HEXAMERINS, THEIR GENE AND BINDING PROTEIN IN RICE MOTH, *CORCYRA CEPHALONICA*

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

P. NAGAMANJU

Department of Animal Sciences
School of Life Sciences
University of Hyderabad
Hyderabad 500 046

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Enrollment no. 98LAPH07

Dedicated to *my*

Parents

(An Abode *of* Affection)

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DECIARATION

1 here by declare that the work embodied in this thesis **entitled "Hexamerins, their gene and binding protein in rice moth, *Corcyra cepltalonica"*** has been carried out by me under the supervision of Prof. Aparna Dutta-Gupta and that this has not been submitted for any degree or diploma of any other university.

P. Nagamanju

Candidate

Prof. Aparna Dutta Gupta

Supervisor

UNIVERSITY OF HYDERABAD

(A central university established in 1974 by an act of Parliament)

Hyderabad 500 046, India

CERTIFICATE

This is to certify that Mrs. P. Nagamanju has carried out the research work embodied in the present thesis under my supervision and guidance for **a** full period prescribed under the Ph.D ordinance of this university. I recommend her thesis entitled **"Hexamerins, their gene and binding protein in rice moth, *Corcyra cephalonica"*** for submission for the degree of **Doctor of Philosophy** of this university.

Prof. Aparna Dutta Gupta 17/5

Head,

Dean, 17/5/03

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ABBREVIATIONS

ALP	Alkaline phosphatase
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BCIP	5-Bromo-4-chloro-3-indoyl phosphate
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
cDNA	Complementary DNA
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Dinucleoside triphosphates
DTT	Dithiothreitol
20E	20-Hydroxyecdysone
EDTA	Ethylene diamine tetra acetic acid
IgG	Gamma immunoglobulin
IPTG	Isopropylthiogalactoside
KCl	Potassium chloride
LB agar	Luria-Bertani Agar
LB medium	Luria-Bertani Medium
MARG	Male Accessory Reproductive Gland
$MgSO_4$	Magnesium sulphate
mM	Millimolar
MOPS	3-(N-morpholino) propane sulphonic acid

mRNA	Messenger RNA
MYA	Million Years Ago
NaCl	Sodium chloride
NBT	Nitro blue tetrazolium
ng	Nanogram
PAGE	Polyacrylamide gel electrophoresis
nM	Nanomolar
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenyl methyl sulphonyl fluoride
POPOP	1, 4-bis [5-(phenyl-2-oxazoly)] benzene
PPO	2, 5, diphenyloxazole
RNA	Ribonucleic acid
RP-HPLC	Reverse phase-HPLC
RT-PCR	Reverse-transcriptase-PCR
SDS	Sodium dodecyl sulphate
TAE	Tris acetate/EDTA buffer
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TE	Tris EDTA
TEMED	N, N, N', N', Tetramethyl ethylene diamine
Tris	Hydroxymethylaminomethane
V/V	Volume/volume

Rat body cell

CHAPTER 1

Introduction

The ontogeny of insects is characterized by metamorphosis, a crucial phenomenon by which structurally different stages such as larvae, pupae and adults are connected with one another. During larval-pupal-adult transformation of holometabolous insects, the larval organs either undergo total degeneration or undergo remodeling to give rise to adult structures. Adult tissues/ structures are also derived from the imaginal discs. These processes involve mobilization as well as synthesis of various molecules like proteins, lipids and carbohydrates. Changes in morphological structure, appearance of new macromolecular entities and altered patterns of macromolecular localization, are some of the hallmarks of the development process. 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 and their expression is of paramount significance.

Fat body is the most conspicuous organ in the body cavity of insects, and it is a multifunctional tissue. 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, 1985). The tissue freely bathes in haemolymph and such an organization facilitates rapid, metabolic exchange of macromolecules between the fat body and haemolymph. Furthermore, during postembryonic as well as adult development there is rapid exchange of proteins between the fat body and haemolymph, which in coordination with each other play a very significant role (Terwilliger *et al,* 1999). The fat body undergoes a chronologically ordered sequence of alterations during postembryonic development (Dean *et al.,* 1985). It is the place of intense biosynthetic

activity. In actively feeding larval stages, the fat body synthesizes various macromolecules including fatty acids, lipids, carbohydrates and wide range of proteins, which are released into haemolymph (Telfer and Kunkel, 1991; Wang and Haunerland, 1991, 1992; Haunerland, 1996). Through the haemolymph, these macromolecules are transported to different tissues, where they perform various physiological functions. At the end of the larval development and during pupal-adult transformation there is considerable change in physiology as well as morphology of fat body cells. The fat body diminishes its activity in intermediary metabolism and protein synthesis and changes from a synthetic organ to a storage organ (Price, 1973; Dean *et ed.,* 1985). This switch is characterized by the appearance of large number of electron dense storage granules and coated vesicles. The fat body at this stage stores various macromolecules which are mobilized during the metamorphosis in holometabolous insects and are used as metabolic fuel and building blocks for pupal-adult development (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 (Boman and Hultmark, 1987; Shahbuddin *et al.,* 1998; Hetru *et al,* 1998; Johns *et al*., 1998, 2001; Vizoli *et al.,* 2000; Nakajima *et al.,* 2001), lipophorins (Sharpiro *et al.,* 1988; Burks *et al.,* 1992; Kanost *et al.,* 1995; Rimoldi *et al*., 1996), diapause proteins (Lewis *et al.,* 2002), hormone-binding or ion-binding proteins (Goodman and Chang, 1985; Huebers *et al,* 1988; Koopsmanchap and de Kort, 1998; Trowell, 1992; Braun and Wyatt, 1996; Harai *et al*., 1998; Maya-Monteiro *et al,* 2000; Gudderra *et al,* 2002), cynoproteins (Chinzei *et al,* 1991a, b; Miura *et al,* 1994), haemocyanins (Van Holde and Miller, 1995; Burmester, 2002) and hexamerins (Telfer and Kunkel, 1991; Haunerland.

1996; Danty *et al.*, 1998). These macromolecules reflect diversified roles played by haemolymph in mediating intercellular communication, transporting metabolites and imposing a defensive barrier against micro-organisms (Kanost *et al*, 1990, Blacklock and Ryan, 1994; Haunerland, 1996; Wang and Nuttal. 1999).

Among all the proteins synthesized by the fat body and secreted into 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*. The ground breaking work on insect hexamerins was done in the dipteran insect, *Calliphora erythrocephala* on the protein originally designated as calliphorin (Munn and Greville, 1969; Munn *et al*, 1967, 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 and Brock, 1981; 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 production of adult proteins.

Hexamerins being ubiquitous, in many insects play a diversified role in the development of insects. Although hexamerins were shown to be haemocyanin related proteins, they do not serve as oxygen carrier in insects. The major function of hexamerins is to serve as 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). 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 the precursors for pupal and adult cuticle deposition in insects (Scheller *et al.,* 1980; Marinotti *et al,* 1988; Peter and Scheller, 1991). They were shown to carry hydrophobic substances such as lipids and riboflavins (Kanost *et al.*, 1990; Miller and Silhacek, 1992; Magee *et al.,* 1994). Hexamerins bind to and also transport hormones in various insects (Enderle *et al.*, 1983; Braun and Wyatt, 1996). Involvement of hexamerins in immune defense was also reported in various insects (Burmester, 1999). They have been found to be associated with diapause (de Kort and Koopmanschap, 1994; Lewis *et al.,* 2002). 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, 1986b) and this feature could be exploited for the site directed delivery of bio-pesticides, toxins and growth regulators for management of insect pests.

Structurally hexamerins are multimeric aggregates of six subunits in the range of 70-90 kDa, which may be homo- or hetero-hexamers, with native molecular mass around 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). Hexamerins have

been biochemically purified and characterized in a number of insects (Telfer and Kunkel, 1991; Korochkina *et al,* 1997a, b; Gudderra *et al.,* 2002). Most documented hexamerins are from orders lepidoptera and diptera. They have also been studied in hymenoptera (Ryan *et al,* 1984, Shipman *et al.,* 1987; Martinez and Wheeler, 1993, 1994,), coleoptera (Rhabe *et al.,* 1990; Koopmanschap *et al.,* 1992) and in a number of hemimetabolous insects (Rehn and Rolim, 1990; Chinzei *et al.,* 1991a, b, 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%). This class includes prototype storage protein calliphorin, from *C. erythrocephala* (Munn *et al.,* 1967, 1971). These are the major haemolymph proteins in diptera (Katsoris and Marmaras, 1979; Brock and Roberts, 1983; de Bianchi *et al.,* 1983; de Bianchi and Marinotti, 1984; Naumann and Scheller, 1991; Burmester *et al.,* 1998a). The second category is the lepidopteran glycoprotein with high aromatic amino acid and low methionine content (Kramer *et al.,* 1980; Riddiford and Hice, 1985; Haunerland and Bowers, 1986a; Palli and Locke, 1987a; Webb and Riddiford, 1988a; Fujji *et al.,* 1989; Karpells *et al.,* 1990; Kunkel *et al.,* 1990) named as lepidopteran arylphorins. Arylphorins are the major haemolymph proteins not only in lepidoptera and diptera, but are also detected in the haemolymph of other insect orders (Ryan *et al.,* 1984; Delobe *et al,* 1992; Martinez and Wheeler, 1993; Koopmanschap *et al.,* 1992). The third group of hexamerin is found in many dipteran insects. which are neither rich in aromatic amino acids nor in methionine (Roberts and Brock, 1981; Benes *et al.,* 1990; Haunerland, 1996; Braun and Wyatt, 1996; Korochkina *et al.,* 1997a, b). The fourth group has relatively high methionine content (about 6%) and low aromatic

amino acid content. They are predominantly expressed in the female larvae of lepidoptera, and they form the yolk protein precursors (Webb and Riddiford, 1988a, b; Bean and Silhacek, 1989; Rehn and Rolim, 1990; Kunkel *et al.,* 1990; Corpuz *et al.,* 1991; Wang *et ed.,* 1993; Jamroj *et al.*, 1996; Pan and Telfer, 1996; Mi *et al.*, 1998; Hwang *et al.,* 2001; Zhu *et al.,* 2002). In addition hexamerins were shown to bind riboflavin and are therefore referred to as riboflavin-binding hexamerins, e.g., *Hylophora cecropia* RbH (Magee *et al.*, 1994). The equivalence of a riboflavin-binding hexamerin and arylphorin as reserves for the development in two saturniid moths was also reported (Pan and Telfer, 1999). It has been observed that insect hexamerins generally undergo post-translational modifications like glycosylation and lipidation and are either glycoproteins or lipoglycoproteins. Lipidation is associated with better storage of these proteins, while gycosylation facilitates secretion of these proteins from the site of synthesis (fat body) in to the haemolymph and their sequestration back into the fat body and other tissues from haemolymph.

With growing importance of hexamerins in insect development, numerous studies concerning their biosynthesis, regulation and evolution have been conducted during recent past (Haunerland, 1996; Burmester, 1999). Hexamerins are the most dramatic examples of developmentally regulated gene function. In both hemi- and holometabolous insects hexamerin synthesis is correlated most often to the larval molting cycles. These proteins accumulate in the haemolymph during inter-molt periods. In some insect species, hexamerins are also found at other developmental stages including adults, reflecting their varied role in various physiological functions (de Kort and Koopmanschap, 1994; Faria *et al.,* 1994; Martinez and Wheeler, 1994; Wheeler and Martinez, 1995; Ancsin and Wyatt, 1996). The synthesis of hexamerin exhibits a precise

developmental sequence in insects (cf. review Leevenbook, 1985; Telfer and Kunkel, 1991). However, significant differences exist between species with respect to timing and duration of synthesis of various hexamerins. In diptera hexamerin synthesis begins only in the penultimate larval stage, simultaneously with the appearance of various mRNAs (Sekeris and Scheller, 1977; Lepesant *et al*, 1986; Schenkel and Scheller, 1986; Patrinou *et al*, 1987), whereas in lepidoptera, they are expressed at a low rate throughout larval life (Ray *et al*, 1987a; Webb and Riddiford, 1988a), *i.e.*, hexamerin synthesis is seen both in the early larval instars as well as in the ultimate larval instar (Kramer *et al*., 1980; Ray *et al*, 1987a; Webb and Riddiford, 1988a; Karpells *et al*, 1990; Kunkel *et al.*, 1990). This could be correlated with the mRNA levels of the fat body. During the final molt, hexamerin mRNA level decreases and finally its synthesis stops (Riddiford and Hice, 1985; Caglayan and Gilbert, 1987).

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 (Kinear and Thomson; 1975; Sekeris and Scheller, 1977; Wyatt and Pan, 1978; 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 become undetectable in the haemolymph during the late pupal and adult stages. In some insect species hexamerins are also expressed in other tissues. In *Calpodes ethlius*, in addition to the fat body small quantities of arylphorins are also synthesized and secreted by the

epidermal tissues and mid gut (Palli and Locke, 1987a, b, c). Pericardial cells (Fife *et al,* 1987; Leung *et al.,* 1989) and haemocytes were reported as the source of hexamerins (Hughes and Price, 1976; Katagiri, 1977). Webb and Riddiford (1988b) reported a broad distribution of hexamerin mRNA in *Manduca sexta.* In fall webworm, *Hyphantria cunea* hexamerins are locally expressed in Malpighian tubules (Hwang *et al.,* 2001). Hexamerin expression was also seen in the gonads of adult insects (Miller *et al.,* 1990; Kumaran *et al.,* 1993). In Colorado potato beetles hexamerin expression was found in short day adults (Koopmanschap *et al.,* 1992). In *Musca domestica* hexamerins are preferentially expressed in adult females, where they serve as amino acid pool for egg formation (Pereira *et al.,* 1989; Capurro *et al.,* 1997, 2000). Zakharkin *et al.* (2001), reported female-specific expression of hexamerin gene in larvae of an autogenous mosquito, *Aedes atropalpus.* Thus, hexamerin gene expression is not only confined to fat body but these genes are also expressed in other tissues at different developmental stages.

Two major morphogenetic hormones JH (juvenile hormone) and 20E (20-hydroxyecdysone) play a very important role in insects and regulate the postembryonic development involving molting, puparium formation and onset of adult metamorphosis. 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 JH and ecdysteroid regulates the stage-specific expression of different genes in various cell types of insects during postembryonic development (Schneiderman and Gilbert, 1964; Riddiford, 1981, 1985; Sehnal 1981; Hiruma *et al.,* 1991).

The influence of these metamorphic hormones on the expression of hexamerins has been reported in several insects. Furthermore,

hexamerins offer excellent model system for the study of hormonally regulated gene expression at both transcriptional and post-transcriptional levels. JH was shown to inhibit the expression of some larval proteins (Jones *et al.,* 1988; Memmel and Kumaran, 1988). It was also reported to inhibit synthesis of one of the hexamerins, in *B. mori* (Tojo *et al.,* 1981), *Spodoptera litura* (Tojo *et al,* 1985) and *Corcyra cephalonica* (Ismail and Dutta-Gupta, 1988). In fall webworm, *Hyphantria cunea* SP-2 expression was suppressed by JH (Hwang *et al.,* 2001). On the other hand, in hemipteran insect, *Riptortus clavatus*. the expression of the cp alpha subunit in the adults has been shown to be enhanced by JH while the expression of the cp beta subunit was suppressed by the same hormone (Miura *et al*., 1998).

The influence of ecdysteroid on fat body protein synthesis was the subject of several studies. Treatment with 20E, stimulated the synthesis of both mRNA and rRNA in the fat body, which enhanced overall protein synthesis (Natori, 1976; Scheller and Karlson, 1977; Lepesant *et al*., 1978; Karlson 1980; Ouellette and Caveney, 1990; Scheller *et al,* 1980; Riddiford, 1985). Ecdysteroids were shown to stimulate the hexamerin synthesis in *D. melanogaster,* mainly through transcriptional regulation (Jowett and Postlethwait, 1981; Lepesant *et al,* 1982, Powell *et al.,* 1984, Mousseron *et al,* 1997). Riddiford and Hice (1985) suggested that the arylphorin synthesis in *M. sexta* was regulated by rising ecdysteroid titer at each molt. In blowfly larvae however, ecdysteroid was reported to inhibit hexamerin synthesis while JH had no inhibitory effect on hexamerin gene regulation under *in vitro* conditions (Pau *et al*., 1979). The inhibitory effect of 20E on lepidopteran hexamerin synthesis has also been reported (Kumaran *et al,* 1987; Ray *et al*., 1987b;; Webb and Riddiford, 1988b).

In addition to hormones, the nutrient supply also appeared to effect the hexamerin expression. In *Bombyx mori*, both methionine rich hexamerin and arylphorin synthesis were negatively affected under conditions of nutrient deprivation (Tojo *et al,* 1981). In contrast, in both *M. sexta* and *S. litura* only synthesis of arylphorin was dependent on nutrient supply (Riddiford and Hice, 1985; Tojo *et al,* 1985; Kumaran *et al,* 1987; Webb and Riddiford, 1988a, b). Recently Telang *et al* (2002) reported that in *H. virescens* the expression of putative arylphorin and one of the methionine rich hexamerins was dependent on the levels of the dietary proteins.

Despite the existence of extensive literature on hexamerins, from both lepidoptera and diptera, there are significant gaps in our understanding of their structure, synthesis, processing, assembly and transport in stored grain pests. This could be due to the diversity of insect species that have served as experimental models presenting an inconsistent and at times contradictory overview of hexamerin expression and their metabolism. One important step towards resolving many of these issues is obtaining cloned DNA molecules encoding the hexamerins from which primary amino acid sequences and sites of post-translational modification can be deduced. These clones could also be useful as probes, for determining steady-state levels of transcript as a function of developmental status or experimental treatment. Furthermore, vector-based expression systems containing hexamerin cDNA/ genes may also prove valuable in dissecting biochemical pathways leading to their metabolism and transport.

To date there are many hexamerins, whose primary sequences have been established (Yashihiro and Okisugu, 1991; Memmel *et al.,* 1992, 1994; Burmester *et al*., 1998a; Gordadze *et al*., 1999; Hwang *et al,* 2001; Zhu *et al,* 2002). Multiple alignment analysis of the deduced amino **acid**

sequence derived from the cDNA sequence data of several species has revealed a significant homology between insect larval hexamerins as well as with other proteins. Three abundant hexamerins have been detected in larval and pupal haemolymph and pupal fat body of the tobacco budworm, *Heliothes virescens*, having sub-unit molecular mass of 74 kDa, 76 kDa and 82 kDa (designated as p74, p76 and p82 respectively). Both, the p82 cDNA and a putative p76 cDNA clones were sequenced. An arylphorin-like amino acid composition was determined for the putative p76 cDNA (Robert and Stephen, 1990). A cDNA clone corresponding to SP-2 from fall webworm, *Hyphantria cuneae* (Hwang *et al.*, 2001) revealed high homology with Tn JHSP-2 of *Trichoplusia ni* (Jones *et al.*, 1990). Bm LSP cDNA identified in *B. mori* (Yashihiro and Okisugu, 1991) showed high homology with microvitellogenin of *M. sexta* (Wang *et al.*, 1988) and protein of *B. mori* (Sakurai *et al*., 1988). Detailed studies revealed that the time of divergence of the lepidopteran and dipteran hexamerins was about 280 million years ago (MYA) (Burmester *et al.*, 1998a). A high homology was also observed between insect hexamerins and arthropod haemocycanins (Naumann and Scheller, 1991), which share about 20-25% sequence identity showing common ancestry (Beintema *et al.*, 1994). It has been reported that arthropodan haemocyanins, prophenoloxidases (PPOs) and insect hexamerins form a super family of haemolymph proteins called AHPH super family (Sanchez *et al.*, 1998; Burmester, 2001). Beintema *et al.* (1994) proposed that hemocyanin is the ancestral arthropod protein and that insect hexamerins lost their copper binding capability after divergence of them from the crustaceans. Phylogenetic studies on hexamerins have reported that arthropod tyrosinase, arthropod hemocyanin, insect hexamerin and dipteran arylphorin receptor share a common origin (Burmester and Scheller, 1996; Burmester *et al.*, 1998a). The derived amino acid sequence from cDNA clone of juvenile hormone-binding protein (JHBP) from the migratory locust revealed that this

protein represents a new group within the hexamerin family of arthropod proteins (Braun and Wyatt, 1996).

The pattern of synthesis of hexamerins in a variety of holometabolous insects including lepidopterans exhibits a precise developmental sequence (Levenbook, 1985). Using these hexamerin cDNA clones as probes for northern blot analysis, expression pattern of hexamerins was widely studied in insects. Using two cDNA hexamerin clones Ray *et al.,* (1987a) clearly demonstrated the presence of hexamerin transcripts only in the fat body of *Galleria mellonella.* Hexamerin transcripts were not detectable in the silk gland, midgut and Malpighian tubules. However, transcripts complementary to both these clones were present in the carcass though much less abundantly than in fat body and this was most likely due to the presence of contaminating fat body in the carcass preparation. Similar kind of tissue distribution for hexamerins was reported *in B. mori* (Yashihiro and Okisugu, 1991). Majority of these studies unequivocally demonstrate that larval fat body is the major tissue where hexamerin genes are expressed. However, Robert and Stephen, (1990) reported that in *H. virescens* p82 and p76 hexamerin genes are transcribed in testis, indicating that metabolically distinct hexamerin pools occur in the haemolymph and testicular fluid. In *M. domestica* Hex-l, larval specific glycoprotein hexamerin disappears from haemolymph after the emergence, and a new hexamerin Hex-f appears in the adult haemolymph (de Bianchi *et al.,* 1983; Marinotti and de Bianchi, 1986; Marinotti *et al.*, 1988). The variation in abundance of Hex-f during female life follows a pattern similar to the one observed for yolk proteins and protein synthesis, attaining its maximum level at about the middle of the gonadotropic cycle, and declining thereafter until the end of the cycle (Capurro *et al,* 2000). Precise developmental, stage specific and independent expression of Hex-1 and Hex-f gene in larvae and adult respectively indicate different

transcriptional control mechanisms which regulate these genes during postembryonic and adult development.

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 (Goldstein *et al*., 1979; Hertel and Perkins, 1987; Lin *et al*., 1998; Santins *et al*., 1998). Among the various proteins reabsorbed by fat body, hexamerins form the majority group. Endocytosis, is also an essential process for the recycling of membranes.

The uptake of hexamerins from insect haemolymph by the fat body cells is an unique feature of the class Insecta involving a receptor which does not belong to the low density lipoprotein (LDL) superfamily (Burmester and Scheller, 1999). These receptors have been recognized in dipteran as well as lepidopteran insects (Ueno and Natori, 1984; Burmester and Scheller, 1992, 1997) and lepidoptera (Wang and Haunerland, 1994a and b; Kirankumar *et al*., 1997). In the flesh fly, *Sarcophaga peregrina*, a 120 kDa receptor was identified. Under the influence of 20E, the receptor acquires 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, 1997). The onset of hexamerin uptake coincides with the third cleavage, which is initiated by ecdysteroids (Burmester and Scheller, 1997, 1999). Recently, the presence of an anterior fat body protein has been reported in *C. vicina* which interacts with the hexamerin receptor and regulates hexamerin uptake by the fat body cells in the posterior part of the organ (Hansen *et al*., 2002). 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, 1994a and b). 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, 1990a). These studies suggest that ecdysteroids activate the hexamerin receptor, which in turn is responsible for sequestration.

With this background on hexamerins from different insects, research in our laboratory concentrated on *Corcyra cephalonica* which belongs to order lepidoptera and family galleriinae/ pyrilidae. 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 in any 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. 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 ah,* 1997). Studies revealed that their synthesis is developmentally as well as hormonally regulated. It has also been observed that hexamerins play a very important role in metamorphosis and reproduction (Dutta-Gupta and Ismail, 1992). To know more about the hexamerin expression and understand the mechanisms that underlie hexamerin endocytosis, present study was conducted with the following set of objectives:

- To identify and isolate the cDNA clones of genes encoding hexamerins
- Sequence analysis and characterization of cDNA clones
- Expression of hexamerin genes during larval development
- Uptake of hexamerins and its mechanism

CHAPTER 2

Materials and Methods

Chemicals:

[^{35}S]-Methionine (specific activity-1,000 Ci / mmol) was obtained from Bhabha Atomic Research Centre (Trombay, India). [a ^{32}P] dATP and [γ ^{32}P] ATP (3,000 Ci / mmol) were obtained from Board of Radiation and Isotope Technology (Jonaki, Hyderabad). Agarose, bovine serum albumin (fraction V), bromophenol blue, Brilliant blue R, Brilliant blue G250, 2-mercaptoethanol, dialysis bags, DTT (dithiothrietol), EDTA (ethylene diamine tetra acetic acid), EtBr (ethidium bromide), IPTG (isopropyl P-D-thiogalactoside), lysozyme, MOPs (3-[N-Morpholino] propane sulphonic acid), PMSF (phenyl methyl sulphonyl fluoride), phenylthiourea, PPO 2, 5, diphenyloxazole, POPOP 1,4-bis [5-(phenyl-2-oxazoly)] benzene, protein A Sepharose, and streptomycin sulphate were obtained from Sigma Chemical Company (St, Louis, MO, USA). Agar, ampicilin, yeast extract and tryptone were purchased from HiMedia Laboratories Limited, (Mumbai). TC-100 insect culture medium (PAA Laboratories GmbH, Austria), sodium dodecyl sulfate (SDS) (Bio-Rad Laboratories, Richmond, CA, USA) and 20-hydroxyecdysone (Rohto Pharmaceutical Co., Osaka, Japan) were also procured. Nitrocellulose and nylon membranes (Millipore, Bedford, USA), X-ray film (Kodak, Rochester, USA), Freund's complete and incomplete adjuvants (Difco Laboratories, Michigan, USA) were used. NucleoSpin Miniprep Kit (#K3063-l, QIAGEN) for plasmid isolation and SMART III cDNA library construction kit (Clone Tech Laboratories, Palo Alto. USA), DIG RNA labeling kit (SP 6/ T7) BM from Boehringer Manheim were procured and used. Anti-rabbit lgG-ALP conjugate, NBT-BCIP, high molecular weight protein marker, DH5a strain of *E. coli*, restriction enzymes (EcoR1, Hind III and Sma I) and random primer labeling kit were supplied by Bangalore Genie (Biological Research Products, Bangalore). Tri-Fast Gold RNA extraction buffer was purchased from Peqlab (Biotechnology BmbH, Germany). Tris, acrylamide, N-N-methylene bis-acrylamide and glycine

from Spectrochem (Bombay, India), silver nitrate, TEMED (N,N,N,N tetramethylethylenediamine), dimethyl sulfoxide (DMSO), glycerol and hydrogen peroxide (30%) from E. Merck (Germany) were obtained. All the other chemicals were obtained from commercial sources in India and were of analytical grade.

Experimental insects:

Corcyra cephalonica:

Corcyra cephalonica (Station), commonly known as the rice moth, belongs to the family galleriinae/ pyrilidae, order Lepidoptera. It is a serious pest of stored cereals, oil seeds and legumes in tropical and sub-tropical regions of the world (Freeman, 1976). It occurs abundantly as a pest of bulk stored sorghum under humid conditions.

Rearing method and life cycle:

The insects were reared in glass troughs in the culture room maintained at 26+1°C temperature, 70+5% relative humidity and a 14:10 h light-dark period. For egg laying an equal number of males and females of C. *cephalonica* were released into glass troughs containing coarsely crushed sorghum. Larvae hatch from the eggs after 4-5 days and begin to feed immediately after emergence. The larval development consisting of five instars was completed in about 25-30 days. The last instar larva entered into a non-feeding stage called prepupa, which extended over 4-5 days. This stage was followed by pupal stadium, which lasted for 7-8 days. The adults lived for about 8-10 days.

Stages used for the present study:

For the present study following larval stages were used and they were categorized on the basis their body weight and head capsule size (Ashok and Dutta-Gupta, 1988; Ismail and Dutta-Gupta, 1988; Lakshmi and Dutta-Gupta, 1990). The last larval instar was classified into three distinct sub-stages. Larvae weighing 56-65 mg with head capsule size 0.80-0.95 mm were recognized as early-last instar (ELI), those weighing from 66-75 mg with head capsule size 0.96-1.03 mm as mid-last instar (MLI) and those weighing from 76-85 mg with head capsule size 1.04-1.08 mm were considered as late-last instar (LLI). Prepupae, which were 2-3 days old, were used. For certain studies, adult males and females were also used.

Bombyx mori:

B*ombyx mori*, commonly known as silk moth belongs to the order Lepidoptera and family Bombycidae. Larval forms of pure Mysore multivoltine strain were obtained from the local breeding center and reared on fresh mulberry leaves under sterile conditions in insect culture room maintained at 26+1˚C temperature, 70+5 % relative humidity and 14:10 h LD period. Staging of larvae was carried out based on their age after the first ecdysis for the second instar and their age after the third ecdysis for fourth instar. The last instar larvae were further classified into ELI (2-3 days old), MLI (4-5 days old) and LLI (6-7 days old) and used for the present study.

Spodoptera litura:

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

Bacterial strains:

(i) **XL1-Blue** (Wood *et al.,* 1985):

In the present study bacterial strain XL1- Blue (*endA*1, *gyrA*96, hsdR17, ***lac',*** *recA*\, *supE*44, *thi*-1, [F' *lacI*^{q}*Z*Δ15, *proAB*, *Tn* 10) was used. *Tn* 10 confers resistance to tetracycline. LB/ tet (15 ug/ml) stock plates were used for library plating and screening. The recombinants were selected by blue/white (p galactosidase) screening. It allowed regulated expression of cloned genes.

(ii) BM25.8 (Palazzolo *et* ***al.,*** 1990):

Bacterial strain BM25.8 (*supE*44, *thi*Δ (*lac-proAB*) *[F'traD36, proAB*$^{+}$, *lacI*^{q}*ZΔM 15] λimm*434 (*kan*^{R}) *hsdR* (r_{k12}-m_{k12}-) was also used in the present study. It is lysogenic for phages *X* and P1, and is used for automatic sub-cloning. Stock plate used was LB/kan (50 ug/ml). BM25.8 bacteria were used for Cre-lox-mediated excision of pTriplEx2 from λTriplEx2.

Methods:

Surgical manipulation:

(i) Thorax ligation:

Larval forms were collected and narcotized by placing them on ice. Ligation was carried out by slipping a loop of silk thread (Ethicon, NJ, USA) around the head of the larvae. The loop was adjusted behind the first **pair** of prolegs and then gradually tightened. The ligated larvae were placed on moist filter paper to maintain humidity and prevent desiccation.

The posterior abdominal part of these larvae were used for experiment after various duration of ligation (12,24, 48, 72 and 96 h)

(b) Microinjection:

The larval forms were narcotized on ice. They were injected with radioisotope and / or hormone using a micro syringe in a volume of 2-5 ul. The wound was sealed with wax-resin mixture **(1:1).**

(c) Organ culture:

For organ culture, different tissues (fat body, ovary, MARG and salivary gland) were dissected out under sterile condition in insect Ringer. The dissected tissues were first rinsed in TC-100 culture medium containing traces of streptomycin sulphate and then in fresh TC-100 medium. All the tissues were preconditioned for 1 h in just TC-100 medium before proceeding with the required experimental set up. They were cultured in 200 ul of fresh TC-100 medium for 4-8 h at 25° C. After culturing, the tissues were rinsed in sterile insect Ringer and processed for homogenate preparation.

Sample preparation:

(i) Tissue homogenates:

The various tissues were dissected out from different developmental stages of insects in cold insect Ringer (130 mM NaCl, 5 mM KCl and 0.1 mM $CaCl_2$). The tissue was weighed and homogenized in a glass-glass homogenizer (Kontes) by hand, in insect Ringer containing 10 mM PMSF at 4°C (Palli and Locke, 1988). The homogenates were centrifuged for 5 min at 4,000 rpm to pellet down the debris. The supernatant was collected, aliquoted and stored at -20°C until use.

(ii) Haemolymph collection:

The prolegs of the mid and late-last instar larvae were cut and they were bled directly into 1.5 ml microfuge tubes pre-rinsed with 0.1% phenylthiourea (phenylthiourea prevents tyrosinase activity and melanisation). All the haemolymph samples were diluted with an equal volume of cold insect Ringer, and centrifuged for 2 min at 4,000 rpm at 4°C to sediment the haemocytes. The supernatant was collected, aliquoted and stored at -20°C until use.

Isolation of Nucleic acid and Protein:

(i) RNA isolation:

For RNA isolation, tissue was dissected out under sterile conditions in ice-cold sterile insect Ringer. It was rinsed in insect Ringer and homogenized in 400 ul Trifast Gold buffer (Peq Lab, Erlangen, Germany). To the homogenate 200 µl of ice cold chloroform was added and gently vortexed. It was incubated for 5 min on ice and centrifuged for 10 min at 12,000 rpm at 4°C. The upper aqueous phase containing the nucleic acid was transferred into a fresh microfuge tube, and re-extracted once again with 200 ul of ice-cold chloroform. The upper aqueous phase was collected and to this 500 ul of isopropanol was added. The sample was vortexed for 15 sec and was allowed to stand for 5 min at room temperature. RNA was pelleted by centrifugation at 12,000 rpm for 10 min. The RNA pellet was washed twice with 70% ethanol and stored in ethanol at -70°C until use.

(ii) Plasmid DNA isolation:

Bacteria containing recombinant plasmids were allowed to grow in LB/ amp broth (1% bacto-tryptone; 0.5% bacto-yeast extract; 0.5% NaCl; 100 ug ampicillin in 10 ml broth) for 14-16 h. The cells were pelleted by

centrifugation for 10 min at 3,000 rpm. Plasmid DNA was isolated using QIAGEN plasmid isolation kit and its protocol. The pellet was suspended in 250 ul of P1 buffer (300 mM NaCl, 100 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.2% mg/ml BSA, and 20 mg/ml RNase A). To the suspension 250 µl of P2 buffer (30% polyethylene glycol (PEG 6000) and 3 M NaCl) was added, inverted gently 4-5 times and incubated at room temperature for 5 min. Then 100 ul of buffer N3 (100 mM NaCl, 100 mM Tris-HCl, pH 7.5 and 25 mM EDTA) was added and inverted for 4-5 times. This was centrifuged for 10 min at 3,000 rpm to separate supernatant from compact white pellet. The supernatant was loaded onto the QIAprep column. The column was washed with 750 ul of PE buffer (10 mM NaCl, 50 mM MOPS, pH 7.0 and ethanol phase) and plasmid DNA was eluted with 50 ul of buffer EB (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

(iii) Radiolabelled hexamerin isolation:

The mid-last instar larvae were narcotized on ice and injected with 10 µCi of [^{35}S] methionine (in 6 µl). The wound was sealed with wax-resin mixture (1:1) and the insects were placed back on their diet (sorghum). After 16 h incubation, haemolymph was drained out in microfuge tube by cutting the prolegs. It was diluted with insect Ringer (1:1) and to pellet down the hemocytes it was centrifuged for 2 min at 4,000 rpm. The diluted haemolymph was subjected to column chromatography (Sephadex G-25) for removal of unincorporated [^{35}S] methionine. The specific activity of the radiolabelled protein was determined by scintillation spectrometry.

Macromolecular quantification:

(i) Protein estimation by micro protein assay **(Bradford** 1976):

Bradford protein reagent was prepared by dissolving Brilliant blue G-250 (10 mg) in 5 ml of 95% ethanol. To this solution, 10 ml of 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 100 ml with double distilled water and filtered through Whatman No. 2 filter paper and stored in an amber colored bottle at 4°C.

For protein estimation, an aliquot of the sample was pipetted out into a 1.5 ml microfuge tube. The volume of the sample was adjusted to 0.1 ml with insect Ringer. One ml of Bradford protein reagent was added and the contents were mixed by gentle inversion. After 15 min, absorbance at 595 nm was measured spectrophotometrically against a blank prepared from 0.1 ml of insect Ringer and 1 ml of protein reagent. Protein concentration in the sample was calculated from a standard curve drawn using BSA (fraction V).

(ii) DNA and RNA quantification:

The concentration of RNA and DNA was determined by measuring absorbance of solution at 260 nm (A_{260}) in a spectrophotometer. The absorbance of the sample was also determined at 280 nm (A_{280}). Purity of the sample was checked by determining the ratio between the readings at 260 nm and 280 nm.

(iii) Radiolabel quantification:

Radiolabel incorporation in various tissues was quantified using scintillation spectrometry. The proteins in tissue homogenate were precipitated by adding equal volume of 10% TCA. The samples were incubated on ice for 15 min, and centrifuged for 5 min at 10,000 rpm. The pellet was collected and washed once with 100 ul of 5% TCA, once with

100 µl ethanol: ether (3:1) and finally with 100 ul of ethanol. The pellet was air dried and dissolved in 50 ul of 0.1 N NaOH. The radiolabel quantification was carried out using 10 µl of solubilised protein, which was added to 5 ml of Bray's mixture (PPO 4 g, POPOP 200 mg, naphthalene 60 g, methanol 100 ml, ethylene glycol 20 ml and volume was made up to 1 liter with 1,4 dioxan). Tri-Carb Packard Liquid Scintillation Analyzer (Model 2100 TR/2300 TR) was used for sample analysis.

Electrophoresis procedures:

(i) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE):

SDS-PAGE was carried out according to the procedure of Laemmli (1970). A 1 cm 3 % stacking gel (pH 6.8) was followed by a 7.5% separating gel (pH 8.8). Tris-glycine buffer (0.025 M) with 0.1% SDS (pH 8.3) was used as electrode buffer.

Preparation of samples:

An aliquot of protein sample was mixed with an equal volume of 2X sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol and 0.002% bromophenol blue) and boiled for 2 min at 100°C, to denature the proteins.

Staining of the polyacrylamide gels:

(a) Coomassie blue staining: Proteins separated on polyacrylamide gels were stained with coomassie blue (0.07% Brilliant blue R in 50% methanol and 7.5% acetic acid) for 4-8 h and destained with destaining solution (5% methanol and 7.5% acetic acid) to visualize the protein bands.

(b) Silver staining: Polyacrylamide gels were silver stained according to the method of Blum *et al.*, (1987) with minor modifications. The gels were incubated in fixative (50% methanol, 12% acetic acid, 50 ul of 37% formaldehyde in 100 ml) for a period of 1 h and were then washed twice with 50% ethanol (30 min each). The gels were pre-treated with sodium thiosulphate (20 mg/100 ml) for 1 min. Then the gels were washed 4-5 times with double distilled water (20 sec each) and impregnated with silver nitrate (0.5% silver nitrate, 187 ul of 37% formadehyde) with gentle agitation on a mechanical shaker for 45 min. After impregnation, the gels were washed 4-5 times with double distilled water (20 sec each) and developed with developer (6% sodium carbonate (w/v), and 0.05% of 37% formaldehyde (v/v)). Color development was stopped by transferring the gels to 12% acetic acid. The stained gels were rinsed twice in double distilled water (20 sec each), and stored in 50% methanol.

(c) Glycoprotein staining: Proteins separated on SDS-PAGE were stained using periodic acid Schiff s stain (Zacharius *et al.*, 1969) with slight modifications. The gels were oxidized with 1 % periodic acid in 3% acetic acid for 1 h and thoroughly leached for 3 h with water. The gels were stained with Schiff's reagent for 30 min in dark. To visualize the glycoproteins, the gels were destained in several changes of 10% acetic acid and stored in 3% acetic acid.

(ii) DNA agarose gel electrophoresis:

Plasmid DNA was electrophoresed on a 1% agarose gel that was casted using 1X TAE buffer (40 mM Tris-acetate and 10 mM EDTA). The same buffer was used as electrode buffer and a voltage of 5V/cm2 was applied. Electrophoresis was carried out until the dye reached 3-4th length of the gel.

Preparation of samples:

An estimated amount (1-5 μg) of DNA was mixed with loading buffer (6X loading buffer: 0.25% bromophenol blue, 0.25% xylene cynol FF, 30% glycerol in water) and loaded into the slots of the gel for separation.

Staining the gels:

For visualizing the DNA, the gels were stained with ethidium bromide (10 mg/ml stock, 3 ul of the stock was added to 100 ml of 1X TAE buffer). The electrophoresed DNA was visualized using transilluminator.

(iii) Denaturing agarose gel electrophoresis for separation of RNA:

RNA was resolved by denaturing agarose gel electrophoresis in the presence of formaldehyde (Sambrook *et al*., 1989)). Agarose (1.2 g) was boiled in 83 ml of 1X MOPS buffer (diluted from 10X stock: 0.2 M MOPS, 0.5 M sodium acetate, 0.5 M EDTA, pH 7.0 adjusted with 2 N NaOH) and when it cooled to 60°C, 17 ml of formaldehyde was added and the gel was casted. Electrophoretic tank was filled with 1X MOPS buffer and a voltage of 70 V was applied.

Sample preparation:

RNA sample (1-5 ug) was mixed with 15.5 ul sample buffer (50 ul 10X MOPS, 175 ul formaldehyde, 500 ul formamide) and heated at 65°C for 10 to 12 min. It was snap cooled for 2 min on ice and 2.5 ul of 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) was added.

Staining the gels:

The gels were stained with ethidium bromide (10 mg/ml stock, 3 ul was added to 100 ml of buffer) and RNA was visualized using transilluminator.

Immunological methods:

(i) Preparation of antisera:

The antibodies were raised against purified hexamerin mixture as well as against individual hexamerin. The purified protein (200 u.g) was injected subcutaneously at multiple sites into three months old male rabbit (New Zealand variety) after emulsifying with Freund's complete adjuvant. Prior to immunization, the blood was collected from these rabbits by giving an incision in lateral ear vein for the separation of pre-immune sera. Booster injections of protein emulsified with Freund's incomplete adjuvant were given every fortnight. After a lapse of one month, 5 to 10 ml of blood was collected and antiserum was separated.

(ii) Purification of **IgG:**

IgG fraction was purified from the above anti-sera using protein A sepharose column. Tris-HCl (1 M, pH 8.0) was added to serum to make a final concentration of 100 mM. A diluted serum was loaded onto a column pre-equilibrated with 100 mM Tris-HCl buffer (pH 8.0). IgG fraction was eluted with a fresh solution of glycine-Cl (0.05 M glycine, 0.15 M NaCl, pH 2.3). The fractions containing IgG were dialyzed for 48 h against 100 mM Tris-HCl buffer (pH 8.0) to remove the glycine. The dialyzed sample was lyophilized and stored at -20°C. It was reconstituted and used for various studies.

(iii) **Immunodiffusion:**

The antibody titer was detected following common immunodiffusion techniques.

(a) Ouchterlony's immunodiffusion:

Immunodiffusion was performed according to Ouchterlony (1959). It was carried out in petri dishes (5.5 cm X 5.5 cm X 0.2 cm) using 1% agar in

PBS-azide (phosphate buffered saline- 40 mM phosphate buffer, pH 7.2, 150 mM NaCl and 0.05% sodium azide). The antigen and antiserum were loaded in opposite wells (3 mm diameter) and diffusion was carried out for 24 to 48 h at 4°C in humid chamber until the precipitin arches were visible.

(b) Single radial immunodiffusion:

It was carried out according to the procedure outlined by Mancini *et al.,* (1965). Agarose (1 %) was prepared in PBS-azide, it was cooled up to 45°C and antiserum was added to it. It was poured on a glass plate (8 cm X 4 cm). Antigen was loaded in circular wells (3 mm diameter) and incubated for 24 to 48 h at 4°C in humid chambers until the precipitation circles became visible.

(iv) Western blotting:

Western blotting was carried out according to the method of Towbin *et al,* (1979). Proteins separated electrophoretically by SDS-PAGE were electro-blotted on to a nitrocellulose membrane using Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for three h at 70 V. The transferred proteins were visualized by staining the blot with ponceau reversible stain (0.1% ponceau S in 5% acetic acid). The blot was thoroughly washed with TBS (50 mM Tris-HCl, pH 7.4 and 150 mM NaCl) before processing to remove the stain. The nonspecific sites on the blot were blocked by incubating it for 1 h in blocking solution (5% milk powder in TBS). After blocking, the membrane was washed in TBS and incubated for 1 h with primary antibody (1:500 dilution with blocking solution). Then the blot was thoroughly washed in TBS (5 changes each of 10 min) and incubated for 1 h in secondary antibody (ALP conjugated anti-rabbit goat IgG) diluted with blocking buffer (1: 10000). The membrane was washed again in TBS buffer (5 changes each of 10 min). To visualize the cross-reacting bands the blot was developed in ALP

substrate solution (containing NBT, BCIP) (Sambrook *et al.,* 1989). Finally, the blot was washed with distilled water, air-dried and stored between two Whatman sheets.

Northern blotting and hybridization (Sambrook *et al.,* 1989):

Total RNA (2 to 5 µg) was separated on 1.2% denaturing agarose formaldehyde gel. After electrophoresis the RNA gel was soaked in 0.05 N NaOH to remove formaldehyde and rinsed in several changes of DEPC treated water. It was equilibrated in 20X SSC (2.9 M NaCl and 0.34 M sodium citrate, pH 7.0) for 45 min. Prior to transfer, the nylon membrane was pre-soaked in 20X SSC for 5 min. Capillary transfer method was employed to transfer the RNA onto the nylon membrane. The transfer was performed for 14-16 h using 20X SSC. After transfer, the membrane was UV cross-linked for 30 sec. The blots were pre-hybridized in pre-hybridization buffer (0.25 M sodium phosphate, 0.001% EDTA, 1% BSA and 7% SDS) for 3-4 h at 55°C using a hybridization chamber. The membrane was probed with radiolabelled hex cDNA. Hybridization was carried out for 14-16 h in the same hybridization chamber maintained at 55°C. The blot was thoroughly washed to remove the left over un-hybridized probe at 55°C. First two washes were given with wash buffer I (0.02 M sodium phosphate, 0.001% EDTA; 0.5% BSA and 5% SDS) and the next two washes were with wash buffer II (0.02 M sodium phosphate; 0.001% EDTA and 1% SDS) al 55°C. To detect the complementary RNA on the blot the membrane was autoradiographed.

Probe preparation:

Hex cDNA present in the plasmids was used as probe for the detection of mRNA transcript on the northern blot. The cDNA was either radiolabeled

by [a P] dATP or transcribed for the synthesis of digoxigenin labeled RNA probe.

For the preparation of the probe the plasmid DNA was digested using restriction enzyme. The digested DNA was subjected to 1 % agarose gel electrophoresis. The cDNA fragment was extracted from the gel by using QIAquick gel extraction kit. This cDNA was used for probe preparation.

The gel purified cDNA (25-50 ng) was radiolabeled using Random Primer Labeling Kit (Bangalore Genei). The reaction was carried out in 1.5 ml microfuge tube mixing heat denatured DNA (25- 50 ng in 10 µl), 10X labeling buffer (10 ul), random primer (4 ul), DTT (20 mM in 10 ul) and dNTP mix (10 ul). The reaction was initiated by adding klenow fragment (4 ul) and 10 u Ci (5 ul) of [a ^{32}P] dATP. The labeling was carried out for 14- 16 h at 25° C. After overnight labeling, the reaction was terminated by adding 1 ul EDTA (0.5 M, pH 8.0). The unincorporated [α^{32}P] dATP was removed by passing the mixture through Sephadex G-25 matrix.

For the preparation of digoxigenin-labeled probe, *in vitro* transcription was carried out using DIG RNA labeling kit (SP 6/ T_7) BM. The transcription was carried out in a microfuge tube by mixing the cDNA (1.5 ug / 1.5 ul), 10X NTP labeling mixture (2 ul), 10X transcription buffer (2 ul) and T7 polymerase (2 ul) along with 1 ul of RNase inhibitor. The transcription was carried out for 2 h at 37° C. It was terminated by adding 1 ul of EDTA (0.5 M, pH 8.0). The RNA was precipitated by adopting standard precipitation protocol (Sambrook *el al.*, 1989). The precipitate was dissolved in 50 ul of DEPC water, from which 10 ul was used for probing the northern blots.

In vitro phosphorylation:

Phosphorylation of fat body protein was carried out according to Shanavas *et al.* (1998). The protein concentration in all the samples was adjusted to 20 µg/20 ul. The total reaction mixture was of 40 ul, containing 20 µl tissue homogenate; 10 ul of 4X reaction buffer (10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM DTT and 1 mM EDTA) and 10 ul (4 µCi) of isotope [γ^{32}P] ATP. The phosphorylation mixture was pre-incubated for 5 min at 30°C. Reaction was initiated by adding *[y^{32}?]* ATP and terminated after 60 seconds by adding 20 ul of 3X SDS sample buffer. The sample (30 ul) was electrophoresed on SDS-PAGE. After the separation of the proteins, the gel was processed for autoradiography to visualize the phosphorylated proteins.

Autoradiography:

Autoradiography was carried out either to detect the phophorylation signal in polyacrylamide gels or to detect signal obtained after northern hybridization. The protein gels were dried under vacuum sandwiched between cellophane sheets for 2 h at 80° C. The nylon filters probed with radiolabelled cDNA were partially air dried and sandwiched between cellophane papers. Both the dry gels and the filters were exposed to Kodak X- Omat- AR X-ray film using intensifying screens (DuPont Cronex) at -70°C.

Fluorography:

Fluorography was carried out according to the method of Bonner and Laskey (1974). It was performed to detect the presence of [^{35}S] methionine labeled polypeptides/proteins in polyacrylamide gels. The gels after

staining were incubated for 2 h in DMSO, followed by another incubation of the gels in fresh DMSO for 2 h. Then the gels were transferred to a solution of PPO in DMSO (24. 8 g PPO in 100 ml DMSO) for 2 h. The gel was washed in distilled water till there was no floating PPO and then transferred on to Whattman No: 3 filter paper. It was dried under vacuum at 80° C using Hoeffer gel drier. The dried gel was exposed to Kodak X-Omat- AR X-ray film using intensifying screens (DuPont Cronex) at -70' C.

CHAPTER 3

Identification, Isolation and Sequencing of Hexamerin cDNA clones

Materials:

Experimental insects and bacterial strains used in the present study are mentioned in chapter 2. The source of chemicals used has also been mentioned in the same chapter.

Methods:

Electrophoretic and immunological methods are described in chapter 2.

Purification of **hexamerins:**

Hexamerins were purified using a protocol standardized in our laboratory (Arif *et al*., 2001). Haemolymph drained from mid- and late-last instar larvae was diluted (1:20) with insect Ringer containing 0.01% phenylthiourea, and centrifuged for 5 min at 4,000 rpm at 4° C to pellet down the hemocytes. The diluted haemolymph was raised to 40% ammonium sulphate saturation and centrifuged at 10,000 X g for 10 min at 4° C to discard the pellet. The supernatant was collected and raised to 60% ammonium sulphate saturation and centrifuged at 10,000 X g for 10 min at 4° C to discard the pellet. The supernatant thus obtained was raised to 70% ammonium sulphate saturation and centrifuged at 10,000 X g for 10 min at 4° C. The pellet was collected and suspended in insect Ringer and passed through Sephadex G-25 column for desalting. The peak fractions were pooled and passed through DEAE-52 column. The proteins were eluted with a linear gradient of 0 to 1 M NaCl and the peak fractions were pooled. Further separation of proteins was carried out by RP-HPLC on Shimadzu 6A HPLC using C-18 Bondapak column (3.9 X 300 mm). Protein (100 ug) was injected in 100% solvent A (0.1% trifluoroacetic

acid in 35% acetonitrile) and eluted using a linear 0 to 10% gradient of solvent B (0.1% trifluoroacetic acid in 85% acetonitrile) over a period of 10 min followed by 100% of solvent B over a period of 30 min. The eluent was monitored at 214 nm for the detection of proteins. At every stage of purification, fractions containing proteins were subjected to 7.5% SDS-PAGE (Laemmli, 1970) and detected by silver staining (Blum *et al*., 1987). The pure hexamerin fraction from DEAE-52 column and the HPLC elutes were lyophilized and stored at -20° C, till further use.

Antibody production:

The DEAE-52 column elute containing a mixture of hexamerins (Hex 1, Hex 2 and Hex 3) was used as antigen for raising polyclonal antibodies in rabbits. It was named as Hex antibody. Antibodies were also generated for RP-HPLC separated subunits Hex 1 and Hex 2 proteins individually. The antibodies for Hex 1 were named as Hex 1 antibody while antibodies for Hex 2 were named as Hex 2 antibody. Hex 3 antibody was kindly provided by Kirankumar (1998). The IgG fractions were purified following the method explained in chapter 2 and were stored at -70° C. The specificity of the antibodies was tested by western blot analysis

Construction of expression library:

cDNA expression library was constructed using SMART cDNA Library construction Kit (CloneTech Company), following the manufacturer's instructions. cDNA was synthesized from late-last instar larval fat body RNA using SMART™ PCR cDNA synthesis kit (CloneTech company). The cDNA was amplified by LD PCR, cleaned by proteinase-K treatment, linearised by Sfi digestion, size fractionated by column chromatography

and ligated into the vector, *X* Tripl Ex2. λ Tripl Ex2 is an ideal phagemid vector for carrying out immunoscreening studies. XL1-Blue strain of *E. coli* was used as host cells to infect the library phages. Part of the library was amplified, while rest of it was stored at 4° C. The amplified library was titred and the titre was found to be 6 X 10^9 pfu / ml. The amplified library was stored in 50% DMSO at - 80° C. In the present study, screening for hexamerin clones was carried out using unamplified library.

Expression library screening for the hexamerin clones:

For identification of cDNA clones encoding hexamerins, immunoscreening was carried out as described by Sambrook *el al*. (1989). A fresh overnight culture of XLl-Blue host cells was prepared in 15 ml of LB/ $MgSO_4$/ maltose (1% bacto-tryptone, 0.5% bacto-yeast extract, 0.5% NaCl, pH 7.0, 10 mM $MgS0_4$, and 0.2% maltose). The culture was centrifuged at 5,000 rpm for 5 min to pellet the bacterial cells. The pellet was suspended in 7.5 ml of 10 mM $MgSO_4$. To obtain 3 X 10^3 library plaques on 100 mm plate or 1.2 X 10^4 library plaques per 150 mm plate the library phages were diluted in lambda dilution buffer (0.01 M NaCl, 0.01 M $MgSo_4.7H_2O$, 0.035 M Tris-HCl, pH 7.5). To 200 ul of the XL1-Blue overnight culture 1 ul of diluted phages were added in sterile 15 ml tubes. The phages were allowed to adsorb to the bacteria at 37° C for 15 min. To each of this phage / bacteria mixture 2.5 ml of melted LB/ $MgSO_4$ (1% bacto-tryptone, 0.5% bacto-yeast extract, 0.5% NaCl, pH 7.0, 10 mM $MgS0_4$) top agar maintained at 42° C was added, gently inverted twice and poured onto pre-warmed LB/ $MgSO_4$ plates. The plates were gently swirled to evenly spread the top agar and kept at room temperature (25-30° C) for the agar to solidify. The plates were incubated at 42° C for 3-4 h. Within 3-4 h colonies appeared on the plates. After 4 h nitrocellulose filters pre-soaked for 20 min in 10 mM IPTG were placed on the cultures.

taking care not to trap any air bubbles. Using waterproof ink, orientation marks were made on the filters and the corresponding plates. The cultures were incubated for another 3 h at 37° C to allow the protein to adhere to the filters. After 3 h the filters were carefully removed and processed for the detection of the positive plaques. The culture plates were wrapped in a plastic wrap and stored at 4° C until use.

The non-specific sites on the filters were blocked by immersing the filters in TBST (20 mM Tris, 500 mM NaCl, pH 7.4, 0.05 % Tween 20) containing 5 % milk powder for 1 h at 60 rpm on a platform rocker. To probe the filters with the primary antibodies, they were washed 4-5 times (each wash 5 min) in TBST and incubated in primary antibody diluted (1: 500) in 5 % milk powder in TBST for 1 h at 60 rpm. After 1 h incubation of the filters in primary antibody, they were washed 4-5 times in TBST. The positive plaques bound to the primary antibody were detected by a secondary antibody which was conjugated to ALP. The nitrocellulose filters were incubated in ALP conjugated secondary antibody (anti-rabbit goat IgG) diluted (1:10,000) in 5 % milk powder in TBST. After 1 h incubation the filters were washed 4-5 times in TBST. To visualize the positive plaques the filters were immersed in 25 ml of ALP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$) containing NBT and BCIP substrates. The filters were developed until the desired signal to noise ratio was visible on the filters. The development was stopped by placing the filters in stop solution (20 mM Tris-HCl, pH 8.0, 5 mM EDTA). The positive plaques were marked with a pencil. The positive plaques showed a very quick and intensive color development, when compared with the false positives. Aligning the filters, orientation marks over the original plates the positive plaques were picked using sterile disposable pipette tips. Each positive plaque was placed in a separate 1.5 ml microfuge tube containing 500 ul 1X lambda dilution buffer. For phage elution, the tubes were vigorously vortexed and incubated overnight at 4° C. The subsequent secondary and tertiary screenings were carried out. The

positive plaques obtained after three rounds of screening were processed for plasmid preparation.

Sub-cloning and plasmid analysis:

Positive plaques *i. e.,* the phage clones (λ Triple Ex) were converted to (p Triple Ex) plasmid clones by excising and circularizing the complete plasmid from the recombinant phage. Fresh overnight culture of BM25.8 host cells was harvested by inoculating 10 ml LB/ $MgSO_4$ broth in a 50 ml culture tubes and incubating at 31° C overnight with shaking at 150 rpm until the absorbance at 600 nm reached 1.1- 1.4. To 10 ml overnight culture of BM25.8, 100 ul of 1 M $MgCl_2$ was added. In a 20 ml culture tube, 150 ul of the phage elute from a positive plaque was mixed with 200 ul of overnight BM25.8 culture containing 10 raM $MgCl_2$. The mixture was incubated for 30 min at 31 ° C without shaking. To the above, 400 ul LB broth was added and incubated for an additional 1 h with shaking (225 rpm). Using a sterile spreader 1-10 ul of infected cell suspension was spread on LB (amp) plate to obtain isolated colonies. Well-isolated colony from each clone was picked for plasmid isolation. Plasmid DNA was isolated as discussed in chapter 2. The plasmid DNA yield from BM25.8 transformants was very low, hence transformation was once again carried out using XL 1-Blue strain following the standard transformation protocol.

Restriction analysis of the plasmid DNA:

Plasmid DNA isolated from the ten positive clones was double digested with EcoR1 and Hind III restriction enzymes. The digestion was carried out at 37° C for 1 h and the reaction was terminated by adding 1 ul of 0.5

M EDTA (pH 8.0). The restriction pattern was analyzed on 1% agarose gel.

Sequencing of the cDNA:

Among the eight clones which showed good restriction pattern, two clones showing exact lengths of vector and insert upon digestion were selected for sequencing. Sequencing was carried out using AmpliTaq® FS *V** Big Dye Terminator kit and protocol. The plasmid DNA (450-500 ng in 1 ul) was mixed with premix DNA template (2 ul), either 5' or 3' sequencing primer (1 ul) and made up the volume to 5 ul with water. It was denatured for 2 min at 94° C. The denatured DNA was amplified in Perkin-Elmer thermocycler by setting up the program- 96° C, 30 sec; 45-60 ° C, 15 sec; 60 ° C, 4 min for 25 cycles. The amplified DNA was ethanol precipitated following standard DNA precipitation protocol (Sambrook *et al*., 1989). The DNA pellet was dissolved in 25 ul of TSR buffer (Template Suppression Reagent) and left on ice for 30 min for the DNA to completely dissolve. It was heat denatured for 2 min at 90° C. The sample was transferred to a special 0.5 ml sequencing tube and was sequenced in Perkin-Elmer 310 Sequencer. Both 5' as well as 3' sequencing was done. Complete sequencing was donc by using two sets of primers (INTERACTIVE Virtual Laboratory). Following are the two sets of primers used for complete sequencing of the cDN A.

I set: Forward: 5'-ACACTGATAAGGCAGTTACTG

Reverse: 5'-CTTCTTGGTAAATGTAAACATCC

II set: Forward: 5'-ACACTGATAAGGCAGTTACTG-3'

Reverse: 5'-CTTCTTGGTAAAATGTAAACATCC

Computer-assisted sequence analysis:

A deduced amino acid sequence for the analyzed cDNA clones was accomplished with the assistance of Molecular Biology Shortcuts (MBS) translator through the MBS-e-mail server. The National Center for Biotechnology Information e-mail server was used to compare the sequence results with other genes available in the Gen Bank database (Altschul *et al.,* 1990). Protean (DNASTAR. Version 1.17) was used to deduce the amino acid composition and MegAlign (DNASTAR, Version 1.05, Madison, WI) was used for the initial multiple amino acid sequence alignment which was adjusted by eye, when necessary. To retrieve protein sequences similar to the cDNA sequences relevant databases were searched using the BLAST (Altschul *et al.,* 1990) and FASTA programs.

Phylogenetic inference:

A multiple alignment of the *C. cephalonica* Hex 2a and Hex 2b amino acid sequences and 33 selected insect hexamerins was constructed using ClustalX (Thompsom *el al.,* 1997) and converted according to Burmester *et al.* (1998b) by the aid of Gene Doc 2.6 (Nicholas and Nicholas, 1997). The signal peptides and c-terminal extensions were eliminated from the final data set. The program package PHYLIP 3.6a2 (Felestein, 2001) was applied for tree calculations. Distances between pairs of proteins were calculated using thc PAM001 matrix implemented in the PHYLIP package. Tree constructions were performed by the neighbour-joining method. A putative insect haemocyanin (Sanchez *et al.,* 1998) was used as the out-group (cf. Burmester, 2001). The reliability of the trees was tested by the bootstrap procedure with 100 replications.

Linearized trees were essentially calculated as described (Burmester, 2001). Briefly, the distance matrix was imported into the Microsoft

EXCEL 97 spread sheet program. Relative rate tests were carried out successively to single proteins or groups of proteins according to the topology of the tree (cf. Burmester *et al*., 1998b). To estimate divergence times, we assumed that the orthoptera diverged from the other neopteran insects 320 million years ago (MYA) (Kukalovà-Peck, 1991). The confidence limits were estimated using the observed standard deviation of the inferred replacement rates.

Results:

Hexamerin purification:

Results presented in figure la show the SDS-PAGE profile of the haemolymph proteins obtained after ammonium sulphate precipitation and Sephadex G-25 steps of purification (40 % supernatant, 40 % precipitate, 60 % supernatant, 60 % precipitate, 70 % supernatant, 70 % precipitate and G-25 column elute). The fractions collected from DEAE-52 column (Fig. 1b) show the presence of pure hexamerins containing all the three *i.e.*, Hex 1, Hex 2, and Hex 3. These were separated on RP-HPLC.

Fig. 1a

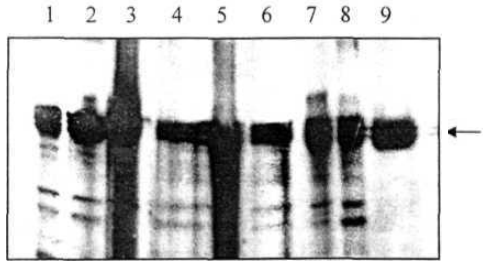

Fig. **1b**

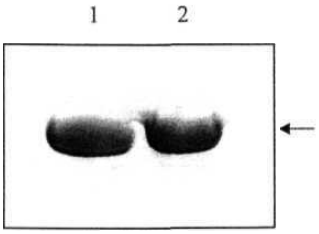

Fig. 1a: SDS-PAGE analysis of haemolymph proteins obtained after various steps of purification. Lane 1- crude haemolymph, lane 2- 40 % ammonium sulphate (AS) supernatant, lane 3- 40 % AS precipitate, lane 4- 60 % AS supernatant, lane 5- 60 % AS precipitate, lane 6- 70 % AS supernatant, lanes 7 & 8- 70 % AS precipitate, and lane 9- G-25 column elute which was later passed through DEAE-52 column.
Amount of protein loaded. Lane 1- 10 µg, and lanes 2 to 9- 5 ug.
Fig. 1b: SDS-PAGE analysis of DEAE-52 column purified hexamerins. Equal amount of protein was loaded in each lane (5 µg).

Figure 2a shows the elution profile obtained from RP-HPLC. The peaks with the retention time 16.875, 17.608 and 19.108 min were collected and concentrated. SDS-PAGE profile (Fig. 2b) of the three **peak** fractions showed the presence of three proteins with molecular masses 86, 84 and 82 kDa. The peak with the retention time of 17.608 showed the presence of Hex 1 (86 kDa), while the one with 16.875 min showed the presence of Hex 2 (84 kDa) and the one with 19.108 min contained Hex 3 (82 kDa).

Fig. 2a

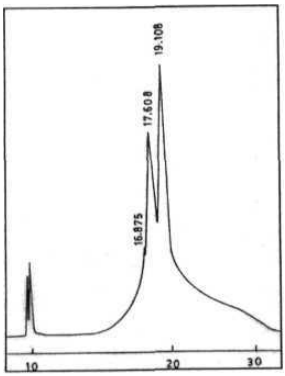

Fig. 2b

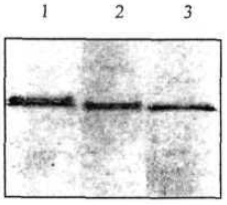

Fig. 2a: Shows the RP-HPLC profile of DEAE-52 column elute. Note the presence of three different peaks corresponding to Hex 1, Hex 2 and Hex 3 with retention time 17.608 min, 16.875 min and 19.108 min.

Fig. 2b: SDS-PAGE analysis of RP-HPLC purified hexamerins. Lane 1-Hex 1 (86 kDa), lane 2- Hex 2 (84 kDa) and lane 3-Hex 3 (82 kDa). Amount of protein loaded 1 µg.

Hexamerin antibody specificity:

Antibodies were generated against a mixture of hexamerins (Hex 1, 2 and 3) as well as individual Hex 1 and Hex 2 and extensive immunoblotting studies were carried out with haemolymph proteins obtained from late-last instar larvae and the results are presented in figure 3. Figure 3a shows the immuno-cross-reactivity of the hexamerin antiserum with all the hexamerins (Hex 1, Hex 2 and Hex 3). Figure 3b shows the specificity of the antibody raised against pure Hex 1 protein. The antibodies cross-reacted strongly with Hex 1 protein while the cross reactivity was extremely feeble with Hex 2 protein. Figure 3c shows the specificity of the antibody raised against pure Hex 2 protein. In this case the antibodies strongly cross-reacted with Hex 2 and only a faint reaction was seen with Hex 1 protein.

Fig. 3a

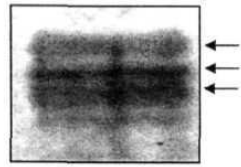

Fig. 3b

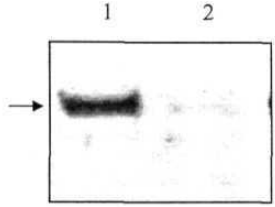

Fig. 3c

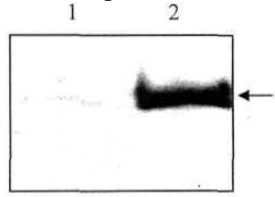

Fig. 3a: Western blot analysis showing the immunological cross-reactivity of hexamerins with antibodies raised against mixture of hexamerins (DEAE-52 column elute). Amount of protein loaded- 5 ug **in** each lane.

Figs. 3b and 3c: Immunoblots showing the immunological cross-reactivity of Hex 1 and Hex 2 with antibodies raised against Hex l(3b) and Hex 2 (3c). Lane 1- Hex 1 and lane 2- Hex 2. (In each lane 1 ug of purified protein was loaded).

Immunoscreening:

Fat body expression library prepared from the fat body RNA of late-last instar larvae of *C. cephalonica* was plated and used for imunnoscreening. Immunoscreening was carried out with the antibodies (Hex antibodies) raised against mixture of hexamerins (DEAE-52 column elute). Figure 4a shows the immunoblot obtained from primary immunoscreening. The positive plaques on the culture plate were detected and the putative positive colonies from the primary screened blots were picked up and processed for secondary immunoscreening. The immunoblot (Fig. 4b) showed nearly 50 % of the plaques positive. After three rounds of immunoscreening, the tertiary immunoblot obtained had all most all the plaques positive when probed with Hex antibodies (Fig. 4c).

Fig. 4a

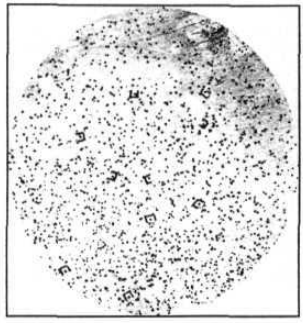

Fig. 4b

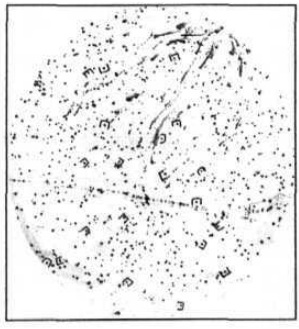

Fig. 4c

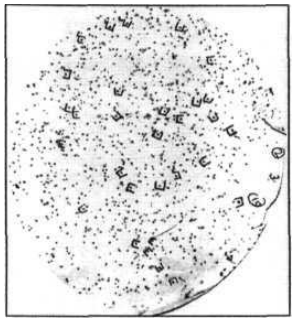

Fig. 4: Immunoscreening analysis showing immunological cross-reactivity of the plaques with antibodies raised against hexamerin mixture (ɔ positive plaques).

4a: immunoblot from primary screening; 4b: immunoblot from secondary screening; 4c: immunoblot from tertiary screening.

Plasmid analysis:

The positive clones obtained from above mentioned screening protocol were used. The phages were isolated and modified as plasmids in BM25.8 to carry out bacterial transformation. Figure 5 shows the agarose gel electrophoretic analysis of the plasmids isolated from the ten positive clones picked after three rounds of immunoscreening. The results showed that there was low yield of plasmids from BM25.8 strain of *E. coli* (Fig. 5a), which might be due to lower degree of amplification, hence transformation was also carried out in XL1-Blue strain of *E. coli*. The plasmid preparation obtained from XL1-Blue strain of *E. coli* gave reasonably good yield of plasmid DNA (Fig. 5b).

Fig. 5a

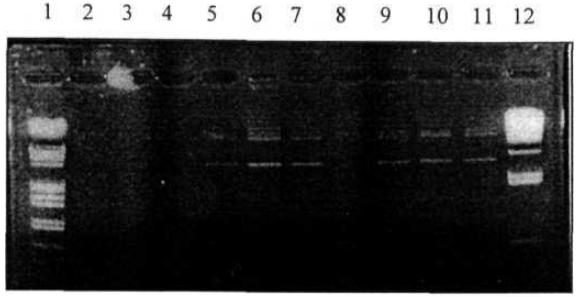

Fig. 5b

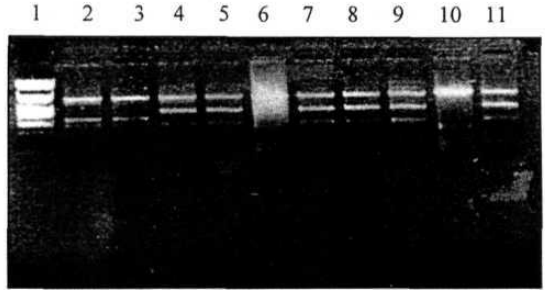

Fig. 5: Agarose electrophoretic analysis of plasmids isolated from the ten positive clones picked up after three rounds of immunoscreening.

Fig. 5a: Plasmids isolated **from** BM25.8 strain. Lanes 1 & 12 DNA marker, lanes 2 to 11 plasmids isolated from the ten positive clones. 500 ng plasmid DNA was loaded in each lane (2 to 11).

Fig. 5b: Plasmids isolated from XL1-Blue strain. Lane 1- DNA marker, lanes 2 to 11 plasmids isolated from the positive clones.

Restriction digestion analysis:

Plasmid DNA was isolated from the above mentioned clones (1 to 10) and was double digested with EcoR1 and Hind III restriction enzymes. The result presented in figure 6 shows the restriction pattern obtained for these plasmids. Six clones (lanes 4, 5, 7, 8, 9 & 11) showed identical pattern while two clones (lanes 2 and 3) and another two clones lanes (6 & 10) showed a different pattern.

Fig. 6

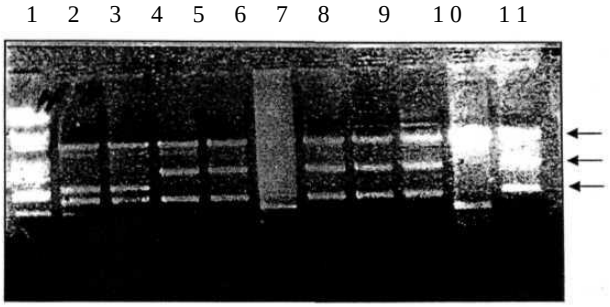

Fig. 6: Restriction pattern of the plasmid DNA isolated from the ten positive clones in which double digestion was carried out with EcoRl and Hind III restriction enzymes.

Lane1- DNA marker, lanes 2 to 11 enzyme restricted plasmid DNA. (500 ng of digested DNA was loaded in each lane).

Sequencing:

Two clones showing the presence of intact insert upon restriction digestion which were picked up from the six positive clones were sequenced using AmpliTaq® FS ¼ Big Dye Terminator kit and protocol. The full-length cDNA was sequenced using two sets of designed primers (details are given in methodology of this chapter). Figure 7 shows the sequencing pattern obtained. The cDNA clones were of full length as evidenced by the

presence of the start codon ATG at 5' end (Fig. 7a) and poly A tail at 3' region of the sequence (Fig. 7b).

Fig. 7a

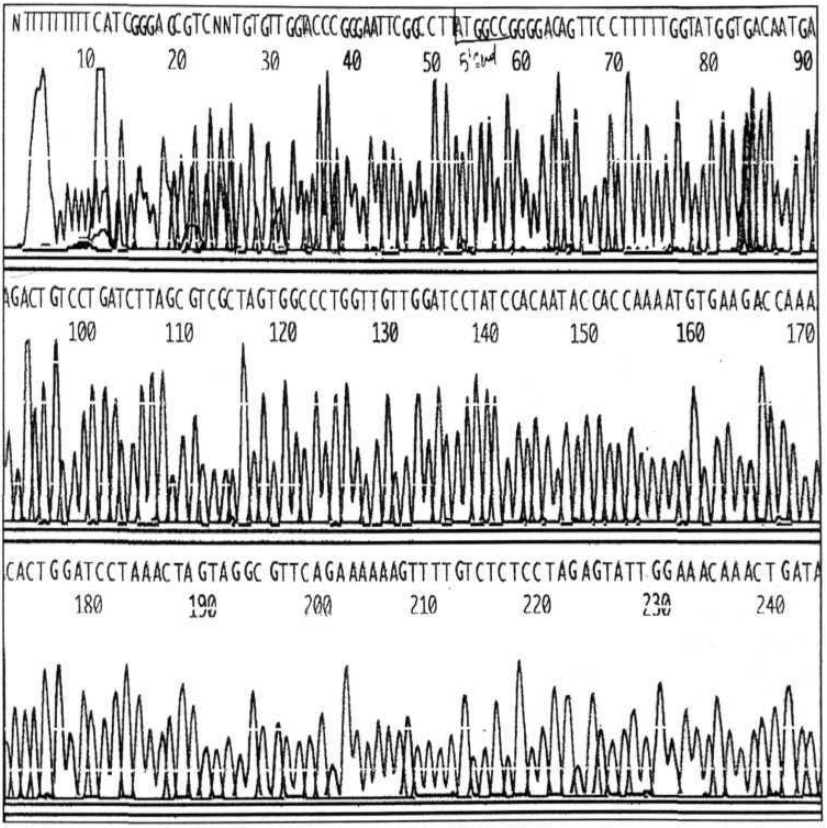

Fig. 7a: 5' sequencing pattern showing the start codon ATG

Fig. 7b

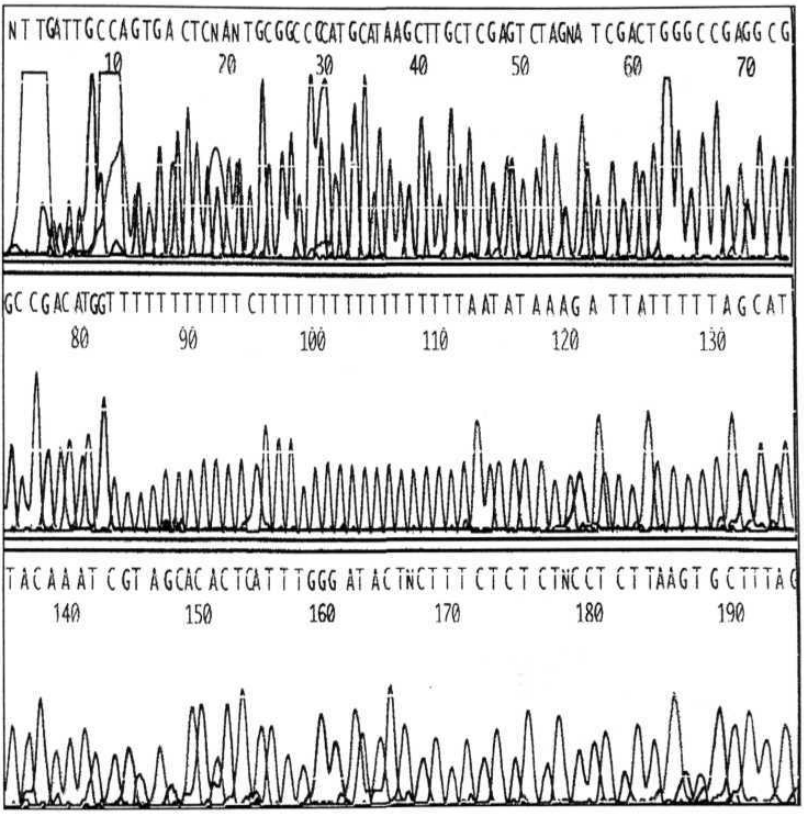

Fig. 7b: 3' sequence pattern showing the poly A tail.

Sequence analysis:

The two Hex cDNA clones comprise of 2,225 (Hex 2a) and 2,230 (Hex 2b) nucleotides, respectively. The sequences include an open reading frame of 2,109 nucleotides, each beginning with the methionine start codon ATG and translation stop codon TAA at positions 2,127 (Figs. 8a and b). A 3' untranslated sequence containing a polyadenylation signal ATAAA is detected in Hex 2a as well as in Hex 2b.

The deduced primary structure of both clones yielded polypeptides of 703 amino acids (including the signal peptides. see below) with estimated molecular mass of 83.4 kDa (Hex 2a) and (Hex 2b) each (Figs. 9a and b), and isoelectric points 5.64 for Hex 2a and 5.59 for Hex 2b. The amino acid sequence shows the presence of high concentrations of the aromatic amino acids phenylalanine (Hex 2a: 6.1% and Hex 2b: 6.4%) and tyrosine (Hex 2a: 11.2% and Hex 2b: 11.4%) while the methionine content is only 1.5 and 1.7% (Tables 1a and 1b).

Figure 10 shows the comparision of Hex 2a and Hex 2b sequences. Analysis of the sequence revealed the presence of a typical 19 amino acid signal peptide which is essential for transmembrane transport and export of the protein from the synthesizing cells (fat body) to the haemolymph (von Heijne, 1986). Hex 2a and Hex 2b show 92.5% identity at the amino acid level. In each of the hexamerin cDNA clones, two putative N-glycosylation sites (NXS/T) were detected at amino acid positions 214 and 482 (Fig. 10).

To retrieve protein sequences similar to Hex 2a and Hex 2b, relevant databases were searched using the BLAST algorithm (Altschul *et al.*, 1990). Comparison of the amino acid sequences from *C. cephalonica* with other lepidopteran insects revealed 73% identity with *Galleria mellonella* Lhp76, 56% with *Manduca sexta* arylphorin, 55% with *Hyalophora cecropia* arylphorin, and 53% with *Bombyx mori* SP2 (Fig. 11).

Fig. 8a

ACAGTTCACTAGTTTTGGTATGGTGACAATGAAGACTGTCCTGATCTTAGCGTCGCTAGTGGC
CCTGGTTGTTGGATCCTATCCACAATACCACCAAAATGTGAAGACCAAAACACTGGATCCTAA
ACTAGTAGGCGTTCAGAAAAAAGTTTTGTCTCTCCTAGAGTATTGGAAACAAACTGATACAGA
AGCCGAGTATTACAATATAGGCAAGTCTTACGATGTCGAAGCAAACATAGAATCCTATACTGA
TAAGGAGGCAGTTACTGAATTTTTGTATTATTATAAAGCTGGTTTCCTGGCGAAAAATGAATT
ATTCTCTATCTTCTATGAAAGGCAGGCTCTGGAAGTAATAGCTTTATATAAACTATTCTACCA
TGCTAAAGAGTTCGAAACATTCTACAAGACTGCTGCTTTCGCTCGTGTTTATTTGAATGAGGG
TCAATTTGTGTATGCGTACTACATGGCAGTCATACAGCGTGAAGATACTAGAGGCATTGTCTT
ACCGCCTCCGTATGAAGTAATGCCAGAATATTTTGTCAACATGGATGTTCTGTTCAAGCTGTA
TCGTATTCAAATGCAAAAGGGCATAATAGTACCAGAGCAAGCAGAAAAGTATGGTATAATTAC
TAAAGACAACGACTATTATTTCTACGCTAACTACTCTGGGCCCTGGACGTACGATAACAACGA
ACATTTGCTATCGTACTTCACAGAAGACATTGGCTGGAATTCGTATTATTACTACTTCAACAT
GAAATCGCCATTCTGGGGGAAAGGCGCAGATGTACTTAAGGGCTTTAAGGGACGTCGTGGTGA
AATTTACTACTACATTTATCAACAAATATTGGCTCGTTATTATCTCGAACGTCTCGCAAACGG
TTTAGGTGAAATACCGAGATTCAGTTGGTTTGAAAAGTTCCCTACTGGTTACTATCCTTCAAT
AGGTCCCTACTTGAGTTCGTTTGTGCATAGAAGTGAAGATTACTACTTGGCTGATGCTGATAA
TATTGATGACATTCAGTTTATTGACTACTATGAGAAGAACTTCTTGCAATATCTGCAAAATGG
GCAGTTTAAGGCTAACAAATATGAAGTTGATTTATACAACTCGAAGTCGATAAATTTCGTTGG
CAACTATTGGCAATCTAACACTGATCTCTACGAAAAAGTACAGCCCAGGAATTACTGGCGATC
ATACGAAGTTGCGGCTCGTCGTGTTCTTGGTGGTGCTCCTAGAAATTATGCCGATCATGTGTA
CATTCCCGCTGCTTTGGACTTCTACCAGACTTCACTTCGTGATCCTGCTTTCTATCAATTATA
CGGGAAGATATTAGAATATATCATACAATACAAAGAACACTTAGAACCCTATACTAAGGATGT
TCTCCATTACGTTGGCGTCAAGGTTAATGACGTGAAAGTAGACAAATTAGTCACCTTCTTCGA
ATATTTCGACTGGAATGCAACTAACGCTTTATATTTCTCTGAGCAACAACTAGAATCTGTTGC
CCCTTCATTCATTGTTCGTCAACCACGTTTGAACCACAAGCCTTTCACTGTAACTATTGACAT
CAAATCCGATGTTGAAGCCGAAGCAGTCTTTAAAATCTTCATTGGTCCCAAATATGATGGCAA
TGGTGTGCCTATTAGCCTAGAAGACAACTGGATGAATTTCGTAGAACTTGATTGGTTTACACA
CAAACTTACATCAGGACAGAACAAGGTTGAACGCAAATCTGAACAGTTCTTCTTCTACAAGGA
GGACTCTGTACCTCTGCGAAAGGTTTACGAGCTTCTGAACAATGGACAGGTGCCACTTTATAT
GGCTGAAAAATTCTTGTTCCCACCGAGGAGGCTAATGTTGCCCAGAGGTACTACCGGCGGATT
CCCATTCCAGTTATATGTTATCGTTTACCCATACCAAGCTCCAGCTGCAGAATGGGGAGAGAT
GAAAGAATATGTGATCGACAACAAGCCTTACGGTTATCCATTCGACCGTCCAGTGCCCGTGGC
TTACCACTTCATTCAGCCTAACATGTACTTTAAGGATGTTTACATTTACCAAGAAGGCGAACA
GTACCCATGGGACACGTCCTACTATAGTCAAAATCTTGTCTCTAAGCAC**TAA**GAGGAGAGAGA
AAGAGTATCCAAATAGTGTCTACGATTTGTAATGCTAAAAATAAATCTTTATATTAATAGAGA
AAAAAAAAAAAAAAAAAAAAAAAAA

Fig. 8a: Complete cDNA (Hex 2a) sequence including the 5' untranslated region comprising of 2,225 nucleotides. The translation strart codon ATG, the stop codon TAA, and the polyadenylation signal ATAAA are shown in bold letters. The 19 amino acid signal peptide and poly A tail are underlined.

Fig. 8b

ACAGTTCACTTTTTGGTATGGTGACAACGAAGACTGTCCTGATCTTAGCGTCGCTAGTGGCCC
TGGTTGTTGGATCCTATCCACAATACCACCAAAATGTGAAGACCAAAACACTGGATCCTAAAC
TAGTAGGCGTTCAGAAAAAAGTTTTGTCTCTCCTAGAGTATTGGAAACAAACTGATACAGAAG
CCGAGTATTACAATATAGGCAAGTCTTACGATGTCGAAGCAAACATAGAATCCTACACTGATA
AGGAGGCAGTTACTGAATTTTTGTATTATTATAAATCTGGTTTCCTGGCAAAAAATGAATTAT
TCTCTATCTTCTATGAAAGGCAGGCTCTGGAAGTAATAGCTTTATATAAACTACTCTACTATG
CTAAAGACTTCGAAACATTCTACAAGACTGCTGCCTTCGCTCGTGTTTATTTGAATGAGGGTC
AATTTGTGTATGCGTACTACATGGCAGTCATACAGCGTGAAGATACTAGAGGCATTGTCTTAC
CGCCTCCGTATGAAGTAATGCCAGAATATTTTGTCAACATGGATGTTCTGTTCAAGCTGTATC
GTATTCAAATGCAAAAGGGCATAATAGTACCAGAGCAAGCAGAAAAGTATGGTATAATTACTA
AAGACAACGACTATTATTTCTACGCTAACTACTCTGGGCCCTGGACGTACGATAACAACGAAC
ATTTGCTATCGTACTTCACAGAAGACATTGGCTGGAATTCGTATTATTACTACTTCCACATGA
AGTCGCCATTCTGGGGGAAAGGCGCAGATGTACTTAAGGGCTTTAAGGGACGGCGTGGTGAAA
TTTACTACTACACTTATCAACAAATATTGGCTCGTTATTATCTCGAACGTCTCGCAAACGGTT
TGGGTGAAATACCGAGATTCAGTTGGTTTGAAAAGTTCCCTACTGCTTACTATCCTTCAATTA
GTCCTTACTTGAGTTCATTTGCTCATAGAAGCGACGATTATTACATGGCTACTCCTGATAATA
TTGAAGATATCCAGTTCATTGACTTTTACGAGAAGAACTTCTTACAGTTTTTGCAAAGGGGCC
AGTTTAAGGTTTACAAACAAGAAGTTGGTTTATACAACTCGAAGTCGATAAACTTTGTTGGCA
ACTATTGGCAATCTAACACTGATCTCTACGAAAAAGTACAGCCCAGGAATTACTGGCGATCAT
ACGAAGTTGCGGCTCGTCGTGTTCTTGGTGCTGCTCCTAACAGTGAACATGAGCACTTAAACA
TTCCTGCTGCTTTGGACTTCTACCAGACATCACTACGTGATCCCGCCTTCTATCAGCTGTATA
GGAAGATCTTAGACTACATCATTCAATACAAAGAATACTTGGAACCGTATTCTAAGGATGTTC
TTCACTATGTCGGTGTCAAGGTTAATGACGTGAAAGTAGACAAACTAGTAACTTATTTCGAAT
ATTTCGACTGGAATGCAACTAACGCCATTTACTTCTCCGAACAACAACTCGAATCTGTTGCTC
CTTCATTCATTGTCCGTCAACCTCGTTTGAACCACCAGCCTTTCACTGTAACTATTGACATCA
AATCGGACGTTGAAGCTGAAGCAGCCTTCAAAATCTTCATTGGTCCTAAATATGATGGCAATG
GTGTGCCTATTAGCCTAGAAGACAACTGGATGAACTTCGTAGAACTCGATTGGTTTACTCACA
AACTTACATCAGGACAGAACAAGGTTGAACGCAAATCCAGTGAATTCTACAACTTCAAGGATG
ACTCTGTACCATTATCAAAGGTTTACGAGCTTCTGAACAGTGGAAAAGTACCACTTGATATGT
CTGAGAAATACTTACACCAACCGAGGAGGTTGATGTTGCCCAGAGGTACCACCTGTGGATTCC
CATTCCAGTTATATGTTATCGTTTACCCATACCAAGCTCCAGCTGCAGAATGGGGAGAGATGA
AAGAATATGTGATCGACAACAAGCCTTACGGTTATCCATTCGACCGTCCAGTACCCGTGCCTT
ACCACTTCATTCAGCCTAACATGTACTTTAAGGATGTTTACATTTACCAAGAAGGCGAACAGT
ACCCATGGGACACGTCCTACTATAGTCAAAATCTTGTCTCTAAGCACTAAGAGGAGAGAGAAA
GAGTATCCGAAATAGTGTCTACGATTTGTAATGCTAAAA**ATAAA**TCTTTATACCAAAAAAAAA
AAAAAAAAAAAAAAAAAAAA

Fig. 8b: Complete cDNA (Hex 2b) sequence including thc 5` untranslated region comprising of 2,230 nucleotides. The translation strart codon ATG, the stop codon TAA, and the polyadenylation signal ATAAA are shown in bold letters. The 19 amino acid signal peptide and poly A tail are underlined.

Fig. 9a

WKQTDTEAEYYNIGKSYDVEANIESYTDKEAVTEFLYYYKSGFLAKNELF
SIFYERQALEVIALYKLLYYAKDFETFYKTAAFARVYLNEGQFVYAYYMA
VIQREDTRGIVLPPPYEVMPEYFVNMDVLFKLYRIQMQKGIIVPEQAEKY
GIITKDNDYYFYANYSGPWTYDNNEHLLSYFTEDIGWNSYYYYFHMKSPF
WGKGADVLKGFKGRRGEIYYYTYQQILARYYLERLANGLGEIPRFSWFEK
FPTAYYPSISPYLSSFAHRSDDYYMATPDNIEDIQFIDFYEKNFLQFLQR
GQFKVYKQEVGLYNSKSINFVGNYWQSNTDLYEKVQPRNYWRSYEVAARR
VLGAAPNSEHEHLNIPAALDFYQTSLRDPAFYQLYRKILDYIIQYKEYLE
PYSKDVLHYVGVKVNDVKVDKLVTYFEYFDWNATNAIYFSEQQLESVAPS
FIVRQPRLNHQPFTVTIDIKSDVEAEAAFKIFIGPKYDGNGVPISLEDNW
MNFVELDWFTHKLTSGQNKVERKSSEFYNFKDDSVPLSKVYELLNSGKVP
LDMSEKYLHQPRRLMLPRGTTCGFPFQLYVIVYPYQAPAAEWGEMKEYVI
DNKPYGYPFDRPVPVPYHFIQPNMYFKDVYIYQEGEQYPWDTSYYSQNLV
SKH

Fig. 9b

MVTMKTVLILASLVALVVGSYPQYHQNVKTKTLDPKLVGVQKKVLSLLEYWKQTD
TEAEYYNIGKSYDVEANIESYTDKEAVTEFLYYYKAGFLAKNELFSIFYERQALE
VIALYKLFYHAKDFETFYKTAAFARVYLNEGQFVYAYYMAVIQREDTRGIVLPPP
YEVMPEYFVNMDVLFKLYRIQMQKGIIVPEQAEKYGIITKDNDYYFYANYSGPWT
YDNNEHLLSYFTEDIGWNSYYYYFNMKSPFWGKGADVLKGFKGRRGEIYYYIYQQ
ILARYYLERLANGLGEIPRFSWFEKFPTGYYPSIGPYLSSFVHRSEDYYLADADN
IDDIQFIDYYEKNFLQYLQNGQFKANKYEVDLYNSKSINFVGNYWQSNTDLYEKV
QPRNYWRSYEVAARRVLGGAPRNYADHVYIPAALDFYQTSLRDPAFYQLYGKILE
YIIQYKEHLEPYTKDVLHYVGVKVNDVKVDKLVTFFEYFDWNATNALYFSEQQLE
SVAPSFIVRQPRLNHKPFTVTIDIKSDVEAEAVFKIFIGPKYDGNGVPISLEDNW
MNFVELDWFTHKLTSGQNKVERKSEQFFFYKEDSVPLRKVYELLNNGQVPLYMAE
KFLFPPRRLMLPRGTTGGFPFQLYVIVYPYQAPAAEWGEMKEYVIDNKPYGYPFD
RPVPVAYHFIQPNMYFKDVYIYQEGEQYPWDTSYYSQNLVSKH

Figs. 9a and b: The deduced amino acid sequence of the Hex 2a (a) and Hex 2b (b) cDNA clones yielding 703 amino acid polypeptide.

Table la

Ala	40	5. 7 %	Lue	**55**	**7. 8 %**
Arg	**25**	3. 6 %	Lys	48	**6. 8 %**
Asn	**36**	5. 1 %	Met	12	**1.7%**
Asp	**37**	5. 3 %	Phe	**46**	6. 5 %
Cys	**0**	**0. 0 %**	**Pro**	**37**	5. 3 %
Gln	**33**	**4. 7 %**	**Ser**	31	4. 4 %
Glu	48	**6. 8 %**	Thr	37	3.6%
Gly	**36**	5. 1 %	Trp	**12**	1.7%
His	l 1	1.6%	Tyr	**80**	11. 4 %
Ile	**36**	5. 1 %	Val	**53**	7. 5 %

Table 1b

Ala	**31**	4. 4 %	Lue	44	6. 5%
Arg	22	3. 1 %	Lys	37	5. 2 %
Asn	**31**	4. 4 %	Met	11	1. 5 %
Asp	**34**	4. 8 %	Phe	43	6. 1 %
Cys	1	0. 1 %	Pro	31	4. 4 %
Gln	**31**	4. 4 %	**Ser**	38	5. 4 %
Glu	**46**	6. 5 %	Thr	27	3. 8 %
Gly	**29**	4. 1 %	Trp	11	1. 5%
His	**12**	1.7%	Tyr	77	10. 9 %
Ile	**32**	4. 5 %	Val	49	6. **9%**

Table 1: Deduced amino acid composition of Hex 2a (a) and Hex 2b (b). Analysis reveals the presence of high content of aromatic amino acids phenylalanine and tyrosine in both Hex 2a as well as Hex 2b sequences.

Fig. 10

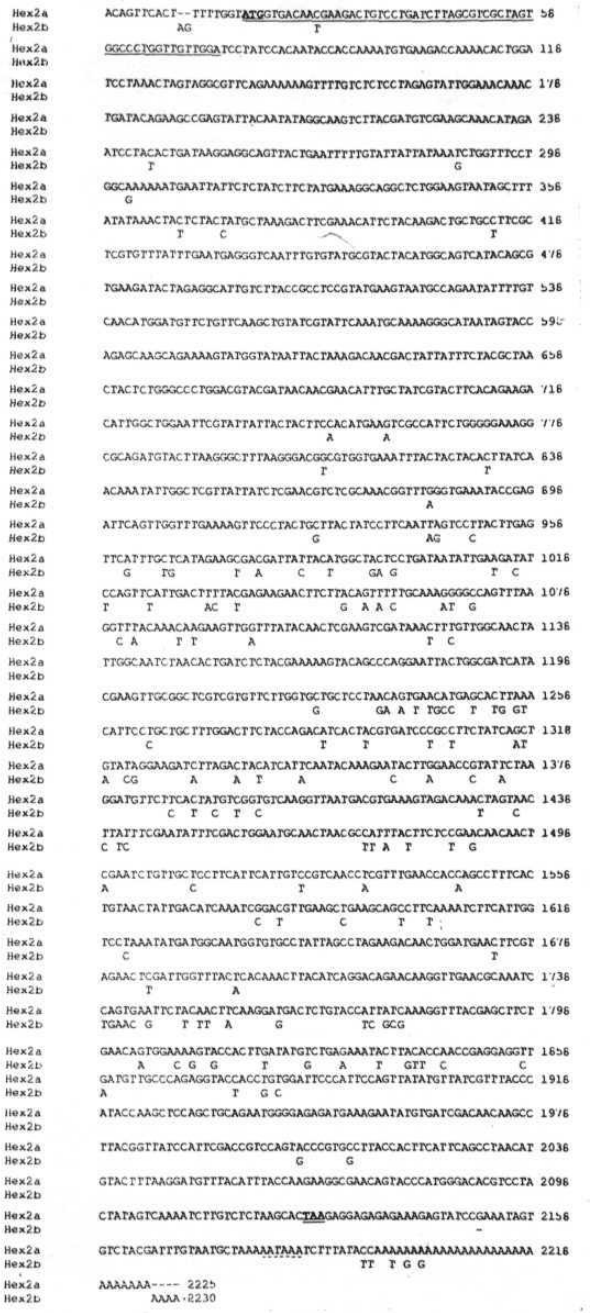

Fig. 10: Comparison of the two Hex cDNA clones, Hex 2a and Hex 2b. The translation start codon is located by 20 (bold letters), the stop codon is present by 2,127 (bold letters) in both the sequences. The polyadenylation signals are at bp 2,178 to 2,186.

Fig. 11

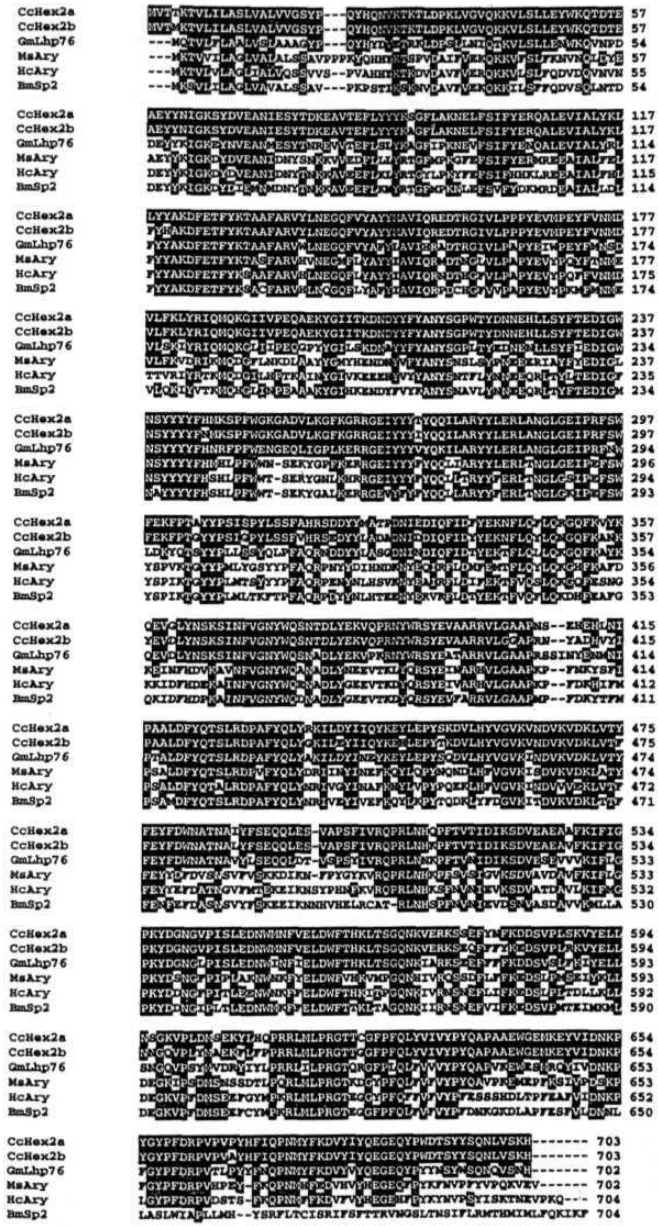

Fig. 11: Alignment of the deduced amino acid sequence of the *Corcyra cephalonica* Hexamerins Hex 2a (CcHex2a) and Hex 2b (CcHex2b) with 4 other lepidopteran hexamerin sequences. *Galleria mellonella* Lhp76 (Accession number 449954), *Manduca sexta* arylphorin a (P14296), *Hyalophora cecropia* arylphorin (AAB86644) and *Bombyx mori* SP2 (A34287). The conserved positions (identical amino acids) are shaded.

Phylogenetic analysis of Hex 2a and Hex 2b:

A phylogenetic tree was constructed using the neighbour-joining method based on the PAM-distances (accepted point mutations per site; Dayhoff *et al*., 1978). The two hexamerins (arylphorins) Hex 2a and Hex 2b of *C. cephalonica* form a well-supported common clad (100% bootstrap value), which is associated with the arylphorin of the wax moth *Galleria mellonella*. The lepidopteran arylphorins themselves are monophyletic (100% support), and are most likely associated with the methionine-rich hexamerins of this taxon, although this topology does not reach the significance level (56% support). As already observed before (Burmester, 1999, 2001), the lepidopteran hexamerins are not monophyletic, but the riboflavin-binding proteins (HceRbH, GmeLHP82, TniAJHSPl) are in basal position within the hexamerins of the other holometabolous insects.

Based on the assumption that the orthoptera (represented here by LmiJHBP) diverged from the other neoptera about 320 MYA (Kukalova-Peck, 1991), the time of divergence of the lepidopteran and dipteran hexamerins was calculated to be about 280 MYA. The emergence of the lepidopteran arylphorins dates back some 255 MYA. The time of divergence of the arylphorins from the galleriinae/ pyralidae {*Galleria* and *Corcyra)* and the ditrysian lepidoptera (higher moths and butterflies) was calculated to be 117 MYA. *Galleria* and *Corcyra* diverged about 54 MYA, and the arylphorins of C. *cephalonica* around 15 MYA (Fig. 12a and Fig. 12b).

Fig. 12a

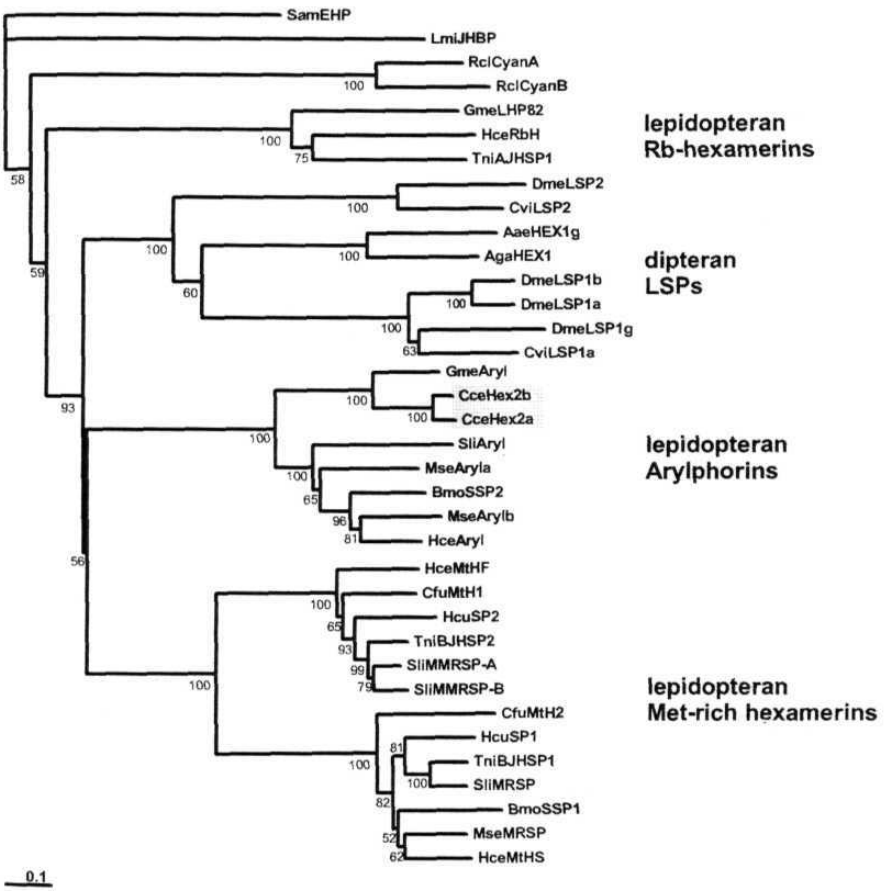
SamEHP
LmiJHBP
RclCyanA
RclCyanB
GmeLHP82
HceRbH
TniAJHSP1
DmeLSP2
CviLSP2
AaeHEX1g
AgaHEX1
DmeLSP1b
DmeLSP1a
DmeLSP1g
CviLSP1a
GmeAryl
CceHex2b
CceHex2a
SliAryl
MseAryla
BmoSSP2
MseArylb
HceAryl
HceMtHF
CfuMtH1
HcuSP2
TniBJHSP2
SliMMRSP-A
SliMMRSP-B
CfuMtH2
HcuSP1
TniBJHSP1
SliMRSP
BmoSSP1
MseMRSP
HceMtHS
lepidopteran Rb-hexamerins
dipteran LSPs
lepidopteran Arylphorins
lepidopteran Met-rich hexamerins
0.1

Fig. 12a: A. Neighbour-joining tree of insect hexamerins. The bar equals 0.1 PAM distance, the numbers at the nodes are the bootstrap support values. Abbreviations: SamHc. *Schistocerca americana* hemocyanin (GenBank accession number AF038569); LmiJHBP, *Locusta migratoria* juvenile-hormone binding hexamerin (U74469); RclCyanA, *Riptortus clavatus* cyanoprotein a (D87272); RclCyanB, *R. clavatus* cyanoprotein b (D87273); CceHex2a, *Corcyra cephalonica* hexamerin 2a (AF294808); CceHex2a, *C. cephalonica* hexamerin 2b (AF294809); GmeLHP82, ***Galleria*** *mellonella* LHP82 (L21997); GmeAryl, *G. mellonella* arylphorin (M73793); BmoSSPl. *Bombyx mori* sex-specific storage protein 1 (P09179); BmoSSP2, *B. mori* sex-specific storage protein 2 (P20613); MseAryla, *Manduca sexta* arylphorin a (P14296); MseArylb, *M. sexta* arylphorin (3 (P14297); MsMRSP, *M. sexta* methionine-rich storage protein (L07609); HceAryl, *Hyalophora cecropia* arylphorin (AF03296); HceRbH, *H. cecropia* riboflavin-binding hexamerin (AF03297); HceMtHF, *H. cecropia* methionine-rich hexamerin F (AF03298); HceMtHS, *H. cecropia* methionine-rich hexamerin S (AF03299); HcuSPl, *Hyphantria cunea* storage protein 1 (U60988); HcuSP2, *Hyphantria cunea* storage protein 2 (AF157013); TniAJHSPl, *Trichoplusia ni* acidic juvenile-hormone-suppressible protein (P22327); TniBJHSPl, *T. ni* basic juvenile-hormone-suppressible protein 1 (L03280); TniBJHSP2, *T. ni* basic juvenile-hormone-suppressible protein 2 (L03281); CfuMtH1, *Choristoneura fumiferana* diapause associated protein 1 (AF007767); CfuMtH2, *C. fumiferana* diapause associated protein 2 (AF007768); SliAryl, *Spodoptera litura* arylphorin (AJ249471); SliMRSP, *S. litura* methionine rich storage protein (AJ249470); SliMMRSP-A, *S. litura* moderately methionine rich storage protein A (AJ249469); SliMMRSP-B, *S. litura* moderately methionine rich storage protein B (AJ249468); DmeLSP1a, *Drosophila melanogaster* larval serum protein la (AE003489); DmeLSP1b, *D. melanogaster* LSP-1β (U63556); DmeLSP1g, *D. melanogaster* LSP-1γ (AE003467); DmeLSP2, *D. melanogaster* LSP-2 (X97770); CviLSPl. *Calliphora vicina* arylphorin (M76480); CviLSP2, *C. vicina* larval serum protein 2 (U89789); AgaHexl. *Anopheles gambiae* hexamerin 1 (U51225); AaeHexl, *Aedes aegypti* hexamerin 1γ (U 86079).

Fig. 12b

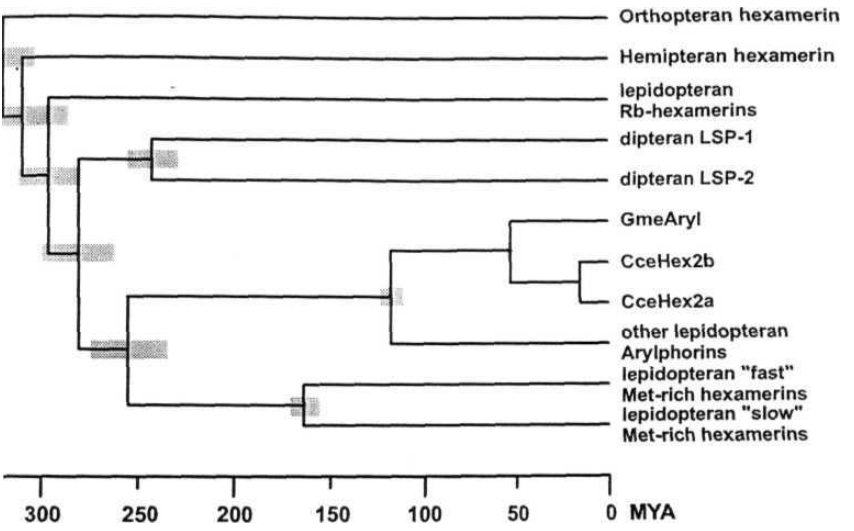

Fig. 12b: Timescale of insect hexamerin evolution. A linearized tree was drawn on the basis of the corrected protein distance data as described in the text. The grey bars are standard errors.

CHAPTER 4

Characterization of hexamerin clones and developmental studies

Methods:

Western blotting and northern hybridization are explained in chapter 2.

Preparation of bacterial lysate:

The positive clones containing the Hex cDNA were cultured overnight in LB/ amp broth in the presence of 10 mM IPTG. After 14-16 h growth, when the absorbance at 600 nm of the cultures reached 1.1-1.4, they were centrifuged at 4,000 rpm for 10 min at 4° C. The bacterial pellet obtained was suspended in 10 mM Tris-HCl (pH 7.4) containing 10 mM PMSF and sonicated (3 pulses, each for 30 sec at 12 amplitude) to disrupt the bacterial cell, for the release of the proteins. The sonicated cell suspension was centrifuged at 4,000 rpm for 10 min at 4° C. The supernatant was collected and western blot analysis was carried out.

Glycoprotein staining:

The haemolymph proteins and bacterial lysates containing expressed hexamerins were separated by SDS-PAGE. Two identical gels were run. One of them was stained with silver stain for detection of proteins and the other was stained with PAS for identification of glycoproteins (as described in chapter 2).

Chemical deglycosylation of hexamerins:

The hexamerins were purified from late-last instar larval haemolymph as described earlier and were chemically deglycosylated by subjecting them to anhydrous hydrogen fluoride (HF) treatment. To 1 mg of hexamerins, 200 µl of anhydrous HF was added and the reaction was carried out at 0° C for 1 h. At the end of 1 h, the reaction was terminated by evacuation of the

visible HF first using a water aspirator (15-30 min) followed by a high vacuum pump for about 2 h. The contents were dissolved in 1 ml of ice cold 0.1 M NaOH to neutralize any traces of residual HF and the pH was readjusted to 7.5 using 0.1 N HC1. The mixture was then incubated overnight at 37° C and chromatographed on Sephadex G-100 column in 0.05 M NH_4HCO_3 at 4° C. The protein eluted in the fractions was pooled and analyzed by immunoblotting along with haemolymph and bacterial lysate proteins.

Amino acid analysis:

HPLC purified hexamerin (Hex 2) was acid hydrolyzed with 6 N HC1 for 24 h at 110° C. After hydrolysis, the sample was analyzed on Shimadzu HPLC amino acid analyzer.

Fluorescence and absorption measurements:

The fluorescence measurements were carried out for HPLC purified hexamerin (Hex 2). The protein was excited at 285 nm, wavelength that excites tyrosine residues. The slit for both excitation and emission was 2 nm. Initial measurements were carried out with amino acid standard containing a mixture of tryptophan and tyrosine (1:10) to obtain a correlation with the spectra obtained for the *C. cephalonica* hexamerin (Hex 2).

Analysis of proteins by immunoblotting:

For developmental profile studies, proteins (30 µg) from whole body homogenates of the 1st, 2nd and 3rd instar larvae, and fat body homogenates of the penultimate, ELI, MLI, LLI larvae and prepupa were

electrophoretically separated and analyzed by immunoblotting (discussed in chapter 2).

For the study of tissue specific expression and / or presence of hexamerins in fat body, carcass, salivary gland and Malpighian tubule from LLI larvae and ovary and MARG from adult insects were used. The tissue proteins (30 µg) were electrophoretically separated and western blot analysis was carried out.

For the study of immunological identity between the hexamerins of C. *cephalonica* and hexamerins of *S. litura* and *B. mori,* the haemolymph proteins from late-last instar larvae of all the three insects were separated by SDS-PAGE, immunoblotted and detected using *C. cephalonica* Hex 2 antibodies.

Northern analysis:

For developmental profile studies, RNA was isolated from fat body of 2^{n}, 3^{rd}, penultimate and final larval instar (discussed in chapter 2). RNA (10 µg) was analyzed by northern blotting.

For the study of tissue specific hexamerin expression, RNA from various larval tissues (fat body, carcass, salivary gland and Malpighian tubule) was isolated and analyzed.

Northern analysis was also carried out with RNA isolated from the larval fat body of *B. mori* and *S. litura,* to find out the similarity if any in hexamerin transcripts of these insects with *C. cephalonica.* The blots were probed with [^{32}P] labeled *C. cephalonica* Hex cDNA.

Effect of ecdysteroids on **hexamerins** synthesis:

***In vivo* studies:**

To understand the effect of 20E on expression of hexamerins *in vivo,* insects from different developmental stages **(ELI,** MLI, LLI larvae and prepupa) were selected. They were thorax-ligated and used after 24 h. The hormone (20E) was topically applied to these isolated abdomens on the dorsal surface (100 ng / insect). Appropriate controls were also maintained. After 24 h of hormone application. the fat body was dissected out from these insects and RNA was prepared. RNA (10 μg) was electrophoresed and transferred to nylon membrane. The blots were hybridized with Dig labeled RNA probes. Probe preparation is explained in chapter 2.

***In vitro* studies:**

To determine the effect of 20E on the expression of hexamerins, *in vitro* incorporation of [^{35}S] methionine by fat body proteins was carried out. The fat body from two mid-last instar insects was dissected out under sterile conditions and cultured in TC-100 medium (200 μl) at 25° C for 8 h following the steps explained in chapter 2 (under organ culture). To experimental culture, 20E (80 nM) was added and to control cultures equal volume of solvent was added. During the last 4 h of incubation 10 μCi of [^{35}S] methionine was added. After incubation. the fat body was dissected out and rinsed thoroughly in cold insect Ringer containing 10 mM PMSF (Palli and Locke, 1988). It was homogenized and centrifuged at 1,000 X g for 5 min to remove debris. Small aliquots of the supernatant **were** quantified for the radioactivity according to the method explained in chapter 2.

Results:

Immunodetection of **hexamerins** in bacterial lysates:

Western blot analysis of the recombinant proteins present in the lysates of XL 1-Blue cells which were transformed with Hex cDNA containing plasmids was carried out using hexamerin antibody that was raised against the DEAE-52 column elute (Hex antibody). All the six lysates, showed strong immunocross-reactivity of a single protein band (Fig. 13a lanes 1-3 lysates from Hex 2a and lanes 4-6 lysates from Hex 2b containing plasmids). The migration of cross-reacting hexamerin present in bacterial lysates (Fig. 13b lane 1- Hex 2a, and lane 3- Hex 2b) was found to be faster than the hexamerin proteins present in the haemolymph of C. *cephalonica* (Fig. 13b lanes 2 and 4). Lysates prepared from DH5a cells transformed with Hex 2a and Hex 2b containing plasmids also showed the presence of fast migrating proteins (Fig. 13c lane 1- Hex 2a, and lane 2- Hex 2b).

Fig 13a

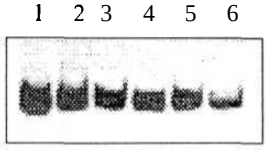

Fig. 13b

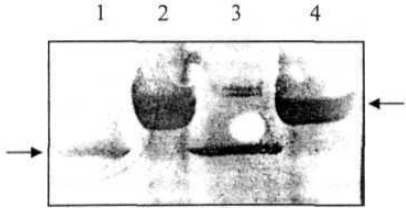

Fig. 13c

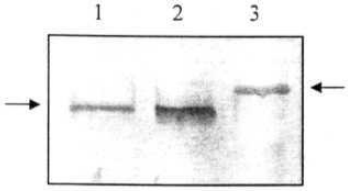

Fig. 13a: Western blot analysis of recombinant proteins present in the lysates prepared from XL 1-Blue cells transformed with Hex cDNA clones. Note the presence of a single (→) cross reacting protein band in all the samples (Hex 2a clones- lanes 1 to 3 and Hex 2b clones lanes 4 to 6). Equal volume of bacterial lysate was loaded in each lane. Hex antibodies raised against a mixture of purified hexamerins were used for analysis. As the experiment was to merely detect the hexamerin, in this study quantification of protein was not carried out.

Fig. 13b: Immunodetection of hexamerins in bacterial lysate and larval haemolymph (lanes 1 and 3 bacterial lysates from Hex 2a and Hex 2b clones, lanes 2 and 4 larval haemolymph). Amount of protein analyzed is 30 ug.

Fig. 13c: Western blot analysis of the recombinant Hex 2a and Hex 2b proteins present in DH5a bacterial lysates. Lane 1- Hex 2a clone, lane 2- Hex 2b clone and lane 3- *C. cephalonica* larval haemolymph protein. Amount of protein loaded in lanes 1 & 2- 30 ug and lane 3- 5 ug.

Chemical nature of hexamerins:

SDS-PAGE of the larval haemolymph proteins as well as proteins from the bacterial lysate showed the presence of many proteins (Fig. 14a) including hexamerins (→), when the identical gel was stained with PAS (Periodic acid Schiff s stain) none of the proteins present in bacterial lysate showed the staining (Fig. 14b lane 2). In contrast, the hexamerins in the larval haemolymph showed the glycoprotein staining (Fig. 14b lane 1). This suggests that the insect hexamerins are glycosylated proteins and hexamerins expressed by the *E. coli* are not glycosylated, hence cannot be detected by this method.

Fig. 14a

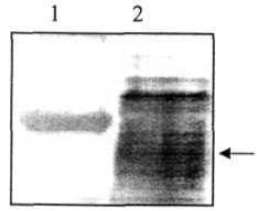

Fig. 14b

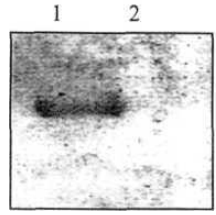

Fig. 14a: Silver stained SDS-PAGE analysis of the bacterial lysate and haemolymph proteins.Hexamerin in the bacterial lysate is shown by (—•). Amount of protein analyzed is 10µg.

Fig. 14b: Periodic acid Schiffs stained SDS-PAGE showing the glycoprotein nature of hexamerins in *C. cephalonica.* Lane 1- haemolymph and lane 2- bacterial lysate. Amount of protein analyzed is 10 ug.

Shift in molecular mass:

Western blot analysis of the deglycosylated larval hexamerins revealed a shift in the molecular mass (Fig. 15a lane 1). The shift matched exactly with the shift in the molecular mass of the recombinant hexamerins from the lysate of bacteria, which expressed Hex cDNA (Fig. 15b lane 1). This further suggests that the shift in the mobility of the hexamerins expressed in *E. coli* cells is primarily due to the absence of glycosylation.

Fig. 15a

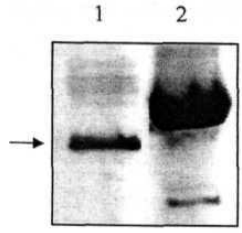

Fig. 15b

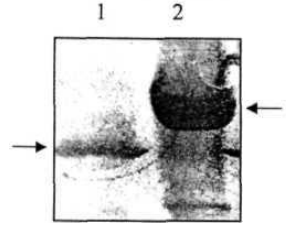

Fig. 15a: Western blot analysis of the deglycosylated *C. cephalonica* hexamerins. Lane 1- deglycosylated hexamerins of *C. cephalonica,* (10 µg was loaded); lane 2- haemolymph from LLI larval stage (30 ug total protein was loaded).

Fig. 15b: Western blot analysis of the bacterial lysate and C. *cephalonica* hexamerins. Lane 1- bacterial lysate, lane 2- haemolymph from LLI larval stage. Amount of protein loaded 30 µg.

Analysis of the hexamerins in the bacterial lysate:

To confirm the identity of the hexamerins in the bacterial lysate, protein blots containing the *C. cephalonica* haemolymph and XL 1-Blue bacterial lysate, which were transformed with the Hex cDNA containing plasmids, were probed separately with three different antibodies (Hex 1 antibody, Hex 2 antibody and Hex 3 antibody). The immunoblots showed a strong cross-reactivity with Hex 2 antibodies (Fig. 16a) while there was no cross-reactivity detected with Hex 1 or Hex 3 antibodies (Figs. 16b and c). Thus based on the strong immunological cross-reactivity of the hexamerins in

the bacterial lysate with Hex 2 antibodies, it was confirmed that hexamerin encoded by the Hex cDNA is Hex 2 of *C. cephalonica*. Hence, the clones were named as Hex 2a and Hex 2b.

Fig. 16a

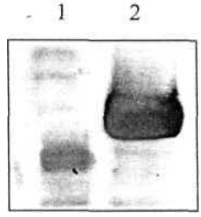

Fig. 16b

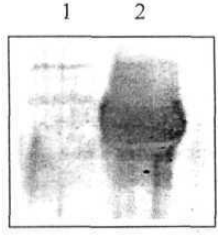

Fig. 16c

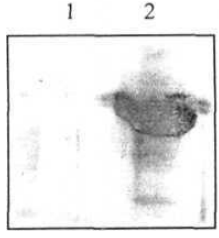

Fig. 16: Western blot analysis of recombinant proteins present in the lysates prepared from XL1-Blue transformed with Hex 2a cDNA clone. Lane 1- bacterial lysate proteins and lane 2 larval haemolymph proteins. The blots were probed with three different antibodies:

16a: blot probed with Hex 2 antibody.

16b: blot probed with Hex 1 antibody.

16c: blot probed with Hex 3 antibody.

Note the presence of (→) cross reacting protein band in the blot probed with Hex 2 antibody.

Amino acid analysis:

Amino acid analysis carried out with HPLC purified *C. cephalonica* Hex 2 revealed the presence of high content of aromatic amino acids, phenylalanine (6.3 %) and tyrosine (12 %) and low content of methionine (Fig. 17). This data corroborates with the amino acid composition obtained from the deduced amino acid sequence of the hex cDN A clones, which also show the presence of high concentration of aromatic amino acids and low methionine content.

Fig. 17

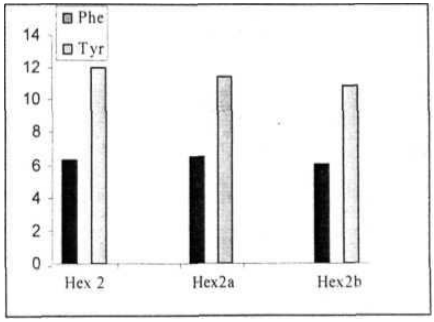

Fig. 17: Histogram showing the phenylalanine (Phe) and tyrosine (Tyr) of *C. cephalonica* Hex 2 protein and hexamerins deduced sequence encoded by the Hex 2a and Hex 2b cDNA clones. Y axis represents % of amino acids.

Fluorescence emission spectra:

Figure 18a shows the fluorescence spectra of control sample containing tryptophan and tyrosine (1:10) excited at 285 nm. A detailed analysis of the emission spectra obtained indicated that the fluorescence emission excited at 285 nm was mainly dominated by tyrosine residues rather than the tryptophan residues. Figure 18b shows the fluorescence emission spectra obtained from acid hydrolyzed Hex 2 of C. *cephalonica* excited at the same wavelength (285 nm). The spectrum obtained showed similar kinds of peaks and matched with the spectra obtained for the standard (Fig. 18a), there by suggesting that Hex 2 has high content of tyrosine.

Fig. 19a

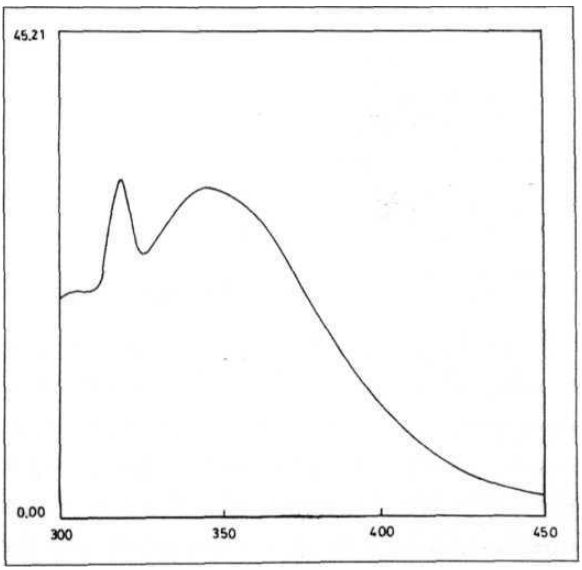

Fig. 18a: Fluorescence emission spectra of standard (a mixture of tyrosine and phenylalanine)

Fig. 19b

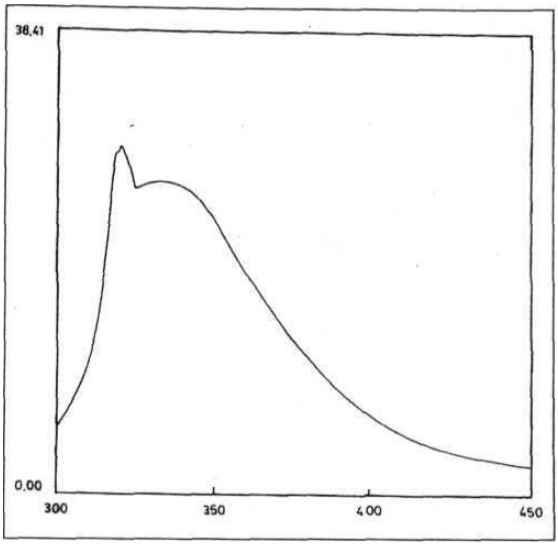

Fig. 19b: Fluorescence emission spectra of Hex 2 of C. *cephalonica.* Note that the spectra is identical to that of standard (Fig. 18a).

Developmental profile of hexamerins:

Immunoblotting studies of whole body proteins (Fig. 19a) and fat body proteins (Fig. 19b) during various stages of larval development of *C. cephalonica* showed the presence of hexamerins from very early stages of postembryonic development. Since dissection of fat body during early stages of larval development was difficult, the studies were carried out with whole body homogenates. As the larval development proceeded from 1st to 3rd instar, there was gradual increase in the hexamerins concentration in the whole body (Fig. 19a, lanes 1- 3). The developmental profile of fat body proteins presented in figure 19b clearly showed the

presence of moderate quantity of hexamerins in penultimate (lane 1), ELI (lane 2), MLI (lane 3) and high quantity in LLI (lane 4) larvae. However, the highest amount of protein was found to be present in the fat body of prepupae (Fig. 20b, lane 6).

Fig. 19a

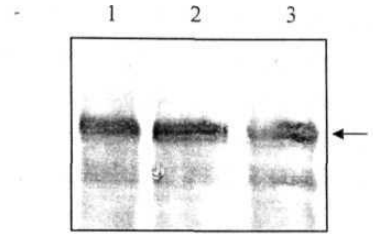

Fig. 19b

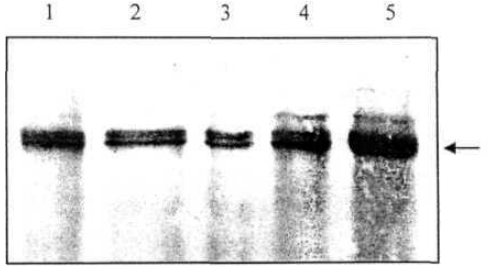

Fig. 19a: Immunoblot showing the developmental profile of hexamerins (→) in the whole body homogenate of 1st, 2n and 3r instar larvae. Lane 1- 1st instar, lane 2- 2nd instar, lane 3- 3rd instar larvae. Equal quantity of whole body homogenate protein (30 μg) was loaded in each well.

Fig. 19b: Immunoblot analysis showing the developmental profile of hexamerins (→) in the fat body homogenates of penultimate (lane 1). ELI (lane 2), MLI (lane 3), LLI (lane 4) larvae and prepupa (lane 5). The blots were probed with Hex antibodies.

Tissue distribution of **hexamerins:**

Proteins from larval fat body, carcass, salivary gland and Malpighian tubule as well as adult ovary and MARG were transferred to nitrocellulose membrane and immunodetected using hexamerin antibodies. Results presented in figure 20a show the presence of hexamerin like proteins in all tissues. It is interesting to note that the amount of hexamerin like proteins present in salivary gland is quite high (lane 3), when compared with other tissues like carcass and Malpighian tubule. The adult tissues ovary as well as MARG showed the presence of hexamerin like proteins (Fig. 20b). However, the molecular mass of the immunoreactive protein present in ovary (lane 2) is higher than the hexamerin band present in the fat body (Fig. 20b). This study clearly suggests that hexamerins are widely distributed in different tissues during postembryonic and adult development.

Fig. 20a

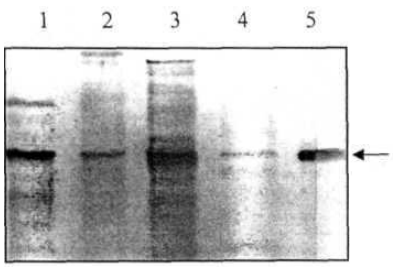

Fig. 20b

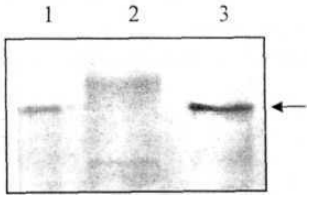

Fig. 20a: Western blot analysis of larval tissues proteins. Lane 1- fat body, lane 2- carcass, lane 3- salivary gland, lane 4- gut and lane 5- MT.

Fig. 20b: Western blot analysis of ovary and MARG proteins from adult insects. Lane 1- MARG, lane 2- ovary and lane 3- fat body.

Equal amount of protein (20 ug) was loaded in all wells. Hex antibodies were used for immunodetection.

Expression of **hexamerins-developmental** profile:

For this study, RNA was isolated from the fat body of different larval stages (2^{nd} to 5^{th} instar) and northern blot analysis was carried out. Probing was **initially** done using Hex 2a cDNA. Later the filter was stripped and reprobed with Hex 2b cDNA. Identical result was obtained with both the clones. The study revealed the presence of Hex- mRNA at all the stages of larval development (Fig. 21). The concentration of the hexamerin 2-transcript increased gradually in fat body from 2^{nd} to final instar larvae and reached a very high level in final instar larvae.

Fig. 22

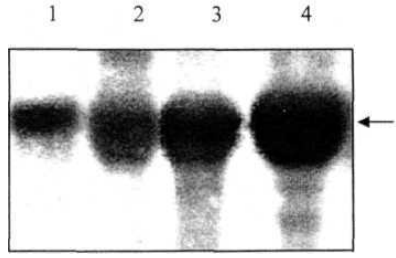

Fig. 21: Northern blot analysis of the Hex 2-mRNA in the fat body during larval development of *C. cephalonica.* Lane 1- 2^{nd} instar, lane 2- 3^{rd} instar, lane 3- 4^{th} instar and lane 4- 5^{th} instar. Amount of RNA loaded was 10 ug per lane. Hex 2a cDNA was used as probe in the study.

Tissue specific expression of the hexamerin gene:

The tissue-specific expression of **Hex** 2-mRNA was investigated by northern blot analysis. From various tissues tested, only the fat body RNA generated a signal when probed with the Hex 2a cDNA (Fig. 22). A single band corresponding to RNA of about 2.2 kb was detected (lane 1) which is in good agreement with the size of the sequenced cDNA (2,127 bp). When the filter was stripped and re-probed with Hex 2b cDNA, the same band appeared (data not presented). The transcript was found to be absent in other tissues like salivary gland (lane 3), gut (lane 4), and Malpighian tubule (lane 5). However, Hex-mRNA was also detected in the carcass preparation (lane 2) but to a lower degree than in fat body and it's mainly due to the contamination, as the carcass cannot be prepared free of fat body tissue.

Fig. 22

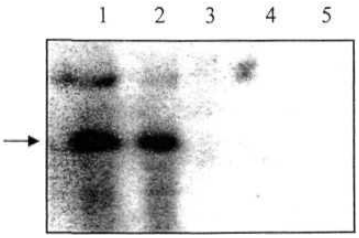

Fig. 22: Northern blot demonstrating the tissue specificity of Hex 2 transcript in ***C.*** *cephalonica* last-instar larvae. Lane 1- fat body, lane 2- carcass, lane 3- salivary gland, lane 4- gut and lane 5- Malpighian tubule.

Species specificity of Hex 2:

Western blot analysis of the haemolymph proteins from *C. cephalonica*, *S. litura* and *B. mori* using Hex 2 antibodies (raised against hexamerin 2 of *C. cephalonica)*, showed no cross reactivity with the hexamerins of *S. litura* and *B. mori* (Fig. 23a). Furthermore, even in northern blot analysis

Hex 2 cDNA did not hybridize with RNA isolated from larval fat body of *S. litura* and *B. mori* (Fig. 23b).

Fig. 23a

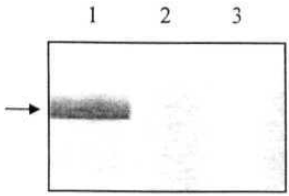

Fig. 24b

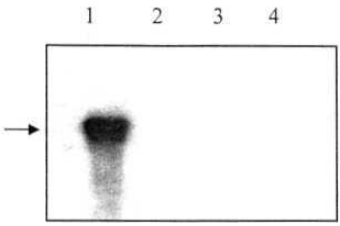

Fig. 23a: Western blot analysis of larval haemolymph proteins from C. *cephalonica*, *B. mori* and *S. litura* showing the immunological specificity of C *cephalonica* hexamerins. Lane 1- *C. cephalonica,* lane 2- *B. mori* and lane 3- *S. litura.* Amount of total protein loaded in each lane was 30 µg. Hex 2 antibodies were used for detection.

Fig. 23b: Northern blot analysis of the fat body RNA from C. *cephalonica, B mori* and *S. litura.* Equal amount of total RNA (10 µg) was loaded in each lane. Hex 2a cDNA was used as probe in the study.

Effect of 20E on the hexamerins mRNA transcript:

For this study, fat body RNA was prepared from different developmental stages (ELI, ML1. LLI larvae and prepupa). which were ligated for 24 h and northern blot analysis was carried out using Hex 2a cDNA probe. Results presented in figure 24b clearly show the decline in the Hex 2 transcript in the LLI larval (lane 3) and prepupal stages (lane 4) compared to the **ELI** (lane 1) and MLI (lane 2) larval stages.

Furthermore, this became more evident when a comparison was made between the unligated control (Fig. 24a lanes 3 and 4) and thorax-ligated insects (Fig. 24 b lanes 3 and 4). Northern blot analysis carried out with fat body RNA isolated from ligated insects after 24 h 20E treatment clearly showed an increase in Hex 2 transcript level in both LLI larval and prepupal stage (Fig. 24c lane 3 and 4) when compared with respective ligated controls (Fig. 24b lanes 3 and 4).

Fig 24a

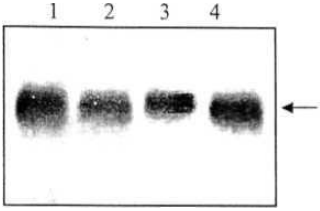

Fig 24b

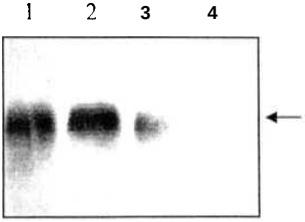

Fig 24c

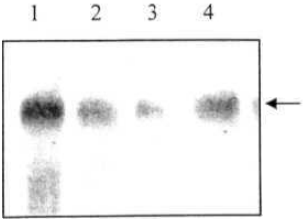

Fig. 24: Northern blot analysis demonstrating the effect of 20E on Hex 2 transcripts at different stages of development. Lane 1- ELI, lane 2- MLI, lane 3- LLI larvae and lane 4- prepupa.

24a: Hex 2-mRNA profile at various developmental stages.

24b: Effect of thorax-ligation on Hex 2-mRNA profile. Fat body was collected 24 h after ligation and RNA was isolated.

24c: Hex 2-mRNA profile after application of exogenous 20E for 24 h. In all the experiments 20 ug of total RNA was loaded. All the three blots were probed with Hex 2a cDNA.

Effect of 20E on the **hexamerins** synthesis:

For this study the fat body, from ligated insects (after 24 h of ligation) was *in vitro* cultured either in presence or in absence of exogenous 20E and [^{35}S] methionine and the radioactivity was analyzed in the proteins. The results presented in figure 25 clearly show that the radioactivity was high in 20E treated fat body proteins as compared to the control. This suggests that 20E stimulates protein synthesis.

Fig. 25

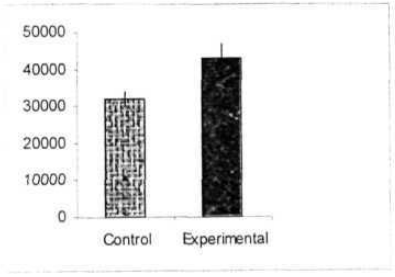

Fig 25: Shows the effect of 20E on the fat body protein synthesis. For experimental set 20E (80 nM) was added to the fat body culture while in control only solvent was added. Y axis represents radioactivity CPM/ ug protein.

CHAPTER 5

Hexamerin binding protein and uptake of hexamerins

Methods:

Detailed protocols for organ culture, preparation of radiolabelled hexamerins, and radiolabel quantification have been explained in chapter 2.

In vitro phosphorylation of fat body proteins:

To understand the phosphorylation status of the 120 kDa hexamerin binding protein (HBP) present in the larval fat body membrane, *in vitro* phosphorylation was carried out following the method explained in chapter 2. Phosphorylated proteins were detected by autoradiography.

To study the *in vivo* effect of 20-hydroxyecdysone (20E) on 120 kDa HBP phosphorylation late-last instar larvae were thorax-ligated. After 24 h ligation, isolated abdomens were treated for 2 and 4 h with exogenous 20E (1 µg / insect). Fat body homogenates were prepared and proteins were *in vitro* phosphorylated.

In vitro effect of 20E on HBP phosphorylation was carried out by culturing the fat body from 24 h ligated late-last instar larvae in presence of hormone (80 nM) for various time durations. Tissue homogenate was prepared and proteins were *in vitro* phosphorylated.

To study the effect of genistein, a tyrosine kinase inhibitor, on phosphorylation of 120 kDa HBP protein, fat body from the 24 h ligated late-last instar larvae were dissected and homogenate was prepared. The homogenate proteins were treated with either genistein, or 20E or 20E and genistein together and then subjected to *in vitro* phosphorylation.

In vitro hexamerin uptake studies:

This was carried out using radiolabelled hexamerins prepared by the method explained in chapter 2. The fat body from late-last instar larvae and prepupae, which were ligated for 24 h was dissected out and cultured under sterile conditions (as described in chapter 2) either in presence or absence of 20E (80 nM). To each of the cultures 100 ug of radiolabelled hexamerin (50,000 cpm) was added. The cultures were incubated for 8 h and tissues were processed for radiolabel quantification (described in chapter 2).

Effect of phosphorylation on the uptake of hexamerins by fat body was designed using 20E and genistein. The fat body from 24 h ligated late-last instar larvae/ prepupae was cultured under sterile conditions. To these cultures either genistein (100 μM) or genistein (100 uM) and 20E (80 nM) along with the 100 ug radiolabelled hexamerin (50,000 cpm) were added. After 8 h incubation, the tissue was collected and processed for radiolabel quantification (described in chapter 2).

In vitro hexamerin uptake by salivary gland, ovary and MARG:

Salivary gland from late-last instar larvae, ovary and MARG from freshly emerged adults were dissected under sterile conditions and cultured in 200 μl of TC-100 medium. To these cultures 100 ug of labeled hexamerins (50,000 cpm) were added and uptake study was carried out for 8 h. After 8 h, the tissues were collected and processed for radiolabel quantification as described in chapter 2.

Results:

Earlier work from our laboratory (Kirankumar *et al.,* 1997) showed the presence of two hexamerin binding proteins (HBP) in the larval and prepupal fat body membranes (Fig. 26). They were identified using biotinylated hexamerins following ligand blotting technique. The molecular masses of these proteins were determined to be 120 and 125 kDa (Figs. 26a and 26b). In the present study an attempt was made to study the phosphorylation of these proteins and its impact if any on the uptake of hexamerins.

Fig. 26a

1 2

Fig. 26b

1 2

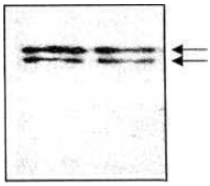

Fig. 26: Hexamerin binding proteins were identified by ligand blotting (Kirankumar *et al.,* 1997). "Uptake of storage protein in Rice Moth, *Corcyra cephalonica:* Identification of storage protein binding proteins in the fat body cell membranes". Photographs are borrowed from above paper.

26a: SDS-PAGE analysis showing the profile of fat body membrane proteins during larval and prepupal stages.

26b: Ligand immunoblot of the above gel showing two hexamerin binding proteins with molecular masses 125 and 120 kDa (→).Lane 1- LLI and lane 2- PP.

Phosphorylation profile of HBP during various stages of development in *C. **cephalonica***:

For this study fat body proteins from MLI, LLI larvae and prepupa were *in vitro* phosphorylated. The results presented in figure 27 clearly showed that only 120 kDa protein undergoes phosphorylation during development from MLI larva to prepupa, furthermore there was profound increase in the amount of [γ^{32}P] ATP incorporated into 120 kDa HPB from MLI to LLI larval stage (Fig. 27b, lanes 1 and 2). However, during prepupal stage, there was a slight decline in [γ^{32}P] ATP incorporation in HBP (Fig. 27b, lane 3). Quantitative densitometric analysis also shows maximum phosphorylation of HBP at LLI larval stage (Fig. 27c).

Fig. 27a

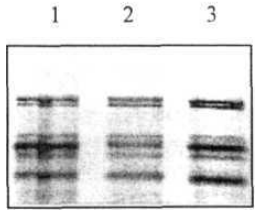

Fig. **27b**

Fig. 27c

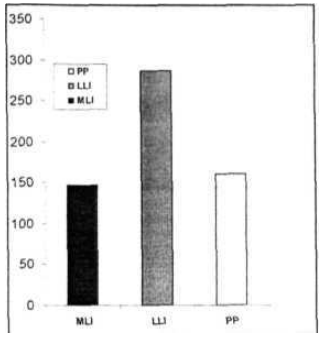

Fig. 27: Phosphorylation profile of 120 kDa HBP during various stages of development in *C. cephalonica.*

27a: SDS-PAGE profile of the fat body proteins.

27b: Autoradiogram of the above gel showing the phosphorylation status of the 120 kDa HBP (→). Lane 1- MLI, lane 2- LLI and lane 3- PP. Amount of phosphorylated protein loaded in each lane was 20 ug.

27c: Quantitative representation of data obtained by densitometric scanning of the above autoradiogram. The values represented on Y axis are in arbitrary units.

In vivo effect of **20E** on HBP phosphorylation:

For this study, late-last instar larvae were thorax-ligated and used after 24 h. The fat body was dissected out from control as well as experimental insects, homogenized and proteins were *in vitro* phosphorylated. The results are presented in figure 28b. Fat body from ligated insects (lane 2) showed lower degree of HBP phosphorylation as compared to the unligated control insects (lane 1). Application of exogenous hormone to the ligated insects (24 h after ligation) for 2 h caused an increase in

phosphorylation of HBP (lane 3) and the effect was more pronounced when the treatment period was extended to 4 h (lane 4). Furthermore, the degree of phosphorylation of HBP was much higher in 20E treated insects (lane 4) when compared with unligated controls (lane 1).

Fig. 28a

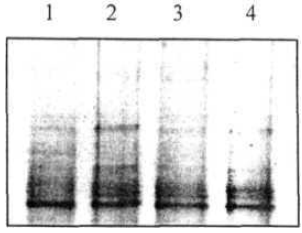

Fig. 28b

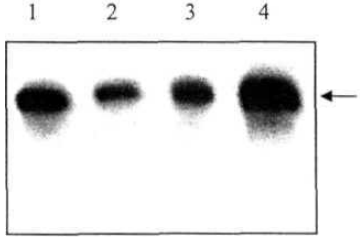

Fig. 28: *In vivo* effect of 20E on the phosphorylation of the 120 kDa HBP (—•) in thorax-ligated late-last instar larvae. The fat body proteins were *in vitro* phosphorylated in presence of {γ^{32}P} ATP.

28a: SDS-PAGE profile of the phosphorylated fat body proteins.

28b: Autoradiogram of the same gel. Lane 1- LLI larvae, lane 2- 24 h ligated LLI larvae, lane 3- 24 h ligated **LLI** larvae with 2 h *in vivo* hormone treatment, lane 4- 24 h ligated LLI larvae with 4 h *in vivo* hormone treatment. Amount of protein loaded in each lane was 20 µg.

In vitro effect of 20E on HBP phosphorylation:

In vitro effect of 20E was studied at homogenate level. Late-last instar larvae were thorax-ligated. The fat body was dissected out from them after 24 h of ligation. Homogenate was prepared and to it either 20E (80 nM) or solvent (10 % ethanol) was added to study the effect. Results presented in figure 29b show that there is an increase in the degree of phosphorylation of 120 kDa HBP after 20E addition (lanes 2 and 3) and the effect was more pronounced with 5 min hormone treatment. However, when the hormone and [γ^{32}P] ATP were added together, HBP showed higher degree of phosphorylation. This study clearly shows that 20E stimulates HBP phosphorylation even in tissue homogenate.

Fig. 29a

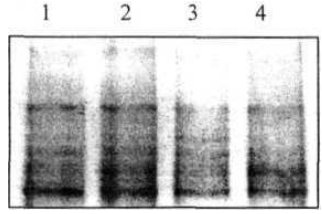

Fig. 29b

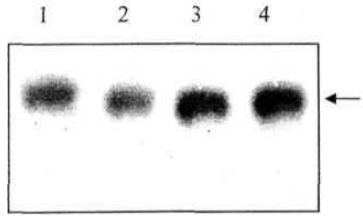

Fig. 29: Shows the *in vitro* effect of 20E (at homogenate level) on the phosphorylation of 120 kDa HBP (→). Late-last instar larvae were thorax-

ligated and fat body was dissected out 24 h after ligation. Tissue homogenate was prepared. To experimental sample hormone (80 nM) and to control sample solvent (10 % ethanol) was added and incubation was carried out. The fat body proteins were *in vitro* phosphorylated for 1 min in presence of [γ^{32}P] ATP.

29a: SDS-PAGE profile of phosphorylated fat body proteins.

29b: Autoradiogram of the same gel. Lane 1- solvent control, lane 2- 1 min 20E treatment. lane 3- 5 min 20E treatment and lane 4- 1 min 20E treatment and simultaneous *in vitro* phosphorylation. 20 ug of phosphorylated protein was loaded in each lane.

Effect of genistein on phosphorylation:

In vitro effect of genistein was studied at homogenate level. The fat body was dissected out from late-last instar larvae after 24 h of thorax-ligation. Homogenate was prepared and to it either 20E or genistein or 20E and genistein were added. Results presented in figure 30b show that 20E, stimulated the phosphorylation of HBP (lane 1), and genistein partially inhibited this 20E stimulated phosphorylation of 120 kDa HBP (lane 3).

Fig. 30a

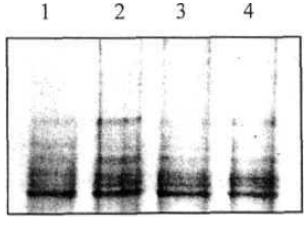

Fig. 30b

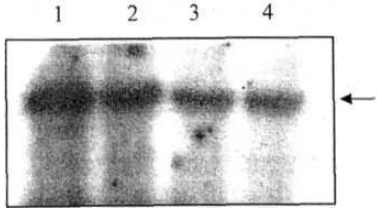

Fig. 30c

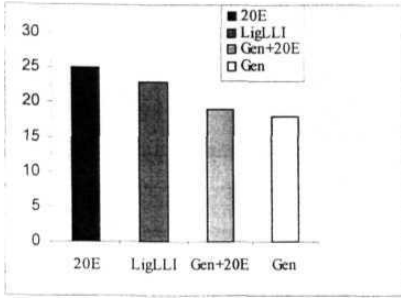

Fig. 30: Shows the *in vitro* effect of 20E and genistein (at homogenate level) on the phosphorylation of 120 kDa HBP (→).

30a: SDS-PAGE profile of phosphorylated fat body proteins.

30b: Autoradiogram of the same gel. Lane 1- 20E (80 mM), lane 2- LL1 ligated control, lane 3- 20E + genistein and lane 4- genistein (100 μM) treatment. Amount of protein loaded 20 μg. Genistein treatment was done for 5 min.

30c: Quantitative representation of data obtained by densitometric scanning of the above autoradiogram. The values represented on Y axis are in arbitrary units.

Effect of **20E** on *in vitro* uptake of **hexamerins** by fat body:

Radiolabelled hexamerins were prepared and analyzed by SDS-PAGE and fluorography. Figures 31 a and b show that the preparations contain intact radiolabelled hexamerins. *In vitro* uptake studies were carried out with the fat body of late-last instar larva and prepupa using organ cultures. Studies revealed that 20E stimulates the uptake of hexamerins and this effect was more profound in the late-last instar larval stage than in the prepupal stage (Fig. 32).

Fig. 31a

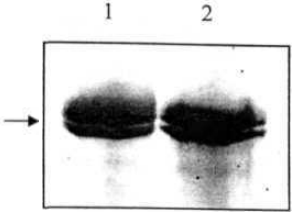

Fig. 31b

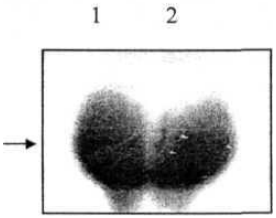

Fig 31a: SDS-PAGE profile of the haemolymph protein from the mid-last instar larvae injected with [^{35}S] methionine and incubated for 18 h. Total protein (20 µg) was loaded in each lane. Note the presence of thick band which represents hexamerins (→).

Fig 31b: Fluorographic study with the above protein sample. For study 20,000 cpm were loaded in each lane. → shows the hexamerin band.

Fig. 32

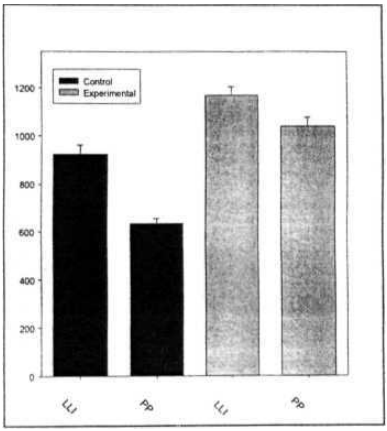

Fig. 32: Effect of 20E on the uptake of radiolabelled hexamerins by the fat body during late-last instar (LLI) larval and prepupal (PP) stages. Insect fat bodies were cultured in the medium under *in vitro* condition for

8 h, either in presence or absence of 20E. To monitor the uptake radiolabelled hexamerins (50,000) cpm were added to the cultures. The values with which the above plot was drawn represent the mean ± S. D of three independent experiments. For each experiment, fat body from two insects was used. Y axis represents the sequestered hexamerin (cpm/ insect fat body).

Effect of genistein and 20E on *in vitro* uptake of hexamerins by the fat body:

Addition of genistein caused a reduction in the uptake of hexamerins, where as in the presence of 20E the inhibitory effect of genistein was abolished to some extent. However, the inhibitory effect of genistein was more profound in the case of late-last instar larvae than prepupae (Fig. 33).

Fig. 33

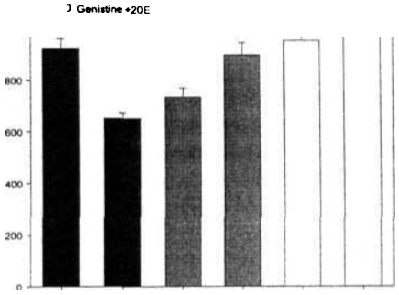

Fig. 33: Effect of 20E and genistein on the uptake of radiolabelled hexamerins by the fat body during late-last instar larval (LLI) and prepupal (PP) stages. Insect fat bodies were cultured in the medium under *in vitro* conditions for 8 h in the presence of either genistein or genistein and 20E. To monitor the uptake, radiolabelled hexamerins were added to culture. The values with which the above plot was drawn represent the

mean ± S. D of three experiments. For each experiment, fat body from two insects was used. Y axis represents the sequestered hexamerin (cpm/ insect fat body).

Hexamerin uptake by larval salivary gland, adult ovary and MARG:

In vitro uptake studies carried out with larval salivary gland, adult ovary and MARG showed that all these three tissues sequester hexamerin (Table 2). However, the sequestration was found to be fairly high in ovary.

Table 2

Tissue/Organ	cpm/ insect tissue
Fat body	**924±38**
Salivary gland	**295±45**
Ovary	**865±50**
MARG	**366±41**

Table 2: Hexamerin uptake by the larval salivary gland, adult ovary and MARG. These tissues were cultured under ***in*** *vitro* condition for 8 h in the presence of radiolabelled hexamerins (50,000 cpm). The values represent the mean ± S. D of three experiments. For each experiment, tissue from two different insects was used.

CHAPTER 6

Discussion and Summary

Extensive work on major haemolymph proteins revealed the involvement of hexamerins in the development and reproduction of insects (Kanost *et al*., 1990; Telfer and Kunkel, 1991). *C. cephalonica* is one of the three most common and the destructive pest among the 23 reported species of insects infecting stored products and packed food in warehouses in different countries (Highland, 1978) and is of great economic importance. Hence, for the last two decades or so our group is working on the physiology and biochemistry of this insect including hexamerins.

liarlicr studies from our laboratory on *C. cephalonica* reported the presence of different hexamerins in the haemolymph (Ismail, 1991) like in any other lepidopteran insect (Haunerland, 1996). On the basis of their electrophoretic mobility and tissue specific expression, they have been identified as three distinct hexamerins with molecular mass of 86 kDa (Hex 1), 84 kDa (Hex 2) and 82 kDa (Hex 3) and that all the three belong to the group of insect storage proteins. Among the three hexamerins, Hex 1 and Hex 2 were found to show similar developmental profile in male as well as female larvae. However, Hex 1 was always present in higher concentration than Hex 2. On the other hand. Hex 3 was synthesized only during the late-last larval instar development and its synthesis was inhibited by the application of exogenous JH (Ismail and Dutta-Gupta, 1988), hence, it was grouped with "juvenile hormone suppressible hexamerins" reported for other lepidopteran insects (Memmel *et al*., 1994; Hwang *et al*., 2001; Zhu *et al*., 2002). 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 larval development (Ismail, 1991; Kirankumar, 1998). Preliminary studies on chemical nature of hexamerins showed that they are lipoglycoproteins.

With this background knowledge on *C. cephalonica* hexamerins, in the present study identification, isolation and characterization of cDNA clones which encode hexamerins was carried out. Developmentally regulated uptake of hexamerins by fat body cells of *C. cephalonica* is widely demonstrated. Although these studies clearly show that 20-hydroxyecdysone regulates hexamerin endocytosis, the exact mechanism and the levels of control are not well understood.

In the present study we identified two full length cDNA clones after three rounds of immunoscreening, from the cDNA expression library constructed from the late-last instar larval fat body RNA using hexamerin antibodies. Our sequence alignment studies showed that these clones encode for proteins related to a number of insect hexamerins. A similar protocol was also used for isolation and identification of a full length cDNA clone (BmLSP) encoding hexamerin (BmLSP) of *B. mori* from the cDNA expression library constructed from fat body poly (A+) RNA isolated from day 2 fourth instar larvae. Antibodies raised against BmLSP were used for immunoscreening (Yoshihiro and Okisugu, 1991). cDNA clones encoding hexamerins of different insects were isolated and sequenced either from cDNA or from cDNA expression library (Sakurai *et al.,* 1988; Corpuz *et al*., 1991; Memmel *et al.,* 1994; Burmester *et al.,* 1998a; Cheon *et al*., 1998; Zheng *et al*., 2001). The cDNA clones isolated in present study were later identified as clones for hexamerin 2 (Hex 2), hence named as Hex 2a and Hex 2b.

Complete sequencing of Hex 2a and Hex 2b cDNA clones was accomplished by using two sets of designed primers. Hex 2a and Hex 2b cDNA clones comprise of 2,225 and 2,230 nucleotides respectively. Size of the clones obtained in the present study matched with the size for other reported insect hexamerin clones (Memmel *et al.,* 1994; Cheon *et al*., 1998; Zheng *et al.,* 2001). Both the sequences show an open reading frame

of 2,109 nucleotides beginning with a methionine start codon ATG and a translation stop codon TAA present at position 2,127 bp. Similar sizes of cDNA clones (PinSPl and PinSP2) encoding hexamerins was also reported for *P. interpunctella* another lepidopteran insect. PinSPl clone consisted of 2,431 nucleotides with an open reading frame of 2,295 nucleotides while PinSP2 clone consisted of 2,336 nucleotides with an open reading frame of 2,250 nucleotides (Zhu *et a!.,* 2002). Even Burmester *et al.* (1998a) reported a similar size of cDNA clone (2,215 nucleotides) encoding LSP2 hexamerin in C *erythrocephala*, with an open reading frame of 2,103 nucleotides beginning with a methionine start codon ATG and ending with a translation stop codon TAA present at position 2,119 bp.

The presence of signal peptide is an essential feature seen in many secretary proteins, which facilitates transmembrane transport and export from the synthesizing cells in to the surrounding fluid (Von Heijne, 1986). This feature is also a characteristic for insect hexamerins. Computer-assisted analysis of Hex 2a and Hcx 2b sequences revealed the presence of a typical 19 amino acid signal peptide. This length of the signal peptide is consistent with that found in other lepidopteran hexamerins, where their length varies between 15 and 20 amino acids (Sakurai *et al.,* 1988; Willott *et al.,* 1989; Jones. 1990; Memmel *et al.*, 1994; de Kort and Koopmanschap, 1994). Recently Zhu *et al.* (2002) reported that PinSPl and PinSP2 cDNA clones encoding for hexamerins in *P. interpunctella* also have a signal peptide length of 17 and 15 amino acids respectively. Furthermore similar size was also reported for dipteran insect. In *Calliphora.* Burmester *et al.* (1998a) reported a typical 20 amino acids signal peptide for LSP-2 hexamerin sequence, which was encoded by LSP-2 cDNA. Our sequence analysis data of Hex 2a as well as Hex 2b cDNA showed the presence of a 3' untranslated sequence containing a polyadenylation signal ATAAA and a polyadenylation tail, which is a

common feature seen in eukaryotic mRNA including other insect hexamerin sequences (Memmel *et al*., 1992; Burmester *et al.,* 1998a; Hwang *et al.,* 2001; Zhu *et al.,* 2002).

When the primary structure for both the clones Hex 2a and Hex 2b was deduced, they yielded polypeptides of 703 amino acid residues with an estimated molecular mass of 83.4 kDa. This molecular mass is in accordance with the molecular mass of *C. cephalonica* hexamerins, which ranges between 82 to 86 kDa. Moreover the size of the polypeptide is also in agreement with the size of the polypeptide encoded for lepidopteran as well as dipteran hexamerins (Memmel *et al*.. 1992; Jamroz *et al.,* 1996; Zakharkin *et al.,* 1997; Lewis *et al*., 2002). Hwang *et al.* (2001) recently reported a similar size of cDNA (2,572 bp), which also yields a polypeptide of 747 amino acid residues with a predicted molecular mass of 88.5 kDa in *Hyphantria cunea*. Even in a dipteran insect, *C. vicina,* Burmester *et al*. (1998a) made a similar report of a cDNA clone (LSP-2) whose deduced primary structure yields a polypeptide of 701 amino acids with a molecular mass of 83.16 kDa.

Deduced amino acid composition obtained from both Hex 2a and Hex 2b cDNA sequences, revealed the presence of high content of aryl groups (17.3% and 17.8% respectively) with low methionine content (1.7%). Based on high content of aryl groups, we classify these proteins, as members of the arylphorin sub-family of insect hexamerins. Arylphorins are a class of storage hexamerins that contain a high proportion of aromatic amino acids (>15%), phenylalanine and tyrosine (Telfer *et al*.. 1983; Scheller *et al.,* 1990). Arylphorins are reported to be the major larval hexamerins present in the haemolymph of lepidopteran as well as dipteran insects and several cDNA clones encoding arylphorins have been identified and reported in these insects (Willott *et al.,* 1989; Robert and Stephen, 1990; Nauman and Scheller, 1991; Memmel *et al.,* 1992; de Kort

and Koopmanschap, 1994; Jamroz *et al*., 1996). Recently Lewis *et al*. (2002) identified and sequenced a cDNA clone AgSP-1 which encodes for diapause-specific arylphorin from a coleopteran insect, *Anthonomus grandis*.

Deduced arylphorin protein sequence obtained from the two cDNA clones (Hex 2a and Hex 2b) also showed two putative N-glycosylation sites, at amino acid positions 214 and 482, suggesting that these proteins might undergo glycosylation and this is in accordance with previous biochemical evidence that hexamerins in *C. cephalonica* are lipoglycoproteins (Ismail, 1991). Recently Hwang *et al*. (2001) and Lewis *et* **al.** (2002) also reported the presence of three potential glycosylation sites for the deduced peptides obtained from cDNA clones SP-2 of *H.cunea* and AgSP-1 of *A. grandis*.

Relevant databases searched, using the BLAST (Altschul *et al*., 1990) and FASTA programs to retrieve protein sequences similar to Hex 2a and Hex 2b cDNA clones, revealed a significant homology of these clones with that of arylphorin clones of other lepidopterans which encode aromatic amino acid rich hexamerins. When comparison was drawn, it was observed that there was 73% identity with *G. mellonella* LHP76, 56% with *M. sexta* arylphorin and 55% with *H. cecropia* arylphorin. Such conserved signature patterns were also reported for PinSPl and PinSP2 cDNA clones encoding *P. interpunctella* hexamerins and 60-70% identity was found with other lepidopteran hexamerin encoding cDNA clones (Zhu *et al*. 2002).

Reconstruction of the phylogenetic relationship based on amino acid distances for Hex 2a and Hex 2b revealed that they are distinct arylphorin genes, that already differentiated about 15 million years ago (MYA). The genera *Galleria* and *Corcyra,* belong to the galleriinae/ pyrilidae, which diverged around 54 MYA at the beginning of the tertiary period. This

estimate predates the earliest fossil record of the pyrilidae around 35 MYA (Ross and Jarzembowski, 1993), and may be an overestimate due to deviations of amino acid replacement rates. On the other hand, the fossil record of the lepidoptera in the middle tertiary or earlier periods is poor, and it is possible that the actual times of divergence are much more ancient than the fossils imply. The other divergence times are in fact in excellent agreement with those published by Burmester *et al*. (1998b) and with the available fossil data (Kukalova-Peck, 1991; Ross and Jarzembowski, 1993).

Earlier [H] palmitate incorporation and PAS staining studies from our laboratory indicated that all the three hexamerins in *C. cephalonica* undergo post-translational modification like lipidation and glycosylation and are lipoglycoproteins (Ismail, 1991). Studies carried out using tunicamycin, an inhibitor of glycosylation showed that unglycosylated hexamerins have a faster mobility on SDS-PAGE and do not get released in to the haemolymph (Ismail, 1991). In the present study the recombinant hexamerins expressed at basal level in the *E. coli* host cells (XL1-Blue and DH5a strains) were of low molecular mass, and their migration on SDS-PAGE was faster than the hexamerins expressed in *C. cephalonica* larvae. Similar shift in mobility was observed in *C. cephalonica* hexamerins after chemical deglycosylation and these deglycosylated larval hexamerins co-migrated with recombinant hexamerins expressed in bacterial host. Furthermore, as expected none of the proteins present in bacterial lysates including recombinant hexamerins were stained with PAS. Thus the shift in the mobility and reduction in the molecular mass of the recombinant hexamerins is most likely due to absence of post-translational modification in the prokaryotic system *(E. coli)*.

The characterization of the isolated clones as Hex 2a and Hex 2b encoding hexamerin 2 (Hex 2) was mainly on the basis of the identity of the

recombinant proteins with that of purified Hex 2 and their selective cross reactivity with Hex 2 antibodies. The fluorescence emission spectra obtained for HPLC purified Hex 2 of *C. cephalonica* was typical of proteins rich in aromatic amino acids. Moreover, even amino acid analysis data obtained with HPLC purified Hex 2 protein showed the presence of high concentration of phenylalanine and tyrosine and this matched with the percentage of these amino acids in deduced amino acid sequence of Hex 2a and Hex 2b cDNA clones. Western blot analysis to find out immunological cross-reactivity of the Hex cDNA encoded proteins with Hex 1, Hex 2 or Hex 3 antibodies clearly showed that the recombinant hexamerins obtained from Hex 2a and Hex 2b cDNA clones cross react specifically with Hex 2 antibody. Thus all these studies suggest that Hex cDNA clones are derived from the Hex 2 mRNA encoding gene.

Expression of hexamerins from very early stages of development has been reported in many insects (Zakharkin *et al.,* 1997; Burmester *et al.,* 1998a; Zhu *et al.,* 2002). A wealth of data has also accumulated over the recent years on the regulation of hexamerin biosynthesis in lepidopteran and dipteran insects. The hexamerin synthesis is regulated in a stage-specific and tissue-specific manner under the influence of the hormones 20E and juvenile hormone. In present study northern analysis using Hex 2a and Hex 2b cDNA clones as probes revealed that hexamerin (Hex 2) is expressed from very early stages of development *i. e.*, from 2nd larval instar in C. *cephalonica*. The mRNA content increased gradually and reached maximum during the "last-instar larvae". Hex 2 transcripts were neither detected in pupa nor in adults. This suggests that the expression of Hex 2 in *C. cephalonica* is developmentally regulated and expressed only in larval stages. Similar pattern was also reported in *P. interpunctella* another lepidopteran insect, where very low level of hexamerin expression was observed in the second instar, which dramatically increased during the third instar, and peaked in the fourth instar (Zhu *et al.,* 2002).

After a long standing research it was concluded that hexamerins are predominantly synthesized by fat body and not by any other tissues except for a few exceptions in which hexamerin genes were shown to be transcribed in other tissues (Capurro *et al.,* 2000; Hwang *et al.*, 2001). It was of interest to determine the tissue specific expression of hexamerin in *C. cephalonica.* Northern blot analysis of total RNA from various tissues, demonstrated that Hex 2a and Hex 2b genes are transcribed only in the larval fat body cells of C *cephalonica.* A faint signal was observed in the carcass that could be due to the presence of contaminating fat body which is associated always in the carcass preparation. In *G. mellonella* and *B. mori* hexamerin transcripts were detected abundantly in the fat body and slightly in carcass (Ray *et al,* 1987b; Yashihiro and Okisugu, 1991). These authors also suggested that this was mainly due to the presence of fat body contamination. Further, the pattern of hexamerin synthesis closely follows the transcriptional activity exhibiting a close parallelism with mRNA levels of the fat body in different insects (Burmester *et al.,* 1998a; Mi *et al.,* 1998).

Ecdysteroids are known to regulate hexamerin gene expression both at transcriptional and translation levels (Benes *et al.*, 1996). Our northern analysis study revealed that hexamerin expression is stimulated in presence of 20E. This is further supported with [^{35}S] methionine incorporation studies. Results from *in vitro* synthesis of hexamerins in presence of 20E showed enhanced synthetic rate, clearly suggesting that 20E stimulates hexamerins synthesis in *C. cephalonica.* Similar observations were also made in other lepidopteran insects (Ray *et al.,* 1987b; Mousseron *et al.,* 1997; Zhu *et al.,* 2002).

Western blot analysis carried out in the present study showed the presence of hexamerins not only in larval fat body, haemolymph and carcass but also in other tissues like salivary gland and Malpighian tubule as well as in

ovary and MARG of adults. However, our northern blot analysis did not give any signal when the mRNA from these tissues was hybridized with Hex 2a or Hex 2b cDNA. Hence, the presence of hexamerins in different tissues is most likely due to the sequestration. This kind of distribution has already been reported earlier by our group in *C. cephalonica* as well as *Chilo partellus* (Bajaj *et al*., 1990; Ismail and Dutta-Gupta, 1991) and in other insects (Korochkina *et al.*, 1997b, Danty *et al*, 1998, Capurro *et al.*, 2000). Wide distribution of hexamerins in different tissues is indicative of its diversified role in insect development (Haunerland, 1996; Burmester. 1999,2001).

Like in all lepidopteran insects even in *C. cephalonica* hexamerins are sequestered into the fat body at the end of larval life during the prepupal stage and it coincides with the rise in ecdysteroid titre (Dutta-Gupta and Ashok, 1998). It was also reported that 20E stimulates the uptake of hexamerin in fat bodies of *C. cephalonica* and that *in vivo* sequestration of hexamerin is 20E dependent (Ismail and Dutta-Gupta, 1990b; Kirankumar **era/.,** 1997, 1998).

Ligand blotting studies carried out with fat body membrane showed the presence of two hexamerin binding proteins (HBP) with molecular masses of 125 kDa and 120 kDa. (Kirankumar *et al.*, 1997). Further, it was shown that the amount of 120 kDa HBP increases significantly at prepupal stage. Increasing evidences now suggest that many steroid induced events are triggered independent of transcription (Hammes, 2003). Various mechanisms for 20E mediated activation of hexamerin receptors have been reported in insects. In larvae of 5. *peregrina*, a 120 kDa receptor protein in cleaved by proteinases into 76 and 53 kDa fragments. which do not bind to hexamerin. It was suggested that during pupation, 20E plays a role in keeping the receptor intact mainly by inhibiting the proteinase and this intact receptor mediates the selective uptake of hexamerins in to the

fat body (Chung *et al.*, 1995). On the other hand, 20E was shown to mediate the cleavage of an inactive precursor into the active receptor in C. *vicina*, followed by the uptake of hexamerins (Burmester and Scheller, 1997). All reports published up to date substantiate that ecdysteroids regulate hexamerin sequestration by mechanisms which do not involve gene activation. However, the nature of the non-genomic effects is unknown.

As protein phosphorylation is recognized as one of the major post-translational modification, which plays role in variety of cellular functions and in many physiological actions (Graves and Kreb, 1999). 20E, like other steroid hormones, is known to exert its action at the post-translational level *e. g.*, by protein phosphorylation. It has been shown to stimulate *in vitro* phosphorylation of a few fat body proteins of *Mamestra brassicae* (Sass, 1988) and *S. peregrina* (Itoh *et al.*, 1985). Several protein tyrosine kinases have been identified in *Drosophila* and *Manduca sexta* (Duffy and Perrimon, 1994; Raabe *et al.*, 1996; Schweitzer and Shilo, 1997; Smith *et al.*, 1997). Some authors (Itoh *et al.*, 1985) suggested that the 20E regulated phosphorylation of a 30 kDa protein was responsible for the conversion of the fat body from a synthetic to storage organ in *S. peregrina.*

Our *in vitro* phosphorylation studies carried out with fat body preparation of C. *cephalonica* showed that among the two HBP, only 120 kDa protein gets phosphorylated and this is developmentally regulated. The highest degree of hexamerin receptor phosphorylation was found at late-last larval stage. The fat body proteins at mid-last larval stage showed a very little phosphorylation while it was moderate at prepupal stage. It has been already demonstrated in *C. cephalonica* that the HBP is synthesized during the final instar of larval development and is present in large quantities at late-last instar larva and prepupa (Kirankumar *et al.*, 1997).

Therefore, the lack of phosphorylation of HBP at mid-last instar larval stage is probably due to the absence of the 120 kDa protein in the fat body membrane at this stage. Further, the high degree of [γ^{32}P] ATP incorporation in late-last instar larvae might be directly related to the presence of higher amounts of the 120 kDa protein with a large number of free sites available for *in vitro* labeling, as the endogenous ecdysteroid concentration is fairly low at this developmental stage (Dutta-Gupta and Ashok, 1998). On the other hand, the lower degree of phosphorylation in prepupa suggests that, in presence of a high 20E titer, the sites of phosphorylation are already occupied, hence there are few sites accessible to [γ^{32}P] ATP during the *in vitro* experiment. This suggests that 20E induces the activation of the 120 kDa HBP through phosphorylation and this phosphorylated protein is responsible for the uptake of hexamerin seen at the prepupal and later developmental stages.

Thorax-ligation significantly reduces the level of ecdysteroid and keeps the posterior part of insect larvae relatively free of endogenous hormone; hence the larvae can be manipulated for hormonal studies (Burmester and Scheller, 1997; Dutta-Gupta and Ashok, 1998). In the present study, thorax-ligation for 24 h showed a significant decline in the incorporation of [γ^{32}P] ATP in HBP, indicating that the phosphorylation of this protein might be mediated by 20E. Application of exogenous 20E to thorax-ligated LLI larvae for various time periods showed an induction in phosphorylation of HBP after 4 h treatment, further, the addition of exogenous 20E to fat body homogenate prepared from 24 h ligated late-last instar larvae induced the phosphorylation of the 120 kDa HBP. This suggests that this process requires neither transcription nor translation. Present study with intact fat bodies, kept in culture, indicates that 20E induced phosphorylation increases the uptake of radiolabelled hexamerin in the fat body cells. This fact strengthens our assumption that the 20E induced phosphorylation of the 120 kDa HBP mediates the hexamerin

uptake and has a physiological significance during larval-pupal transformation in *C. cephalonica*.

It has been demonstrated that the cleavage of the hexamerin receptor in *Calliphora* was essential for the uptake of hexamerins, but was not dependent on new protein synthesis or RNA transcription (Burmester and Scheller, 1997). In the present study, the phosphorylation of the 120 kDa HBP of *C. cephalonica* is significantly enhanced by 20E not only in intact tissue but also in homogenate, suggesting that the receptor phosphorylation is not dependent on *de novo* protein synthesis.

Pathways that control the tyrosine phosphorylation were shown to be important for axon guidance in insects during embryonic and postembryonic development (Callahan *et al*., 1995, Menon and Zinn, 1998). Recently, Arif *et al*. (2002) reported a juvenile hormone dependent tyrosine kinase mediated phosphorylation of a 48 kDa protein which is developmentally regulated in different tissues including fat body of *Bombyx mori*. In the present study the phosphorylation of 120 kDa protein was inhibited by genistein, a broad spectrum inhibitor affecting many receptor and non-receptor tyrosine kinases (Akiyama *et al*., 1987). Genistein also blocked the hexamerin uptake by cultured larval fat body. Furthermore, when genistein treatment was carried out in the presence of 20E, the inhibitory effect on phosphorylation of 120 kDa protein was less pronounced. Recently communicated work from our laboratory shows that the 120 kDa phosphoprotein of *C. cephalonica* cross-reacts with anti-phosphotyrosine monoclonal antibody (Arif *et al.,* 2003). Based on these results, we suggest that the 20E induced phosphorylation of the 120 kDa HBP is a tyrosine kinase mediated process. Present study also shows that phosphorylation is essential for HBP activation and is a prerequisite for the uptake of hexamerin in *C. cephalonica* fat body. Further, the phosphorylation of 120 kDa hexamerin receptor in *C. cephalonica* is

developmentally regulated by 20E probably through a non-genomic action. Further studies have to be carried out to substantiate this concept.

Earlier studies from our laboratory have convincingly demonstrated that intact hexamerins are sequestered and secreted by the MARG of C. *partellus* (Ismail and Dutta-Gupta, 1991; Ismail *et al.*, 1993). In the present study larval salivary gland and adult tissues like ovary and MARG of *C. cephalonica* were shown to sequester hexamerins. However, the amount of hexamerin incorporated in adult ovary was much higher than in other tissues like larval salivary gland and adult MARG. Hence, the present study further strengthens the concept, that hexamerins most likely play a wide role during both larval as well as adult development of insects.

Summary and Conclusions

1. Two cDNA clones Hex 2a and Hex 2b were identified and isolated by immunoscreening from expression library prepared from fat body mRNA.

2. Complete sequence of Hex2a and Hex2b comprises of 2,225 and 2,230 nucleotides respectively, with an open reading frame of 2,109 nucleotides and a 3' untranslated sequence containing a polyadenylation signal ATAAA.

3. 92.5% homology was seen between Hex2a and Hex2b and they are considered as two genes.

4. The deduced primary structure for both the clones yielded polypeptides of 703 amino acids with an estimated molecular mass of 83.4 kDa each.

5. The amino acid composition of both the clones showed the presence of high concentration of aromatic amino acids, phenylalanine (Hex2a: 6.1% and Hex2b 6.4%) and Tyrosine (Hex2a: 11.2% and Hex2b 11.4%) with low methionine content of 1.7%.

6. The amino acid analysis for the purified *C. cephalonica* hexamerin (Hex 2) also revealed the presence of high aromatic amino acids (tyrosine 12%. phenylalanine 6.3%) and low methionine content of 1.4% which suggests that the cDNA encodes for Hex 2.

7. Multiple amino acid sequence alignment studies revealed 73% homology with *Galleria mellonella* LHP76, 56% with *Manduca sexta* arylphorin, and 55% with *Hylophora cecropia* arylphorin, thus the two clones Hex2a and Hex2b group with the lepidopteran hexamerins.

8. A phylogenetic tree constructed using the neighbour-joining method based on the PAM-distances revealed that Hex2a and Hex2b diverged 15 million years ago.

9. Hexamerins expressed in *E.coli* are of low molecular mass compared to Hex 2 proteins in the *C. cephalonica* which is due to the lack of post translation modifications like glycosylation and lipidation in the prokaryotic system like *E. coli*.

10. Northern and western blot studies revealed that hexamerins are expressed from very early stages of development, *i. e.*, from 2nd instar larvae.

11. Northern blot studies clearly showed that fat body is the only site of hexamerins synthesis and a slight cross-reactivity in carcass is only because carcass is always associated with fat body.

12. Western blot studies showed the presence of intact hexamerins in other larval tissues like carcass, salivary gland and Malpighian tubules, and adult tissues like ovary and MARG, which are a result of sequestration of hexamerins by these tissues.

13. The 120 kDa HBP protein involved in the sequestration of hexamerins gets phosphorylated which is stimulated by 20E and

inhibited by genistein there by suggesting the kinase involved in the phosphorylation to be a tyrosine kinase.

14. Uptake of hexamerins in fat body is stimulated by 20E and inhibited genistein, hence it suggests that phosphorylation of HBP might be a requisite for the uptake of hexamerins by fat body.

CHAPTER 7

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APPENDIX

Publications from the work

Research paper published:

P. Nagamanju, I. A. Hansen, T. Burmester, S. R. Meyer, K. Scheller, A. Dutta-Gupta (2003) Complete sequence, expression and evolution of two members of the hexamerin protein family during the larval development of the rice moth, *Corcyra cephalonica*. *Insect Biochem Mol. Biol.* 33: 73-80.

Sequences submitted to the Gen Bank:

1. Accession No. AF29808: 2225 bp *Corcyra cephalonica* clone 2a hexamerin 2 (HEX2) mRNA complete cds.
2. Accession No. AF29809: 2230 bp *Corcyra cephalonica* clone 2b hexamerin 2 (HEX2) mRNA complete cds.

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Complete sequence, expression and evolution of two members of the hexamerin protein family during the larval development of the rice moth, *Corcyra cephalonica*

P. Nagamanju [a], I.A. Hansen [b], T. Burmester [c], S.R. Meyer [b], K. Scheller [b], A. Dutta-Gupta [a,*]

[a] *Department of Animal Sciences, School of Life Sciences. University of Hyderabad, Hyderabad 500 046, India*
[b] *Department of Cell and Developmental Biology, Biocentre of the University, D-97074 Würzburg, Germany*
[c] *Institute of Zoology, University of Mainz, D-55099 Mainz, Germany*

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Abstract

Three distinct types of storage hexamerins are expressed in the "last-instar" larvae of the rice moth, *Corcyra cephalonica*. A cDNA expression library was constructed from fat body-RNA and screened with a polyclonal antibody raised against purified hexamerin (SP2) of *Corcyra cephalonica*. Two slightly different "full-length" hexamerin cDNA clones (Hex2a and Hex2b) were isolated and sequenced. Both include open reading frames of 2109 bp which are translated into polypeptides of 703 amino acids with 92.5% identity. Signal peptides of 19 amino acids are present at the N-termini. The 684 amino acids native proteins have a high content of aryl groups (17.6%). According to both the criteria for amino acid composition and the phylogenetic analysis, Hex2a and Hex2b belong to the lepidopteran arylphorins. Northern blot studies revealed that the Hex2 genes are species- and tissue-specifically expressed in fat body cells of "last-instar" (= 5th) larvae.

Keywords: Corcyra cephalonica; Rice moth; Fat body; Hexamerin; Arylphorin; cDNA; Developmental profile

1. Introduction

During the post-embryonic life of holometabolous insects, high amounts of specific lipoglycoproteins accumulate in the haemolymph of the "last-instar" larvae. These proteins form hexamers in the 500 kDa range and, therefore, are referred to as hexamerins (Telfer and Kunkel, 1991). According to the biochemical properties of their six subunits (M_r = 70–90 kDa per subunit), they may be categorized into three major classes: (i) the arylphorins, polypeptides rich in the aryl groups tyrosine and phenylalanine (for review, see Scheller et al., 1990), (ii) female-specific methionine-rich proteins, which may be needed as a sulfur reserve for egg development in Lepidoptera (Telfer and Kunkel, 1991; Pan and Telfer, 1996), and (iii) proteins which are neither rich in aromatic amino acids nor in methionine (Haunerland, 1996; Braun and Wyatt, 1996). Some hexamerins were identified to bind riboflavin and are therefore referred to as riboflavin-binding hexamerins, e.g. *Hyalophora cecropia* **RbH** (Magee et al., 1994). The equivalence of a riboflavin-binding hexamerin and arylphorin as reserves for adult development in two saturniid moths was also reported (Pan and Telfer, 1999)

The biosynthesis of hexamerins is developmentally regulated and generally restricted to the larval fat body. During the feeding period, the fat body cells secrete hexamerins into the haemolymph where they accumulate to high concentration and finally may account for about 80% of total haemolymph proteins (Scheller et al., 1990). Insect pupae do not feed during metamorphosis. Therefore, they depend on material that has been accumulated during the larval life. At the end of this period, shortly before pupation, a rise in titer of ecdysteroid hormones induces the incorporation of a large

* Corresponding author. Tel.: + 91-40-30-10-052; fax: +91-40-30-10-145.
E-mail address: apdgsl@uohyd.ernet.in (A. Dutta-Gupta).

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fraction of hexamerins from the body fluid into the fat body cells. In two dipteran insects investigated so far (*Sarcophaga peregrine*, *Calliphora vicina*), the transport process through the fat body cell membranes is mediated by a specific receptor, whose activity is controlled by ecdysteroid hormone (Ueno and Natori, 1984; Burmester and Scheller, 1999). The mechanism of receptor-mediated, hormonally controlled endocytosis is conserved during evolution in diptera (Burmester and Scheller, 1997) and has also been demonstrated in two lepidopteran species, *Corcyra cephalonica* (Ismail and Dutta-Gupta, 1990a; KiranKumar et al., 1997, 1998) and *Helicoverpa armigera* (Haunerland, 1996). Besides their function as storage proteins, the hexamerins have been shown to be a constituent of the sclerotizing system of the cuticle (Peter and Scheller, 1991) and serve as an ecdysteroid carrier in the haemolymph (Enderle et al., 1983). In *C. cephalonica*, hexamerins play also an important role for the reproduction of the moth (Dutta-Gupta and Ismail, 1992).

Corcyra cephalonica is a major stored grain pest of cereals and oilseed in semiarid tropics and causes serious damage. With this background, our quest to know more about these proteins has provoked us to characterize the proteins at the molecular level and to identify the hexamerin encoding sequences. Here we report the isolation of two complete hexamerin-cDNA clones and their deduced amino acid sequences in C. *cephalonica*. Furthermore, we show that the hexamerin-mRNA is selectively expressed in the larval fat bodies, and that its biosynthesis is developmentally regulated.

2. Material and methods

2.1. Experimental insects

The rice moth, *Corcyra cephalonica*, was reared at 27 ± 1°C, 60-65% relative humidity and 14:10 L:D period on crushed sorghum seeds. In the present study, 2nd, 3rd, 4th, and 5th (= last) instar larvae were used. The staging of the larval forms was carried out as reported earlier (Ashok and Dutta-Gupta, 1988). The larval forms of *Bombyx mori*, *Spodoptera litura* and *Helicoverpa armigera* were maintained on their natural diet and the "last-instar" larvae were used. All animals were water-anaesthetized before dissection.

2.2. RNA isolation

Fat bodies from "late-last" instar larvae of *C. cephalonica* were dissected out in a drop of phosphate buffered saline and subsequently washed in the same buffer. Total RNA was isolated using the *TriFast Kit* (PeqLab, Erlangen, Germany) and used for the cDNA preparation. RNA was also isolated from fat bodies of *Bombyx mori*, *Spodoptera litura* and *Helicoverpa armigera* "last-instar" larvae, and furthermore, from salivary gland, gut, Malpighian tubules and carcass of C. *cephalonica*.

2.3. Preparation of cDNA expression library and immunoscreening

The cDNA was generated and amplified with the SMART III cDNA Library construction Kit (Clonetech Laboratories, Palo Alto, USA). The library was cloned in a pTriplEx2 vector and screened with the IgG fraction of a polyclonal antisera raised against purified hexamerin (SP2) of C. *cephalonica* (Arif et al., 2001). The screening was performed according to standard protocols (Sambrook et al., 1989). After three rounds of immunoscreening, ten positive clones were picked up and used for further studies.

2.4. Sequencing of hexamerin clone

Ten positive clones obtained after immunoscreening were used for in vivo excision. The plasmids were grown in XL1-Blue cells (Stratagene, LaJolla, USA) and the inserts were sequenced on a Perkin-Elmer 310 sequencer. All the clones proved to contain hexamerin-like sequences (confirmed by BLAST search). Two clones were analyzed in detail designing appropriate primers. Two "full-length" cDNA sequences were obtained.

2.5. Northern blotting

Two micrograms of total RNA isolated from the desired tissue were separated on a 1.2% denaturating agarose formaldehyde gel. RNA was then blotted onto a nylon membrane (Sambrook et al., 1989). The filters were prehybridized in 6 x SSC, 5 x Denhardt's solution, 1% SDS, 100 μg/ml denatured salmon sperm DNA and 50% formamide for 4 h at 42°C. The membrane was probed with a ^{33}P labeled (random primer kit of Bangalore Genci, India) Hex-cDNA sequence. Hybridization was carried out at 42°C for 16 h. The stringency of the final wash was 0. 1% SSC. 0.1% SDS at 65°C. The filter was exposed to a Kodak XOMAT X-ray film with intensifying screen.

2.6. Immunoblotting

Insect haemolymph as well as bacterial lysate proteins were dissolved in SDS sample buffer (60 mM Tris-HCl, pH 6.8 containing 2.5% SDS, 0.36 M β-mercaptoethanol, 0.5 mM EDTA, 10% glycerol) and were separated by SDS-PAGE. The proteins were electrophoretically transferred onto nitrocellulose membranes. Non-specific binding sites were blocked with 5% non-fat dry milk powder diluted in Tris-buffered saline (TBS: 0.15

M Tris-HCl, pH 7.0 containing 0.5 M NaCl) for 1 h at room temperature. The membranes were incubated overnight at 4°C with primary antibody raised against HPLC purified hexamerin (SP2) (IgG fraction, 1:1000). After washing three times with TBS containing 0.5% Tween-20 (TTBS), the membranes were incubated with the secondary antibody, an alkaline phosphate-conjugated anti-rabbit IgG developed in goat (Banagalore Genei, India), 1:5000 diluted in 3% milk powder/TTBS for 1 h at room temperature. Following TTBS and TBS rinsing, the immunodetection was carried out by using NBT-BCIP as substrates.

2.7. Amino acid analysis

HPLC purified hexamerin (SP2) was acid hydrolyzed with 6N HC1 and was analyzed using the Shimadzu HPLC amino acid analysis system.

2.8. Phylogenetic inference

A multiple sequence alignment of the *C. cephalonica* amino acid sequences Hex2a, Hex2b, and 33 selected insect hexamerins was constructed using CLUSTALX (Thompson et al., 1997) and corrected according to a previously published alignment (Burmester et al., 1998) by the aid of GeneDoc 2.6 (Nicholas and Nicholas, 1997). The signal peptides and C-terminal extensions were eliminated from the final data set. The program package PHYLIP 3.6a2 (Felsenstein, 2001) was applied for tree calculations. Distances between pairs of proteins were calculated using the PAM001 matrix implemented in the PHYLIP package. Tree constructions were performed by the neighbor-joining method. A putative insect hemocyanin (Sanchez et al., 1998) was used as the outgroup (cf, Burmester, 2001). The reliability of the trees was tested by the bootstrap procedure with 100 replications.

Linearized trees were essentially calculated as described (Burmester, 2001). Briefly, the distance matrix was imported into the Microsoft EXCEL 97 spread sheet program. Relative rate tests were carried out successively to single proteins or groups of proteins according to the topology of the tree (cf. Burmester et al., 1998). To estimate divergence times, we assumed that the Orthoptera diverged from the other neopteran insects 320 million years ago (MYA) (Kukalovà-Peck, 1991). The confidence limits were estimated using the observed standard deviation of the inferred replacement rates.

3. Results

3.1. Structure of the hexamerin DNA clones and hexamerin proteins

By screening the fat body expression library with specific anti-hexamerin (SP2) antibodies, we identified two positive hexamerin-cDNA clones (Hex2a-cDNA and Hex2b-cDNA) with similarity to other hexamerin sequences present in GenBank. Both cDNA clones were therefore subcloned and sequenced. (GenBank Accession nos. AF294808 and AF294809). The two HexcDNA clones comprise of 2225 and 2230 nucleotides, respectively. The sequences include the open reading frames of 2109 nucleotides, each beginning with the methionine start codon ATG and translation stop codon TAA at positions 2127 (Fig. 1). A 3' untranslated sequence containing a polyadenylation signal ATAAA is detected in Hex2a as well as in Hex2b.

The deduced primary structure of both clones yielded polypeptides of 703 amino acids (including the signal peptides. see below) with estimated molecular mass of 83.4 kDa each. The calculated isoelectric points are 5.64 for Hex2a and 5.59 for Hex2b. The amino acid sequence shows the presence of high concentrations of the aromatic amino acids phenylalanine (Hex2a: 6.1% and Hex2b: 6.4%) and tyrosine (Hex2a: 11.2% and Hex2b: 11.4%) while the methionine content is only 1.7%. Amino acid analysis with purified *C. cephalonica* hexamerin (SP2) also revealed the presence of high aromatic amino acids (tyrosine 12%, phenylalanine 6.3%) and a low methionine content (1.4%).

Computer analysis of the sequence revealed the presence of a typical 19 amino acid signal peptide necessary for transmembrane transport and export from the synthesizing cells into the hemolymph (von Heijne, 1986). Hex2a and Hex2b show 92.5% identity at the amino acid level. In each of the hexamerins, two putative N-glycosylation sites (NXS/T) were detected at amino acid positions 214 and 482 (Fig. 2).

To retrieve protein sequences similar to Hex2a and Hex2b, relevant databases were searched using the BLAST algorithm (Altschul et al., 1990). Comparison of the amino acid sequences (Fig. 2) revealed 73% identity with *Galleria mellonella* Lhp76, 56% with *Manduca sexta* arylphorin, 55% with *Hyalophora cecropia* arylphorin, and 53% with *Bombyx mori* SP 2..

3.2. Tissue specific expression of the hexamerin gene

The tissue-specific appearance of the Hex-mRNA was investigated by Northern blot analysis. From various tissues tested only the fat body RNA generated a signal when probed with the Hex2a-cDNA (Fig. 3). We detected one single band corresponding to RNA of about 2.2 kb. This is in good agreement with the size of the sequenced cDNA (2.127bp). When the filter was stripped and re-probed with Hex2b-cDNA. the same band appeared (not shown). The transcript was found to be absent in other tissues like salivary gland, gut, and Malphigian tubules. Hex-mRNA was also detected in the carcass preparation but to a lower degree than in fat body as the carcass cannot be prepared free of fat body tissue.

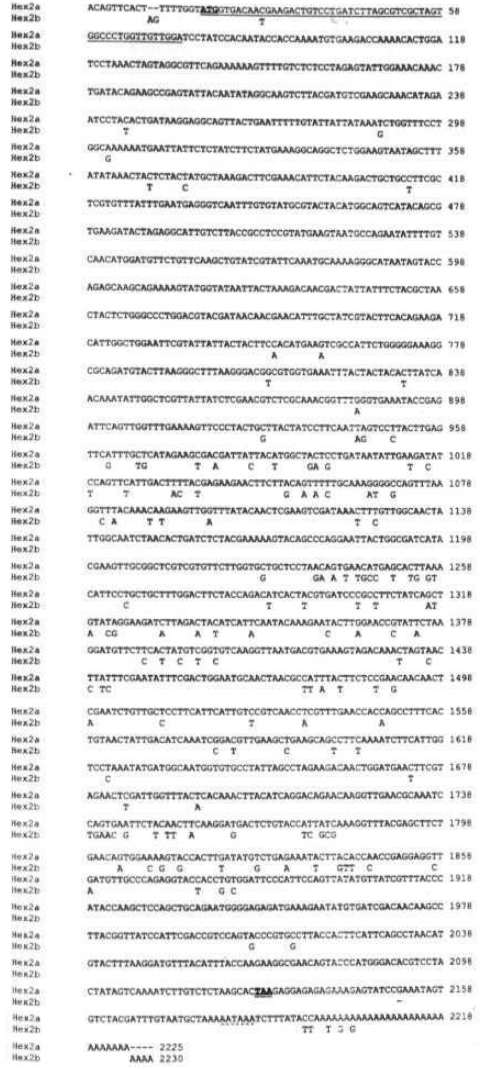


Fig. 1. Comparison of *Corcyra cephalonica* hexamerin-cDNA sequences Hex2a and Hex2b. The translation start is located at by 20 (bold letters), the stop codon is present at by 2127 (bold letters) in both sequences. The polyadenylation sites are at bp 2178 to 2186. The putative signal peptide sequence is underlined.

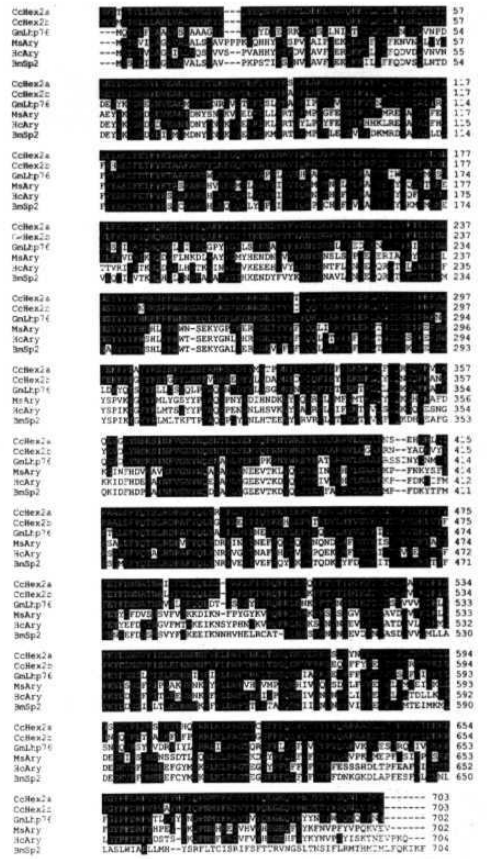


Fig. 2. (a) Alignment of the deduced amino acid sequence of the *Corcyra cephalonica* hexamerins CcHex2a and CcHex2b with 4 other lepidopteran hexamerin sequences. *Galleria mellonella* Lhp76 (Accession number 449954); *Manduca sexta* arylphorin α (P14296); *Hyalophora cecropia* arylphorin (AAB86644); *Bombyx mori* Sp2 (A34287). The conserved positions (identical amino acids) are shaded.

3.3. Developmental expression of hexamerin gene

To examine the developmental pattern of the Hex-2a, gene. RNA was isolated from fat bodies of 4th and 5th instar larvae. Because it was difficult to prepare sufficient quantities of RNA from fat bodies of 2nd and 3rd instar larvae we prepared the RNA from whole *C. cephalonica* larvae.

From Fig. 4 it can be seen that Hex2a-mRNA was present at all stages of the larval development of C. *cephalonica* and that the mRNA content reached a

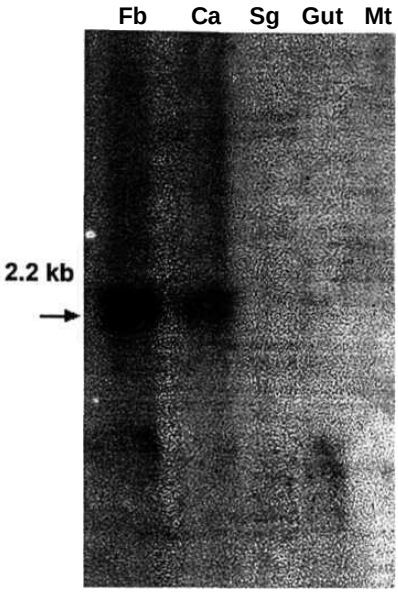


Fig. 3. Northern blot demonstrating the tissue specificity of the Hex2 transcript in *C. cephalonica* last-instar larvae. Fb—fat body, Ca—carcass, Sg—salivary gland, Gut—gut, and Mt—Malphigian tubules. 2 µg of total RNA was applied to each lane.

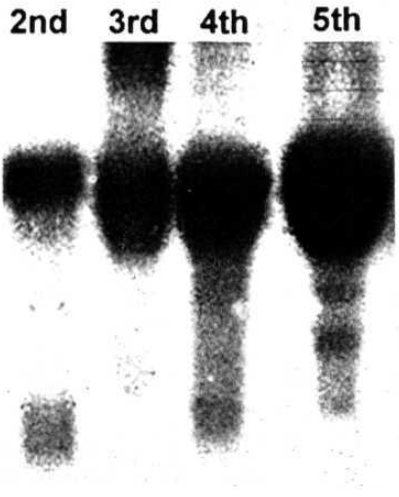


Fig. 4. Content of Hex2-mRNA in the fat body during larval development (2nd to 5th instar) of *C. cephalonica*.

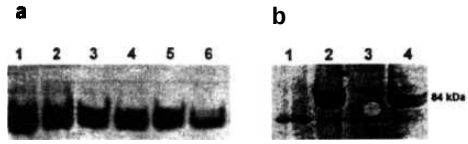


Fig. 5. a. Western blots showing the presence of *C. cephalonica* Hex2a (1–3) and Hex2b (4–6) in six different recombinant bacterial lysates. b. Comparison of the recombinant Hex2a (1) and Hex2b (3) with the respective hexamerins in the haemolymph of "last-instar" larvae (2, 4).

maximum in "last-instar" larvae. Northern blot analyses demonstrated that the Hex2-cDNA did not hybridize to mRNA isolated from fat bodies of *Bombyx mori*, *Spodoptera litura* and *Helicoverpa armigera.*

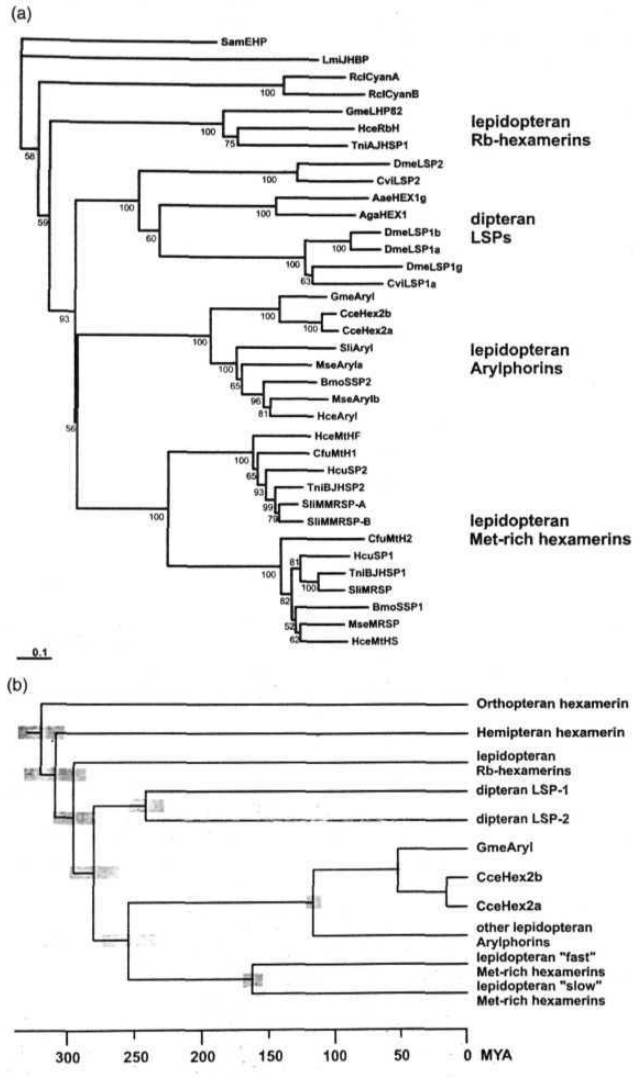
3.4. *E. coli expression of hexamerin*

The recombinant proteins from lysates of XL 1-blue cells in which the Hex-cDNA containing plasmids were grown were separated by SDS-PAGE and probed with hexamerin (SP2) specific antibodies. The Western blot demonstrates a strong cross-reactivity of the antibody with a single protein band (Fig. 5a) migrating slightly faster than Hex2 protein present in the haemolymph of "last-instar" larvae of *C. cephalonica* which migrates at 84 kDa (Fig. 6b). These experiments demonstrate that the isolated cDNA clones encode the Hex2a and Hex2b proteins.

3.5. *Phylogenetic analysis of Hex2a and Hex2b*

A phylogenetic tree was constructed using the neighbor-joining method based on the PAM-distances (accepted point mutations per site; Dayhoff et al., 1978). The two arylphorins Hex2a and Hex2b of *C. cephalonica* form a well-supported common clade (100% bootstrap value) which is associated with the arylphorin of the waxmoth *Galleria mellonella.* The lepidopteran arylphorins themselves are monophyletic (100% support), and are most likely associated with the methionine-rich hexamerins of this taxon, although this topology does not reach the significance level (56% support). As already observed before (Burmester, 1999, 2001), the lepidopteran hexamerins are not monophyletic, but the riboflavin-binding proteins (HceRbH, GmeLHP82, TniAJHSPl) are in basal position within the hexamerins of the other holometabolous insects.

Based on the assumption that the Orthoptera (represented here by LmiJHBP) diverged from the other Neoptera about 320 MYA (Kukalovà-Peck, 1991), the time of divergence of the lepidopteran and dipteran hexamerins was calculated to be about 280 MYA. The emergence of the lepidopteran arylphorins dates back some 255 MYA. The time of divergence of the arylphor-



ins from the Pyralidae *(Galleria* and *Corcyra)* and the ditrysian lepidoptera (higher moths and butterflies) was calculated to be 117 MY A. *Galleria* and *Corcyra* diverged about 54 MYA, and the arylphorins of C. *cephalonica* around 15 MYA.

4. Discussion

On the basis of their electrophoretic mobility, we have previously identified three distinct storage proteins in *Corcyra cephalonica* with molecular masses of 86 kDa

Fig. 6. a. Neighbour joining tree of insect hexamerins. The bar equals 0.1 PAM distance, the numbers at the nodes are the bootstrap support values. Abbreviations: SamHc, *Schistocerca americana* hemocyanin (GenBank accession number AF038569); LmiJHBP, *Locusta migratoria* juvenile-hormone binding hexamerin (U74469); RclCyanA, *Riptortus clavatus* cyanoprotein a (D87272); RclCyanB, *R. clavatus* cyanoprotein b (D87273); CceHex2a, *Corcyra cephalonica* hexamerin 2a (AF294808); CceHex2a, *C. cephalonica* hexamerin 2b (AF294809); GmeLHP82, *Galleria mellonella* LBP82 (L21997); GmeAryl, *G. mellonella* arylphorin (M73793); BmoSSP1, *Bombyx mori* sex-specific storage protein 1 (P09179); BmoSSP2, *B. mori* sex-specific storage protein 2 (P20613); MseAryla, *Manduca sexta* arylphorin a (P14296); MseArylb, *M. sexta* arylphorin (5 (P14297); MSMRSP. *M. sexta* methionine-rich storage protein (L07609); HceAryl. *Hyalophora cecropia* arylphorin (AF03296); HceRbH, *H. cecropia* riboflavin-binding hexamerin (AF03297); HceMtBF. *H. cecropia* methionine-rich hexamerin F (AF03298); HceMtHS, *H. cecropia* methionine-rich hexamerin S (AF03299); HcuSPI. *Hyphantria cunea* storage protein 1 (U60988); HcuSP2, *Hyphantria cunea* storage protein 2 (AF157012); TniAJHSPI, *Trichoplusia ni* acidic juvenile-hormone-suppressible protein (P22327); TniBJHSPI, *T. ni* basic juvenile-hormone-suppressible protein 1 (L03280); TniBJHSP2. *T. ni* basic juvenile-hormone-suppressible protein 2 (L03281); CfuMtHl, *Choristoneura fumiferana* diapause associated protein 1 (AF007767); CfuMtH2, *C. fumiferana* diapause associated protein 2 (AF007768); SliAry1, *Spodoptera litura* arylphorin (AJ249471); SliNMSP, *S. litura* methionine rich storage protein (AJ249470); SliMMRSP-A, *S. litura* moderately methionine rich storage protein A (AJ249469); SliMMRSP-B, *S. litura* moderately methionine rich storage protein B (AJ249468); DmeLSPla, *Drosophila melanogaster* larval serum protein la (AE003489); DmeLSPlb. D. *melanogaster* LSP-1β (U63556); DmeLSPlg, *D. melanogaster* LSP-1γ (AE003467); DmeLSP2, *D. melanogaster* LSP-2 (X97770); CviLSPI, *Calliphora vicina* arylphorin (M76480); CviLSP2, *C. vicina* larval serum protein 2 (U89789); AgaHexl, *Anopheles gambiae* hexamerin 1 (U51225); AaeHexl, *Aedes aegypti* hexamerin 1γ (U86079). B. Timescale of insect hexamerin evolution. A linearized tree was drawn on the basis of the corrected protein distance data as described in the text. The grey bars are standard errors.

(SP1), 84 kDa (SP2), and 82 kDa (SP3). All three belong to the family of insect hexamerins. They are lipoglycoproteins, synthesized by the larval fat body and released into the haemolymph. SP1 and SP2 show similar developmental profiles in males as well as in females, but SP1 is a little bit more abundant than SP2. SP3 is synthesized only during the last larval instar and the synthesis is inhibited by the application of juvenile hormone (Ismail and Dutta-Gupta, 1988). Hence, this protein belongs to the group of "juvenile hormone suppressible hexamerins" (Memmel et al.. 1994; Hwang el al.. 2001).

In the present study we have focused on SP2 and have identified two cDNA clones (Hex2a and Hex2b) which encode each a polypeptide of 703 amino acid residues. From the high content of aryl groups (17.3% and 17.8%, respectively) and low methionine percentage (1.7%) which also corroborates with amino acid analysis data, we classify these proteins as members of the arylphorin subfamily of the hexamerins. This classification is validated by our phylogenetic analyses.

The two hexamerins Hex2a and Hex2b from *C. cephalonica* group with the lepidopteran hexamerins. Although the sequences are closely related (92.5% identity on the amino acid level), they most likely do not correspond to different allels within the population. Rather they are distinct arylphorin genes that already differentiated about 15 MYA. The genera *Galleria* and *Corcyra,* which both belong to the Pyralidae:Galleriinae, diverged around 54 MYA at the beginning of the Tertiary period. This estimate predates the earliest fossil record of the Pyralidae around 35 MYA (Ross and Jarzembowski, 1993), and may be an overestimate due to deviations of amino acid replacement rates. On the other hand, the fossil record of the lepidoptera in the middle Tertiary or earlier periods is poor, and it is possible that the actual times of divergence are much more ancient than the fossils imply. The other divergence times are in fact in excellent agreement with those published by Burmester et al. (1998) and with the available fossil data (Kukalovà-Peck, 1991; Ross and Jarzembowski, 1993).

A wealth of data has accumulated over recent years that the fat body is the source of hexamerin biosynthesis in lepidoptran and dipteran insects. Our Northern analyses demonstrate that the genes encoding Hex2a and Hex2b are solely transcribed in the fat body cells of C. *cephalonica* larvae. In other tissues of larvae as well as in pupae and adults, we could not detect even traces of Hex2 mRNA. We have previously reported the selective sequestration of SP1 and SP2 by male accessory glands of several lepidopteran insects, including *C. cephalonica,* and their release into the seminal fluid (Ismail and Dutta-Gupta, 1990b, 1991). This suggests the role of SP1 and SP2 in reproduction of lepidopteran insects. In the fall web worm, *Hyphantria cunea,* hexamerin-encoding transcripts were also detected in Malphigian tubules, suggesting an unknown role for the hexamerin in this organ (Hwang et al., 2001). However, this protein belongs to the class of juvenile hormone-suppressible hexamerins.

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AF294808. Corcyra cephaloni...[gi:12006900]

Links

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  TITLE     Complete sequence, expression and evolution of two members of the
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  JOURNAL   Insect Biochem. Mol. Biol. 33 (1), 73-80 (2003)
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  TITLE     Molecular cloning of Corcyra cephalonica hexamerins
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AF294809. Corcyra cephaloni...[gi:12006902] Links

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