Hexamerins: Expression regulation and their uptake in Rice moth, Corcyra cephalonica

Thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

by

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Dedicated to
My Grand Mother

Contents

DECLARATION

I hereby declare that the work embodied in this thesis entitled "**Hexamerins: Expression, regulation and their uptake in rice moth, *Corcyra cephalonica***" has been carried out by me under the supervision of Prof. Aparna Dutta Gupta and this has not been submitted for degree or diploma of any other university earlier.

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CERTIFICATE

This is to certify that **Mr. Manohar Damara** has carried out research work embodied in this thesis under my supervision and guidance for a full period prescribed under the Ph.D ordinance of this University. We recommended his thesis **"Hexamerins: Expression, regulation and their uptake in rice moth, *Corcyra cephalonica*"** for submission for degree of Doctor of Philosophy of this University.

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(Manohar Damara)

Abbreviations

°C	:	degree centigrade / celsius
20E	:	20-Hydroxyecdysone
ALP	:	Alkaline phosphatase
ATP	:	Adenosine 5` triphosphate
Aza	:	Azadiractin
BCIP	:	5-Bromo-4-chloro-3-indolyl phosphate
BSA	:	Bovine serum albumin
cDNA	:	Complementary DNA
cpm	:	Count per minute
2-D GEL	:	Two Dimentional Gel Electrophoresis
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
HEPES	:	N-(2-hydroxyethyl)piperazine-N`-(2-ethanesulfonic acid)
IgG	:	γ Immunoglobulin
IPTG	:	Isopropyl β-D-thiogalactoside
JH	:	Juvenile hormone
kDa	:	Kilodalton
LB	:	Luria-Bertani medium
LLI	:	Late-last instar larvae
MARG	:	Male accessory reproductive gland
mg	:	Milligram
MLI	:	Mid-last instar larvae
mM	:	Millimolar
MOPS	:	3-Morpholinopropanesulfonic acid
mRNA	:	Messenger ribonucleic acid
NBT	:	Nitrotetrazolium blue
Ng	:	Nanogram
nM	:	Nanomolar
NP-40	:	Nonidet P-40 (Nonylphenyl polyethylene glycol)
PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction

PMSF	**:**	**Phenylmethylsulfonyl fluoride**
PP	**:**	**Prepupae**
RNA	**:**	**Ribonucleic acid**
RACE	**:**	**Rapid amplification of cDNA Ends**
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**
mg	**:**	**microgram**
µM	**:**	**micromolar**

The ontogeny of holometabolous insects is characterized by metamorphosis, a crucial phenomenon by which structurally different stages such as larvae, pupae and adults are connected with one another. In this group during larval-pupal-adult transformation, the larval organs undergo either total degeneration or remodeling to give rise to adult structures. These processes, primarily involve mobilization of various molecules like proteins, lipids and carbohydrates and *de novo* synthesis of variety of macromolecules. Proteins are the most important macromolecules which make their manifestation during development and morphogenesis of insects (Schmidt and Schwankl, 1975), hence studies on tissue specific proteins, their expression, regulation as well as their sequestration by various tissues are of paramount significance.

The fat body is a multifunctional tissue and it is the most conspicuous organ in the larval body cavity of holometabolous insects. It exhibits regional differentiation and is typically located in two body regions that reflect its embryonic origin. The peripheral or sub-cuticular fat body is found adjacent to the body wall external to the musculature, while the visceral fat body surrounds the alimentary canal (Dean *et al.,* 1985; Keeley *et al*., 1985). The tissue freely bathes in haemolymph and such an organization facilitates rapid, metabolic exchange of macromolecules between the fat body and the haemolymph in coordination with each other play a significant physiological role (Terwilliger *et al.,* 1999).

The fat body is known to be the major site of biosynthetic activity in insects and undergoes a chronologically ordered sequence of alteration during post-embryonic development (Dean *et al*., 1985). In actively feeding larval stages, the fat body synthesizes various macromolecules including fatty acids, lipids, carbohydrates and wide range of proteins, which are released in to the haemolymph (Telfer and Kunkel, 1991; Wang and Haunerland, 1991, 1992; Haunerland, 1996). Through the haemolymph, these macromolecules are transported to different tissues, where they are sequestered to perform various

physiological functions. At the end of the larval development and during pupal-adult transformation there is considerable change in morphology as well as physiology of fat body cells. The tissues intermediary metabolic and protein synthetic activities diminish and from a synthetic organ it becomes a storage tissue (Price, 1973; Dean *et al*., 1985). This switch is characterized by the appearance of large number of electron dense storage granules and coated vesicles inside the cytoplasm of the cell. The fat body at this stage stores various macromolecules which are later mobilized during the metamorphosis and are used as metabolic precursors and building blocks for pupal-adult transformations mainly in holometabolous insects including Lepidoptera (Leevenbook, 1985; Inagaki and Yamashita, 1986; Bean and Silhacek, 1989; Kanost *et al*., 1990; Telfer and Kunkel, 1991; Haunerland, 1996; Burmester, 1999).

Larval haemolymph contains a diverse array of peptides and proteins, such as immune response proteins (Shahbuddin *et al*., 1998; Hetru *et al*., 1998; Johns *et al*., 1998, 2001; Vizoli *et al*., 2000; Nakajima *et al*., 2001; Roy *et al*., 2004; Bang *et al*., 2005), lipophorins (Sharpiro *et al*., 1988; Burks *et al*., 1992; Kanost *et al*., 1995; Rimoldi *et al*., 1996), hormone-binding and ion-binding proteins (Koopsmanchap and de Kort, 1998; Trowell, 1992; Braun and Wyatt, 1996; Harai *et al*., 1998; Maya-Monteiro *et al*., 2000; Gudderra *et al*., 2002), haemocyanins (van Holde and Miller, 1995; Burmester, 2002; Zacharieva *et al*., 2008) and hexamerins (Telfer and Kunkel, 1991; Haunerland, 1996; Danty *et al*., 1998; Burmester, 2007; Bitondi, 2008). Presence of these proteins/peptides clearly reflects diversified roles played by haemolymph in mediating intercellular communication, transporting metabolites and imposing a defensive barrier against micro-organisms.

Among all the proteins synthesized by the fat body and secreted in to the haemolymph, hexamerins are the most important proteins which play a vital role in the development of insects. Hexamerins were first reported by Lauffer (1943)

in the larval haemolymph of *Bombyx mori*. However, the ground breaking work on insect hexamerins was done in the dipteran insect, *Calliphora erythrocephala* on the protein originally designated as calliphorin (Munn *et al*., 1971). Subsequently, presence of hexamerins in the haemolymph of several insect species was reported (cf. review. Wyatt and Pan, 1978; Levenbook, 1985; Haunerland, 1996; Burmester, 1999). Considering their localization predominantly in the larval haemolymph they have been earlier termed as larval serum proteins (LSPs) (Roberts *et al*., 1987) or larval haemolymph proteins (LHPs) (Chrysanthis *et al*., 1981; Leevenbook, 1985). They are often called as storage proteins, because they accumulate in dense protein granules indicating their proposed function as amino acid reserve for the synthesis of proteins during pupal-adult transformation.

Hexamerins being ubiquitous, in many insects play an array of roles not only during post-embryonic development but also in adults. Although hexamerins were shown to be haemocyanin related proteins, they do not serve as oxygen carrier in insects (Beintema *et al*. 1994). The major function of hexamerins was shown to be as a source of nitrogen and amino acid pool to support *de novo* protein synthesis, during metamorphosis and reproduction (Kanost *et al*., 1990; Telfer and Kunkel, 1991; Burmester and Scheller, 1995, 1996; Pan and Telfer, 1996; Wheeler and Buck, 1996; Seo *et al*., 1998; Wheeler *et al*., 2000). Hexamerins presumably support egg formation primarily by providing precursors for the synthesis of yolk proteins by the fat body and chorion proteins by the follicle cells (Wyatt, 1991; O'Brien, *et al.,* 2002*)*. Earlier studies from our laboratory have clearly demonstrated that hexamerins play a role not only in female reproduction but also in male reproduction of lepidopteran insects and make a major component of seminal secretion (Bajaj *et al*., 1990). Hexamerins were reported to be incorporated in cuticle during the pupal-adult development (Marinotti *et al.*, 1988; Peter and Scheller, 1991). In the ant *Componatus festinates*, hexamerins were shown to play an important role in brood nourishment

and colony founding (Martinez *et al*., 2000). Hexamerins also bind to potentially toxic xenobiotics (Haunerland and Bowers, 1986) and this feature could be exploited for the site directed delivery of bio-pesticides, toxins and growth regulators for management of insect pests. Recently hexamerins have been shown to play a role in the caste differentiation and regulation in an ant, *Reticulitermes flavipes* (Scharf *et al.*, 2006, 2007a, b)

Structurally hexamerins are multimeric aggregates of six subunits in the range of 70-90 kDa, which may be homo- or heteromers with native molecular mass around 450- 500 kDa (Telfer and Kunkel, 1991; Haunerland, 1996; Zhakarkin *et al*., 1997). This characteristic seems to be retained in several orders of holometabolous insects including lepidoptera (Haunerland, 1996; Wheeler *et al*., 2000) and even in hemimetabolous insects (Faria *et al*., 1994; Ancsin and Wyatt, 1996; Benes *et al*., 2003). Hexamerins have been biochemically purified and characterized in a number of insects (Telfer and Kunkel, 1991; Korochkina *et al*., 1997; Gudderra *et al*., 2002). Most documented hexamerins are from orders lepidoptera and diptera. They have also been studied in hymenoptera (Martinez and Wheeler, 1993, 1994; Silke Hagner-Holler, 2007), coleoptera (Koopmanschap *et al*., 1992; Sohn *et al*., 2004) and in a number of hemimetabolous insects (Rehn and Rolim, 1990; Chinzei *et al*., 1992; Faria *et al*., 1994; Ancsin and Wyatt, 1996; Jamroj *et al*., 1996).

Based on amino acid composition hexamerins have been classified into four main classes (Telfer and Kunkel, 1991). The first category of protein is characterized by exceptionally high content of aromatic amino acids (18-26%) and low methionine content (2%) and is generally called as arylphorins. This class includes prototype storage protein calliphorin, from *C. erythrocephala* (Munn *et al*., 1971). These are the major haemolymph proteins in diptera (Naumann and Scheller, 1991; Burmester *et al.*, 1998). The second category is the lepidopteran glycoprotein with high aromatic amino acid and low methionine content and

named as lepidopteran arylphorins (Riddiford and Hice, 1985; Palli and Locke, 1987; Webb and Riddiford, 1988; Karpells *et al*., 1990; Kunkel *et al*., 1990). The third group of hexamerin is found in many dipteran insects, which is neither rich in aromatic amino acids nor in methionine (Benes *et al*., 1990; Haunerland, 1996; Braun and Wyatt, 1996; Korochkina *et al*., 1997). The fourth group has relatively high methionine content (about 6%) and low aromatic amino acid content and they are predominantly expressed in the female larvae of lepidoptera, and they form the yolk protein precursors (Webb and Riddiford, 1988a; Bean and Silhacek, 1989; Rehn and Rolim, 1990; Kunkel *et al*., 1990; Corpuz *et al*., 1991; Wang *et al*., 1993; Jamroj *et al*., 1996; Pan and Telfer, 1996; Mi *et al*., 1998; Hwang *et al*., 2001; Zhu *et al*., 2002). Insect hexamerins generally undergo post-translational modifications like glycosylation and lipidation and are either glycoproteins or lipoglycoproteins.

Hexamerins are predominantly synthesized by the larval fat body and released simultaneously in to the surrounding body fluid (haemolymph). In the haemolymph their concentration increases gradually from penultimate larval to final larval instar, where they may account for 80% of the total haemolymph proteins by weight (Kramer *et al*., 1980; Tojo *et al*., 1980; Palli and Locke, 1987a; Kanost *et al*., 1990; Telfer and Kunkel, 1991; Haunerland, 1996). Their concentration in the haemolymph begins to decline during prepupal and early-pupal development, concurrently with the accumulation of these proteins in the fat body, and they generally become undetectable in the haemolymph during the late pupal and adult stages.

To date there are many hexamerins, whose gene and amino acid sequences have been established (Yashihiro and Okisugu, 1991; Memmel *et al*., 1994; Burmester *et al*., 1998; Gordadze *et al*., 1999; Hwang *et al*., 2001; Zhu *et al*., 2002). Previously our group has cloned and characterized an arylproin hexamerin cDNA from rice moth, *C. cephalonica* (Nagamanju *et al.*, 2003). Multiple

alignment analysis of the deduced amino acid sequence derived from the cDNA sequence showed its homology to most of the insect arylphorins. Phylogenetic studies on hexamerins have reported that arthropod tyrosinase, arthropod hemocyanin, insect hexamerin (lepidoptera, diptera) and dipteran arylphorin receptor share a common origin (Burmester and Scheller, 1996; Burmester *et al*., 1998).

Two major morphogenetic hormones (i) juvenile hormone (JH) and (ii) 20-hydroxyecdysone (20E) play a very important role in insects and regulate the postembryonic development involving molting, metamorphosis and pupal-adult transformations. Ecdysteroids induce molting, where as JH determines the status of each molt *i.e*., whether it is a larval-larval molt or larval-pupal molt (Riddiford, 1994, 1996; Gilbert *et al*., 2000). A precise interplay between the titer of JH and ecdysteroid regulates the stage-specific expression of different genes, thus regulating postembryonic development of the insects (Hiruma *et al*., 1991; Brey *et al*., 1999; Palli *et al.,* 2008; Tobe *et al.*, 2008).

Hexamerins offer excellent model system for the study of hormonally regulated gene expression at both transcriptional and post-transcriptional levels. The influence of the metamorphic hormones on the expression of hexamerins has been evaluated in several lepidopteran as well as dipterian insects. JH was shown to inhibit the expression of some larval proteins (Ray *et al*., 1987; Jones *et al*., 1988; Memmel and Kumaran, 1988). It was also reported to inhibit synthesis of one of the hexamerins, in *Trichoplusia ni* (Schelling *et al.*, 1993), *Galleria mellonella* (Cymborowski *et al.*, 2003) and *Corcyra cephalonica* (Ismail and Dutta-Gupta, 1988). In fall webworm, *Hyphantria cunea* SP-2 expression was suppressed by JH (Hwang *et al*., 2001). Ecdysteroids were shown to stimulate the hexamerin synthesis in *Apis mellifera* (Bitondi *et al.*, 2008), *Drosophila melanogaster*, mainly through transcriptional regulation (Mousseron *et al*., 1997; Kumar *et al.*, 2004; Mintzas *et al*., 2008). Riddiford and Hice (1985) suggested

that the arylphorin synthesis in *M. sexta* was regulated by rising ecdysteroid titer at each stage. The hormonally induced expression of hexamerins in lepidopteran insects had been shown, however the precise regulation of hexamerin synthesis by specific hormones has not been studied in detail.

Selective uptake or receptor mediated uptake has now been recognized as a widely prevalent mechanism by which functionally important macromolecules such as nutrients, hormones, vitamins and many others are acquired by animal cells (Lin *et al*., 1998; Santini *et al*., 1998; van Der Horst *et al*., 2005). Among the various proteins sequestered by fat body, the larval hexamerins form the major group.

The uptake of hexamerins from haemolymph by the fat body cells is an unique feature of the class "Insecta". It involves a receptor which does not belong to the low density lipoprotein (LDL) superfamily (Burmester and Scheller, 1999). These receptors have been recognized in dipteran (Ueno and Natori, 1984; Burmester and Scheller, 1992, 1997) as well as lepidopteran insects (Wang and Haunerland, 1994; Kirankumar *et al*., 1997). In the flesh fly, *Sarcophaga peregrina*, a 120 kDa receptor was identified. Under the influence of 20E, the receptor acquired the ability to sequester hexamerins (Ueno and Natori, 1984; Chung *et al*., 1995). Studies in the blow fly, *Calliphora vicina*, revealed that the hexamerin receptor is synthesized as a precursor (130 kDa) which is subjected to a three-fold post-translational cleavage, to give rise to the active receptor (Burmester and Scheller, 1997a, b). The onset of hexamerin uptake coincides with the third cleavage, which is initiated by ecdysteroids (Burmester and Scheller, 1997, 1999). It has also been shown, that a rise in the ecdysteroid titer at the end of the larval life triggers the incorporation of hexamerins in the fat body of the fruit fly, *D. melanogaster*. The fat body protein 1 (Fbp-1) was identified as the responsible receptor (Burmester *et al*., 1999). In the boll worm, *Helicoverpa zea*,

a single 80 kDa receptor protein was reported to mediate the uptake of VHDL and storage proteins (Wang and Haunerland, 1993, 1994).

Using ligand binding studies, our group has demonstrated the presence of 120 kDa hexamerin receptor in the fat body membrane of the rice moth, *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 stages. However, 20E treatment induced a precocious uptake of hexamerins in the late-last instar (LLI) larval fat body (Ismail and Dutta-Gupta, 1990). Further studies suggested that ecdysteroids activate the hexamerin receptor through phosphorylation and the phosphorylation is mediated by tyrosine kinase (Kirankumar *et al*., 1997; Arif *et al*., 2003) and finally activated receptor is responsible for sequestration.

In the present study, *Corcyra cephalonica* which belongs to order lepidoptera and family galleridae was used as the insect model. It is commonly known as rice moth, is a serious pest of cereals, oil seeds and legumes in tropical and sub-tropical regions of the world. In *C. cephalonica*, like many other lepidopteran insect, characteristic changes occur during growth and metamorphosis. Three hexamerins, Hex 1 (86 kDa), Hex 2 (84 kDa) and Hex 3 (82 kDa) were identified from the haemolymph of the actively feeding larvae (Ismail and Dutta-Gupta 1993) and one of the hexamerin subunit *Hex2* cDNA was cloned and completely characterized. This was found to be an arylphorin rich hexamerin subunit (Nagamanju *et al*., 2003). Like other lepidopteran insects hexamerins, they are synthesized by the actively feeding larval fat body cells and secreted in to the surrounding haemolymph. During the prepupal and pupal stages, they are sequestered by the fat body which is mediated by receptor present on the plasma membrane (Ismail and Dutta-Gupta, 1991; Kirankumar *et al*., 1997, Arif *et al*., 2003, 2007).

Why present work?

The role played by insect hexamerins in the pupal–adult transformation, reproduction, caste regulation and immunity is well demonstrated in various insects including lepidoptera. Further the importance of the morphogenetic hormones in the regulation of the hexamerin synthesis and their uptake is well documented, however a comprehensive study on the developmental regulation of hexamerin(s) gene expression and synthesis in the stored grain pests which have a totally different environmental milieu and which are exposed to various physiological stresses, is an unexplored area.

Studies from various research groups have shown the presence of the hexamerin receptor in different lepidopteran insects, the mechanism of there activation and the role played by them in hexamerin sequestration. Our group has already demonstrated that in *Corcyra cephalonica* the hexamerins are sequestered into the fat body by receptor mediated endocytosis. Further, the phosphorylation of the receptor is prerequisite for the uptake of hexamerins which is mediated by 20E, and this phosphorylation is tyrosine kinase dependent. Till date the identification of the receptor and its functional characterization was done by most groups including our's at the homogenate level. However, to the best of our knowledge till now the hexamerin receptor is neither purified nor cloned from any lepidopteran insect. Hence based on the above the present work was carried out to get a detailed insight into the regulation of hexamerin synthesis, its gene expression and molecular characterization of hexamerin receptor of *Corcyra cephalonica.* An attempt was also made to study the distribution of hexamerin receptor in other tissues, which were reported to sequester hexamerins.

Objectives:

(i). Hormonal regulation of hexamerin synthesis and its gene expression during the larval /post-embryonic development

(ii). Cloning and characterization of methionine rich hexamerin cDNA from *C. cephalonica*

(iii). Studies on hexamerin uptake, cloning and characterization of hexamerin receptor from *C. cephalonica*

Materials & Methods:

Chemicals:

[γ^{32}P] ATP (3000 Ci/mmol), and [α^{32}P] dATP (3000 Ci/mmol) were purchased from Board of Radiation Isotope and Technology (BRIT), Trombay India. Monoclonal anti-phosphotyrosine antibody (PY-99) was procured from Santa Cruz Biotechnology, USA. Nitrocellulose membrane was from Pall Biosciences, USA. The charged nylon membrane Hybond, Sephadex G-25, Sephadex G-50 and DEAE Sephacel were procured from Amersham Biosciences (USA). Plasmid isolation, gel elution and PCR amplification kits were procured from QIAGEN (USA). Random primer labeling kit and restriction enzymes were from MBI Fermentas (USA). Whatman No. 1 and 3 filter papers and cellulose phosphate P-81 sheets were procured from Whatman, UK. X-Omat AR X-ray film was supplied by Kodak, USA. 20-Hydroxyecdysone, brilliant blue G-250 and Brilliant blue R-250, bovine serum albumin (BSA), bromophenol blue, dithiotreitol (DTT), ethylene di-amino tetra acetic acid (EDTA), ethylene glycol tetra acetic acid (EGTA), freund's complete and incomplete adjuvants, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), high and low molecular weight mass protein markers, leupeptin, phenylthiourea, phosphatidylserine, phenylmethylsulphonyl fluoride (PMSF) streptomycin sulfate, N,N,N',N'-Tetramethylethylenediamine (TEMED) and triton X-100 were obtained from Sigma Chemical Co. USA. TC-100 insect culture medium was purchased from JRH Biosciences Inc. USA. Alkaline phosphatase (ALP) conjugated secondary antibodies (anti- rabbit and anti- mouse) and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) mix were procured from Bangalore Genei, India. Acrylamide, N-N`-methylene bisacrylamide, 2-mercaptoethanol, glycerol, sodium dodecyl suplhate (SDS), glycine, silver nitrate and Tris base were purchased from Sisco Research Laboratories Pvt. Ltd.. India. Agar, 3-(N-morpholino) propanesulfonic acid (MOPS), ampicillin, tetracycline, tryptone and yeast extract were obtained from HiMedia Laboratories, India. All other chemicals used were of analytical grade and were procured from local sources in India.

Primers used:

All the custom designed primers were either procured from Sigma (India) or from MWG Primers (India), while the sequencing was done using the service of Bioserve Biotechnologies (INDIA). The list of primers used for the following study is given below.

(i) For Hexamerin genomic clone isolation:

Hex genomic clone F1	5' AAGACTGTCCTGATCTTAGCGTCG 3'
Hex genomic clone R1	5' TTACTACTTGGCTGATGCT 3'
Hex genomic clone F2	5' AGCATCAGCCAAGTAGTAA 3'
Hex genomic clone R2	5'GACACGTCCTACTATAGTCAA 3'
Genome walking R (gene specific)	5'AAATGTGAAGACCAAAACACTGGC 3'
Genome walking F (adaptor)	5' GTAATACGACTCACTATAGGGC 3'

(ii) For Methionine rich hexamerin cloning:

Met F (degenerate):	5' TACGAAATCTWCCCRTMSTWC 3'
Met R (degenerate):	5' CTTRAADGGMTGGTGRTTSA 3'
Met F (RACE):	5' TCGAACGTCTATCGAACGGATTAGGT 3'
Met R (RACE):	5' TGGATTACGTCTCCCACGGACGAA 3'

(iii) For Hexamerin receptor cloning:

Hex receptor F (degenerate):	5' DFNATGTTYGTNTAYGCNYTNAC 3'
Hex receptor R (degenerate):	5' GAVTAVSWBATGGAYATNACNA 3'
Hex receptor F(RACE):	5' CTTGATGCCAGCGTCCACTGAGGC 3'
Hex receptor R (RACE):	5'GACATCACGAATGCTATGTATCTC 3'
Hex receptor full length F (gene specific):	5' TTCGACTGAGCACGAGGACACTGAC 3'
Hex receptor full length R (gene specific):	5' TTTGTTCCTGTGTCCACC 3'
Hex receptor Expression F (gene specific):	5' GATCCATGGGACTGAGGAGTAGAAAA 3'
Hex receptor Expression R (gene specific):	5' CTGCAGTTAGGTACATCGGTGGAACTT 3'

(iv) RACE primers from BD biosciences (Manufacturer):

GeneRacer F (adaptor**)**	5’ CGACTGGAGCACGAGGACACTGA	3**’**
GeneRacer R (adaptor**)**	5’ GCTGTCAACGATACGCTACGTAAGC	3’

Experimental insect:

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 moth 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 (V^{th}) 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.

Stages of insect 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 (Lakshmi and Dutta Gupta, 1990). 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.

Surgical manipulations and organ culture:

(i) Thorax-ligation:

This was carried using the last (V^{th}) instar larvae to deplete or reduce the titer of endogenous hormone (Dutta-Gupta and Ashok, 1988). 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. The tissues anterior to ligation were cut with sterile scissors and wound was dressed with traces of antibiotic mixture [Penicillin and streptomycin 1:1] and phenylthiourea (0.025%). Finally it was sealed with wax (paraffin and bees wax, 10:1). These ligated larvae were kept in petridishes covered with moist filter paper to prevent desiccation.

(ii) Microinjection:

The appropriate larvae to be microinjected were narcotized on ice and were injected with radioisotope [^{35}S] methionine or hormones (20E and JH) using a microsyringe in a volume of 2-5 µl. The control insect received equal volume of the carrier. The wound was dressed with traces of antibiotic mixture and sealed using sealing wax (Mentioned in above section).

(iii) Organ culture:

The tissues (fat body, salivary gland, ovary and male accessory reproductive gland) were dissected under sterile condition using ice cold insect Ringer (130 mM NaCl, 0.5 mM KCl, 0.1 mM $CaCl_2$). They were rinsed in 100 µl of TC-100 insect culture medium (JRH Biosciences Inc., USA) containing traces of streptomycin sulfate. This was followed by transfer of tissues to fresh 200 µl medium for 1 h for 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 under sterile conditions. After incubation, tissues were rinsed in ice cold insect Ringer and used for homogenate preparation. The choice of TC-100 insect culture medium was mainly because it does not contain insect haemolymph (important for the present study) and was developed to support the growth of lepidopteran cells in culture and is widely used for insect tissue culture (Gardiner and Stockdale, 1975).

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 microfuge 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-Cl (pH 7.4) and were centrifuged at 1,000 Xg for 3 min at 4°C to sediment the haemocytes. This supernatant i.e., the cell free haemolymph samples were used immediately.

Preparation of homogenate sample:

A 2-5% homogenate of desired tissue from appropriate developmental stage was prepared from the ligated or unligated larvae of *C. cephalonica*. The tissues were rapidly dissected in cold insect Ringer homogenized in 150 µl homogenization buffer (10 mM Tris-Cl pH 7.4, 0.1% Triton X-100, 1 mM PMSF, 1 mM EDTA and 1 mM DTT) and centrifuged at 1,000 Xg for 5 min for removal of larger debris. The aliquots of the supernatant were used for various studies such as protein estimation, protein profiling and protein phosphorylation.

Preparation of membrane fractions from different tissues (fat body, salivary gland, MARG, ovary and gut):

The tissues were dissected in ice cold insect Ringer and homogenized (as mentioned earlier) in buffer A [5 mM HEPES (pH 8.5) and 0.1 mM $CaCl_2$]. The membrane fractions

from the total tissue homogenate were prepared according to the method of Fischer *et al.*, (1980) with slight modifications. The fat body homogenate was centrifuged at 1,000 Xg for 10 min at 4°C and the resultant supernatant which was free of cell debris was decanted. The supernatant was further centrifuged at 30,000 Xg 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 [10 mM HEPES (pH 7.0), 2 mM $CaCl_2$, 10% sucrose (w/v) and 0.1% Triton X-100 (v/v)]. This particular fraction was used as membrane preparation and stored at -70°C.

Hormone treatment:

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 working 20E solution 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.

Protein estimation by microprotein assay method:

Protein content of various samples was estimated using the microprotein assay method of Bradford (1976).

(i) Preparation of protein reagent:

10 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 till further use.

***(ii) 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-Cl (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).

Protein profiling by polyacrylamide gel electrophoresis:

(i) Denaturing gel electrophoresis (SDS-PAGE):

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) was carried according to the procedure of (Laemmli, 1970), using 1 cm long, 2.1 % stacking gel (pH 6.8) followed by a 7.5 long, 10% resolving gel (pH 8.8) for hexamerins and 5% resolving gel for the membrane fraction. 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 samples were prepared by mixing an aliquot of the protein sample with sample buffer containing 0.125 M Tris-CI (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.002% bromophenol blue followed by incubation at 100°C for 1 min.

***(ii) 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 resolving gel containing 5% acrylamide, 66 mM Tris-citrate, (pH 9), 0.05% ammoniumpersulfate and 0.045% N, N, N', N'-tetramethylenediamine. The electrophoresis was carried out using disodium tetraborate buffer (4 g/L) at 4 °C and 20 mA for 2-3 h.

(iii) 2- Dimensional gel electrophoresis:

The 2- dimensional electrophoresis was carried according to the method of O'Farrell (1975) with slight modifications using Bio-rad PROTEAN II xi 2-D Cell apparatus.

Gel polymerization: The polymerization of the gels for IEF was done according to the manufactures protocol. The capillary tubes were polymerized using tube gel monomer solution (9.2 M urea, 3 ml acrylamide- bis-acrylamide (30:1), 0.2 ml Biolyte 5/7 ampholyte, 0.8 ml Biolyte 3/10 ampholyte, 1 ml detergent solution containing CHAPS/NP40 made up to the volume of 20 ml with double distilled water).

Pre-focusing: The polymerized capillary tubes were placed in the 2-D cassette and prefocusing was done at 200V/ 1h, with 20 mM NaOH as the upper buffer and 10 mM H_3PO_4 as the lower running buffer.

Sample preparation: Approximately 100 µg of haemolymph, fat body and membrane proteins and 30 µg of pure hexamerin protein sample from rice moth were prepared using 2-D cleanup kit (Amersham Biosciences). These processed samples were dissolved in rehydration buffer (8 M urea, CHAPS, 40 mM DTT, 0.5 % 5/7 and 3/10 ampholyte, 0.002 % bromophenol blue) and used.

Isoelectric focusing: The samples dissolved in the rehydration buffer were loaded on top of the prefocused gel and IEF was carried out with the following voltage, 200V/ 2 h, 500V/ 2 h and 800V/16 h.

Equilibration of the IEF gels: The gels from the tubes were removed according to manufacturer's protocol and were placed in equilibration buffer (6 M urea, 75 mM Tris-HCl pH 8.8, 29.3 % glycerol, 2 % SDS and 0.002 % bromophenol blue) for 10 min. The gels were washed extensively to remove the excess buffer and were transferred on top of the SDS-PAGE. The 2-dimention was run at an initial voltage of 60 for 30 min followed by 100V till the dye front reached the bottom. The gels were removed and silver stained for visualization of protein spots.

Vizualization of electrophoretically separated proteins on polyacrylamide gels:

(i) Silver staining:

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 three washes in 50% ethanol. Subsequently the gel was pretreated with sodium thiosulphate (20 mg/100 ml) for 1 min and rinsed thrice (30 sec each) with distilled water. The gel was impregnated in silver nitrate [0.2% $Ag(NO)_3$) 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) containing 50 µl of 37% formaldehyde (v/v). Finally, the stained gel/s was thoroughly rinsed with distilled water and stored in 50% methanol.

***(ii) 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- R 250 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.

Mass spectroscopic analysis:

Mass spectroscopic analysis and protein identification was carried out using Bruker ultraflex MALDI TOF-TOF using the manufacturer's standard protocol.

Electroelution:

The sample containing the pure hexamerins was separated on a standard 7.5 % SDS-PAGE. The proteins were allowed to overrun for a period of 4 h so that there was a maximum separation. A reference lane containing the same protein sample was coomassie stained for identification of the band of interest. The subunit protein bands thus separated and identified were excised from approximately twenty gels. The gel pieces containing the individual subunit band were placed in a dialysis tube (10 kDa cut-off) individually with elution buffer (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 protein along with the buffer from the dialysis tube was 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 for the generation of antibodies.

Production of polyclonal antibodies:

The antibody was raised against the native hexamerins as well as individual subunits of hexamerins. Three month old male rabbits (New Zealand variety) were injected with 100 µg of protein (emulsified with 500 µl of complete Freund's adjuvant) subcutaneously into several sites on the back. Prior to injection, the lateral ear vein was bled for collection of pre-immune serum. After a fortnight, first 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 second booster injection. The collected blood was left overnight at 4°C for clotting and serum was separated by centrifugation at 5,000 Xg for 20 min. The serum was aliquoted and stored at -20 °C after adding 25% glycerol and 0.001% azide.

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 in 100 ml of 5% acetic acid). The stain was removed by 3-4 washes with TBST [Tris buffered saline with Tween-20, 10 mM Tris-Cl (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 thorough 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 out 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-Cl (pH 9.5), 5 mM $MgCl_2$ and 10 mM NaCl] for color reaction.

In vitro phosphorylation of proteins:

In vitro phosphorylation of homogenate/membrane fractions of fat body and other tissues was carried out as described in Shanavas *et al.,* (1998) with slight modification. A 40 µl reaction mixture contained 10 mM Tris-Cl (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) and/or 20E (80 nM) 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-Cl (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 Xg for 5 min, the supernatant that contained phosphorylated proteins (10 µg) was 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 20E 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).

Autoradiography:

This was employed for detection of [^{32}P] labeled polypeptides or nucleic acids. The gels were sandwiched between cellophane sheets and dried under vacuum at 80°C for 1 h using a Hoefer gel drier. While 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 screen at -70°C, which was then developed and fixed as per standard procedure.

Plasmid DNA isolation:

The bacterial strain containing the recombinant plasmid was allowed to grow in LB/amp (100 µg ampicillin/100 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 Xg 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, the tube was 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-Cl (pH 7.5) and 25 mM EDTA] and mixing the solution by inversion for 4-5 times. The mix was centrifuged at 3,000 Xg for 10 min to separate supernatant which contained plasmid DNA from compact white pellet 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-Cl, (pH 8.0) with 1 mM EDTA].

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 Whatmann-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 was 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 Xg 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 Xg 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 Xg 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-Cl (pH 8.0) with 1 mM EDTA] and stored until use.

RNA isolation:

The desired tissue was dissected under sterile condition in RNase free 10 mM Tris-Cl (pH 7.4). It was rinsed with the same buffer and homogenized (~2-4 mg tissue) in 1 ml of TRI-reagent (Sigma, USA). The homogenate was allowed to stand for 5 min at room temperature followed by addition of 200 µl chloroform and incubation on ice for 5 min. The mixture was centrifuged at 12,000 Xg 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 Xg 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 RNAase contamination by treating the solutions and glasswares with diethyl pyrocarbonate (DEPC) followed by sterilization.

Agarose gel electrophoresis:

(i) ***Plasmid DNA:***

Sample preparation: 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) for electrophoretic separation.

Electrophoresis of plasmid DNA: 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, and polymerized. The electrophoresis was carried out using the same buffer at voltage 5 V/cm^2 until the dye front reached $3/4^{th}$ of the length of the gel. Electrophoretically separated DNA samples were visualized and analyzed using UVP-gel documentation system (Bio-Rad USA).

(ii) Genomic DNA:

Sample preparation: The samples of genomic DNA, digested or undigested were prepared as mentioned above. The digestion of genomic DNA was carried out overnight at 37°C with the chosen restriction enzyme(s).

***Gel electrophoresis of genomic DNA*:** The genomic DNA was electrophoresed using 0.8% agarose which was prepared as mentioned above in plasmid DNA electrophoresis section.

(iii) RNA electrophoresis:

Sample preparation: The samples were prepared by mixing (15-20 µg) of RNA, 12.5 µl formamide, 2.5 µl 10x 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.

***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 35 ml of DEPC-treated sterile distilled water. It was boiled at 100°C and cooled till 60-65°C. To it, 5 ml of 10x gel buffer and 9 ml of formaldehyde were added and the volume of the sample was made up to 50 ml after adding ethidium bromide (0.5 µg/ml). This was followed by polymerization of 10 cm long gel. The electrophoresis was carried using 1x gel buffer at voltage 5 V/cm^2 until the dye reached the end of the gel. The ethidium bromide stained gels were visualized analyzed using UVP-gel documentation system (Bio-Rad, USA).

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-Cl (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 dampened 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 3 M NaCl.

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 (175.3 g NaCl, 88.2 g sodium

citrate dehydrate for 1 liter) 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.

Preparation of radiolabeled probe:

PCR amplified or restriction digested inserts of the DNA of interest were eluted from agarose gel and used for preparation of the probe. The DNA was radiolabeled using hexaLabel^{TM} DNA labeling kit from MBI Fermentas and [αP^{32}] dATP following the manufacturer's instructions. The labeled DNA was either used for hybridization reaction directly or stored at -20^0C for future use within the period of first half-life of the radiolabel. Before adding the probe to the hybridization buffer, the probe DNA was denatured in a boiling water bath for 5 min and snap cooled on ice.

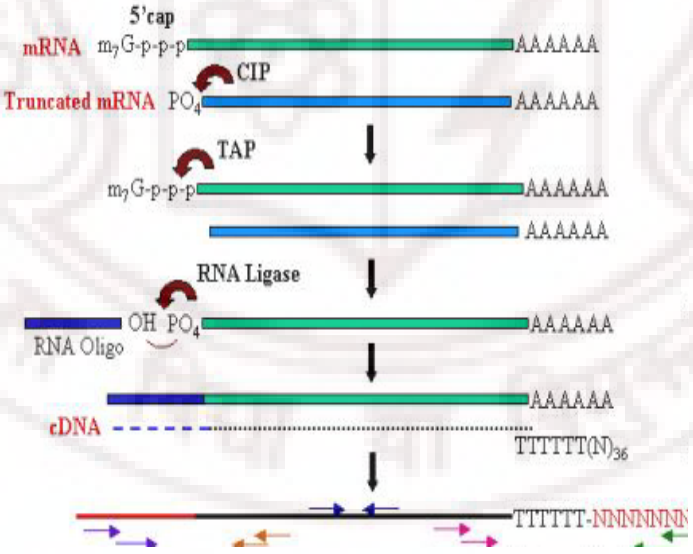
Nucleic acid hybridization:

Hybridization of both, Southern and northern blots, was performed in bottles in a rotisserie device at 65^0 C. For hybridization, modified Church and Gilbert buffer comprising of 0.5 M phosphate buffer, (pH 7.2), 7% sodium dodecyl sulfate and 10 mM EDTA (Church and Gilbert, 1984) was used. Appropriate volume of buffer, atleast 70 µl/cm^2 of the blot, was used for hybridization. The blot was pre-wetted in water in a dish then transferred to 2x SSC, rolled and kept in a clean, dried hybridization bottle with the nucleic acid side upward. Usually 20 ml of buffer was pre-warmed and poured in the bottle. Sonicated salmon sperm DNA (200 µg) was boiled for 5 min, quickly chilled on ice and added in the prehybridization buffer to reduce the background and nonspecific binding. The blot was prehybridized for 4 h along with rotation at 2-3 rpm. A small volume of prehybridization buffer was taken out in a 15 ml disposable tube and denatured probe was mixed with it and was poured back in the hybridization bottle. The hybridization was carried out for 12-16 h. Blots were washed with excess of (at least 1-5 ml/cm^2) wash buffer twice 5 min each in 2x SSC containing 0.1% SDS, followed by 1x SSC, containing 0.1% SDS for 15 min, and finally with 0.1x SSC containing 0.1% SDS for 10 min, at 65^0 C. Blots were exposed to Kodak X- OMAT X-ray film with

intensifying screen and stored in -70^0C for required time period. Cassettes were removed from -70^0 C and allowed to come to room temperature before the films were developed and fixed, using x-ray film developer and fixer. Autoradiogram thus obtained were washed thoroughly under running tap water and dried for storage.

5' and 3' amplification of cDNA ends (RACE):

5' and 3' RACE was carried out following the manufacturer's instructions (Invitrogen). A flow chart for describing the different steps is presented below. The internal primers designed for this purpose from the *Hex 2* cDNA are presented in "primers used" section of this chapter. The RACE adaptor primers were supplied with kit. The total RNA (5 µg) was taken and sequentially subjected to dephosphorylation, decapping and RNA–oligo ligation. The processed RNA was subjected to reverse transcription using anchored oligo dT primer. The first PCR reaction for 5' RACE was performed with gene specific reverse primer and Generacer™ 5' primer, while that of 3' RACE was carried out with gene specific forward primer and Generacer™ 3' reverse primer. The RACE products obtained so were cloned in pTZ57R/T vector and sequenced.



Diagrammatic representation of the strategy followed for RACE and cloning of RACE products: 5 µg of the total RNA was treated sequentially with calf intestine phosphate (CIP) for the removal of the exposed 5' PO_4 groups from the truncated RNAs, followed by tobacco mosaic acid phosphatase (TAP) for the removal of the cap structure from the 5' ends of intact RNAs, and finally the ligation of an RNA oligomer of known sequence to the newly exposed 5' PO_4 end. Finally the reverse transcription was carried out using anchored oligo dT primer. The reverse transcribed product was used in the PCR reaction.

Polymerase chain reaction (PCR):

The basic protocol, which served as a guideline and a starting point for the PCR amplification is stated below. However, the optimal conditions (incubation time, temperatures, concentration of $MgCl_2$, template DNA and Taq DNA polymerase) were altered according to the need. The following components were added to a sterile 0.2 ml microcenntrifuge tube kept in ice. 5µl 10x buffer mix, 1.5 µl 50 mM $MgCl_2$, 1 µl of 10 mM dNTP mix , 1 µl primers (10 pMol each) and 1 µl Taq DNA polymerase. The reaction was finally made to the volume of 50 µl by the addition of nuclease free water.

The reactions were carried out according to the following protocol with the necessary changes in incubation time if needed. (i) initial denaturation at 94^0 C for 2 min, (ii) denaturation at 94^0 C for 45 sec, (iii) primer annealing at 50- 60^0 C for 30 sec, (iv) primer extension at 72^0 C for 1min /kb and (v) final extension at 72^0 C for 15 min. The steps (ii) – (iii) were repeated for 34 cycles. The PCR reaction products were either stored at -20^0 C or analyzed on agarose gel electrophoresis**.**

Reverse transcription for preparation of cDNA:

For reverse transcription of RNA to cDNA, the following components were mixed in a RNase-free sterile 0.2 ml microcentrifuge tube, keeping all the components on ice. 1-5 µl of total RNA (1 µg/µl), 1 µl oligo-d $(T)_{18}$ (500 ng/µl), 1µl 10 mM dNTP mix and DEPC-treated water to a final volume of 12 µl**.** The components were mixed thoroughly by pipetting up and down and briefly spun down. The RNA sample was denatured at 65^0C for 5 min, snap chilled on ice for 2 min and spun down briefly. To this 8 µl of master mix containing 4 µl of 5x first-strand buffer, 2 µl 0.1 M DTT, 1 µl RNAse Out (40 U/µL) and 1 µl M MuLV reverse transcriptase (200 U/µl) was added. Once again components were mixed well by pipetting up and down and centrifuged briefly. The reaction mixture was incubated at 42^0C for 50 min using a thermal cycler, with a heated lid at 100^0C. Subsequently RT reaction was inactivated at 70^0C for 15 min, chilled on ice for 2 min and centrifuged briefly. This RT product was used directly for PCR. After inactivation, 1 µl of RNAase H (2 U/µl) was added to each tube and

incubated at 37^0C for 20 min to degrade the mRNA. The final product was either stored at -20^0C or used immediately for PCR reaction.

Gel elution of DNA:

Gel Cleanup kit from Sigma (USA) was used for extracting DNA from agarose gel, exactly following manufacturer's instructions. The DNA was eluted in 20-40 µl (depending on the initial concentration of DNA) of prewarmed sterile water. 2 µl of eluted DNA sample was run on an agarose gel for checking the concentration and quality before using it for further work. The DNA samples were then stored at -20^0C.

Cloning of PCR product into InsT/Aclone™ vector pTZ57R/T:

DNA polymerases (e.g. *Taq*) that are lacking 3'-5' exonuclease activity possess deoxynucleotidyl transferase activity in addition to primer extension activity, which frequently results in the addition of extra adenines at 3'-ends of amplified DNA molecules. InsT/AcloneTM PCR product cloning kit was used for one-step cloning of *Taq* amplified, gel purified PCR fragments into a specialized vector pTZ57R/T, containing 'T' overhangs at the ends of the linearized vector for easy ligation with PCR products having 'A' overhangs. PCR amplified fragments were either column purified or separated on a 0.8% agarose gel, required fragments were excised and DNA was purified using DNA binding affinity matrices. The products were finally eluted in 20-30 µl of sterile water and used in next step for cloning. About 0.165 mg (0.18 pmol ends) of pTZ57R/T DNA in 3 µl was taken into a 1.5 ml centrifuge tube. To this, 12 µl (approximately. 0.54 pmol ends) of PCR product was added and mixed thoroughly by pippetting up and down. Then 2 µl of 10x ligation buffer [400 mM Tris-Cl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP (pH 7.8) at 25^0 C], 2 µl of 50% (w/v) PEG 4000 and 1 µl of T4 DNA ligase enzyme [5 U/µl in storage buffer 20 mM Tris-Cl (pH 7.5), 1 mM DTT, 50 mM KCl, 0.1 mM EDTA and 50 % glycerol] were added. Contents of the tubes were mixed thoroughly, briefly spun down and incubated at 22^0C for 16 h. Ligation products were stored at -20^0C until used for transformation of DH5α cells.

Preparation and transformation of competent cells:

Competent cells of *E. coli* [DH5α and BL-21 (DE3) pLysS strains] were prepared following the protocol of Ausubel *et al*. (1994), with slight modification using $CaCl_2$ method. Competent cells of DH5α and BL-21 (DE3) pLysS (200 µl) were transformed using 10 µl of ligation product and transformed cell were selected using appropriate marker following blue white selection.

Transformation of BL-21 (DE3) pLys S cells with cloned plasmid and over expression of recombinant protein in bacterial system:

Competent cells of BL21 (DE3) pLys S cells were prepared as mentioned above. The bacterial culture (without vector) was grown in chloramphenicol (17 µg/ml of LB). Following transformation, colonies were observed after 16 h. For over expression of the recombinant protein, 1 ml over night grown inoculum was used for inoculation of 100 ml LB with appropriate antibiotics till OD_{600} reached 0.6-0.8. A stock solution of 1 M IPTG was prepared in sterile water and from this a required volume for obtaining a final concentration of 1 mM added to the grown culture for induction. The bacterial culture was induced for 1-12 h, depending on the requirement. The bacterial cells were harvested and then resuspended in PBS ($1/20^{th}$ volume of original culture) and sonicated (30 sec for 3-5 pulses). The presence of recombinant protein was analyzed by SDS-PAGE.

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 for comparision of the sequence results with other available cDNAs in the database by BLAST search (Altschul *et al.*, 1990). A multiple sequence alignment of *C. cephalonica,* hexamerin receptor and the methionine rich hexamerin deduced amino acids in FASTA format with best matching invertebrate sequences was carried out 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) were used for determination of 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 hexamerin receptor.

Purification of hexamerins from the haemolymph:

The hexamerins were purified using two step purification protocol of (Arif *et al.,* 2003) that includes gel filtration and anion exchange chromatography (Fig. 1). The crude haemolymph (lane 1) when subjected to gel filtration yielded fraction which shows the presence of hexamerins with minor contaminants (lane 2). When, the fraction fraction was loaded on to DEAE- Sephacel ion exchanger and eluted using a salt gradient pure hexamerin protein was obtained (lane 3).

Specificity of polyclonal antibodies generated against hexamerins:

For this the haemolymph and the fat body proteins were electrophoresed and transferred on to the nitrocellulose membrane and probed with the polyclonal antisera generated against pure hexamerin(s). The result shows that the antibodies generated are fairly specific as they crossreacted selectively with hexamerins in haemolymph (lane 1) and fat body (lane 2) respectively (Fig. 2b).

Western analysis of haemolymph proteins separated by 2-D electrophoresis:

The haemolymph proteins were separated using 2-D gel electrophoresis and transferred on to the nitrocellulose membrane and detected with the hexamein antibodies. The results presented in figure 3 show the 2-D profile of haemolymph proteins (a) and the cross reactivity of two distinct spots with hexamerin antibodies (b) which corresponds with two distinct subunits of hexamerin.

Identification of hexamerins in Corcyra as heteromers:

Hexamerins from other insects have been reported to be either homomers or heteromers, however there nature in *C. cephalonica* is not known. So, we made an effort to find out whether the hexamerins in the present insect are homomers or heteromers. The purified hexamerins were subjected to native gel electrophoresis,

which shows a single band (Fig. 4a). This native band was cut, electro-eluted and analyzed on SDS-PAGE under reducing condition. The separation shows two protein bands with molecular weight of 84 and 86 kDa suggesting that hexamerins of Corcyra are heteromers. The 84 kDa subunit was earlier identified as arylporin, for which cDNA is already available (Nagamanju *et al.,* 2003)

Developmental profile of hexamerins in haemolymph:

The haemolymph from different developmental stages (ELI, LLI, PP and pupa) was collected and equal quantity of protein (30 µg) was loaded and analysed by SDS-PAGE and western blotting. The results (Fig. 5) show that the concentration of hexamerins in haemolymph is fairly high at the LLI stage (lane 2) as compared to the ELI stage (lane 1) or (lane 3) PP or pupal (lane 4) stage, suggesting that the maximum synthesis as well as its release occurs at the LLI stage.

Developmental profile of hexamerins in fat body:

The results obtained from the SDS-PAGE and western analysis of fat body proteins from different developmental stages (Fig. 6) show the presence of high concentration of hexamerins at pre-pupal stage (lane 4) when compared with other developmental stages (lanes 1, 2, and 3). The presence of large quantity of hexamerins in the fat body at pre-pupal stage is mainly due to the sequestration of the protein from haemolymph like many other lepidopteran insects (Ismail and Dutta- Gupta, 1988).

Isolation of hexamerins subunits:

Since the hexamerin(s), as a native protein showed a precise developmental pattern of synthesis, release and uptake, we wanted to see whether the subunits also follow the same suit. In addition to it, the pure subunits were also required for preparation of affinity matrix for the purification of hexamerin receptor. The hexamerin subunits (84 and 86 kDa) were purified by overrunning the pure hexamerins on a standard SDS-

PAGE. As seen in the figure 7 (a) the two subunits of hexamerins resolved fairly well on the 7.5 % gel. The bands were cut and eletro-eluted individually. Figure 7(b) shows the purity of isolated subunits with molecular mass of 84 (lane 2) and 86 kDa (lane 3).

Specificity of the polyclonal antibodies developed against individual subunits:

The polyclonal antibodies were raised against the purified subunits and the cross reactivity was checked against both the subunits. As seen in figure 8 (b) the antibodies generated against the 86 kDa subunit specifically cross reacted with the 86 kDa subunit, whereas the antibodies raised against the 84 kDa subunit cross reacted with both of them (Fig. 9b).

Developmental profile of 84 and 86 kDa hexamerin subunits in fat body and haemolymph:

This was carried out using the antibodies generated against 84 and 86 kDa subunits. The results presented in figure 10a and b reveal that the developmental pattern obtained was similar to that of the native protein. There is a stage specific increase in the concentration of individual subunit. The LLI larval stage shows the highest hexamerin concentration when compared with the ELI and MLI larval stages (Fig. 10a and b). Further the fat body profile (Fig. 11a and b) also suggest, that the synthesis and release during the late-last instar larval development is higher than the ELI and MLI larval stages. This was true in case of both the subunits

In vivo effect of JH and 20E on the haemolymph hexamerin profile:

The above studies showed a developmental regulation of hexamerins, hence we wanted to find out the role of morphogenic hormones 20E and JH if any on the synthesis of hexamerins. Physiological concentrations of 20E and JH were injected into the LLI larvae. After an incubation of 6 h the haemolymph was collected and analyzed using SDS-PAGE. The results presented in the figure 12 (a) show that in the JH treated

insects (+JH) there is a marked decline in the hexamerin content of haemolymph when compared with the control (c) which was treated with carrier (acetone). On the other hand in the 20E treated insect (Fig. 12 b) there is an increase in hexamerin content (+ 20E) suggesting that 20E has a stimulatory effect on synthesis and release of hexamerins.

In vivo effect of 20E on the hexamerin synthesis and release into the haemolymph:

As the 20E treatment caused significant increase in the hexamerin content in the haemolymph, the work was further extended to study the 20E dependent hexamerin synthesis and regulation during the last instar larval development. For this study either MLI or LLI larvae were ligated, and 24 h post ligated larva was injected with 10 µl of 20E (80 nm). The control insects received equal volume of solvent (10 µl of 0.05% ethanol). The insects were incubated for a period of 6 h followed by isolation of haemolymph and the fat body. Electrophoresis was carried out using equal concentration of protein and the results are presented in figure 13 (a and b). The results clearly show that 20E treatment causes an increase in the hexamerin content of the haemolymph (Fig. 13a) at both the MLI and LLI stages (lanes 2 and 4 respectively). Similar results were also seen with the fat body (Fig. 13b) where the hexamerin content of 20E treated larvae (lanes 1and 3) is higher than that of the control (lanes 2 and 4).

In vitro effect of 20E on the hexamerin synthesis and release using organ culture:

The fat body from the MLI and LLI larvae was dissected under sterile condition. The tissue was rinsed thoroughly in insect ringer for removal of adhered hexamerins. After this the tissue was incubated in the TC-100 insect culture medium for 2 h as a pre-conditioning step for the removal of endogenous hormone. Once this was achieved the tissue was transferred to fresh medium (200 µl) and 20E at a final concentration of 80 nM was added in the experimental cultures, while equal volume of solvent (0.05%

ethanol) was added to the control. The samples were incubated at 26° C with agitation for 6 h. After the incubation the tissues were removed and homogenized. Equal amount of protein was loaded on to a SDS- PAGE and electrophoresis was carried out (Fig 14). There was a distinct increase in the hexamerin content of 20E treated fat body cultures (lanes 1 and 3) when compared with the controls (lanes 2 and 4) in both the MLI and LLI larval stages, suggesting that 20E stimulates the synthesis of hexamerins in *C cephalonica.*

Radiolabelled methionine incorporation studies to show the effect of 20E on the hexamerin synthesis and release:

The experiment was carried out to show that the increase in haemolymph hexamerin concentration is due to the accelerated synthesis under the influence of 20E. For this radiolabelled ***[*** ^{35} *S]* methionine was injected into the 24 h post-ligated LLI larvae (50,000 Cpm). After an incubation of 2 h the experimental larvae were injected with 20E (80 nM), while controls received equal volume of carrier for a period of either 4 or 6 h. This was followed by the isolation of the fat body. The tissue was rinsed thoroughly in 10mM Tris–Cl buffer (pH 7.4) containing 0.1 % Triton X-100, for removal of any residual proteins that were adhered to it. The tissue was homogenized, protein was quantified and equal amount of protein was loaded on to the gel. The gel was electrophoresed, stained, vacuum dried and exposed to the X-ray film. Finally autoradiogram was developed. The results in figure 15 (a) clearly show that 20E treatment for a period of 4 and 6 h caused a significant increase in hexamerin synthesis (lanes +4 h and +6 h) compared with the controls (lanes -4 h and -6 h). In the continuing experiment, the haemolymph from the above insect were collected and proteins were immunopreicipitated with the hexamerin antibodies. The immunoprecipate was analyzed by SDS-PAGE and autoradiography (Fig.15 b). The intensity of the radioactive signal present in the hexamerin band of hormone treated larvae is significantly high (lanes +4 h and +6 h) when compared with the controls

(lanes -4 h and -6 h). This clearly suggests that the 20E stimulates the hexamerin synthesis and its release in to the haemolymph during late larval development.

Effect of RH 5992 and Azadiractin on hexamerin profile:

Agonist and its inhibitors are widely used for evaluation of various effects of hormones. In the present study the effect of RH 5992 (a non-steroidal ecdysone agonist) and azadiractin a neem based insecticide which blocks the release of 20E was evaluated. Late last instar larvae of *C. cephalonica* were injected with 10 µl of RH 5992 dissolved in DMSO at a final concentration of 80 nM, the control insects received 10 µl of DMSO. After application the larvae were incubated for the required period of time. The haemolymph and fat body samples were collected and analyzed. The result in the figure 16 shows a time dependent (lanes 2 and 4) increase in the hexamerin concentration of the fat body as well as haemolymph. This clearly suggests that the agonist has a stimulatory effect on hexamerin synthesis. For the next experiment 10 µl of azadiractin at a final concentration of 30 ppm dissolved in acetone was applied topically, whereas 10 µl of acetone was applied topically to the controls. The haemolymph was collected over a period of 24, 48, and 72 h after treatment and the samples were subjected to SDS-PAGE. The result in the figure 17 clearly shows that the azadiractin treatment (for 24, 48 and 72 h) causes a time dependent decline in the hexamerin content of the haemolymph as compared to the controls.

Northern analysis showing the effect of 20E and RH 5992 on the hexamerin (Hex 2b) transcript:

As above mentioned experiment shows a profound increase in the hexamerin content after 20E as well as RH 5992 treatment, we wanted to check whether the increase is due to the increase in translational or due to increased expression associated with higher level of transcript. For this the northern analysis was carried out using fat body tissue from 20E and RH 5992 treated insects. The probe used for the purpose was Hex 2 cDNA clone. The results in the figure 18 show the effect of 20E on MLI as well as LLI

larval stages. The amount of *Hex 2* transcript present in the fat body of 20E treated insects was much higher than in carrier treated controls (-20E). Identical results were also seen with RH 5992 treatment (Fig. 18c and d) in which transcript level was fairly high in experimental insects when compared with controls. For all the experiments rRNA was used as equal loading internal control.

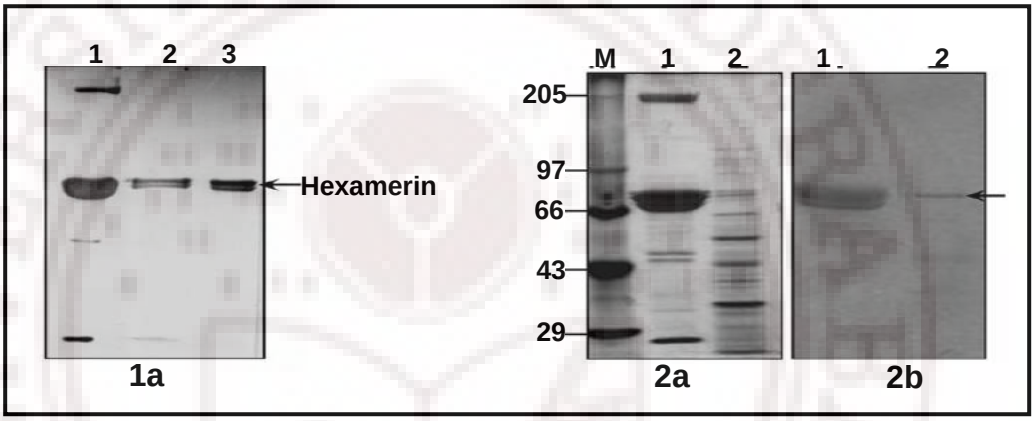
Isolation of Hex 2b genomic clone:

Preceding section clearly suggest that the effect of 20E on the hexamerin synthesis was not regulated at the proteome level, and it was rather transcriptional regulation. Hence an attempt was made for the isolation of the genomic clone of *Hex 2b* gene, for which we already have the cDNA. Two sets of specific primers were designed (i) F_1, R_1 which covered the sequence from the start to middle and (ii) F_2, R_2 which denoted the sequence from the middle to the end region. PCR amplification was carried out using larval fat body DNA as the templete. The result in the figure 19a (lane 1) shows the specific amplification of 1.9 kb fragment with F_1R_1 and 1.6 kb fragment (lane 2) with F_2R_2 respectively. The sequence analysis of these two clone's revealed the presence of complete coding sequence, with 4 exons and 3 introns. The total length of the sequence is 3.5 kb. The three introns that intersperse the coding sequence show conserved intron - exon boundaries.

Genome walking analysis with Hex 2b genomic clone:

Once the full length genomic clone for Hex 2b was obtained, an attempt was made to clone the 5' untranscribed region using genome walking analysis. This was carried out basically to find out if there are any ecdysteriod binding sites (ecdysteriod responsive elements) at the 5' upstream sequence, which regulate the expression of the hexamerin genomically at the transcriptional level. The PCR amplicon obtained using adaptor specific forward primer and gene specific reverse primer is of 800 bp (Fig. 20a). The sequence analysis of the fragment (Fig. 20b) showed the presence of TATA box, E box,

CAAT box and more importantly the ecdysone responsive elements (similar to *Drosophila* hsp 27).



Purification of hexamerins from larval haemolymph of C. cephalonica and generation of polyclonal antibody against it:

Figure 1: SDS-PAGE showing a comparative profile of proteins at various steps of purification from *C. cephalonica* larval haemolymph. (Lane 1- crude haemolymph, lane 2- Sephadex G-150 elute and lane 3- pooled peak fraction elute from DEAE Sephacel ion exchange column.

Figure 2: SDS profile (a) and corresponding western blot (b) of the total haemolymph and fat body proteins. The western blot was probed with antibody raised in rabbit, against the purified hexamerins shown in figure 1, lane 3. Lane M- Molecular weight marker, lane 1-crude haemolymph and lane 2- is the fat body homogenate.

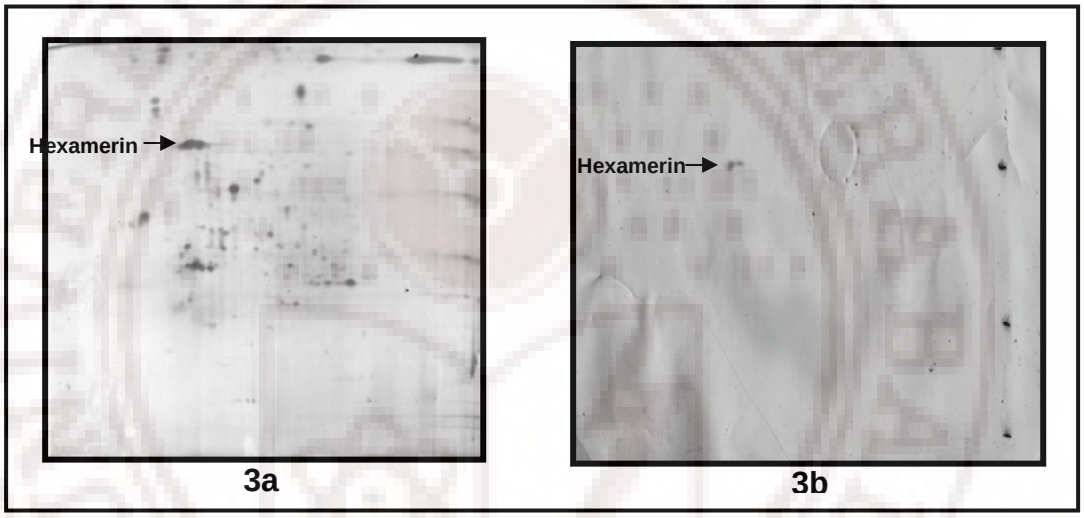


Figure 3: Western analysis of haemolymph proteins separated by 2-D analysis using hexamerin antibodies:

a: 2-D profile of crude haemolymph proteins from last instar larvae.

b: Corresponding western blot, which was probed with the polyclonal hexamerin antibodies. Please note the presence of two distinct spots (subunits) of hexamerin (⟶).

Figure 4: Identification of hexamerins in Corcyra as heteromers:

a: Purified hexamerins were separated on a native PAGE using 5% resolving gel without SDS and reducing agents.

b: SDS-PAGE of the electro-eluted native protein which was resolved as a single band on the native gel. The separation was carried out using 7.5% SDS-PAGE.
Note the presence of two distinct polypeptides (⟶).

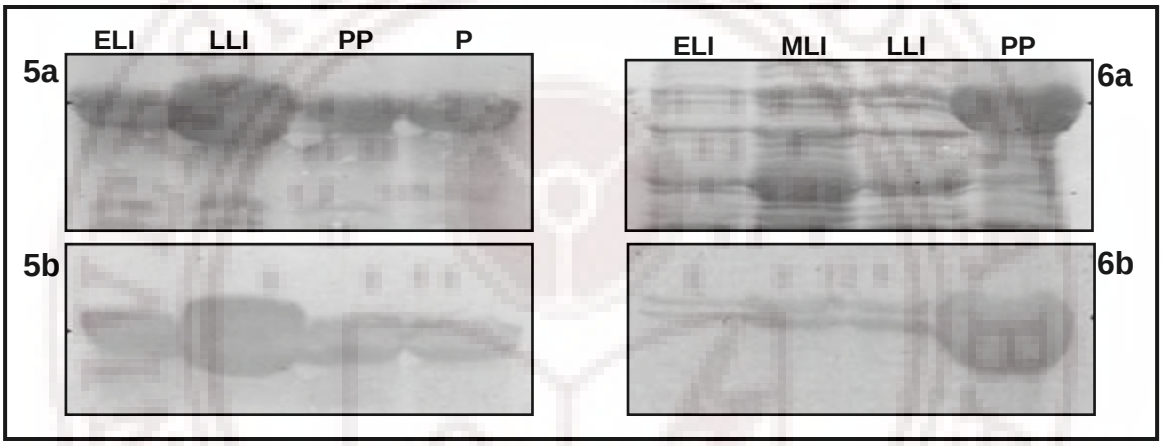


Figure 5: Developmental profile of hexamerins in haemolymph:

a: SDS-PAGE profile of hexamerins at different developmental stages.

b: Corresponding western blot. Detection was carried out using the hexamerin antibodies. Equal quantity of protein was loaded in each lane (30 µg). The western blot shows that the amount of hexamerin present is higher in LLI stage as compared to the ELI stage**.**

Figure 6: Developmental profile of hexamerins in fat body:

a and b: SDS-PAGE profile at different developmental stages (a) and its corresponding western blot (b). The figure shows that there is a higher concentration of hexamerin in the prepupal (PP) stage as compared to the other stages.

ELI- Early-last instar larvae; LLI- late last instar larvae; and PP- prepupae.

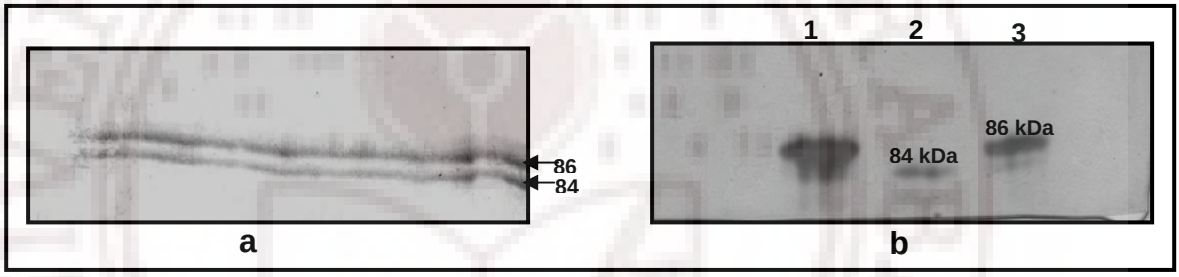
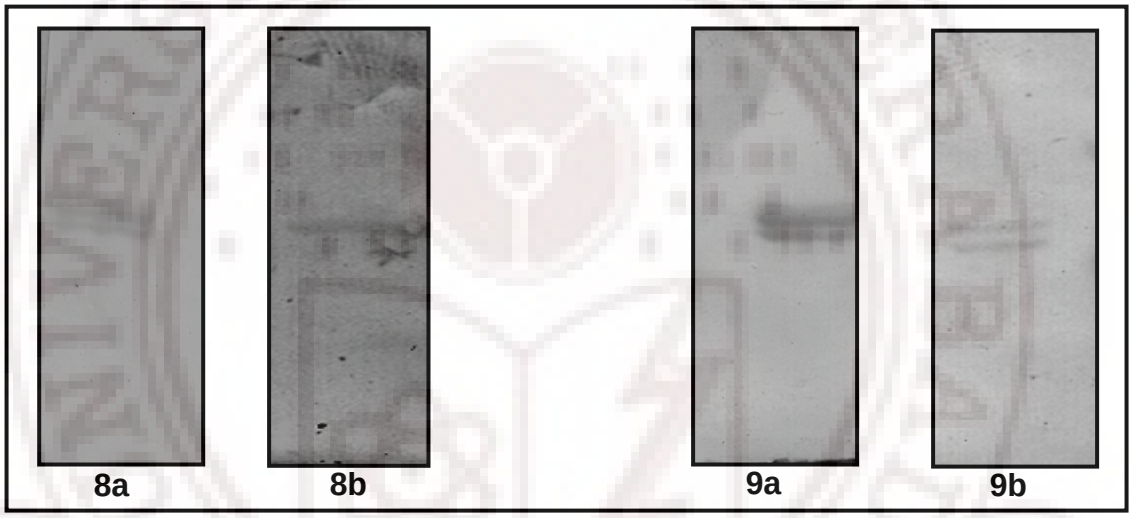


Figure 7: Separation of hexamerins subunits:

a: SDS-PAGE showing the separation of the two subunits of hexamerin. The sample was allowed to over run so that the maximum separation can be achieved. For this experiment the protein were separated on a 20 cm long 7.5% SDS-PAGE, and stained with coomassie blue.

b: SDS-PAGE showing the purity of the electroeluted subunits. Lane 1 is the pure hexamerin with both the subunits, lanes 2, and 3 are 84 and 86 kDa subunits respectively.



Figures 8 and 9: Analysis of the specificity of the polyclonal antibodies generated against individual subunits:

8a and 9a are the Ponceau S stained membrane blots showing proper transfer of hexamerins subunits, while 8b and 9b are the immunoblots using antisera generated against 84 kDa (8b) and 86 kDa (9b). The antibody generated against 86 kDa protein specifically reacted with the 86 kDa subunit alone, whereas the 84 kDa antibodies cross reacted with both the subunit (84 and 86 kDa)

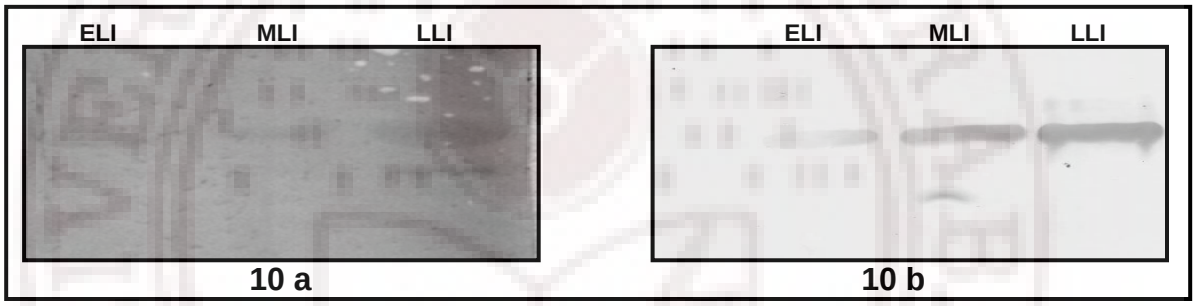


Figure 10: Hexamerin profile in haemolymph detected by 84 and 86 kDa subunit antibodies:

a and b are the western blots showing the amount of hexamerin subunits present at different developmental stages. The analysis clearly shows that the subunits concentration is increasing in a stage specific manner. The concentration of hexamerin is higher at LLI stage as compared to the ELI. The results are similar to the earlier results that involved hexamerins as a whole.

Figure 11: Hexamerin profile in fat body tissue identified by 84 and 86 kDa hexamerin subunit antibodies:

a and b show the western blot analysis of the fat body using 84 kDa subunit and 86 kDa subunit antisera, respectively. The analysis shows that the concentration of subunits increases in a stage specific manner and is similar to the profile of hexamerin in fat body as a whole.

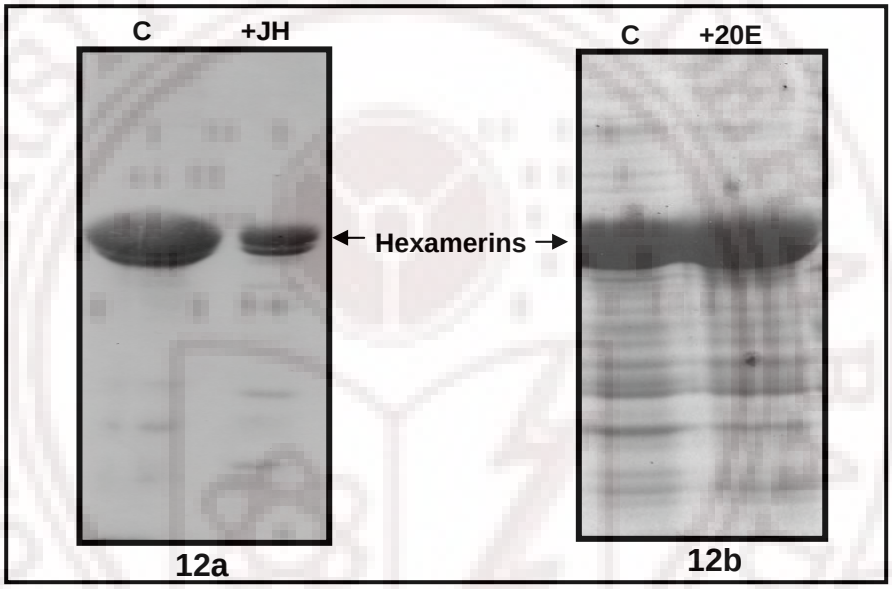


Figure 12: In vivo effect of JH and 20E on haemolymph hexamerin profile:

a: SDS-PAGE showing the heaxamerin profile of haemolymph in the JH treated (+ JH) and control insects (C). A reduction in the hexamerin concentration was observed in the hormone treated insects.

b: The profile of the haemolymph proteins treated with 20E as seen in a SDS-PAGE. Lane (+ 20E) shows an increase in hexamerin concentration when compared with the control (C).

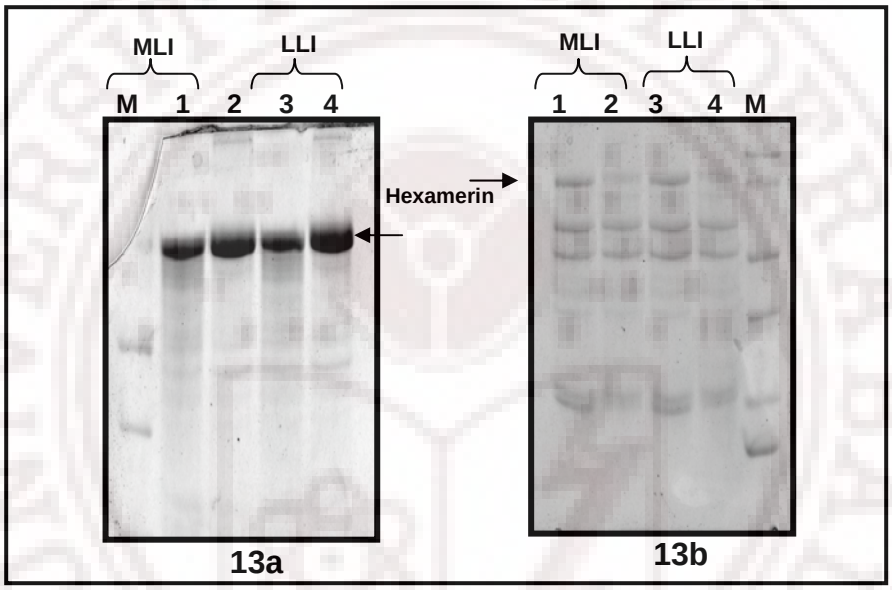


Figure 13: In vivo effect of 20E on synthesis and release of hexamerins:

SDS-PAGE showing the *in vivo* effect of 20E on the hexamerin profile. The 24 post ligated larvae (MLI and LLI) were treated with physiological concentrations of the hormone (80 nM) and incubated for 6 h.

a: Haemolymph profile: Lanes 2 and 4 show the effect of 20E treatment while lanes 1 and 3 are respective controls.

b: Fat body profile: Lanes 1 and 3 are 20E treated while lanes 2 and 4 are respective controls.

Lanes 1 and 2 are from MLI stage, while lanes 3 and 4 are from LLI stage.

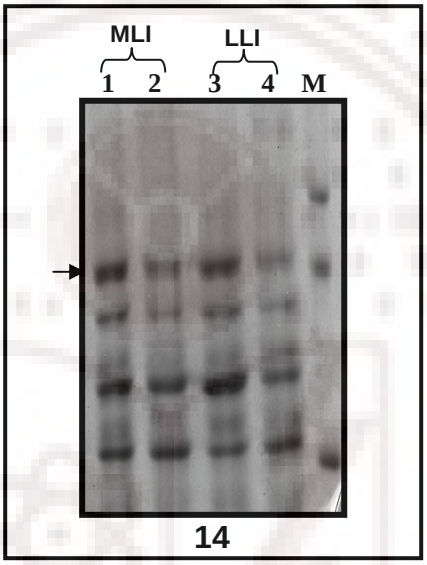


Figure 14: In vitro effect of 20E on hexamerin synthesis and release using organ culture:

Shows the *in vitro* effect of 20E on the heaxmerin synthesis in fat body cultures of 24 h post ligated insects. Lanes 1 and 3 are fat body cultures treated with 20E from MLI and LLI and lanes 2 and 4 are respective controls. M is the molecular weight marker. The figure clearly shows an increase in hexamerin amount (→) in hormone treated cultures.

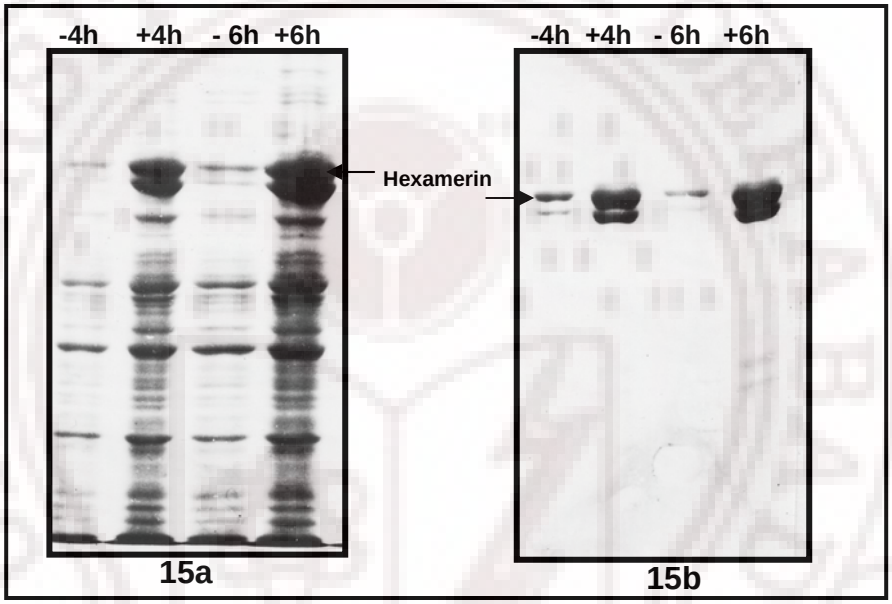


Figure 15: [^{35} S] methionine incorporation studies to show the effect of 20E on the hexamerin synthesis:

a: Autoradiogram showing the 20E induced hexamerin synthesis in the 24 h post-ligated insects that were injected for a period of 4 and 6 h. The lanes +4 h & +6 h are 20E treated and lanes -4 h & -6 h are controls.

b:- Autoradiogram showing the immuno-predicated radiolabelled hexamerin with the hexamerin antibodies. In this equal concentration of haemolymph protein was taken for the precipitation reaction. Lanes +4 h & +6 h and lanes -4 h & -6 h are 20E treated and controls respectively. The 20E treated insects showed an increase in the hexamerin synthesis as compared to the controls, suggesting the stimulatory role of 20E on hexamerins synthesis.

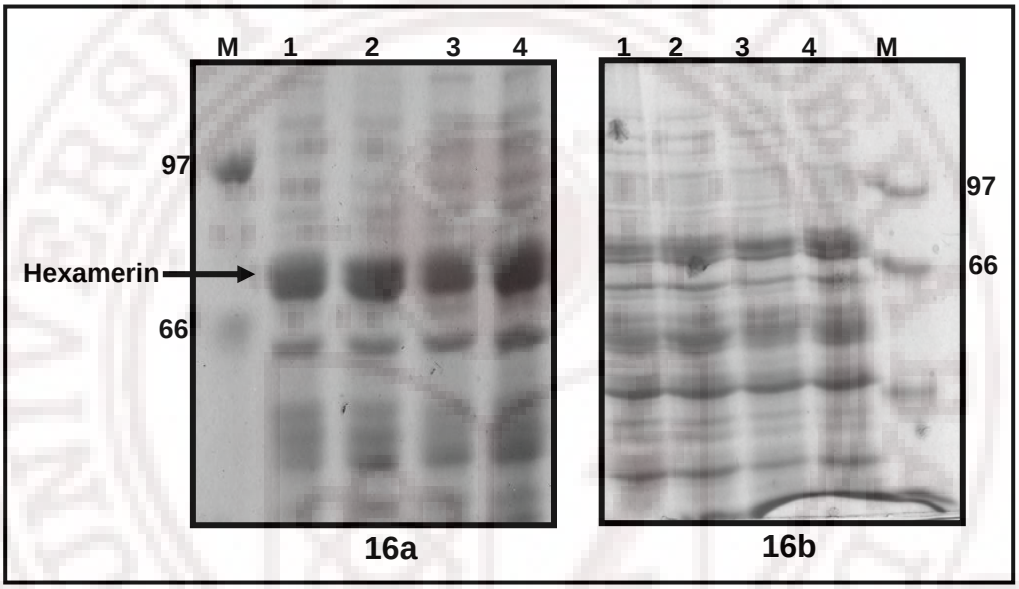


Figure 16: In vivo effect of 20E agonist RH 5992 on the hexamerin synthesis:

SDS-PAGE analysis of haemolymph (a) and fat body proteins (b) after RH 5992 treatment. Note the increase in hexamerin concentration in agonist treated insects (lanes 2 and 4) when compared with the control insects (lanes 1 and 3).

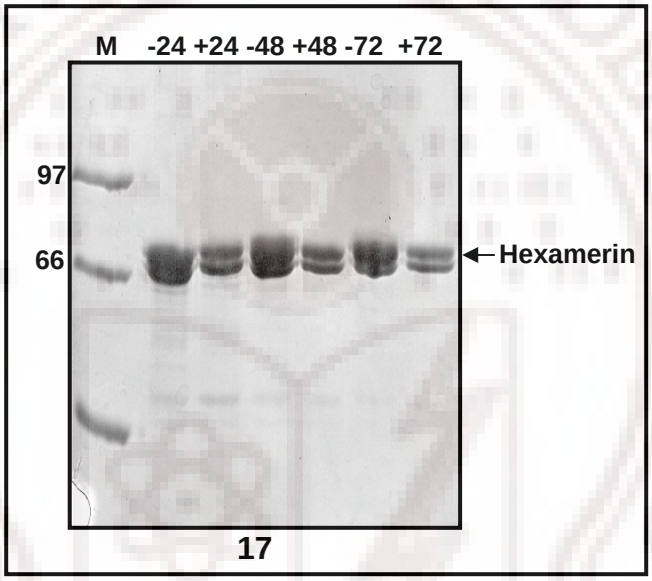


Figure 17: In vivo* effect of Azadiractin on haemolymph hexamerin profile*:

The SDS-PAGE analysis showing the time dependent decline in the hexamerin concentration in the haemolymph after 24 h (+24), 48 h (+48) and 72 h (+72) treatment.

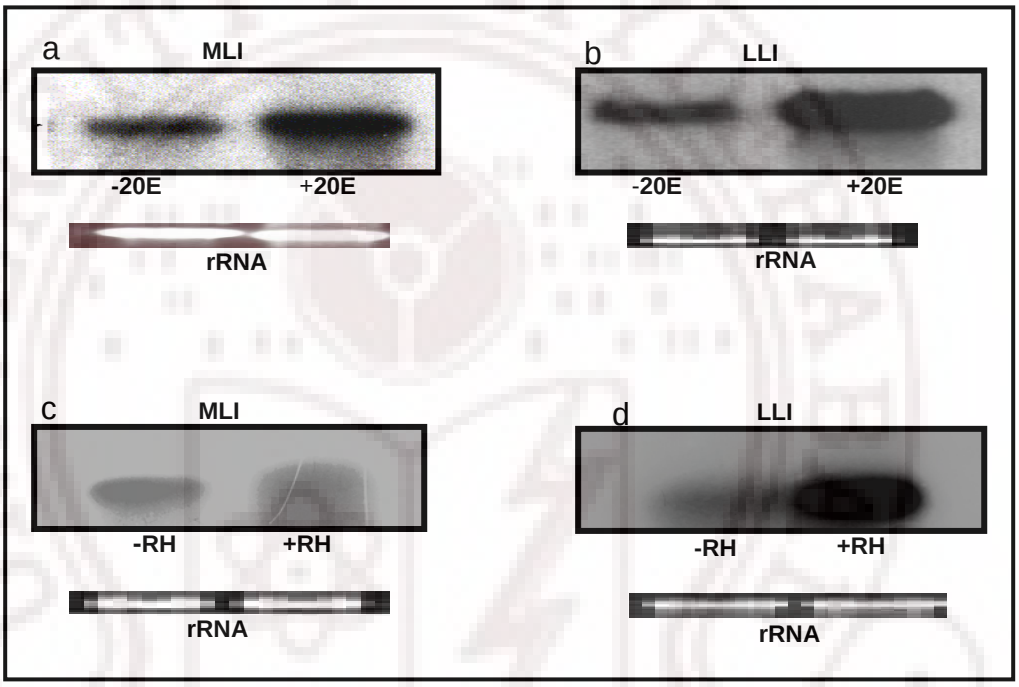


Figure 18: Northern analysis showing the effect of 20E and RH 5992 on the hexamerin transcript:

a and b are the northern blots showing the effect of 20E on the hexamerin transcript in both MLI and LLI larval stages. Higher transcript levels are observed in 20E treated insects (+ 20E) as compared to the controls (- 20E). Identical results were obtained after agonist RH 5992 treatment (c) and (d). Hex 2b cDNA clone was used as a probe (Nagamanju *et al.*, 2003)

Note: rRNA was loaded to show the equal loading of samples.

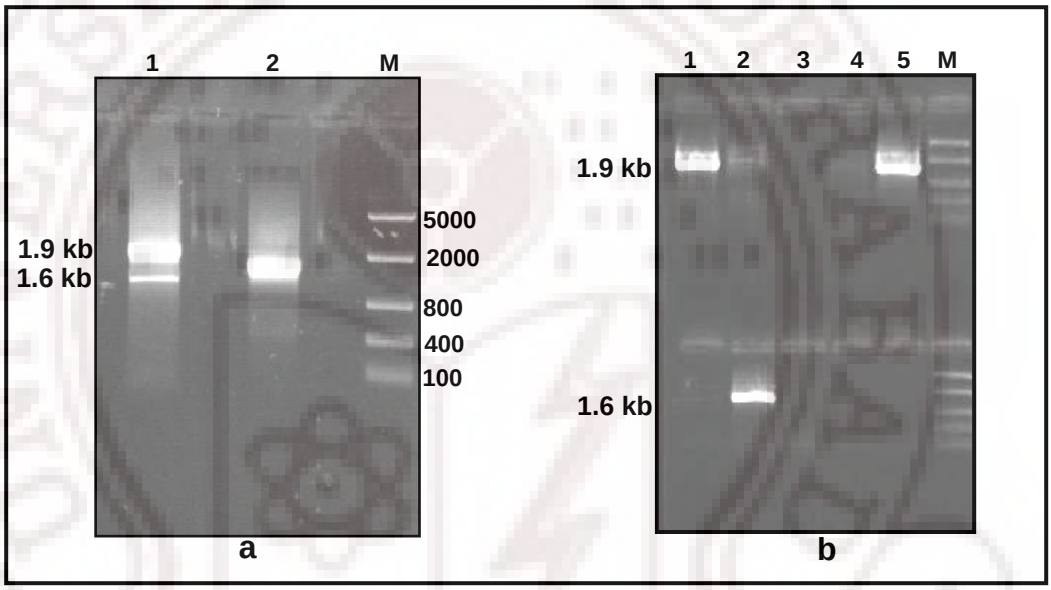


Figure 19: Isolation of Hex 2b genomic clone from C. cephalonica:

a: PCR amplification of 1.9 and 1.6 kb fragments with the primer sets F_1R_1 and F_2R_2 respectively that were designed using the full length cDNA of the hexamerin clone (Hex 2b).

b: Colony PCR of the corresponding clones that were amplified. Lanes 1 and 5 show the colonies with 1.9 kb fragment, while lane 2 shows colony with 1.6 kb fragment. In lanes 3 and 4 there is no amplification.

Figure 19 c: Complete genomic sequence of the Hex 2b hexamerin gene.

The total length of the clone is 3.5 kb, it has 4 exons and three introns with the characteristic intron-exon boundaries.

Blue (exon), black (intron), red (forward primer F_1) brown (reverse primer R_1, the complementary sequence of R_1 primer was used as forward primer F_2) and pink (reverse primer R_2). Intron – exon boundaries are represented as green.

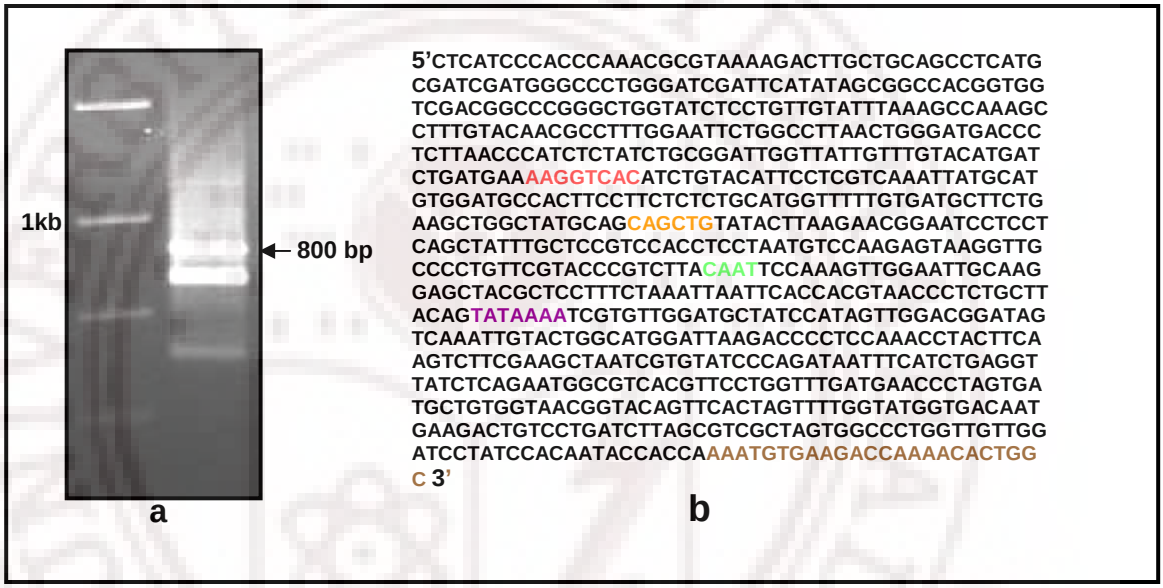


Figure 20: Genome walking analysis:

a: The genomic walking analysis was carried out using the forward adaptor primer (from manufacturer's kit) and gene specific reverse primer. A 880 bp fragment was amplified (→).

b: Sequence of 800 bp amplicon showing various regulatory elements/sequences:

The sequence had the following regulatory sequences.
Red … ecdysone response element (hsp27 form *Drosophila*)
Brown … primary primer
Florescent green ….. CAAT box
Orange ….. E box (CANNTG)
Violet ….. TATA box

Figure 20c: The complete sequence of the Hex 2b genomic clone along with its 5' UTR:

The sequence in the small alphabets is the 5' UTR whereas the capital letters denote the gene sequence with the exons (blue) and introns (black).

Amplification of Corcyra methionine rich hexamerin partial cDNA:

Methionine rich hexamerins are known to be present in large number of lepidopteran insects (Jamroj *et al*., 1996; Pan and Telfer, 1996; Mi *et al*., 1998; Hwang *et al*., 2001; Zhu *et al*., 2002) and their role in reproduction has already been reported (Pan and Telfer, 1996; Seo *et al*., 1998; Wheeler *et al*., 2000). As our group has earlier shown that hexamerins play important role not only in female reproduction but also in male reproduction, an attempt was made to clone methionine rich hexamerin from *C. cephalonica*. For the isolation of cDNA clone different sets of degenerate primers were designed using the homology analysis of the already existing sequences of methionine rich insect hexamerins from the Genbank. Using fat body RNA, reverse transcript was synthesized and PCR amplification was carried out. Figure 21a shows amplification of a 800 bp fragment (lane 1). The fragment was cloned and reconfirmed using colony PCR (Fig. 21b) prior to sequencing. The homology blast and sequence analysis revealed that the fragment belongs to a methionine rich hexamerin (Fig. 21c).

Isolation of full length cDNA clone of methionine rich hexamerin using 5' and 3' RACE:

For the isolation of the full length sequence we employed the rapid amplification of cDNA ends (RACE) strategy using the kit from (Invitrogen Inc.,USA). The 5' end was amplified using the adaptor specific forward primer provided by the manufacturer and the gene specific reverse primer designed from the 800 bp methionine rich hexamerin cDNA clone. Figure 22 shows the PCR amplification of a 600 bp DNA fragment (a) which was cloned and reconfirmed using colony PCR (b). Figure 22(c) shows the nucleotide sequence of 600 bp fragment, which is rich in methionine.

Once the 5' and the middle fragment were available, an attempt was made to clone the remaining fragment using the 3'RACE. This was done using the adaptor specific reverse primer provided in the kit and the gene specific forward primer (designed from the 800 bp fragment), which yielded a 1 kb fragment (Fig. 23a). The fragment was

cloned and its identity was confirmed using colony PCR (Fig. 23b). Figure 23c shows the sequence of 1 kb fragment which was amplified by 3'RACE reaction.

Figure 24a shows the complete cDNA sequence of methionine hexamerin gene of *C. cephalonica.* Detailed analysis of the sequences revealed that the percentage of methionine amino acid present in the sequence is 4.92 %, which is far above than 3.2 % required for a storage protein to be included in the methionine rich hexamerin class. The total length of the cDNA is of 2.5 kb with an ORF of 2.3 kb. It has ATG as the start codon and TAA as the stop codon with the polyadenylation signal. The pair wise alignment of the amino acid sequence of *C. cephalonica* methionine rich hexamerin, with other insect methionine rich hexamerins (Fig. 24b) shows that it has fairly high homology with *C. fumiferena* (75%), followed by *P. interpunctella* (71%) and *P. xylostella* (70%). Detailed phylogenetic analysis (Fig. 24c) further confirms that these proteins have a common origin.

Southern and northern analysis of the methionine rich hexamerins:

The Southern analysis was carried out to find out the copy number of the methionine rich hexamerin gene in *C. cephalonica*. For this the genomic DNA isolated from the larval fat body was digested with different restriction enzymes (Eco R1, Bam H1, Hind III and DRA 1), was electrophoresed and transferred on to nylon membrane. The hybridization was carried out using 5' methionine rich cDNA fragment which was radiolabelled using [α^{32} P]. The results in the figure 25a show a single band in all the lanes (1- 4) suggesting that the methionine rich hexamerin is a single copy gene in *C. cephalonica*. Northern analysis was carried out using fat body RNA isolated from late-last instar larvae, and the results presented in the figure 25b show the presence of a single transcript with a molecular sige of 2.3 kb.

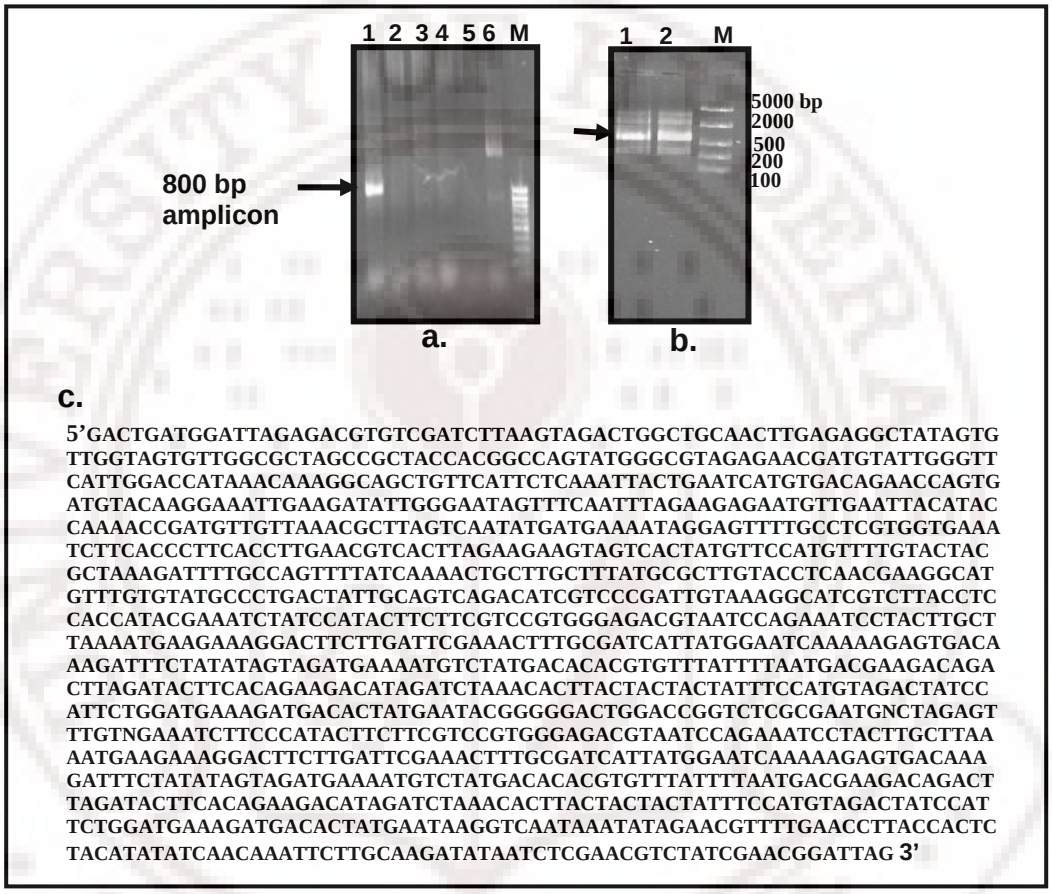


Figure 21: Amplification of 800 bp methionine rich hexamerin cDNA fragment:

a: shows the PCR amplification of an 800 bp amplicon of methionine rich hexamerin cDNA using the degenerate primer (lane 1). The cDNA prepared from the fat body of late last instar larvae was used as the template. No specific amplification was observed in (lanes 2, 3, 4, 5 and 6) where different annealing conditions were employed.

b: Colony PCR showing the presence of the fragment in the vector.

c: The nucleotide sequence of the 800 bp fragment which was amplified.

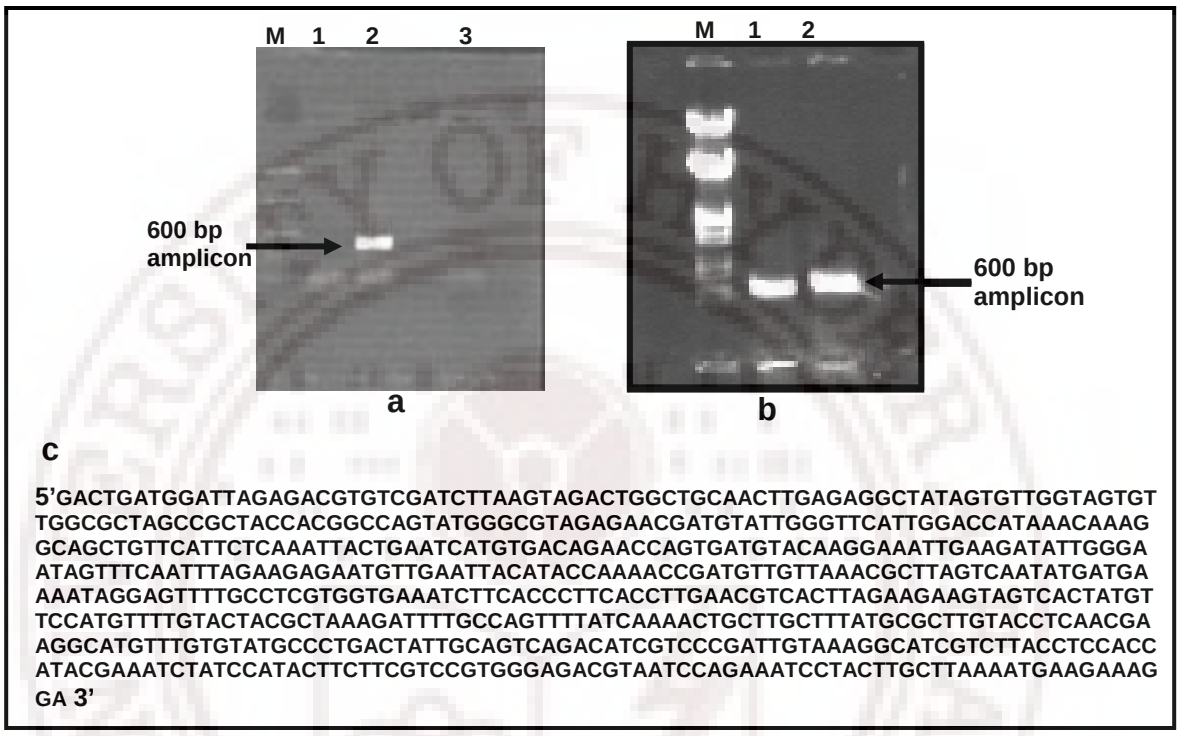


Figure 22: 5' RACE amplification of the methionine rich hexamerin:

a: PCR analysis showing an amplicon of 600 bp (lane 2), which was obtained after 5' RACE reaction using forward adaptor specific primer (5'CGACTGGAGCACGAGGACACTGA3') and reverse gene specific primer (5'TGGATTACGCTCCCACGGACGAA3').

b: Colony PCR confirming the presence of the fragment in the vector (lanes 1 and 2).

c: Nucleotide sequence of 600 bp fragment obtained from 5' RACE reaction.

**5'GACTGATGGATTAGAGACGTGTCGATCTTAAGTAGACTGGCTGCAACTTGAGAGGCTATAGTGTTGGT
AGTGTTGGCGCTAGCCGCTACCACGGCCAGTATGGGCGTAGAGAACGATGTATTGGGTTCATTGGACCA
TAAACAAAGGCAGCTGTTCATTCTCAAATTACTGAATCATGTGACAGAACCAGTGATGTACAAGGAAATT
GAAGATATTGGGAATAGTTTCAATTTAGAAGAGAATGTTGAATTACATACCAAAACCGATGTTGTTAAACG
CTTAGTCAATATGATGAAAATAGGAGTTTTGCCTCGTGGTGAAATCTTCACCCTTCACCTTGAACGTCACT
TAGAAGAAGTAGTCACTATGTTCCATGTTTTGTACTACGCTAAAGATTTTGCCAGTTTTATCAAAACTGCTT
GCTTTATGCGCTTGTACCTCAACGAAGGCATGTTTGTGTATGCCCTGACTATTGCAGTCAGACATCGTCCC
GATTGTAAAGGCATCGTCTTACCTCCACCATACGAAATCTATCCATACTTCTTCGTCCGTGGGAGACGTAA
TCCAGAAATCCTACTTGCTTAAAATGAAGAAAGGA 3'**

Forward adaptor specific primer: 5'CGACTGGAGCACGAGGACACTGA 3'
Reverse gene specific primer : 5'TGGATTACGCTCCCACGGACGAA -3'

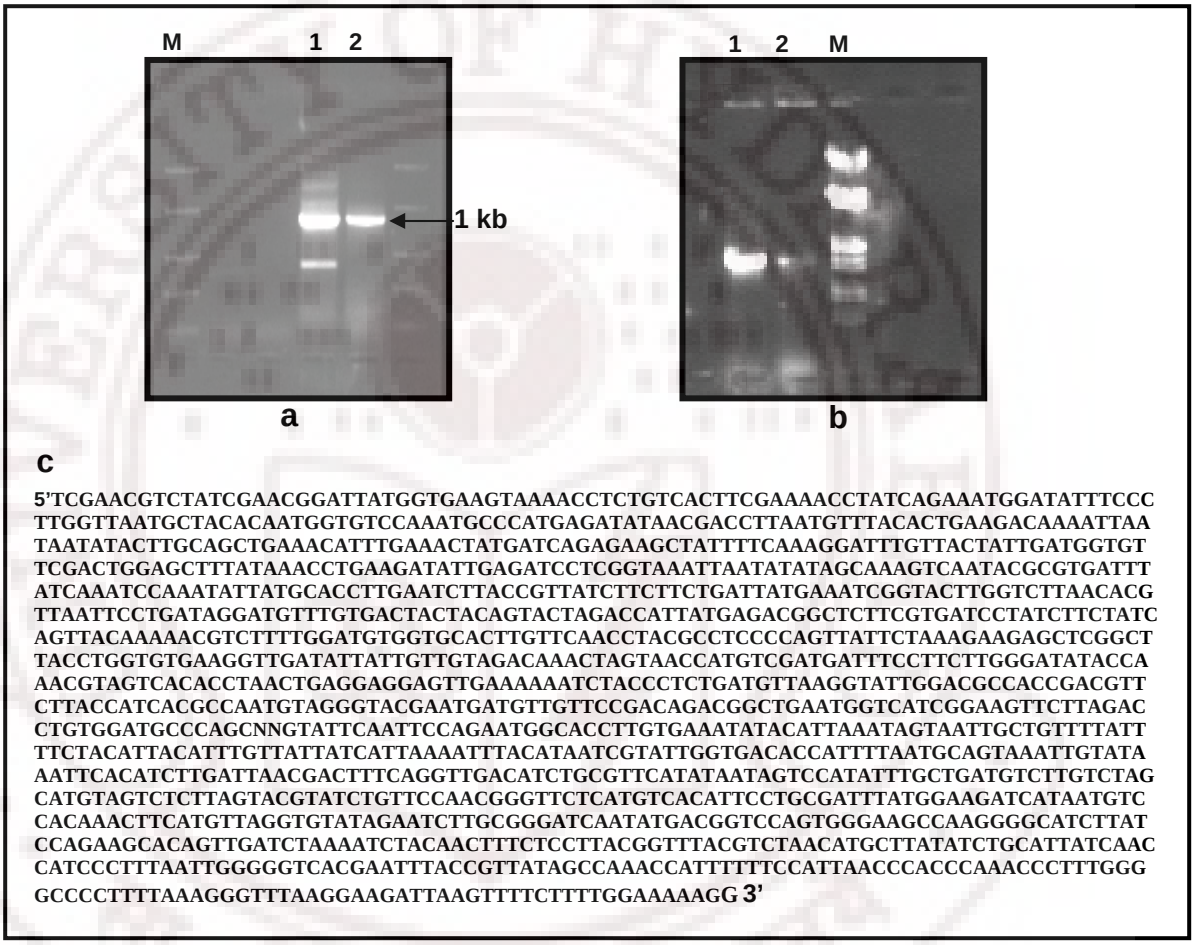


Figure 23: 3' RACE amplification of the methionine rich hexamerin:

Figure a: Amplification of 1 kb fragment (lanes 1 and 2) using the gene specific forward primer (5'TCGAACGTCTATCGAACG 3') and adaptor specific reverse primer (5' GCTGTCAACGATACGCTAGTAACG 3').

b: Colony PCR showing the presence of correct size (1 kb) insert in the vector lanes (1 and 2). M is the molecular weight marker.

c: Nucleotide sequence of the 1 kb fragment obtained by 3' RACE reaction.

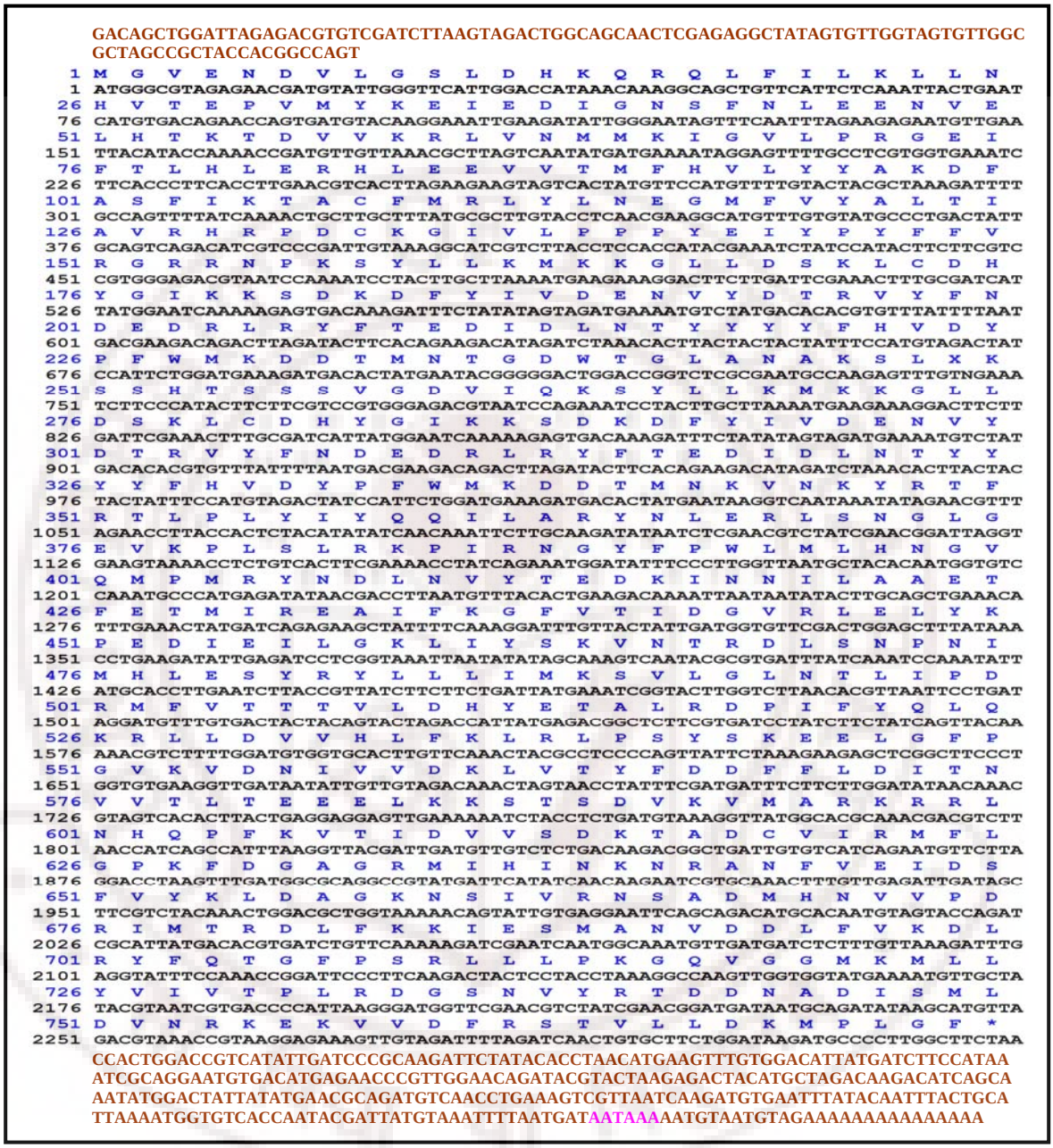


Figure 24a: Complete cDNA clone of the methionine rich hexamerin of C. cephalonica with deduced amino acid sequence:

The isolated clone was 2.5 kb long. It has an ORF of 2.31 kb and short 5' and 3' truncated UTR's.

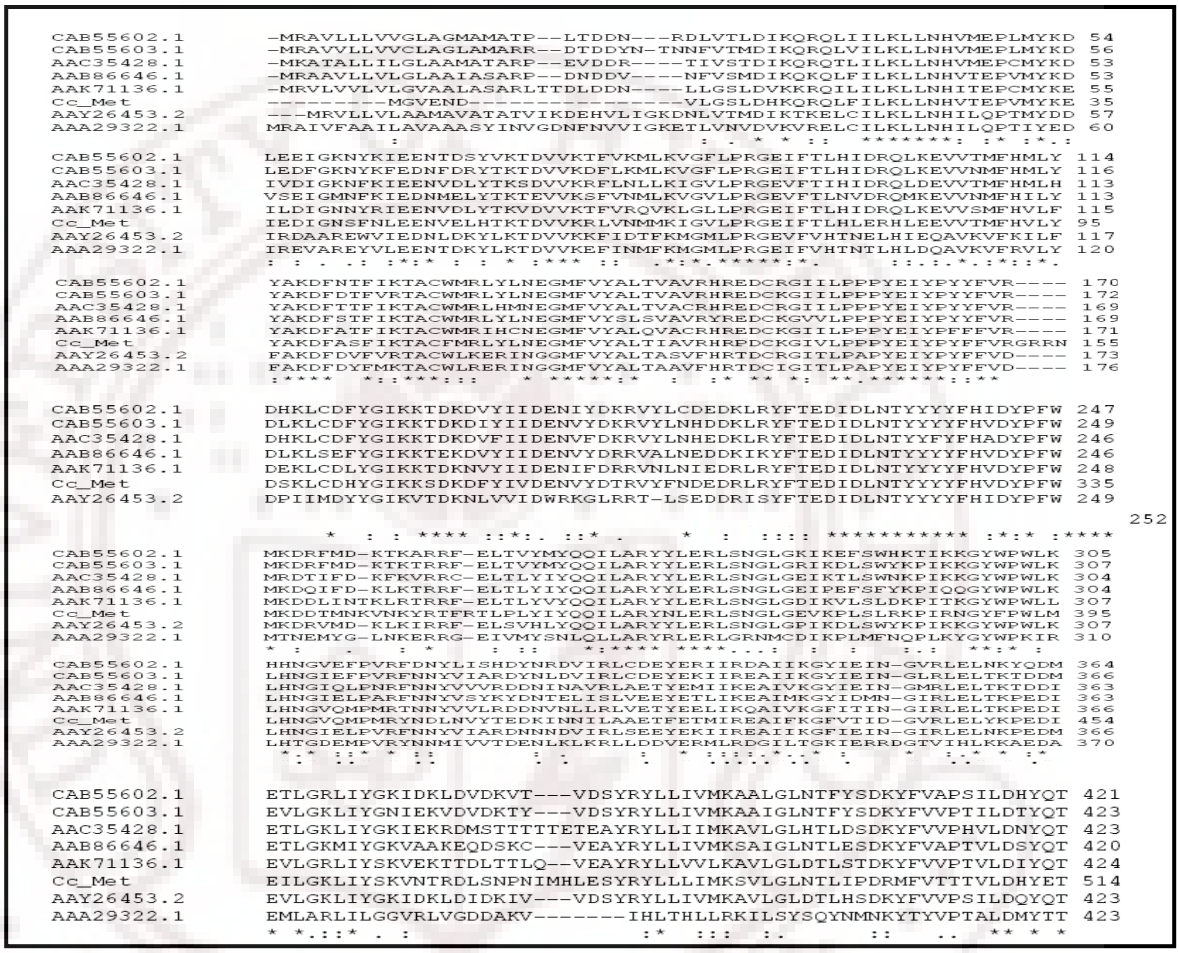


Figure 24b: Alignment of the deduced amino acid sequence of C. cephalonica methionine rich hexamerin with other insect methionine rich hexamerins present in the databank:

CAB55602…… *S. litura* moderatelymethionine rich hexamerin
CAB55603……*S. litura* SP1 methionine rich hexamerin
AAC35428…… *C. fumiferana* SP1
AAB86646…… *H. cunea* methionine rich hexamerin
AAK71136…… *P. interpunctella* methionine rich hexamerin
AAY26453…… *S. nonagrioides* moderately methionine rich hexamerin
AAA29322…… *M. sexta* methionine rich hexamerin

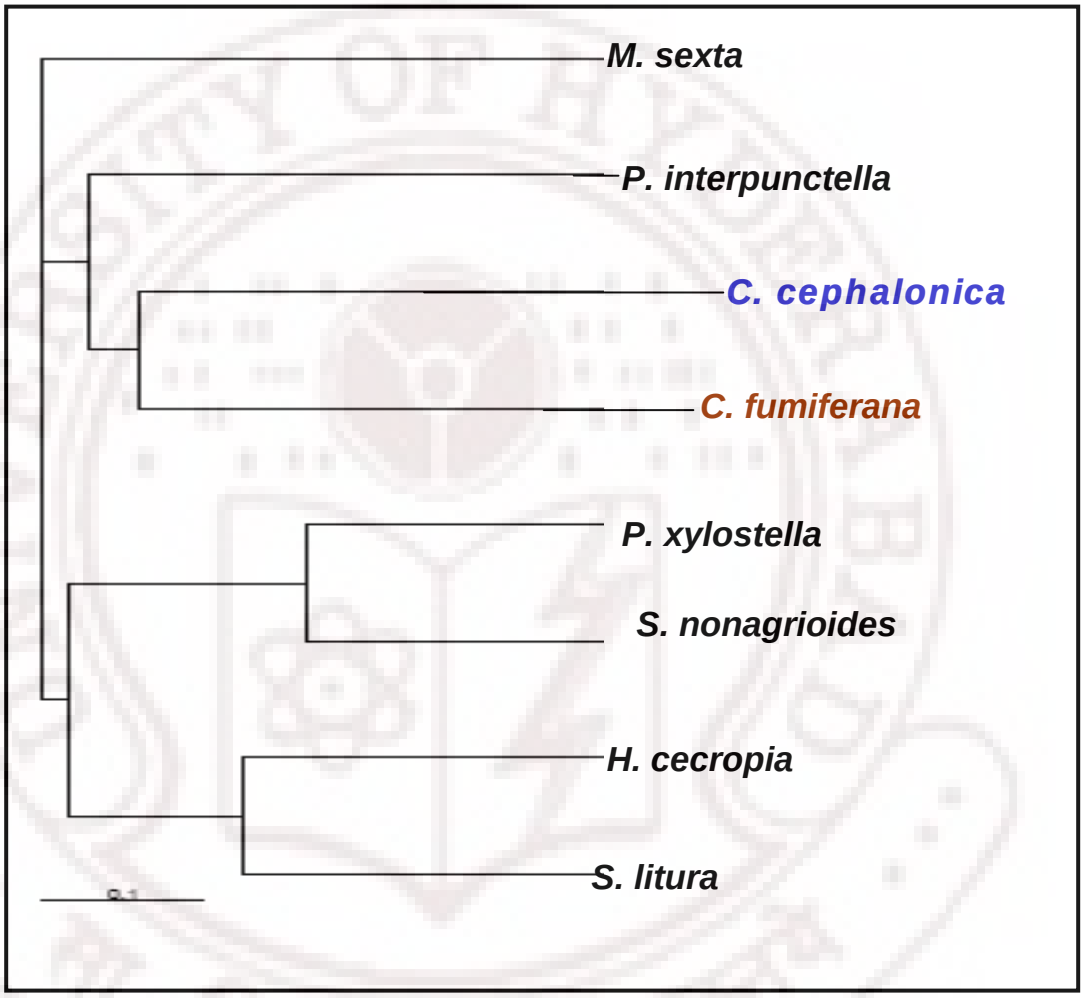


Figure 24c: Phylogenetic tree of methionine rich hexamerin from various lepidopteran insects:

Please note that the sequence has highest homology with *C. fumiferana* and both fall in the same clad.

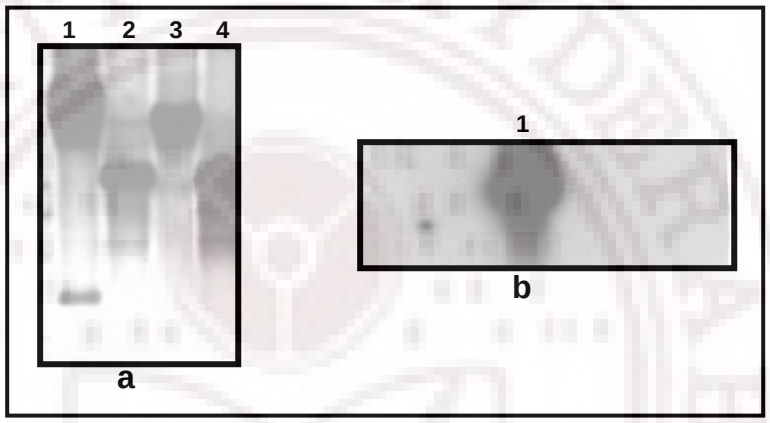


Figure 25: Southern and northern analysis of the Corcyra methionine rich hexamerin:

a: Southern analysis of the methionine rich hexamerin. For this experiment the fat body DNA was isolated from late last instar larvae. The DNA was digested with 4 different restriction enzymes EcoRI –lane 1, Bam HI –lane 2, Dra 1 –lane 3 and Sma 1-lane 4. The digested genomic DNA was electrophoresed and transferred to nylon membrane which was probed with 800 bp cDNA fragment of methionine rich hexamerin of *C. cephalonica.*

b: Northern analysis showing the presence of the transcript of methionine rich hexamerin in the fat body of *C. cephalonica*.

Identification of hexamerin receptor from the fat body of C. cephalonica:

Earlier studies from our laboratory reported that a 120 kDa polypeptide located in the plasma membrane of late larval and prepupal fat body cells of *C. cephalonica* binds to hexamerin and facilitates its sequestration (Kirankumar *et al*., 1997). It was further shown that phosphorylation was pre-requisite for sequestration, which was regulated by 20E (Arif *et al.,* 2003). In the present study, experiments were carried out to unambiguously identify the phosphorylated 120 kDa protein 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 its incubation with biotinylated hexamerin. The results revealed that the biotinylated hexamerin selectively bound to 120 kDa protein (Fig. 26b lanes –E and +E) and the intensity of binding was higher in 20E treated membrane fraction. This ligand blot, when autoradiographed, showed that the same 120 kDa protein was highly phosphorylated in presence of 20E (Fig. 26c, lane-+E), suggesting that the 120 kDa is the hexamerin receptor and the phosphorylation of the receptor is indeed 20E mediated. Since the above *in vitro* studies suggested that the phosphorylation of hexamerin receptor is 20E mediated and phosphorylation is pre-requisite for the uptake of hexamerins, we wanted to see whether in the presence of 20E, a higher uptake of hexamerins occurs in organ cultures of fat body. For accomplishing this [^{35} S] methionine labeled hexamerin were prepared (Ismail *et al*., 1993) and fat body from different stages (ELI, LLI and PP) were organ cultured in the presence and absence of 20E with a known quantity of radiolabelled hexamerins. After incubation the tissues was rinsed thoroughly to remove the residual hexamerins that were bound on the surface and was homogenized. The proteins were TCA precipitated and radioactivity was analyzed using scintillation counter. The results show that there was a stage dependent increase in the uptake of the hexamerins with the maximum sequestration at the pre-pupal (PP) stage (Fig. 26d). The uptake of hexamerin in ELI larvae was fairly low when compared with LLI larvae and prepupa. Furthermore, the results clearly show a significant increase in hexamerin uptake by LLI larval fat body in presence of 20E, as compared to control tissue.

Detection of hexamerin protein in different tissues of C. cephalonica:

This was carried out basically to find out whether there are other tissues which either synthesize and/or sequester hexamerins. For this different tissue like salivary gland, male accessory reproductive gland (MARG), ovary, and gut were analyzed. The tissue proteins were subjected to SDS-PAGE and western analysis was carried out using the hexamerin antibodies. The results in the figure 27a (lanes 2, 3, and 4) show the presence of hexamerins in salivary gland, MARG and ovary respectively. Lane 1 is the fat body tissue which was used as the positive control. Immunoreactivity was absent in the gut suggesting that gut doest not posses the hexamerins (lane 5). Further, to confirm whether the presence of the hexamerins in these tissues is due to its synthesis by the respective tissue, PCR analysis was carried out using gene specific primers of *Hex 2b* clone. Total RNA was isolated from the different tissues and cDNA was prepared. The results presented in figure 27b (lane 1) show the amplification of a 2.3 kb fragment only in fat body, but not in any other tissue (lanes 2, 3 and 4), which showed the presence of hexamerins. Thus the study clearly suggests that hexamerins, detected by the western analysis were not due to the result of synthesis by these tissues.

Identification of hexamerin receptor in different hexamerin sequestering tissues:

In the above experiment it has been shown that the hexamerins present in various tissues like salivary gland, MARG, and ovary are not synthesized as there was no transcript of hexamerin in these tissues, hence they are likely to be sequestered from the haemolymph. Earlier studies from our group had shown that in fat body hexamerin sequestration is a receptor dependent phenomenon (Kirankumar *et al*., 1997). Hence, to find out whether or not it is a receptor mediated process in these tissues, ligand blot analysis was carried with the membrane proteins of different tissues (salivary gland, MARG, ovary and gut) using biotinylated hexamerins. The results show that

biotinlyated hexamerins specifically bound to the membrane fraction (Fig. 28a). The binding was in the range of 120 kDa (lanes 2, 3, and 4) which was similar to that of fat body hexamerin receptor (lane 1) which was used as a (+ve) control. gut did not show any signal (lane 5). This was followed by the study of phosphorylation profile of the binding protein, since we know that the fat body receptor undergoes phosphorylation which is tyrosine kinase mediated (Arif *et al.,* 2003). The membrane fraction from the tissues was *in vitro* phosphorylated, subjected to SDS-PAGE analysis and the autoradiogram was developed for the gel. The results (Fig. 28b) show that like in fat body (lane 1) the receptor in other hexamerin sequestering tissue was phosphorylated (lanes 2, 3 and 4) while in gut it was absent (lane 5).

Western analysis using anti-phosphotyrosine antibody further revealed that the 120 kDa receptor protein indeed undergoes tyrosine phosphorylation in all the tissues (Fig. 28c) other than gut. Once it was confirmed that there was a receptor in these tissues, which was similar to that of fat body, *in vitro* uptake studies using organ cultures were carried out to ascertain the sequestration of the [^{35} S] methionine labelled hexamerins. The results show that fairly good amount of hexamerin is sequestered in all other tissues than gut (Fig. 29). However, as expected the uptake was highest in fat body, followed by salivary gland, MARG and ovary.

Ecdysteriod dependent phosphorylation of fat body membrane proteins:

For this study the membrane proteins were prepared from the late-last instar larval fat body and *in vitro* protein phosphorylation was carried out using [γ^{32}P] labelled ATP either in presence or absence of 20E. The results presented in figure 30b clearly show that the degree of phosphorylation is fairly high in presence of 20E (+20E) when compared with control (-20E).

2-D analysis of the in vitro phosphorylated fat body membrane proteins:

Once the 120 kDa protein was identified as the hexamerin receptor attempts were made to purify the receptor using conventional methods like affinity purification and pull down assays. However, we were unsuccessful with all the attempts made using the above methods, hence we started to look for other possible properties of receptor, which could be exploited for designing the protocol. Since it is known that the hexamerin receptor in *C. cephalonica* is 120 kDa protein and undergoes phosphorylation, which is 20E dependent (Arif *et al*., 2003) we took advantage of this property of the receptor. The rationale behind the experiment was that under the influence of 20E the hexamerin receptor undergoes phosphorylation. Hence the difference in the degree of phosphorylation of a 120 kDa protein in presence of 20E would probably allow us to visualize a highly phosphorylated protein spot in 2-D electrophoretic separation as hexamerin receptor.

Keeping the above in view fat body membrane fraction proteins were *in vitro* phosphorylated either in presence or absence of 20E. The phosphorylated proteins were subjected to SDS-PAGE as well as 2-D gel electrophoresis. The stained gels were vacuum dried and autoradiogram of the corresponding gels was obtained. Differential phosphorylation of hexamerin receptor can be seen in the 20E treated compared to control. Figures 30 and 31 (a, c) shows the protein separation on SDS-PAGE and 2-D gel electrophoresis in the hormone treated and control preparations while, figures 31b and d are their corresponding autoradiograms. Detailed analysis of the autoradiogram revealed increased phosphorylation of two proteins in presence of 20E (Fig. 31b) when compared with figure 31d. The spots designated as 1and 2 on the gel were picked by superimposing the autoradiogram on the dried gel and were analyzed by MALDI TOF – TOF. The spot 1, which had a molecular weight around 23 kDa showed homology to adenosine phosphorybosyl transferase of *Drosophila* (Fig. 32a). The spot 2, which had the molecular weight around 48 kDa, revealed its identity to GTP-binding protein - rab3A of mouse and GTP binding protein smg-25 of bovine (Fig. 32b). Reports show that this protein present in bovine brain is involved in the secretory pathway (Verhage,

2007). Whereas adenosine transferase, participates in the 20E mediated signaling pathways in the *Drosophila* (Kessler, 1993). As the results are preliminary further work has to be carried out to show the function of these proteins in *C. cephalonica*

PCR amplification of the putative hexamerin receptor:

Since the 2-D electrophoretic analysis of phosphorylated fat body membrane fraction did not provide the desired results other avenues were explored. One of them was the genomic approach, which was based on two reports: (i) Scheller *et al.,* (1996) proposed that in diptera the hexamerin receptor evolved from their ligands, and (ii) Haunerland *et al.,* (2003) showed that the VHDL receptor binds to storage proteins in *Helicoverpa zea*. With the above background information and that the larval hexamerin receptor of *C. cephalonica* undergoes phosphorylation, which is tyrosine kinase dependent, an array of degenerate primers using the above mentioned criteria were designed for amplification. PCR analysis was carried out using fat body mRNA isolated from 20E treated larvae with different combinations. The result in figure 33b shows an amplification of 850 bp fragment, whose sequence analysis revealed its homology with insect storage proteins (hexamerins) and more importantly with the VHDL receptor of *H. zea* (Haunerland *et al.,* 2004). Once the fragment was cloned, the attempt was made to obtain the full length sequence using the RACE strategy. The 5' and 3' RACE reactions were carried out using the adaptor specific forward primer and gene specific reverse primer for the 5' RACE and gene specific forward primer and adaptor specific reverse primer for the 3' RACE. Results reveal in and showed an amplification of 680 bp fragment in the 5' RACE reaction (Fig. 33a) and 900 bp amplicon in 3' RACE reaction (Fig. 33c). The sequence analysis of the 5' product showed a phosphotyrosine kinase domain at the N-terminal end (Fig. 33d).

PCR amplification of the full length putative hexamerin receptor:

Once the three fragments were obtained containing the total cDNA sequence of the putative hexamerin receptor the next logical step was to clone the complete cDNA sequence as a single clone. For this gene specific primers were designed at the extremes of the 5'and 3'end and PCR analysis was carried out. Figure 34a shows the amplification of a 2.6 kb fragment. The sequence analysis of this fragment revealed that it was identical to the initial three fragments, with an open reading frame (ORF) of 2.5 kb (Fig. 34b).

Cloning and restriction digestion analysis of 5' (N-terminal) sequence of putative hexamerin receptor:

To elucidate the function of the putative hexamerin receptor, the N-terminal sequence was expressed because it contained the phosphotyrosine kinase domain. For this purpose gene specific primers were designed along with the restriction sites (EcoRI and Bam HI). Figure 35a shows the amplification of the N-terminal fragment which is about 618 bp. This was followed by confirmation of the clone in the expression vector by single and double restriction digestion. Figure 35b and c show the single and double digestion pattern respectively. Further the N-terminal product of 618 bp which was released as a result of double digestion can be clearly seen figure 35c. This fragment was cloned in an expression vector for further studies.

Detection of the expressed N-terminal protein using anti His –tag antibodies:

The expression of the recombinant protein was carried out using the overnight culture. one ml of the overnight culture was added to the fresh medium and incubated until the O.D reached 0.8 nm, then it was induced with IPTG (where the total concentration of IPTG was 0.8 mM). The culture was incubated for 3 h while doing so 1 ml of the culture was removed for every 1 h to check the expression. The different fractions were

electrophoresed and transferred on to a nitrocelluose membrane and the expression of the receptor fragment was detected using anti His-antibodies. The result in figure 36 clearly show the expression of a protein, which matched with the size of deduced amino acid sequence of cloned N-terminal fragment of putative hexamerin with His-tag. Further, the expression was slightly higher after 2 h of induction (lane 2) when compared with expression to the 1st h and the 3rd h culture.

Ligand blotting analysis confirming the hexamerin receptor:

After recombinant expression of truncated N-terminal fragment of putative hexamerin receptor, the binding studies were performed for confirmation. For these simultaneous experiments *i.e.* ligand blotting and western analysis were carried out. The expressed proteins from both the induced and un induced cultures were electrophoresed and transferred on to a nitrocellulose membrane. The blots were cut into two equal halves having, both the induced and the un induced samples. One of them was used for western analysis and the other was used for ligand blotting. The ligand blotting was carried out using the pure hexamerins, which were over layed on the membrane blot overnight. Then the membrane was washed thoroughly for removal of unbound hexamerins. This was followed by addition of hexamerin antibodies and incubated for 2 h, after which the detection was carried out using anti-rabbit Ig G secoundary antibodies. The result in figure 37 (lane 2) shows a specific band, which corresponds with to the molecular weight of 28 kDa which was identical to the one detected using the His –tag antibodies. Further in un induced cultures this band was absent, (lane 1) suggesting that the hexamerins bound specifically to the expressed recombinant protein which is probably the truncated hexamerin receptor.

Identification of hexamerin receptor transcript in different hexamerin sequestering tissues:

Once it was seen that the expressed hexamerin receptor bound specifically to the hexamerins. We further wanted to confirm that the isolated clone was actually a hexamerin receptor clone. For this PCR analysis was carried out using the gene specific primers designed from fat body hexamerin receptor cDNA. Since our earlier studies have already shown that the receptor in other tissues is similar in size and function to its counterpart the fat body. Hence if the primers that are specific to the fat body receptor should amplify a similar size product. This would further strengthen our finding that the complete cDNA that we have isolated in the present study is authentically is the hexamerin receptor cDNA. For this cDNA was prepared from (MARG, ovary, and salivary gland) and PCR analysis was carried out. Figure 38 shows the amplification in all the above mentioned tissues (lanes 2-4) and the amplicon size is similar to the one found for the fat body (lane 1).

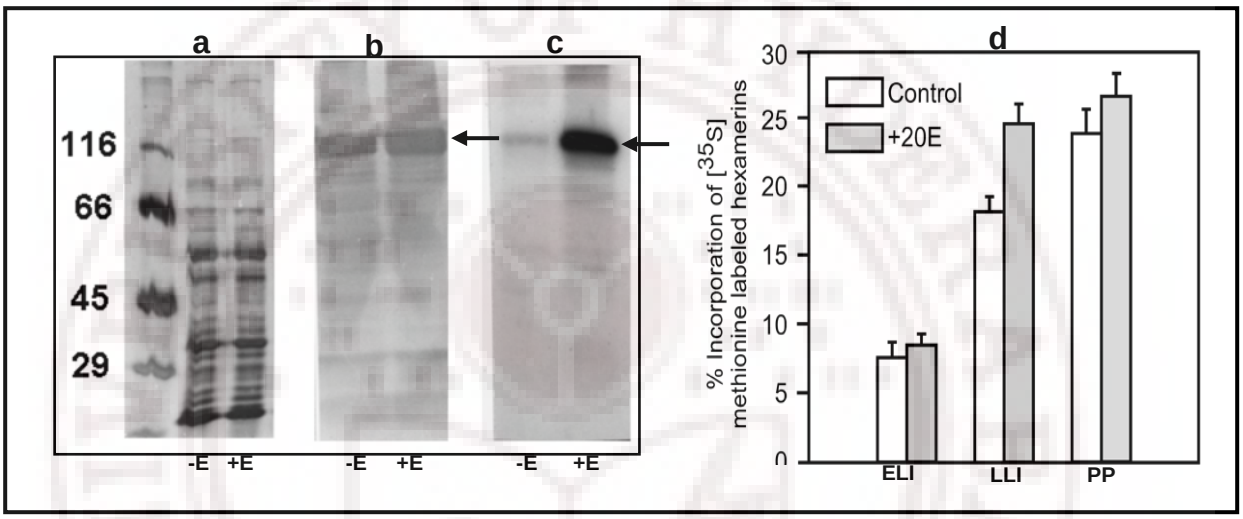


Figure 26: Identification of hexamerin receptor in the fat body of C. cephalonica:

a: SDS-PAGE profile of the fat body membrane fraction used for *in vitro* phosphorylation either in absence or presence of 20E. The lanes which are denoted with (–E) are controls and (+E) are 20E treated.

b: Ligand blot probed with the biotinylated hexamerins. (→) Note the binding of biotinylated hexamerins with 120 kDa protein.

c: The blot was subsequently processed for autoradiography. (→) Note the increase in degree of phosphorylation of 120 kDa protein which binds with biotinylated hexamerin in presence of 20E

d: Shows the percentage incorporation of the radiolabelled hexamerins by the fat body of different developmental stages in organ culture studies either in the presence or absence of 20E.

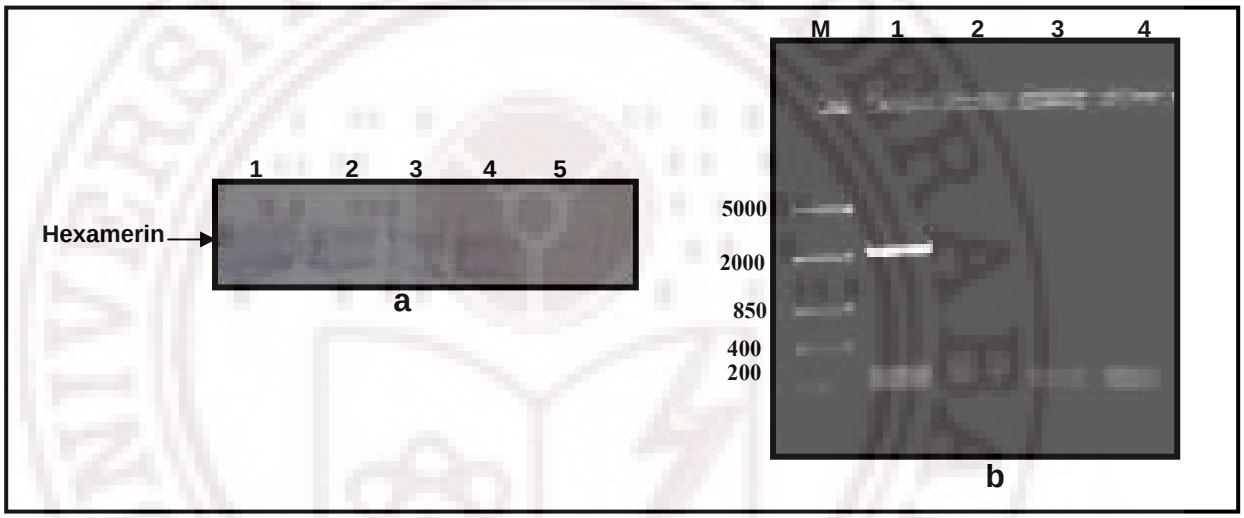


Figure 27: Detection of hexamerin protein in different tissues of C. cephalonica:

a: Western blot analysis showing the presence of hexamerins in different tissues. Lanes 1, 2, 3, 4 and 5 represent fat body, salivary gland, MARG, and ovary and gut respectively.

b: Shows the results of PCR analysis of hexamerin transcript from various tissues using specific primers designed from *Hex 2b* gene of *C. cephalonica.* Please note the presence of amplicon (2.3 kb) only in the fat body (lane 1) which is absent in all other tissues, salivary gland (lane 2), MARG (lane 3) and ovary (lane 4).

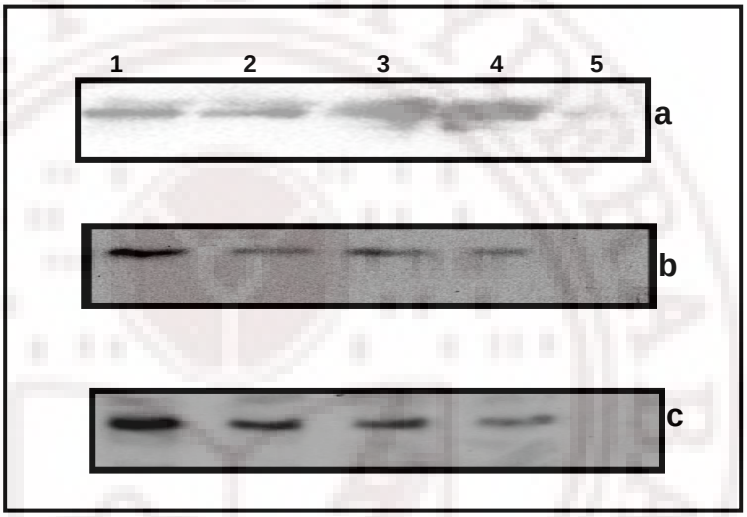


Figure 28: Identification of hexamerin receptor in different hexamerin sequestering tissues:

a: Ligand blot analysis using the biotinlyated hexamerins shows the binding of labeled hexamerins with 120 kDa membrane protein in all the tissues except gut.

b: Autoradiogram showing the phosphorylation of 120 kDa protein in all the hexamerin sequestering tissues.

c: Western blot analysis showing the detection of 120 kDa protein using anti-phospo tyrosine antibody. The antibody detected hexamerin receptor in all the tissues except gut.

Lane-1 Fat body, lane -2 salivary gland, lane-3 MARG lane- 4 oocyte and lane – 5 gut.

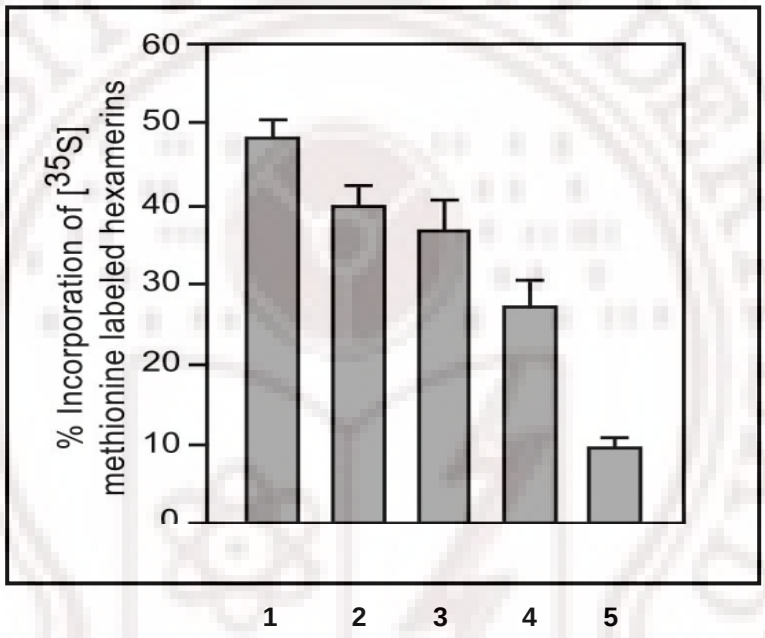


Figure 29: In vitro uptake of radiolabelled hexamerins:

For this various organs were cultured under sterile conditions in TC 100 culture medium as described in materials and methods, to this cultures a known quantity of radiolabelled hexamerin were added. After 4 h of incubation the tissues were collected rinsed thoroughly and homogenized. Proteins were TCA precipitated, washed and radioactivity was quantified using scintillation spectrophotometer.

Lane 1- Fat body, 2- salivary gland, 3- ovary, 4- MARG and 5- gut

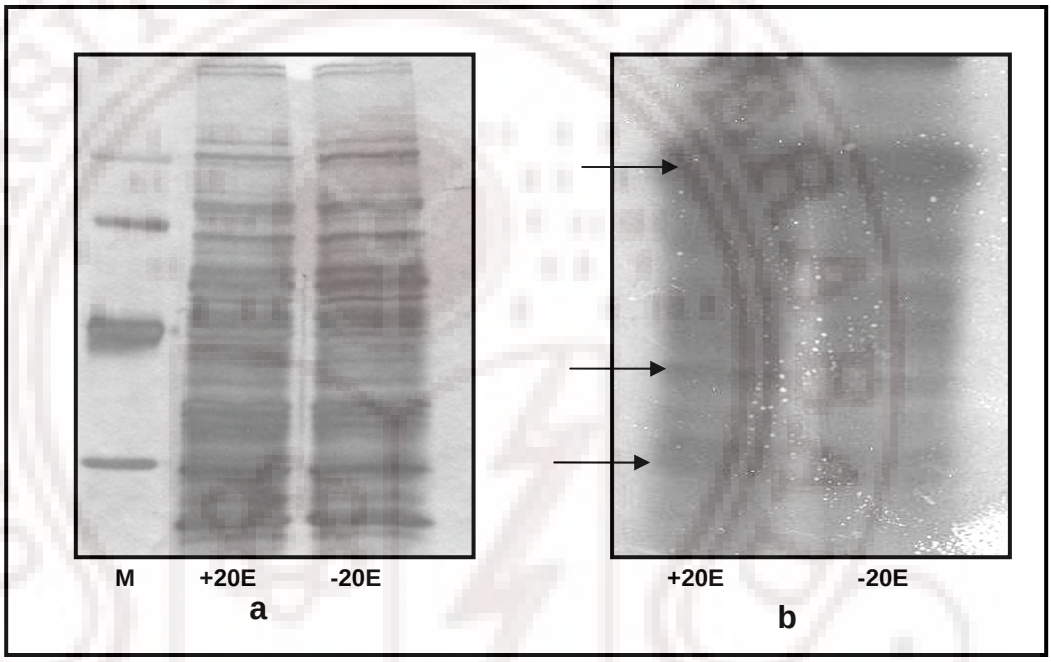


Figure 30: 20E dependent in vitro phosphoryaltion of fat body membrane fractions:

a: SDS-PAGE profile of the *in vitro* phosphorylated membrane fractions in presence of 20E (+20E) and control (-20E). 20 µg of protein was loaded on the gel. M is the molecular weight marker

b: Corresponding autoradiogram of the vacuum dried gel showing the differential phosphorylation pattern**.**

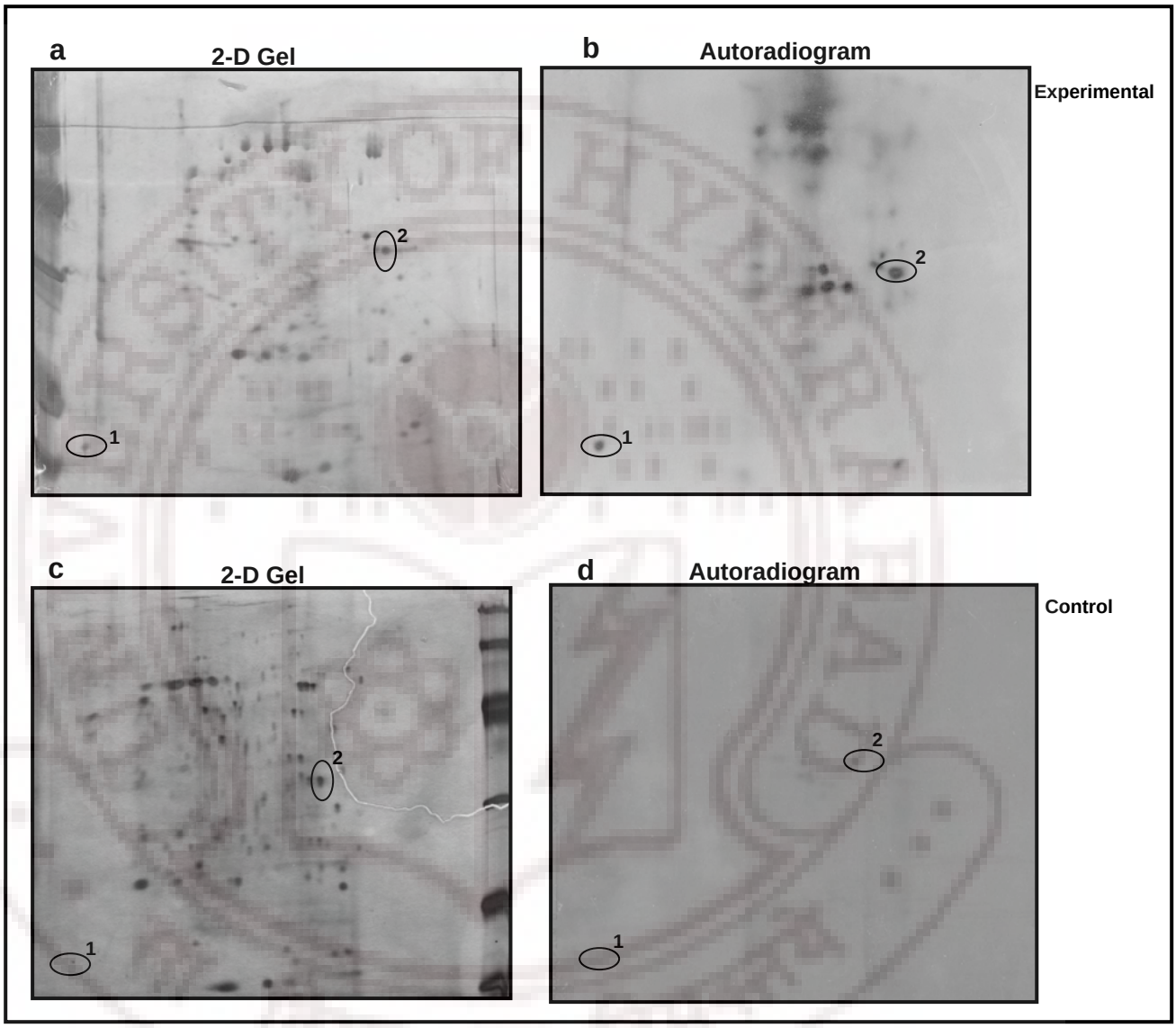


Figure 31: 2-D analysis of the in vitro phosphorylated fat body membrane proteins:

a and c: Show the 2-D separation of *in vitro* phosphorylated fat body membrane proteins in presence(a) or absence (c) of 20E and without 20E respectively.

b and d: Are the corresponding autoradiogram of a and c. Note the spots marked as 1 and 2 which show an increased phosphorylation in the 20E treated samples when compared to untreated samples.

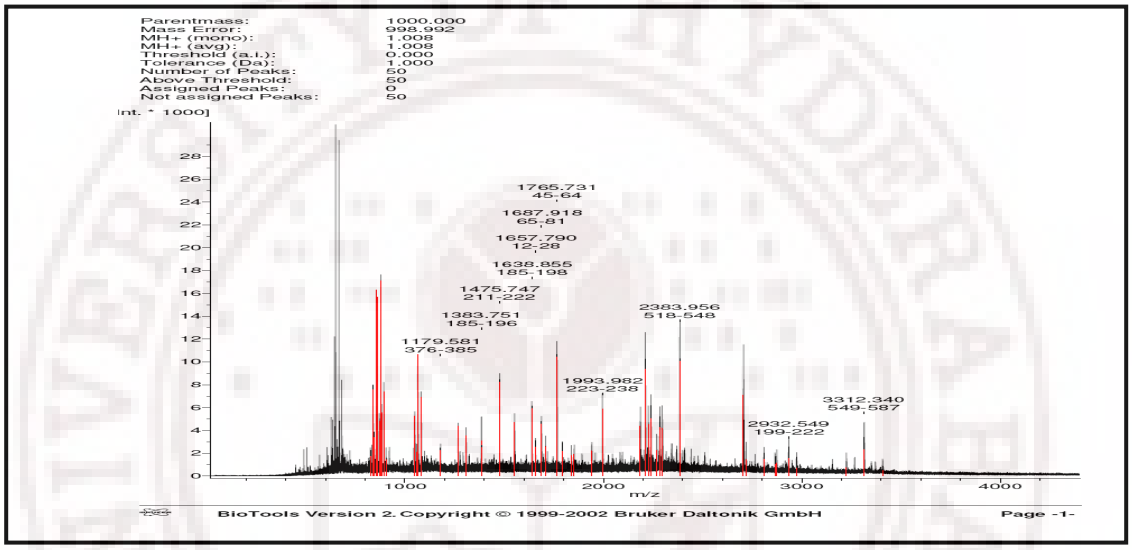


Figure 32a: MALDI TOF-TOF analysis of the spot denoted as 1 in figure 31a.

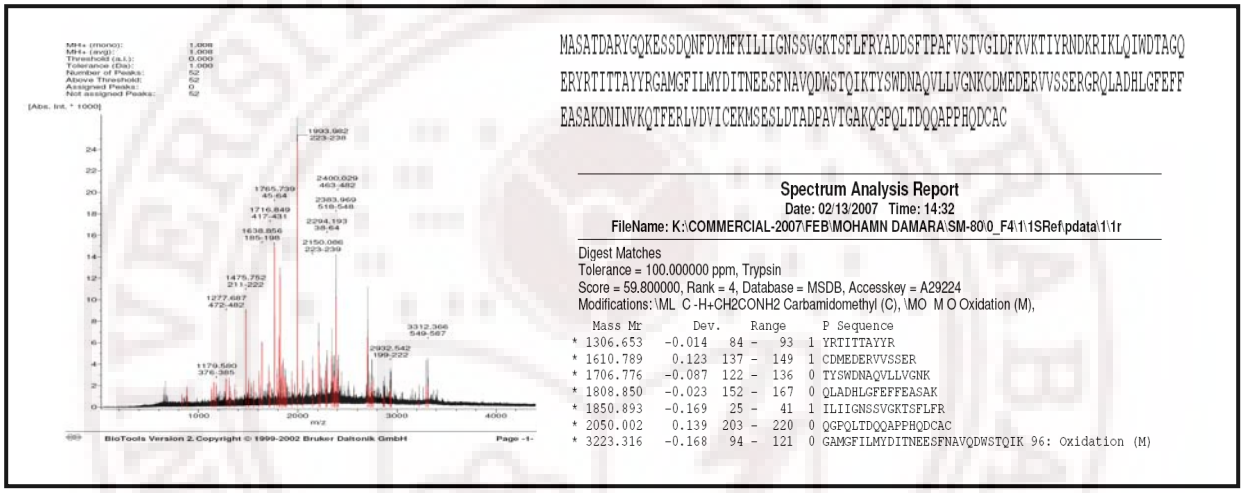
adenine phosphoribosyltransferase (EC 2.4.2.7) - fruit fly (Drosophila melanogaster)

MSPSISAEDKLDYVKSKIGEYPNFPKEGILFRDIFGALTDPKACVYLRDLLVDHIRESAPEAEIIVGLDSRGFLFNLLIAT
ELGLGCAPIRKKGKLAGEVVSVEYKLEYGSDTFELQKSAIKPGQKVVVVDDLLATGGSLVAATELIRKVGGVVVESLVVVM
ELVGLEGRKRLDGKVHSLIKY

Digest Matches
Tolerance = 100.000000 ppm, Trypsin
Score = 45.300000, Rank = 4, Database = MSDB, Accesskey = A29596
Modifications: \ML C -H+CH2CONH2 Carbamidomethyl (C), \MO M O Oxidation (M),

	Mass Mr	Dev.	Range		P	Sequence
*	1550.685	-0.098	2 - 15	1		SPSISAEDKLDYVK
*	1837.928	-0.012	33 - 48	1		DIFGALTDPKACVYLR
*	2210.097	-0.155	127 - 148	0		VVVVDDLLATGGSLVAATELIR
*	2238.136	-0.017	107 - 126	1		LEYGSDTFELQKSAIKPGQK
*	2267.128	-0.164	150 - 171	1		VGGVVVESLVVVMELVGLEGRK

Figure 32b: The MALDI TOF-TOF analysis of the spot denoted as 2 in figure 31b.



GTP-binding protein smg -25 bovine

MASATDSRYGQKESSDQNFDYMFKILIIGNSSVGKTSFLFRYADDSFTPAFVSTVGIDFKVKTIYRNDKRIKLQIWDTAGQ
ERYRTITTAYYRGAMGFILMYDITNEESFNAVQDWSTQIKTYSWDNAQVLLVGNKCDMEDERVVSSERGRQLADHLGFEFF
EASAKDNINVKQTFERLVDVICEKMSESLDTADPAVTGAKQGPQLTDQQAPPHQDCAC

Digest Matches
Tolerance = 100.000000 ppm, Trypsin
Score = 59.800000, Rank = 4, Database = MSDB, Accesskey = S34070
Modifications: \ML C -H+CH2CONH2 Carbamidomethyl (C), \MO M O Oxidation (M),

	Mass Mr	Dev.	Range	P	Sequence
*	1306.653	-0.014	84 - 93	1	YRTITTAYYR
*	1610.789	0.123	137 - 149	1	CDMEDERVVSSER
*	1706.776	-0.087	122 - 136	0	TYSWDNAQVLLVGNK
*	1808.850	-0.023	152 - 167	0	QLADHLGFEFFEASAK
*	1850.893	-0.169	25 - 41	1	ILIIGNSSVGKTSFLFR
*	2050.002	0.139	203 - 220	0	QGPQLTDQQAPPHQDCAC
*	3223.316	-0.168	94 - 121	0	GAMGFILMYDITNEESFNAVQDWSTQIK 96: Oxidation (M)

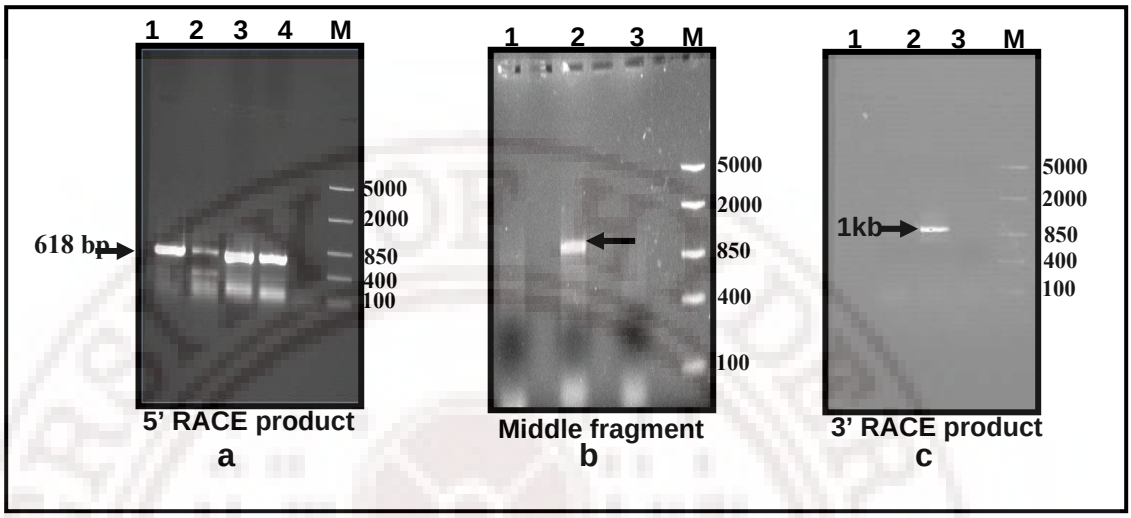


Figure 33: PCR amplification of the putative hexamerin receptor:

a: Lanes 1- 4 show the amplification of 618 bp PCR product obtained using gene specific reverse primer and adaptor specific forward primer. Lane– M is Molecular marker.

b: Lane 2- shows the PCR amplification of 850 bp fragment using the degenerate primers.

c: Lane 2- shows the PCR amplification of 1kb fragment using 3' RACE strategy.

5'TTCGACTGAGCACGAGGACACTGACATGGGACTGAGGAGTAGAAAAGTGTTGTGGATGGTCAAGATGGTGATCCTAGTGATGC
TGCTGGTAACGGTGGCGACGGCTTCCGTCGTTCCCGATGACTCTAAAGTCCTAATTACTAAGGAACCGATGGTAAACTTGGACGT
GAAGACAAAGGAATTATTTATACTTAAGCTGCTCAACCACATCCTACAGCCATCAATGTACGAGGACATCAGATCGATCGCAAGA
GAATATACCATCGAGGATAATATGGACAAATATGTGAAAGTGGAAGTAGTTAAGGCATTCATCGCCACATACAAGCTGGGCATGC
TGCCTCGAGGTGAGGTCTTCGTGGACATGGACGTGAAGCAAGCAGAAGAAGCGATCAAAGTATTCCAACTCCTATATTTCGCTAA
AGACTTCGATATCTTTGTGCGGACCGCCTGCTGGTTACGAGAACGCATCAACGGAGGCATGTTTGTCTACGCTTTGACTGCCGCC
GTTTTTCACAGACCTGATTGCAATGGCATCAGTCTTCCTGCGCCTTATGAAATCTATCCACACTACTTCGTCGACAGTCACA
TTCTACACAAAGCATTCATGATGAAAATGACAAAAGCCTCAGTGGACGCTGGCATCAAG 3

Putative hex recp	GAGCACGAGGACACTGACATGGACTGAAGGAGTAGAAAAGT
Biomphalaria cal binding ptn 2mRna	GAGCACGAGGACACTGACATGGACTGAAGGAGTAGAAA
Gly Max SOS-like kinasemRna	GAGCACGAGGACACTGACATGGACTGAAGGAGTAGAAA
M.sexta putative octopmine recp	GAGCACGAGGACACTGACATGGACTGAAGGAGTAGAAAA
*T.castane*um recp tyrosine kinase	GAGCACGAGGACACTGACATGGACTGAAGGAGTAGAAAA
L.esculentum WAK-like kinase	GAGCACGAGGACACTGACATGGACTGAAGGAGTAGAAAAGT

d: Sequence of 618 bp fragment amplified using the 5' RACE strategy. It showed the presence of a tyrosine kinase domain (it is represented in blue colour).

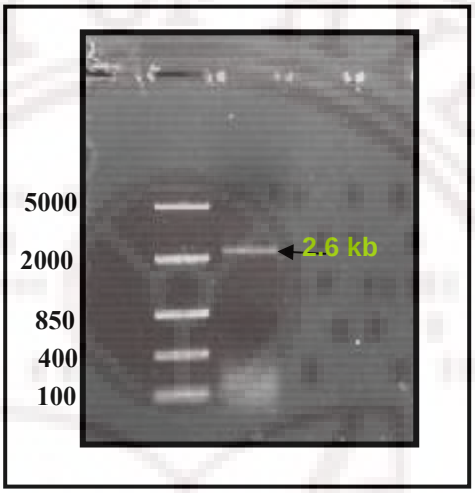


Figure 34a: PCR amplification of the full length putative hexamerin receptor:

Amplification of 2.6 kb full length putative hexamerin receptor. The PCR reaction was carried out using gene specific primers.

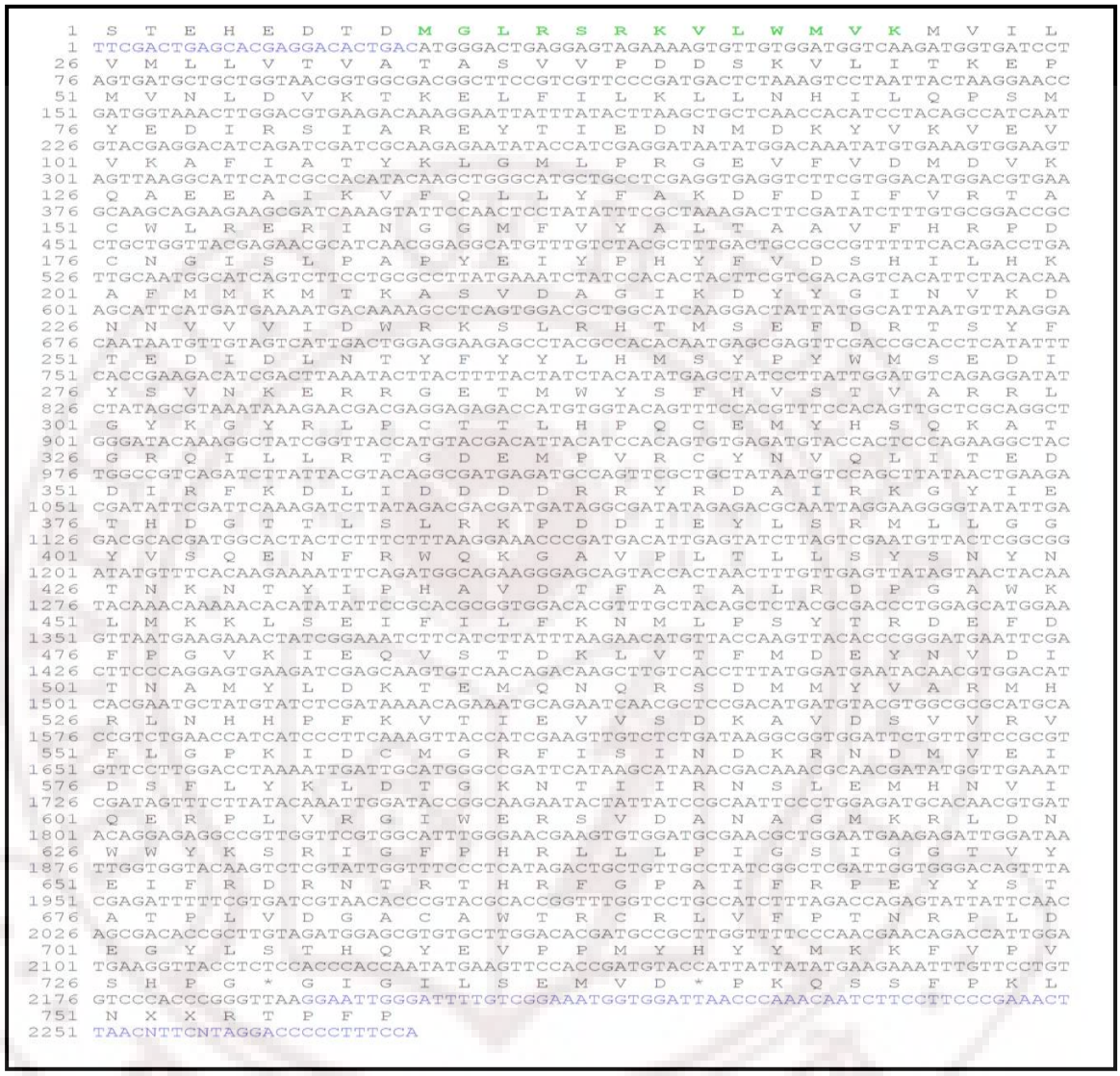


Figure 34b: Complete sequence of the putative hexamerin receptor.

The full length cDNA is 2.6 kb with an ORF of 2.5 kb.

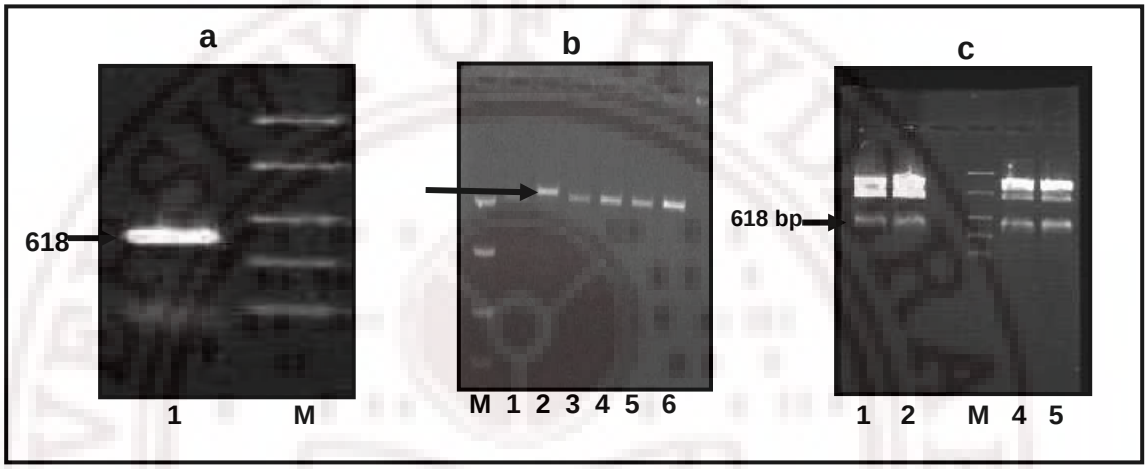


Figure 35: Cloning and restriction digestion analysis of 5' (N-terminal) sequence of putative hexamerin receptor:

a: PCR amplification of the 618 bp N-terminal fragment with the EcoR1 and BamH1 restriction sites (lane 1).

b: Single restriction digestion with EcoRI was carried out. Lane 2 shows the positive digestion which is indicated by a retarded movement on the gel. Whereas lanes 1, 3, 4, 5 and 6 do not show any digestion, indicating the absence of the fragment in the expression vector.

c: Double restriction digestion with EcoRI and BamHI. As seen in the figure the digestion released the fragment of 618 bp (lanes 1, 2, 4 and 5) confirming the presence of the clone in the vector.

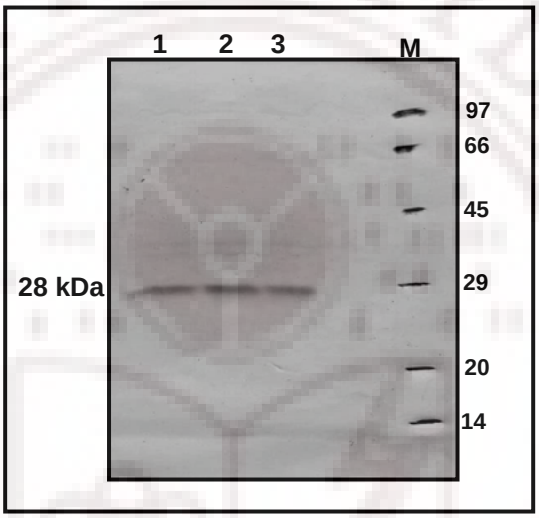


Figure 36: Detection of the expressed His-tagged N-terminal truncated putative hexamerin receptor using anti His antibodies:

Western blot analysis using anti-His antibodies. The antibodies detected a band which is around 28 kDa. Slightly a higher expression of the recombinant protein was observed after 2 h of IPTG induction (lane 2) compared with other time periods 1 h (lane 1) and 3 h (lane 3).

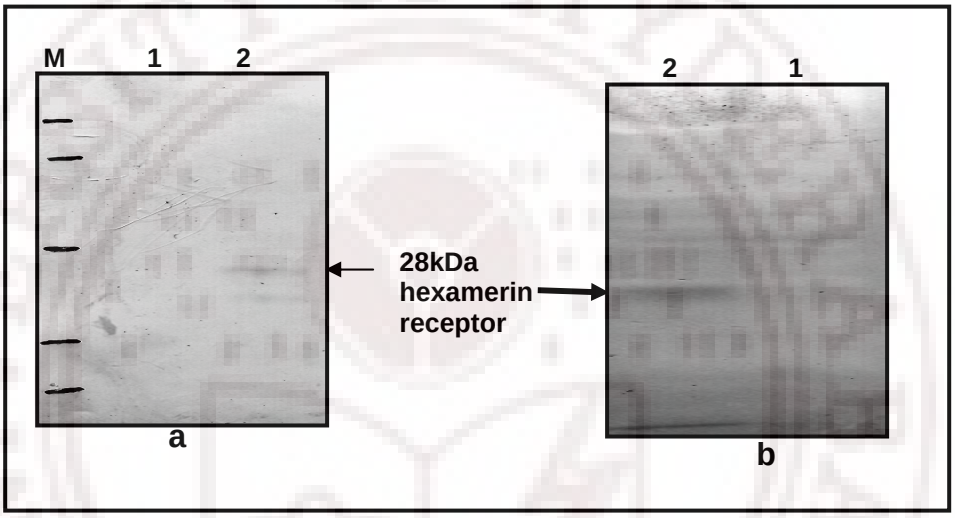


Figure 37: Ligand blotting analysis confirming the hexamerin receptor:

a: Detection of the recombinant protein expressed using anti- His antibodies (lane 2). Lane 1 is the uninduced vector lysate.

b: Ligand blot analysis of the expressed proteins with the hexamerins. The detection was carried out using hexamerin antibodies. Lane 2 (induced culture) shows a specific band whose molecular weight corresponds to 28 kDa, whereas the un induced vector lysate did not show any cross reactivity suggesting the absence of ligand binding.

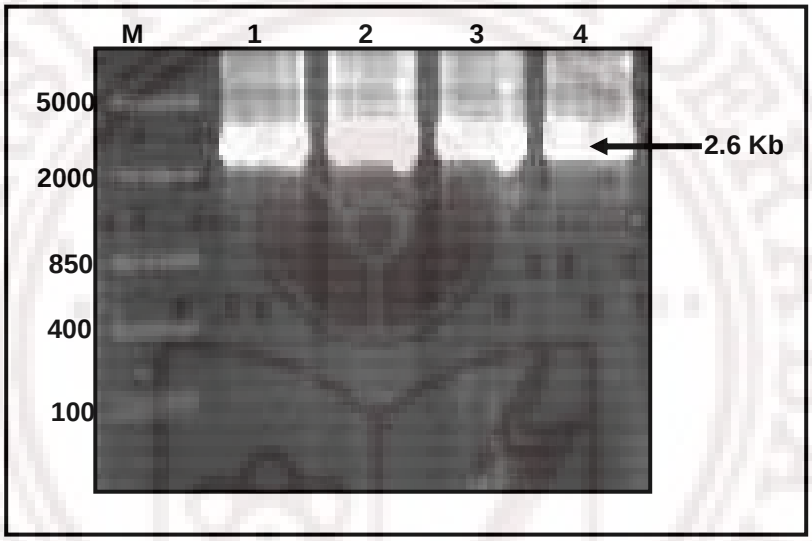


Figure 38: Amplification of hexamerin receptor cDNA from different hexamerin sequestering tissues:

The PCR analysis was carried out using RNA from different hexamerin sequestering tissues. The primers used for the amplification were the gene specific primers designed from the fat body hexamerin receptor. The results clearly show amplification of 2.6 kb amplicon in lanes 2 salivary gland, 3 MARG, and 4 ovary which was similar to that of the fat body hexamerin receptor in size (lane 1).

Rice moth *C. cephalonica* is of great economic importance as this is one of the three most common and the destructive pests among the 23 reported species of insects infecting stored grains and packed food in different countries including India (Highland, 1978). With an intension of obtaining the detailed information on the physiology and biochemistry of this insects our research group has been working for more than two decades with the objective of exploring the structure and function of hexamerins their expression and sequestration, the present study was carried out.

Hexamerins are the most dramatic examples of developmentally regulated gene function. The major function of the hexamerins is to serve as nitrogen and amino acid pool to support *de novo* protein synthesis during metamorphosis (Kanost *et al.*, 1990; Telfer and Kunkel, 1991; Burmester and Scheller, 1995, 1996; Pan and Telfer 1996). In recent times it has been shown that hexamerin do have a role in many other functions like reproduction (Wheeler and Buck 1996; Seo *et al.,* 1998; Wheeler *el al.,* 2000), Community organization (Scharf, 2007a, b), and immunity (Krishnan *et al.*, 2008) etc.

Earlier studies from our laboratory on *C. cephalonica* reported the presence of different hexamerins in the haemolymph (Ismail, 1991) like in other lepidopteran insects (Haunerland, 1996). On the basis of there electrophoretic mobility and molecular mass they were identified as 86 kDa (Hex1), 84 kDa (Hex2) and 82 kDa (Hex3) proteins. Furthermore, like all other insects even in *C. cephalonica*, fat body is reported to be the major site for the synthesis of hexamerins, from where they are released in to the haemolymph during the larval development (Ismail, 1991; Kirankumar, 1998). Nagamanju *et al.,* (2003) cloned the full length cDNA of 84 kDa protein (Hex2) and based on the percentage of aromatic amino acid classified it as arylphorin.

In the first part, the work was carried out to unravel the hormonal regulation of hexamerins in *C. cephalonica* during last-instar larval development. For initial experiments 20E and/or JH was injected to the insects to analyze the effect of these hormones on the hexamerin synthesis. We observed an increase in the synthesis of hexamerins after 20E treatment indicating that it has a stimulatory effect. On the other hand results with JH

treatment showed a decline in the synthesis of hexamerins suggesting the inhibitory role of the hormone.

Once the stimulatory effect of 20E was observed we further analyzed the details of it by using the thorax-ligated larvae. Thorax ligation is known to eliminate/reduce the endogenous circulating hormone hence the model is commonly used for analyzing the effect of hormones that are applied externally. The *in vivo* studies using the 16 h postligated larvae showed an increase in the hexamerin synthesis after 20E treatment. Identical results were also obtained, when the 16 h ligated larvae were treated with RH 5992 (a non-steroidal agonist of 20E). Similar stimulation of hexamerin synthesis was also observed when fat body in organ cultures was exposed to exogenous 20E. This was followed by challenging the insects with azadiractin which is shown to block the release of 20E. We observed a time dependent inhibitory effect suggesting that the reduction of hexamerin synthesis in the treated insects is most likely due to the blockade of ecdysteriod release leading to a lowered hormone titer in haemolymph.

As we assume that the ecdysteroid mediated regulation of hexamerin synthesis could be genomic, in the next phase of our work we carried out extensive northern profiling analysis using *Hex 2b* cDNA as probe. Our results using thorax- ligated larvae showed that 20E as well as its agonist RH 5992 treatment enhance the hexamerin transcript level in the fat body clearly suggesting that ecdysteroids elicits their effect on hexamerin at the transcriptional level, which could be due to the presence of specific regulatory sequences. For which we cloned the full length genomic clone of hexamerin *(Hex 2b)* which is 3.5 kb in length. It has 3 introns and 4 exons. Once the full length of the genomic clone was obtained, the 5' UTR region was isolated as most of the regulatory sequences are known to be present at UTR's. Detailed analysis of the 5' UTR of the hexamerin gene (*Hex 2b*), revealed the presence of an ecdysone responsive element (ERE's), which is homologous to the *Drosophila* Hsp27 (Pelham *et al*., 1987). Apart from ERE, we noticed the presence of other sequences like TATA, CAAT and E boxes. The presence of ecdysone responsive elements further suggests that the hexamerin gene is probably transcriptionally regulated by 20E.

It is well documented that the hexamerin concentration increases in stage specifically in both lepidoptera and diptera and is maximum at the LLI stage, however this is in reference to the total native protein (Burmester and Scheller, 1998). We wanted to see whether the same is true for the individual subunits. For this the hexamerin subunits were purified by gel elution and polyclonal antibodies against them were raised individually. We found that the antibodies raised against the 86 kDa cross reacted with both the subunits, where as the 84 kDa antibody specifically cross reacted with the 84 kDa subunit. The reason behind the cross reactivity of the 86 kDa antibodies might be due to its polyclonal nature as well as the carbohydrates which are commonly associated with the hexamerins (hexamerins are glycoproteins). The stage specific expression pattern of the subunits shows that they closely follow the native hexamerin protein profile. The concentration of the subunits increases gradually in the haemolymph during the early stage and reaches the highest at late-last instar larvae.

In the present work the methionine rich hexamerin cDNA was cloned using the degenerate primers and the RACE strategy. The full length cDNA obtained has a size of 2.45 kb with an ORF of 2.26 kb and probably codes for 86 kDa hexamerin subunit of *C. cephalonica*. The deduced amino acid sequence contained 4.9 % methionine suggesting that it belongs to the methionine rich class of hexamerins. The sequence shows homology with the methionine rich hexamerins reported in the databank, having the highest to *P. interpunctella* (75 %) and *C. funiferana* (72%). The Southern analysis revealed that the methionine rich hexamerin of *C. cephalonica* is a single copy gene, while northern analysis detected a single transcript of about 2.3 kb.

The histogenesis of adult tissue during metamorphosis requires large amounts of energy and building blocks, which are normally provided by "storage proteins" (Haunerland, 1996), and the major fraction of which consists of hexamerins. These are sequestered by the larval fat body through a receptor mediated endocytosis, stored in vacuoles, hydrolysed by the activity of lysosomal enzymes for utilization (Telfer and Kunkel, 1991; Haunerland, 1996; Burmester and Scheller, 1999, Nagamanju *et al.*, 2003). The storage protein receptor has been identified in different insects at the homogenate level. In *Calliphora* the cleavage of inactive precursor receptor was essential for the uptake of hexamerins (Burmester and

Scheller, 1997). Whereas in *S. peregrine*, the 120 kDa receptor was synthesized and cleaved during the early larval stages of development when the ecdysteriod titer is low, but during the late larval stages the high haemolymph titer of ecdysteroid inhibited the protease activity thus allowing the receptor to sequester hexamerins (Chung *et al.*, 1995).

Previous studies from our laboratory showed that the fat body hexamerin receptor in *C. cephalonica* is a 120 kDa protein (Ismail and Dutta-Gupta, 1990b; Kirankumar *et al.*, 1997). Furthermore activation of the receptor was dependent on phosphorylation which is regulated by an increase in ecdysteroid level during late-larval development. It was also shown that the phosphorylation is mediated by tyrosine kinase (Arif *et al.*, 2003.)

In the present study an attempt was made to purify the hexamerin receptor from the larval fat body of rice moth *C. cephalonica*. Initial experiments to purify the receptor were based on the conventional methods like affinity purification and pull down assays. As we failed in getting the desired results, alternative efforts were made exploiting the known structural and functional features of hexamerins. Based on the fact that the hexamerin receptor in *C. cephalonica* undergoes phosphorylation (Arif *et al.*, 2003) and on a report that the storage protein/hexamerin receptors in Diptera are evolved from their ligands (Scheller and Burmester, 1997), a genomic apporoach was made by designing several sets of degenerate primers. Finally we were successful in isolating a 800 bp clone whose sequence was homologous to storage proteins of different insects as well as to a part of VHDL receptor of *H. zia (*Haunarland *et al.*, 1996*).* Incidentally Haunarland had reported that the VHDL receptor in *Helicoverpa zia* does bind to its storage proteins. Driven by this initial cue we went further ahead and isolated a full length cDNA clone of the putative fat body hexamerin receptor of *C. cephalonica*. The 5' and the 3' RACE yielded the products of 618 bp and 1 kb respectively. Since the sequence analysis of the amplified fragment revealed the presence of the phosphotyrosine kinase domain at the 5' end and the homology of the sequence with other insect storage proteins we assumed that this might be the receptor clone as it fulfilled the two above criteria. The length of complete cDNA sequence of the clone was found to be 2.6 kb with and ORF of 2.4 kb.

Once the full length cDNA clone of putative hexamerin receptor was available we tried to express the recombinant protein for further analysis of the receptor. However, all our attempts to do so failed, in spite of repeated trials. Hence we expressed the N-terminal fragment containing the phosphotyrosine kinase domain. The induction of the culture with IPTG at a concentration of 0.8 mM resulted in the expression of the recombinant protein. Upon western analysis with anti-His antibodies we detected a protein of about 28 kDa, which fits perfectly well with the deduced molecular weight of the cloned N-terminal fragment along with the histidine chain. The observed ligand binding of hexamerins with the recombinant 28 kDa protein present in the induced cultures unambiguously suggests that the protein expressed is the hexamerin receptor. Further, the ligand binding was not seen in un induced cultures.

Apart from this we also analysed other tissues for the presence as well as synthesis of hexamerins. For this purpose tissues like salivary gland, MARG, ovary and gut were used. The western analysis using the hexamerin antibodies, showed the presence of hexamerins in all the tissues except gut. Detailed PCR analysis using the gene specific primers of fat body hexamerin subunit (Hex 2b) revealed that except the fat body no other tissue expresses *Hex 2b* RNA transcript, Hence the accumulation of hexamerin is most likely due to sequestration. Which is clearly supported by radiolabelled hexamerin uptake studies. Further ligand blot analysis showed the binding of the biotinlyated hexamerins to the membrane fraction of the salivary gland, MARG and ovary at around 120 kDa, matched perfectly with the size of fat body hexamerins receptor. Phosphorylation profile of these tissues, revealed phosphorylation of the 120 kDa hexamerin binding protein which is mediated by a phospotyrosine kinase. All the above evidences indicate that these tissues do contain a hexamerin receptor, which is similar in size and mode of its activation to larval fat body hexamerin receptor.

Finally, PCR analysis using the gene specific primers of the fat body hexamerin receptor, revealed an amplified product in all the hexamerin sequestering tissues which is of similar size to that of the fat body counterpart. Thus cementing the fact that identical hexamerin receptor is present in all the hexamerin sequestering tissues of *C. cephalonica*

In *C. cephalonica* the influence of morphogenetic hormones on hexamerin synthesis were carried at both the proteomic as well as genomic level. The hormonal treatment showed that 20E had a stimulatory effect whereas JH showed an inhibitory effect on hexamerin synthesis. The *in vitro* and *in vivo* treatment with 20E and its agonist RH 5992 on both the ligated and unligated insects showed an increase in the hexamerin content when compared to the controls. Whereas the treatment of the insects with azardiractin (blocks the release of 20E) blocked the hexamerin synthesis in a time dependent manner.

Complete genomic clone of the hexamerin (hex 2b) was cloned using the gene specific primers. A 3.6 kb genomic clone was obtained which had three introns. The 5' upstream sequence was obtained by genome walking analysis. The analysis of the upstream sequence revealed presence of Ecdysone responsive elements' (ERE). The northern analysis with 20E treated insect fat body showed an increased hexamerin transcript level. This study suggests a transcriptional regulation of hexamerin synthesis in the rice moth, which is under the control of ecdysteriods.

The full length methionine rich hexamerin cDNA was cloned using the degenerate primers and the RACE strategy. The full length cDNA obtained had a size of 2.45 kb with an ORF of 2.26 kb. The sequence showed homology to most of the methionine rich hexamerins reported in the databank with the maximum homology to *P. interpunctella* (75 %) and *C. funiferana* (72%). The deduced amino acid sequence contained 4.9 % methionine suggesting that it belongs to the methionine rich class of hexamerins.

The Southern analysis revealed that the methionine rich hexamerin is a single copy gene. The northern analysis showed that the probe was specific and it detected a transcript around 2.3 kb. The methionine rich DNA fragment with an ORF of 2.26 kb corresponded to the molecular weight of 86 kDa.

The full length cDNA clone of the putative hexamerin receptor was amplified using the RACE strategy. The PCR analysis with the combination of different primers yielded initially an amplicon of 800 bp whose sequence analysis revealed that the fragment has homology with storage proteins of different insects as well as to a part of VHDL receptor of *H. zea.*

The sequence analysis of the 5’ fragment showed a phosphotyrosine kinase domain at the N-terminal end, whereas the 3’ sequence showed homology to most of the storage proteins in insects.

We expressed the N-terminal fragment containing the phosphotyrosine kinase domain in pRSET A expression vector. The western analysis carried out with anti-His antibodies resulted in the detection of recombinant protein at a size around 28 kDa which fits perfectly well with the deduced molecular weight of the N-terminal fragment along with the His-tag.

Recombinant protein was expressed, ligand blotting analysis was carried out to confirm that the expressed protein is the truncated hexamerin receptor. The ligand blotting analysis showed that the hexamerins specifically bound to the 28 kDa protein.

Presence of a hexamerin receptor identical to that of fat body was confirmed in tissues like salivary gland, MARG and ovary using ligand blotting and uptake of biotinlyated hexamerins, western and PCR analysis.

Conclusions:

In short the full length genomic clone of a hexamerin (Hex 2b) was cloned and the role of 20E in the regulation of hexamerin at both the proteomic and genomic level was determined. The presence of ecdysone responsive elements (ERE ‘s) at the 5’ upstream region shows that the probable mode of hexamerin regulation by 20E is genomic and is transcriptionally regulated. The full length cDNA (2.3 kb) of methionine rich hexamerin was cloned using the RACE strategy and the sequence analysis of the clone showed its homology to most of the reported methionine rich hexamerins in insects. Finally the full length hexamerin receptor was cloned and an N-terminal fragment containing the phosphotyrosine domain was expressed as recombinant truncated protein. The specificity of ligand blotting analysis using hexamerins confirmed that the expressed protein is a hexamerin receptor.

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