat body and its release as well as accumulation in

haemolymph is highest at LLI larval stage (Fig. 51a and b, lane 2). It is well documented that hexamerins are synthesized only during the feeding larval stage (Haunerland, 1996; Burmester and Scheller, 1999). Therefore, the appearance of nearly identical intensity hexamerin protein band in the fat body of prepupae (Fig. 51a, lane 3) suggests that uptake starts at prepupal stage. However, the uptake is low as compared to the high uptake by the pupal fat body (Fig. 51a, lane 4). Due to hexamerin sequestration by the fat body, there is a gradual decline in its level in haemolymph (Fig. 51b, lanes 3 & 4).

Studies with regard to *in vitro* uptake of hexamerin were carried to find whether the 20E induced phosphorylation of the hexamerin receptor has any role in hexamerin sequestration. For the uptake study, [^{35}S] methionine labeled hexamerin was partially purified and used (Fig. 52). The developmental regulation of hexamerin uptake (Fig. 53a) as well as the phosphorylation of hexamerin receptor was checked (Fig. 53b). The study shows that the phosphorylation of 120 kDa hexamerin receptor is developmentally regulated (Fig. 53b). The lack of phosphorylation of receptor at ELI larval stage is most likely due to the absence of receptor at this stage. The slightly higher phosphorylation of receptor at LLI larval stage as compared to prepupa is largely due to the presence of well developed receptor with a large number of free sites available for *in vitro* labeling as the endogenous 20E titer is fairly low at this developmental stage (Dutta-Gupta and Ashok, 1998). Figure 53a shows the incorporation of [^{35}S] methionine labeled hexamerin by the fat bodies kept in culture from different developmental stages. The incorporation was low at ELI but high at LLI larval and prepupal stages. The higher incorporation of [^{35}S] methionine labeled hexamerin in LLI stage as compared to other stages suggests the 20E regulated hexamerin receptor activation for the uptake.

In order to check if the 20E induced phosphorylation of the hexamerin receptor is essential for hexamerin endocytosis, i*n vitro* uptake of bio-labeled hexamerin was carried (Fig. 54). The results obtained, revealed that uptake of hexamerin were highest in the LLI larval fat body in presence of ATP and 20E when compared with either 20E alone or ATP alone (Fig. 54a). Furthermore, the 20E stimulated uptake of hexamerin was significantly blocked by genistein treatment. On the other hand, actinomycin D or cycloheximide had no effect on this 20E stimulated uptake. The solvent treatment (0.05% ethanol) did not alter the uptake of hexamerin and it was more or less the same as in the control. The fluorogram (Fig. 54b) further confirmed this result because when the fat bodies kept in culture is supplemented with 20E and ATP there was significant uptake of radiolabeled hexamerin, which is

unaffected by the transcriptional or translational inhibitors. Further, this uptake of radiolabeled hexamerins in presence of 20E and ATP is inhibited by genistein. These studies indicate that the phosphorylation is mediated by a tyrosine kinase and is a pre-requisite for the activation of receptor by 20E for hexamerin uptake.

Developmental and hormonal regulation of fat body tyrosine kinase activity-

As the phosphorylation of hexamerin receptor was mediated by tyrosine kinase, the study was extended on endogenous fat body tyrosine kinase using synthetic peptide as substrate. The rate of phosphorylation of the peptide substrate by tyrosine kinase shows normal Michaelis-Menten kinetics with respect to the concentration of ATP and peptide substrate (Fig. 55a and b) in the presence of saturating concentration of substrate the Km value for ATP was 154 μM. The synthetic peptide proved to be a very effective substrate for *C. cephalonica* fat body tyrosine kinase with an apparent Km of 67 μM and a Vmax of 0.934 nmol/min/mg of fat body homogenate protein. The fat body tyrosine kinase activity is developmentally regulated with highest activity at LLI larval stage (Fig. 56a). Thorax ligation for different time periods showed gradual decline in the enzyme activity (Fig. 56b), and 24 h of ligation provided a fat body tissue, ideal for hormone manipulation studies. The hormone 20E induced the enzyme activity when injected to 24 h post-ligated larvae or when added in the culture medium along with the fat bodies kept in culture for 4 h (Fig. 56c).

Possible identification of intrinsic tyrosine kinase activity in hexamerin receptor of fat body -

ATP is known to be cell impermeable but even then the phosphate moiety from the ATP was transferred to the hexamerin receptor, in the fat bodies kept in culture and the receptor was phosphorylated (Fig. 54). Therefore, it is assumed that the phosphorylation event is probably a cell surface phenomenon and occurs due to the intrinsic kinase activity of the receptor. The results presented in figure 57 provide evidence to this assumption. When the fat bodies kept in culture was incubated along with [γ^{32}P] ATP, the receptor was found to be phosphorylated under the influence of 20E (Fig. 57a). Immunohistochemical staining of fat body sections using monoclonal antibody against the phosphotyrosine residue, further suggested the presence of protein phosphorylated at tyrosine residue (Fig. 57c and d). Further, the use of series of synthetic receptor tyrosine kinase inhibitors, tyrphostins (Ohmichi *et al.,* 1993) shows (Fig. 57e-i) that one of the inhibitor AG 879, significantly

inhibited the phosphorylation of the receptor at 100 μM concentration (Fig. 57i). Though the inhibitor concentration required for inhibition was fairly high but it does suggest that probably the receptor undergoes autophosphorylation, due to the intrinsic tyrosine kinase activity.

The haemolymph protein, HP19 inhibits the 20E induced phosphorylation of hexamerin receptor-

The back phosphorylation experiment result presented in figure 58 shows that the 19 kDa haemolymph protein, HP19 from *C cephalonica* inhibits the 20E stimulated phosphorylation of hexamerin receptor. Back phosphorylation experiment, where the fat body tissue was cultured in absence or presence of 20E with or without HP19 / genistein in order to allow the sites of phosphorylation to be occupied by the endogenous ATP present in the fat body cells prior to the *in vitro* phosphorylation experiment using [γ^{32}P] ATP was carried out. In such case (Fig. 58a and b), the incorporation of [γ^{32}P] in 120 kDa hexamerin receptor was low in presence of 20E (lane 2) as compared to control (lane 1). The incorporation of [γ^{32}P] in presence of 20E and HP19 (lane 4) was higher as compared to 20E alone (lane 2) or HP19 alone (lane 5). It is interesting to note that the autophosphorylation of 60 kDa CaM kinase II (Shanavas *et al.,* 1998) was also inhibited by HP19 in presence of 20E, hence there is higher incorporation of [γ^{32}P] (Fig. 58, lane 4). Furthermore, a broad spectrum tyrosine kinase inhibitor, genistein had no inhibitory effect on the 60 kDa protein, and the sites of this fat body protein were occupied by endogenous ATP, hence no [γ^{32}P] incorporation was seen after the *in vitro* phosphorylation reaction (lane 3) and the results were comparable with 20E treatment (lane 2). In case of back phosphorylation studies, the pattern of tyrosine kinase activity in these tissues (Fig. 58c), prior to the *in vitro* phosphorylation reaction clearly showed a higher activity in 20E treated fat body, which in turn might be responsible for the phosphorylation of sites leading to low incorporation of [γ^{32}P] in 120 kDa receptor during the *in vitro* phosphorylation reaction (Fig. 58 a and b). The results of figure 59 clearly reveal that HP19 not only inhibits the phosphorylation in intact fat body tissue but also in the fat body homogenate preparation. These results together indicate that HP19 blocks the 20E regulated hexamerin receptor phosphorylation at the larval stages of development in *C. cephalonica*.

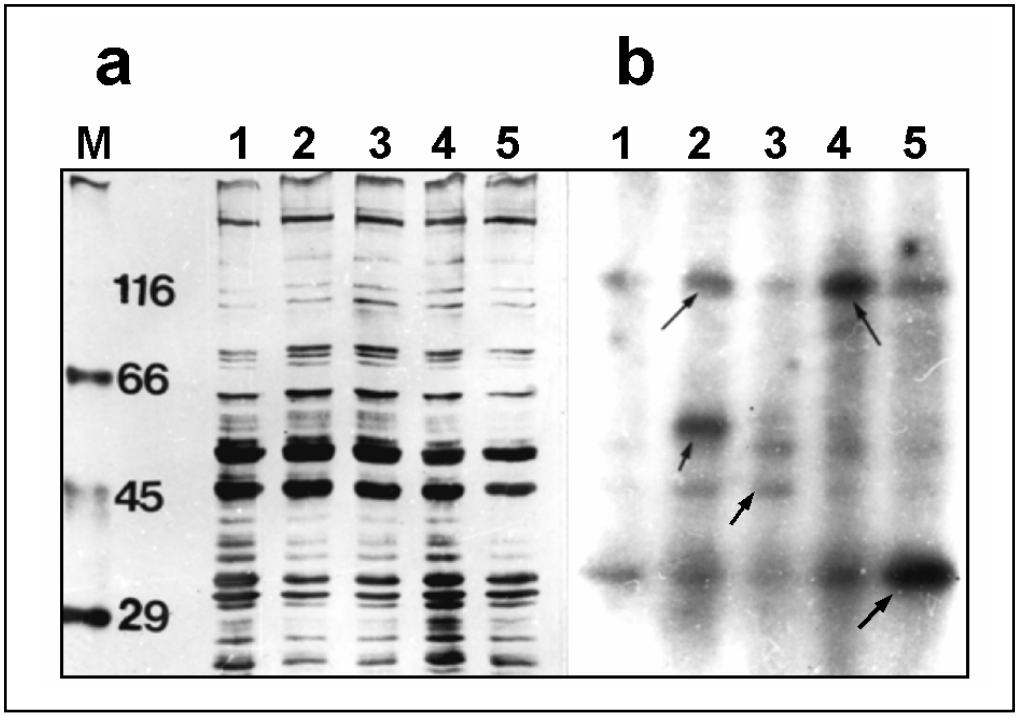


Fig. 39: *In vitro* incorporation of [γ^{32}P] ATP into the fat body proteins of *C. cephalonica* under different phosphorylating conditions:-

(a) 10% SDS-PAGE and (b) Autoradiograph. Lanes- 1 mM $CaCl_2$ (1), 1 mM $CaCl_2$ + 80 nM 20E (2), 1 mM EGTA (3), 1 mM EGTA + 80 nM 20E (4) and 1 mM $CaCl_2$ + 100 µM phosphatidylserine + 10 µM diolein (5). The phosphorylation of the 60 kDa (arrow) and 120 kDa (arrow) bands was stimulated in the presence of 20E. Lane M- protein marker (kDa).

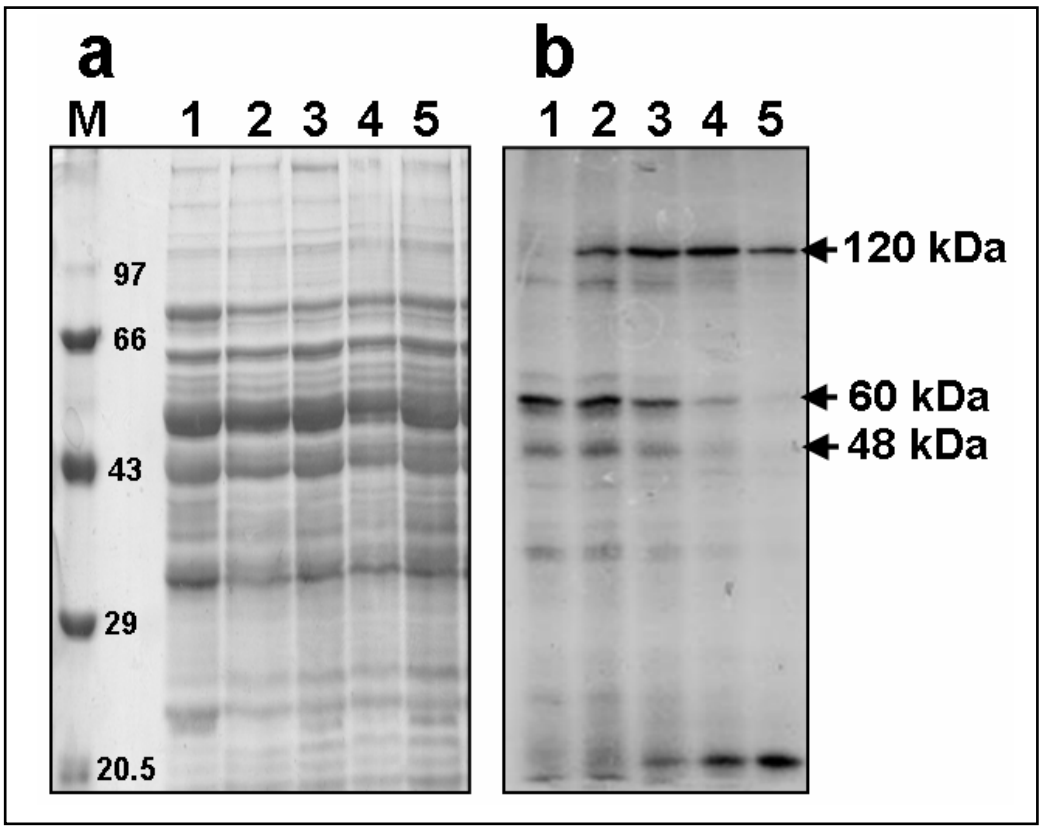


Fig. 40: Effect of different time incubations on the *in vitro* incorporation of [γ^{32}P] ATP into the fat body proteins of *C. cephalonica*:-

(a) 10% SDS-PAGE and (b) Autoradiograph. The *in vitro* phosphorylation reaction was initiated by addition of [γ^{32}P] ATP for different time periods, 10 sec (lane 1), 30 sec (lane 2), 1 min (lane 3), 2 min (lane 4) and 5 min (lane 5). After the desired time period, the reaction was stopped with addition of 3 X SDS sample buffer followed by immersion in boiling water bath for 2 min. Equal amount of protein was used for 10% SDS-PAGE and subsequently processed for autoradiography.

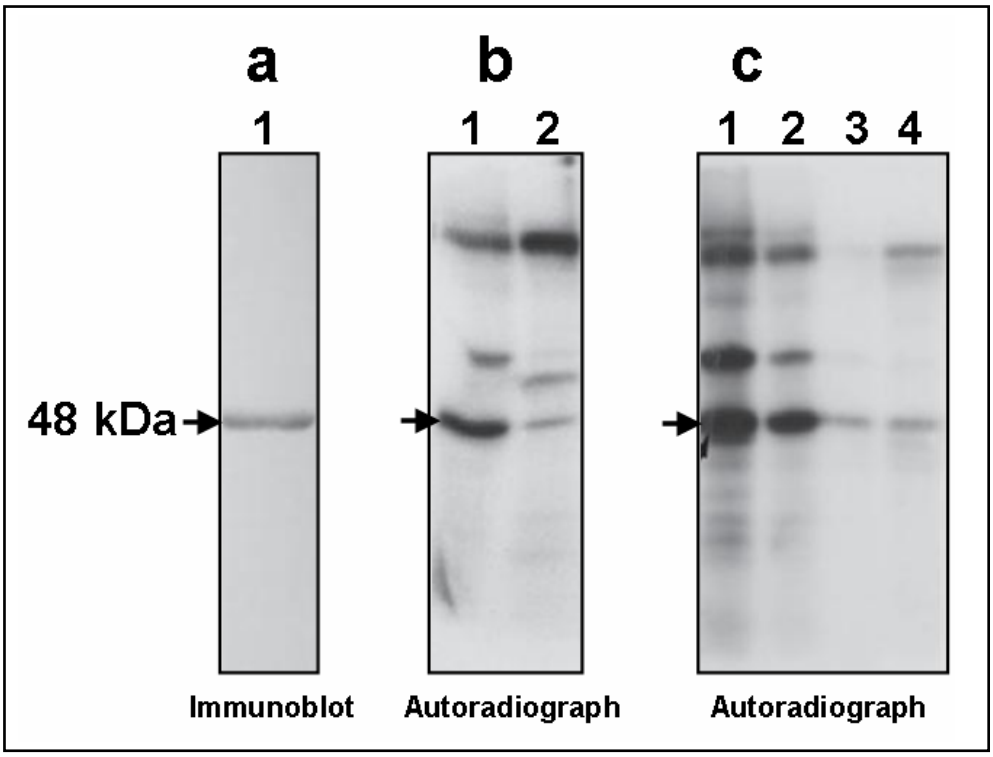


Fig. 41: Phosphorylation of 48 kDa protein in the CNS of silkworm *Bombyx mori*:-

(a) Western blot showing the immunocrossreactivity (arrow) of the phosphorylated 48 kDa CNS protein from LLI larvae probed with monoclonal anti-phosphotyrosine antibody.

(b) Autoradiograph showing the effect of genistein on the phosphorylation of 48 kDa protein (arrow). The *in vitro* phosphorylation reaction was carried in presence of 25 μM genistein. Lane 1- control (− genistein), lane 2- experimental (+ genistein).

(c) Autoradiograph showing the phosphorylation of 48 kDa protein (arrow) in different tissues of *B. mori*. An equal amount of protein (10 μg) was loaded on each lane. Lanes- CNS (1), salivary gland (2), fat body (3) and epidermis (4).

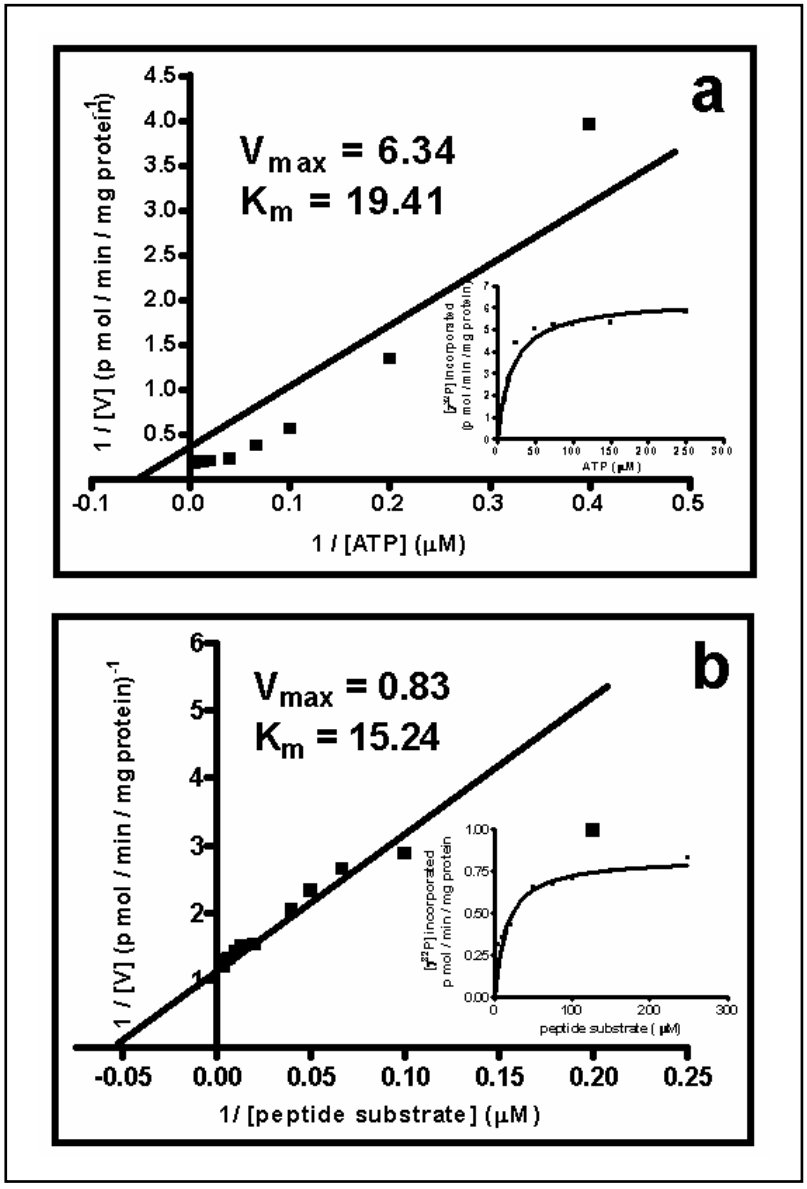


Fig. 42: Lineweaver-Burk and Michaelis-Menten plots of the phosphorylation of a peptide substrate pGLU[4]- myelin basic protein fragment 4-14 (MBP_{4-14}) by endogenous protein kinase C (PKC) of *C. cephalonica* fat body. Initial rates were measured under standard condition for 15 min by filter paper assay. The ATP (a) and synthetic peptide substrate (b) concentration were varied as indicated.

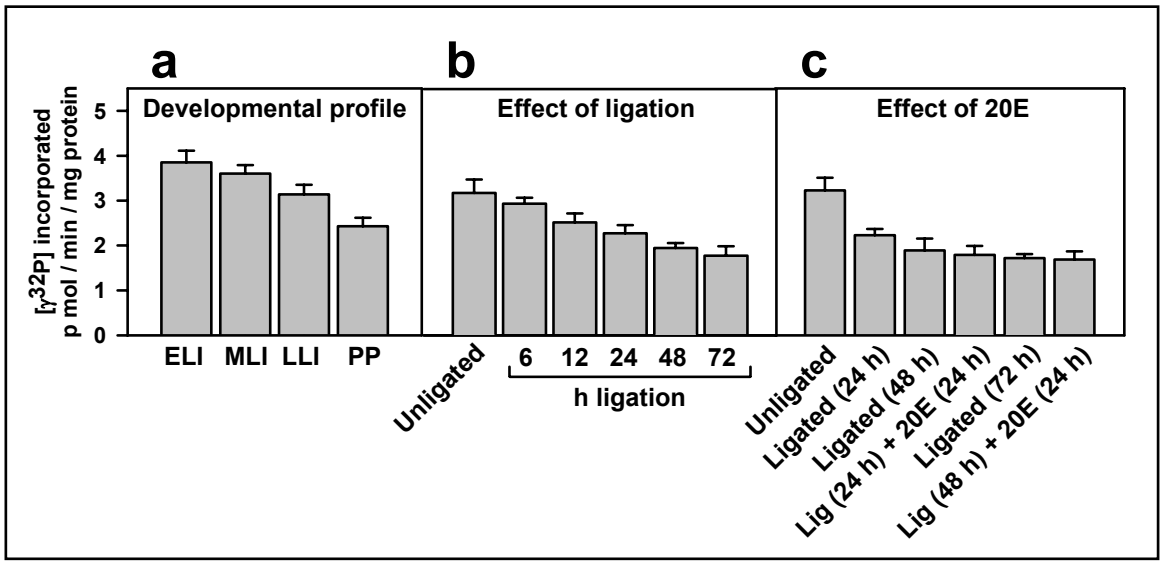


Fig. 43: Endogenous fat body protein kinase C (PKC) activity in *C. cephalonica*:-

Changes in the fat body PKC activity during different developmental stages (a), upon ligation of LLI larvae for different time periods (b) and *in vivo* effect of 20E (hormone injection) to 24 h and 48 h post-ligated LLI larvae (c). The values are mean ± S.D. of four independent determinations.

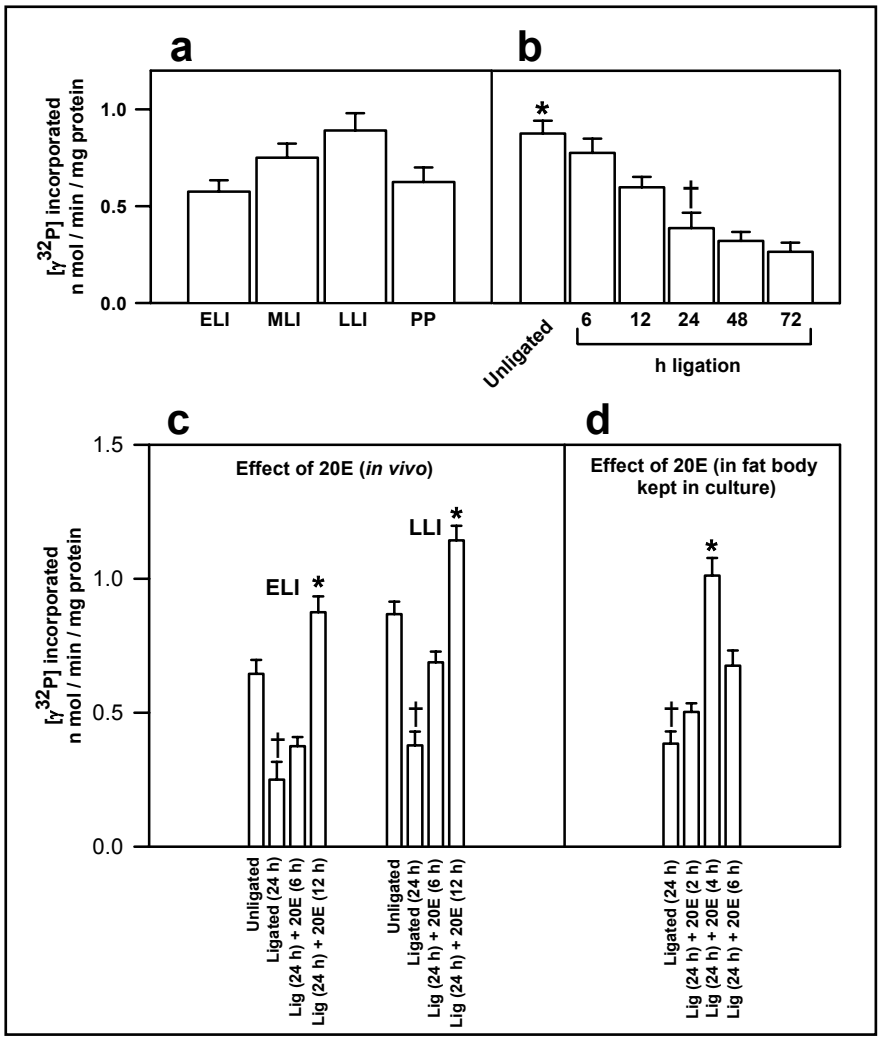


Fig. 44: Calcium/calmodulin dependent protein kinase II (CaM kinase II) activity in fat body of *C. cephalonica* and its regulation:-

(a) Changes in the activity of fat body CaM kinase II activity during different developmental stages.

(b) Effect of thorax-ligation for different time period on the fat body CaM kinase II activity. For this experiment, LLI larvae were thorax-ligated and used.

(c) *In vivo* effect of 20E (by injection) on fat body CaM kinase II activity in 24 h post-ligated ELI or LLI larvae.

(d) Effect of exogenous 20E on the CaM kinase II activity in fat bodies kept in culture. The fat body from two 24 h post-ligated LLI larvae was incubated for different time periods with 20E (80 nM in 0.05% ethanol) at 25°C with gentle shaking. At the end of incubation, the fat bodies were removed from the culture medium, rinsed thoroughly in insect Ringer, homogenized and assayed for CaM kinase II activity as described in materials and methods.

Each value is the mean ± S.D. of four independent determinations. * Significantly different over † ($p < 0.05$).

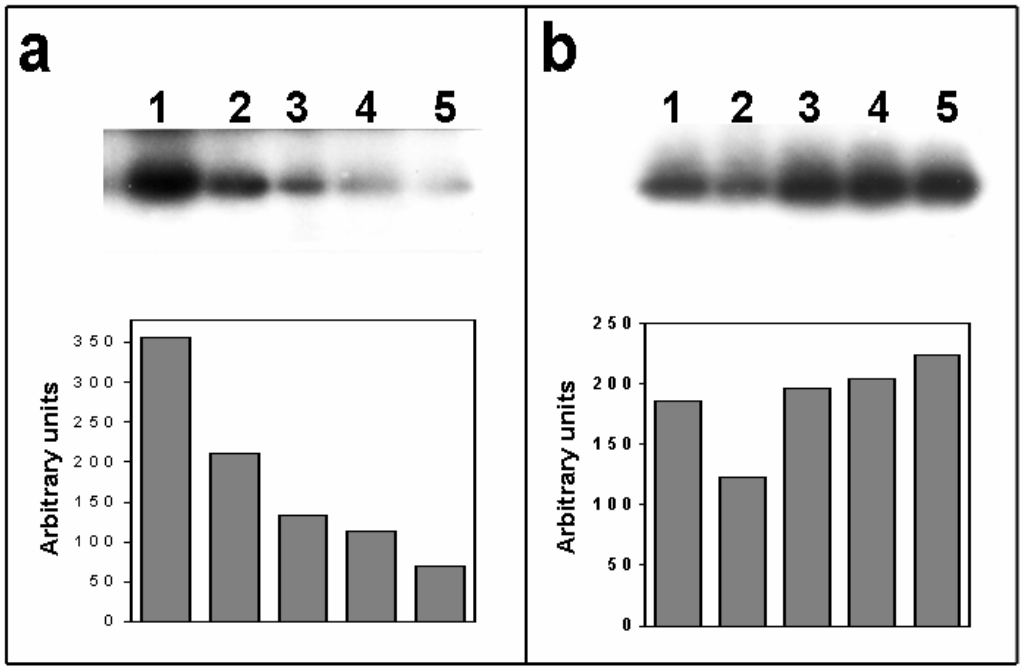


Fig. 45. Autoradiograph and densitogram showing the effects of thorax-ligation (a) and 20E (b) on the *in vitro* phosphorylation of the 120 kDa protein:-

(a) The fat bodies were collected after different time periods of thorax-ligation, homogenized and used for *in vitro* phosphorylation. Lanes- control *i.e.,* unligated (1). post-ligated (2 to 6) *viz.,* 6 h (2), 12 h (3), 24 h (4) and 48 h (5) post-ligated. Note the reduction in incorporation of [γ^{32}P] ATP in the 120 kDa polypeptide.

(b) Effect of 20E on *in vitro* phosphorylation of the 120 kDa protein in fat body homogenate prepared from 24 h post-ligated LLI larvae. Lanes- unligated (1), 24 h ligated *i.e.,* untreated control (2), 20E treated (3 to 5) *viz.,* 40 nM (3), 80 nM (4) and 150 nM (5) 20E. For this study fat body was dissected from 24 h post-ligated LLI larvae, homogenized and used. Note the enhanced phosphorylation of the 120 kDa protein in presence of 20E. Quantitation of the phosphorylated protein was done using UN-SCAN-IT gel software.

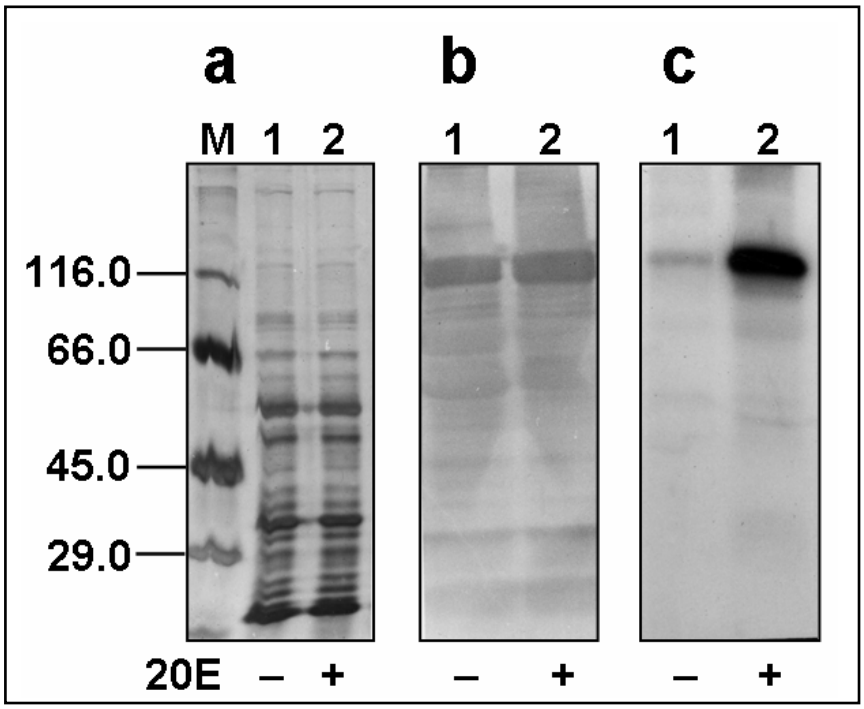


Fig. 46: Demonstration of phosphorylated 120 kDa protein as hexamerin receptor:-

SDS-PAGE (a), ligand blot (b) and autoradiograph of ligand blot (c). Fat body proteins isolated from 24 h post-ligated larvae were first *in vitro* phosphorylated either in absence or in presence of 20E, resolved on 10% SDS-PAGE, blotted on to nitrocellulose membrane and probed with biotinylated hexamerin. The membrane was subsequently processed for autoradiography. Lanes- protein marker in kDa (M), phosphorylated fat body proteins in absence of 20E (1) and in presence of 80 nM 20E (2).

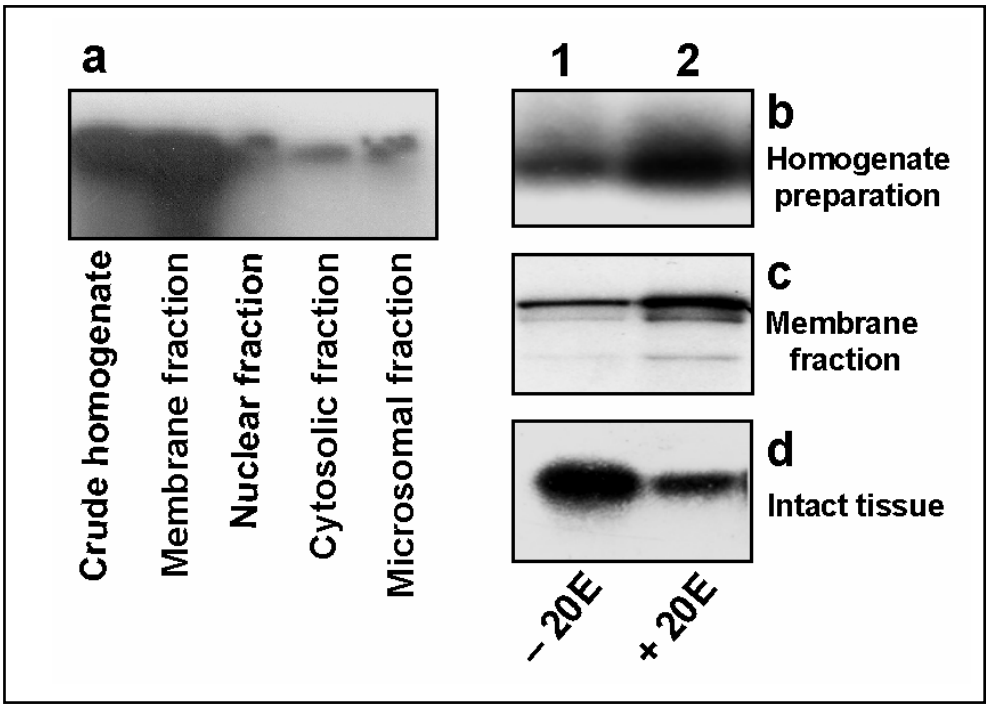


Fig. 47: Independence of tissue integrity for the phosphorylation of 120 kDa hexamerin receptor in *C. cephalonica*:-

(a) Autoradiograph showing extensive phosphorylation of the hexamerin receptor in membrane fraction prepared from fat body tissue of LLI larvae.

(b) and (c) Autoradiographs showing the effect of 20E on the *in vitro* phosphorylation of the fat body homogenate and membrane fraction prepared from LLI larvae. Lanes- control *i.e.,* 0.05% ethanol (1) and 80 nM 20E treated (2). The phosphorylation of the 120 kDa protein was stimulated in the presence of 20E.

(d) Autoradiograph showing the effect of 20E on the phosphorylation of 120 kDa protein in intact fat body tissue of 24 h post-ligated LLI larvae by back phosphorylation study. The fat bodies kept in culture were incubated with 80 nM 20E (in 0.05% ethanol) for 4 h at 25°C. The control contained equal volume of 0.05% ethanol. At the end of incubation, the fat bodies were removed from the culture medium, rinsed, homogenized and subjected to *in vitro* phosphorylation using [γ^{32}P] ATP.

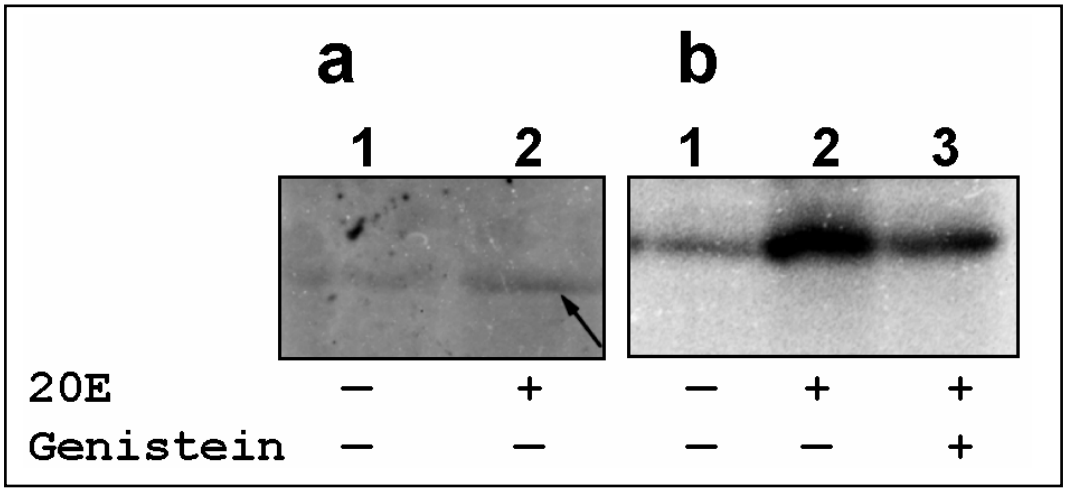


Fig. 48: Evidence of tyrosine kinase mediation for the phosphorylation of 120 kDa hexamerin receptor:-

(a) Immunoblot demonstrating the cross-reaction of the 120 kDa receptor with monoclonal anti-phosphotyrosine antibody of *in vitro* phosphorylated proteins of fat body homogenate from 24 h post-ligated larvae in absence (lane 1) and presence of 80 nM 20E (lane 2).

(b) Autoradiograph showing the effect of genistein on the phosphorylation of the 120 kDa hexamerin receptor. The fat body homogenate from 24 h post-ligated LLI larvae was first incubated either with 80mM 20E (lane 2) or with 80 mM 20E + 40 μM genistein (lane 3) for 5 min at 30°C followed by *in vitro* phosphorylation for 1 min with [γ^{32}P] ATP. Lane 1- control.

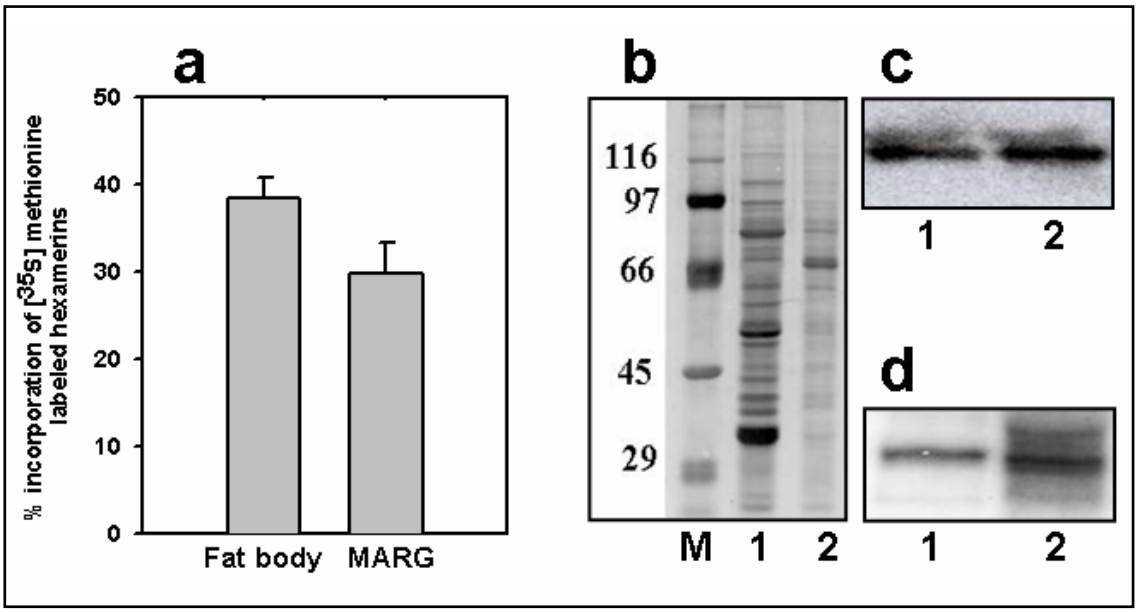


Fig. 49: Identification of hexamerin receptor and its phosphorylation in other hexamerin sequestering tissue MARG:-

Uptake of hexamerins (a), identification of 120 kDa hexamerin receptor by ligand blotting (c) and its phosphorylation (d) in fat body (lane 1) and MARG (lane 2) of *C. cephalonica*. The uptake of [^{35}S] methionine labeled hexamerins (a) was carried by incubating the fat bodies kept in culture and MARG tissue (from freshly eclosed males) for 4-6 h (for details see materials and methods). Membrane proteins from these tissues were resolved on SDS-PAGE (b) and probed with biotinylated hexamerins for ligand blotting (c). To check for the phosphorylation, the proteins were *in vitro* phosphorylated and processed for autoradiography (d).

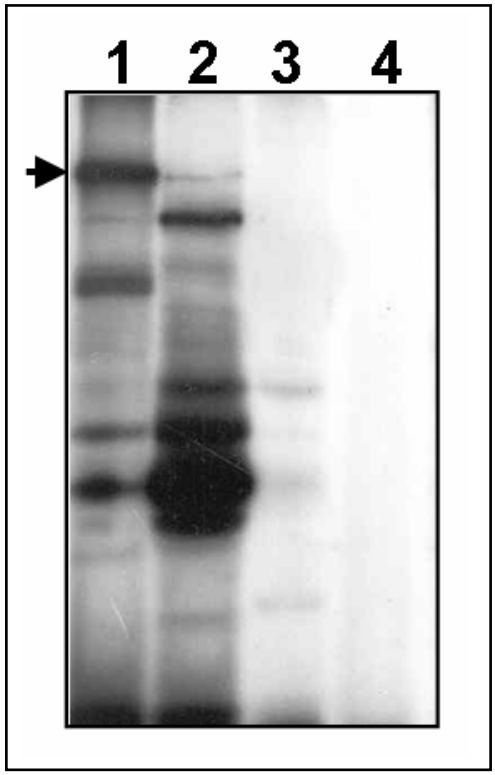


Fig. 50: Autoradiograph showing lack of phosphorylation of 120 kDa protein (arrow) in non-hexamerin sequestering tissues:-

Different insect tissues from LLI larval stage was homogenized and used for *in vitro* phosphorylation reaction as described in materials and methods. Lanes- fat body (1), epidermis and central nervous system (2), gut and Malpighian tubule (3) and haemolymph (4). Note the presence of a phosphorylated hexamerin receptor in fat body (lane 1) of *C. cephalonica.* Presence of a faint band in lane 2 is mostly due to the contaminating fat body, which is normally associated with epidermis.

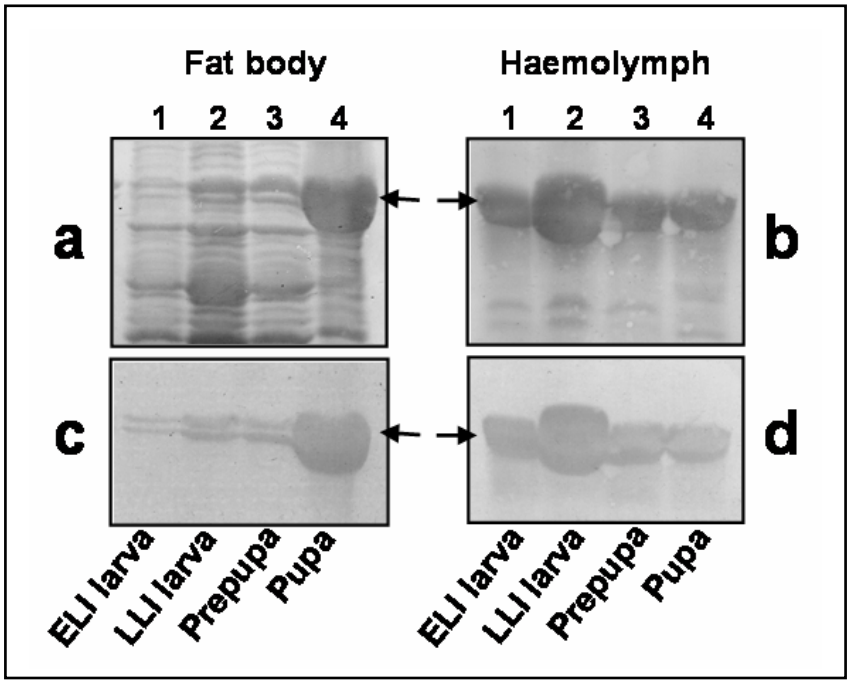


Fig. 51. Demonstration of hexamerins synthesis by larval fat body, release into the haemolymph as well as uptake by the prepupal and pupal fat body of *C. cephalonica*:-

Western analysis of hexamerins in the haemolymph and the fat body of different developmental stages of *C. cephalonica*. Note the relatively high rate of synthesis, release and accumulation of hexamerins at LLI as compared to ELI larval stage. These hexamerins are sequestered by the prepupal and pupal fat body. (a and b) Proteins were separated on SDS-PAGE transferred to nitrocellulose membrane and stained using Ponceau S stain. (c and d). Immunostaining was carried out using antibodies generated against hexamerins.

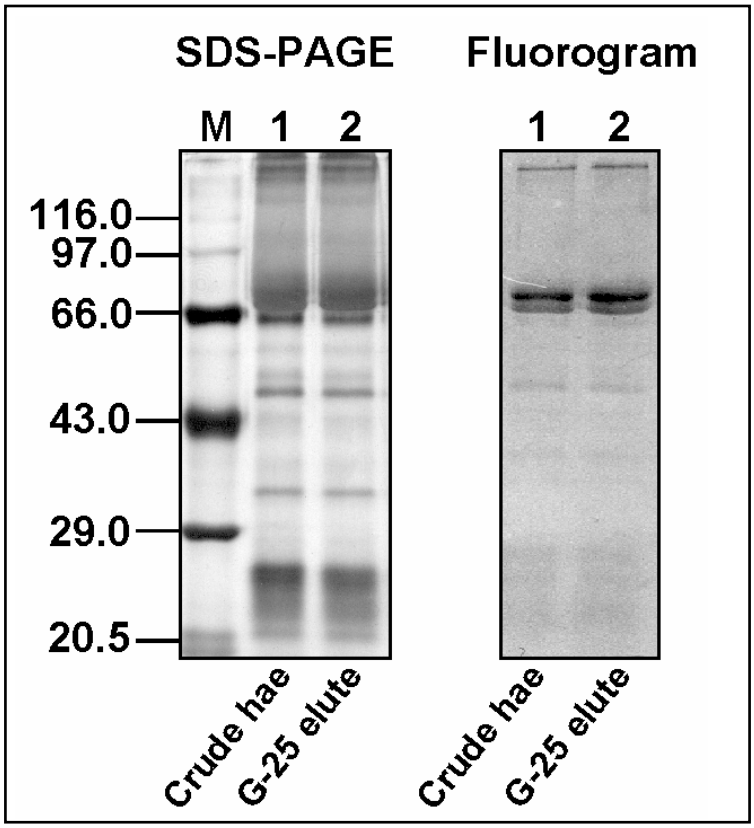


Fig. 52: [^{35}S] methionine labeling (bio-labeling) of hexamerins from larval forms of *C. cephalonica*:-

The LLI larvae were injected with 10 μCi [^{35}S] methionine and allowed to grow in normal culture condition. After 16 h the haemolymph was collected and hexamerins were partially purified by passing the crude haemolymph fractions through sephadex G-25 column in order to remove unincorporated [^{35}S] methionine, salts and other low mass proteins. The radiolabeled proteins were resolved on 7.5% SDS-PAGE and incorporation was visualized by fluorography. Note a predominant labeling of hexamerins in both crude (lane 1) as well as sephadex G-25 eluate (lane 2).

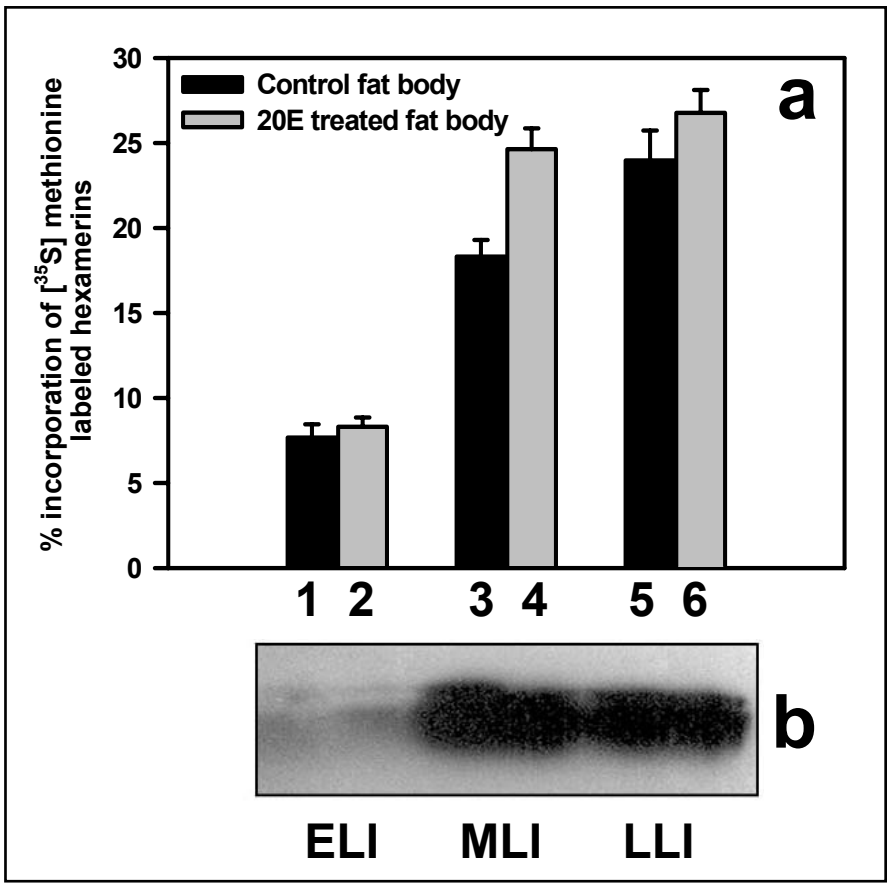


Fig. 53: Evidence of ecdysteroid dependent activation of hexamerin receptor for the uptake of hexamerins in *C. cephalonica*:-

(a) The uptake of hexamerins by the fat bodies kept in culture from different developmental stages of *C. cephalonica* in absence or presence of 20E. Intact fat bodies from two 24 h post-ligated larvae or prepupae were cultured and incubated with 80 nM 20E (0.05% ethanol) for 1 h prior to the addition of [^{35}S] methionine labeled hexamerins (50,000 cpm) for uptake studies. For the uptake, the fat bodies were incubated for 6 h as described in materials and methods chapter. After the incubation the tissue was removed from the incubation medium, rinsed thoroughly with insect Ringer, homogenized and equal quantity of protein was used to determine the radioactivity. Numbers 1, 3 and 5 are controls (−20E), while 2, 4 and 6 are 20E treated fat body cultures. Each value is the mean ± S.D. of four independent determinations.

(b) The autoradiograph shows the *in vitro* phosphorylation of the hexamerin receptor in the fat body of the above-mentioned developmental stages. For this study, the fat body homogenates prepared from the control (−20E *i.e.,* sample number 1, 3 and 5) in the above experiment were used.

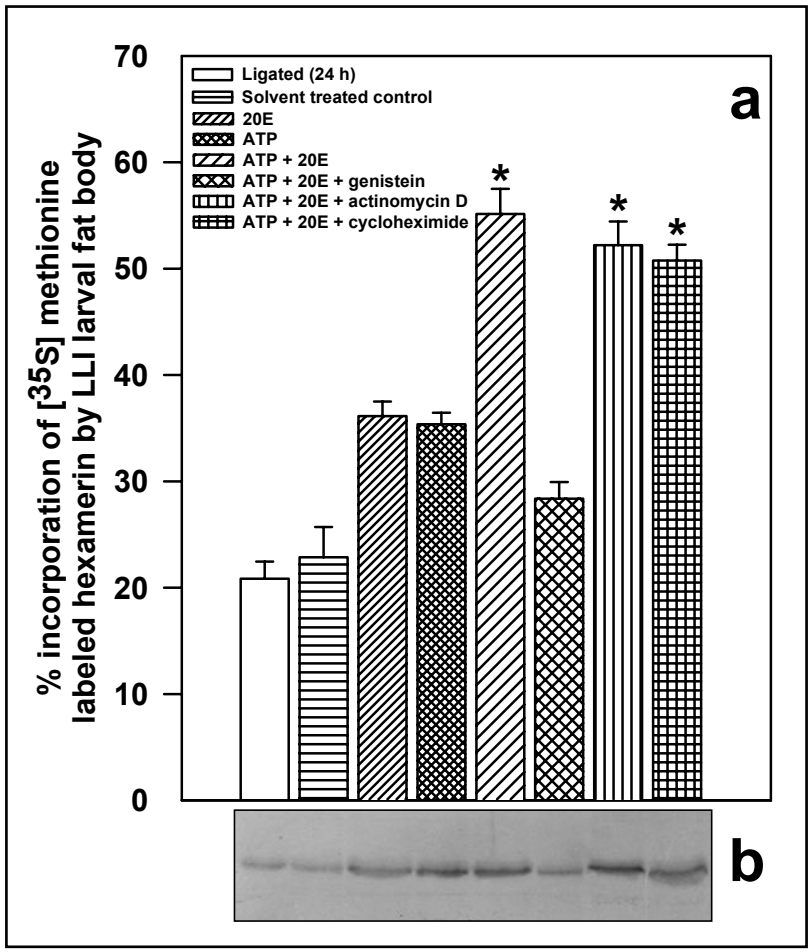


Fig. 54: Effect of hexamerin receptor phosphorylation on the uptake of hexamerins by LLI larval fat bodies kept in culture:-

(a) Effect of 80 nM 20E / 1mM ATP / 40 μM genistein / 1 mM actinomycin D / 1 mM cycloheximide / or a combination of them on the uptake of bio-labeled hexamerins by intact fat bodies of 24 h post-ligated LLI larvae. Note: The incorporation of hexamerin was high in the 20E + ATP treated fat body as compared to untreated, solvent treated, 20E alone or ATP alone treated fat body. The uptake was inhibited in the genistein treated fat body, while actinomycin D or cycloheximide had no effect. The data was statistically analysed by one way ANOVA followed by comparisons of means by Student-Newman-Keuls multiple comparison test using Sigma Stat software. *$p<0.05$ was defined as the criterion for statistical significance. The values represent the mean $\pm$ S.D. of duplicate determinations from three separate experiments.

(b) Corresponding fluorograph showing similar hexamerin uptake pattern as (a).

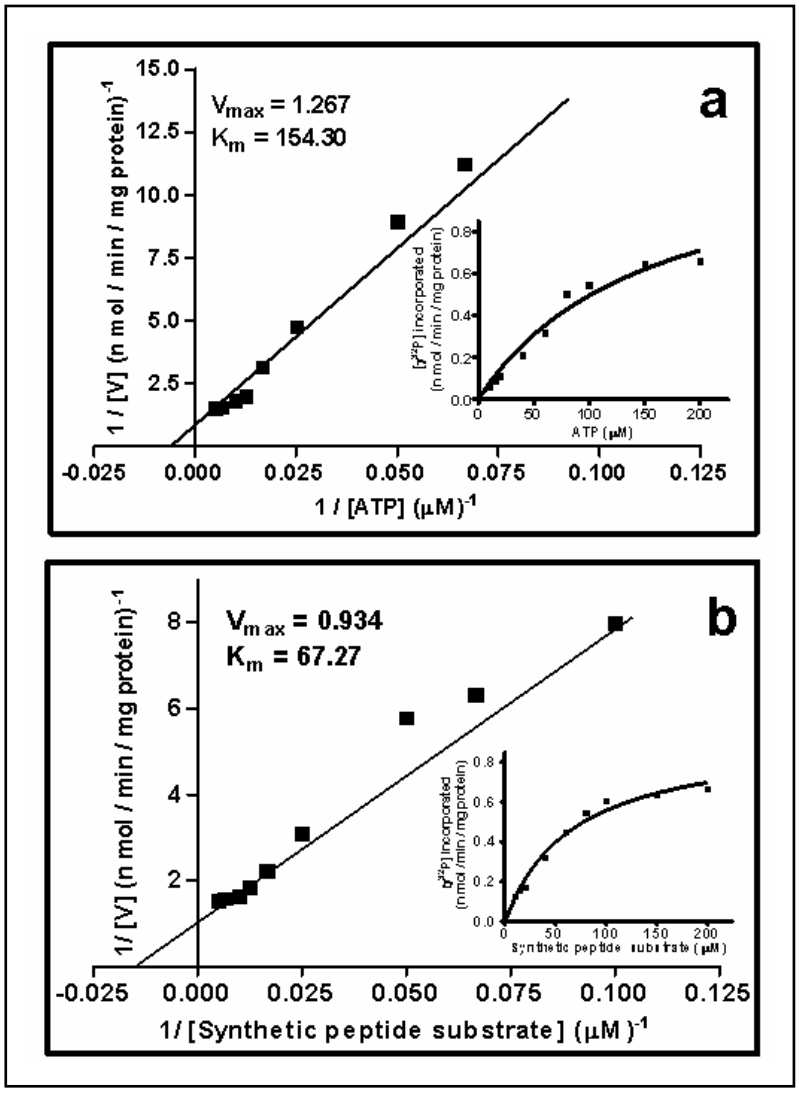


Fig. 55: Lineweaver-Burk and Michaelis-Menten plots of the phosphorylation of a synthetic peptide substrate (A-7433, Sigma) by endogenous tyrosine kinase of *C. cephalonica* fat body. Initial rates were measured under standard condition for 2 min by filter paper assay. The ATP (a) and synthetic peptide substrate (b) concentration were varied as indicated.

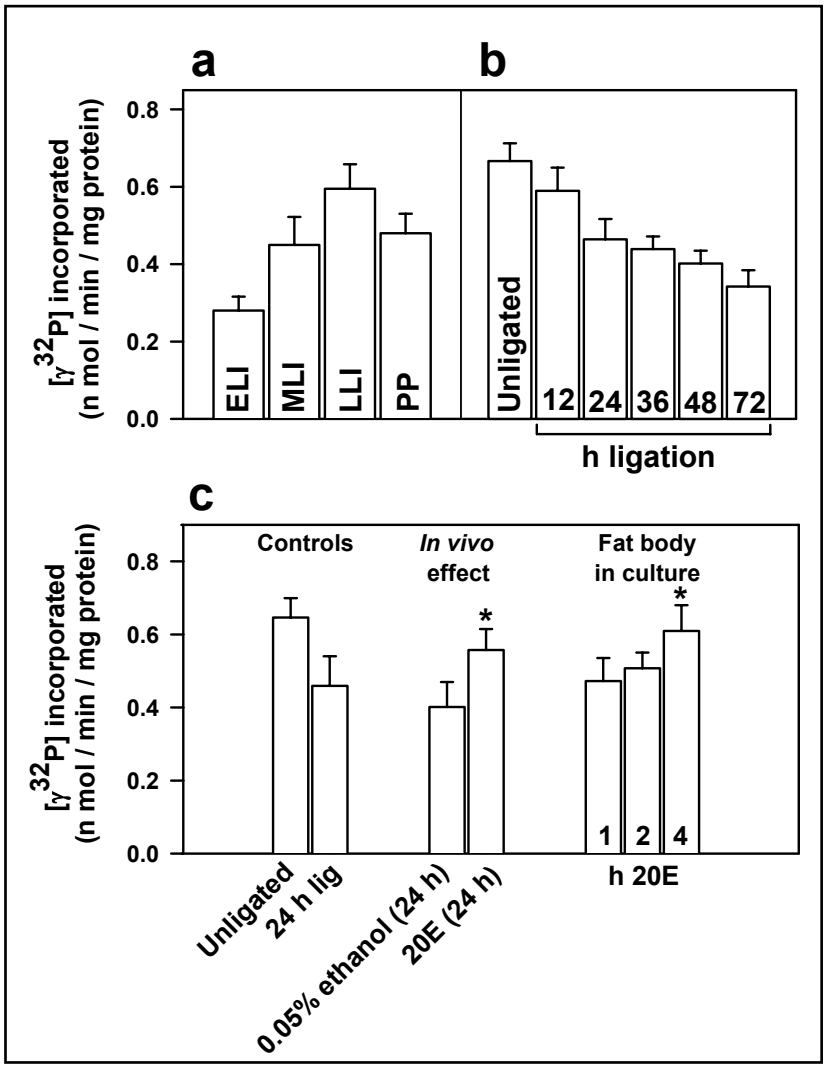


Fig. 56: Endogenous fat body tyrosine kinase activity of fat body of *C. cephalonica*:-

Changes in the activity of fat body tyrosine kinase of *C. cephalonica* during different developmental stages (a), upon ligation of LLI larvae for different time periods (b) and stimulation in the activity by 20E *in vivo* (by injection to 24 h post-ligated LLI larvae) as well as in the fat bodies kept in culture (c). * A significant increase in enzyme activity was seen upon 24 h under *in vivo* and 4 h under *in vitro* hormone treatment. The values represent mean ± S.D. of four independent determinations.

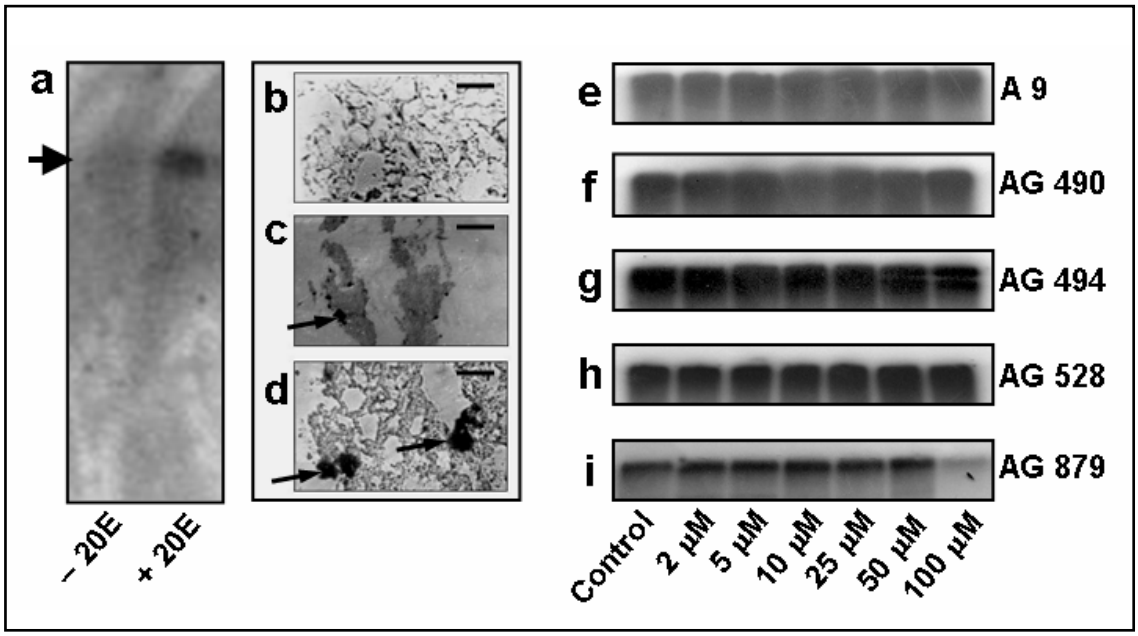


Fig. 57: Possible identification of receptor tyrosine kinase mediated phosphorylation of hexamerin receptor:-

(a) Autoradiograph showing phosphorylation of hexamerin receptor in intact fat body tissue. The fat body tissue in culture was incubated with [γ^{32}P] ATP for 1 h at 25°C followed by homogenization, separation of proteins on SDS-PAGE and autoradiography.

(b-d) Immunohistochemical localization of protein phosphorylated at tyrosine residues in the fat body tissue using monoclonal anti-phosphotyrosine antibody. Light micrograph of fat body section showing immunocrossreactivity (arrow head, c- low and d- high magnification). The control section was processed with mice serum (b). Scale bar (b & d) 10 μm = 0.48 cm and (c) 10 μm = 0.12 cm.

(e-i) Effect of tyrphostins, synthetic tyrosine kinase inhibitor on the phosphorylation of hexamerin receptor. The fat body homogenates were incubated with different concentration of these inhibitors for 10 min at 30°C, prior to *in vitro* phosphorylation.

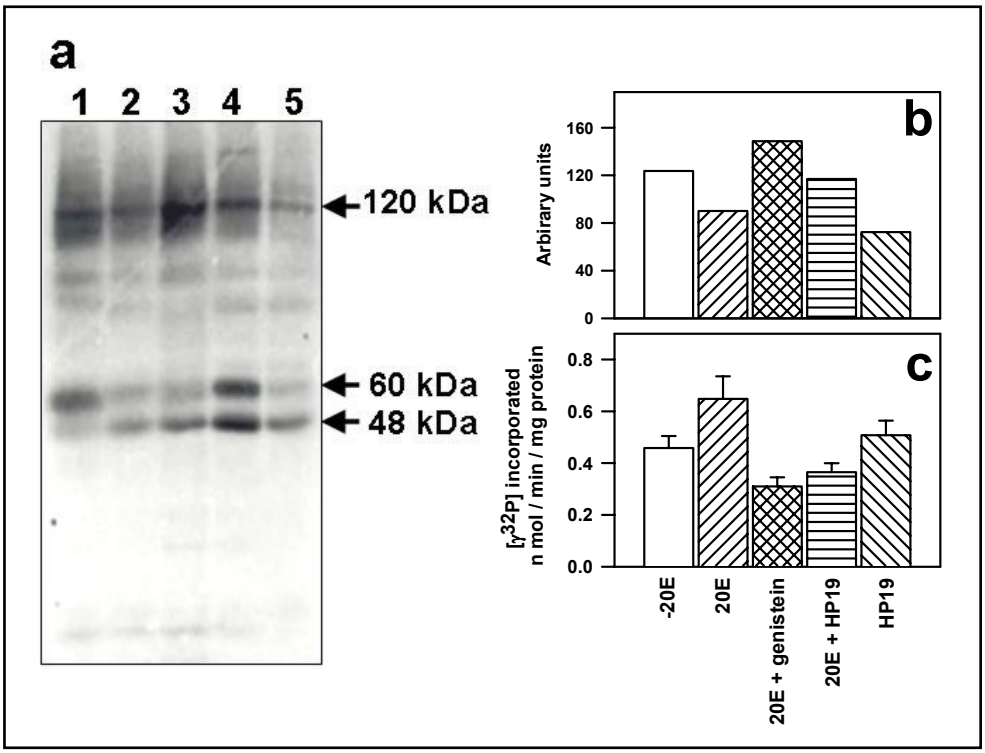


Fig. 58: Effect of HP19 on the 20E dependent phosphorylation of 120 kDa hexamerin receptor and autophosphorylation of 60 kDa CaM kinase II in fat body of *C. cephalonica*:-

Autoradiograph (a), the corresponding densitogram (b) and the endogenous tyrosine kinase activity in the fat body (c) from the back phosphorylation of fat body proteins. The fat body tissue from 24 h post-ligated LLI larvae was rinsed thoroughly in TC-100 insect culture medium. The tissue was then incubated for 4 h in absence (lane 1) or presence of 20E (lane 2) and 20E with 40 µM genistein (lane 3) or 40 ng HP19 (lane 4) or HP19 alone (lane 5). The incorporation of [γ^{32}P] ATP was found to be relatively high in control (−20E), genistein and HP19 along with 20E treated fat body tissue as compared to the 20E alone or HP19 alone treated fat body. The endogenous tyrosine kinase activity in these tissue as expected show reverse pattern compare to (a) and (b). The quantitation of the phosphorylated protein band intensity was done using UN-SCAN-IT gel software. For (c) the values are mean ± S.D of three independent determinations.

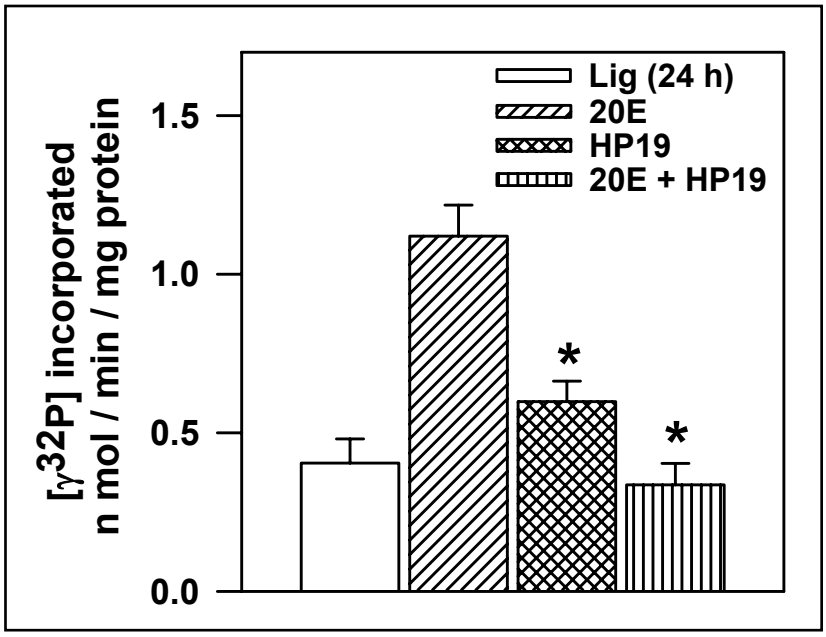


Fig. 59: Inhibitory effect of HP19 on the 20E dependent stimulation of endogenous tyrosine kinase activity in fat body homogenate:-

The fat body homogenate prepared from 24 h post-ligated LLI larvae was incubated with 80 nM 20E (in 0.05% ethanol) / 40 ng HP19 / 20E + HP19 for 30 sec followed by assay of tyrosine kinase activity. For control, the homogenate protein was incubated with an equal volume of 0.05% ethanol. Note the inhibition in the activity by HP19 in presence as well as in absence of 20E and is significantly different from 20E treated samples ($p<0.05$). Each value is the mean ± S.D. of four independent determinations.

Results- Chapter V

Mechanism of 20-hydroxyecdysone regulated actions that is mediated by HP19

Part of this work has been published in-

Arif *et al.,* (2004). The insect haemolymph protein HP19 mediates the nongenomic effect of ecdysteroids on acid phosphatase activity. ***J. Biol. Chem.*** 279, 28000-28008.

Background-

A wealth of data on the molecular mechanism of ecdysteroid action shows that the transcriptional cascade leading to molting and metamorphosis is initiated when 20E binds to its nuclear receptor complex (Riddiford *et al.,* 2001). This corroborates with all other steroid hormones action that regulate most of the biochemical pathways and the temporal sequence of developmental processes in vertebrates as well as in invertebrates, usually at the transcriptional level (Beato and Klug, 2000; Scheller *et al.,* 2003). However, for about four decades ample evidence has accumulated that some of the hormonally induced effects seemed to be too rapid for the classical model (Falkenstein, 2000a; Losel and Wehling, 2003). This evidence casts doubt on the so-called genomic pathway as the sole mode of steroid action. Today, several modes for nongenomic steroid actions are examined. Most of them are thought to continuously modulate the long-term program allowing cells or organs to adapt rapidly to environmental changes. Numerous experiments with many different species show that insect metamorphosis in general is under the genomic control of ecdysteroids. A few studies indicate that some events, necessary for and accompanying metamorphosis, are controlled by 20E at a nongenomic level. However, studies on these mechanisms are restricted to a small number of experimental systems *e.g.,* the activation of lysosomal enzymes and the hexamerin receptor (Verkuil, 1979; Burmester and Scheller, 1997a).

In *S. peregrina* and *C. vicina,* the ecdysteroid mediated activation of the hexamerin receptor was found to be independent of transcription and protein synthesis (Ueno and Natori, 1984; Burmester and Scheller, 1997a). These studies suggest that the receptor activation by the hormone occurs at a post-translational level. Earlier studies conducted in *C. cephalonica* do not reveal the cleavage of receptor, hence it was assumed that 120 kDa hexamerin receptor protein is activated post-translationally for the uptake of hexamerins under the influence of 20E. To learn more about the mechanisms that underlie hexamerin endocytosis in *C. cephalonica*, following were studied in the present work- (i) the receptor phosphorylation, (ii) its role in receptor activation, (iii) the action of 20E and the role of HP19 in regulation of this process. The detailed results on the phosphorylation of hexamerin receptor and its significance in hexamerin uptake are presented in chapter IV of results section. The results obtained suggest that HP19 inhibits the 20E induced phosphorylation of hexamerin receptor that is mediated by a tyrosine kinase. The hexamerin receptor phosphorylation is found to be required as a pre-requisite for hexamerin uptake during the non-feeding stages of insects. Additionally it is also shown in the earlier section of thesis that HP19 also mediates the 20E

dependent stimulation of ACP activity. Present study indicates possible nongenomic action of ecdysteroid for the regulation of these functions. In this section of thesis, an attempt is made to further understand the regulation of these 20E mediated actions and to establish if these actions are regulated at nongenomic level.

Nongenomic regulation of 20E stimulated ACP activity and role of HP19: evidence at protein level-

When the fat bodies kept in culture were incubated with 20E and HP19 for 4 h, we observed that the stimulation of ACP activity remained unaffected in the presence of transcriptional or translational inhibitors (Fig. 60a). The results in figure 60b further indicate the nongenomic regulation of 20E stimulated ACP activity by HP19 because the addition of protein directly to the fat body homogenate also mediated the steroid stimulated action. This effect was rapid and could be observed within 30 sec to 1 min. We further confirmed the nongenomic regulation of 20E dependent ACP activity by incubating the fat bodies kept in culture first with [^{35}S] methionine for 2 h followed by incubation with hormone, HP19 and transcriptional or translational inhibitors. The results presented in figure 61 show that the total protein synthesis is induced under the influence of 20E and this induction is inhibited by actinomycin D and cycloheximide (Fig. 61a). However, the inhibitors did not affect the ACP activity. The HP19 enhanced the 20E stimulated ACP activity (Fig. 61b).

Characterization of C. cephalonica ACP (CcACP) cDNA-

After establishing the HP19 mediated nongenomic effect of ecdysteroid on ACP activity, effort was made in the present study to understand this phenomenon at RNA level. For this, the approach was taken to clone the ACP of *C. cephalonica*. Incidentally while screening for the hexamerin cDNA from the fat body expression library using polyclonal antibody against *C. cephalonica* hexamerins (Nagamanju *et al.,* 2003), one of the false positive showed sequence homology with phosphatidic acid phosphatase (PAP). This clone was *in vitro* transcribed to get RNA probe that was used for hybridization screening of the fat body cDNA library and thus one clean positive was picked (Fig. 62a) and used for second round of screening. After two rounds of screening two positive clones were picked (Fig. 62b). Restriction analysis of these clones suggested it to be of identical size (Fig. 63, lanes 2 & 5). One of the clones was subcloned and partially sequenced (GenBank Accession AF317884). The partial cDNA sequence of *C. cephalonica* PAP (Fig. 64) showed ~38% sequence identity with *Drosophila melanogaster* phosphatidic acid phosphatase (PAP), wunen and tunen gene

(Fig. 65). When this clone was used for Southern hybridization, it showed that CcPAP is a product of multiple copy gene (Fig. 66a, lanes 1 & 2). Expression of this gene in various larval tissues of *C. cephalonica* (Fig. 66b) matched with the activity profile of ACP (Fig. 66c). The highest level of expression (Fig. 66b, lane 2) as well as activity (Fig. 66c) was obtained in the visceral fat body. The total larval body also showed high level of expression (Fig. 66b, lane 1) as well as the ACP activity compared to other tissues. This is because of the presence of visceral fat body in this preparation that constitutes a large fraction of total insect body (Keeley, 1985). The results presented in figure 67a show the gradual increase in the expression level of ACP gene from ELI larvae to prepupal stage and this is consistent with the activity profile seen in the fat bodies from these developmental stages (Fig. 67b).

Nongenomic regulation of 20E stimulated ACP activity and role of HP19: evidence at transcript level-

To understand the nongenomic regulation of ecdysteroids, mediated by HP19 at molecular level, the RNA from fat body, which was cultured with 20E and HP19 along with the control (unligated larvae that were not kept in culture) were subjected to northern analysis. The results indicate that the presence of HP19 that mediates the 20E stimulation of ACP activity (Fig. 68b) did not cause any increase in the transcript of the PAP (Fig. 68a). Although it proves the hypothesis of nongenomic regulation of ACP activity, *i.e.,* the rapid regulation without any change in transcript level, however these studies gives no clue if the ACPs that are regulated by HP19 are lysosomal and are required for autophagy during metamorphosis or they are membrane bound protein required for other signalling such as the phospholipase D activation.

Nongenomic regulation of hexamerin receptor phosphorylation by 20E: role of HP19-

In the earlier results section (Chapter IV), it was shown that 20E stimulates the phosphorylation of *C. cephalonica* hexamerin receptor present in fat body cells as well as other tissues and it is partly mediated by a tyrosine kinase. This phosphorylation occurs in homogenate, membrane as well as in intact tissue and is a pre-requisite for hexamerin uptake during the prepupal and pupal stages. Further, this phosphorylation is unaffected by inhibitors of transcription and translation. The study was extended to understand the rapid nongenomic regulation of receptor phosphorylation by 20E and role of HP19 in this process. The results in figure 69a show that the addition of HP19 either in absence (lane 1) or in presence (lane 2) of

20E inhibited the basal (lane 3) as well as 20E stimulated (lane 4) phosphorylation of the 120 kDa hexamerin receptor. For this experiment, the fat body homogenate was incubated with 20E and / or HP19 for 30 sec prior to the *in vitro* phosphorylation. This rapid inhibition was also seen whether the homogenate was incubated simultaneously along with 20E and HP19 (Fig. 69b) or initially incubated with 20E for different time periods followed by fixed time incubation with HP19 (Fig. 69c) or vice versa (Fig. 69d). However, with longer incubation periods, there was no significant inhibition by HP19 in the phosphorylation status of receptor protein (Fig. 69b-d). This study indicates that HP19 rapidly blocks the 20E regulated hexamerin receptor phosphorylation in larval fat body.

Nongenomic regulation of fat body tyrosine kinase activity by 20E: role of HP19-

The results in figure 70 show that endogenous fat body tyrosine kinase activity, which is stimulated by 20E is inhibited by HP19, both in fat bodies kept in culture (Fig. 70a) and in homogenate (Fig. 70b). When the fat body cultures were incubated with 20E and HP19 for 4-6 h, we observed that the 20E stimulated activity was inhibited by the presence of HP19 but remained unaffected in the presence of transcriptional or translational inhibitors (Fig. 70a). The results in figure 70b once again indicate that, this HP19 assisted 20E dependent tyrosine kinase activity is nongenomically regulated because the inhibition by HP19 was also seen in the fat body homogenate preparation and was also rapid (in 30 sec). However, HP19 alone had more or less no inhibitory effect on the activity in the fat body homogenates. The partial inhibition by HP19 alone in fat body cultures, suggest that the basal level of 20E present in the 24 h post-ligated larvae is enough for HP19 to act and inhibit the activity to certain extent, though for significant inhibition physiological concentration of 20E is required.

Nongenomic regulation of fat body CaM kinase II activity by 20E: role of HP19-

The results presented in figure 71 show that like the fat body tyrosine kinase, HP19 also inhibits the endogenous fat body CaM kinase II, which is stimulated by 20E both in fat bodies kept in culture (Fig. 71a) and in homogenate (Fig. 71b). In the earlier section of the results presented in figure 58 (Chapter- IV), HP19 has been shown to inhibit the CaM kinase II autophosphorylation.

Regulation of HP19-

Present study indicated a multiple role of HP19 on few of the 20E dependent actions. The HP19 mediated the 20E stimulation of ACP activity and inhibited the 20E induced

phosphorylation of hexamerin receptor, activity of tyrosine kinase and CaM kinase II activity as well as its autophosphorylation. Present study suggests that HP19 is either a kinase inhibitor or a phosphatase activator, however, at the moment it is not very clear. Earlier studies from our laboratory do suggest that the regulatory action of HP19 like protein might be due the possession of protease like property (Vasanthi, 1999). The low concentrations of the protein required to regulate the 20E dependent actions also suggest probable regulatory nature of the protein. In the present study effort was also made to understand the relative stability of HP19 (shelf life) in the total haemolymph as well as in the partially purified fraction. It was found to be modestly stable for a period of upto 30 days upon proper storage condition (−4°C). However, stability in terms of its interaction with hormone to regulate various actions remains to be tested. Comparisons with other ubiquitously distributed regulatory molecules like 14-3-3, stathmin and ubiquitin, which have known multiple roles in cellular functions, HP19 did not show any immunological similarity effect at western level (Figs. 20 and 21 in chapter II of results section). In order to understand whether HP19 was itself phosphorylated to regulate these 20E dependent actions, *in vitro* phosphorylation of haemolymph proteins were carried out (Fig. 72). The results indicated that there is no phosphorylation of HP19 in absence or presence of calcium either in HGLFB (Fig. 72b), a tissue that synthesizes this protein or in the haemolymph (Fig. 72a) where it is released.

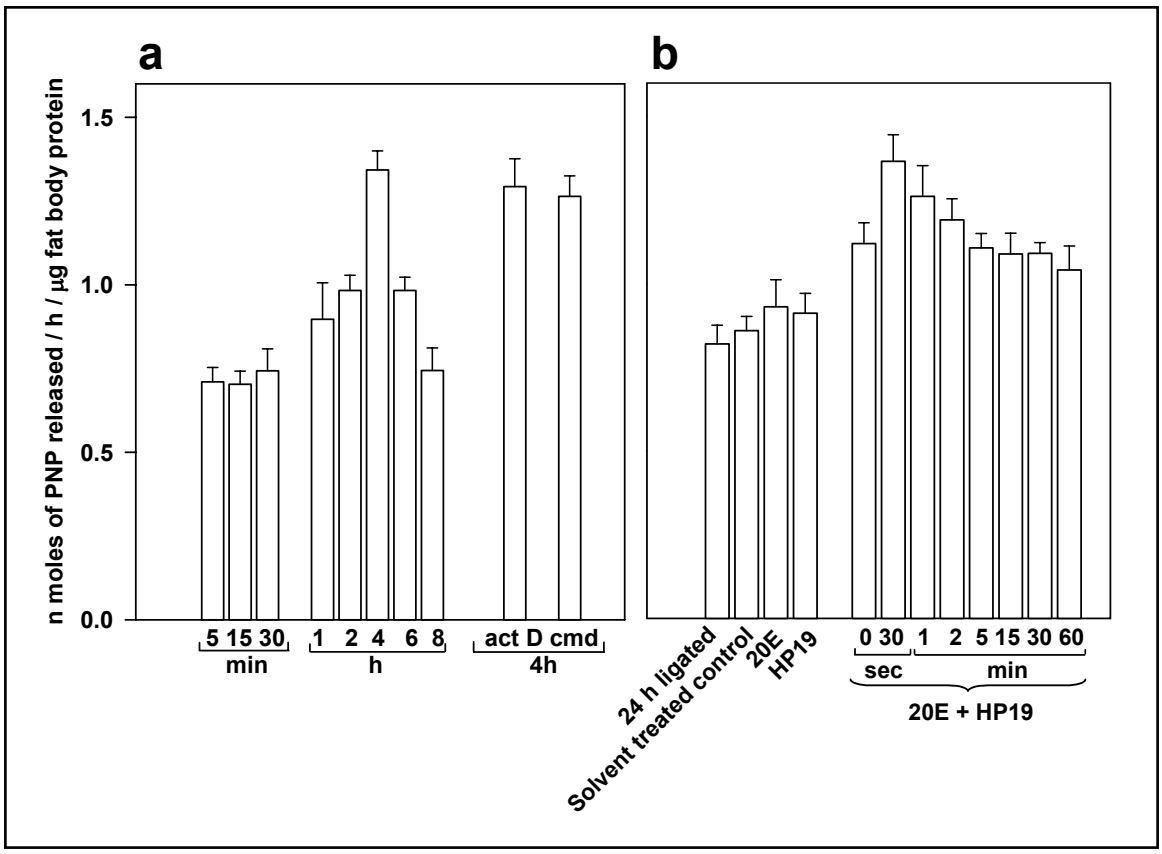


Fig. 60: Nongenomic regulation of fat body ACP activity by 20E in presence of HP19:-

(a) The fat body cultures prepared from two 24 h post-ligated LLI larvae were incubated with 80 nM 20E + 40 ng HP19 without or together with actinomycin D (1 mM) or cycloheximide (1 mM). Addition of HP19 along with 20E caused a gradual time dependent increase in the ACP activity, which reached a high value after 4 h. Note that the transcriptional and translational inhibitors do not block the 20E stimulated ACP activity of fat bodies kept in culture. Actinomycin D (act D) and cycloheximide (cmd).

(b) Rapid in *vitro* effect of HP19 on the 20E dependent ACP activity in fat body homogenates. For this experiment, the fat bodies were dissected from two 24 h post-ligated LLI larvae and homogenates were prepared. The homogenate was incubated with 80 nM 20E and / or 40 ng HP19 for different time incubation. Note HP19 alone did not have any stimulatory effect.

All the values are mean ± S.D. of four independent determinations.

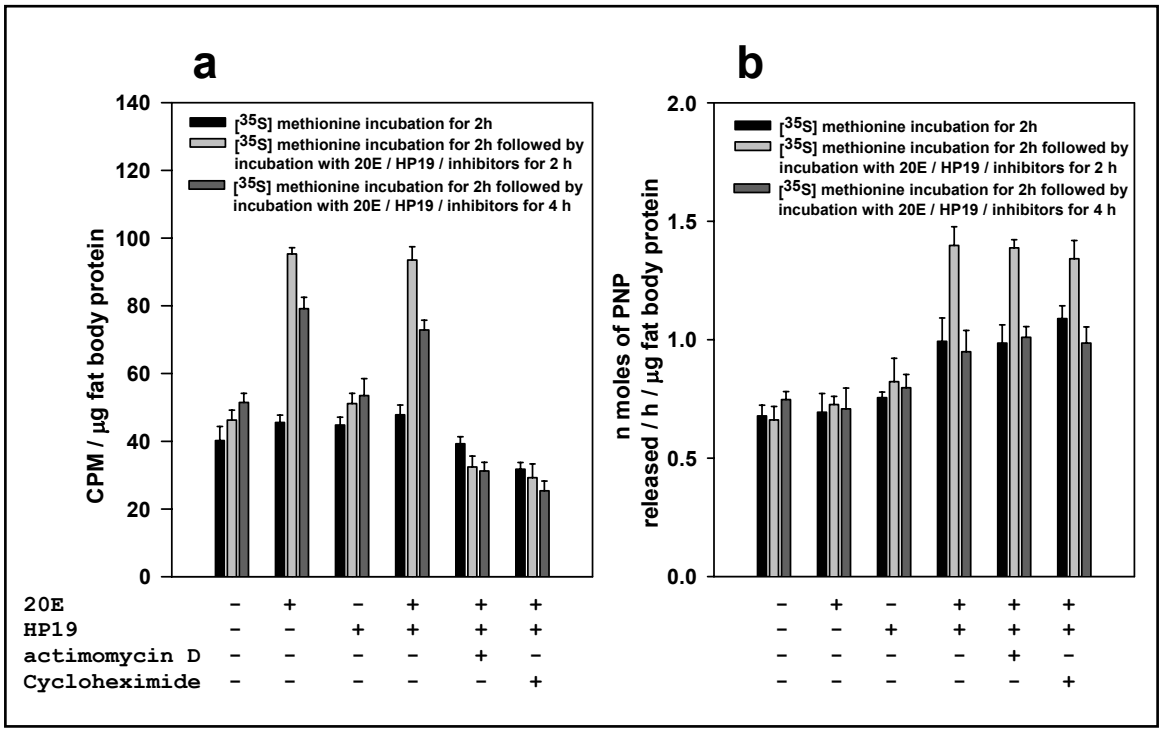


Fig. 61: Effect of protein biosynthesis inhibitors on HP 19 action:-

The fat bodies from two 24 h post-ligated LLI larvae were cultured initially with 10 µCi of [^{35}S] methionine for 2 h followed by an additional incubation with 80 nM 20E / 40 ng HP19 / 1 mM actinomycin D / 1 mM cycloheximide for 2 and 4 h. The 20E stimulated protein synthesis is inhibited by the inhibitors (a). ACP activity is stimulated by 20E in presence of HP19 and is not blocked by inhibitors (b). The values are mean ± S.D. of four independent determinations.

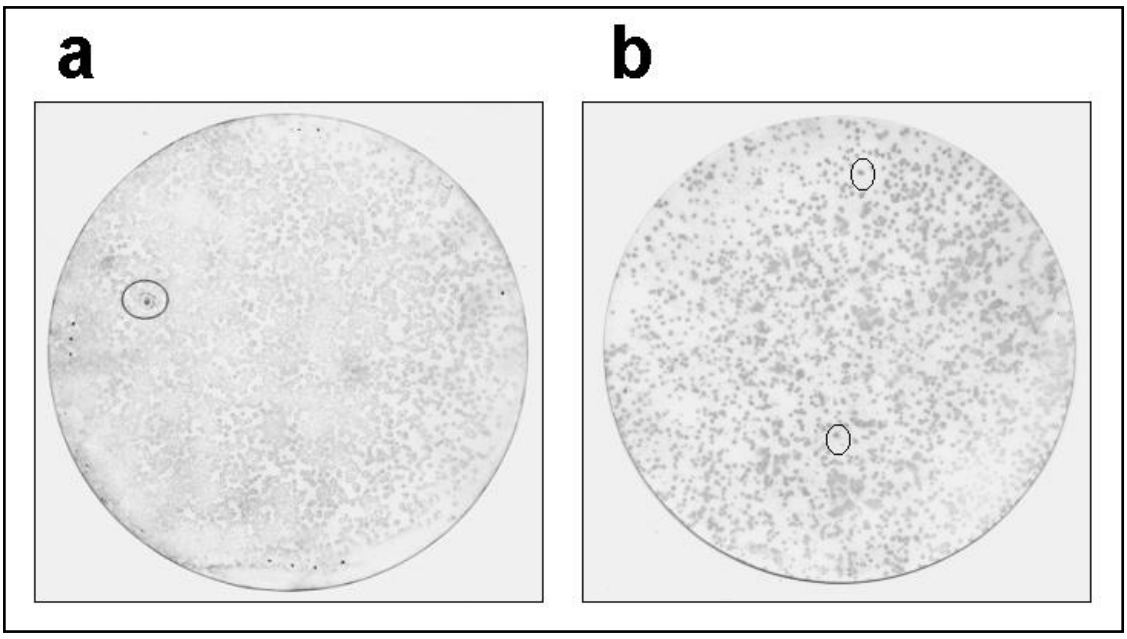


Fig. 62: Hybridization screening of fat body cDNA expression library using RNA probe to pick cDNA encoding ACP:-

(a and b) are respectively the hybridization screened blots of 1st and 2nd round of screening. While immunoscreening the *C. cephalonica* fat body expression library for hexamerin encoding genes using hexamerin antibody (Nagamanju *et al.,* 2003), one of the false positive upon sequencing showed homology with phosphatidic acid phosphatase (PAP). This PAP clone was used as the primary bait to clone ACP of *C. cephalonica* after *in vitro* transcription. The RNA probe from PAP cDNA clone was then used for hybridization screening of fat body cDNA expression library. Note that in (a) there was one clean positive after 1st round of screening which was used for 2nd round of screening (b) and after two rounds of screening, 2 positive clones were picked and used for further analysis.

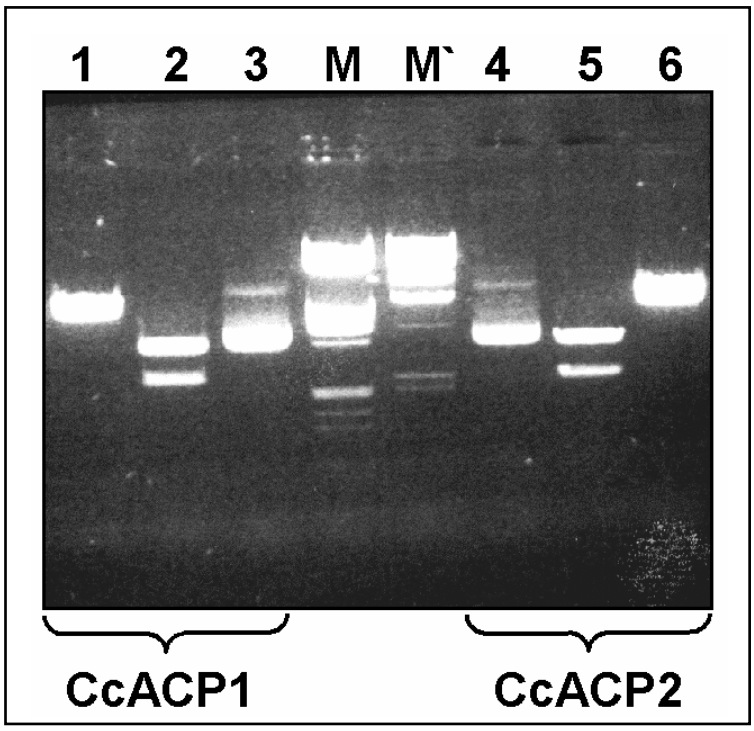


Fig. 63: Restriction analysis (double digest) of the positives obtained by hybridization screening of HGLFB-cDNA expression library:-

By hybridization screening of 6 x 10^9 recombinant phage plaques two positives were obtained after two rounds of screening, *in vivo* excised, converted into plasmids and used for XL-1Blue cell transformation Approximately 1 µg of plasmid DNA was subjected to *Eco RI* + *Not I* digestion. Lanes- λDNA *Eco RI / Hind III* double digest (M), λDNA *Hind III* digest (M`), plasmid DNA with the ACP insert (1 & 4), *Eco RI* + *Not I* double digested plasmid DNA (2 & 5) and *Eco RI* digested plasmid DNA (3 & 6). The restriction analysis (double digest) revealed both the clones to be of identical size.

```
1       atagttagtg ataagccaat acaacataca cgacaggatg tcgcgacgag
51      acgcgtccgt acacgtgctg agaaagattg tcttggattt gctattattg
101     tctgcactga ttggatgcat attcataacc gaactttctc tgggagccgt
151     tcgctcgcgg attcttctgt ggggatgaaa gtcttatgtt tccatacaag
201     aaagacacgg tttccagtac catgttgaga atagttggac tcggattgcc
251     tatgttatct ataataatct gcgaatgggt cctgctaagg aaggaacaga
301     gcgacgaagt atgcttcggg attcgtatcc cagcgtgggt tcgtggggcg
351     tattgcgcgc tcgcatcctt cggtttgggc gtttgtttca tggaactcac
401     tgccaatgta gctaaaaaca ctattggaag accgcgacct catttctttt
451     ctgtatgtca accttccgtg gactgcaact cgttagaatg gcggaacagg
501     tacatccagt cccacgagta ccactgcacc ggtgatcaga aggagctatt
551     caaggatatg aggatgtcat tcctgagtgg acattcctcc tgggctgctt
601     acactatggt gtacttggct ctttacctag aaaagcgcat ggtctggcgc
651     ggtactcgag tgctcaggca taccctacag ttttctgcca tcatgctgag
701     ctggtt
```

Fig. 64: The partial cDNA nucleotide sequence of *C. cephalonica* ACP phosphatase (CcACP):- The GenBank accession number for the sequence is AF317884.

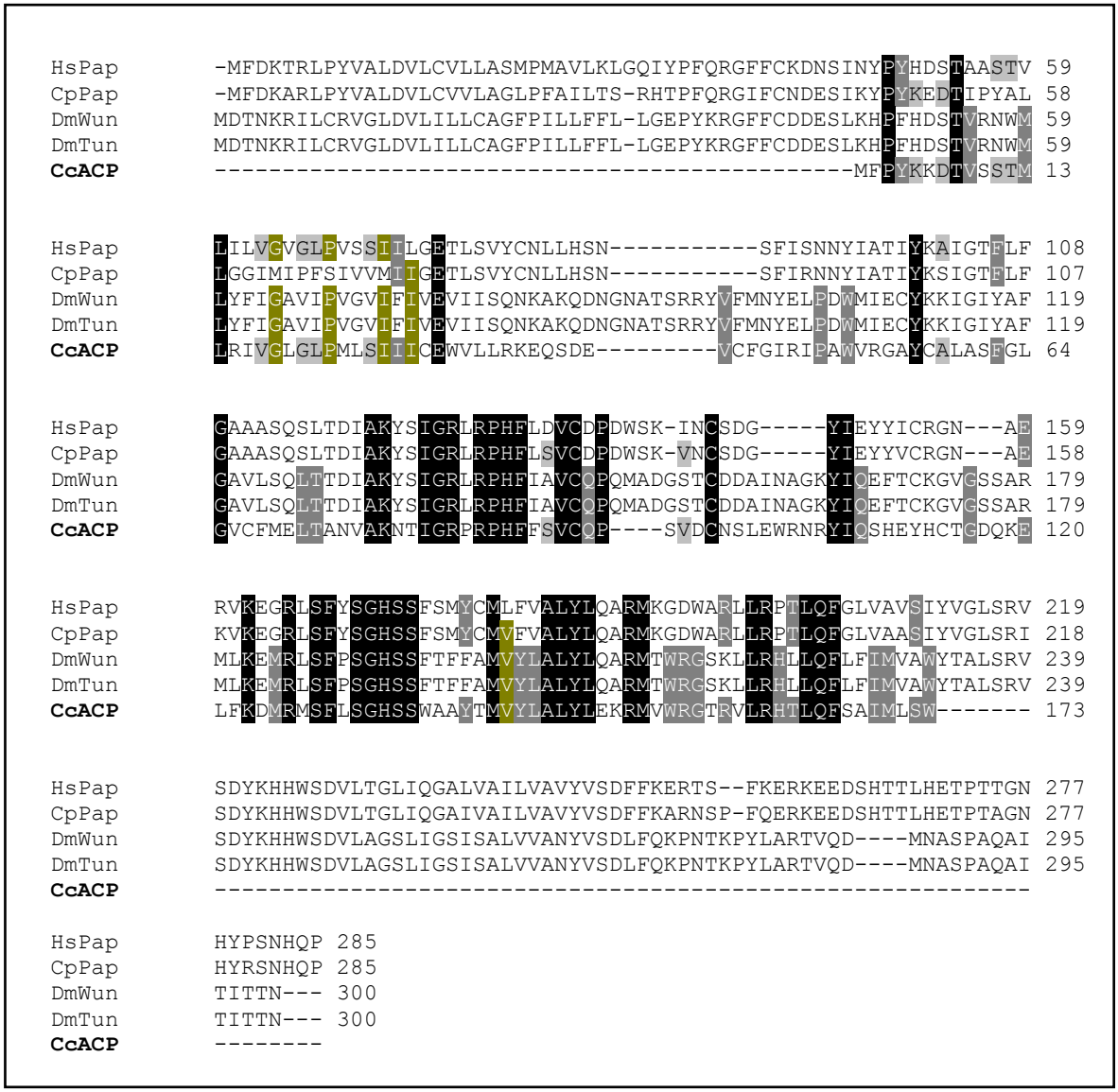


Fig. 65: Alignment of the deduced amino acid sequence of *C. cephalonica* ACP (CcACP) with ACP sequences (PAP) of other insects (BLAST search):-

Drosophila melanogaster tunen- DmTun (AF236058); *D. melanogaster* wunen- DmWun (AF145595); *Homo sapiens* type 2 phosphatidic acid phosphatase 2a2- HsPap (AF014403) and *Cavia porcellus* phosphatdic acid phosphatase 2a2- CpPap (AF088284). The identical amino acid positions are shaded and gaps are indicated by dashes. CcACP showed 38% identity with DmTun and DmWun, 36% with HsPap and 33% CpPap.

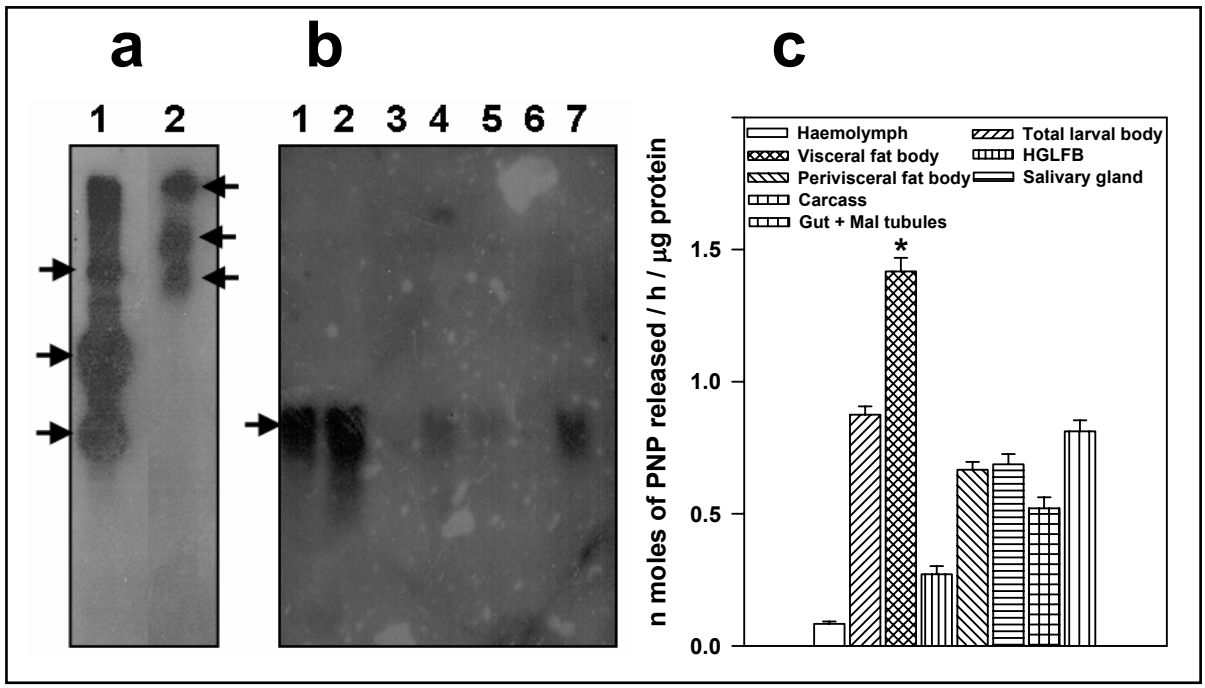


Fig. 66: Characterization of ACP of *C. cephalonica*:-

(a) Southern blot analysis showing multiple gene copy (arrow) of ACP. The genomic DNA (30 µg) from total larval body was digested with *EcoRI* (lane 1) or *Bam HI* (lane 2) and probed with CcACP partial cDNA as described in materials and methods.

(b) Northern blot showing the presence of ACP transcripts (arrow) in different larval tissues of *C. cephalonica*. Equal quantity of total RNA from various tissues were probed with CcACP partial cDNA. Note that ACP is expressed in majority tissues along with HGLFB. Lanes- total larval body (1), visceral fat body (2), HGLFB (3), perivisceral fat body (4), salivary gland (5), carcass (6) and gut + Malpighian tubule (7)

(c) ACP activity in different LLI larval stage tissues. Note that the activity profile is comparable to the northern profile (b) though activity was negligible in haemolymph and significantly low in HGLFB as compared to the activity in visceral fat body. The results presented are mean ± S.D. of four independent determinations. * Significantly different over all other values ($p<0.05$).

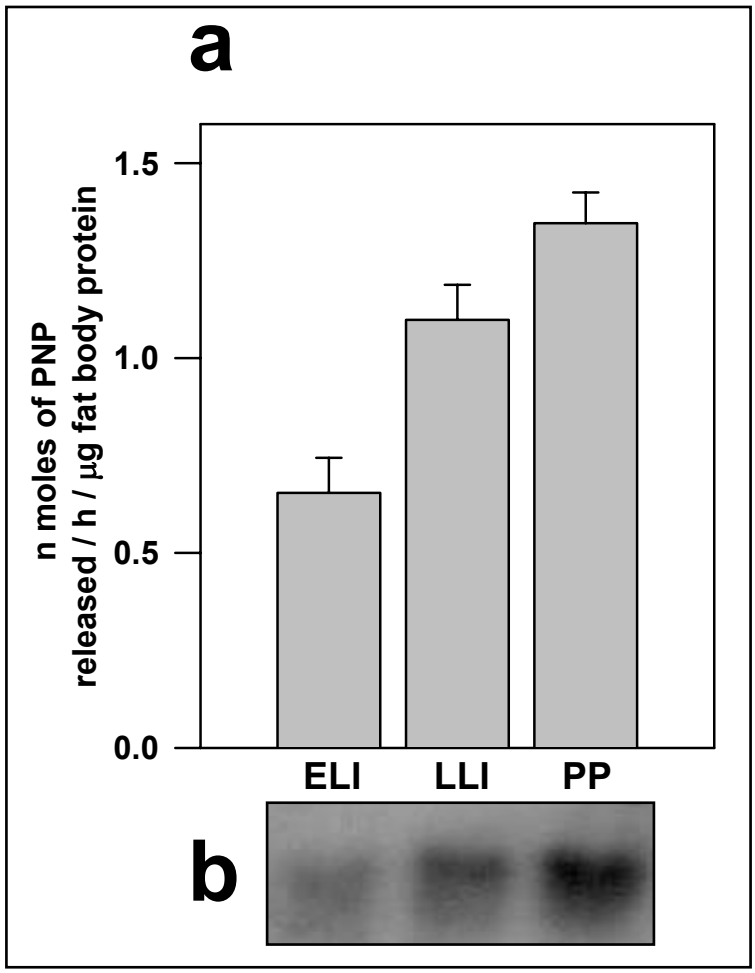


Fig. 67: Differential activity (a) and northern expression profile (b) of ACP at different developmental stages of last (Vth) instar larvae:-

Equal quantity of total RNA from various developmental stages was probed with CcACP partial cDNA. Note the parallel increase in enzyme activity and transcript level from early-last instar to prepupal stage. For (a), the results presented are mean ± S.D of four independent determinations.

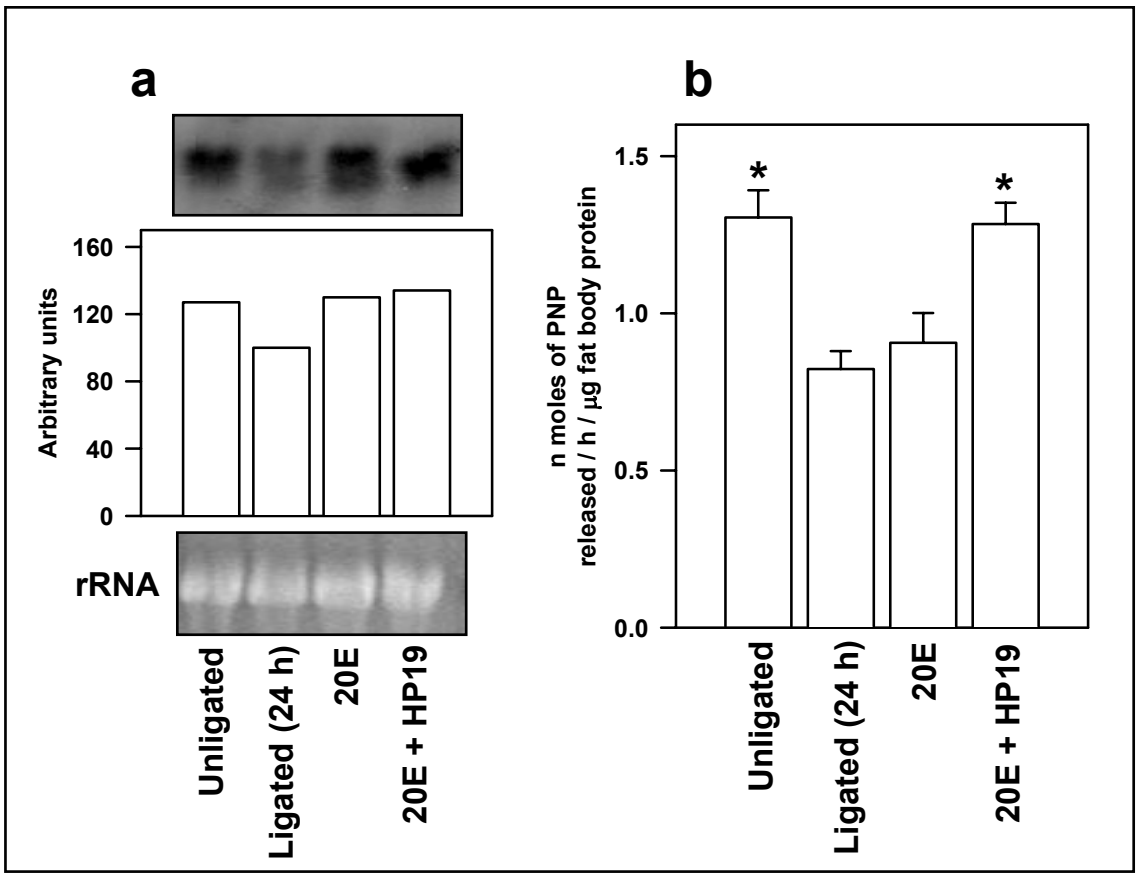


Fig. 68: Nongenomic action of 20E on ACP activity mediated by HP19: evidence at transcript level:-

Northern expression, the corresponding densitogram (a) and activity profile of ACP (b) in the fat bodies kept in culture that was incubated with 20E alone or along with HP19. The fat bodies from six 24 h post-ligated LLI larvae were cultured as described in materials and methods with 20E (80 nM in 0.05% ethanol) or 20E + HP19 (40 ng) for 4 h. The control contained equal volume of 0.05% ethanol. The fat body from unligated larvae was used directly for the assay. At the end of the incubation, the fat bodies were removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenized and part of the homogenate was used for RNA isolation and rest for the ACP activity assay. For northern study, equal quantity of total RNA from various tissues was probed with CcACP partial cDNA. For activity study, each value is the mean ± S.D. of four independent determinations. * Significantly different over all other values ($p<0.05$). Note that equal quantity of total RNA from different tissues (represented as ribosomal RNA) showed no significant change in transcript level though there was significant increase in the activity when the fat bodies kept in culture were incubated with 20E and HP19.

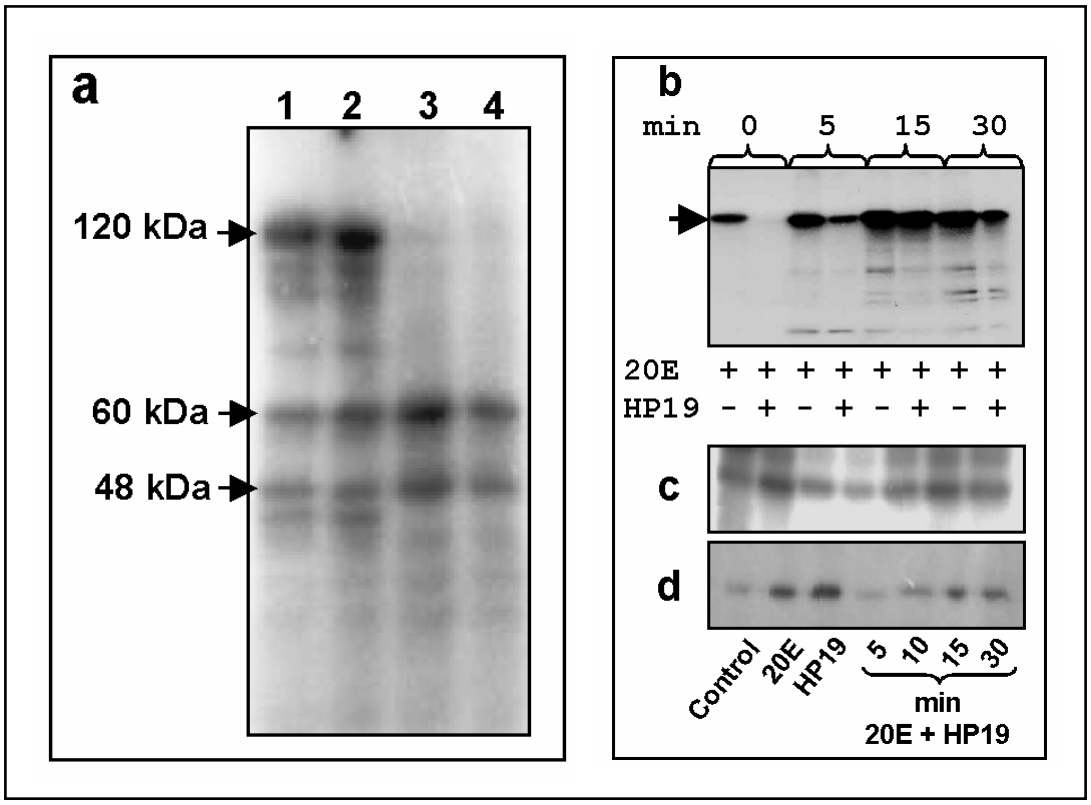


Fig. 69: Nongenomic regulation of 120 kDa hexamerin receptor phosphorylation by 20E in presence of HP19:-

(a) Effect of HP19 on the 20E dependent phosphorylation of the 120 kDa hexamerin receptor in the fat body homogenate preparation from LLI larvae. The fat body homogenate was incubated with 80 nM 20E (lane 2) / 40 ng HP19 (lane 3) / 20E + HP19 (lane 4) for 30 sec followed by *in vitro* phosphorylation, revealed inhibition of the basal (lane 3) as well as 20E stimulated (lane 4) phosphorylation of 120 kDa hexamerin receptor (arrow) by HP19. For control, the homogenate protein was incubated with an equal volume of 0.05% ethanol (lane 1).

(b-d) Rapid inhibition of 20E induced phosphorylation of hexamerin receptor by HP19. The 20E induced phosphorylation of hexamerin receptor was rapidly inhibited by HP19 (within 0-5 min) in either of the conditions *i.e.,* (b) when 20E and HP19 were added simultaneously to fat body homogenate and incubated for different time periods or (c) when the fat body homogenate was preincubated with 20E for different time periods followed by incubation with HP19 for 15 min or (d)-vice-versa. After the incubation the fat body homogenates were subjected to *in vitro* phosphorylation and processed for electrophoresis followed by autoradiography.

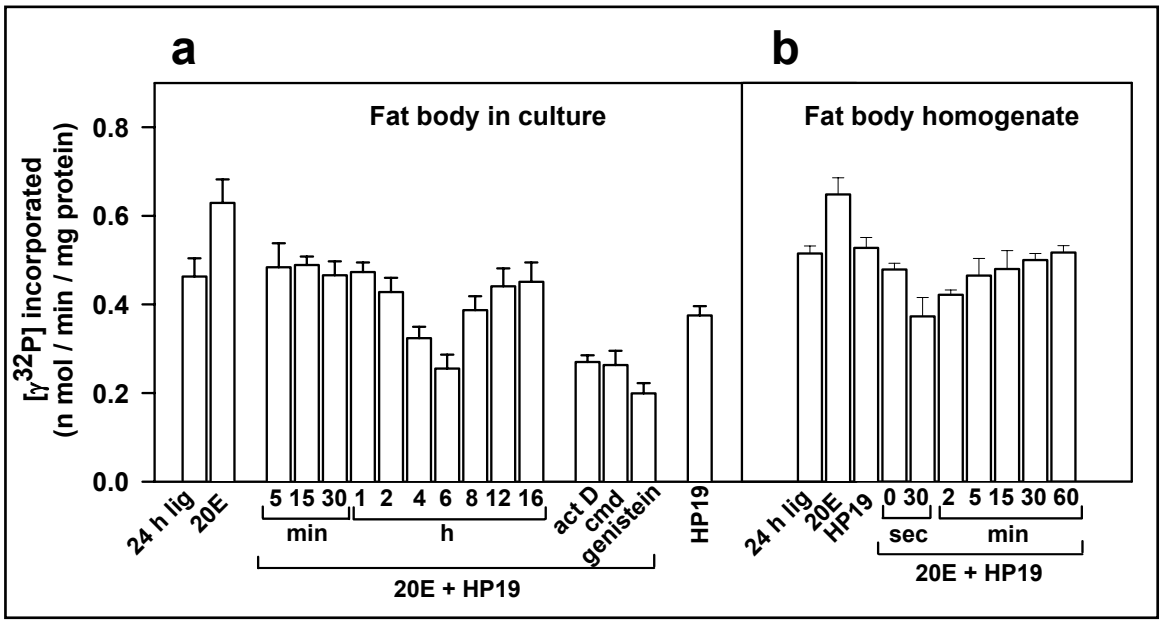


Fig. 70: Nongenomic regulation of fat body tyrosine kinase activity by 20E in presence of HP19:-

(a) The fat bodies from two 24 h post-ligated LLI larvae were incubated with 20E (80 nM) and HP19 (40 ng) for different time periods. The inhibition of 20E induced activity (induction by 20E at 4 h incubation was maximum, also see figure 56 in Chapter- IV) by HP19 was maximum at 6 h when incubated together with 20E (80 nM). The presence of inhibitors either actinomycin D (1 mM) or cycloheximide (1 mM) does not block the inhibition. Genistein (40 μM) also inhibited the tyrosine kinase activity of fat bodies kept in culture. The degree of inhibition by HP19 alone was low at 6 h time incubation. Actinomycin D (act D) and cycloheximide (cmd).

(b) Rapid *in vitro* inhibition of 20E induced tyrosine kinase activity by HP19 in the fat body homogenates prepared from 24 h post-ligated LLI larvae. In the homogenate, 20E induced the activity in 2 min and HP19 alone does not affect this inhibition.

All the values are mean ± S.D. of four independent determinations.

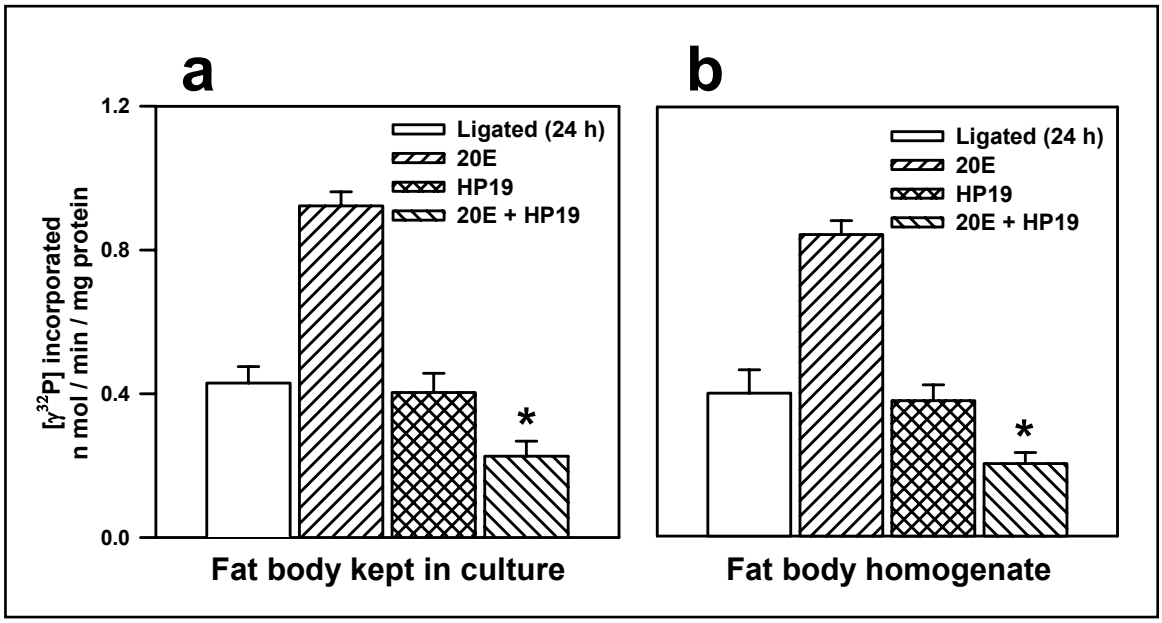


Fig. 71: Evidence of nongenomic regulation of fat body CaM kinase II of *C. cephalonica*:-

(a) Shows the effect of 20E and / or HP19 on CaM kinase II activity in fat body tissues kept in culture. The fat bodies from two 24 h post-ligated LLI larvae were cultured as described in materials and methods with 20E (80 nM in 0.05% ethanol) or HP19 (40 ng) or 20E + HP19 (40 ng) for 4 h. 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 used for CaM kinase II assay.

(b) Shows the effect of 20E and / or HP19 on CaM kinase II activity in fat body homogenate. The fat body homogenate prepared from the tissue obtained from 24 h post-ligated LLI larvae was incubated with 20E (80 nM) or HP19 (40 ng) or 20E + HP19 for 1 minute at 30ºC followed by assay of CaM Kinase II activity.

Note both in fat bodies kept in culture and in homogenate, HP19 inhibited the activity. The inhibition in activity by HP19 was more pronounced in combination with 20E. Each value is the mean ± S.D. of four independent determinations. * Significantly different from 20E + HP19 treated group ($p<0.05$).

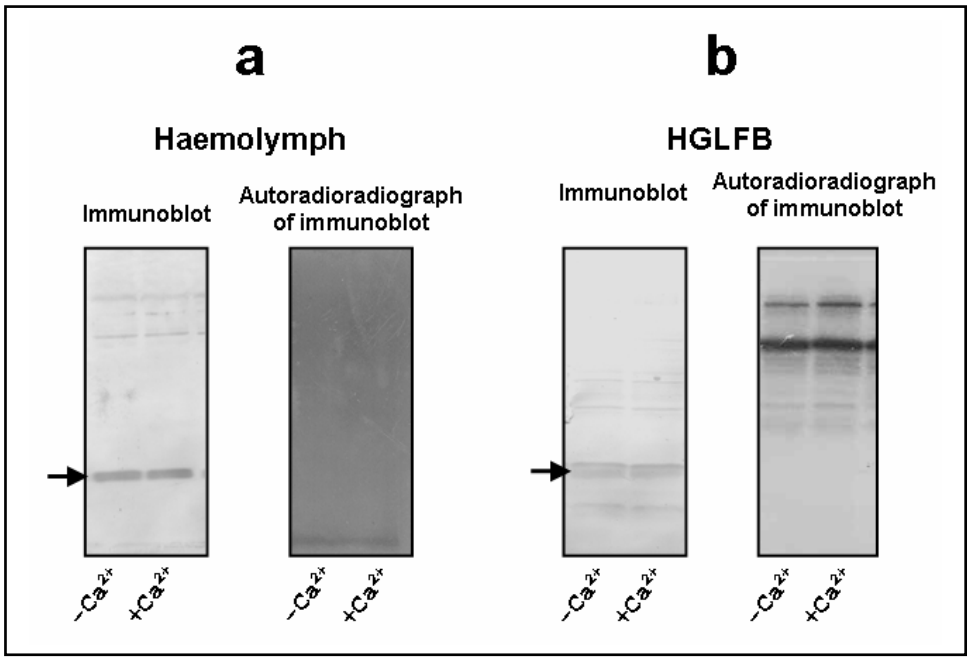


Fig. 72: Phosphorylation of total haemolymph and HGLFB proteins:-

The total haemolymph (a) as well as HGLFB (b) proteins from *C. cephalonica* were subjected to *in vitro* phosphorylation as described in materials and methods in absence or presence of Ca^{2+} (0.1 mM $CaCl_2$). The phosphorylated proteins were resolved on 12% SDS-PAGE, transferred onto nitrocellulose membrane, probed with anti-HP19 IgG fraction, processed for western analysis for the detection of HP19 in these tissues. Finally, the processed blots were autoradiographed to check the autophosphorylation of HP19. Note in both the tissues, HP19 (arrow) remained unphosphorylated in absence or presence of Ca^{2+}.

Discussion

Growth, development, metamorphosis and reproduction in insects are mainly controlled by interplay of two groups of hormones, the sesquiterpenoid juvenile hormones and ecdysteroids (Nagasawa *et al.,* 1990; Riddiford, 1996; Gilbert *et al.,* 2000; Riddiford *et al.,* 2001; Truman and Riddiford, 2002). The prominent functions of ecdysteroid hormones are their ability to trigger the temporal sequence of developmental process underlying molting and metamorphosis, one of the most dramatic events in the animal kingdom. (Truman, 1996a, b; Henrich *et al.,* 1999; Riddiford *et al.,* 2001; Trumann and Riddiford, 2002).

For molting and metamorphosis, the ecdysteroids are known to have wide ranged effects on various tissues including the metabolically most active tissue, the fat body during the larval stages of development in the holometabolous insects including lepidoptera (Schenkel and Scheller, 1986; Gilbert *et al.,* 1996; Riddiford *et al.,* 2001; Hansen *et al.,* 2002). It is well known, that ecdysteroids initiate the breakdown of larval structures during metamorphosis (Trumann, 1996b), either by initiating the formation of autophagic vacuoles for autophagy of group of cells (Lockshin and Beaulton, 1974; Dean, 1978; Sass and Kovacs, 1975, 1977, 1980) or by apoptosis *i.e.,* the programmed death of individual cells (Abrams, 1999). Steroid triggered metamorphosis by regulation of autophagy is well reported in *Drosophila* (Lee and Baehriecke, 2001; Thummel, 2001). 20-Hydroxyecdysone (20E) 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 laboratory have also revealed that ecdysteroids stimulate the 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; Shanavas *et al.,* 1996). The uptake of storage proteins (hexamerins) by the fat body cells (Ismail and Dutta-Gupta, 1990b; Dutta-Gupta and Ismail, 1990, 1992; KiranKumar *et al.,* 1997, 1998) as well as by the male accessory reproductive gland (MARG) (Ismail and Dutta-Gupta, 1990c, 1991; Dutta-Gupta and Ismail, 1992; Ismail *et al.,* 1993) was also reported to be regulated by ecdysteroids. Literature survey suggests that regulation of most of these actions by ecdysteroids is by modulation of transcription (Scheller and Karlson, 1977; Schenkel and Scheller, 1986; Henrich *et al.,* 1999; Riddiford *et al.,* 2001). However, some of these actions have also been shown to be regulated at post-translational level (Verkuil, 1979; Ueno *et al.,* 1983; Ueno and Natori, 1984; Chung *et al.,* 1995; Tomaschko, 1999; Burmester and Scheller, 1997a, 1999).

The ecdysteroids are synthesized by PGL 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 haemolymph *i.e.,* the insects "blood" is known as a 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 *G. mellonella* proved to be effective in causing the shortening of interganglionic connections *in vivo* (Pipa, 1969). However, *in vitro* studies in which isolated connectives from final instar larval stages were cultured in the presence of 20E, the hormone alone could not initiate the process, but it did maintain the shortening in culture if the process had already begun *in vivo* (Robertson and Pipa, 1973; Robertson, 1974). This suggest about the involvement of additional factors in the initiation of nerve cord shortening. In *Drosophila,* injection of 20E under *in vivo* conditions to the larval salivary glands caused alterations in the ultra-structure of the gland finally leading to its degeneration. However, 20E could not stimulate 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. *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 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 the *in vitro* cultured mid gut stem cells of *H. virescens* by the extracts from perivisceral fat body of freshly ecdysed *M. sexta* pupae stimulation in the mid gut stem cells. Several other studies also suggested about the presence of factor(s) in the

haemolymph, which regulate ecdysteroid synthesis as well as the ecdysteroid dependent action (Stall, 1982; Meola and Gray, 1984; Gray *et al.,* 1987, Watson *et al.,* 1985; 1987; 1988; Shiraishi and Natori, 1989). Majority of these factors are proposed to be synthesized in fat body and released into the haemolymph. However, no convincing report on the characterization of any of these factors is yet available.

One of the major events occurring during the postembryonic development of holometabolous insects, including lepidopterans, is the histolysis of larval organs (Lockshin and Beaulton, 1974). The process of cellular destruction and / or remodeling is mediated among others by lysosomal activity (Sass *et al.,* 1989). Acid phosphatase (ACP) is one of the most commonly used marker enzymes for studying the lysosomal activity (Schin and Clever, 1965; Konichev, 1982; Kutuzova, 1991). The autophagic process or the lysosomal activity in the whole insect as well as in the fat body exhibits a specific pattern during the postembryonic development. 20E has been shown to elicit effects on autophagic processes of the fat body by stimulating the ACP enzyme activity (Verkuil *et al.,* 1979; Sass and Kovacs, 1980; Verkuil, 1980; Ashok and Dutta-Gupta, 1988).

In the present study, thorax-ligation that renders the posterior part in larvae relatively free of endogenous hormone (Priester *et al.,* 1979; Burmester and Scheller, 1997; Dutta-Gupta and Ashok, 1998) caused a gradual decline in larval fat body ACP activity, pointing to its hormonal dependence in *C. cephalonica*. The injection of a physiological dosage of exogenous 20E into ligated larvae caused a stimulation of ACP activity. However, when 20E alone was added to fat bodies kept in culture, the hormone could not stimulate the enzyme activity. Earlier it was demonstrated that the administration of exogenous 20E, stimulated the fat body ACP activity in the ligated larvae of *Spodoptera litura* (Sridevi *et al.,* 1987) and *C. cephalonica* (Ashok and Dutta-Gupta, 1988). However, the addition of 20E to *in vitro* cultures did not alter the fat body ACP activity (Ashok and Dutta-Gupta, 1991). Similar observations were also reported in *M. sexta*, where the ACP activity remained unchanged in fat body cultures in response to 20E (Caglayan, 1990). These results clearly suggest that some additional factors mediate the 20E regulated stimulation of the ACP activity.

In the present study, only co-treatment of the cultured fat bodies with 20E together with larval haemolymph of *C. cephalonica* enhanced the ACP activity. The results confirmed the earlier finding of the presence of some haemolymph factor that mediated the 20E dependent ACP activity because only when the fat body culture was supplemented with

haemolymph, a stimulation of the ACP activity by 20E could be observed (Ashok and Dutta-Gupta, 1991). In the present study, this haemolymph factor is identified and characterized as 19 kDa protein, HP19. Attempts were made to understand the synthesis and developmental regulation of HP19 and ascertain its role during the postembryonic development of insect as well as its effect on selected 20E dependent actions.

The haemolymph factor was identified as a protein by physico-chemical treatments of haemolymph prior to its assessment for the ability to mediate the 20E dependent ACP activity stimulation. Present study revealed that the factor from *C. cephalonica* was heat labile as well as sensitive to acid, alkali, ethanol and V_8 protease treatment. This suggested the proteinaceous nature of haemolymph factor, and henceforth, termed haemolymph protein, HP. Several lines of studies have suggested that the regulatory molecules present in haemolymph are generally peptides or proteins (Stone and Mordue, 1980; Candy, 1981; Keeley *et al.,* 1985).

The novel function of the protein prompted to devise a protocol for purification. Although a few nanograms of the protein was found to be sufficient for stimulation even in crude or partially purified fractions, the yield of the purified protein was very low. Other limitations were the requirement of a large quantity of haemolymph of a specific developmental stage (LLI) and removal of the major contaminating protein, hexamerin that constitutes 75-80% of total haemolymph protein (Telfer and Kunkel, 1991; Haunerland, 1996). Therefore, an antibody against HP19 was raised by electroeluting HP19 and its specificity against HP19 was confirmed. When this antibody was added together with haemolymph to cultures of LLI larval fat body, the haemolymph failed to mediate the 20E dependent action. Similarly, when the haemolymph was first immunoprecipitated using HP19 antibody and the resulting complex and the supernatant were added to the fat body cultures, HP19 action was suppressed.

Western analysis of denatured as well as non-denatured PAGE demonstrated that HP19 is a monomeric protein in *C. cephalonica,* while Southern analysis clearly suggested that HP19 is the product of a single copy gene. Four independent methods, co-culturing, western analysis, immuno-histochemistry and *in situ* immuno-detection, revealed that HP19 is synthesized by the larval HGLFB, from where it is released into the haemolymph. This is further confirmed by the tissue specific gene expression of HP19 only in HGLFB. Western analysis, revealed a difference of approximately 5 kDa in the mass of HP19 in HGLFB (*i.e.,*

24 kDa) and in haemolymph (19 kDa). The predicted mass of the unmodified translated protein from the HP19 cDNA was close to the HP19 synthesized in HGLFB. Northern analysis showed the presence of 0.66 kb HP19-mRNA which further indicates a synthesis of a protein with mass about 24 kDa by HGLFB. The identification of HGLFB tissue as the site for HP19 synthesis is in agreement, with the assumption that fat body is the major tissue in insect larvae that synthesizes the majority of proteins including the humoral factors (Dutkowski and Oberlander, 1974; Gray *et al.,* 1987; Watson *et al.,* 1987; Shiraishi and Natori, 1989; Csikos and Sass, 1997; Farkas and Sutakova, 1998; Kawamura *et al.,* 1999; Smagghe *et al.,* 2003).

The amino acid sequence deduced from HP19 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 (Feng *et al.,* 1999). Therefore, we conclude that HP19 might be cleaved before its release into the haemolymph. 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.

In *C. cephalonica,* biosynthesis of HP19 takes place in the HGLFB during the total last larval instar development. The protein is rapidly released into the haemolymph. The maximal HP19 concentration in the tissue as well as in the haemolymph could be observed in LLI larvae. It is notable that only haemolymph from this developmental stage is capable of stimulating the 20E effect on ACP activity, *i.e.,* the activity of HP19 is developmentally regulated. However, the molecular mechanism of activation of ACP by ecdysteroid hormone is unclear at this point. This might be either due to a post-translational or conformational changes in the protein. The stage specific synthesis of HP19 is further confirmative by the results that the presence of protein was not detected in the HGLFB at prepupal (PP) stage by western blotting, a stage later than the active stage. The detection of HP19 protein band in haemolymph at PP stage is probably due to the transfer of already existing protein present in the fat body of larval stage. However, at present we have no clue about the mechanism of HP19 inactivation at PP developmental stage.

To gain more insight into the nature and function of HP19, we produced and characterized CcHP19-cDNA clones and compared the sequence with those of related proteins, particularly with that of CfGST, which exhibits 67% identity (Feng *et al.,* 1999). The CcHP19 does not contain specific amino acids that are responsible for glutathione

binding or are involved in modulating the specific activity of mammalian mu, pi and sigma GSTs (Reinemer *et al.,* 1991; Ji *et al.,* 1992, 1995; Feng *et al.,* 1999). The HP19 sequence also showed other putative post-translational modification sites like PKC (189-191), casein kinase II (81-90, 124-127, 142-145 and 161-164) and tyrosine kinase (19-25 and 33-41) phosphorylation sites as well as N-myristoylation sites (138-143 and 150-155). However, *in vitro* phosphorylation studies with haemolymph did not reveal any phosphorylation of HP19.

Glutathione S-transferases (GSTs) with hormone regulating actions are unknown, although few studies on 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, the possibility of GST exhibiting HP19 function was analysed. For this purpose, effort was made to replace HP19 with affinity purified GST and tested whether GST can exhibit the same effect as of HP19. The CfGST shows 67% sequence identity with HP19 and the CfGST antibody showed immunocrossreactivity with different tissues of *C. cephalonica* proteins in the mass range of HP19. However, the detailed studies carried with the affinity purified GST did not show any potentiation in 20E stimulated ACP activity like that of HP19 purified from the haemolymph or recombinant HP19. In addition, the GST like activity in the haemolymph was found to be extremely low and negligible in the purified HP19. All these studies together suggest that HP19 has no GST enzymatic activity. During the purification of cytosolic GST of *C. cephalonica* by affinity chromatography, the flow through fractions that did not bind to the affinity matrix mediated the 20E effect on ACP activity, further indicates that HP19 is different from GSTs.

Results obtained with specific HP19 antibody indicated that the antibody completely removed the mediation ability of haemolymph, which is required by 20E to stimulate ACP activity in the fat bodies kept in culture. In the present study experiments were designed to gain insight in the role of HP19 on the growth and development during the postembryonic life 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*. Keeping the above in view, the specific antibodies were raised against HP19 and used in the present study, basically to understand the role of HP19 in insect growth and development. In the presence of antibody the protein would be immuno-complexed, hence it would not be able to mediate the 20E dependent action. The results obtained in the present study suggest that the injected antibodies interfered with the physiological action of the protein and caused the development of either nonviable larval, larval-pupal or pupal-adult intermediates. A detailed analysis on various parameters revealed, significant changes in experimental insects when compared with the controls. The mortality was more or less the same in the experimental and control groups, however, the antibody injected larvae showed reduced salivation, delayed reduction in body length and small head capsule size. Although the duration required for pupation was identical but upon antibody injection most of the larvae developed into abnormal non-viable larvae or larval-pupal intermediates and some of them could not metamorphose into adult and gave rise to non-viable pupal-adult intermediates.

Extensive studies from our group suggest that ACP activity increases gradually during the postembryonic development of *C. cephalonica* and reaches a peak value at pupal stage, where larval organs undergo histolysis (Ashok and Dutta-Gupta 1988). In the present study the control insects showed the same pattern, however, in antibody injected insects, this increase in ACP activity was totally blocked and it remained more or less the same after different time periods. This study further strengthens the view that the ACP has an important role in insect growth and regulates the histolysis of larval organs during larval-pupal-adult transformation. The HP19 plays a specific role in this 20E mediated stimulation, hence the unavailability of HP19 in antibody injected larvae caused defective development.

Present study on the protein profile of fat body and haemolymph revealed that HP19 not only regulates fat body ACP activity but also has a role in regulating the hexamerin sequestration. 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). These hexamerins are later taken back via a receptor mediated endocytotic process by the non-feeding prepupal or pupal fat body cells to meets its energy requirement (Ismail and Dutta-Gupta, 1990b; Haunerland, 1996; Burmester and Scheller, 1999). Present study clearly shows that HP19 does not have any role in the

synthesis of hexamerins but plays important function during its sequestration, hence in the fat body of 14 day post-antibody injected larvae, very little or no hexamerin was sequestered. Immunohistochemical analysis further confirmed that HP19 injected insects had improper sequestration suggesting that HP19 not only has effect on ACP activity but plays important role in 20E regulated hexamerin sequestration. Formation of protein granules in the fat body, followed by the uptake of hexamerin in multivesicular bodies and storage granules has been well reported in the fat body of insect (Locke and Collins, 1965, 1968; Marx, 1983; Levenbook, 1985). Present study also revealed a significant reduction in the cytoplasmic granules in the fat body of antibody injected larvae when compared with controls, suggesting that hexamerins were not sequestered in these insects.

A wealth of data on the molecular mechanism of ecdysteroid action shows that the transcriptional cascade leading to molting and metamorphosis is initiated when 20E binds to its nuclear receptor complex (Henrich *et al.,* 1999; Riddiford, 2001). For about four decades evidence has accumulated that some of the hormonally induced effects are too rapid for the classical model (Tomaschko, 1999; Falkenstein *et al.,* 2000a; Valverde and Parker, 2002, Losel and Wehling, 2003). It also casts doubt on the so-called genomic pathway as the sole mode of steroid action. Today, several modes for nongenomic steroid actions are examined. Most of them are thought to continuously modulate the long-term effects allowing cells or organs to adapt rapidly to environmental changes. Extensive studies with many different species show that insect metamorphosis, in general is under the genetic control of ecdysteroids. Few studies do indicate that some events, necessary for and accompanying metamorphosis, are controlled by 20E at a nongenomic level. However, studies on these mechanisms are restricted to a small number of experimental systems *e.g.,* the activation of lysosomal enzymes and the hexamerin receptor during larval-pupal transformation (Verkuil, 1979; Ueno *et al.,* 1983, Ueno and Natori, 1984; Chung *et al.,* 1995; Burmester and Scheller, 1997a, 1999).

To learn more about the molecular mechanisms of ecdysteroid in regulating HP19 assisted ACP activity, the fat bodies kept in culture were incubated for several time periods with HP19 and 20E. A minimum incubation of 4 h was required for the stimulation of enzyme activity by 20E, time enough for a genomic hormone action. However, the measured stimulation was unaffected by transcriptional or translational inhibitors, indicating the independence of gene activation. Furthermore, the *in vitro* study with fat body homogenate showed a rapid stimulation (within seconds to 1 min) of the enzyme activity. Since the

homogenate preparation is essentially a cell or nucleus disintegrated fraction, this suggests that cell or nuclear integrity is not an essential requirement for the effect of 20E on ACP activity. This possible nongenomic regulation was further strengthened by the results that in presence of 20E, fat body cultures showed a higher incorporation rate of [^{35}S] methionine, which was inhibited by inhibitors of transcription and translation. This, however, had no effect on the ACP activity and presence of HP19 in cultured tissue rendered 20E to stimulate the enzyme activity even in the presence of inhibitors of transcription and translation.

We further confirmed the HP19 assisted 20E dependent ACP activation at RNA level. The *C. cephalonica* ACP (CcACP) cDNA cloned and sequenced in the present study, revealed the presence of transcript in all the major larval tissues and the level of expression matched with the activity profile in these tissues. HP19 mediated the 20E dependent ACP stimulation in the fat body cultures, however, northern analysis of RNA isolated from these cultures, revealed that the stimulation in ACP activity in presence of HP19 was not associated with an increase in transcript level. This further confirmed nongenomic regulation of ACP activity by 20E that is assisted by HP19. However, the exact nature of phosphatase remains to be identified and characterized because the CcACP cDNA clone obtained in the present study showed sequence identity in the range of 30-40% with the type 2 phosphatidic ACP, which is known to have different function than the lysosomal acid phosphatases (Martin *et al.,* 1987; Moolenar *et al.,* 1992; Kai *et al.,* 1996; Zhang *et al.,* 1997).

The histogenesis of adult tissue during metamorphosis requires large amounts of energy and building blocks, which are normally provided by "storage proteins" (Haunerland, 1996). The major fraction consists of hexamerins, which are sequestered by the larval fat body by receptor mediated endocytosis, stored in vacuoles and utilized by the activity of lysosomal enzymes (Telfer and Kunkel, 1991; Haunerland, 1996; Burmester and Scheller, 1999, Nagamanju *et al.,* 2003). The developmentally regulated synthesis and the uptake of hexamerins by the fat body cells of dipteran as well as of lepidopteran insects are widely demonstrated (Telfer and Kunkel, 1991; Haunerland, 1996; Burmester and Scheller, 1999; Nagamanju *et al.,* 2003). Although various studies demonstrated that ecdysteroids regulate hexamerin endocytosis, the mechanisms and levels of control are not well understood.

Our laboratory has earlier demonstrated that 20E stimulates the uptake of hexamerin in fat bodies of *C. cephalonica* and that the precocious *in vivo* sequestration of hexamerin is 20E dependent (Ismail and Dutta-Gupta, 1990b; KiranKumar *et al.,* 1997). Furthermore, the

uptake is mediated by a membrane-bound receptor (KiranKumar *et al.,* 1997). Various mechanisms for 20E mediated receptor activation have been reported in different insects. In the larvae of *S. peregrina*, a 120 kDa receptor protein is cleaved by proteinases into 76 and 53 kDa fragments, which do not bind to hexamerin. It was suggested that during pupation, 20E plays a role in keeping the receptor intact mainly by inhibiting the proteinase and this intact receptor mediates the selective uptake of hexamerin into the fat body (Chung *et al.,* 1995). On the other hand, 20E was shown to mediate the cleavage of an inactive precursor into the active receptor in *C. vicina*, followed by the uptake of hexamerin (Burmester and Scheller, 1997a). All reports published up to date substantiate that ecdysteroids regulate hexamerin sequestration by post-translational mechanisms, which do not involve gene activation. However, the nature of the nongenomic effect is unknown.

The pleiotropic effects of hormones are due to several distinct processes. In eukaryotes, the modulation of transcription and thus the changes at the level of gene regulation is not the only mode of action for steroids. There are also the multiple possibilities of post-transcriptional and post-translational events that are rapid and do not involve any change in gene expression is also rapidly emerging (Valverde and Parker, 2002; Losel and Wehling, 2003). Protein phosphorylation is now recognized as the major regulatory mechanism by which the activity of various proteins is controlled by external physiological stimuli (Browning *et al.,* 1985, Perronnet *et al.,* 1986; Graves and Krebs, 1999, Davies *et al.,* 2000). In insects, Rauch *et al.,* (1998) demonstrated that ecdysteroid receptor (EcR) and ultra spiracle (USP) proteins from *Chironomus tentans* are phosphoproteins, and are differentially regulated by ecdysteroids. Further, 20E treatment does not alter the extent and status of phosphorylation of EcR, although the concentration of EcR increases. In contrast, the phosphorylation of USP is significantly enhanced by 20E but the concentration remains more or less the same

In the present work it was observed that the 120 kDa hexamerin receptor of *C. cephalonica* is phosphorylated and the phosphorylation is mediated partly by a tyrosine kinase which is activated by 20E. Ligand blotting of phosphorylated fat body proteins with bio-labeled hexamerin and subsequent autoradiography of the same blot revealed that the phosphorylated 120 kDa protein is the hexamerin receptor.

In the present study, thorax-ligation for different periods showed a gradual decline in the incorporation of [γ^{32}P] in hexamerin receptor, indicating that the phosphorylation of this

protein might be mediated by 20E. Addition of exogenous 20E either to fat body homogenate or to membrane fraction prepared from 24 h post-ligated LLI larvae, induced the phosphorylation of the 120 kDa receptor. This suggested that this process requires neither transcription nor translation *i.e.,* the receptor phosphorylation is not dependent on *de novo* protein synthesis. Back phosphorylation study further confirmed that 20E induces the 120 kDa protein phosphorylation in intact fat body.

In *Calliphora vicina,* the cleavage of the hexamerin receptor was shown to be essential for the uptake of hexamerin, but was not dependent on new protein synthesis or RNA transcription (Burmester and Scheller, 1997a). In the present study, ligand blot studies with phosphorylated fat body proteins further suggested the probable activation of the hexamerin receptor through a nongenomic action of ecdysteroid hormone. However, there was appreciable binding of hexamerin to its receptor even in absence of 20E. This suggests that the receptor phosphorylation probably affects steps other than ligand binding in hexamerin uptake by the fat body cells.

The western analysis of hexamerins corroborated with the earlier finding that hexamerins are synthesized by the actively feeding larval stages and released into the haemolymph where they accumulate and account for >75% of total haemolymph proteins (Telfer and Kunkel, 1991; Haunerland, 1996). The fat body cells of *C. cephalonica* and *C. partellus* sequester this protein during the non-feeding prepupal and pupal stages (Ismail and Dutta-Gupta, 1990b, 1990c 1991; Dutta-Gupta and Ismail, 1990, 1992; Ismail *et al.,* 1993; KiranKumar *et al.,* 1997, 1998). In addition to fat body, other insect tissue such as male accessory reproductive gland (MARG) was also shown to sequester hexamerin during the non-feeding stages (Ismail *et al.,* 1993). In the present study, the search for hexamerin receptor in MARG resulted in identification of similar mass binding protein (120 kDa) like that of fat body. Furthermore, the 120 kDa protein present in MARG was also phosphorylated. However there was no phosphorylation of 120 kDa protein in non-hexamerin sequestering tissues like gut, Malpighian tubules, epidermis, central nervous system, and haemolymph. This indicates that the 120 kDa binding protein in MARG that undergoes phosphorylation has a possible role in hexamerin sequestration.

The increase in hexamerin uptake by LLI larval fat body in presence of 20E, a stage at which endogenous ecdysteroid titer is low (Dutta-Gupta and Ashok, 1998) indicates the ecdysteroid dependent activation of hexamerin receptor. In the present study, the highest

degree of hexamerin receptor phosphorylation was found at late-last larval stage. The fat body proteins at early-last larval stage showed very little phosphorylation while it was moderate at prepupal stage. It has been already demonstrated in *C. cephalonica* that the 120 kDa hexamerin receptor is synthesized during the final instar of larval development and is present in large quantities in late-last instar larvae and prepupae (KiranKumar *et al.,* 1997). Therefore, the lack of phosphorylation of hexamerin receptor at early-last instar larvae is most likely due to the absence of the 120 kDa protein in the fat body membrane at this stage. Furthermore, at the ELI larval stage, the hexamerin uptake by fat body is very low even in the presence of 20E. The high degree of [γ^{32}P] incorporation in LLI larvae obtained in present study might be directly related to the presence of higher amounts of the 120 kDa protein with a large number of free sites available for *in vitro* labeling, as the endogenous ecdysteroid concentration is fairly low at this developmental stage (Dutta-Gupta and Ashok, 1998). On the other hand, the lower degree of phosphorylation in prepupae suggests that, in presence of a high 20E titer, the sites of phosphorylation are already occupied, hence there are fewer sites accessible to [γ^{32}P] during the *in vitro* experiment. This suggests that 20E induces the activation of the 120 kDa hexamerin receptor through phosphorylation.

The phosphorylation of 120 kDa hexamerin receptor is developmentally regulated and mediated by 20E through a nongenomic action. However, it was not yet clear whether the hexamerin receptor is autophosphorylated because of its intrinsic kinase activity, as the ATP provided in the culture medium phosphorylated the receptor present in fat body. Hence in the present study, work was further extended to investigate the phosphorylation of hexamerin receptor, and the kinase involved to understand its physiological role in hexamerin uptake.

In the present study, the 120 kDa phosphorylated hexamerin receptor of *C. cephalonica* cross-reacted with anti-phosphotyrosine monoclonal antibody and the phosphorylation of the protein was inhibited by genistein, a broad spectrum inhibitor affecting many receptor and non-receptor tyrosine kinases (Akiyama *et al.,* 1987). Genistein also partially blocked the hexamerin uptake by cultured larval fat body. This suggests that the 20E induced phosphorylation of the 120 kDa hexamerin receptor is partly mediated by a tyrosine kinase.

The phosphorylation of hexamerin receptor in membrane fraction of fat body of *C. cephalonica* indicated that tissue integrity is not an essential requirement and that this post-translational phosphorylation is independent of gene activation. The phosphorylation of

hexamerin receptor in the fat bodies kept in culture prior to the incubation of these tissues with [^{35}S] methionine labeled hexamerin suggested that the phosphorylation of receptor is a prerequisite for hexamerin uptake because only when the receptor was phosphorylated there was significant uptake of hexamerins. This uptake was partly inhibited by the broad-spectrum tyrosine kinase inhibitor, genistein, but remained unaffected by the inhibitors of transcription and translation. As the cell impermeable ATP is able to phosphorylate the receptor in intact fat body tissues kept in culture, it clearly suggests that hexamerin receptor is possibly a cell surface receptor, which is autophosphorylated due to its intrinsic tyrosine kinase activity and that the phosphorylation is probably essential for receptor activation and occurs prior to the hexamerin uptake. The uptake of [^{35}S] labeled hexamerins in presence of exogenously added 20E and ATP even in the presence of transcriptional or translational inhibitors further provided the evidence of a post-translational activation mechanism *i.e.,* the nongenomic regulation of hexamerin receptor activation by ecdysteroid for hexamerin uptake in *C. cephalonica.*

Earlier studies by biochemical means for the receptor mediated endocytosis of hexamerins in *C. vicina* revealed the presence of one receptor cleavage product physiologically outside (Burmester and Scheller, 1997a). However, no typical transmembrane domains or endocytosis sorting signal was found in the hexamerin receptor sequences (Burmester and Scheller, 1995). Therefore, the linking of receptor to the cell membrane is not clear. Some recent data based on yeast two-hybrid-system suggest that a transmembrane binding protein may be involved (Hansen *et al.,* 2002, 2003).

The repetition of the experiment of phosphorylation of hexamerin receptor in the fat bodies kept in culture with [γ^{32}P] ATP further strengthens the hypothesis of autophosphorylation of receptor due to its intrinsic tyrosine kinase activity as the receptor was found to be phosphorylated in presence of 20E. Immuno-histochemical localization study showed the presence of phosphotyrosine residue in the fat body cells. Previously several receptor tyrosine kinases (RTKs) have been identified in *Drosophila* that includes the RTK homologous to the mammalian epidermal growth factor and insulin receptor like tyrosine kinase (Yamamoto, 1994; Freeman, 1996; Raabe *et al.,* 1996; Schweitzer and Shilo, 1997, Menon and Zinn, 1998). Katsoris *et al.,* (1991) also demonstrated the presence of phosphotyrosine containing proteins in various tissues of mediterrenian fruit fly *Ceratitis capitata*. Further, study with a series of synthetic tyrosine kinase inhibitors, tyrphostins which selectively blocks receptor autophosphorylation and also discriminates between receptor and

non receptor kinases (Ohmichi *et al.,* 1993) indicated that one of the tyrphostin inhibitor, AG 879 at a concentration of 100 μM significantly inhibited the phosphorylation of *C. cephalonica* hexamerin receptor. The tyrphostins inhibit several receptor tyrosine kinase *e.g.,* epidermal growth factor, platelet derived growth factor *etc.,* the intracellular domains of which encodes for intrinsic tyrosine kinase activity (Lyall *et al.,* 1989; Posner *et al.,* 1989; Levitzky, 1992; Osherov, 1993; Doron *et al.,* 1998); The AG 879 tyrphostin was originally shown to selectively block nerve growth factor (NGF) dependent $pp140^{c\text{-}trk}$, a receptor tyrosine kinase autophosphorylation (Ohmichi *et al.,* 1993) indicating that possibly *C. cephalonica* hexamerin receptor is autophosphorylated due to intrinsic tyrosine kinase activity. However, the nature and similarities of hexamerin receptor with NGF- $pp140^{c\text{-}trk}$ is yet to be elucidated.

The endogenous fat body tyrosine kinase activity was checked using synthetic peptide as a substrate. There is a developmental regulation of tyrosine kinase, with LLI larval stage displaying highest activity. There was a gradual decline in the activity upon thorax-ligation and application of exogenous 20E both *in vivo* and in fat bodies kept in culture, stimulated the activity.

As demonstrated, the hexamerin receptor of *C. cephalonica,* a 120 kDa protein is activated through phosphorylation by a tyrosine kinase. The phosphorylation occurred in the intact fat body as well as in homogenate and membrane fraction. Therefore it was tempting to extend the study to check the effect of HP19 on the 20E stimulated phosphorylation of the hexamerin receptor. In the present study, it was demonstrated that HP19 inhibits the 20E induced phosphorylation of hexamerin receptor. The study also showed that HP19 inhibits the autophosphorylation of 60 kDa protein, which was previously identified as CaM kinase II in the CNS of *B. mori* (Shanavas *et al.,* 1998) and the fat body of *C. cephalonica* (Vasanthi, 1999). The CaM kinase II activity as well as its autophosphorylation is proposed to be involved in variety of Ca^{2+} mediated processes in non-neural tissue like fat body and muscles (Hanson and Schulman, 1992a).

The inhibition in phosphorylation of *C. cephalonica* hexamerin receptor by HP19 was rapid in homogenate preparation. This once again suggests that the tissue integrity is not an essential requirement for 20E regulated phosphorylation and it is a nongenomic phenomenon. Although a minimum incubation of 6 h was required for significant inhibition in the 20E stimulated activity by HP19 in fat bodies kept in culture but the inhibition was fairly rapid

(within 30 sec) in the fat body homogenate preparations, further strengthens our assumption of nongenomic regulation.

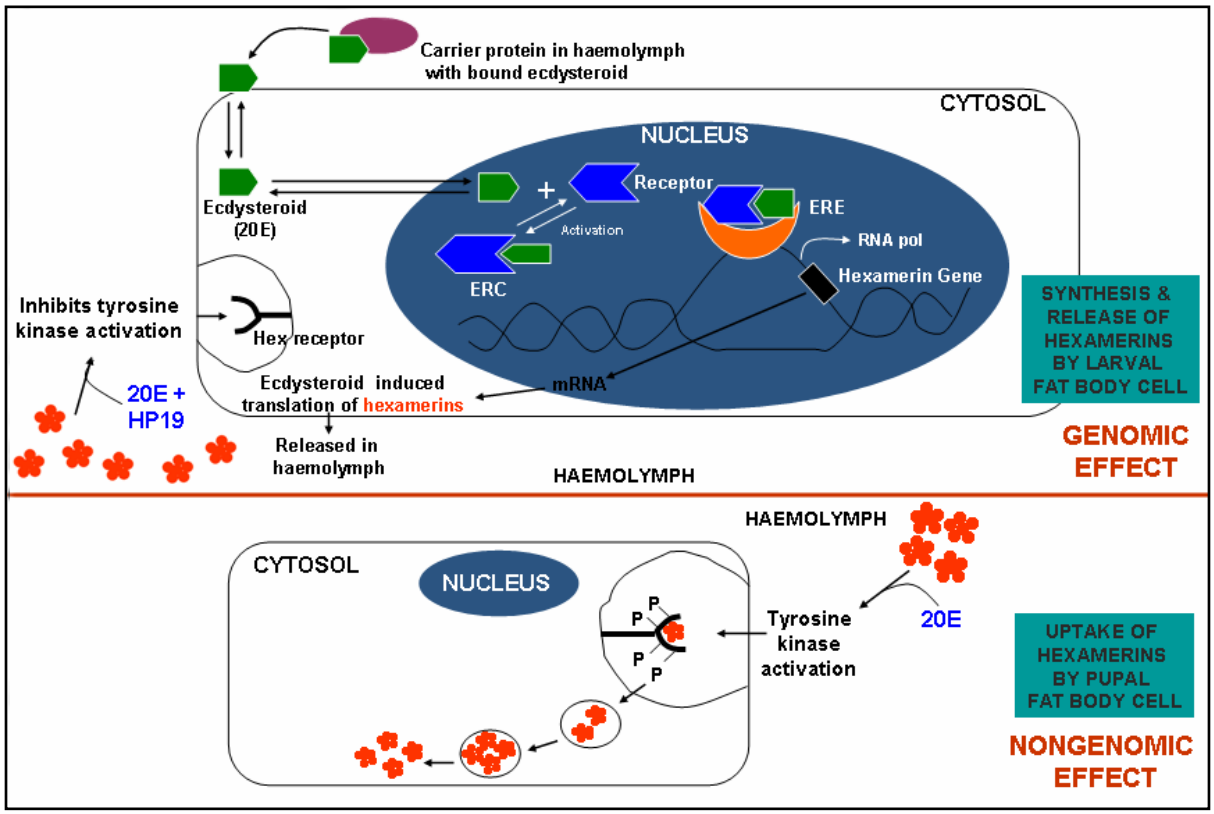
The physiological significance of this inhibition lies in the experimental evidence, that uptake of hexamerin is a stage specific event (Haunerland, 1996; Burmester and Scheller, 1999). Previous studies have shown that the receptor is present during the last larval instar in an inactive form and is not capable of sequestering hexamerins *i.e.,* binding its ligand (KiranKumar *et al.,* 1997). Furthermore, it is well documented that the ecdysteroid titer is very low during the last larval instar but increases rapidly (10 fold or more) before pupation (Dutta-Gupta and Ashok, 1998). In the present study, it could be demonstrated that the hexamerin receptor becomes phosphorylated at late larval and prepupal stages and is able to bind hexamerins. Moreover, the present study also shows that 20E is responsible for the phosphorylation (receptor activation) by enhancing the activity of a tyrosine kinase. From these studies, it is evident that phosphorylation of the receptor is inhibited by HP19 in the larval stage, when hexamerin is not sequestered by the fat body. This suggests the existence of an inbuilt regulatory mechanism to prevent the ecdysteroid dependent hexamerin uptake by the larval fat body cells, a stage when the fat body tissue is actually synthesizing both the hexamerin and its receptor. Later, at the prepupal and pupal stage when hexamerin uptake occurs, HP19 gets inactivated which is evident by its inability to mediate the 20E regulated ACP activity at this stage.

In the present study, like ACP, the dual requirement of both HP19 and 20E remains elusive in the regulation of 20E dependent phosphorylation of hexamerin receptor as well as the fat body tyrosine kinase activity as HP19 alone could also inhibit the tyrosine kinase activity and receptor phosphorylation. Co-relating this study with the reported endogenous titer of hormones in different insect species including *C. cephalonica* (Calvez, 1976; Plantevin, 1987; Dutta-Gupta and Ashok, 1998), a possible explanation can be given for HP19 action on receptor phosphorylation even when there is no exogenous 20E added during the *in vitro* studies. At larval stage, HP19 is functionally active to mediate the 20E regulated actions, however; the endogenous ecdysteroid titer is low, which is further reduced upon thorax ligation. Thorax-ligation brings down the hormone titer significantly but a basal level of hormone at a given stage of any insect is always maintained. Hence, it is assumed that the inhibition of receptor phosphorylation by HP19 without the exogenous 20E is probably due to the availability of this basal level of ecdysteroid. This assumption is further strengthened by the experimental proof that HP19 is inactive at prepupal stage and hence there is no

inhibition of tyrosine kinase or the hexamerin receptor phosphorylation, though the endogenous titer of ecdysteroid is very high during this stage. The selective inhibition of the receptor phosphorylation in the fat bodies kept in culture as well as in the homogenate preparations, suggests that HP19 most likely has a kinase inhibitory or phosphatase activating effect on the phosphorylation of the hexamerin receptor and possibly acts by inhibiting the fat body tyrosine kinase activity.

On the basis of above results, it is speculated that 20E and HP19 in combination regulate the hexamerin uptake, represented as a hypothetical model (Model 1 & 2). The hexamerins which are synthesized under the influence of ecdysteroid (Wang *et al.,* 1995) by the fat body of actively feeding larval stages and released into haemolymph are sequestered by the non-feeding prepupal (Ismail and Dutta-Gupta, 1990b, c) and pupal fat body (Haunerland, 1996; Burmester and Scheller, 1999). In *C. cephalonica* larval stage, where the endogenous titer of ecdysteroid is low (Dutta-Gupta and Ashok, 1998), HP19 is active and it inhibits the fat body tyrosine kinase activity. Hence, the phosphorylation of hexamerin receptor is blocked. Whereas at non-feeding stage, the ecdysteroid level is high and the HP19 gets inactivated and there is ecdysteroid mediated phosphorylation of the receptor, which in turn is responsible for hexamerin uptake. The present study also suggests that hexamerin receptor most probably has intrinsic kinase activity due to which it undergoes autophosphorylation in hormone dependent manner. However, extensive studies have to be carried to find out the exact nature of tyrosine kinase involved in autophosphorylation of hexamerin receptor in *C. cephalonica*.

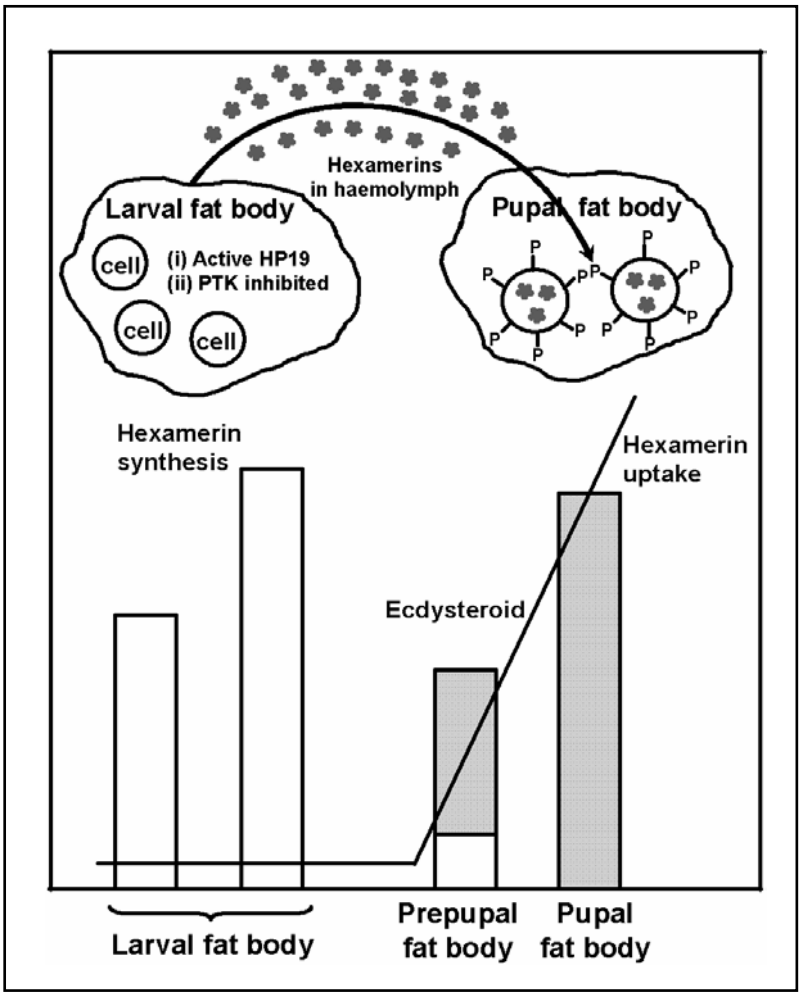
In the present study, the results indicated that in addition of regulation of ACP activity and phosphorylation of hexamerin receptor, 20E also induces the autophosphorylation and activity of CaM kinase II. Earlier studies from our laboratory revealed that the CaM kinase II activity and its autophosphorylation in the CNS and fat body of *B. mori* is developmentally regulated (Shanavas *et al.,* 1998; Vasanthi, 1999). In the present study the results indicated that the fat body CaM kinase II in *C. cephalonica* is developmentally regulated with highest activity at LLI larval stage. Injection of 20E to ELI and LLI thorax-ligated larvae stimulated the CaM kinase II activity which is inhibited by HP19. Similarly, 20E induced autophosphorylation of CaM kinase II is also inhibited by HP19.



Model. 2: Proposed model representing the stage specific hexamerin synthesis and uptake by the fat body cells of *C. cephalonica*: Demonstration of the modulation of genomic and nongenomic actions of ecdysteroids by a cross-talk:-

Upper panel shows the regulation of hexamerin synthesis at genomic level by ecdysteroids. The ecdysteroid binds to specific receptor and modulates transcription and translation of hexamerin gene (Wang *et al.,* 1995). These hexamerins are synthesized in bulk by the actively feeding larval fat body cells, and released into the haemolymph, where they accumulate and account for >75% of total haemolymph proteins (Telfer and Kunkel, 1991; Haunerland, 1996; Burmester and Scheller, 1999). Despite of the presence of hexamerin receptor (KiranKumar *et al.*,, 1997), there is no hexamerin sequestration at this stage, because HP19 is active. The active HP19 inhibits the tyrosine kinase, therefore no phosphorylation of receptor, resulting in lack of the ability of receptor to sequester hexamerins.

Lower panel shows the regulation of hexamerin uptake at nongenomic level by ecdysteroids. At the non-feeding pupal stage the fat body cells sequester the hexamerins from haemolymph again under the influence of ecdysteroids. At this stage, the HP19 is inactive, hence there is no inhibition of tyrosine kinase, thus hexamerin receptor phosphorylation occurs under the influence of high endogenous titer of ecdysteroids, resulting in hexamerin sequestration.



Model. 2: Hypothetical model representing the stage specific hexamerin synthesis and uptake by the fat body cells of *C. cephalonica*:-

White open bar indicates hexamerin synthesis by larval fat body cells. The shaded bar indicates hexamerin uptake by prepupal and pupal fat body cells. A simplified curve shows the ecdysteroid titer. At larval stage, the ecdysteroid is in low level (Dutta-Gupta and Ashok, 1998) and haemolymph protein, HP19 is active, resulting in inhibition of protein tyrosine kinases required for mediation of hexamerin receptor phosphorylation. At pupal stage, the ecdysteroid level is high and HP19 is inactive, hence there is no inhibition of tyrosine kinase activity and so on the phosphorylation of hexamerin receptor, thus hexamerin uptake occurs.

In order to understand the effect of HP19 on few more 20E regulated actions and to ascertain, if HP19 has any effect on them, the study was extended on phosphorylation of a 48 kDa protein and fat body protein kinase C (PKC) activity. These were selected as they were found to undergo or stimulate phosphorylation of proteins under different phosphorylating condition in the fat body of *C. cephalonica.* PKC has been earlier reported to be involved in the regulation of hormone titer especially the JH (Sevala and Davey, 1989; Gilbert *et al.,*

1996). It is also suggested to be involved in the regulation of JH mediated action in male accessory glands of *Drosophila* (Yamamoto *et al.,* 1988)

Earlier studies from our laboratory indicated phosphorylation of a 48 kDa protein in the CNS of *B. mori,* which was induced by JHI (Shanavas, 1997). Present study also revealed the phosphorylation of a 48 kDa protein which is mediated by a tyrosine kinase as the monoclonal antibody against phosphotyrosine identified the phosphorylated 48 kDa protein. The present study indicated the presence of tyrosine kinase activity in the fat body, which is developmentally regulated. In addition to its involvement in the phosphorylation of 120 kDa hexamerin receptor for uptake of hexamerin in 20E dependent manner, the tyrosine kinase is also found to be involved in the phosphorylation of 48 kDa protein in the fat body of *C. cephalonica*. However, the degree of this phosphorylation was not influenced by the presence of 20E.

In the present study on PKC, the results indicated that although thorax-ligation shows reduction in activity but it was highest during ELI stage and gradually decreased at the LLI and pre-pupal stage. This indicate that PKC activity is possibly JH dependent and not on 20E. This was confirmed with the *in vivo* injection of 20E to the thorax-ligated larvae that showed no stimulation in the fat body PKC activity.

The studies *i.e.,* the effect of HP19 on 20E mediated action such as ACP activity stimulation, phosphorylation of hexamerin receptor, tyrosine kinase activity and CaM kinase II activity as well as autophosphorylation in the fat body of *C. cephalonica* unequivocally indicate, multiple roles of HP19 on these 20E dependent actions. Its involvement in mediation of 20E stimulated ACP activation as well as selective inhibition of the 20E induced phosphorylation of hexamerin receptor and 20E dependent CaM kinase II activity as well as its autophosphorylation, suggest that the protein to be either a phosphatase activator or a kinase inhibitor. These studies further suggest that HP19, is probably a regulatory protein in the signalling events for few of the cellular actions in response to hormone, 20E.

Small molecular mass proteins with kinase inhibitory activities have been well documented in mammalian system (Fraser and Walsh, 1991; Eyster *et al.,* 1993). In the present study an attempt was made to detect the similarities of HP19 with few of the highly conserved well characterized regulatory molecule involved in signal transduction pathways. However, the results of these studies did not reveal any immunological identity with other highly conserved proteins such as 14-3-3 (Shaw, 2000; Fu *et al.,* 2002), stathmin (Sobel,

1991; Ozon, 1997) and ubiquitin (Jentsch and Pyrowolakis, 2000; Weissman, 2000). Despite of 67% sequence identity of the deduced amino acids of HP19 cDNA (CcHP19) with that of *C. fumiferana* GST (Feng *et al.,* 1999), there were no GST like enzymatic activity in HP19 and the affinity purified GST had no effect on ACP activation. Hence the study clearly suggests that though HP19 belongs to the glutathione S-transferase gene family but is different from GST. Furthermore, these studies do not rule out the possibility of HP19 being a regulatory molecule.

The characteristic feature of any regulatory molecule is (i) its presence in very low amounts and (ii) effective at very low concentration (Gilbert, 1994). The *C. cephalonica* HP19 satisfies these two criteria effectively. As demonstrated, HP19 is present in extremely low concentration in haemolymph and is effective in mediating the 20E dependent actions in nanogram range, even in the crude preparation. Present study revealed that addition of low concentration of HP19 (40 ng) along with the physiological dosage of 20E *i.e.,* 80 nM (Dutta-Gupta and Ashok, 1998), was sufficient to cause a ~70% stimulation in ACP activity. This combination of hormone and HP19 also caused a significant decline in the CaM kinase II activity (~150%) as well as its autophosphorylation, tyrosine kinase activity (~130%) and the hexamerin receptor phosphorylation. Other results also indicate about the hypothesis of HP19 being a regulatory protein. Although HP19 required a time incubation of 4 h to mediate the 20E dependent ACP activation in the fat bodies kept in culture. However, the factor lost its mediation effect gradually from 6-8 h. In the fat body homogenate, HP19 was effective within 30 sec to 1 min, but like the fat bodies kept in culture, HP19 lost its ability to sustain the effect at longer time incubation. Furthermore, HP19 that inhibited the 20E induced phosphorylation of hexamerin receptor within 30 sec to 5 min in the fat body homogenate, failed to show the effect with longer incubation periods. Similar results were also obtained with the fat body tyrosine kinase activity, further indicating about the short half-life of HP19. The results also revealed that the addition of haemolymph collected from the anterior part of 24 h post-ligated LLI larvae, could not mediate the 20E dependent ACP activation in cultured fat body (Fig. 1d). This further substantiates that HP19 has a short half-life. However, the fractionated or purified HP19 had relatively long shelf life upon appropriate storage. These studies suggest that HP19 is a regulatory molecule, which works in combination with 20E and brings about the unidentified conformational changes to effect its action on hormone dependent cellular responses. There is also a possibility of some additional regulatory molecule along with HP19 being involved in these signal transduction pathways that play role in controlling the rapid or nongenomic effect of ecdysteroids. Unique and previously

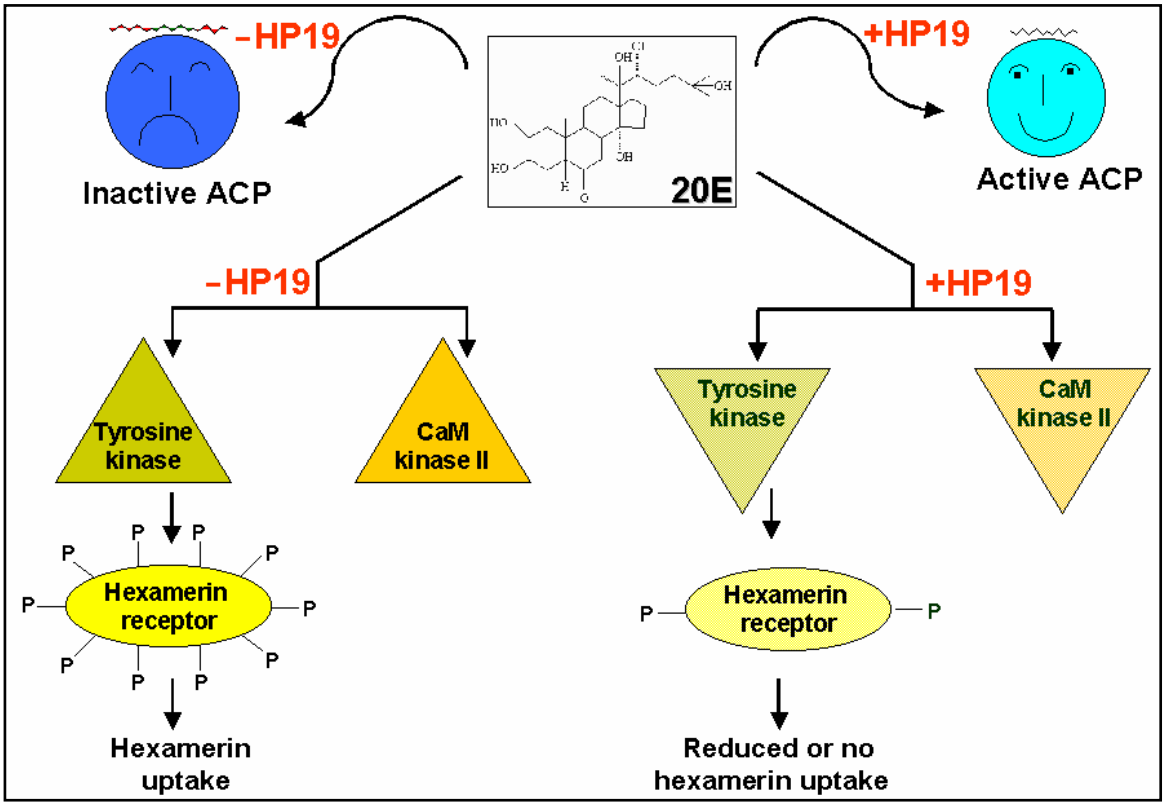
undescribed proteins or chimeras of typical steroid receptor domain with other unique or known protein domain have been suggested to be responsible for membrane initiated steroid actions (Watson and Gametchu, 2003).

The presence of regulatory molecule in haemolymph, the insect blood that is practically exposed to all other tissues, suggests the possibility of similar 20E action on other larval tissues. This however remains to be tested. However, the present study does indicate the presence of HP19 like protein in the haemolymph of few more lepidopteran insect. Addition of haemolymph from other lepidopteran species namely, *B. mori, G. mellonella, S. litura, A. janata* and *P. demoleus,* to the fat bodies of *C. cephalonica* caused increase in 20E dependent ACP activity. Further, in these lepidopteran species, western analysis with HP19 antibody of *C. cephalonica* not only revealed cross-reactivity but also suggested that unlike *C. cephalonica* HP19, the protein is multimeric in nature. However, the presence of such a protein could not be detected in *C. vicina*, a dipteran insect species.

Detailed studies with haemolymph from *B. mori* revealed that only the LLI larval haemolymph could mediate the 20E dependent ACP activity. Attempt of purification of this protein in *B. mori,* revealed the mass to be of approximately 26 kDa. Hence, the present study clearly suggests that like *C. cephalonica*, a similar regulatory mechanism may be operative in lepidoptera and the protein is not species specific at least for the lepidopteran insects investigated so far. Furthermore, the HP19 like protein is stage specifically regulated for its synthesis as well as for the mediation of 20E regulated actions.

Conclusively, the present study suggests a multiple role of a tissue and stage specifically regulated protein, HP19 (Model 3). The protein mediates the ecdysteroid dependent actions. When the protein is absent, 20E is unable to stimulate the ACP activity. Only when HP19 is available to mediate the process, there is an induction in enzyme activity. Whereas in case of kinases *viz.,* tyrosine kinase and CaM kinase II, the absence of HP19, allows 20E to induce the activity and autophosphorylation of CaM kinase II and the activity of tyrosine kinases. However, in the presence of HP19, the 20E shows a reverse effect, and there is inhibition in the activity of both the kinases as well as the phosphorylation associated with it. The HP19 not only renders the 20E to inhibit the tyrosine kinases activity but also inhibits the phosphorylation of hexamerin receptor that is mediated by tyrosine kinase, (probably a receptor tyrosine kinase) due to which there is low or no hexamerin uptake. In absence of HP19, 20E induces the tyrosine kinase which in turn phosphorylates the receptor

and thereby induces the uptake of hexamerins. Though, from the present study, we have enough evidence for the nongenomic regulation of these ecdysteroid dependent actions but at the moment the molecular mechanism for the regulation of these nongenomically regulated events are not clear. However, the study does suggest convincingly about the role of HP19 protein in the regulation of ecdysteroid dependent signalling.



Model. 3: Hypothetical model representing the multiple role of HP19 in mediating the 20E dependent actions:-

In the absence of HP19 20E is unable to stimulate the ACP activity and only when HP19 is available there is an induction in the ACP activity. For the kinases *i.e.,* tyrosine kinase and CaM kinase II, in absence of HP19, there is a stimulation in the activity and the phosphorylation dependent on these kinases. Whereas, in the presence of HP19, the 20E has a reverse effect, and there is inhibition in the activity as well as the phosphorylation associated with it. The model also represents that in absence of HP19, the tyrosine kinases are induced which in turn phosphorylates the hexamerin receptor, hence there is uptake of hexamerins. On the other hand, in presence of HP19, the tyrosine kinase activity is inhibited resulting in low or no phosphorylation of receptor and thus there is reduced or no hexamerin uptake.

Summary

- Injection of 20-hydroxyecdysone (20E) into 24 h post-ligated late-last instar (LLI) larvae of *C. cephalonica* stimulated the ACP activity. While, in the fat bodies kept in culture, 20E alone did not have any stimulatory effect on ACP activity and only in the presence of haemolymph from LLI larvae, the hormone could stimulate the ACP activity. This suggests that some factor(s) from haemolymph is required by 20E for ACP activity stimulation.

- Physico-chemical treatments of haemolymph rendered it inefficient in mediating the stimulatory effect of 20E on ACP activity, suggesting the proteinaceous nature of haemolymph factor. The purification of protein by salt precipitation, fractionation with specific cut-off membrane filters and gel-filtration chromatography revealed a contaminant free pure polypeptide of 19 kDa mass, henceforth the active protein was named as HP19.

- The specificity of HP19 antibody (IgG fraction) generated using electroeluted HP19 was confirmed by functional assays as well as by western analysis. HP19 is found to be a monomeric protein. Southern analysis also revealed it as a product of single copy gene.

- HP19 is synthesized tissue specifically by hind gut associated lobular fat body (HGLFB) and are released into the haemolymph. The protein is developmentally regulated with maximum concentration at LLI larval stage in HGLFB as well as haemolymph and only the protein from this stage was capable of mediating the 20E dependent ACP activation. Detailed studies with other insect further suggested that HP19 protein is stage specific but not species specific at least for lepidopteran insects.

- Cloning and sequencing of HP19 cDNA by immunoscreening of a HGLFB-cDNA expression library showed that HP19 belongs to the family of GST like proteins. The HP19 cDNA was 634 nucleotide long with an open reading frame of 585 bp, which encodes a protein of 195 amino acids. The theoretical mass of the translated unmodified protein was 22.95 kDa, which was close to the mass of HP19 detected in HGLFB. Northern analysis revealed the tissue specific expression of 0.66 kb HP19 transcript only in HGLFB. The transcript size matched with the calculated mass of HP19 detected in HGLFB.

- Despite of high sequence identity (67%) of the deduced amino acid of *C. cephalonica* HP19 (CcHP19) cDNA with *Choristoneura fumiferana* GST (CfGST) cDNA, the affinity purified cytosolic GST from *C. cephalonica* had no enhancing effect on the 20E dependent ACP activity when compared with purified or recombinant HP19. Further, the haemolymph as well as the purified HP19 had negligible GST enzymatic activity. These studies together

suggest that HP19 is not a GST enzyme and that GST function is not required for ecdysone mediated stimulation of ACP activity.

- Injection of HP19 antibody to LLI larvae suppressed the physiological action of the protein probably by interfering with the HP19 molecule and caused the development of nonviable larvae or larval-pupal or pupal-adult intermediates. The antibody injection blocked hexamerin sequestration, suggesting that HP19 not only regulates the ACP activity but also has a role in hexamerin sequestration.

- *In vitro* phosphorylation of *C. cephalonica* fat body proteins revealed that 20E induced the phosphorylation of 120 and 60 kDa proteins. The 48 kDa and a PKC dependent protein phosphorylation were found to be independent of 20E.

- The autophosphorylation as well as activity of 60 kDa protein that has been identified previously as CaM kinase II is stimulated by 20E. Further, the CaM kinase II activity is developmentally regulated with highest activity at LLI stage. The HP19 inhibited the 20E stimulated autophosphorylation as well as activity of CaM kinase II and the process is nongenomically regulated as it occurs in fat bodies kept in culture as well as in homogenate.

- The 20E induced phosphorylation of 120 kDa protein that is identified as hexamerin receptor occurs in intact fat body, homogenate as well as in the membrane fraction suggesting that tissue integrity is not an essential requirement for phosphorylation. The identification of identical mass hexamerin binding protein in another hexamerin sequestering tissue, MARG also undergo phosphorylation, suggests the physiological significance of receptor phosphorylation in hexamerin uptake. The phosphotyrosine antibody identified the phosphorylated hexamerin receptor and genistein partly inhibited the phosphorylation, indicating that the process is mediated partly by a tyrosine kinase.

- The fat body tyrosine kinase of *C. cephalonica* is developmentally and hormonally regulated. The LLI larval fat body showed maximum enzyme activity. 20E induced the activity in fat bodies kept in culture as well as in homogenate preparation.

- Present study suggest that 20E induces the hexamerin uptake in fat body, possibly by the post-translational modification of receptor *i.e.,* phosphorylation. Incubation of fat bodies kept in culture with [γ^{32}P] ATP revealed phosphorylation of 120 kDa hexamerin receptor in presence of 20E suggesting that the receptor phosphorylation is a cell surface event due to which increase in hexamerin uptake occurs by the LLI larval fat body culture when the

medium is supplemented with 20E and ATP. This also suggest that phosphorylation of hexamerin receptor under the influence of 20E is a pre-requisite for hexamerin uptake. Inhibition of hexamerin receptor phosphorylation by tyrphostin (AG 879), a receptor tyrosine kinase inhibitor, suggests it as an autophosphorylation due to the intrinsic tyrosine kinase activity of the receptor.

- The HP19 protein inhibits the 20E induced phosphorylation of receptor as well as the 20E stimulated activity of tyrosine kinase in intact fat body as well as in the homogenate preparation indicating the nongenomic regulation by 20E that is assisted by HP19.

- Although minimum incubation of 4 h was required to mediate the 20E dependent ACP activation, however, this stimulation remained unaffected by the inhibitors of transcription and translation. Further, at homogenate level, HP19 mediated the stimulation rapidly within 30 sec to 1 min. Studies of [^{35}S] methionine incorporation in proteins of fat bodies kept in culture showed that 20E stimulates the total protein synthesis that was inhibited by actinomycin D and cycloheximide, however, ACP activity in these tissues were stimulated by 20E in presence of HP19 and is not blocked by these inhibitors. Using *C. cephalonica* ACP (CcACP) cDNA clone, it was also shown that there is no change in the expression of ACP transcript when 20E induced the activity in presence of HP19 in the fat bodies kept in culture. These studies together suggest the nongenomic regulation of ACP activity by 20E that is mediated by HP19.

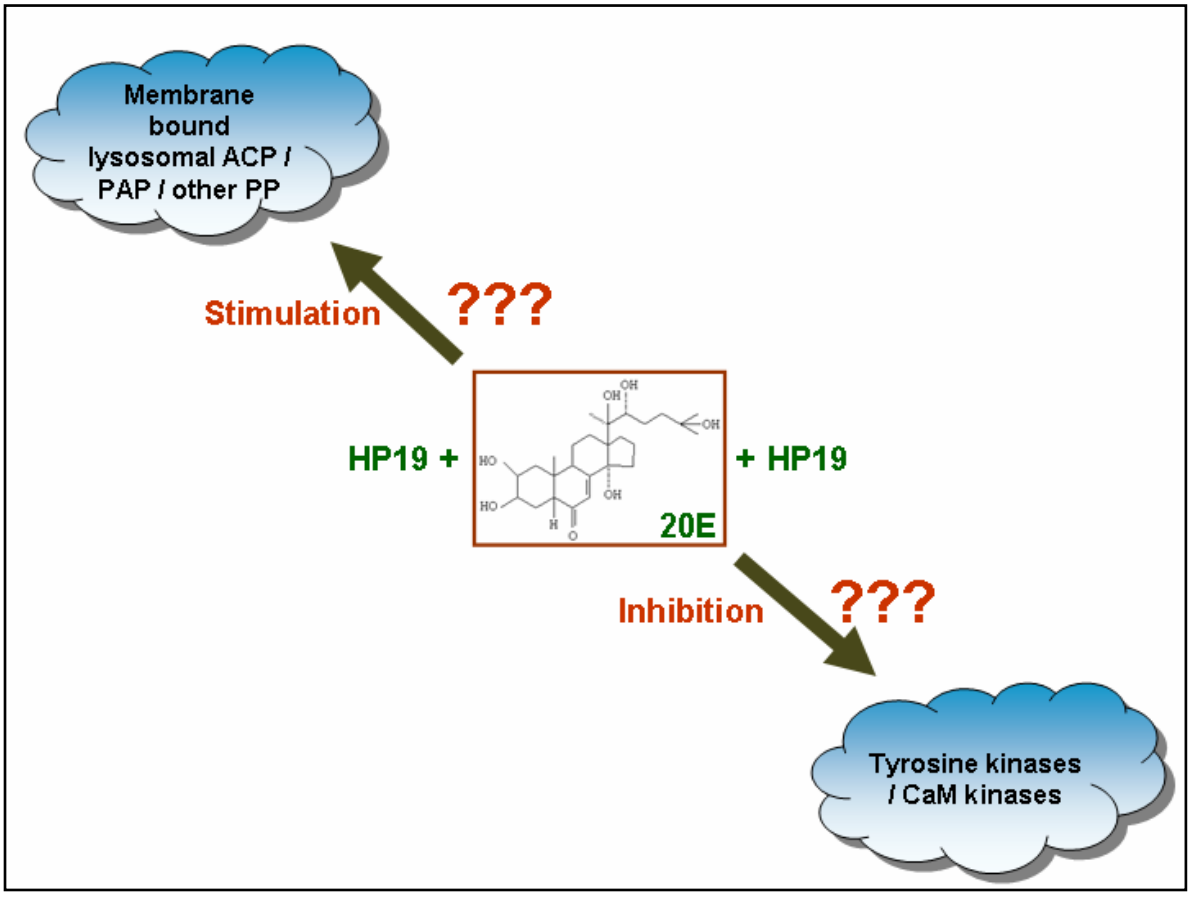
- Cloning and sequencing of CcACP cDNA, showed sequence identity with *D. melanogaster* wunen and tunen gene, which is a type-2 phosphatidic acid phosphatase. Southern analysis of CcACP revealed ACP to be a product of multiple copy gene. Northern analysis showed developmental regulation of transcript expression in all the major larval tissues and the level of expression matched with the ACP activity profile in these tissues.

- The study also indicates the short half-life of HP19 because once it is in combination with hormone and the target tissue *i.e.,* fat body, the effect on 20E regulated actions such as- ACP activity stimulation or inhibitory effect on kinases and receptor phosphorylation is seen only for a limited time, after which there is recovery in the event. However, the shelf-life of HP19 is relatively long. Furthermore, HP19 is effective in extremely low concentration to mediate all the above mentioned 20E dependent actions. However, HP19 is not autophosphorylated to regulate these actions.

- Present study clearly suggests that HP19 in combination with 20E regulates the hexamerin uptake (see Model 1 and 2). It further indicates the nongenomic regulation of few of the selected 20E dependent actions that is mediated by HP19 (see Model 3). However, these data give no convincing clue that regulatory role of HP19 on ecdysteroid action is either due to the kinase inhibitory or phosphatase activating effect (see Model 4).

Unanswered Questions and Conclusion

Unanswered questions-



Model. 4: Hypothetical model representing the missing link of HP19 mediated ecdysteroid signalling in insect:-

The HP19 protein mediates the 20E stimulation of ACP activity and in combination with hormone inhibits the kinases activity and the associated phosphorylation through a nongenomic mode of action. However, the exact pathway or mechanisms for the regulation of these actions are unclear. Further, it is not clear if HP19 mediates the action of 20E on lysosomal acid phosphatase (ACP) or the membrane bound phosphatidic acid phosphatase (PAP) or any other protein phosphatases (PPs).

The present study addresses a very important aspect in the hormone regulated actions by a heamolymph protein, HP19 during the postembryonic life of insects. However, this study fails to answer many questions and opens up a new avenue for further study to understand several aspects such as- (i) Is there an interaction of 20E with HP19? (ii) Does the hexamerin receptor possess intrinsic tyrosine kinase activity that autophosphorylates the receptor for hexamerin uptake? (iii) Is HP19 a kinase inhibitor or a phosphatase activator? and (iv) What are the mechanisms or pathways by which the HP19 mediates the nongenomic effect of ecdysteroids? In addition to it, though the study suggests a nongenomic regulation of

ACP, but gives no clue regarding the nature and type of phosphatase(s), which is / are regulated by the interplay of ecdysteroid and HP19. Hence, further work needs to be done to broaden the understanding of nongenomic regulation by ecdysteroids and the regulatory role of HP19 on the 20E stimulated ACP activity and herewith on the phosphorylation of hexamerin receptor in insects.

Conclusion-

In short, the purification and characterization of haemolymph protein, HP19 of rice moth *Corcyra cephalonica* is related in sequence to glutathione S-transferase and is required for the rapid modulation of ecdysteroids on ACP activity, tyrosine kinase activity, CaM kinase II activity as well as autophosphorylation and hexamerin receptor phosphorylation. The characterization of HP19 suggests that, it may be a previously unidentified component of one of the proposed nongenomic mechanisms or perhaps in some new pathways of nongenomic action. These findings are thus of broad interest to understand the mechanism of steroid hormone action.

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CHARACTERIZATION OF A HAEMOLYMPH PROTEIN HP19 AND ITS POSSIBLE ROLE IN NONGENOMIC ACTIONS OF 20-HYDROXYECDYSONE DURING THE POSTEMBRYONIC DEVELOPMENT OF RICE MOTH, *CORCYRA CEPHALONICA*

Synopsis of the thesis submitted for the degree of

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Layout of the thesis-

The thesis deals with the appearance of a stage and tissue specifically regulated haemolymph protein HP19, identified and characterized during this study. The thesis is written with a general introduction and methods used followed by results. The results section is divided into five chapters, indicating the stepwise approach adopted to carry out the study. Finally a comprehensive discussion and few hypothetical models on the basis of the findings of the study are discussed.

The results section which is divided into five chapters is as follows:

Chapter I- Identification, isolation and characterization of HP19 in *Corcyra cephalonica*

Chapter II- Cloning, sequencing and molecular characterization of HP19

Chapter III- Role of HP19 during the postembryonic development

Chapter IV- Effect of HP19 on 20-hydroxyecdysone dependent actions

Chapter V- Mechanism of 20-hydroxyecdysone regulated actions that is mediated by HP19

Introduction-

Postembryonic development in insects involves growth, molting and metamorphosis. These events are controlled by the hormones that are mainly secreted by the brain, corpora cardiaca, corpora allata and prothoracic glands. Further, the roles of these hormones have been widely studied during the postembryonic development especially in the holometabolous insect resulting in vast literature. However, knowledge regarding the regulation of various hormone mediated actions during the postembryonic development of insects is limited and the field remains largely unexplored.

According to the basic model of the endocrine control, the neurosecretory cells in brain in response to appropriate stimuli synthesize and secrete various neuropeptides such as allatotropic and prothoracicotropic hormone, which in turn stimulate the corpora allata and prothoracic glands to synthesize and secrete the morphogenetic hormones namely juvenile

hormones (JHs- sesquiterpenes) and ecdysteroids (a group of steroid hormones) respectively. The relative titre and interplay of these hormones orchestrates the progression of one developmental stage to the next. The ecdysteroids regulate the onset and the timing of the molt, whereas JHs regulate the quality of the molt (Gilbert *et al.,* 1996).

The haemolymph ecdysteroid titre at any given stage is reflection of several metabolic processes. The alteration in haemolymph ecdysteroid titre is responsible for eliciting the change in commitment. Hence, the ecdysteroids are necessary for metamorphosis as well as for the regulation of critical sequence of behavioral, physiological and biochemical changes which takes place in insects. Extensive studies have been carried out on the regulation of ecdysteroid titre at the synthesis level. However, studies on other regulatory mechanisms are very limited and one of the major thrust area open for research. In lepidopterans like in most holometabolous insects, the ecdysteroid that can elicit molting and metamorphosis is 20-hydroxyecdysone (20E), the active form of prohormone ecdysone (Karlson, 1996).

Steroid hormones mostly propose to act through its interaction with specific intracellular receptor. These receptors are DNA-binding proteins of the nuclear receptor superfamily. The steroid-receptor complex binds to hormone-responsive elements on the chromatin and regulates gene transcription and translation. This mode of cellular action is generally referred to as a genomic action (Beato and Klug, 2000). Ashburner *et al.,* (1974) proposed similar genomic mode of action for 20E. Extensive studies based on this model, over the past few decades have provided insight into the molecular mechanism of 20E action. From these studies has emerged, a clearer understanding of the mechanism by which a systemic hormonal signal is refined into stage and tissue specific developmental responses.

Apart from the traditional genomic mode of action of steroid hormones there are several reports that suggest rapid nongenomic effect of steroids and which acts independently of gene transcription or translation. Unlike genomic effect the nongenomic effect is rapid and the response is apparent within few seconds to 10 minutes. Further, this response is unaffected by the transcriptional or translational inhibitors (Losel and Wehling, 2003).

A major area with regard to understanding the regulation of 20E action is that of tissue specificity. The hormone has different effects on the same tissue at different developmental stages. Thus, studies focused on molecular basis of differentiation during development and built-in regulatory mechanisms at the tissue level largely aid in understanding the regulation of 20E action and such studies call for immediate attention. The fat body in insects is an important metabolic centre and can be compared to the vertebrate liver, in view of the complex functions performed by it. The tissue is structurally organized to provide maximal exposure to the haemolymph and because of its changing metabolic role & integral position in maintaining metabolic homeostasis, the fat body serves with increasing frequency as a model for examining endocrine regulation (Kunkel, 1981; Riddiford *et al.,* 2001; Hansen *et al.,* 2002).

Extensive studies have revealed that 20E stimulates synthesis of various proteins in different tissues during postembryonic development of holometabolous insects. In lepidopteran insects the uptake of hexamerins by fat body (Kirankumar *et al.,* 1997, 1998) as well as the male accessory reproductive gland (Ismail and Dutta-Gupta, 1990, 1991; Dutta-Gupta and Ismail, 1992; Ismail *et al.,* 1993) is also shown to be regulated by ecdysteroids. Detailed studies have shown that the lysosomal activity in the whole body as well in the fat body exhibits a definite pattern during postembryonic and adult development (Ashok and Dutta-Gupta, 1988).

As insects do not feed during pupal stage, they depend on nutrients that have previously accumulated in the larval period. In all holometabolous insects investigated so far, amino acids and energy are supplied by proteins that have been selectively taken up by the fat body from the haemolymph (Haunerland, 1996; Burmester and Scheller, 1999). Most of the incorporated proteins belong to the family of hexamerins named according to their composition of six identical or closely related subunits (Telfer and Kunkel, 1991). The hexamerins are synthesized mainly by the fat body of actively feeding larval stage and are sequestered back into the fat bodies of prepupal and non-feeding pupal stage. This sequestration or uptake of hexamerins is a receptor mediated endocytotic process. The uptake is unique relative to the endocytosis process of eukaryotes because the hexamerin receptor does not belong to the low density lipoprotein (LDL) superfamily (Burmester and Scheller, 1999).

The hexamerin receptors have been recognized in dipteran as well as lepidopteran insects and ecdysteroids have been suggested to activate the receptor for hexamerin uptake (Ueno and Natori, 1984; Wang and Haunerland, 1993, 1994; Chung *et al.,* 1995; Burmester and Scheller, 1997, 1999). Using ligand binding studies, our laboratory has earlier demonstrated the presence of 120 kDa hexamerin receptor in the fat body membrane of rice moth, *Corcyra cephalonica* (Kirankumar *et al.,* 1997). The receptor was found to be present in the last larval instar, and - at maximal concentration - in the prepupal stage. The sequestration of hexamerin in *C. cephalonica*, like in other lepidopteran insects, was not observed during the larval stage (Ismail and Dutta-Gupta, 1990). However, 20E treatment induced a precocious uptake of hexamerins in the late-last instar (LLI) larval fat body (Ismail and Dutta-Gupta, 1990). These studies suggest that ecdysteroid hormone activates the hexamerin receptor prior to hexamerin uptake.

As mentioned, metamorphosis in insects is the transition from the larval to the adult stage and the events are controlled by ecdysteroid hormones (Riddiford *et al.,* 2001; Trumann and Riddiford, 2002; Gilbert *et al.,* 2002). It involves the breakdown of larval structures and the formation of new tissues. The former occurs either by the apoptosis of individual cells or autophagy of group of cells (Thummel, 2001). As a part of cell remodeling during metamorphosis, acidic autophagic vacuoles accumulate in the fat body cell and the activity of several lysosomal enzymes such as acid phosphatases 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 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. Acid phosphatase (ACP) (EC 3.1.3.2) is one of the commonly used marker enzyme to study the lysosomal activity in insect and is found in every organism studied to date. The ACP exists in multiple forms and different isozymes. 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.,* 1979a; Verkuil, 1980; Ashok and Dutta-Gupta, 1988; Sass *et al.,* 1989; Kutuzova *et al.,* 1991). Some data also provide evidence that the induction in lysosomal activity by ecdysteroids is governed at a nongenomic level (Verkuil, 1979b). However, no clear evidence has been provided to support the hypothesis. In the present study an attempt has been made to understand the activation of acid phosphatase by 20E in the fat body cells of the rice moth, *C. cephalonica*. Apart from the

lysosomal acid phosphatases there exists another acid phosphatase, the phosphatidic acid phosphatases or PAPs (3.1.3.4). The PAPs are of type-1 PAP (PAP1) and type-2 PAP (PAP2). The PAP1 (38-43 kDa) is the cytosolic or membrane bound acid phosphatase and is involved in the supply of diacylglycerols in the classical pathway of glycerolipid biosynthesis by dephosphorylating phosphatidic acid phosphatase whereas the PAP2 (35-50 kDa) is membrane bound and is involved in signal transduction that is mediated by phospholipase D (Kai *et al.,* 1996).

Literature search suggests that 20E not only exerts its action through the modulation of transcription but also by its action at the post-translational level *e.g.,* by protein phosphorylation (Itoh *et al.,* 1985; Sass, 1988). Based on the earlier studies from our laboratory on the effects of 20E, CaM kinase II, hexamerin receptor and tyrosine kinase were identified as the marker proteins for the present study. These markers offered a system to find out if their phosphorylation/activity was regulated by 20E and was there an alteration in the phosphorylation status/activity of these proteins in the presence of haemolymph protein HP19.

Why present study?

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 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 activity. Therefore an attempt was made in the present study to analyze the possible role of haemolymph factor(s) if any, in mediating 20E action on selected marker proteins. Thus, a protein in the haemolymph named **HP19 *i.e.* haemolymph protein of mass 19 kDa** was identified as a possible molecule in regulating few of the 20E mediated actions in some of the lepidopteran insect species.

Objectives of the present study-

Postembryonic development in insects involves growth, molting and metamorphosis. These events are controlled mainly by two morphogenetic hormones namely ecdysteroids and juvenile hormones. There is vast information about the regulation of these hormones at synthesis as well as at its titer level. The information also exists about the interaction of these hormones among themselves to regulate metamorphosis. However, knowledge regarding the regulation of hormone dependent actions is limited and the field remains largely unexplored. In the present study, an attempt has been made to understand this important aspect of the molting hormone, ecdysteroids. The study discusses the appearance of a stage and tissue specifically regulated protein HP19. This protein is identified in the present study and is found to be responsible for the regulation of few of the 20-hydroxyecdysone dependent actions.

The specific objectives of the dissertation work are given below:

- Confirmation of the presence of factor in haemolymph required for 20E stimulation of acid phosphatase.
- Understand the nature of the haemolymph factor, its isolation, characterization and developmental regulation.
- Molecular characterization of HP19.
- Role of HP19 in postembryonic development of *Corcyra cephalonica*.
- Effect of HP19 on 20-hydroxyecdysone dependent actions.
- Mechanism of 20-hydroxyecdysone regulated actions that is mediated by HP19.

Methodologies-

Some of the methods adopted to achieve the above mentioned objectives are given below-

- Denaturing PAGE - Laemlli (1970)
- Non denaturing PAGE - Burmester *et al.,* (1999)
- *In vitro* phosphorylation - Combest & Gilbert (1986); Shanavas *et al.,* (1998)
- Back phosphorylation - Forn and Greengard (1975)
- Purification of IgG - Protein A-affinity column
- Immunoprecipitation - Protein A-Sepharose beads
- Histochemical studies - Standard procedure
- Immunohistochemical studies - Meltzer *et al.,* (1997)
- *In vivo* immobilization of proteins- Del pino *et al.,* (1998)
- Western Hybridization - Towbin *et al.,* (1979) and BCIP/NBT detection
- Southern Hybridization - Sambrook *et al.,* (1989)
- Northern Hybridization - Sambrook *et al.,* (1989)
- cDNA library construction - Clontech and Stratagene manual
- Immunoscreening - Clontech manual
- Hybridization screening - Clontech manual
- Acid phosphatase assay - Henrickson and Clever (1972)
- Tyrosine kinase assay - Casnellie *et al.,* (1982)
- CaM kinase II assay - Fukunaga *et al.,* (1989)

Results and Discussion-

Chapter I- Identification, isolation and characterization of HP19 in *Corcyra cephalonica*

Earlier studies from our laboratory revealed that 20-hydroxyecdysone (20E) under *in vivo* condition stimulates the fat body acid phosphatase (ACP) activity in *Spodoptera litura* (Sridevi *et al.,* 1987) and *Corcyra cephalonica* (Ashok and Dutta-Gupta, 1988), but fat body cultures require factor or factors from haemolymph to show the stimulatory effect on enzyme activity (Ashok and Dutta-Gupta, 1991). In the first part of my doctoral study, I confirmed the

presence of factor required by the 20E to stimulate the fat body ACP activity of rice moth, *Corcyra cephalonica.* Different pretreatments of haemolymph prior to its addition to the fat body culture rendered the haemolymph impotent in mediating the 20E regulated ACP activity, suggesting the factor to be proteinaceous in nature.

In the attempts to purify the protein, the total haemolymph protein was fractionated on Sephadex G-50 and thus the active fraction was found to be a protein of ~22 kDa calculated from the elution profile, or 19 kDa, calculated from the mobility on SDS-PAGE. On the basis of these results the purification of the active haemolymph protein was carried out, first by fractionating the total haemolymph protein using 30 kDa cut-off membrane filters followed by gel filtration chromatography. Thus, a contaminant free pure polypeptide band of 19 kDa was identified that mediated the 20E regulated ACP activity and was named as **HP19**. Starting with 50 mg total haemolymph protein, we obtained a 98.5-fold purification with 0.05% yield.

Although nanogram of the protein was found to be sufficient for mediation even in crude or partially purified fractions, the yield of the purified protein was very low. Other limitations in purification were the requirement of large amounts of haemolymph of a specific developmental stage (LLI) and the removal of the major contaminating protein, hexamerin, that constitutes 75-80% of total haemolymph proteins (Haunerland, 1996). Therefore, an antibody against HP19 was raised by electro-eluting HP19 and was confirmed to be specific against HP19. When this antibody was added together with haemolymph to cultures of LLI fat body, the haemolymph failed to mediate the 20E dependent action. Similarly, when the haemolymph was first immunoprecipitated and the resulting complex and the supernatant was added to the culture, HP19 action was suppressed.

Western analysis of denatured as well as of non-denatured PAGE demonstrated that HP19 is a monomeric protein without any subunits in *C. cephalonica* and is probably the product of a single copy gene. Four independent methods, co-culturing, western analysis, immuno-histochemistry, and *in situ* immuno-staining, revealed that HP19 is synthesized by the hind gut associated lobular fat body (HGLFB) from where it is released into the haemolymph. This is further confirmed by the tissue specific gene expression only in HGLFB. Western analysis evidences a difference of ~5 kDa in the mass of HP19 in HGLFB (i.e 24 kDa) and in haemolymph (19 kDa). The predicted mass of the unmodified translated HP19 cDNA was close to the HP19 synthesized in HGLFB.

The results indicate that the biosynthesis of HP19 takes place in the HGLFB during the total last larval instar *i.e* the early-last (ELI), mid-last (MLI) and late-last instar (LLI) stage.

The protein is rapidly released into the haemolymph. The maximal HP19 concentration in the tissue as well as in the haemolymph could be observed in LLI. It is notable that only haemolymph from this LLI developmental stage is capable of mediating the 20E effect on ACP activity. Hence, the activity of HP19 is developmentally regulated, although the molecular mechanism of activation of ACP by ecdysteroid hormone is unclear at this point.

Apart from *C. cephalonica,* similar HP19 like protein was found in few other lepidopteran insects investigated but could not be detected in a diptera, *Calliphora vicina*. However, western analysis revealed that the mass of the protein in these lepidopteran species was slightly different and some of them had additional subunits. The studies, clearly suggest that HP19 is not species specific at least for lepidopteran insects but is definitely stage specific in its activity.

Chapter II- Cloning, sequencing and molecular characterization of HP19

To get more insight into the nature and function of HP19, we produced and characterized *C. cephalonica* HP19 cDNA (CcHP19). For the identification of the cDNA encoding the HP19 protein, a cDNA expression library, prepared from the RNA of HGLFB of LLI larva was immunoscreened using polyclonal HP19 antibody. This HP19 cDNA was 634 nucleotides long, with an open reading frame of 585 bp, which encodes a protein of 195 amino acids. The calculated molecular mass of the translated unmodified protein was 22.95 kDa, which is close to the mass of HP19 detected in HGLFB, a tissue that synthesizes the protein. The polypeptide comprises 12.3% basic (9 arg, 1 his and 14 lys) and 13.3% the acidic residues (10 asp, 16 glu). The estimated isoelectric point (pI) is 5.36. There is no cys residue in the sequence. The cDNA sequence begins with the methionine start codon at position 1 and translation stop codon at 586. A 3` untranslated sequence containing a polyadenylation signal AATAAA is located at 588 nucleotide followed by a poly $(A)_{29}$ tract. The polyadenylation signal overlapped the translation stop codon TAA by one base.

The amino acid sequence deduced from HP19 cDNA did not show a typical signal peptide necessary for transmembrane transport (Von Heijne, 1986.), probably due to the lack of any hydrophobic sequence (Feng *et al.,* 1999). Therefore, we conclude that might be a cleavage occurs before the release into the haemolymph. 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 is secreted from the synthesizing cells.

The comparison of the *C. cephalonica* HP19 (CcHP19) cDNA with the sequences in GenBank, showed 67% identity with *Choristoneura fumiferana* GST (CfGST) (Feng *et al.,* 1999). Similarities with other invertebrate GST were found to be less than 38%. Although the CcHP19 cDNA sequence revealed 67% identity with CfGST, affinity purified GST from *C. cephalonica* had no enhancing effect on the 20E dependent ACP activity when compared with purified HP19 or recombinant HP19. Further, the haemolymph as well as the purified HP19 had negligible GST activity. It was also found while purification of GST by affinity chromatography, the protein fractions that did not bind to glutathione affinity matrix enhanced the 20E dependent ACP activity, again suggesting that it is not a GST molecule.

Chapter III- Role of HP19 during the postembryonic development

In order to understand the role of HP19 in insect growth and development during the postembryonic life of *Corcyra cephalonica,* the protein was *in vivo* immuno-complexed and rendered unavailable for mediation of 20E dependent actions. This was done by injecting HP19 antibody to final (Vth) instar larvae, an approach frequently used to understand the physiological processes (Hiraoka and Hayakawa, 1990; Del Pino *et al.,* 1998). This injection of HP19 antibody interfered with the proteins physiological action and led to the growth of either nonviable larval-pupal or pupal-adult intermediates as compared to controls where the larvae grew normally and gave a normal healthy adult. Further analysis on various parameters suggested that compared to controls the antibody injected larvae had several alterations. Although the difference in % mortality was not significant when compared with controls but other changes were significant. The antibody injected larvae, showed reduced salivation, delayed reduction in body length and head capsule size. The time of pupation though was normal but most of the pupae that developed were abnormal, non-viable larval-pupal intermediates and some of the larvae which could metamorphose into pupae finally gave rise to non-viable pupal-adult intermediates. The ACP activity profile demonstrated gradual increase in the activity of control sets of fat body collected after different days of post-injection corroborating with the reported developmental profile of ACP (Dutta-Gupta and Ashok, 1988). However antibody injected insects had almost static fat body ACP activity after different days of post injection. This suggested that the acid phosphatase has role in insect growth and development by possibly regulating the histolysis of larval organs and HP19 plays role in the regulation of ACP activity.

The protein profile of fat body and haemolymph revealed that HP19 not only regulates ACP activity but also has a role in regulating hexamerin sequestration. Hexamerins are high molecular weight multimeric proteins synthesized by actively feeding larval fat body cells and released into haemolymph. These hexamerins are later sequestered back via a receptor mediated endocytosis process by the non-feeding pupal fat body cells to meet its energy requirement (Haunerland, 1996). In the present study, HP19, although did not interfere with the synthesis of hexamerins but as compared to control the antibody injected larvae showed no sequestration of hexamerins after 14 days of post injection. Immunohistochemical staining of the fat body tissue sections collected after different days of post-injection further confirmed that HP19 antibody injected insects had improper sequestration confirming the hypothesis that HP19 not only has effect on ACP activity but also regulates hexamerin sequestration. The macroscopic view of the whole mount fat body demonstrated that in HP19 antibody injected sets, there was a distinct change in the destruction and tissue remodeling as compared to controls further confirming the hypothesis of a possible role of HP19 in regulating insect growth and development.

Chapter IV- Effect of HP19 on 20-hydroxyecdysone regulated actions

Studies from our laboratory have revealed that in addition to the increase in lysosomal activity in the whole body as well as in the fat body by 20E, it also stimulates the synthesis of various proteins in different tissues during the postembryonic development of the lepidopteran insects (Ray *et al.,* 1987 a,b; Sridevi *et al.,* 1988, 1989; Ismail and Dutta-Gupta 1990; Shanavas *et al.,* 1996). The uptake or sequestration of storage proteins (hexamerins) by the fat body (KiranKumar *et al.,* 1997) as well as the male accessory reproductive gland (Ismail and Dutta-Gupta, 1990, 1991; Dutta-Gupta and Ismail, 1992; Ismail *et al.,* 1993) was also shown to be regulated by ecdysteroids. Earlier studies in dipteran insects suggested a post-translational activation of hexamerin receptor by 20E. Studies from our laboratory suggested that hexamerin receptor undergoes protein phosphorylation that is regulated by 20E.

In the present study an attempt was made to understand some of the 20E regulated actions and effect of HP19 on them. For this three proteins namely hexamerin receptor, tyrosine kinase and CaM kinase II were identified as the potential markers. In addition to this attempt was also made to understand the protein kinase C (PKC) regulation.

A. Effect on hexamerin receptor phosphorylation- Previous studies from our laboratory suggests that the 120 kDa hexamerin binding protein in the fat body cell membranes of *C. cephalonica* is activated by ecdysteroid hormone (Kirankumar *et al.,* 1997). The study further suggested the post-translational modification of the receptor by protein phosphorylation (Kirankumar, 1998). In the present study attempt was made to understand the role of 20E in the phosphorylation of 120 kDa hexamerin receptor. The results suggested that 20E stimulates the phosphorylation of 120 kDa hexamerin binding protein that has been demonstrated to represent the receptor. The 20E stimulated phosphorylation is mediated partly by a tyrosine kinase as monoclonal phosphotyrosine antibodies cross-react with the receptor and phosphorylation is blocked partly by genistein. The receptor phosphorylation is developmentally regulated. Back phosphorylation study provides additional evidence for 20E regulation of hexamerin receptor phosphorylation in intact fat body. Phosphorylation of the receptor was found to be essential prerequisite for hexamerin uptake. Since this 20E stimulated phosphorylation of hexamerin receptor occurs in intact tissue, membrane as well as in the fat body homogenates and the uptake of hexamerin by the phosphorylated receptor is unaffected by the inhibitors of transcription and translation, suggest that the activation of hexamerin receptor for the uptake occurs at a nongenomic level and is unaccompanied with the increase of transcript or protein synthesis.

In addition to this the hexamerin receptor was also identified by ligand binding studies in another hexamerin sequestering tissue *i.e*., male accessory reproductive glands which was found to undergo phosphorylation like the receptor present in the fat body. The phosphorylation of the hexamerin receptor was found to be inhibited by HP19.

B. Effect on tyrosine kinase activity- As discussed above that the phosphorylation of 120 kDa hexamerin receptor is mediated partly by a tyrosine kinase and the phosphorylated hexamerin receptor was found to be essential prerequisite for activation followed by hexamerin uptake. Since ATP is cell impermeable hence the phosphorylation of hexamerin receptor in cultured fat body followed by increased uptake of [^{35}S] methionine labeled hexamerin suggested that the hexamerin receptor is probably a cell surface receptor with intrinsic kinase activity. Hence we checked the *in vitro* uptake of hexamerins by carrying the phosphorylation of hexamerin receptor with [γ^{32}P] ATP. The results revealed 20E induced phosphorylation of 120 kDa hexamerin receptor in cultured fat body. The immunohistochemical staining of the 20E supplemented cultured fat body using monoclonal anti-phosphotyrosine antibody indicated the

presence of the phosphorylated tyrosine residue in the fat body cells. We extended the study to check if the hexamerin receptor phosphorylation is mediated by receptor tyrosine kinase (RTK). For this we used a series of RTKs inhibitor tyrphostins (AG9, AG490, AG94, AG528 & AG879) but only one of the inhibitor AG879 at high concentration (100 μM) inhibited the phosphorylation of hexamerin receptor. This suggests that probably a RTK mediates the phosphorylation of hexamerin receptor. However, the result is inconclusive to understand if the hexamerin receptor is a cell surface receptor with intrinsic kinase activity.

The study indicated that tyrosine kinase is present in the fat body, which is developmentally regulated. Fat body tyrosine kinase activity study with a synthetic peptide as substrate indicated that the LLI stage has highest tyrosine kinase activity and ligation reduces the activity indicating the hormonal dependence. The 20E was found to induce the fat body tyrosine kinase activity both *in vivo* and cultured fat body. The fat body tyrosine kinase activity was inhibited by the presence of HP19 both in cultured fat body as well as in the homogenate. In addition to this, the study also revealed a tyrosine kinase mediated phosphorylation of 48 kDa protein. The effect of HP19 on the phosphorylation of 48 kDa phosphorylation was not carried out as it was found to be independent of 20E and dependent on juvenile hormone (JH).

C. Effect on CaM kinase II activity and autophosphorylation- Earlier studies from our laboratory revealed that the CaM kinase II activity and its autophosphorylation in the CNS and fat body is developmentally regulated (Shanavas *et al.,* 1998; Vasanthi, 1999). In the present study, the results indicate that CaM Kinase II is developmentally regulated with highest activity at LLI stage. The activity as wells as autophosphorylation is 20E dependent. Addition of 20E to ELI and LLI ligated larva stimulates the CaM kinase II activity which is inhibited by HP19. Similarly 20E induces the autophosphorylation of CaM Kinase II, but is inhibited by HP19.

D. Effect on protein kinase C (PKC)- In order to understand the effect of HP19 on other 20E regulated actions the study was extended on PKC regulation in the fat body of *C. cephalonica*. The results indicated that although thorax-ligation shows reduction in PKC activity but the activity was highest during ELI stage and gradually decreased at the LLI and pre-pupal stage. This indicate that PKC activity is possibly JH dependent and not on 20E. This was confirmed with the *in vivo* injection of 20E to the thorax-ligated larvae that showed no stimulation in the

fat body PKC activity. Since the PKC activity was unaffected by 20E, it was not considered worthwhile to check the effect of HP19 on the fat body PKC activity.

These studies on the effect of HP19 on 20E mediated action such as stimulation of ACP activity, phosphorylation of hexamerin receptor, tyrosine kinase activity and CaM kinase II activity and its autophosphorylation in the fat body of *C. cephalonica* suggested that HP19 most likely has either a kinase inhibitory/phosphatase activating effect. This however remains to be studied. Present study suggests that HP19 has a regulatory role on the 20E stimulated ACP activity, phosphorylation of hexamerin receptor and the kinases (tyrosine kinase and CaM kinase II).

Chapter V- Mechanism of 20-hydroxyecdysone regulated actions that is mediated by HP19

A wealth of data on the molecular mechanism of ecdysteroid action shows that the transcriptional cascade leading to molting and metamorphosis is initiated when 20E binds to its nuclear receptor (Beato and Klug, 2000). For about four decades evidence has accumulated that some of the hormonally induced effects seemed to be too rapid for the classical model (Falkenstein *et al.,* 2000; Losel and Wehling, 2003). This evidence casts doubt on the so called genomic pathway as the sole mode of steroid action. To date, several modes for nongenomic steroid actions are being examined. Most of them are thought to continuously modulate the long-term program allowing cells or organs to adapt rapidly to environmental changes. Numerous experiments with a huge number of different species display that insect metamorphosis in general is under the genetic control of ecdysteroids. Studies also indicate that some events, necessary for and accompanying metamorphosis, are controlled by 20E at a nongenomic level. However, studies on these mechanisms are restricted to a small number of experimental systems *e.g.* the activation of lysosomal enzymes and the hexamerin receptor.

A. Nongenomic regulation of acid phosphatase activity-

To learn more about the molecular mechanisms of ecdysteroid in regulating the ACP activity assisted by HP19, the fat bodies were incubated for several time periods with HP19 and 20E. A minimum incubation of 4 h was essential for the stimulation in the enzyme activity by 20E; time enough for a genomic hormone action. However, the measured

stimulation was unaffected by transcriptional or translational inhibitors, indicating the independence of gene activation. Furthermore, the *in vitro* study with fat body homogenate showed a rapid stimulation (within seconds to 1 min) of the enzyme activity. Since the homogenate preparation is essentially a cell- or nucleus disintegrated fraction, this suggests that cell/nuclear integrity is not an essential requirement for the effect of 20E on ACP activity. This possible nongenomic regulation was further strengthened by the results that in the presence of 20E, fat body cultures showed a higher incorporation rate of [^{35}S] methionine which was inhibited by transcriptional or translational inhibitors. This, however, had no effect on the ACP activity and the presence of HP19 in the tissue culture rendered 20E to stimulate the enzyme activity even in the presence of inhibitors of transcription and translation.

After confirming the regulation of ACP activity by 20E in presence of HP19 at protein level, the study was extended to understand the regulation at RNA level. For this, the approach was taken to clone the ACP of *C. cephalonica.* Incidentally while screening for the hexamerin cDNA from the fat body expression library using polyclonal antibody against *C. cephalonica* hexamerins, one of the false positive showed sequence homology with phosphatidic acid phosphatase (PAP). This clone was *in vitro* transcribed to get RNA probe which was used for hybridization screening of the library and thus one clean positive was picked and used for second round of screening. In this way two identical positive clones were picked and were sequenced after restriction analysis. The partial cDNA sequence of *C. cephalonica* PAP showed ~38% sequence homology with *Drosophila* PAP (wunen gene). When this clone was used for southern hybridization, it showed that PAP is product of multiple copy gene. Activity profile showed highest ACP activity in visceral fat body and this matched with the northern profile from the RNA of different tissues of *C. cephalonica*.

To understand the nongenomic regulation of ecdysteroid mediated by HP19 at molecular level, the RNA from fat body which was cultured with 20E and HP19 along with the controls were subjected to northern analysis. The results indicated that the presence of HP19 that mediates the 20E stimulation of ACP activity did not show increase in the transcript of the PAP. This suggests the nongenomic regulation of ACP. These studies however gives no clue if the ACPs that are regulated by HP19 is lysosomal and required for autophagy during metamorphosis or it is a membrane bound protein required for the phospholipase D activation in signal transduction.

B. Nongenomic regulation of hexamerin receptor phosphorylation, tyrosine kinase activity and CaM kinase II activity and autophosphorylation- The inhibitory effect of HP19 on the 20E induced fat body tyrosine kinase activity in cultured fat body, although occurs at longer time incubation but is unaffected by the inhibitors of transcription and translation. This further confirms the nongenomic regulation of 20E mediated by HP19. The nongenomic regulation of fat body tyrosine kinase activity occurs even in the homogenate preparation. Similar inhibition was also seen with the 20E induced autophosphorylation and activity of CaM kinase II indicated that 20E nongenomically regulates the CaM kinase II activity in the fat body of *C. cephalonica*.

Regulation of HP19-

Present study indicated a multiple role of HP19 on some of the 20E dependent actions. The HP19 mediated the 20E stimulation of ACP activity and inhibited the 20E induced phosphorylation of hexamerin receptor, activity of tyrosine kinase and CaM kinase II activity and autophosphorylation. Present study suggests that HP19 either is a kinase inhibitor or a phosphatase activator. However, it is not clear at present and further studies are required to confirm this. The results also indicate that HP19 might be a protease like molecule to regulate the 20E dependent actions. We also compare this relatively stable HP19 (good shelf life) with other ubiquitously distributed regulatory molecules that have multiple roles in cellular functions. However, HP19 did not show any similarity at western level with proteins like 14-3-3, stathmin and ubiquitin. In order to understand if HP19 was itself phosphorylated, to regulate the selected 20E dependent actions. The results indicated that there is no phosphorylation of HP19 in absence or presence of calcium either in HGLFB, a tissue that synthesizes this protein or in the haemolymph where it is released.

Summary and conclusion-

In short a haemolymph protein HP19, identified and characterized in the present study appears to be an important regulatory protein in the postembryonic life of *C. cephalonica.* It mediates the 20E stimulated fat body ACP activity. Further, 20E induced activity and autophosphorylation of CaM kinase II are inhibited by HP19. In the absence of HP19, the hormone (20E) induces the tyrosine kinase activity which in turn stimulates the phosphorylation of hexamerin receptor which is followed by increased hexamerin uptake.

When HP19 is present, there is an inhibition of tyrosine kinase activity, which results in reduction of phosphorylation of hexamerin receptor. Hexamerins are synthesized by actively feeding larval fat body cells and released into haemolymph. The hexamerin synthesis is regulated by the morphogenetic hormones (juvenile hormone and ecdysteroids) through their interaction with nuclear receptors (Wang *et al.,* 1995). The presence of active HP19 at late-last larval instar stage possibly inhibits the fat body tyrosine kinase activity, hence there is no phosphorylation of hexamerin receptor, and hexamerins are not sequestered by larval fat body cells. At pupal stage, the HP19 is inactive, hence there is stimulation of tyrosine kinase by 20E (endogenous titer at this stage is high) which in turn mediates the phosphorylation of hexamerin receptor and the uptake of hexamerins occurs.

Unanswered questions-

The present study addresses a very important aspect in the hormone regulated actions by a heamolymph protein, HP19 during the postembryonic life of insects. However, this study fails to answer many questions and opens up a new avenue for further study to understand several aspect (i) Is there an interaction of 20-hydroxyecdysone (20E) with HP19? (ii) Does the hexamerin receptor possess intrinsic tyrosine kinase activity that autophosphorylates the receptor for hexamerin uptake? (iii) Is HP19 a kinase inhibitor or a phosphatase activator? and (iv) What are the mechanisms or pathways by which the HP19 mediates the nongenomic effect of ecdysteroids?

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