Silk genes and their regulation in stored grain pest, *Corcyra cephalonica*

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Department of Animal Sciences

By

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DECLARATION

I, R. K. Chaitanya, hereby declare that this thesis entitled "**Silk genes and their regulation in stored grain pest, *Corcyra cephalonica***" submitted by me under the guidance and supervision of Professor Aparna Dutta Gupta is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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CERTIFICATE

This is to certify that this thesis entitled "**Silk genes and their regulation in stored grain pest, *Corcyra cephalonica***" is a record of bonafide work done by Mr. R. K. Chaitanya, a research scholar for Ph.D. programme in Department of Animal Sciences, University of Hyderabad under my guidance and supervision.

The thesis has not been submitted previously in part or full to this or any other University or Institution for the award of any degree or diploma.

Prof. Aparna Dutta Gupta
(Guide & Supervisor)

(Head of the Department)

(Dean of the School)

This thesis is dedicated to my mother for all the hardships she faced and sacrifices she made to bring me till this stage.

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Chaitanya....✍

Contents

Abbreviations

°C	degree centigrade / celsius
ALP	Alkaline phosphatase
ATP	Adenosine 5` triphosphate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
cDNA	Complementary DNA
DAB	Diamino benzidine
DEPC	Diethyl pyrocarbonate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphates
DTT	1, 4-Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELI	Early-last instar larvae
HEPES	N-(2-hydroxyethyl)piperazine-N`-(2-ethanesulfonic acid)
HRP	HorseRadish Peroxidase
IgG	γ Immunoglobulin
IPTG	Isopropyl β-D-thiogalactoside
JH	Juvenile hormone
kDa	Kilodalton
KLH	Keyhole Limpet Hemocyanin
LB	Luria-Bertani medium
LD	Light:Dark
LLI	Late-last instar larvae
MLI	Mid-last instar larvae
MOPS	3-Morpholinopropanesulfonic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mRNA	Messenger ribonucleic acid
NaCl	Sodium Chloride
NBT	Nitrotetrazolium blue
ng	Nanogram

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pg	Picogram
PGL	Prothoracic gland
PMSF	Phenylmethylsulfonyl fluoride
PP	Prepupa
PTTH	Prothoracicotropic hormone
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SSC	Saline sodium citrate
TAE	Tris-Acetate-EDTA buffer
TBST	Tris buffered saline-Tween20
TE	Tris-EDTA
TEMED	N, N, N`, N`, tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultra Violet
v/v	Volume/volume
w/v	Weight/volume
X-Gal	5-Bromo-4-chloro-3-indolyl β-D-galactoside
µg	Microgram
20E	20-Hydroxyecdysone

INTRODUCTION

1. Insect metamorphosis

a) Evolutionary origin

Metamorphosis in insects is marked by abrupt changes in the form and/or structure during the postembryonic development. Issues of interest in insect metamorphosis include its evolutionary origin, hormonal control, the source of adult structures and the identity of genes that might regulate metamorphosis and code for proteins that form the structures of the different metamorphic stages.

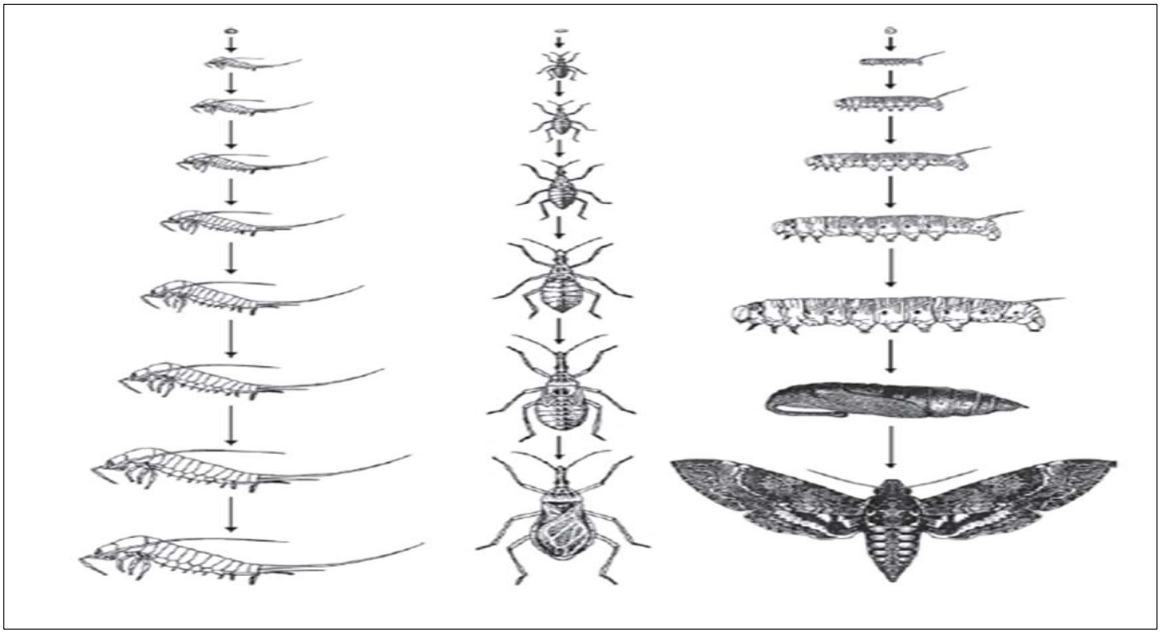

Fig. 1. Metamorphosis. Three types of development in insects. *Left*, ametabolous development illustrated by *Machlis* (Thysanura). *Center*, hemimetabolous development illustrated by *Rhodnius* (Hemiptera). *Right*, holometabolous development illustrated by the moth *Manduca* (Lepidoptera). (Source: from H. E. Evans, Insect Biology, 1984)

Among the orders of insects, pattern of cellular participation in metamorphosis vary significantly. In the simplest case, the adults are only slightly different from larvae. Majority of their structures arise from the larval cells while in the most advanced form, the differentiated larval cells undergo programmed cell death and are replaced by the new

cells arising from undifferentiated precursors commonly known as stem cells (Buszczak and Segraves, 2000). A few "primitive" insects do not undergo metamorphosis (are ametabolous). Thysanura (silverfish), for example, hatch from the egg looking much like the adult, and only undergo subtle anatomical changes between molts. Further, they continue to molt as sexually mature adults. Incomplete metamorphosis (hemimetabolous development) is found in Blattodea (cockroaches), Orthoptera (grasshoppers), Hemiptera (bugs) and others. Here the larval and adult forms resemble each other. After several molts from one larval stage (called instar) to the next, major changes appear at the final molt from larva to adult. These include the development of functional wings, genitalia and internal changes that accompany sexual maturation. At larval stage, these insects have wings, which are small and non-functional and are visible as pads on the thorax. Such insects are called exopterygotes because of their external wings (Truman and Riddiford, 1999). In insects with complete metamorphosis (holometabolous development), there is a dramatic change in appearance, physiology and behavior between larvae and adults. Further, a physiologically and morphologically distinct pupal stage intervenes the final larval instar and the adult stage. Available evidences indicate that there was a common ancestor for all of the holometabolous insects. This group includes the orders, Diptera (flies), Hymenoptera (bees and wasps), Coleoptera (beetles) and Lepidoptera (butterflies and moths). Such holometabolous insects account for about 90 % of all insect species. Their success is presumably due to their ability to exploit radically different environmental resources at different stages in their lives (Wigglesworth, 1939, 1954; Sehnal et al., 1996).

b) Morphology and physiology

The morphological changes in an individual that accompany metamorphosis can be created in one of the two ways (i) a single lineage of cells can form larval, then pupal and

finally adult structures and (ii) change may involve the death of most of the larval cells, followed by their replacement with the proliferation and differentiation of clusters of "stem cells". An example of the first type, persistence of cells throughout the life of an insect, is the abdominal epidermis of lepidopteran larvae. Here a single layer of epithelial cells secrete initially the cuticle of each larval stage, then switches to form the pupal cuticle, and finally, accompanied by special differentiative cell divisions that forms the scales, sockets and background cuticle of the adult (Andersen et al., 1995; Willis, 1996). The second type of change involves groups of cells that contribute little or nothing to larvae, but are used to build the structure of pupa and adult. An example of the second type is seen in holometabolous insect wing development. The cells that contribute to the wings are present as imaginal discs in larvae. Imaginal discs are infoldings of the single layer of cells that constitute the surface epithelium. Once formed, they do not contribute to the external structure of the larva but evaginate to the surface at pupation, forming first pupal and then adult cuticle. Insects with such hidden, undifferentiated larval wing pads are also called endopterygotes, a synonym for holometabolous insects.

In addition to the creation of these morphological structures definitive for the pupa and adult, complex changes also occur in the internal organs in the transition from larva to adult. As the food source changes in many cases from leaves to nectar, changes occur in the mouthparts from cutting and chewing via strong mandibles to sipping via an extensible proboscis. Accompanying these changes in food source are changes in structure and physiology of the digestive system. Different enzymes are needed to handle the altered ingredients. In lepidopterans, the abdominal prolegs and their muscles of larvae, useful in walking, are lost. Flight muscles develop and the nervous system is rewired to accommodate this. Some neurons die; others develop from neuroblasts; while

some others change their outgrowths, (axons and dendrites) to establish connection with new targets (Gilbert et al., 1996; Willis, 1996; Truman, 2005).

c) Hormonal control

There is far less variation in the hormonal control of metamorphosis than in the morphological changes themselves and the cellular events that underlie them. Essentially two classes of hormones (juvenoids and ecdysteroids) are involved, and the same mechanisms of metamorphic controls apply to both hemi- and holo-metabolous insects. Briefly, juvenoids determine what insects molt into, while ecdysteroids make them molt (Sehnal and Mayer, 1968; Nijhout, 1994; Truman and Riddiford, 2002). When juvenoids are absent at a critical period early in a molt cycle, the cells respond to ecdysteroids by forming the structures of the next metamorphic stage instead of making another larval instar (Gilbert et al., 1996; Truman and Riddiford, 2002). In this case, unimpeded by juvenoids, ecdysteroids also cause metamorphosis. Final instar hemipteran or lepidopteran larvae that receive an extra dose of juvenoids by injection or by implantion of active corpora allata (the glands that make juvenoids) molts into a giant extra instar larva rather than undergoing a metamorphic molt (to an adult in the hemipteran insect or to a pupa in the lepidopteran insect), while if a lepidopteran pupa receives excess juvenoids, it will molt into another pupa, something not seen in nature. This action of juvenoids results in their being recognized as "*status quo*" hormones, for this they direct the repetition of the pattern of syntheses necessary to reform the characteristics of the previous stage (Riddiford, 1996; Davey, 2000).

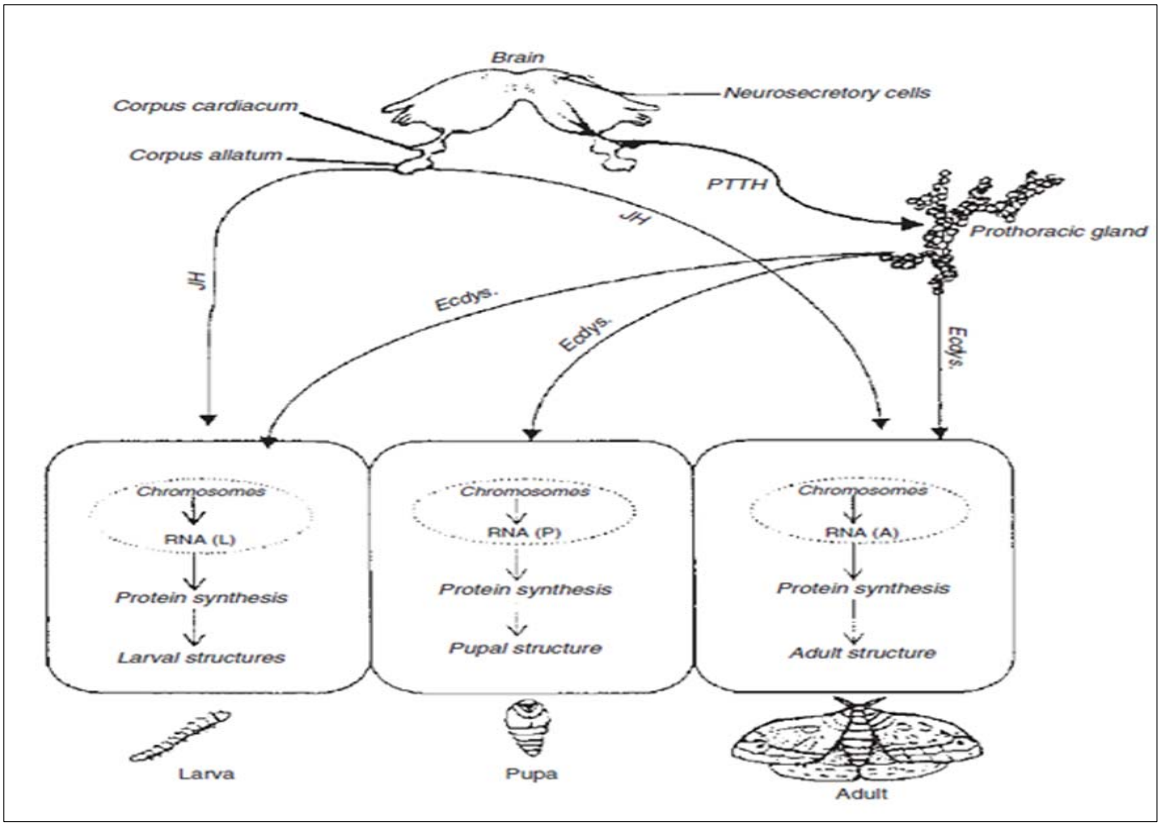

Fig. 2. Schematic diagram of the principal endocrine organs of lepidopteran insects and the regulation of metamorphosis by their hormones. Ecdys. = ecdysteroids; JH = juvenile hormone. (Source: from John L. Capinera, Encyclopedia of Entomology, 2nd edition, 2008)

d) Regulatory and Effector genes

Ecdysteroids initiate a cascade of gene activity that involves both early and late genes. Most of these genes code for transcription factors that activate a series of genes for the proteins that build the structures of the various stages. One such gene, named *broad* was first identified in *Drosophila* (DiBello et al., 1991; Zhou and Riddiford, 2002), where it is expressed in response to ecdysteroids as they initiate the larval-pupal molt, but it is not expressed during larval-larval molts. A gene with a similar sequence and expression pattern was reported in *Manduca sexta* (Zhou and Riddiford, 2002). Also of interest are the genes that code for proteins, which contribute directly to the form and function of the

morphologically distinct metamorphic stages of the holometabolous insects. These can be structural proteins such as cuticular proteins or enzymes that participate in physiological activities such as hydrolysis and intermediary metabolism, or even enzymes necessary for the formation of pigments found at different stages (Andersen et al., 1995; Willis, 1996). It is the complex orchestration of spatial and temporal activities of similar hormones and genes, which results in the vast diversity of form and function that is displayed by metamorphosing insects.

2. Ecdysteroids

a) Brief history

Ecdysteroids are hormones that regulate a wide variety of cellular processes in the life cycle of arthropods including insects. Pulses of ecdysteroids coordinate the complex events of molting and metamorphosis during the postembryonic development, which is essential in the life of the insects. Two of the most common and studied ecdysteroids are ecdysone (α-ecdysone) and 20-hydroxyecdysone (ecdysterone, crustecdysone) (Horn and Bergamasco, 1985). Research on insect hormones began in 1934, when Wigglesworth (1934), in his study on the physiology of ecdysis in the hemipteran *Rhodnius prolixus* described the metamorphosis in the blood sucking bug. One year later, Fraenkel (1935) published a study of the hormone, causing pupation in the blowfly *Calliphora erythrocephala*. From Karlson's autobiography, we learn that, in 1943 he started the work on the isolation of the molting hormone of insects, and it was only two decades later, Butenandt and Karlson (1954) had succeeded in isolating a crystalline form of ecdysone. Later, in the year 1966, the structure of 20-hydroxyecdysone, which is regarded in most arthropods as the predominant active hormone, was sequentially established by Dennis

Horn' s group as crustecdysone and Hoffmeister group as ecdysterone (Hampshire and Horn, 1966; Hoffmeister, 1966). The concept of steroid hormone action on gene expression was first developed by studies on the mode of action of ecdysteroids in Dipteran flies. This was due to the findings of Clever and Karlson (1960), that ecdysteroids can activate transcription in polytene chromosomes of the midge, *Chironomus tentans*. Evidence to support this concept was the visible enlargement of specific regions of polytene chromosome puffs of the target cells (Ashburner et al., 1974; Ashburner and Richards, 1976).

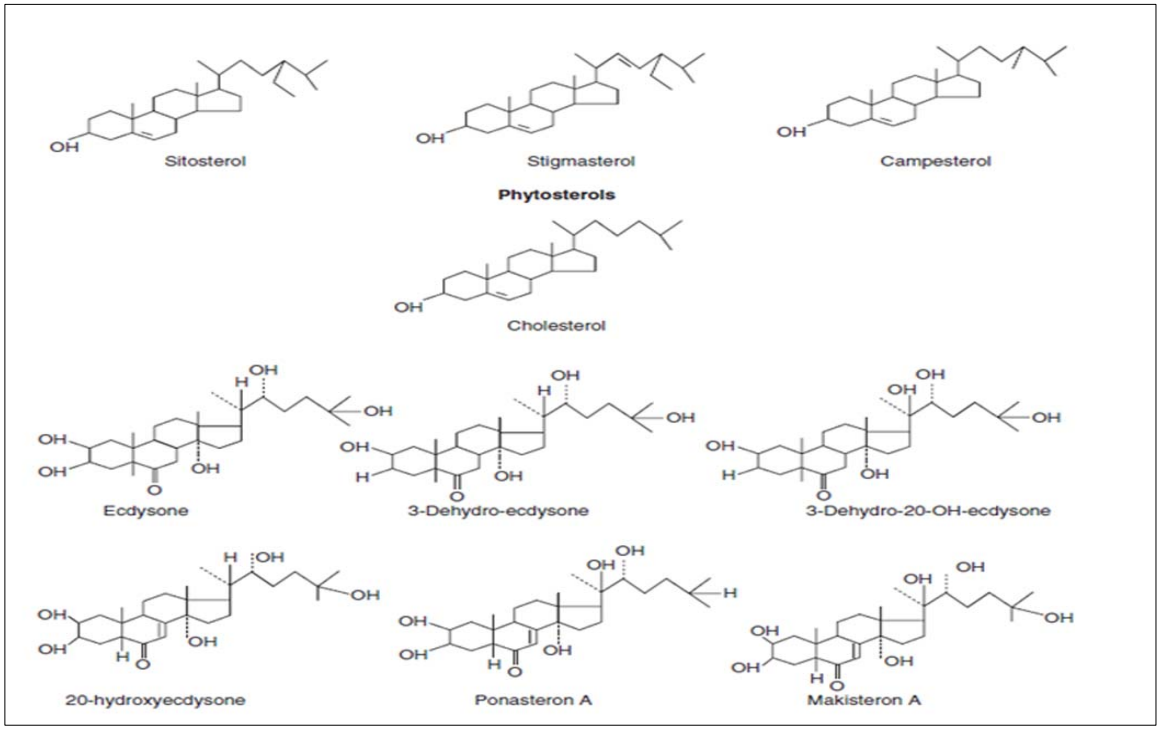

Fig. 3. Ecdysteroids. Structural formulae of some common ecdysteroids of insects which are derived from phytosterols and cholesterol.

b) Origin and site of synthesis

Cholesterol, originating either from the diet or from the conversion of dietary C28 and C29 phytosterol, is the common precursor of ecdysteroids. Insects as well as other arthropods fail to synthesize the *de novo* steroid nucleus, therefore require exogenous or

dietary source of sterol for normal growth. The prothoracic glands, comprising a single steroidogenic cell type in most insects, are the predominant site of ecdysteroids biosynthesis during postembryonic development (Chino et al., 1974, Sakurai and Gilbert, 1990). In higher insects, the prothoracic glands are a part of an organ, the ring gland, which also includes the corpus cardiacum and corpus allatum (Ciancio et al., 1986). In some insects, epidermal, oenocytes and gonadal cells are alternative sites for ecdysteroids production, especially during adult life, at a time when prothoracic glands are no longer present (Swevers and Iatrou, 2003). In female ovaries, the epithelium of the follicle cells produces ecdysteroids, which play a crucial role in the induction of vitellogenesis. During embryogenesis, in the absence of any differentiated site of biosynthesis, many insects are able to elevate their ecdysone titer by metabolizing maternal ecdysteroid conjugates to active hormones (Rees, 1995; Gilbert et al., 1997, 2002). In crustaceans, ecdysteroids are produced by the Y-organ glands, and control a number of processes in the life cycle of the animals, similar to insects (Grieneisen, 1994).

c) Regulation of ecdysteroid synthesis

The regulation of ecdysteroid synthesis is very complex, and under the control of peptide hormones as well as the sesquiterpenoid juvenile hormone (JH). Ecdysteroids are mainly synthesized by the prothoracic glands and released into the hemolymph upon stimulation by the prothoracicotropic hormone (PTTH), a neuropeptide produced by the insect brain. The periodic increase in ecdysteroids' titer critical to insect development, reflect to a large degree of activation of the prothoracic gland by PTTH, which is synthesized as a preprohormone and it is expressed in a number of median neurosecretory cells of the brain. It occurs in multiple forms large and small, some are active others not, depending on the insect species (Agui, 1975; Smith and Sedlmeeir, 1990). Binding of PTTH to the

membrane receptor elevates intracellular Ca^{2+} influx, and the action in turn enhances cAMP formation. As a consequence cAMP-dependent protein kinase is stimulated. The activated kinase stimulates ecdysteroid production. PTTH acts at critical steps in the regulation of ecdysteroids levels in developing insects. The concentration of active ecdysteroids is modified by target cell specific hormone metabolism (Smith and Gilbert, 1989; Smith et al., 2003). The prohormones ecdysone, 3-dehydroecdysone and 3-dehydro-20-hydroxyecdysone, which are secreted into the hemolymph, are metabolized to the more active compound 20-hydroxyecdysone (20E), which is in most arthropods the predominant active hormone (Warren et al., 1988 a, b). Although the ecdysteroids have been characterized from several insects, their biosynthetic pathways and enzymes involved in the hormones biosynthesis are not yet fully elucidated. Also, little is known about the genes involved in the biosynthesis of the hormones.

Further, PTTH is not the sole regulator of ecdysone biosynthesis. JHs exert an indirect tropic effect on the prothoracic glands, which is probably mediated by a stimulatory protein in the hemolymph, possibly a precursor steroid carrier. Also, the developmental state of the prothoracic glands to respond to the regulatory effectors appears to be a key factor in the regulation of ecdysone synthesis. During larval-pupal development, regulatory effectors, hormone interactions, and competence of the prothoracic glands may all be integrated to regulate the ecdysteroid titers (Kiguchi and Riddiford, 1978; Smith and Sedlmeeir, 1990). In crustaceans, ecdysteroid synthesis is regulated by molt-inhibiting hormone (MIH). This neurohormone exerts opposite effects compared to PTTH in insects by inhibiting ecdysteroid synthesis in the Y-organs. MIH also increases cAMP second messenger which activates protein kinases, following alteration of cellular Ca^{2+} levels (Smith and Sedlmeeir, 1990). The contrasting steroidogenic effects of PTTH and MIH

probably arise from differences in the cellular kinase substrates. In insects such substrates enhance ecdysteroid secretion by increasing the translation of granular protein. In crustaceans, MIH stimulates changes leading to the inhibition of both protein synthesis and steroidogenesis (Henrich et al., 1999; Lafont, 2000).

d) Mode of action of ecdysteroids: molecular aspects

Metamorphosis transforms the insect larva into a reproductive adult through a complex series of developmental events involving cell proliferation, differentiation, remodeling of structures for new functions, and finally programmed cell death. These developmental processes are coordinated by pulses of ecdysteroids that include the principle hormone 20E along with JH. However, in some insects' ecdysteroids, which have morphogenetic functions of their own, and also JHs regulate the processes. The ecdysteroids cause molting and also are responsible for the changes in the genetic programs that are necessary for metamorphosis, whereas the presence of JH in the larva prevents these changes from taking place, but does not prevent the molting response. Thus, metamorphosis ensues when ecdysteroids rise in the absence of JH in the final larval instar (Sehnal and Mayer, 1968; Sehnal, 1985; Dubrovsky, 2005). In most insects, exogenous JH at this time causes the formation of a supernumerary larva (Kaduno-Okuda et al., 1986).

The target cells/tissues of ecdysteroids contain proteins which bind to the hormone usually. These binding proteins have high affinity for the hormone. Further, a limited number of binding sites are available per target cell, which is characteristic of steroid hormone receptors. In addition, the binding proteins exhibit a differential affinity for the various ecdysteroids, depending on their biological activity. Determination of the mode of action of ecdysteroids at the genomic level was based on a series of pioneering studies

using *Chironomus* and *Drosophila* which showed that in polytene chromosomes certain stage-dependent puffs could be induced by exogenous ecdysone (Clever and Karlson, 1960; Ashburner and Richards, 1976). This model interaction of the hormone with the hormone-receptor complex recognizes specific DNA response elements, and triggers a cascade of gene activity that directs the molting process. Over one hundred different genes are known to be regulated by ecdysteroids (Cherbas, 1993; Thummel, 2000). Hormonal regulation is gene and tissue specific, and is modified according to the developmental stage of the insect. The general molecular mechanism of steroid hormone action is quite diversified to adapt hormone action according to the physiological needs. Target cell specific hormone metabolism is one way to adapt molting hormone action in a tissue specific manner. Besides molting, ecdysteroids regulate a large number of processes including spermatogenesis, oogenesis, reproduction, embryogenesis, diapause, change in insect color, behavior, metabolism and cell death. Ecdysteroids also regulate the activity as well as concentration of enzymes responsible for the synthesis and degradation of 20E, and thus modulate ecdysteroid titer; regulate the central nervous system sensitivity to eclosion hormone and ecdysis triggering hormone; and the regulation of melanization by the induction of the enzyme dopa decarboxylase (Pipa, 1969; Radford and Misch, 1971, Peronnet et al., 1986; Lanot et al., 1989; Shanavas et al., 1996; Lee and Baehriecke, 2001). The relatively simple ecdysone-inducible genes of salivary gland glue proteins or larval serum proteins-1 of fat body, for example, have a single transcript. However, other ecdysone-inducible genes show a complex pattern of transcription and stretch over regions of 50 kb or more (Cherbas, 1993; Thummel, 2002).

e) Phytoecdysteroids

Analogues of ecdysteroids occur in a variety of plants: pteridophyta, gymnosperms, and angiosperms. The ecdysteroids isolated from plants are ecdysone, 20E, 3-epi-20-

hydroxyecdysone, 20-hydroxyecdysone 22 acetate, ponasterone A and many others of less importance. The physiological relevance of these phytoecdysteroids in the plants, which are identical to the zooecdysteroids, is not thoroughly studied. A high concentration of ecdysteroids may contribute to their protection against arthropod pests by mimicking the natural hormones and disturbing the hormonal balance within these animals. They can also act as antifeedants and antagonize or mimic the action of the ecdysteroid hormones (Dinan, 2001).

f) Genomic action of ecdysteroids

The action of ecdysteroids on target cells, as is the case with steroids in general, can be divided into fast and ephemeral, as well as slow and systemic. The two modes of ecdysteroid action eventually converge and give rise to the integral cellular response. Fast effects are generally traced back to a not yet well defined target on the cell membrane (Wehling, 1997; Nakagawa and Henrich, 2009). On the other hand, the systemic effects involve an intracellular receptor, namely the ecdysteroid receptor (EcR) (Truss and Beato, 1993; Freedman, 1997). The EcR is a member of the nuclear receptor superfamily and exhibits the typical modular structure composed of the N-terminal A/B domain, the DNA-binding C domain, the hinge (D) region, the ligand-binding E domain, and the C-terminal F domain. The ligand-binding domain is multifunctional and includes ligand-dependent dimerization and transactivation functions, while ligand-independent transactivation and dimerization functions are found in the terminal domains as well as in the region spanning the DNA binding domain and the N-terminal region of the hinge, respectively. The EcR heterodimerizes with other members of the nuclear receptor superfamily, noticeably with the ultraspiracle protein (USP), which is an orthologue of the vertebrate retinoic acid X receptor (RXR). The EcR/USP heterodimers bind to the ecdysteroid response elements

(ERE) present in the promoter regions of ecdysteroid responsive genes and regulate their transcription (Oro et al., 1990; Koelle et al., 1991; Yao et al., 1992; Antonieweski et al., 1993; Yao et al., 1993; Mangelsdorf et al., 1995). Most of the nuclear hormone receptors, including EcR, function as ligand-controlled transcription factors, a characteristic that renders these receptors or their key regions (i.e., the ligand- and DNA-binding domains) especially suitable as constituents of gene switches (Allgood and Eastman, 1997). Several nuclear receptors, including glucocorticoid receptor (GR), progesterone receptor (PR), estrogen receptor (ER), and EcR, are being used to develop gene switches for applications in medicine and agriculture. Since the EcR and its ligands are not found in vertebrates, they are attractive targets for the development of gene switches to be used in mammalian system (Allgood and Eastman, 1997).

3. Juvenile hormones

a) Brief history

Evidence for the existence of a blood-borne factor that influenced or modified the molting process was provided for the first time by Wigglesworth in the 1930s. His innovative experiments on the bloodsucking bug, *Rhodnius prolixus*, demonstrated that corpora allata (CA) which are small (usually) paired glands found in close association with the corpora cardiaca, posterior to the insect brain were the source of this factor, which was later termed "juvenile hormone" (JH) due to its *status quo* action on the molt (Wigglesworth, 1934, 1936). It took a fairly long time before the chemical structure of a natural juvenile hormone (JHI) was successfully determined by Roller et al., (1967).

b) Forms and synthesis

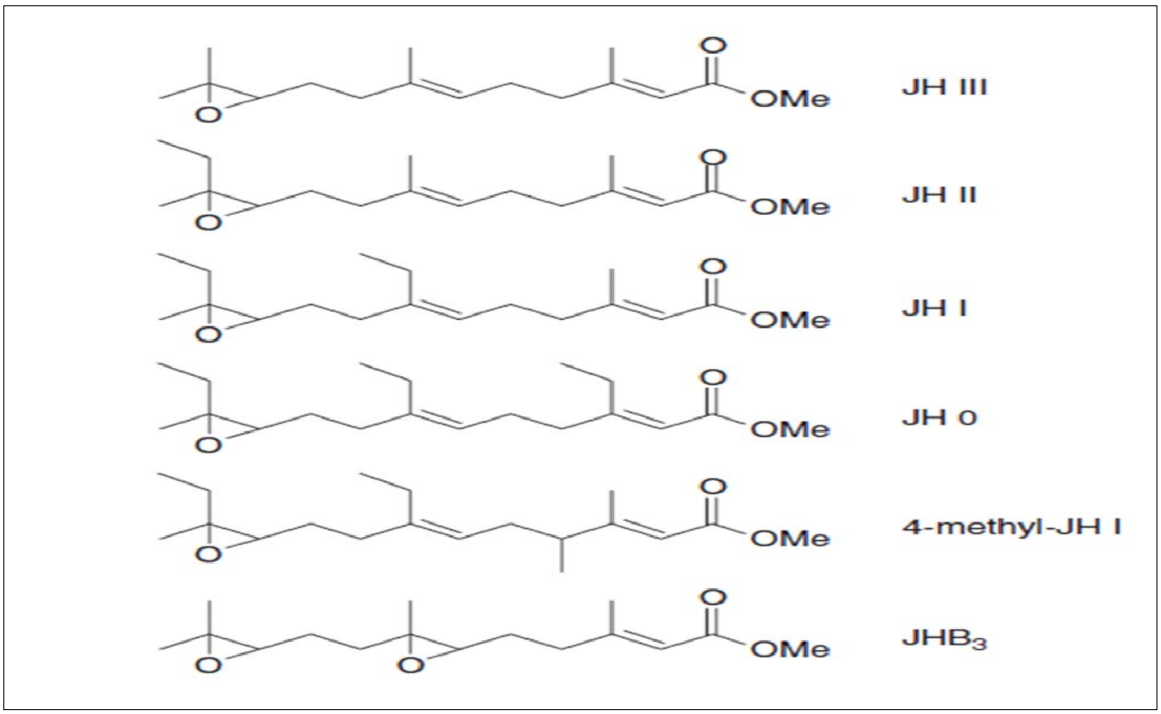

Fig. 4. Juvenile hormone. Chemical structure of the six known juvenile hormones; Me, Methyl group.

Juvenile hormones make up a group of lipophilic sesquiterpenes produced by the CA and released into the hemolymph. Six different forms of JHs have been identified so far from insect tissues and hemolymph. All known JHs are methyl esters of epoxyfarnesoic acid or of one of its homologs i.e., with one or several methyl and/or ethyl side chains. The simplest and most ubiquitous one is JHIII found in the majority of insects (Meyer et al., 1970; Judy et al., 1973). In the Lepidoptera, however, five of them have been described, including JH III and the homologous JH 0, JH I, JH II, and 4-methyl-JH I. In addition, the CA of cyclorrhaphous Diptera secretes both JH III and a bis-epoxide form of this hormone (JHB3) (Richard et al., 1989 a, b). Biosynthesis of juvenile hormone proceeds through the mevalonate pathway that uses acetyl-CoA as the principal building block. In the case of JHs with ethyl side chains, one unit of propionyl-CoA, derived from valine and isoleucine, substitutes for one of the three acetyl-CoA units that make up mevalonate,

thus leading to the formation of homomevalonate. Condensation of three isoprene units by farnesyl diphosphate synthase results in the formation of the JH backbone (i.e., farnesyl diphosphate or one of its homologs). This diphosphate is then sequentially converted to an alcohol then to an acid, followed by the formation of a methyl ester and epoxidation (Schooley and Baker, 1985).

c) Functions of JH

One of the most intensively studied functions of JH is its juvenilizing effect during larval development, whereby high levels of the hormone during larval molts modify the type of cuticle secreted at a molt, thus maintaining the insect in a juvenile state. Consequently, the JH titer is maintained at a relatively high level during most of the larval period until the final instar, at which time it drops precipitously, allowing the subsequently released ecdysteroids to induce the larval-pupal or nymphal-adult transformation. With the exception of a rise in its levels during the prepupal stage (at least in some Lepidoptera), the JH titer usually remains low until adult emergence, when the CA are reactivated again (Tobe and Feyereisen, 1983; Riddiford, 1994). In adult females of most insects, JH becomes a reproductive hormone and induces vitellogenin synthesis in the fat body as well as its uptake by the developing oocytes, making it an absolute requirement for egg maturation. In these insects, JH titers fluctuate as a function of the various phases of the ovarian cycle. Sex pheromone production and sexual receptivity is often coordinated with the ovarian cycle and are regulated by JH in insects (Bownes and Blair 1986; Bownes, 1989; Belgacem and Martin, 2002). In males, JH was shown to regulate the production of accessory sex gland secretions and control courtship behavior. Other processes that JH has been shown to govern in some adults include migration, polyphenism, caste

differentiation and reproductive diapause (Shemshedini and Wilson, 1990; Tompkins, 1990; Herndon et al., 1997; Pursley et al., 2000; Wilson et al., 2003).

d) Regulation of JH synthesis

The principal factors regulating the levels of JH in insect hemolymph are the rate of hormone synthesis by the CA and its degradation in the hemolymph and tissues. These glands do not store JH, but they release the hormone into the blood as soon as it is synthesized. The biosynthetic activity of the CA is controlled chiefly by neuropeptides that either stimulate (allatotropins), or inhibit (allatostatins, allatinhibins) JH production. These neuropeptides are produced by neurosecretory cells and are delivered to the CA via neural connections to the brain (Richard et al., 1990; Rachinsky and Tobe, 1996; Lenz et al., 2000). The biosynthesis of JH is modulated by factors released in the hemolymph by organs or tissues that are anatomically distant from the CA, e.g., ovary, neurosecretory cells in the ventral nerve cord, etc. (Gilbert et al., 1996). Catabolism significantly affects the hemolymph titer of JHs during various stages of insect development. In this respect, JH esterase (JHE) is instrumental in the critical lowering of juvenile hormone titers during the final instar of lepidopteran insects. The same enzyme also plays an important role in JH titer regulation following mating in adult females of certain lepidopteran species (Roe and Venkatesh, 1990; Campbell et al., 1992, 1998).

Within tissues, the degradation of JH is mediated by an epoxide hydrolase (JHEH). Experimental evidence suggests that JH modulates gene transcription in insects, e.g., induction: transcription of the JHE gene; suppression: transcription of the *Broad Complex* gene, implying that it most likely binds to an intracellular receptor in a fashion similar to that described for steroid hormones, including ecdysone (Palli et al., 1990; Casas et al.,

1991; Feng et al., 1999; Zhou and Riddiford, 2002). To date, only few cytosolic or nuclear JH binding proteins have been isolated, and their role as a JH receptor has either been questioned or remains unconfirmed (Riddiford, 2008). Some of the effects attributed to JH, including the induction of vitellogenin uptake by developing oocytes through the hormone-dependent formation of openings between follicular cells (patency), appears to be mediated by a cell-surface receptor, in a manner similar to that described for peptide hormones (Zhu et al., 2003).

4. Insect growth regulators

This group is comprised of a diverse group of chemical compounds with varying molecular structures. However, they are grouped together here because they all affect insect growth in some manner. These chemicals are comparatively new tools used in insect control. Interestingly, these chemicals illustrate the evolution of insect control methods, which are becoming target specific have fairly reduce environmental impact and improve efficacy.

a) Ecdysteroid analogs

Over the decades several attempts have been made to use ecdysteroid analogs for insect control (Smagghe and Degheele, 1998). The most dramatic discovery occurred in the early 1980s, while chemists at Rohm and Haas Company synthesized 1, 2-diacyl-1-substituted hydrazines, which had potent insecticidal activity. The diacylhydrazines are a novel class of chemically and mechanistically new insect control agents (Wing et al., 1988). The first member of this group was RH-5849 (N-tert-butyl-N'-benzohydrazide) that had interesting foliar and root-systematic insecticide activities against a range of lepidopteran,

coleopteran and dipteran pests. More recently, another potent commercial analog, tebufenozide [N-tert-butyl-N'-(4-ethylbenzoyl)-3, 5-dimethylbenzo-hydrazide; RH-5992] has been introduced as selective foliar caterpillar control agent (Carlson, 2000). In addition, halofenozide [N-tert-butyl-N'-(4-chlorobenzoyl) benzohydrazide; RH-0345] that is a more effective soil insecticide for white grub and caterpillar control in turf (Dhadialla, 1998) and methoxyfenozide [N-tert-butyl-N'-(3-methoxy-o-toluoyl)-3, 5-xylohydrazide; RH-2485] which exhibits high insecticidal efficacy and selectivity against insects of various families of Lepidoptera including Pyralidae, Pieridae, Tortricidae and Noctuidae, has also been released (Carlson et al., 2001).

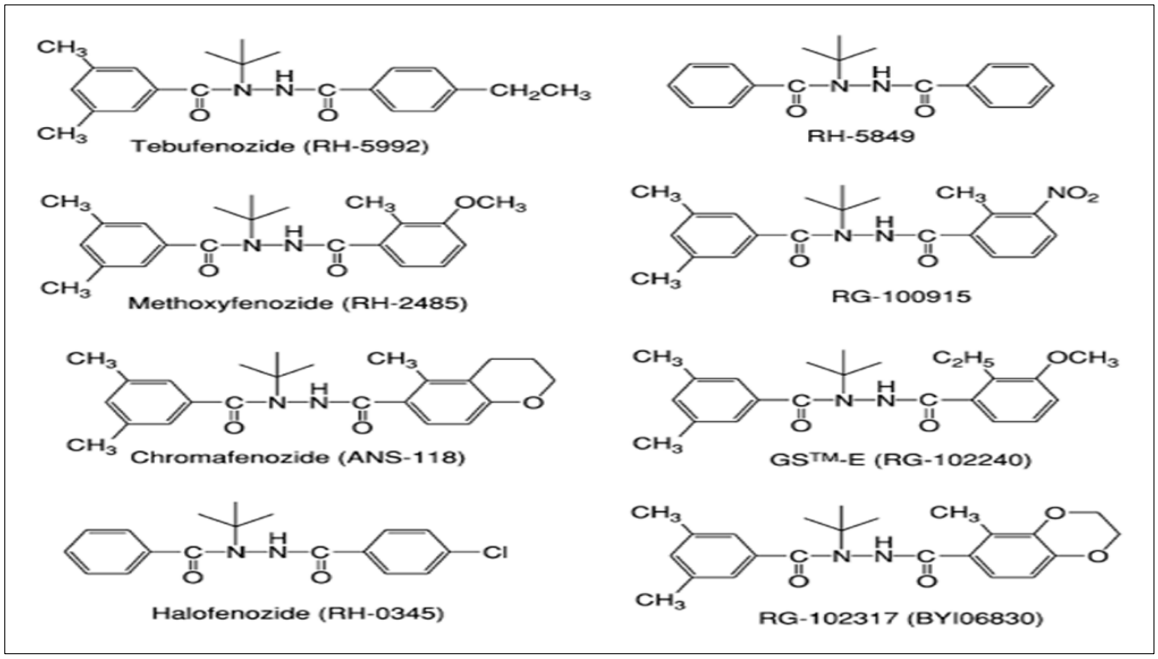

Fig. 5. Structures of diacylhydrazine congeners

The dibenzoylhydrazines can be readily synthesized from tert-butylhydrazine hydrochloride and the corresponding substituent benzoyl chloride using Schotten-Bauman conditions. The presence of the bulky tert-butyl group on the hydrazine allows the acid chlorides to be reacted in a sequential and highly region-specific manner (Hsu, 1991;

Nakagawa et al., 1998). The dibenzoylhydrazines, although not steroids, mimic actions of the insect molting hormone, (20E) by binding directly to the binding sites and act as full agonist at that site. As a consequence, treated larvae express all the classic symptoms of an untimely and severe overdose with 20E, called hyperecdysonism. Treatment also induces premature apolysis, which is the primary mode of action, and larvae stop feeding. Within 3-12 h after uptake, molting is initiated, and by 24 h, intoxicated larvae prematurely slip their old head capsules in an attempt to ecdyse. However, normal successful ecdysis is inhibited. In addition, abnormal cuticle deposition and other molting irregularities are seen, such as a lack of sclerotization and tanning of the new cuticle, absence or conspicuous low number of endocuticular lamellae, hindgut extrusion and loss of hemolymph and molting fluid that result in desiccation and ultimate death (Dhadialla et al., 1998).

The dibenzoylhydrazines manifest these typical effects via interaction with the ecdysteroid receptor complex that consists of a heterodimer of two steroid receptor superfamily members, i.e., EcR and the Ultraspiracle proteins. This hormone-receptor complex binds then to the ecdysone responsive element on the DNA. These chemicals permit the expression of genes and behavioral events that are dependent upon the presence of 20E (Dhadialla et al., 1998, Palli and Retnakaran, 2001). In adults, the dibenzoylhydrazines cause a reduction of egg production and fertility in various target insects belonging to orders Lepidoptera, Coleoptera and Diptera (Trisyono and Chippendale, 1997, 1998). However, the exact mechanism of action is not yet known.

The dibenzoylhydrazines were tested against larvae and adults from different insect orders, and the toxicity was most pronounced when exposure and uptake occured via

ingestion. RH 5849, Tebufenozide and methoxyfenozide exhibit high selective toxicity against Lepidoptera. Further, these compounds are marketed around the world for control of important agricultural caterpillar pests in cotton, vegetables, top fruit, grapes, ornamentals, forestry and rice. The insect pests which are being targeted are beet armyworm, cabbage looper, codling moth, *Cydia pomonella*, *Spodoptera exigua*, grape berry moth, *Lobesia botrana*, spruce budworm, *Choristoneura fumiferana*, rice leafroller, *Cnaphalocricis medinalis*, and others (Darvas et al., 1992).

Tebufenozide and methoxyfenozide show very little or no effect on a panel of non-lepidopteran pests (Coleoptera, Hemiptera, mites and nematodes). Likewise, laboratory and field experiments showed that there are little or no adverse effects at normal rates on a wide range of non-lepidopteran beneficial insects such as honeybees and bumblebees (Hymenoptera), many predatory insects (Hemiptera, Coleoptera, Neuroptera, Odonata, Plecoptera, Trichoptera and Ephemeroptera) and mites (Acarina), several caterpillar endoparasitoids (Hymenoptera) and certain insect predators such as spiders (Arachnida). These compounds appear to be extremely specific for lepidopteran caterpillars, compatible for integrated pest management (IPM). Further, these compounds are found to be safe towards several non arthropod invertebrates such as earthworms and nematodes, and have a low toxicity for a representative crustacean (*Daphnia magna*) (Kunkel et al., 1999). The vertebrates, several representative mammals, bird and fish species (rat, quail and trout) show very little susceptibility to methoxyfenozide and tebufenozide. Extensive tests showed that these compounds are non-irritating (rabbit), non-sensitizing (guinea pig), non-mutagenic and non-oncogenic (mouse, rat and dog). In retrospect, this relative lack of vertebrate toxicity is anticipated, since such organisms do not synthesize/express and/or utilize 20E, the ecdysone receptor complex (EcR and USP) or any other closely

homologous molecules (Smagghe and Degheele, 1994; Song et al., 1997; Dhadialla et al., 1998; Kunkel et al., 1999).

b) Juvenile hormone analogs

The disruption of JH functions in insects has long been viewed as a promising strategy for the development of environmentally safe, insect-specific pest control products. Williams (1967) was the first to suggest the use of JH or JH-like for controlling insect pests, reasoning that an artificial increase in the levels of JH at times when such levels are normally low would result in a fatal disruption of insect development. Various compounds, collectively referred to as JH analogs (JHAs) or "juvenoids" have, indeed, been developed for this purpose, and a few have enjoyed commercial success for the control of insects whose pest status is restricted to the adult stage (e.g., mosquitoes) (Retnakaran et al., 1985). Compounds that mimic the insect JH include methoprene and Kinoprene (Harding, 1979). These juvenoids keep the insect in its immature form which prevents the production of future generations and often results in death from conflicting hormonal messages during ecdysis to the adult stage. Fenoxycarb, a carbamic acid derivative without anticholinesterase activity (Grenier and Grenier, 1993) and pyriproxyfen (Langley et al., 1990) also are juvenoids, however, unlike methoprene and kinoprene they do not resemble the structure of JH. All of these compounds act as JH agonists (mimics). These insecticides, however, do not usually provide adequate control of crop and forest pests that feed as larvae, because death usually is not immediate and larvae continue to feed and cause damage, dying only near the end of larval life or at metamorphosis (Retnakaran, 1973 a, b). For this reason, current research efforts are aimed at finding ways of artificially lowering JH titers, or antagonizing JH function at the receptor level; if

successful, such strategies would result in the precocious (and fatal) induction of metamorphosis in larval forms and in the inhibition of reproduction in the adult.

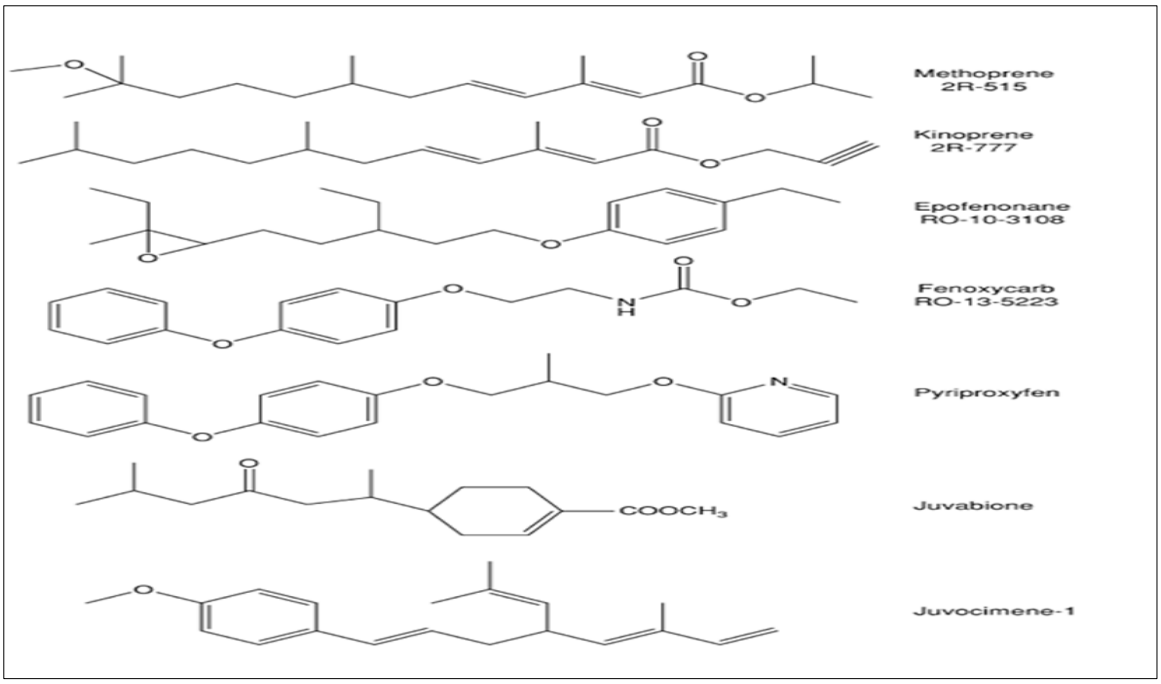

Fig. 6. Juvenile hormone analogs

5. Insect silks

Silks are ectodermal secretions that are stored as a hydrated jelly within cells or, more often, in multicellular cavities; the gels polymerize into water-insoluble filaments during passage to the external environment. Materials that meet this definition occur in all terrestrial arthropod subphyla: Chelicerata (in three orders of Arachnida, i.e., spidermites, pseudoscorpions and spiders), Myriapoda (in Symphyla, Pauropoda and primitive millipedes, absent in the centipedes) and Hexapoda. Insect silks are produced by a variety of dermal glands, Malpighian tubules, colleterial sex glands, the gut and the labial glands. The latter typically produce saliva but half of each gland in the Psocoptera and the entire glands in the larvae of many Holometabolous insects, are specialized for the production of silk. Labial silk glands develop during embryogenesis as ectodermal tubular

invaginations composed of several tens to a few hundred cells. They grow by polyploidization, extend into the abdomen and often reach a large size, second only to the gut and the fat body (Sehnal and Akai, 1990; Craig, 1994).

Spinning from the labial glands of larvae probably evolved in the ancestor of the Lepidoptera and the Trichoptera, more than 250 million years ago. Caterpillars of some species spin virtually continuously and live in silky tubes or domiciles; other species produce just a small pad or a girdle that provides support during moulting. Many species spin durable cocoons in which the larvae pupate. Silk composition has been analysed in some detail in the suprafamilies Yponomeutoidea, Pyraloidea and Bombycoidea. The conclusions are probably valid for the entire clade Ditrysia that harbours 98% of about 160,000 described lepidopteran species (Kristensen et al., 2007). The silk fiber of Ditrysia is a highly organized structure composed of several proteins that are derived from the posterior and middle silk gland sections (PSG and MSG, respectively). The fiber consists of two filaments (one from each gland) that are polymers of a large protein called heavy chain fibroin (H-fibroin, 350-500 kDa in different species) (Inoue et al., 2000; Hwang et al., 2001; Zurovec and Sehnal, 2002; Yonemura et al., 2006). It is produced in the PSG along with two other proteins, light chain fibroin (L-fibroin, 25 kDa) and P25 glycoprotein (also called fibrohexamerin) that occurs in two forms due to various levels of glycosylation (27-31 kDa) (Tanaka et al., 1993; Zurovec et al., 1998 a; Tanaka and Mizuno, 2001). These three proteins are assembled into elementary units in the endoplasmic reticulum (Inoue et al., 2003), form microfibers in the Golgi vacuoles and are released as secretory granules into the gland lumen (Akai et al., 1993), where they accumulate as a highly concentrated gel. The gel moves into the MSG and becomes enveloped by several layers of sericins. Major sericins of the domestic silkworm, *Bombyx mori*, include a 150 kDa protein

produced in the most distal part of MSG, a 400 kDa protein from the central and a 250 kDa protein from the proximal MSG region (Takasu et al., 2002). Silk proteins polymerize into a fiber during spinning when they pass through the anterior silk gland section and the spinneret. Filament polymerization is based on cross-linking of aligned and closely packed H-fibroin molecules. This process apparently requires the withdrawal of water and application of a shearing force. Sericins polymerize with a delay: inner sericins (from the distal MSG) seal the filament doublet into a single fiber and outer sericins glue the fibers to substrates or to one another during cocoon construction. To release the fiber, cocoons are soaked in hot and slightly alkaline water that dissolves the outer sericin layer. Several fibers with a sticky surface are reeled together into a raw silk thread (Jin and Kaplan, 2003). Measurements of the physical properties and structural silk analyses by X-ray diffraction and other techniques are typically done on fibers liberated from the cocoons in hot water bath; it is assumed that the established values characterize the filaments, i.e., primarily the H-chain fibroin. Sericins and additional, minor silk components are not considered (Zurovec et al., 1998 b; Nirmala et al., 2001).

At least 6 and as many as 15 sericin-type proteins have been extracted from the silk of *B. mori* (Sprague 1975; Gamo et al., 1977). Some of them have identical peptides but differ in the degree of glycosylation. It was suggested that *B. mori* harbors upto five sericin genes but identified only *ser1* and *ser2* (Grzelak, 1995). The *ser1* gene of 9 exons is expressed in the distal and central MSG and yields 4-5 mRNAs by differential splicing (Hamada et al., 1987; Michaille et al., 1989). A large central exon encodes 60 copies of a 38 amino acid repeat (Garel et al., 1997). Recombinant proteins based on a common version of this repeat (SRTSGGTSTYGYSSSHRGGSVSSTGSSSNTDSSTKNAG) were shown to self-assemble into β-sheets and crystallites (Michaille et al., 1990). Based on this observation,

filament sealing into a fiber may be attributed to hydrogen bonding between serine residues of the H-fibroin and serine residues of the inner-layer sericins. Genes similar to *ser1* and *ser2* in the overall structure and the splicing patterns, but different in repetitive sequences, are also found in the wax moth *Galleria mellonella* of Pyraloidea (Zurovec et al., 1992). However, sericins have not been sufficiently examined in any other species.

The investigations on *B. mori* showed that H-fibroin, L-fibroin and P25 assemble in the endoplasmic reticulum in a ratio of 6:6:1 into elementary secretory units (Inoue et al., 2000). H-fibroin interacts with the two other proteins by its nonrepetitive N- and C-termini. These terminal sequences are conserved across Lepidoptera and Trichoptera. The N-terminus harbors an amphiphilic proline-flanked region of 35 residues and the C-terminal with 26-27 residues include three Cys (only one in the Saturniidae family) in conserved positions. The redox state of the endoplasmic reticulum (Hwang et al., 1992) favors formation of disulphide bonds: four internal in P25, one within L-fibroin, one within H-fibroin and one linking Cys170 of the L-fibroin to Cys-22 in the H-fibroin C-terminus (Tanaka et al., 1999 a). Disulfide linkage between L-fibroin and H-fibroin is indispensable for the secretion of both proteins (Takei et al., 1987). Non-covalent interaction of P25 with the H-fibroin N-terminus facilitates transport and secretion of the highly insoluble H-fibroin/L-fibroin heterodimers (Tanaka et al., 1999 b). At the same time, L-fibroin plays a protective role in P25 glycosylation-altered deglycosylation causes disintegration of the elementary secretory unit (Inoue et al., 2000). Studies revealed that only a portion of the 30 kDa P25 and none of the less glycosylated 27 kDa form of P25 is present in the elementary secretory units (Inoue et al., 2003), but both forms occur in the spun-out silk. It is therefore not excluded that filament formation is associated with a partial deglycosylation or other changes in P25. The conservation of L-fibroin, P25 and

the non repetitive H-fibroin ends in the basal superfamily Yponomeutoidea, suggests strongly that their interaction represents a plesiomorphic mechanism of the silk secretion and gel/filament conversion in Ditrysia. It is proposed that silk filament construction from the three protein types is fairly conserved because the hydrophobic nature of H-fibroin requires association with the L-fibroin and P25 (Sehnal and Zurovec, 2004). However, the H-fibroin of *Yponomeuta evonymella* is amphiphilic (Yonemura and Sehnal, 2006). A different mechanism of the gel/filament conversion evolved in the family Saturniidae (Bombycoidea) that possesses relatively small H-fibroins (200-250 kDa) with alternating hydrophilic and hydrophobic motifs in their repeats (Sezutsu and Yukuhiro, 2000; Hwang et al., 2001). This amphiphilicity probably allows gel formation and the gel/filament transition without the L-fibroin and the P25 that have been lost in this family (Tamura et al., 1987; Tanaka and Mizuno, 2001). *Bombyx* and *Antheraea* represent families Bombycidae and Saturniidae that are characterized by low silk production in early larval instars and high production for the construction of extensive cocoons after the termination of feeding. During cocoon construction, up to 20% of the body biomass is converted to silk.

The silk gland morphology and the silk composition in Trichoptera are similar to those of Lepidoptera. Histological evidence demonstrates the presence of sericin-like proteins, but none have yet been characterized (Engster et al., 1976). PSG proteins of the silk filament have been analysed in all three trichopteran suborders and found to be similar, in spite of differences in the silk use. The larvae of *Hydropsyche angustipennis* (suborder Annulipalpia, family Hydropsychidae) spin catching nets and hiding tubes and, at the end of larval development, construct small domes from the sand grains. The larvae of *Limnephilus decipiens* (Integripalpia, Limnephilidae) use silk to stick together pieces of plants and other materials (depending on the species) into protective cases; at the end of

larval development they close the cases and pad them with silk lining. The larvae of *Rhyacophila obliterata* (Spicipalpia, Rhyacophilidae) use silk only before pupation to spin parchment-like cocoons. The silk of all three above mentioned species contains homologues of lepidopteran H-fibroin and L-fibroin but not of P25 (Yonemura et al., 2006). Terminal regions of the H-fibroins and the entire L-fibroin sequences contain conserved spacing of residues with specific properties (hydrophobicity, charge), including the cysteines that link H-fibroin and L-fibroin in the silkworm silk. The structures of both the *H-fibroin* and *L-fibroin* genes are also similar to those of lepidoptera, suggesting that the H-fibroin/L-fibroin interaction evolved in the ancestor of trichoptera and lepidoptera. P25 does not occur in trichoptera but it is uncertain whether it has been secondarily lost in this order or if it represents an innovation specific to Lepidoptera-Ditrysia. Since the silk produced by caddishflies persists in water, one would expect H-fibroins with hydrophobic repeats, but those identified so far are amphiphilic (Engster et al., 1976). The high content of hydrophilic residues, both neutral and charged, probably facilitates the secretion and storage of the covalently linked L-fibroin/H-fibroin dimer in the absence of P25. However, the mechanism which stabilizes the spun-out silk in water remains to be discovered.

Rationale of the present study

A wealth of information is available on principal insect hormones and their wide array of functions. Besides molting, ecdysteroids regulate a large number of processes including spermatogenesis, oogenesis, reproduction, embryogenesis, diapause, change in insect color, behavior, metabolism and cell death. Juvenile hormone has been shown to govern insect molting, metamorphosis, vitellogenesis, sex pheromone production, sexual receptivity, polyphenism, caste differentiation and reproductive diapause. However, the role played by these hormones in insect silk gland regulation is scarcely understood.

Insect silks are secretions of labial glands. The silk core in insects is generally constituted of a complex of three proteins namely, H-chain fibroin (H-fibroin), L-chain fibroin (L-fibroin) and P25. Disulfide linkage between L-fibroin and H-fibroin is indispensable for the secretion of both proteins. Non-covalent interaction of P25 with the H-fibroin N-terminus facilitates transport and secretion of the highly insoluble H-fibroin/L-fibroin heterodimers. At the same time, L-fibroin plays a protective role in P25 glycosylation. Saturniid silks possess relatively small H-fibroins (200-250 kDa) with alternating hydrophilic and hydrophobic motifs in their repeats, without the L-fibroin and the P25 that have been lost in this family. P25 does not occur in Trichoptera but it is uncertain whether it has been secondarily lost in this order or if it represents an innovation specific to Lepidoptera. Hence, it's necessary to examine L-fibroin and P25 in many other insect species for better understanding. Also, a detailed study of hormonal regulation of these silk genes would be vital in unraveling the significance of cocoon formation associated with pupae of holometabolous insects.

The mechanism of silk secretion, silk composition and the silk structure has been examined most systematically in Lepidoptera, being stimulated by the interests of commercial sericulture. Commercial silks are products of the domestic silkworm *Bombyx mori*, several silkmoth species (most of them from the genus *Antheraea*) and a few other moths whose larvae spin large and closed cocoons. However, there are no studies on silk produced by insect pests so far. Insects can be conveniently divided into three groups i.e., harmless, injurious and beneficial. The injurious insects referred as pests annually destroy between 6-30% of agricultural harvest in developing countries (USDA-Agricultural Research Service Information Bulletin, 1995). These losses become even more significant for stored cereal products than pre-harvest losses because post-harvest costs are much higher than the cost of production. A wide range of lepidopteran pests cause damage and constitute a major factor that reduce the agricultural harvest globally including India. Food grains are being spoiled after harvest due to lack of sufficient storage and processing facilities. The annual loss in stored product has been given as 10 percent of all stored grain - about 13 million tons of grain loss due to insects alone (Singh and Satapathy, 2003). Economic losses include actual consumption of product by pests; product quality degradation through biochemical changes, product contamination with toxins, webbing, cast skins, hairs or feces, and the cost of control (Gorham, 1991). Management of stored product pest through fumigation, chemical pesticides and plant-based deterrents saves only a portion of the product from damage (Page and Lubatti, 1963, Lemon, 1967). The control of stored grain pest by using synthetic chemicals has resulted in toxicity even to non-target organisms. Safe and efficient stored product pest management is essential to protect the grains from infestation by insects (Kiruba et al., 2006). Therefore, increased emphasis on developing safer, more effective and eco-friendly methods through modern biotechnology advances are required to control crop loss.

For the present investigation, *Corcyra cephalonica* was chosen as the experimental insect. It is a lepidopteran pest of stored cereals such as wheat, rice, sorghum, maize, millet, etc. in tropical and sub tropical region of the world. In the current study, we cloned and characterized the silk genes from *C. cephalonica* and made an attempt to study their hormonal regulation during the last intar larval development as the cocoon formation is initiated soon after this stage. Insect growth regulators such as RH-5849 and RH-5992 (20E analogs) and methoprene (JH analog) that interfere with the molting and metamorphosis are in use currently. These are selectively toxic to lepidopteran pests and environmentally benign. Hence, an understanding of the mechanism of hormone regulation of silk genes of *C. cephalonica* through the present work would predictably provide an insight to efficiently target the larval forms of this insect other such lepidopteran silk producing insect pests.

Specific objectives

- **Cloning, expression, characterization and developmental regulation of H-fibroin, L-fibroin and P25 genes from *Corcyra cephalonica***

- **Effect of hormones (20E and JH analog, methoprene) on H-fibroin, L-fibroin and P25 gene expression during last-instar larval development**

- **20E regulation of H-fibroin gene, its role in insect development and a probable target for control**

MATERIALS & METHODS

1. Chemicals

Anti-His, ALP-, HRP-, Biotin-conjugated antibodies, DAB development system, IgG purification kit were procured from Bangalore GENEI. KLH-conjugated polyalanine peptide was commercially synthesized from USV Limited Custom Peptide Synthesis service. GeneRacer^{TM} kit for rapid amplification of cDNA ends, Superscript III® first strand synthesis system for RT PCR, EcR-293 cells was from Invitrogen. InsTAclone^{TM} PCR cloning kit, DNA ligation kit, Hexalabel^{TM} DNA labeling kit, PCR master mix and all DNA, RNA and protein markers were obtained from Fermentas, Life Sciences. QIAprep® spin miniprep kit for plasmid isolation and QIAquick® gel extraction kit were purchased from QIAGEN. Genomewalker^{TM} universal kit was from Clonetech. Dual luciferase reporter system was bought from Promega. Enzyme immunoassays were performed using Enzyme immunoassay kit from Cayman Chemicals. Supersignal® west-pico chemiluminiscent substrate was obtained from Pierce protein research products. Hybond^{+} nylon and nitrocellulose membranes were purchased from Amersham Biosciences. SYBR Green master mix and other real-time PCR reagents were obtained from Applied Biosystems. DMEM medium, FBS, Bleocin, Geneticin were purchased from GIBCO-BRL. TRI Reagent, DEPC, MOPS, 20-hydroxyecdysone, methoprene, TC-100 medium, RH-5849, RH-5992, DTT, Freund's complete and incomplete adjuvants, Agarose, HEPES, TEMED and protease inhibitors were procured from Sigma-Aldrich Chemicals. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and the chemicals required for bacterial culture are from HIMEDIA chemicals. [γ^{32}P] ATP and [α^{32}P] dATP were purchased from Board of Radiation Isotope and Technology (BRIT), India. PET 28a expression vector was from Novagen. All other chemicals used were of analytical grade and were obtained from local sources in India.

2. Experimental insects

a) *Corcyra cephalonica* (Stainton)

Corcyra cephalonica is commonly known as rice moth and belongs to the order Lepidoptera and family Galleridae. It is a serious lepidopteran pest of stored cereals such as wheat, rice, sorghum, maize, millet, etc. in tropical and sub tropical region of the world. The larval forms were reared in culture troughs that contained coarsely crushed sorghum seeds. The cultures were maintained at 26±1º C, 60±5 % relative humidity (RH) and 14:10 h light: dark (LD) photoperiod. The larval development proceeds through five instars and is completed in about 45-50 days. The final (5^{th}) larval instar was further classified based on their body weight and head capsule size into early-last (ELI), mid-last (MLI) and late-last instar (LLI) (Lakshmi and Dutta-Gupta, 1990) followed by the non-feeding prepupal (PP) stage that was conveniently divided into wandering or early-prepupal (EPP) and late-prepupal (LPP) stages. The prepupal stage extends over 4-5 days followed by the pupal stage which lasts for 7-8 days. The adult moths normally survive for 8-10 days. For the present study, the above mentioned stages were used.

b) *Bombyx mori*

Bombyx mori is commonly known as silk moth and belongs to the order Lepidoptera and family Bombycidae. The IV^{th} instar larvae of pure Mysore strain were obtained from local breeding centre and were reared on fresh mulberry leaves under sterile conditions in insect culture room maintained at 26±1 ºC, 60±5 % relative humidity (RH) and 14:10 h light:dark (LD) photoperiod. Staging of *B. mori* larvae was done based on their age after the fourth ecdysis. One to two days old last instar were designated as early-last instar

(ELI), 5 to 6 days old as mid-last instar (MLI) and 9-10 days old as late-last instar (LLI). Silk glands were collected from the larval stage and used for the present study.

3. RNA isolation

The desired tissue was dissected under sterile conditions in RNase free 10 mM Tris-HCl (pH 7.4) and was rinsed in the same buffer and homogenized (100 mg tissue) in 1 ml of TRI Reagent (Sigma chemicals). The homogenate was allowed to stand for 5 min at room temperature followed by addition of 200 µl chloroform and incubated for 15 min at room temperature. The mixture was centrifuged at 12,000 g for 15 min at 4 ºC. The upper aqueous phase that contained RNA was collected, to which 500 µl of isopropanol was added, mixed thoroughly and incubated at room temperature for 10 min. The RNA in the form of pellet was collected after centrifugation at 12000 g for 20 min at 4 ºC. The RNA pellet was washed thrice with 80 % ethanol. The pellet was air dried and was dissolved in 20 µl of diethyl pyrocarbonate (DEPC) treated water and stored at -70 ºC until further use. During the entire preparation, care was taken to avoid RNase contamination by treating the solutions, plastic ware and glassware with DEPC followed by sterilization. This protocol was adopted from the manufacturer (Sigma Chemicals).

The purity and quantity of RNA was checked using Nanodrop (ND-1000) spectrophotometer. A solution with an A_{260} of 1 contains ~ 40 µg of single stranded RNA/ml. Using this, the concentrations of RNA was calculated in various samples isolated. The absorbance of the sample was also determined at 280 nm (A_{280}) to check the interference with protein. The purity of the sample was determined by the ratio of A_{260}/A_{280}.

4. Agarose gel electrophoresis of RNA

The RNA sample (15-20 µg) was prepared by mixing 12.5 µl formamide, 2.5 µl 10 x formaldehyde gel buffer [0.2 M MOPS, 80 mM sodium acetate and 10 mM EDTA (pH 8.0)] and 4 µl formaldehyde in a total volume of 25 µl. The mix was heat denatured at 65° C for 5 min followed by snap cooling on ice for 2 min. To this, 2.5 µl of gel loading dye (50 % glycerol, 1 mM EDTA, 0.25 % bromophenol blue and 0.25 % xylene cyanol) was added and used. The RNA samples were electrophoresed on 1.2 % agarose-formaldehyde denaturing gel. The agarose was suspended in DEPC-treated sterile distilled water. It was boiled at 100° C and cooled till 60-65 °C. To it, 1 x formaldehyde gel buffer and formaldehyde (17 ml/100 ml) were added along with ethidium bromide (0.5 µg/ml). This was followed by polymerization of 10 cm long gel on horizontal gel electrophoresis system. The electrophoresis was carried using 1 x formaldehyde gel buffer at voltage 5V/cm^2 until the dye reached the end of the gel. The ethidium bromide stained gels were visualized using UV-transilluminator and analyzed using UVP-gel documentation system.

5. First strand cDNA synthesis

The first strand cDNA was synthesized using 5 µg of total RNA. Total RNA, 1 µl of oligo$(dT)_{20}$ (50 µM), 1 µl of dNTP (10 mM) mix and DEPC water were added to make a final volume of 10 µl in a PCR tube. The tube was incubated at 65 ºC for 5 min and was then placed on ice. To this tube, a mix of 2 µl 10 x RT buffer, 4 µl of $MgCl_2$ (25 mM), 2 µl of DTT (0.1 mM), 1 µl of RNase OUT™ (40U/µl) and 1 µl of Superscript™ III RT (200U/µl) was added to make a 20 µl reaction mixture. The mixture was incubated at 50 ºC for 50 min and later the reaction was terminated at 85 ºC for 5 min. To this, 1 µl of RNaseH (2U/µl) was added and incubated at 37 ºC for 20 min. The cDNA synthesized

was stored at -20 ºC till further use. This protocol was adopted from the manufacturer (Invitrogen life technologies).

6. Cloning of partial fragments of H-fibroin, L-fibroin and P25

Partial clones of H-fibroin were amplified using, F1 [5'-ATG AGA GTC ACA ACC TTC-3'] as the forward primer, R1 [5'-TAT AAC TAC GTC TTC TTC GTA-3'] as the reverse primer and F2 [5'-GGAGGACTCGGACTTGGTGGAT-3'] as the forward primer and R2 [5'-GGCCTCTGCAGTCGACGG-3'] as the reverse primer. The cycling conditions were set as 94 ºC for 3 min, 94 ºC for 30 sec, 50 ºC for 30 sec, 72 ºC for 30 sec and 72 ºC for 5 min. Partial clone of L-fibroin was obtained using, F1 [ACG AGC GCA TTW GCM GCY CC] as the forward primer and R1 [GCG GMT RTT GAA GGC RTT GTA CAG] as the reverse primer. The cycling conditions were set as 94 ºC for 3 min, 94 ºC for 30 sec, 55 ºC for 45 sec, 72 ºC for 45 sec and 72 ºC for 5 min. Partial clone of P25 was obtained using, F2 [TTC AAC GCT WCK TAC ATM GAC] as the forward primer and R2 [GGC AGA GCR RYG ACG TCG G] as the reverse primer. The cycling conditions were set as 94 ºC for 3 min, 94 ºC for 30 sec, 52 ºC for 30 sec, 72 ºC for 45 sec and 72 ºC for 5 min. The PCR products were cloned into p-GEM-T easy vector (Promega).

7. Rapid Amplification of cDNA Ends (RACE) of L-fibroin and P25

The RACE technique is based on oligo-capping and RNA ligase-mediated RACE methods. A modified oligo-dT cellulose procedure was used to purify mRNA from high quality total RNA isolated from the tissue. The mRNA was treated with calf intestinal phosphatase (CIP) to eliminate the 5' phosphates from truncated mRNA and non-mRNA.

Dephosphorylated RNA was treated with tobacco acid pyrophosphatase (TAP) to remove the 5' cap structure from intact full length mRNA, which leaves a 5' phosphate required for ligation to the GeneRacer^{TM} RNA Oligo. The GeneRacer^{TM} RNA Oligo was ligated to the 5' end of the full length, decapped mRNA using T4 RNA ligase. The GeneRacer^{TM} RNA Oligo supplies, a known priming site for GeneRacer^{TM} 5' PCR primers after the mRNA was transcribed into cDNA. The ligated mRNA was reverse-transcribed using Superscript^{TM} III RT and the GeneRacer^{TM} Oligo dT primer, which supplies a priming site for the GeneRacer^{TM} 3' PCR primers. The reaction was heat inactivated and the mRNA strand was degraded by RNaseH treatment for more efficient RACE PCR. The cDNA was diluted in 10 mM Tris-Cl, 0.1 mM EDTA (pH 8.0) and stored at -20 ºC for further use. The primers designed for L-fibroin 5'RACE and 3'RACE were L1 [GCG GAT GTT GAA GGC GTT GTA CAT GCG T] and L2 [CTG TCT CCA GCT ACT TAA CGG AC]-3' respectively. The primers designed for P25 5'RACE and 3'RACE were P1 [GCA CCC AAA GTC GCA AAT GAG AGA G] and P2 [GGC TGA CAC ATC AGT GCT GTC CGT AGA] respectively. All the PCR conditions were programmed as specified in the manufacturer's protocol (Invitrogen).

8. Agarose gel electrophoresis of DNA

The DNA was electrophoresed on 0.8 % (10 cm long) agarose gel. The gel was polymerized using 1 x TAE [40 mM Tris-acetate and 1 mM EDTA (pH 8.0)]. The required quantity of agarose was suspended in appropriate volume of 1 x TAE and boiled for solubilization. The solution was allowed to cool till 60-65 °C to which, ethidium bromide (0.5 µg/ml) was added. This was followed by polymerization on the horizontal gel electrophoresis system. The electrophoresis was carried using the same buffer at voltage 5V/cm^{2} until the dye reached 3/4th of the length of the gel. All the ethidium

bromide gels were visualized using UV-transilluminator and analyzed using UVP-gel documentation system.

An estimated amount of plasmid DNA sample (1-2 µg/10 µl), undigested (circular), linearized or double digested with appropriate restriction enzymes was mixed with 2 µl of 6 x DNA loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol and 30 % glycerol) and loaded into the gel for electrophoretic separations.

9. Gel extraction

This protocol was designed to extract and purify DNA of 70 bp to 10 kb from standard agarose gels in TAE buffer. All the PCR products were gel purified prior to any further application. The gel piece corresponding to the amplified DNA was excised and transferred to a microcentrifuge tube. To this, 3 volumes of Buffer QG was added (Buffer QG contains Guanidium thiocyanate) that solubilizes the agarose gel slice and provides appropriate conditions for binding of DNA to the silica membrane of the QIAquick spin column. This sample was applied onto the QIAquick spin column for adsorption of DNA. The column was then washed with 0.75 ml of PE buffer [10 mM NaCl, 50 mM MOPS (pH 7.0) and ethanol phase] that removes any unwanted primers and impurities such as salts, enzymes, unincorporated nucleotides, agarose dyes and ethidium bromide. The DNA was then eluted with 50 µl of buffer EB [10 mM Tris-HCl (pH 8.0) with 1 mM EDTA]. This protocol was adopted from the manufacturer (Qiagen).

10. Ligation

A reaction mixture of 6 µl of gel purified DNA (100 ng/µl), 3 µl of vector pTZ57R/T (55 ng/µl), 4 µl of 5 x ligation buffer, 1 µl of T4 DNA ligase (5U/µl) and 6 µl of nuclease-free water was set

up. The ligation reaction was terminated after overnight incubation at 16 °C in the ligation bath. After ligation, the ligation mixture was transformed into DH5α competent cells. The transformation reaction was carried out at 42 °C for 90 sec. The mixture was plated onto LB agar plates containing ampicillin at a final concentration of 100 µg/µl. Positive colonies were picked up based on blue-white screening. This protocol was supplied by the manufacturer (Fermentas)

11. Bacterial strains

In the present study, for cloning studies, *E. coli* bacterial strain DH5α [*supE*44 *ΔlacU* 169 (ϕ 80 *lacZ ΔM* 15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1] was used. This is a recombinant deficient amber suppressing strain used for plating and growth of plasmids. The LB broth (pH 7.0) contained 10 g/L bacto-tryptone, 5 g/L bacto-yeast extract and 5 g/L NaCl. For LB agar plate, 15 g/L agar was included in the LB broth and autoclaved. The recombinants were selected by blue white (β-galactosidase) screening. For expression studies, BL21 (DE3) strain [*hsdS gal* (*ʎcIts*857 *ind*1 Sam7 *nin5 lac*UV5-T7 gene *1*)] was used. This strain is used for high level expression of genes cloned into expression vectors containing the bacteriophage T7 promoter.

12. Bacterial transformation

A single bacterial colony [DH5α or BL21 (DE3)] was picked from a plate that has been incubated for 16-20 h at 37 ºC. This colony was transferred into 100 ml of LB broth in a conical flask. The culture was incubated for 3 h at 37 ºC with vigorous agitation, monitoring the growth of the culture. For efficient transformation, it was essential that the number of viable cells not exceed 10^8 cells/ml, which for most strains of *E. coli* is equivalent to an OD_{600} of ~ 0.4. The culture was cooled to 0 ºC by storing the tube on ice for 10 min. The cells were recovered by

centrifugation at 4,100 rpm for 10 min at 4 ºC. The medium was decanted from the cell pellet. The pellet was resuspended by gentle vortexing in 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution (80 mM $MgCl_2$ and 20 mM $CaCl_2$). The cells were recovered again by centrifugation at 4,100 rpm for 10 min at 4 ºC. The cell pellet was resuspended freshly in 2 ml of ice-cold 0.1 M $CaCl_2$ for each 50 ml of original culture. At this point the cells were either directly used for transformation or dispensed into aliquots and frozen at -70 ºC for further use.

13. Plasmid isolation

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

14. Genomic DNA isolation

The excised tissue (100 mg) was homogenized in 1 ml of lysis buffer [10 mM Tris-Cl (pH 8.0), 0.1 M EDTA, 0.5 % (w/v) SDS, 20 µg/ml DNase free pancreatic RNase]. To this lysate, proteinase K was added to a final concentration of 100 µg/ml. The lysate was incubated in a water bath for 3 h at 50 ºC, swirling the viscous solution from time to time. Equal volume of phenol equilibrated with 0.1 M Tris-Cl (pH 8.0) was added and mixed gently for 10 min. The mixture was separated into two phases by centrifugation at 5000 g for 15 min at room temperature. The viscous aqueous phase was collected carefully and to this, 0.2 volume of 10 M ammonium acetate and 2 volumes of absolute alcohol were added at room temperature. The DNA in the form of precipitate was centrifuged at 5000 g for 5 min at room temperature and was washed thrice with 70 % ethanol. The DNA pellet was then dissolved in 100 µl of TE buffer [10 mM Tris-Cl (pH 8.0) with 1 mM EDTA (pH8.0)].

10 µl of isolated DNA sample was diluted with 0.25 ml of TE (pH 8.0) and vortexed vigorously for 1-2 min. The absorbance of the diluted sample was read at 260 nm. A solution with an A_{260} of 1 contains ~ 50 µg of DNA/ml.

15. Southern blotting

The total larval body genomic DNA was digested with different enzymes and subjected to 0.8 % agarose gel electrophoresis. After electrophoresis, the DNA was denatured by soaking the gel for 45 min in several volumes of 1.5 M NaCl and 0.5 M NaOH with constant agitation, followed with brief washing in sterile distilled water. The gel was neutralized for 30 min in 1 M Tris-Cl (pH 7.4) and 1.5 M NaCl followed by transfer of

DNA to nylon membrane (Hybond, Amersham Biosciences) by capillary transfer method (Sambrook et al., 2001). The membrane was soaked in 10 x SSC prior to the transfer. The transfer was performed for 16-18 h using 10 x SSC. After transfer, the blot was once rinsed in 6 x SSC and the damped blot was UV cross-linked (between thymidine residues in the DNA and positively charged amine groups on the membrane surface) using 0.15 J/cm^2 UV irradiation at 254 nm. This blot was stored at -20 °C until further use. The 20 x SSC used for Southern and Northern blotting contained 0.3 M sodium citrate and 3 M NaCl (pH 7.0).

16. Northern blotting

The total RNA samples were resolved on 1.2 % agarose-formaldehyde gel as described above and used for blotting on to nylon membrane (Hybond+, Amersham Biosciences) by capillary transfer. Prior to transfer, the formaldehyde from the gel was removed by several changes of RNase free DEPC-treated sterile water followed by equilibration in 20 x SSC for 45 min. The nylon membrane was also presoaked in 20 x SSC for 5 min. The capillary transfer was performed for 16-18 h using 10 x SSC. After the transfer, the blot was once rinsed in 6 x SSC and the damped blot was UV cross-linked. The blot was stored at -20 °C until further use.

17. Preparation of [α^{32}P]dATP labeled probe for Southern and Northern hybridization

This was employed to determine the L-fibroin and P25 gene copy number on Southern blot and their mRNA transcript on northern blot. For this, cDNA insert corresponding to the whole open reading frame of L-fibroin and P25 were gel purified and approximately

100 ng was random prime labeled using DNA labeling kit (Fermentas) and [α^{32}P]dATP (3,000 Ci/mmol, BRIT, India). The reaction was carried in step wise manner. 10 µl of cDNA template (~100 ng) was mixed with 10 µl random (hexamer) primer [7.5 o.u/ml in 300 µl of 0.25 M Tris-Cl (pH 8.0), 25 mM $MgCl_2$ and 5 mM DTT] and 40 µl sterile distilled water, vortexed and spun at 10,000 g for 5 sec. The mix was incubated in boiling water bath for 10 min followed by quick cooling on ice. The mix was again spun down quickly. To this, dNTP mix (15 µM each) without d ATP along with 50 µCi [α^{32}P]d ATP and 1 µl Klenow fragment (5U/µl) was added, mixed thoroughly and incubated for 20 min at 37 °C. The reaction was stopped by 1 µl of 0.5 M EDTA (pH 8.0). The unincorporated dNTPs and [α^{32}P]dATP were removed by passing the mixture through Sephadex G-25 column. The probe thus prepared, had specific activity of >10^9 dpm/µg. The radiolabeled probe was stored at -20 °C until further use.

18. Southern and Northern hybridization

Prehybridization was carried out for 3 h in Church buffer [0.5 M sodium phosphate buffer (pH 7.2), 1 mM EDTA, 7 % SDS and 1 % BSA] at 65 ºC. Hybridization with the prepared probes was carried out for 16 h at 65 ºC. After hybridization, first wash was given at room temperature with 5 x SSC for 5 min, second low stringent wash was done using 2 x SSC, 0.1 % SDS for 10 min, third medium stringent wash was done with 1 x SSC, 0.1 % SDS for 10 min and the final high stringent wash was performed using 0.1 x SSC, 0.1 % SDS for 15 min. All the washes were carried at 65 ºC. Finally the blots were dried and exposed to Kodak X-OMAT X-ray film followed by autoradiography to detect the hybridization.

19. Recombinant expression of L-fibroin and P25 clones

The PET 28a expression vector as well as the L-fibroin and P25 full-length cDNA clones were digested with BamHI and HindIII restriction enzymes. The inserts were cloned into PET 28a expression vector and were transformed into non-expression host strain DH5α. The positive clones were identified and the reading frame was verified by sequencing. The recombinant plasmid was then cloned into expression host strain BL21 (DE3). The expression of the recombinant clones was induced and optimized by various concentrations of IPTG. The overexpressed target proteins were loaded on denaturing polyacrylamide gel and were confirmed using anti-His antibody.

20. Gel elution of L-fibroin and P25 recombinant proteins

The gel containing the overexpressed protein was cut into fine pieces. The gel pieces were incubated in elution buffer [Tris-Cl (pH 8.8), 1 % SDS, 0.1 M Glycine and 2 % w/v Glycerol] at 37 ºC overnight with constant agitation. The eluted protein was concentrated using PEG 4000. The protein was dialyzed against 1 x PBS (pH 7.0). This protein was used for generation of antibody.

21. Peptide synthesis

Based on the H-fibroin partial sequence obtained from *C. cephalonica* and comparison with the H-fibroin sequences from other insect species, a peptide consisting 10 alanine residues was conjugated to Keyhole Limpet Hemocyanin (KLH) carrier protein commercially (USV Limited). This hapten (polyalanine peptide) conjugated carrier protein (KLH) was dissolved in PBS (pH 7.4) and used for antibody generation.

22. Production of polyclonal antibodies

The antibodies were individually raised against the recombinant expressed L-fibroin and P25 proteins that were eluted after slicing the protein band as well as against the KLH conjugated polyalanine peptide. Three month old male rabbits (New Zealand variety) were injected with 100 µg of protein (emulsified with 500 µl of complete Freund's adjuvant) by subcutaneous injections into various sites on the back. Prior to injection, the lateral ear vein was bled to collect pre-immune serum. After a fortnight, 1st booster injection was given followed by a 2nd booster injection after seven days. For booster injections, 50 µg protein emulsified with Freund's incomplete adjuvant was used. The blood was collected after a week of 2nd booster injection. The collected blood was left overnight at 4 °C for clotting and serum was obtained by centrifugation at 5,000 g for 20 min. The serum was aliquoted and stored at -20 °C after adding 25 % glycerol and 0.001 % azide.

23. Purification of IgG

The IgG fraction of H-fibroin, L-fibroin and P25 antibodies were purified by affinity chromatography using protein-A agarose column (Bio-Rad) according to the manufacturer's protocol. The binding of F_c regions of IgG to protein-A in the crude mixture of serum proteins was utilized for the purification. The serum protein was diluted (1:1) with the wash or binding buffer [20 mM sodium phosphate (pH 7.4) and 150 mM NaCl] and was loaded on to the protein A-agarose column prequilibrated with wash or binding buffer. This was followed with the washing of column using the same buffer until the absorbance of elutes at 280 nm approaches the background level. The bound IgG fraction was eluted with 4 column volumes of elution buffer [100 mM glycine-HCl (pH

3.0)] to tubes that contained 100 µl of neutralization buffer [1 M Tris-HCl (pH 9.0)] for immediate neutralization of the eluted purified IgG fraction. The purified IgG fraction was lyophilized and stored at -20 °C till further use.

24. Preparation of homogenate and protein estimation

A 2-5 % homogenate of desired tissue was prepared. For this, the tissues were rapidly dissected in cold insect Ringer (130 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2$ and 1 mM PMSF) and were homogenized in 150 µl homogenization buffer [10 mM Tris-Cl (pH 7.4), 0.1 % Triton X-100, 1 mM PMSF, 1 mM EDTA and 1 mM DTT] followed by centrifugation at 1,000 g for 5 min to remove larger debris. The aliquots of the supernatant were used for various studies.

Protein content in various samples was estimated according to the micro protein assay method (Bradford, 1976). Bradford reagent was prepared by dissolving ten mg of brilliant blue G-250 (Sigma) in 5 ml of 95 % ethanol. To this solution, 10 ml of 85 % (w/v) orthophosphoric acid was added. The resulting solution was diluted to a final volume of 100 ml with distilled water, filtered through Whatman No. 1 filter paper and stored in an amber colored bottle at 4 °C.

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

25. Polyacrylamide gel electrophoresis

Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with acrylamide : N,N'-bisacrylamide (30:1) was carried out according to the procedure of Laemmli (1970), on a 1 cm long, 2.1 % stacking gel (pH 6.8) followed by a 7.5 or 15 cm long, 12 % or 10 % or 7.5 % or 5 % resolving gel (pH 8.8) for resolution. Tris-glycine [25 mM Tris and 192 mM glycine (pH 8.3)] with 0.1 % SDS was used as the electrode buffer. Electrophoresis was carried out at 100 V until the tracking dye reached 1 cm above the base of the resolving gel. The sample was prepared by mixing an aliquot of the protein sample with the sample buffer containing 0.125 M Tris-CI (pH 6.8), 4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol and 0.002 % bromophenol blue followed by incubation at 100 °C for 1 min.

26. Coomassie staining of polyacrylamide gels

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

27. Silver staining of electrophoretically separated proteins on polyacrylamide gel

This was carried according to the procedure of Blum et al., (1987). The gel was incubated in fixative (50 % methanol, 12 % acetic acid and 50 µl of 37 % formaldehyde/100 ml) for 1 h followed with 3 washes in 50 % ethanol. Subsequently the gel was pretreated with sodium thiosulphate (20 mg/100 ml) for 1 min and rinsed thrice (20 sec each) with distilled water. The gel was impregnated with silver nitrate (0.2 % containing 187 µl of 37

% formaldehyde) with gentle agitation for 30 min. It was rinsed with distilled water and developed with 6 % sodium carbonate (w/v) and 50 µl of 37 % formaldehyde (v/v). Finally, the stained gel was thoroughly rinsed with distilled water and stored in 50 % methanol.

28. Western blotting and immunostaining

The electrophoretically separated polypeptides were transferred (electro-blotted) onto nitrocellulose membrane using Trans-Blot apparatus (Bio-Rad) according to the procedure of Towbin et al., (1979). For this, the gel was first equilibrated in Towbin buffer (25 mM Tris, 192 mM glycine and 20 % methanol) for 30 min followed by transfer to the membrane for 3 h at 70 V with 250 mA current limit. The transfer of protein to membrane was checked by reversible Ponceau S staining (100 mg Ponceau S in 5 % acetic acid). The stain was removed by 3-4 washes with Tris-buffered saline with Tween-20 (TBST) [10 mM Tris-Cl (pH 7.4), 150 mM NaCl and 0.1 % Tween-20 (v/v)). For immunostaining, the protein blot was processed with 3 % BSA (w/v) in TBST for 1 h at room temperature to block the non-specific binding sites followed by washing with TBST (10 min x 5 changes). The blot was then incubated with the primary antibody diluted in TBST containing 3 % BSA (w/v) for 2 h to overnight. This was again followed by a through wash in TBST (10 min x 5 changes). Thereafter, the blot was incubated with ALP (alkaline phosphatase) or HRP (horse radish peroxidase) conjugated anti-mouse or antirabbit IgG for 1 h. Once again the blot was washed in TBST (10 min x 5 changes). The visualization of the specific cross-reactivity was carried with the substrates of ALP i.e., NBT/BCIP [0.0033 % nitroblue tetrazolium and 0.0165 % 5-bromo-4-chloro-3-indoly-l-phosphate in 10 mM Tris-HCl (pH 9.5), 5 mM $MgCl_2$ and 10 mM NaCl] for color reaction. The HRP-antibody incubated blots were developed using

chemiluminiscent reagents (Pierce) and exposed to Kodak X-OMAT X-ray film for 1-5 min followed by autoradiography to detect the signal.

29. Autoradiography

This was employed for detection of radiolabeled polypeptides or nucleic acids. The stained gels were sandwiched between cellophane sheets and dried under vacuum at 80 °C for 1 h using a Hoefer gel drier. The Southern, Northern, chemiluminiscent or EMSA blots were wrapped in a saran wrap. These gels or blots were exposed to Kodak X-Omat X-ray film for 1-3 days depending on the radiation counts on Geiger Muller counter using DuPont Cronex intensifying screens at -70 °C and were developed and fixed.

30. Hematoxylin-eosin staining

The salivary glands from the larvae were dissected out and fixed in Bouin's fluid (saturated picric acid:formaldehyde:acetic acid- 15:5:1) for 4 h at room temperature. These tissues were then dehydrated through a water-ethanol series, cleared in xylene and embedded in paraffin wax that was premelted at 60 ºC. Care was taken to avoid formation of any air bubbles during embedding. The embedded tissue was blocked onto a wooden or plastic support. The sections (5 µm thick) were cut on microtome (Leica) and mounted on glass slides coated with Meyer's albumin (ovalbumin:glycerol:water- 1:1:1). These sections were spread using water film at 40 ºC, air dried and stored at 37 ºC or room temperature for further processing. The sections were then deparafinized by three changes of xylene and were rehydrated in ethanol-water series just prior to the processing of slides for staining. The staining with hematoxylin and eosin was carried by first treating with nuclear stain hematoxylin for 2 min followed by a brief wash with water and

differentiation under microscope. The same slide was subsequently stained with cytosolic stain eosin (1 % in 10 % ethanol). This was done by dehydrating the slide in water-ethanol series. After the slides were treated with 80 % ethanol, it was stained with eosin for 15 min followed by quick dehydration with 90 % ethanol and absolute ethanol. The slides were finally dipped in xylene and mounted with DPX mountant (A synthetic resin mixture of a polystyrene- distyrene, a plasticizer- tricresyl phosphate and xylene). Microscopic examinations and photography was carried out using Nikon EFD-3 microscope fitted with a Nikon NFX-35 automatic camera (Nikon, Japan).

31. Immunohistochemical studies

The immunohistochemical localization of H-fibroin in the lumen of the salivary gland sections was carried with anti-rabbit polyalanine antibody. The deparaffinized sections were rehydrated in water-ethanol series and treated with blocking solution (1 % non-immune goat serum in PBS) for 1 h at 4 °C. This was followed by antibody treatment (1:250 dilutions in blocking solution) for 24 h at 4 °C with gentle shaking. The slides were then treated with goat anti-rabbit IgG conjugated with Biotin (1:500 dilutions in blocking solution) for 2 h at 4 ºC. Washing after each step was done with three changes of PBS. Streptavidin conjugate was added to these slides and incubated for 30 min at 4 ºC. Then the slides were finally processed for color development using diamino-benzidine (DAB) development system and mounted with DPX mountant. The specificity of antibody was checked by processing parallel tissue sections using pre-immune serum.

32. Organ culture of salivary glands

Salivary glands dissected under sterile condition in ice cold insect Ringer (130 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2$ and 1 mM PMSF) were rinsed in 100 µl of TC-100 insect

culture medium (Sigma) with traces of streptomycin sulfate. This was followed by transfer of the gland to fresh 500 µl medium for 1 h as preconditioning prior to the required experimental set up. Based on the experiment, the tissue was incubated in the culture medium along with different concentrations of hormones/analogs/agonists for varying time periods in an incubator (95 % relative humidity, 5 % CO_2 and 25 ºC). After incubation, the tissue was rinsed in ice cold Ringer and processed for RNA isolation. The choice of TC-100 medium for culture was basically due to less complex composition as compared to other insect media, contains no insect haemolymph (important for present study) and developed to support the growth of lepidopteran cells in culture (Gardiner and Stockdale, 1975).

33. Hormone treatments

The stock solutions of 20-hydroxyecdysone (20E), non-steroidal hormone agonists RH-5849 and RH-5992 (tebufenozide) was prepared by dissolving 1 mg of hormone compound individually in 100 µl of ethanol, which was finally diluted to 1 ml with distilled water. This was aliquoted into small volume and stored at -20 °C till use. This stock solution was further diluted as per requirement. The final concentration of ethanol in 20E never exceeded 0.05 % in any of the experiments. Control insects received equal volume of carrier solvent (0.05 % ethanol). Similarly, a stock solution of juvenile hormone analog methoprene was prepared by dissolving 1 mg in 100 µl of acetone, which was finally diluted to 1 ml of distilled water. Control insects received equal volume of carrier solvent (acetone). For *in vivo* studies, various concentrations of RH-5849 and RH-5992 were given along with the feed. 0.05 % ethanol was served as control.

34. MTT staining

The MTT assay measures the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT (yellow colored) enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored (dark purple) formazan product. Since reduction of MTT can only occur in metabolically active cells, this assay was used to measure the viability of cells/tissue. The MTT dye (Himedia) was dissolved (5 mg/ml) in PBS (pH 7.0). The salivary glands cultured in 500 µl of TC-100 medium were incubated with 50 µl of MTT solution in dark for 1 h and were visualized under the microscope. Freshly excised salivary glands were used as positive control, while heat killed salivary glands served as negative control.

35. Enzyme immunoassay

The protocol of the manufacturer was followed (Cayman Chemicals). Briefly, the 96-well plate that was precoated with mouse monoclonal anti-rabbit was incubated with acetylcholine esterase linked to 20E (tracer), 20E antiserum and either standard or unknown sample. Washes were performed to remove all unbound reagents. The assay is based on the competition between 20E (present in the haemolymph) and 20E tracer for a limited amount of 20E antiserum. The Ellman's reagent that contains the substrate for acetylcholine esterase was added to the wells. The product of this enzymatic reaction has a distinct yellow color and was measured at 412 nm. For this assay, the hemolymph samples were collected from narcotized larvae and to those, 5 volumes of diethyl ether was added. The upper ether layer that contained the hormone was transferred into a new tube and the extraction procedure was repeated twice. The combined ether extract was

evaporated by heating at 30 ºC under a gentle stream of nitrogen. The dried extract was dissolved in the appropriate buffer for estimation.

36. Real-time PCR analysis

The purity and quantity of RNA was checked by using Nanodrop spectrophotometer (ND-1000). Five micrograms of total RNA was reverse transcribed using Superscript III^{TM} first strand synthesis system (Invitrogen). PCR reactions (20 µl) were performed using Power SYBR Green Master mix (Applied Biosystems). Reactions were set up using primer sets for L-fibroin (sense 5'-TAT TAC TCG TCG CGT CGA GCG C-3' and antisense 5'-CAG CTC ACC GAG AGC TGC AAC-3'), P25 (sense 5'-TCC TTG TGC TAG CTG GCA CG-3' and antisense 5'-TGA ACC GGG GGA TGG TGT ACT-3'), H-fibroin (sense 5'-CCT TCG TGA TCT TGT GCT GTG CC-3' and antisense 5'-CTG ACG ATC TTG TCC TCA CCG GA-3') and *C. cephalonica β-actin* gene (sense 5'-GGT AGT AGA CAA TGG CTC CGG-3' and antisense 5'-CCC AGT TAG TGA CGA TTC CGT G-3'). For real-time PCR, reactions were measured using Fast 7500 Real time PCR system (Applied Biosystems). PCR conditions were optimized to generate >95 % PCR efficiency. Dissociation curve analysis was performed after the last cycle to confirm amplification of a single product. The real time results are presented as change in expression relative to control using target gene Ct values normalized to that of *β-actin* gene Ct values based on the $2^{(-\Delta\Delta C(T))}$ method (Livak and Schmittgen, 2001).

37. Construction of genome walking library and isolation of upstream elements

The basic strategy: Separate aliquots of DNA are completely digested with different restriction enzymes that leave blunt ends. Each batch of digested genomic DNA is then

ligated separately to the GenomeWalker Adaptor. These pools of uncloned, adaptor-ligated genomic DNA fragments are referred to for convenience as GenomeWalker "libraries." After the libraries have been constructed, the first or primary PCR uses the outer adaptor primer (AP1) provided in the kit and an outer, gene-specific primer (GSP1). The primary PCR mixture is then diluted and used as a template for a secondary or "nested" PCR with the nested adaptor primer (AP2) and a nested gene-specific primer (GSP2). This generally produces a single, major PCR product from at least three of the four libraries. Each of the DNA fragments which begin in a known sequence at the 5' end of GSP2 and extend into the unknown adjacent genomic DNA can then be cloned and further analyzed.

Genomic DNA was isolated from the larval salivary glands. Genome walking library was constructed using Universal Genome Walker kit (Clontech) following manufacturer's instructions. Twenty five micrograms of genomic DNA was digested overnight with *EcoRV*, *PvuII*, *DraI* or *StuI*. Digested DNA was purified using phenol chloroform extraction method. Genomic DNA digested with each restriction enzyme was ligated to adaptors separately. H-fibroin upstream region was isolated using gene specific primer 1 (5'-GCA AGG CAC AGC ACA AGA TCA CGA AGG-3') and the adapter primer-1 (provided in the kit). Touchdown PCR was used for the amplification. Cycling conditions were 94 °C 30 sec, 72 °C 3 min, 5 cycles, 94 °C 30 sec, 68 °C 30 sec, 72 °C 3 min for 30 cycles. A nested PCR was carried out using gene specific primer 2 (5'-GCA CAA GAT CAC GAA GGT TGT GAC TCT C-3') and adaptor primer-2 (provided in the kit). All the amplicons were cloned into pGEM-T Easy vector (Promega) and sequence was determined bi-directionally.

38. PCR based progressive deletion constructs

Progressive 5' deletion constructs were amplified by PCR. Resultant constructs were cloned in *Kpn*I and *Hind*III sites of pGL2 basic firefly luciferase vector (Promega). The identity of each construct was verified by double digestion and the absence of cloning artifacts was determined by nucleotide sequencing.

39. Transient transfection studies

EcR-293 cell line [derived from HEK(human embryonic kidney) cells by incorporation of the regulatory vector pVgRXR which encodes both the RXR and EcR receptor proteins] was obtained (Invitrogen) and cultured using DMEM (Dulbecco's modified Eagle medium) supplemented with 10 % fetal bovine serum, 2 mM L-Glutamine, 30 µg/ml Bleocin and 300 µg/ml of Geneticin. All the cell culture reagents were purchased from GIBCO-BRL. Cultures were grown in an incubator at 37 ºC, 5 % CO_2 and 95 % relative humidity. Cultures were maintained at low passage number (n<10). Cells were plated 48 h before transfection in 24 well plates. 4 pg/µl of Ponasterone A was used for induction. Reporter gene constructs (Firefly luciferase) and the pRL-TK plasmids (Renilla luciferase) were co-transfected into 80-90 % confluent cell cultures. Promoter activation leads to expression of secreted luciferase protein. Hence, 48 h after co-transfection, cells were collected and washed with phosphate buffered saline solution. The cells were lysed by addition of 100 µl (per well) passive lysis buffer (Promega). Luciferase reporter activity was determined with the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. Light intensity was measured using Turner Design 20/20 luminometer and the promoter activity was shown in relative luciferase units.

40. Nuclear extract preparation

Silk glands were excised and homogenized gently in ice-cold PBS. The homogenate was incubated in 0.4 ml of hypomolar HEPES buffer [10 mM HEPES (pH 7.9)] containing 10 mM KCl, 0.1 mM EDTA, 0.1mM EGTA, 1 mM DTT, 1 mM PMSF, 2 µg/ml Leupeptin, 2 µg/ml Aprotinin and 0.5 mg/ml Benzamidine for 15 min on ice. Then, 12.5 µl of 10 % NP-40 was added for lysis of the cells. The sample was vortexed vigorously for 10 sec and was centrifuged for 30 sec at 2000 rpm. The supernatant (cytoplasmic extract) was collected and stored at -70° C. The nuclear pellet was resuspended in 25 µl of ice-cold nuclear extraction buffer [20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 µg/ml Leupeptin, 2 µg/ml Aprotinin and 0.5 mg/ml Benzamidine]. The tubes were then incubated on ice for 30 min. This nuclear extract was then centrifuged for 5 min at 2,000 rpm and was stored at -70 °C for further use.

41. Preparation of [γ^{32}P]dATP labeled probe for gel shift assay

The commercially synthesized wild type as well as mutated 43-mer oligonucleotides which have a - OH group at their 5'-end, were labeled using [γ^{32}P]dATP and T4 polynucletide kinase. A reaction mixture of 2 µl of oligonucleotides (final concentration of 2 pmoles), 5 µl of 10 x polynucleotide kinase buffer, 2 µl of [γ^{32}P]dATP (7000 µCi/mmole; 100 µCi/µl) and 4 µl of T4 polynucleotide kinase (10U/ul) was set to make the final volume upto 50 µl with nuclease free water. The reaction mixture was incubated at 37 ºC for 30 min. The reaction was stopped on addition of 2 µl of 0.5 M EDTA. The radiolabeled mix was then purified using G-50 Sephadex column to remove any unincorporated [γ^{32}P] dATP. The radioactivity was detected (Cerenkov counting) and it

was found to be 8 x 10^6 counts. For elution, 1 µl of corresponding complementary oligonucleotide was added (final concentration of 100 pmoles/ul). The mix was boiled in a water bath for 3 min and left at room temperature for 30 min to obtain a double stranded [γ^{32} P] dATP labeled probe.

42. Preparation of polyacrylamide gel for gel shift assay

A 7.5 % gel was casted by mixing the following solutions: 12.5 ml of acrylamide:bis acrylamide (29.2:0.8), 10 ml of 5 x EMSA buffer [0.25 M Tris, 2 M glycine, 0.01 M EDTA, (pH 8.5)], 27.06 ml of water, 0.4 ml of 10 % ammonium persulphate and 0.04 ml TEMED to make upto a final volume of 50 ml. The gel was allowed to polymerize for 45 min. The gel was prerun in 1 x EMSA buffer at 150 V and later the samples were loaded.

43. Gel shift assay

Gel shift assay was performed by incubating 5 µg of nuclear extract with 15 femto moles of [^{32} P] end labeled double stranded wild or mutated ERE oligonucleotide (wild type oligo: 5'ACG AAA TAG TGG ATT T**TC A**GT ATA AAA AGC CTT GGA AAT CTG G3'; mutated: 5'ACG AAA TAG TGG ATT T**GT G**GT ATA AAA AGC CTT GGA AAT CTG G3') in the presence of 0.5 µg of poly dI:dC in a binding buffer [20 mM HEPES (pH 7.9), 0.5 mM EDTA, 0.4 mM DTT, 5 % glycerol] for 15 min at 37 °C. The DNA-protein complex formed was separated from free oligonucleotide on a 6 % native polyacrylamide gel. 0.5 ng/µl of 20E was used for induction. For supershift, 1 µg of sheep anti *Chiromous*-ecdysone receptor antibody was incubated with the nuclear extract at room temperature for 10 min prior to addition of radiolabelled probe and was detected

detected goat anti-rabbit IgG-HRP conjugate. The exposed film was developed for detection of radioactivity.

44. Scanning electron microscopic studies

Silk secreted out from control as well as treated insects was collected manually and was mounted onto the stubs containing carbon tape, in a sterile environment. These were then loaded onto the specimen stub and were made electrically conductive using gold metal coating. The surface of the silk fibers was scanned and analyzed.

45. Statistical analysis

Data were expressed as mean ± SEM (n=3). Significance between groups was analyzed by ANOVA followed by Student New-man-Keuls test using Sigmastat. Values were considered significant at $P<0.05$.

46. Computer assisted analysis

BankIt program was used to submit nucleotide sequences of H-fibroin, L-fibroin and P25. **ClustalW** program was used to perform multiple alignments on a set of DNA or protein sequences. **BLAST (Basic Alignment Search Tool)** algorithm was primarily used for similarity searches in sequence databases. **CorePromoter** program was used to predict the transcriptional start sites and localized them into 50-100 bp core-promoter regions. **Transfac** is a relational database of transcription factor cis-acting binding sites and transcription factors from many organisms, and also includes PSSMs (position specific scoring matrices) representing the observed variation in each column of the aligned binding sites for one factor. **TESS (Transcription Element Search System) and**

MatInspector programs helped in finding potential transcription factor binding sites in DNA sequences. **WU-BLAST** program performs similarity searches of proteins and nucleotide sequence databases; similar to NCBI BLAST but developed independently and can produce different results. **ExPASy (Expert Protein Analysis System)** proteomics server of the Swiss Institute of Bioinformatics is dedicated to the analysis of protein sequences and structures. **LaserGene and GENERUNNER** softwares were used for primer designing, sequence assembly and analysis. **PHYLIP** software was used in phylogenetic tree construction and analysis. **VecScreen** system from NCBI was used for identification of vector contamination in nucleic acid sequences.

RESULTS AND DISCUSSION

PART I

Cloning, expression, characterization and developmental regulation of *Corcyra cephalonica* H-fibroin, L-fibroin and P25 genes

Initially, primers were designed to obtain partial fragments of L-fibroin, P25 and H-fibroin. The PCR amplification of L-fibroin and P25 with degenerate primers yielded 550 bp and 400 bp fragments respectively. These sequences were used further to design primers to obtain full-length cDNA. The 5' and 3' cDNA ends were cloned using rapid amplification of cDNA ends (RACE) strategy and were sequenced. Two partial fragments of H-fibroin were cloned. The details of primers used as well as the reaction conditions are given in materials and methods section (pages 39 and 40). These full-length cDNAs of L-fibroin and P25, partial cDNAs of H-fibroin were used to carry out further studies.

The coding sequence of *C. cephalonica* L-fibroin consisted of 804 nucleotides and codes for a polypeptide of 267 amino acids. The 5' UTR region consisted of 41 bases and the 3' UTR region consisted of 280 nucleotide bases that contained a single polyadenylation signal sequence. The transcript of L-fibroin accounted for 1125 bases (Fig. 7). ClustalW multiple alignment showed clear homology between the L-fibroin of *C. cephalonica* and other insect L-fibroins known, which have three conserved cysteine residues. The positions of these cysteines, short stretches of amino acids in their vicinity, and the spacing of certain other residues were conserved in all L-fibroins including *C. cephalonica*. In addition, high similarity was also found in the signal peptide as well as adjacent sequence (Fig. 8). The pI value for the deduced L-fibroin peptide was 4.8. L-fibroin nucleotide sequence has been submitted to GenBank under accession no. GQ901975.

The coding sequence of *C. cephalonica* P25 consisted of 657 nucleotides and its translation yielded a protein of 218 amino acid residues. Two putative glycosylation sites were present in its coding sequence. The 5' UTR region consisted of 53 nucleotides. The

3' UTR region was found to be 330 bases long and contained two polyadenylation signal sequences (Fig. 9). The total sequence of 1040 nucleotides was deposited in GenBank under accession no. GQ901976. The eight cysteine residues present in the coding sequence were well conserved among all the P25 sequences reported. Protein motifs CRVSEFF, YPLIRLTT and CDFGC were present in all species (Fig. 10). The pI value of the deduced P25 peptide was 8.3.

Phylogenetic analysis revealed *C. cephalonica* L-fibroin's relatedness to that of *G. mellonella* and showed 71 % homology between the two (Fig. 11). *C. cephalonica* P25 showed 78% homology with *G. mellonella* and was found phylogenetically close to it (Fig. 12).

The identified 5' end of the H-fibroin gene of *C. cephalonica* encoded 98 amino acid residues (Fig. 13a). The alignment of the deduced peptide with the N-terminus of lepidopteran H-fibroins, revealed homology in the predicted signal peptide region which covered 18 residues. The non-repetitive sequence that follows the signal peptide was characterized by frequent grouping of two non-polar residues (Ile, Leu, Val, Phe, Pro) flanked by one or two charged residues (Asp, Glu, Lys, Arg). These motifs (VRTFVIETD and IYEEDVVI), the overall negative charge, and the similar distribution of charged residues resembled the N-terminus of lepidopteran H-fibroin (Fig. 14). This sequence has been submitted to GenBank under accession no. GQ901977.

Another partial fragment of H-fibroin gene of *C. cephalonica* was also cloned which encoded 119 amino acid residues. The deduced amino acid sequence contained polyalanine motifs (Fig. 13b). Similar motifs were found in *G. mellonella*, *Antheraea*

pernyi, *Plodia interpunctella* and *Anagasta kuehiniella* (Accession nos. AF095239, AF083334, AY253533 and AY253535 respectively). Based on this motif, a Keyhole Limpet Hemocyanin (KLH) conjugated polyalanine peptide was synthesized and used for generation of polyclonal antibody and used for various studies.

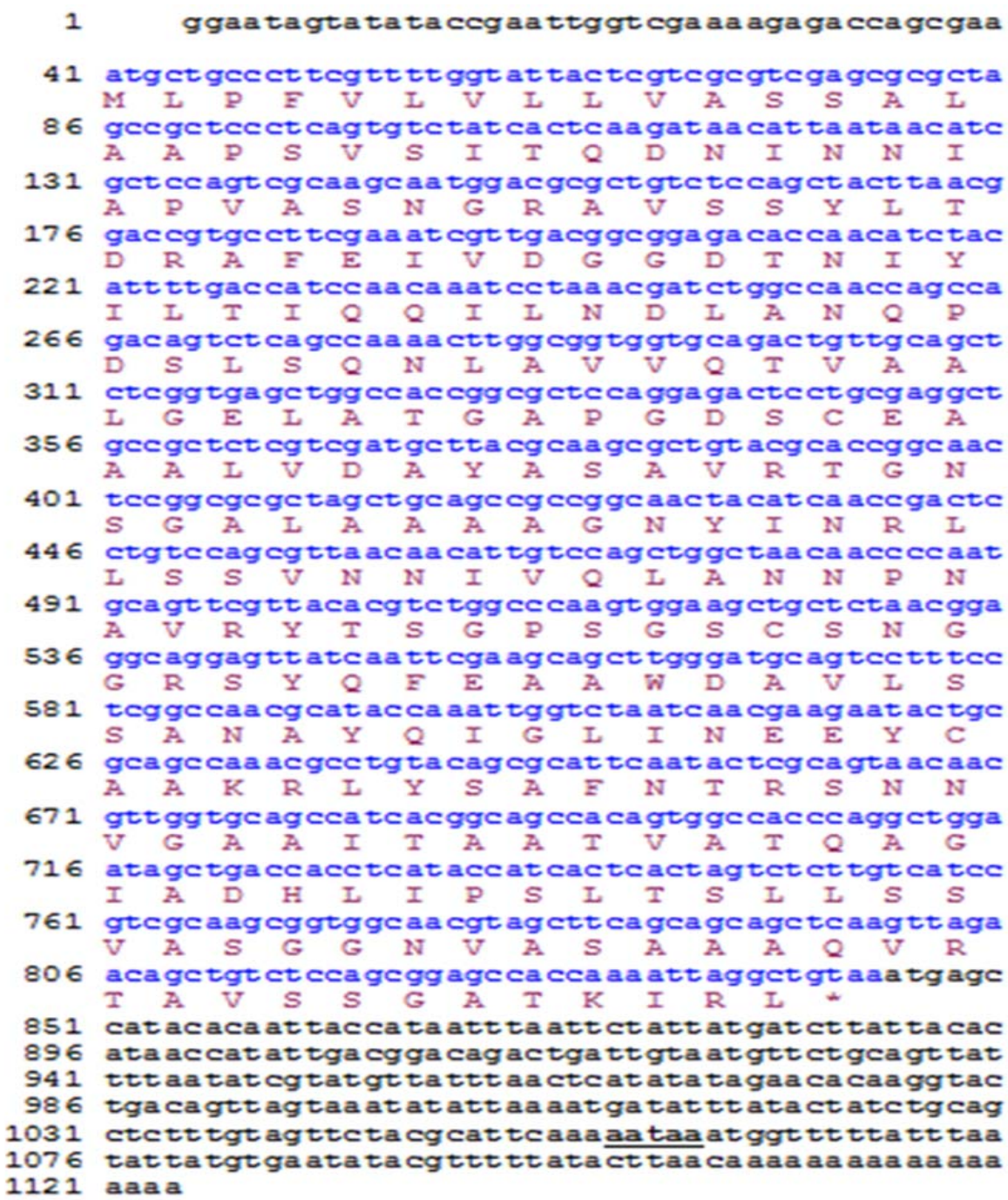

Fig. 7. Full length cDNA sequence and deduced amino acid sequence for *C. cephalonica* L-fibroin. The 5' UTR and 3'UTR regions are represented in black color. The coding sequence and the deduced amino acid sequence are represented in blue and red colors respectively. The polyadenylation signal sequence (AATAA) in the 3' UTR region is underlined.

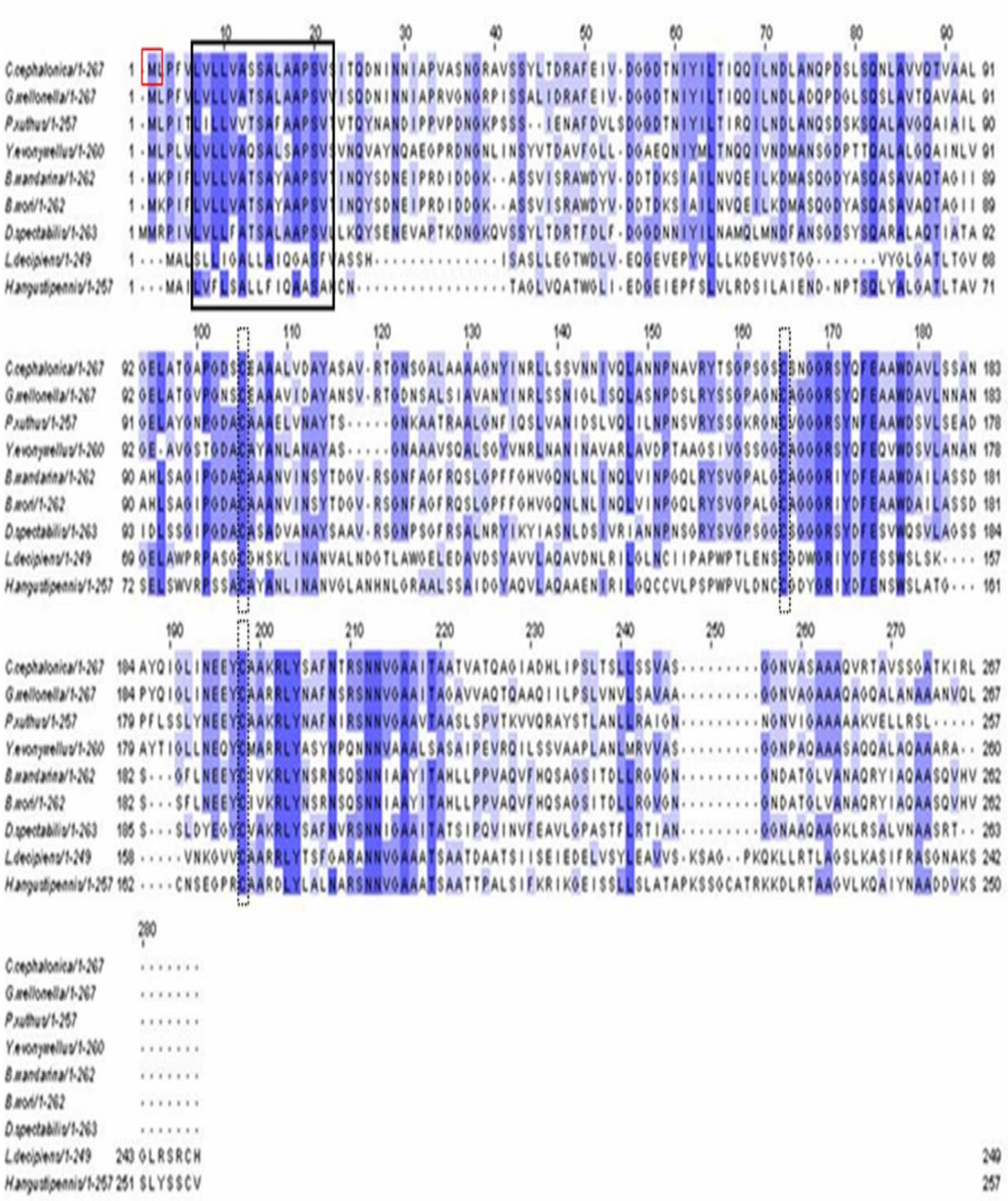

Fig. 8. ClustalW multiple alignment of L-fibroin from *C. cephalonica* (Accession no. : GQ901975) with other lepidopteran insects in *G. mellonella*, *P. xuthus*, *Y. evonymella*, *B. mandarina*, *B. mori*, *D. spectabilis*, *L. decipiens* and *H. angustipennis* (Accession nos. : S77817, AB001824, AB195977, AB001820, X17291, AB001822, AB214510 and AB214508 respectively). Conserved cysteine residues in all the L-fibroin sequences are shown in dotted boxes and the conserved N-terminal is represented in rectangle.

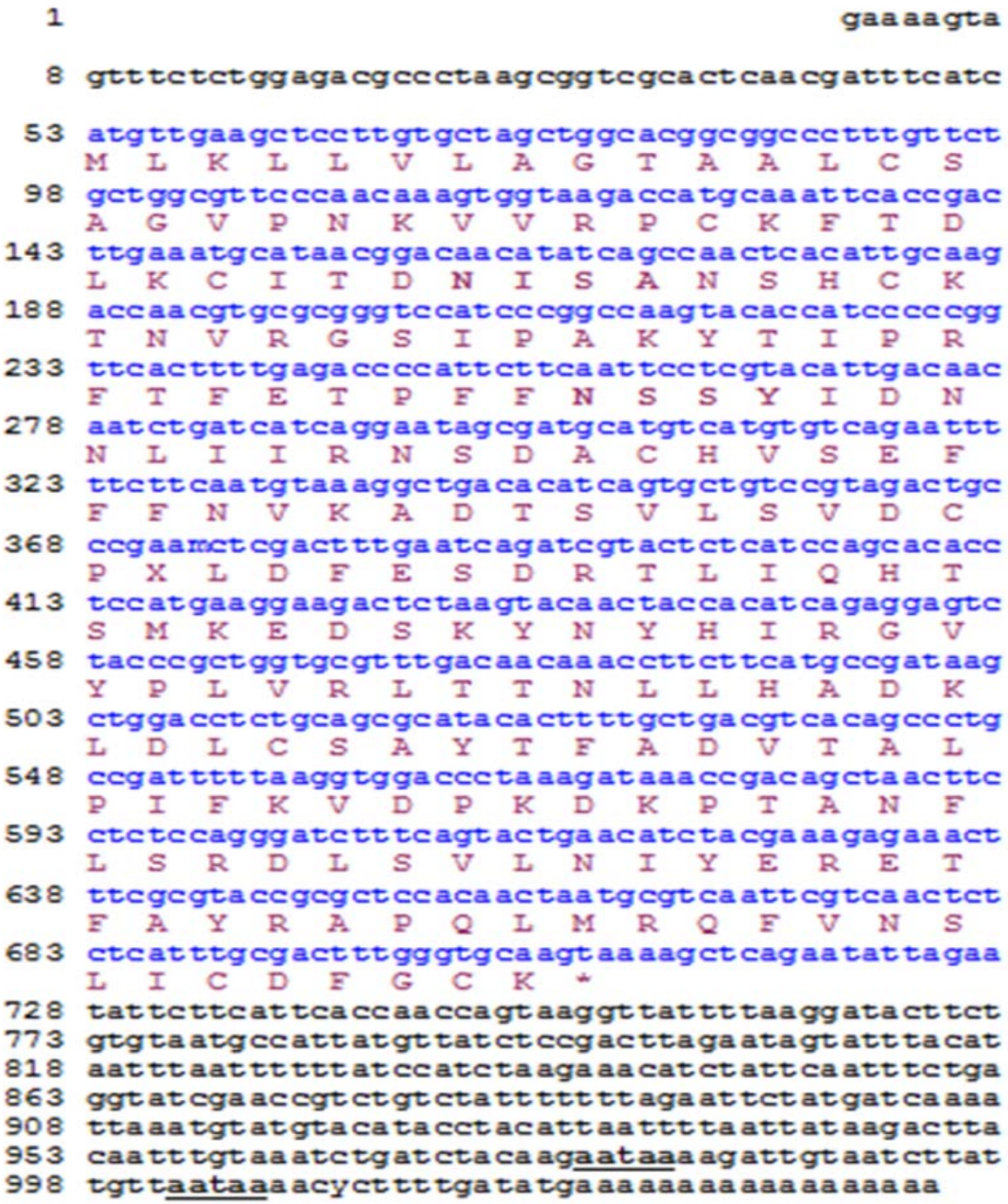

Fig. 9. Full length cDNA sequence and deduced amino acid sequence for *C. cephalonica* P25. The 5' UTR and 3'UTR regions are represented in black color. The coding sequence and the deduced amino acid sequence are represented in blue and red colors respectively. The polyadenylation signal sequences (AATAA) in the 3' UTR region are underlined.

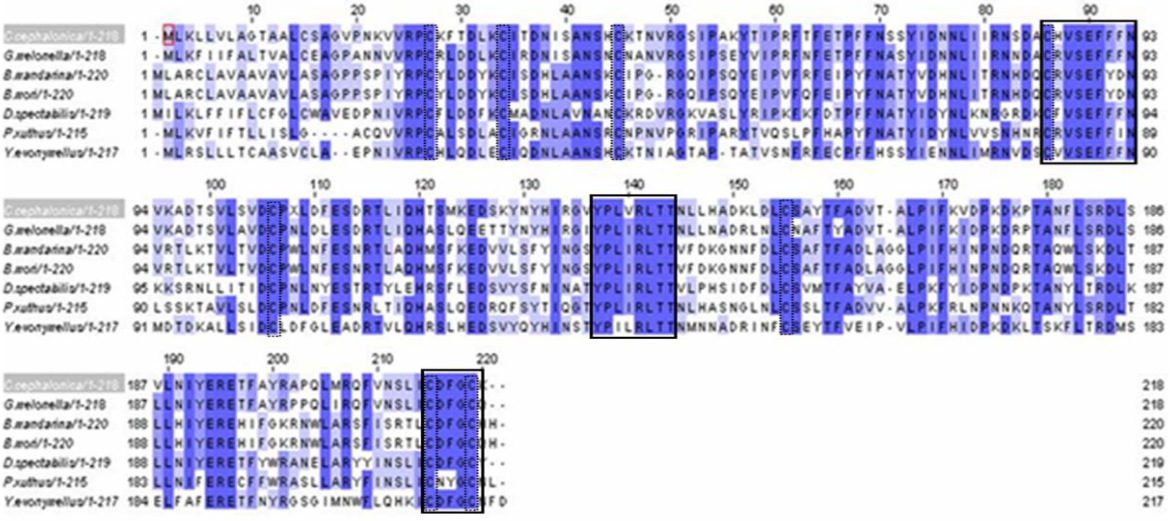

Fig. 10. ClustalW multiple alignment of P25 from *C. cephalonica* (Accession no. : GQ901975) with its counterparts in *G. mellonella*, *B. mandarina*, *B. mori*, *D. spectabilis*, *P. xuthus* and *Y. evonymella* (Accession nos. : AF009827, AB001821, X04226, AB001823, AB001825 and AB195976 respectively). Conserved cysteine residues are shown in dotted boxes and the conserved amino acid motifs are represented in rectangles.

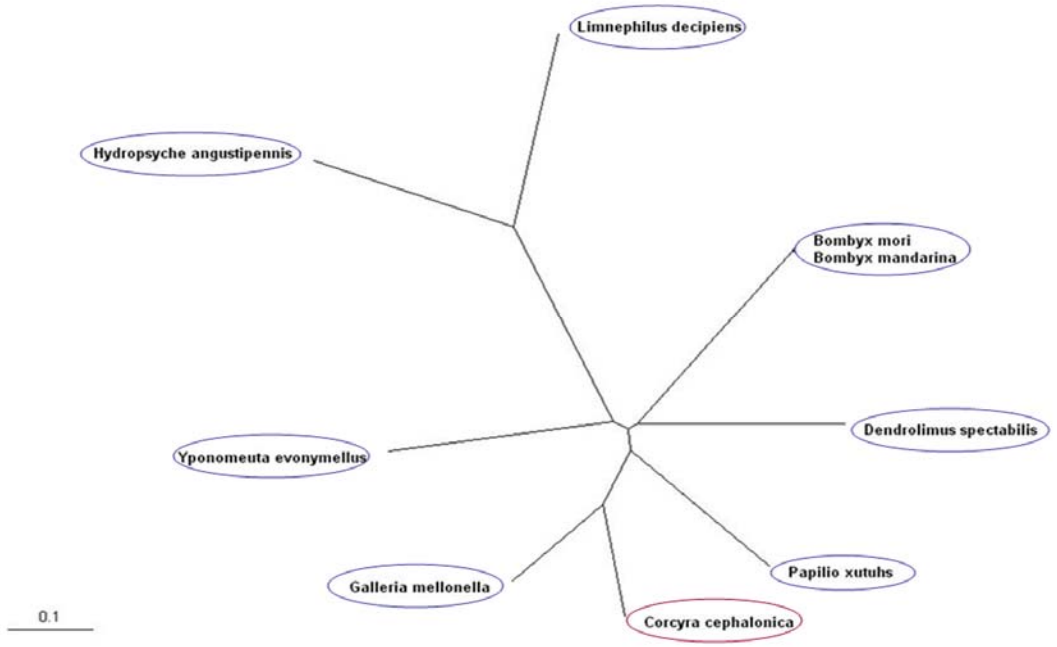

Fig. 11. The phylogenetic tree showing the evolutionary relatedness of *C. cephalonica* L-fibroin protein (red) with that of other known insect species (blue). The scale is depicted on lower left side.

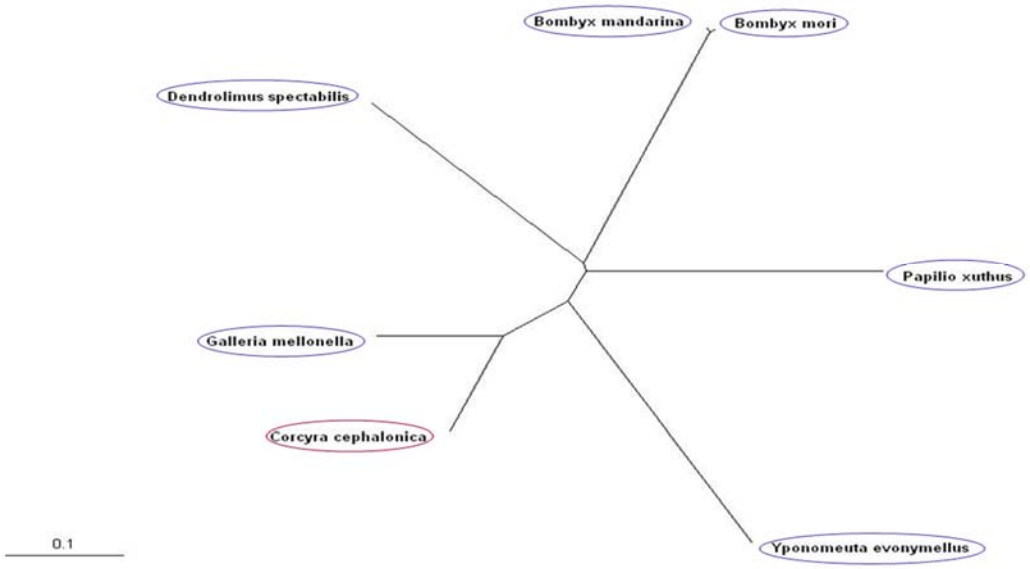

Fig. 12. The phylogenetic tree showing the evolutionary relatedness of *C. cephalonica* P25 protein (red) with that of other known insect species (blue). The scale is depicted on lower left side.

a)

```
  1 atgagagtcacaaccttcgtgatcttgtgctgtgccttgcagtatgtcactgcagatgct
    M  R  V  T  T  F  V  I  L  C  C  A  L  Q  Y  V  T  A  D  A
 61 atcgatgataacattattaacattggcaatgcattcactgaattaagtgaaacaacaacg
    I  D  D  N  I  I  N  I  G  N  A  F  T  E  L  S  E  T  T  T
121 aatgaagttgacgtagaaaatgggactgtatacaaatcgactactcggaaaaagttcgaa
    N  E  V  D  V  E  N  G  T  V  Y  K  S  T  T  R  K  K  F  E
181 agagaaggagactttacaccgaatatatccggtgaggacaagatcgtcagaacttttgtc
    R  E  G  D  F  T  P  N  I  S  G  E  D  K  I  V  R  T  F  V
241 atagaaactgatgcatctggtcatgaaacgatttacgaagaagacgtagttata
    I  E  T  D  A  S  G  H  E  T  I  Y  E  E  D  V  V  I
```

b)

```
  1 ggaggactcggacttggtggatatggaccatacggaggatggggactcggaggagccggt
    G  G  L  G  L  G  G  Y  G  P  Y  G  G  W  G  L  G  G  A  G
 61 ggagctggtggatctcctgtcatcgtcattgaagacaacgcatctgcagccgcagcagca
    G  A  G  G  S  P  V  I  V  I  E  D  N  A  S  A  A  A  A  A
121 gctgccgcatctgccgcaggcgctggaggactcggacttggtggatatggaccatacgga
    A  A  A  S  A  A  G  A  G  G  L  G  L  G  G  Y  G  P  Y  G
181 ggatggggactcggaggagccggtggagctggtggatctgcagctgcagctgcggcagct
    G  W  G  L  G  G  A  G  G  A  G  G  S  A  A  A  A  A  A  A
241 gcaggcgtaagccgagtagtggcccgaccaactgtccgaggagcttgcagccttacacgt
    A  G  V  S  R  V  V  A  R  P  T  V  R  G  A  C  S  L  T  R
301 agacaattcgttgttaaggaattcaatcggatcccgggcccgtcgactgcagaggcc
    R  Q  F  V  V  K  E  F  N  R  I  P  G  P  S  T  A  E  A
```

Fig. 13. Nucleotide (blue) and deduced amino acid (red) sequences of **a)** 5' end and **b)** a partial fragment of *C. cephalonica* H-fibroin. The polyalanine motifs can be seen.

Fig. 14. ClustalW alignment of the deduced 5'-N-terminal regions of H-fibroin of *C. cephalonica*, *G. mellonella*, *Y. evonymella* and *B. mori* (GenBank Accession nos. GQ901977, AF095239, AB195979 and AF226688 respectively). Maximum aligned motifs are represented in rectangles. Arrow (↓) shows putative signal peptide cleavage site.

So far, three types of silk fibroin structures have been elucidated in insects. The H-L/P25 structure was well characterized in *B. mori* (Inoue et al., 2000) and *G. mellonella* (Zurovec and Sehnal, 2002), while in Saturniid moths; the H-H fibroin structure was predominant (Hwang et al., 2001). In case of Trichoptera larvae the silk core comprised of H-fibroin and L-fibroin, but not P25 (Yonemura et al., 2006). Our study confirms the presence of L-fibroin and P25 in *C. cephalonica*. Further, the partial sequences of H-fibroin gene obtained, infer the presence of H-L/P25 elementary unit type in this species. The occurrence of H-fibroin, L-fibroin and P25 in *Y. evonymella* suggests a mechanism of silk fiber formation based on the interaction of these three components evolved at lease 150 million years ago when the ancestors of Yponomeutoidea separated from the rest of Lepidoptera (Yonemura and Sehnal, 2006).

The present study and the published results revealed that some essential features of H-fibroin, L-fibroin and P25 were remarkably well conserved among their homologues. The conserved locations of cysteines and several other residues and the distribution patterns of charge and hydrophobicity indicate which parts of the H-fibroin, L-fibroin and P25 molecules play important role in their mutual interactions (Yonemura and Sehnal, 2006). Also, the high conservation of the signal sequences in the H-fibroin and L-fibroin probably facilitated their entry into the endoplasmic reticulum, but is also important for the linkage of these proteins (Inoue et al., 2003). Moreover, the presence of signal peptide cleavage sites, indicate the secretory nature of all the three proteins.

Extensive investigations in *B. mori* revealed that the first two cysteine residues of L-chain fibroin form intramolecular disulfide bond and the third cysteine residue forms an intermolecular disulphide bond with the H-chain. It is thus likely that the secondary structure is affected by the intramolecular disulphide bond and availability of the most C-terminally situated cysteine residue. Further, the site of disulphide linkage with the H-chain is maintained among L-chain homologues of different Lepidopteran species (Takei et al., 1984, 1987; Tanaka et al., 1999 a). Earlier studies revealed differences in the number as well as the position of glycosylation sites in the amino acid sequence of P25, among the insects. The P25 of *B. mori* contained three separate sugar moieties and it has been proposed that each of them interacts noncovalently with the N-termini of two H-fibroin molecules, so that six H-fibroin proteins are linked to one centrally located P25 protein (Inoue et al., 2000). This model might not be applicable to our insect model, in which P25 contains two glycosylation sites. However, the presence of L-fibroin and probably two glycosylated P25 forms in diverse species further suggests that their interaction/coupling with H-fibroin is a common phenomenon.

The pI value of deduced amino acid sequence of *C. cephalonica* P25 was found to be basic. These values varied among various insect species. In case of *B. mori* and *G. mellonella* it was found to be acidic, while in *Papilio xuthus* and *Dendrolimus spectabilis*, reports suggest its basic nature. These differences might probably facilitate their interaction with the H-fibroin and ensure its proper folding (Tanaka et al., 1999 b; Tanaka and Mizuno, 2001). The presence of purine base at -3 position and AUG as the start codon in the sequences of L-fibroin and P25 infers the presence of Kozak sequence in their leader sequence (Kozak, 1984).

Once the sequences of H-fibroin, L-fibroin and P25 were cloned and analyzed, these genes were characterized for gene copy number, size and number of transcripts, their presence in various tissues and stage dependent expression. However, in case of H-fibroin gene of *C. cephalonica*, the size of transcript as well as the gene copy number was not determined as it is a large protein of 391 kDa in *B. mori* (Inoue et al., 2000) and 500 kDa in *G. mellonella* (Zurovec and Sehnal, 2002) and its corresponding gene would range between 15-20 kb (Lizardi and Brown, 1975). The developmental regulation and tissue expression studies of H-fibroin were performed by real-time PCR using the primers designed from its 5' partial fragment.

Southern analyses identified a single band when digested with *EcoRI, BamHI, HindIII* and *KpnI* and probed with ORF region of either L-fibroin (Fig. 15a) or P25 (Fig. 15b). These results suggest that both these genes exist as a single copy per haploid genome. Northern analyses showed a single 1.1 kb transcript of L-fibroin in the silk gland of *C. cephalonica*, the size of which matches exactly with the corresponding full length cDNA

sequence of 1,125 nucleotides (Fig. 16a). A single 1 kb transcript of P25 was obtained which was expected from its full length cDNA sequence (Fig. 16b). The intensity of the signal observed shows that these genes are highly expressed in the silk gland. The sizes of the transcripts were determined with respect to radiolabeled RNA marker. Further, it was shown that the transcripts for L-fibroin (Fig. 17a) and P25 (Fig. 18a) were specifically found in the silk gland and were not detected in fat body, gut tissue and the remaining body carcass. This finding was reconfirmed by northern blotting (Figs. 17 and 18 b). Similarly, using quantitative PCR, it was shown that H-fibroin mRNA is expressed selectively in silk glands (Fig. 21).

To investigate the stage dependent expression of *C. cephalonica* H-fibroin, L-fibroin and P25, we analyzed transcript levels by quantitative RT-PCR from the total RNA isolated from the silk glands of different stages of insect cycle. The quantitative PCR analysis depicts that the expression of all the three genes (Figs. 19, 20 and 22) increased gradually during larval development from 1^{st} to 5^{th} instar. Thereafter, the expression began to decline during the wandering early-prepupal (EPP) stage and was significantly low at late-prepupal (LPP) stage. The maximum expression of the genes was observed during the late-last instar larval stage.

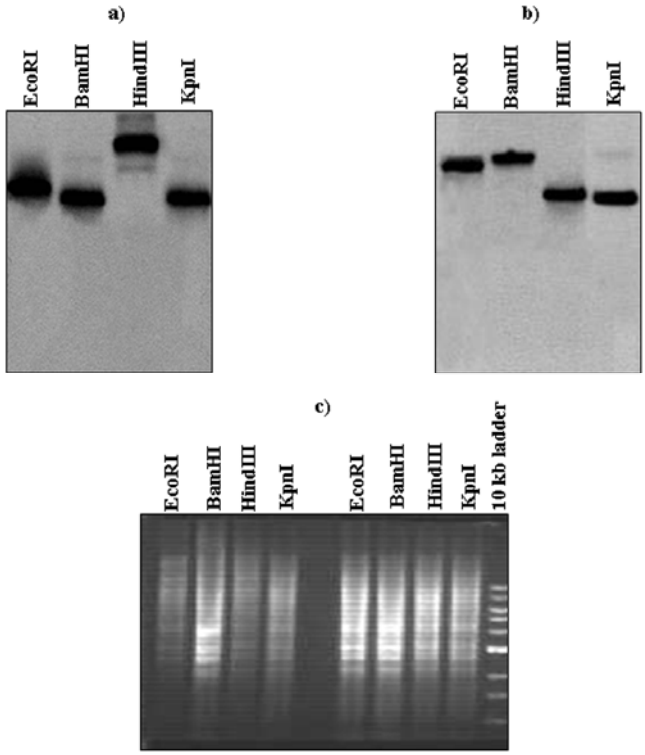

Fig. 15. Southern blot analysis of genomic DNA extracted from *C. cephalonica* salivary gland, digested with *EcoRI* (lane 1), *BamHI* (lane 2), *HindIII* (lane 3) and *KpnI* (lane 4) respectively and probed with plasmid containing full length cDNA of **a)** L-fibroin and **b)** P25. **c)** The profile of DNA digested with *EcoRI* (lanes 1 & 5), *BamHI* (lanes 2 & 6), *HindIII* (lanes 3 & 7) and *KpnI* (lanes 4 & 8) along with the 10 kb ladder (lane 9) is shown.

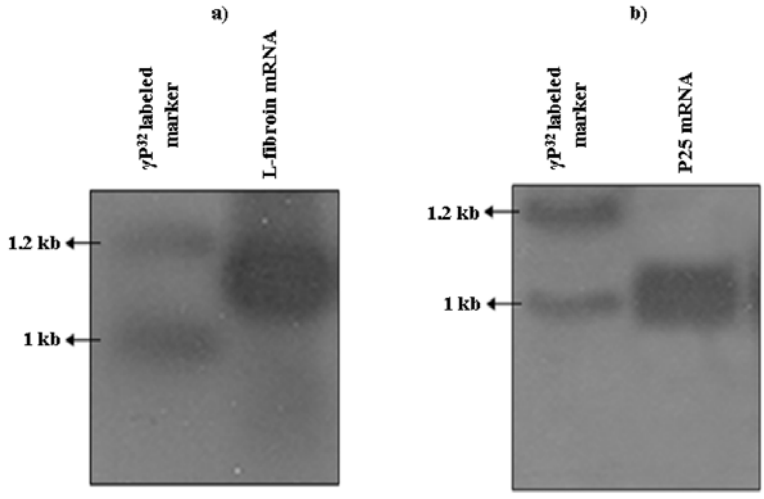

Fig. 16. Northern blots showing the transcript sizes of **a)** L-fibroin (lane 1: marker and lane 2: ~1.1 kb transcript) and **b)** P25 (lane 1: marker and lane 2: ~1 kb transcript).

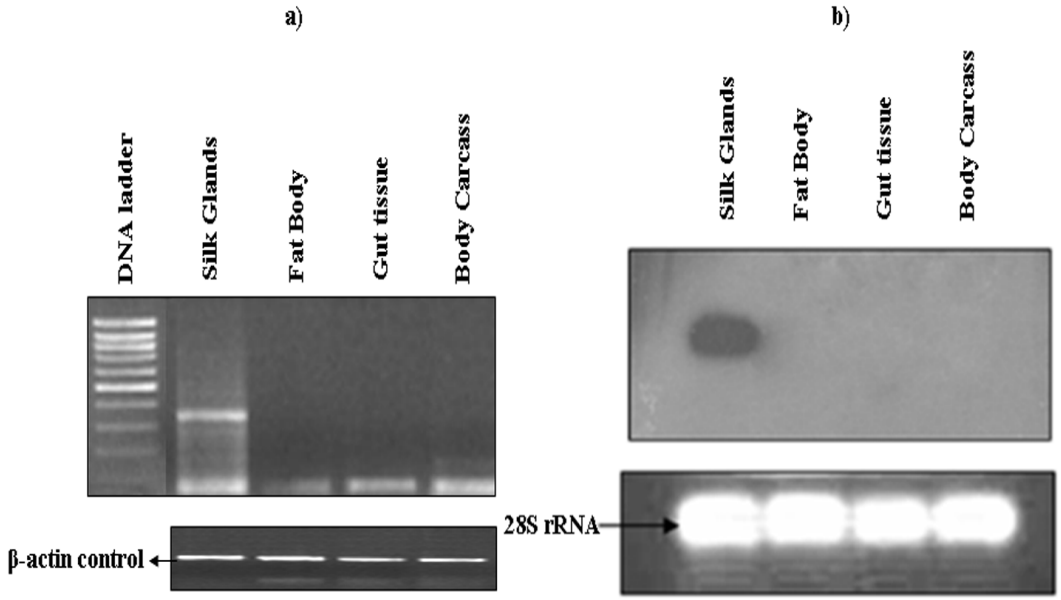

Fig. 17. Tissue specific expression of L-fibroin shown by **a)** semi-quantitative PCR and **b)** northern blotting. The expression is seen in the larval silk glands of *C. cephalonica*. Please note that signal was not detected in the fat body, gut tissue and the remaining body carcass. Equal loading of β-actin and 28S rRNA are shown in lower panel.

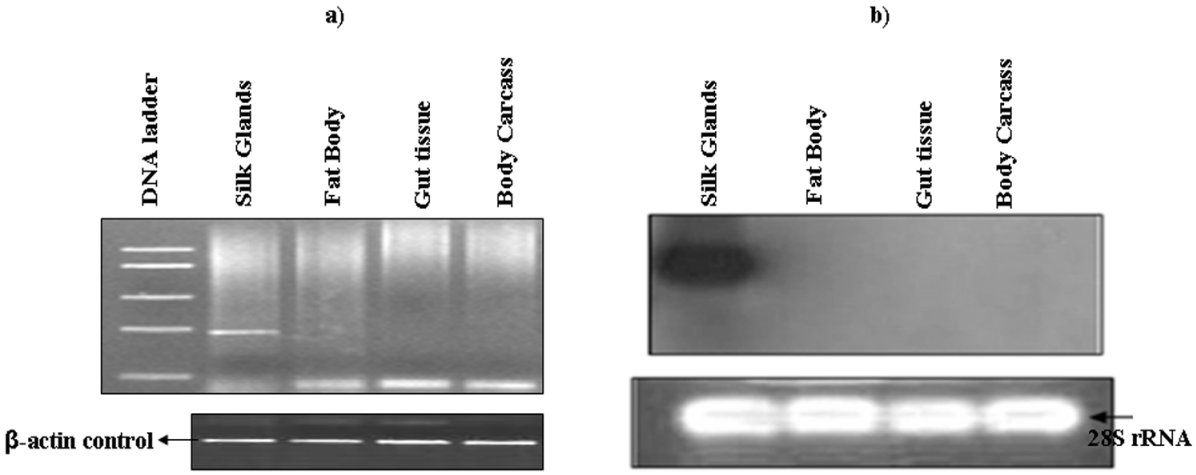

Fig. 18. Tissue specific expression of P25 shown by **a)** semi-quantitative PCR and **b)** northern blotting. The expression is seen in the larval silk glands of *C. cephalonica*. Please note that signal was not detected in the fat body, gut tissue and the remaining body carcass. Equal loading of β-actin and 28S rRNA are shown in lower panel.

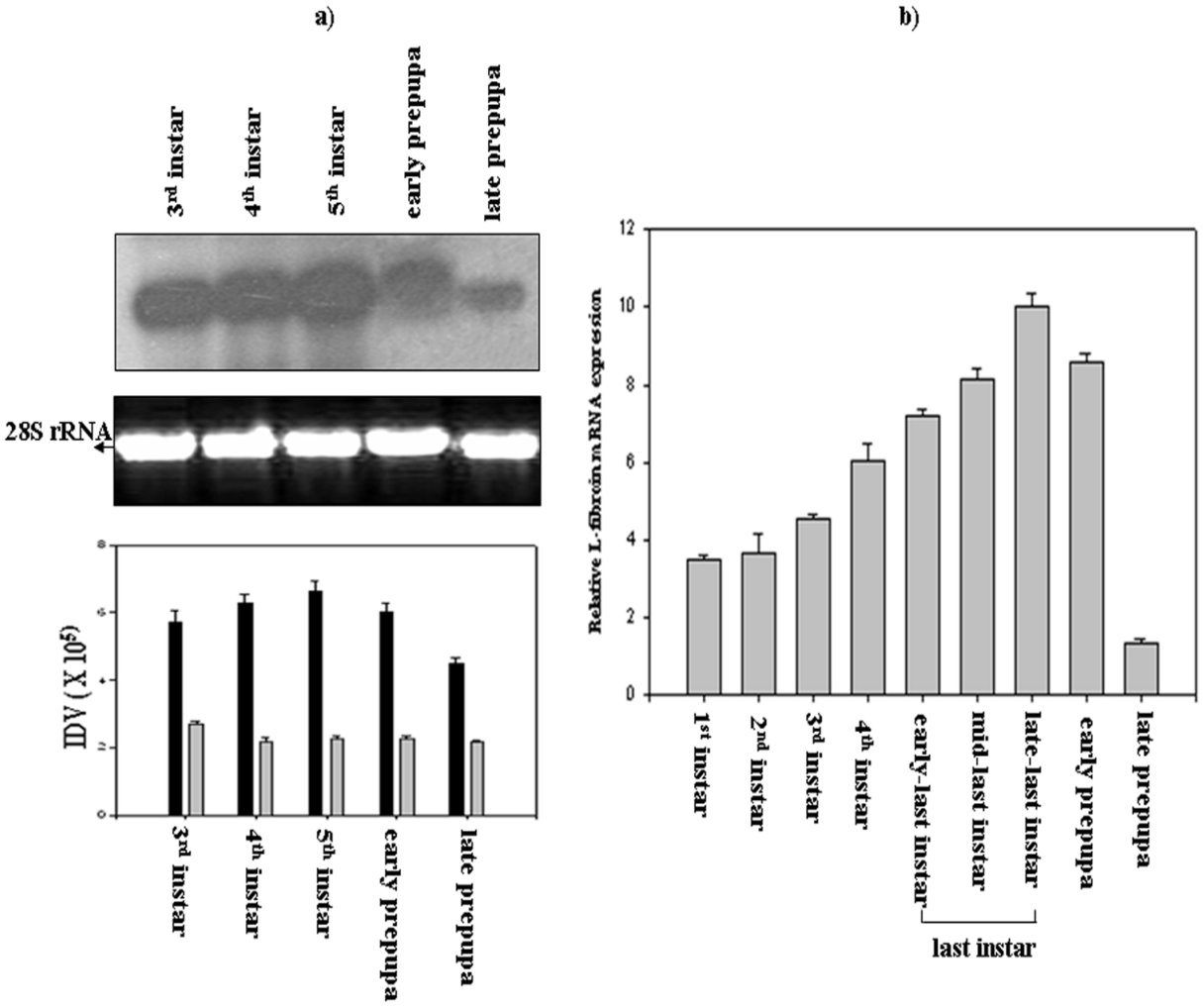

Fig. 19. Analysis of stage-dependent expression of L-fibroin in *C. cephalonica* silk glands by **a)** northern blotting and **b)** real-time PCR. In case of northern blot, equal loading of total RNA (28S rRNA) from all the represented stages is shown in the lower panel. Densitometric analysis was carried out using α- imager software. X-axis represents various developmental stages and the Y-axis represents integrated density values (IDV) multiplied by 10^5. In case of real-time PCR, L-fibroin expression is presented as fold change, relative to late-last instar larvae Each value is expressed as mean ± S.E.M., $n = 3$.

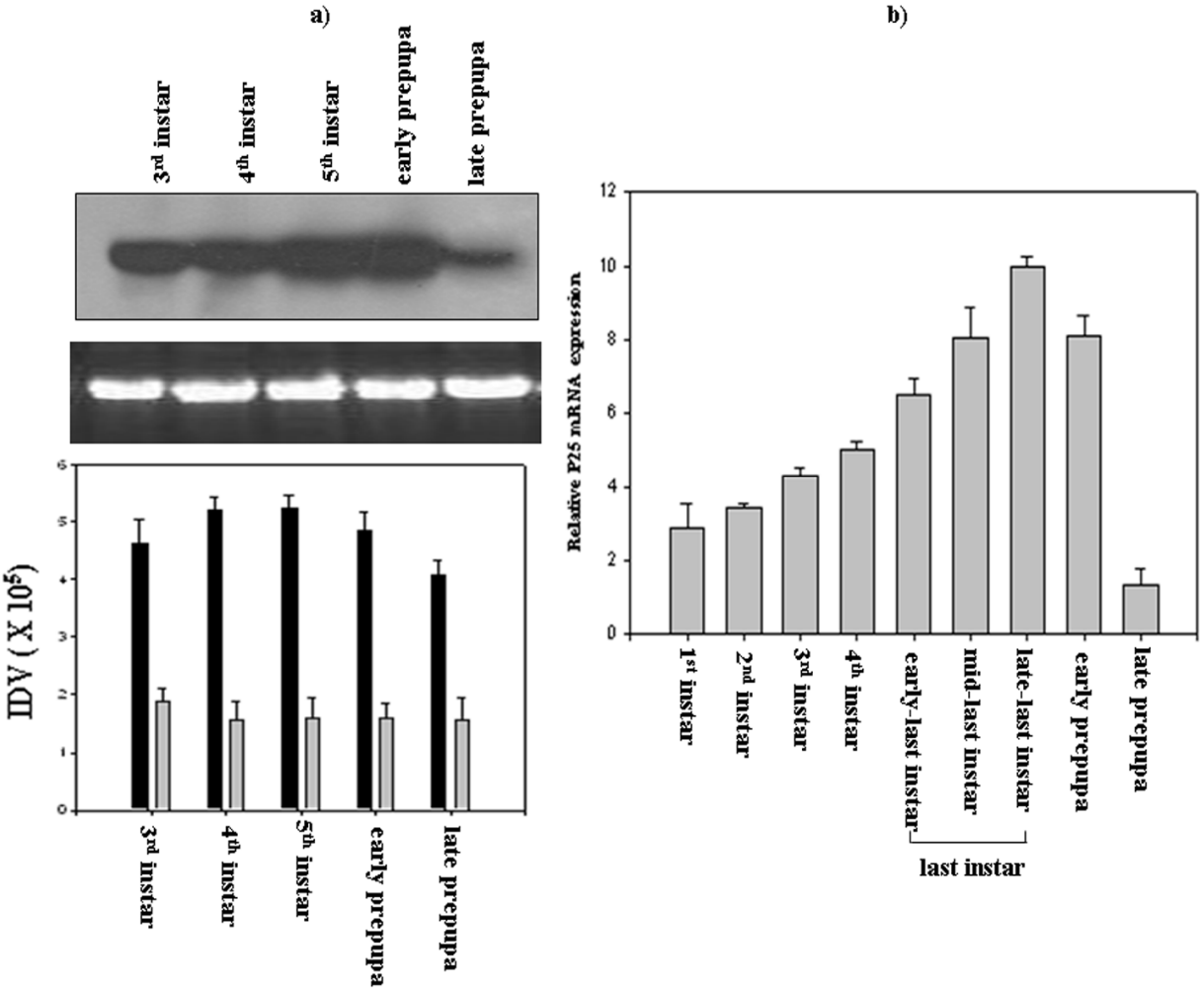

Fig. 20. Analysis of stage-dependent expression of P25 in *C. cephalonica* silk glands by **a)** northern blotting **b)** real-time PCR. In case of northern blot, equal loading of total RNA (28S rRNA) from all the represented stages is shown in the lower panel. Densitometric analysis was carried out using α- imager software. X-axis represents various developmental stages and the Y-axis represents integrated density values (IDV) multiplied by 10^5. In case of real-time PCR, P25 expression is presented as fold change, relative to late-last instar larvae. Each value is expressed as mean ± S.E.M., $n = 3$.

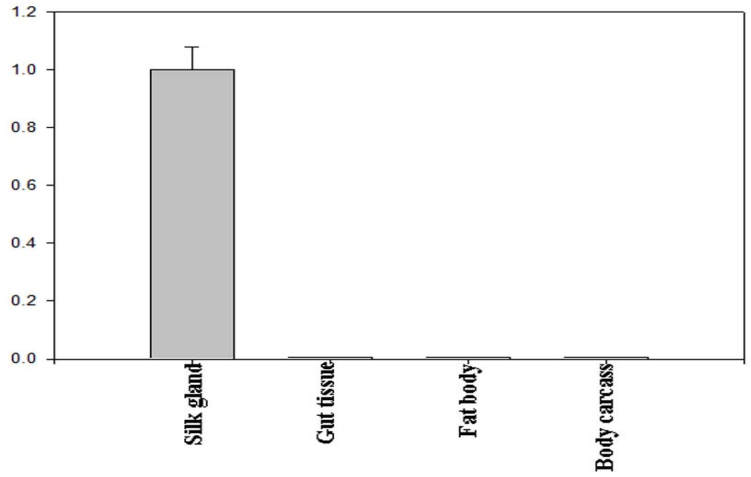

Fig. 21. Tissue specific expression of H-fibroin shown by real-time PCR. The expression is seen in the larval silk glands of *C. cephalonica*. Please note that signal was not detected in the fat body, gut tissue and the remaining body carcass (CS). H-fibroin expression is reported as fold change relative to silk glands. Each value is expressed as mean ± S.E.M., $n = 3$.

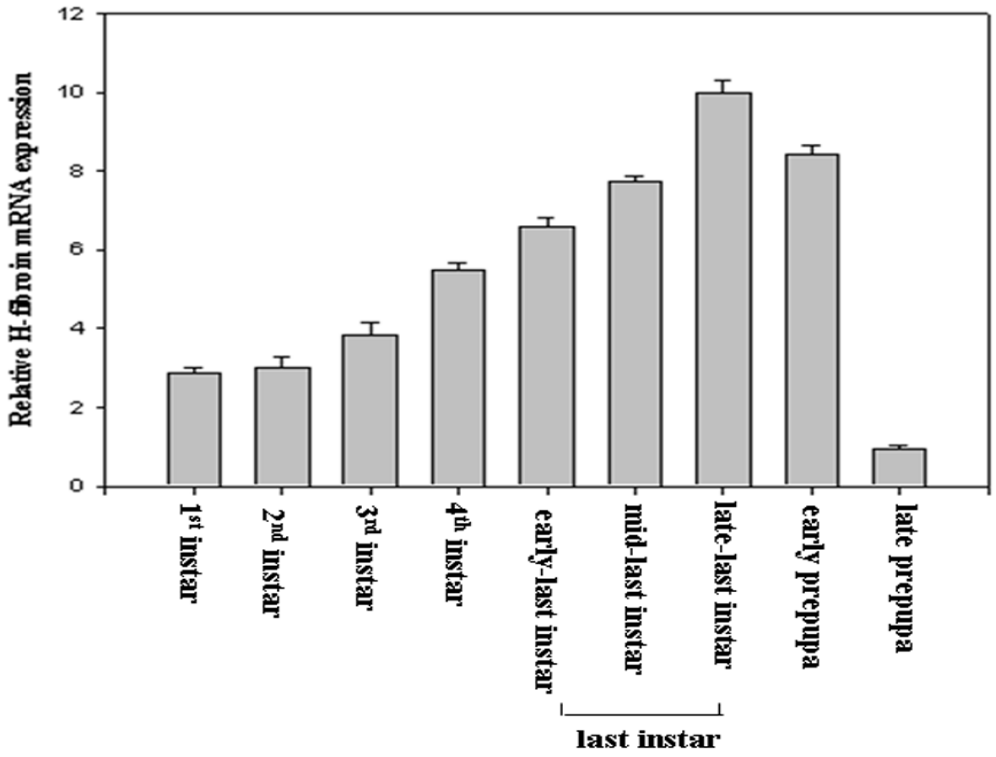

Fig. 22. Analysis of stage-dependent expression of H-fibroin in *C. cephalonica* silk glands by real-time PCR. H-fibroin expression is presented as fold change relative to late-last instar larvae. Each value is expressed as mean ± S.E.M., $n = 3$.

The structure of the L-fibroin gene was elucidated in *B. mori*. The single-copy gene was shown to consist 13,472 nucleotides and its coding region was derived from six exons. (Kikuchi et al., 1992). In addition, a single copy L-fibroin gene was also reported in *G. mellonella* (Zurovec et al., 1995). The P25 gene of *B. mori* was reported to be a single copy gene with 3500 nucleotides (Couble et al., 1985), while in *G. mellonella*, the single copy gene of P25 consisted of about 4,440 nucleotides (Yang et al., 1998; Zurovec et al., 1998 a). The finding in the present study that L-fibroin and P25 are single copy genes in *C. cephalonica* is in accordance with aforementioned reports.

Our studies clearly show that a single 1.1 kb transcript of L-fibroin is expressed in silk glands of *C. cephalonica*. The gene of *B. mori* also transcribed a single mRNA (1180 nucleotides) and homologous transcripts of similar length were found in *B. mandarina*, *D. spectabilis* and *P. xuthus*. However, *G. mellonella* salivary glands showed the presence of two distinct transcripts one of 1192 nucleotides and a shorter one of 1104 nucleotides, that is truncated at the non-translated 3' terminus (Tanaka and Mizuno, 2001). The presence of only one polyadenylation site in L-fibroin gene of *C. cephalonica* which is identical with that of *B. mori* might account for the presence of single transcript. In case of *G. melonella,* L-fibroin gene was shown to contain multiple polyadenylation sites and therefore exhibit alternative splicing and hence form two different transcripts (Zurovec et al., 1995).

The P25 mRNA of *C. cephalonica* is found to be approximately 1 kb and it is shown to be expressed as a single transcript in silk glands. Earlier studies in *B. mori* also revealed a single P25 mRNA of about 1.3 kb, (Tanaka and Mizuno, 2001) while the homologous mRNA of *G. mellonella* was around 2 kb due to the extension at the non translated 3' end

(Yang et al., 1998; Zurovec et al., 1998 a). A much longer 3' extension occurred in one of the two P25 mRNAs of *D. spectabilis*, whose sizes were reported to be 4.4 kb (major) and 1.2 kb (minor) respectively (Tanaka and Mizuno, 2001).

Northern and real-time analyses revealed the tissue-specific expression of all the three genes, which is seen only in the silk glands of *C. cephalonica*. The expression was found to be specific to silk gland. In lepidopteran insects, several putative cis-elements were identified. The binding sites for BMFA, SGFB, PSGF and homeoproteins are considered to be responsible for silk gland specific expression of all the three silk protein genes (Hui et al., 1990). BMFA is a ubiquitous protein, proposed to be involved in the repression of fibroin genes at molting (Durand et al., 1992). SGFB is a silk gland-specific regulatory protein expressed in both PSG and MSG, but has access to its target sequence only in PSG cells. PSGF is a factor deduced from DNase I protection assay, supposed to be expressed only in PSG and to facilitate the recruitment of SGFB (Horard et al., 1997). Durand et al., (1992) introduced modified P25 gene of *Bombyx* into *Drosophila* and identified the region - 437 to + 76 as sufficient to drive the tissue specific expression of this silk gene. In addition, in *B. mori*, a 691 bp (- 650 nt to + 41 nt) functional *L-fibroin* promoter with PSG transcriptional specificity, in which the binding sites of SGFB, BMFA, and PSGF could be recognized was reported (Guo et al., 2005). The upstream region analysis of H-fibroin gene of *C. cephalonica* showed the presence of SGFB and homeoprotein binding sites which is in accordance with the earlier reports. However, the presence of such sites in the upstream of L-fibroin and P25 genes need to be investigated.

In the present study, developmental expression of all the three genes revealed a synchronous pattern. Highest expression was seen during the LLI larval stage, which

declined towards the end of prepupal stage (LPP) where the insect stops feeding and commits to pupa formation. Earlier, it was shown that lepidopteran silk glands become secretory prior to hatching. During the larval life these glands increase in their secretory potential by both, growth as well as increase in ploidy. Silk proteins are alternatively expressed (intermoult) and repressed (moult), leading to periodic bursts of silk production. Their transcription is highest during the fifth intermoult, especially in those species that use the silk primarily or exclusively for cocoon formation (Sehnal and Akai, 1990). These studies further strengthen our data, both northern and real-time studies, show a gradual increase in the expression of silk genes from 1st larval instar to LLI larval stage of *C. cephalonica* and clearly suggest that the transcription of these genes is developmentally regulated. The expression of these genes during 1st and 2nd instars was not shown in northern blots as it was practically impossible to dissect the silk glands for RNA isolation from these stages. Our study becomes the first report of developmental regulation of these genes at molecular level.

In order to characterize H-fibroin, L-fibroin and P25 proteins, we carried out further studies. Restriction enzyme digested ORF regions of L-fibroin and P25 were cloned into expression vector PET28a and the conditions were optimized for expression with various concentrations of IPTG, in the host cells, BL21 (DE3). The expressed proteins were gel eluted and were injected into the New Zealand white rabbit for generation of antibodies. The generated anti-L-fibroin and anti-P25 antibodies were purified and used for further characterization studies. In case of H-fibroin, a different strategy was used to generate antibodies. Based on the deduced amino acid sequence of the H-fibroin partial fragment, a polylanine peptide conjugated with KLH carrier protein was commercially synthesized and used. The antibody raised was used for further characterization of H-fibroin protein.

C. cephalonica L-fibroin recombinant protein was over expressed upon induction with 0.2 mM IPTG concentration (Fig. 23a). The expressed protein was predominantly present in the pellet fraction after sonication. The pellet was dissolved in PBS (pH 7.4). The molecular weight of the protein matched with the expected size of 30 kDa including the short tag of histidine amino acid residues similar to 5 kDa on SDS-PAGE (10 %). This protein was immunoblotted and further probed with anti-His-antibody to confirm its expression (Fig. 23b). This protein was eluted from the polyacrylamide gel using the protocol (described in materials and methods section) and was injected into the New Zealand white rabbit for generation of polyclonal antibody. The sera was collected and purified for IgG fraction (Fig. 24a). The silk gland protein homogenate that was blotted onto nitrocellulose membrane was probed using the anti-L-fibroin antibody and the molecular weight of L-fibroin was found to be 25 kDa (Fig. 24b).

As mentioned in the above paragraph, *C. cephalonica* P25 recombinant protein was over expressed. The optimal induction was found with 0.1 mM IPTG concentration (Fig. 25a). The expression was prominent in the pellet fraction after sonication. The pellet was resolubilized in PBS (pH 7.4). When probed with anti-His antibody, the recombinant protein was shown to be approximately 30 kDa on a 12 % SDS-PAGE that includes a short tag of histidine amino acid residues similar to 5 kDa (Fig. 25b). This expressed protein was separated on polyacrylamide gel and eluted out (described in materials and methods section) for generation of polyclonal antibodies. The generated polyclonal antiserum against P25 protein was purified for IgG fraction (Fig. 26a). The silk gland protein homogenate that was blotted onto nitrocellulose membrane was probed using the anti-P25 antibody and the molecular weight of P25 was found to be 28 kDa (Fig. 26b).

In case of H-fibroin, the commercially procured synthetic peptide (KLH-conjugated polyalanine) was dissolved directly in PBS (pH 7.4) and was injected into the rabbit. After the primary as well as two booster doses was given, the rabbit was bled and the serum was collected. The serum that contained anti-polyalaine antibody was purified for IgG fraction (Fig. 29a). The silk gland protein homogenate was separated by SDS-PAGE and electroblotted onto a nitrocellulose membrane. Immunodetection using the generated antibody revealed the molecular weight of *C. cephalonica* H-fibroin to be around 350 kDa (Fig. 29b). Immunocytochemical analysis with this anti-polyalanine antibody also demonstrated its strong cross-reactivity with the polyploid glandular cells as well as lumen of the silk glands (Figs. 31 and 32).

Fat body, gut, hemolymph, silk gland and body carcass tissues of *C. cephalonica* were western blotted and probed with all the three generated polyclonal antibodies, the signals were detected in the lane that was loaded with silk gland protein homogenate only (Figs. 27 and 30). The developmental profile of H-fibroin, L-fibroin and P25 proteins follow a similar pattern as that of their respective transcripts. The expression gradually increased from 3rd instar to LLI larval stage where the highest expression was observed during the LLI stage. Thereafter, the expression declined towards the end of prepupal stage (Figs. 28 and 33).

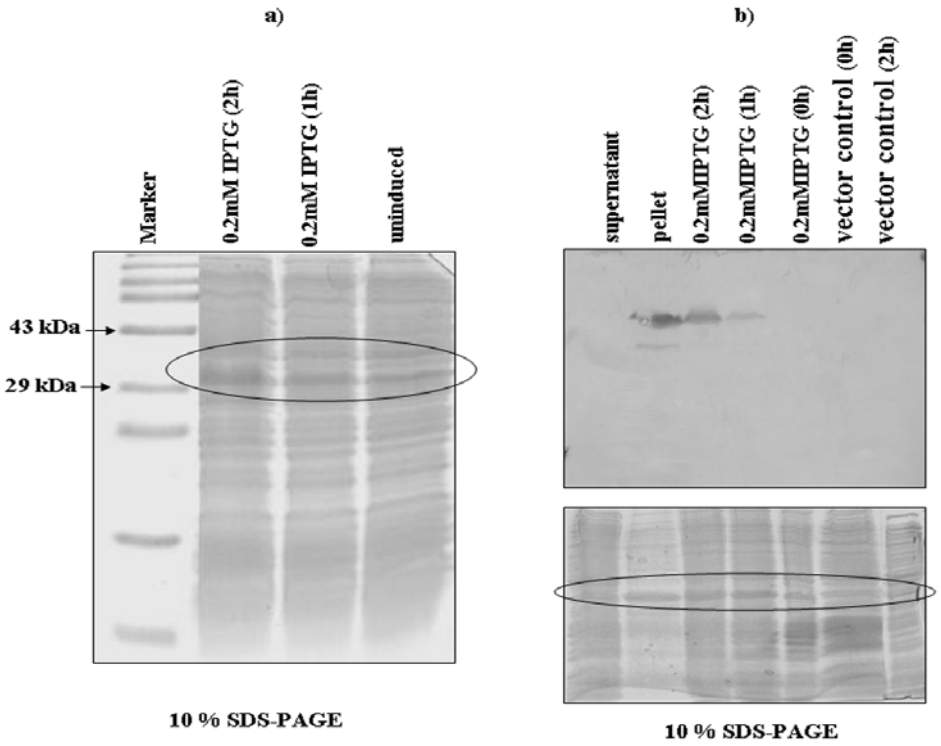

Fig. 23. a) Expression of recombinant L-fibroin protein of *C. cephalonica*. Please note the over expression in 2 h IPTG induced cultures **b)** Confirmation of the expressed protein shown by mouse anti-His antibody detection. The cross reactivity is exclusively seen in the pellet and the IPTG induced bacterial lysate while it's totally absent in vector control bacterial lysates. The corresponding protein gel is shown as control in the lower panel.

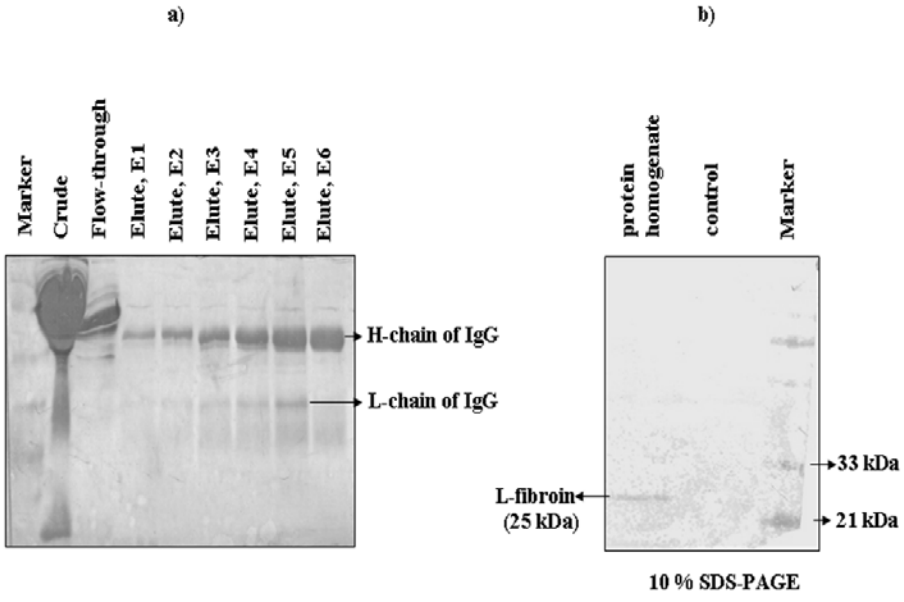

Fig. 24. a) SDS-PAGE showing the purification profile of serum IgG fraction generated against recombinant L-fibroin. **b)** Western blot analysis of salivary gland L-fibroin using anti-rabbit L-fibroin IgG. For control lane protein homogenate from gut was loaded.

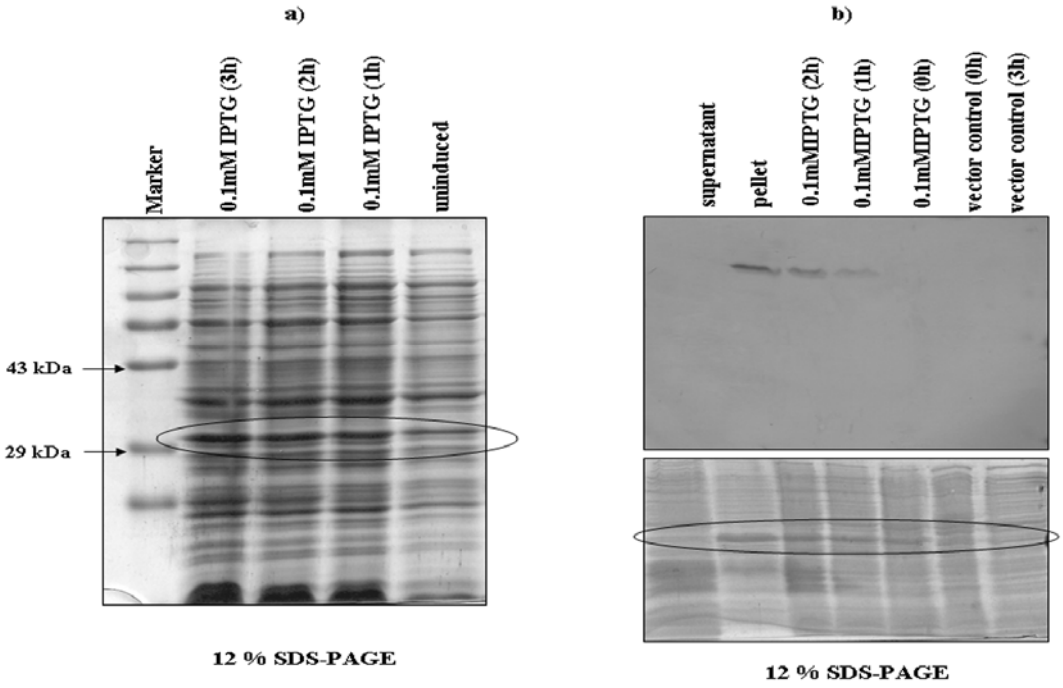

Fig. 25. a) Expression of recombinant P25 protein of *C. cephalonica*. Please note the overexpression in 3 h IPTG induced cultures **b)** Confirmation of the expressed protein shown by mouse anti-His antibody detection. The cross reactivity is exclusively seen in the pellet and the IPTG induced bacterial lysate while it's totally absent in vector control bacterial lysates. The corresponding protein gel is shown as control in the lower panel.

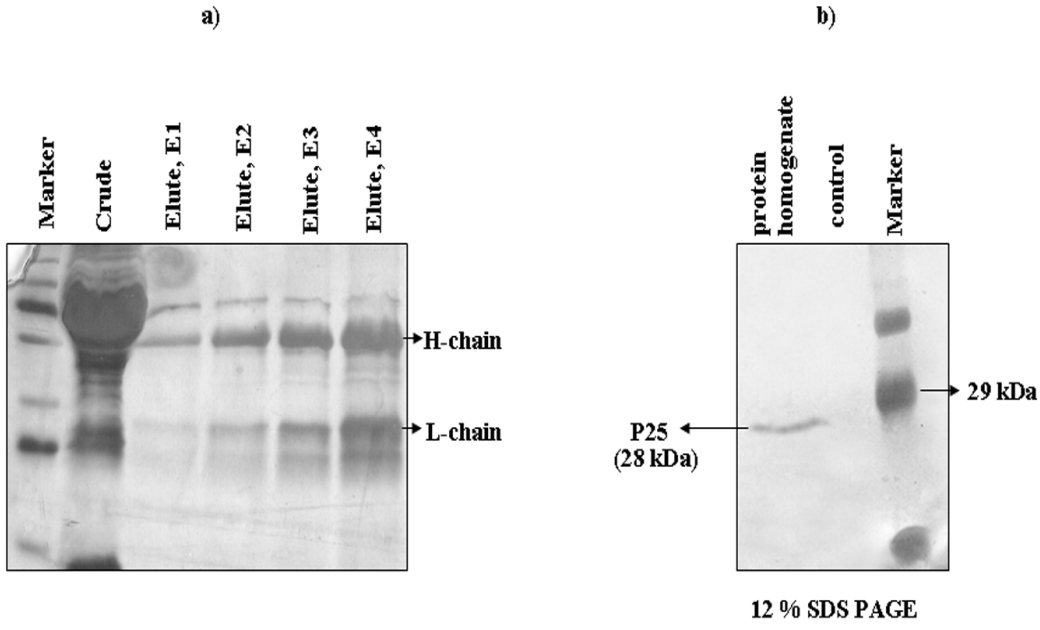

Fig. 26. a) SDS-PAGE showing the purification profile of serum IgG fraction generated against recombinant P25. **b)** Western blot analysis of salivary gland P25 using anti-rabbit P25 IgG. For control lane protein homogenate from gut was loaded.

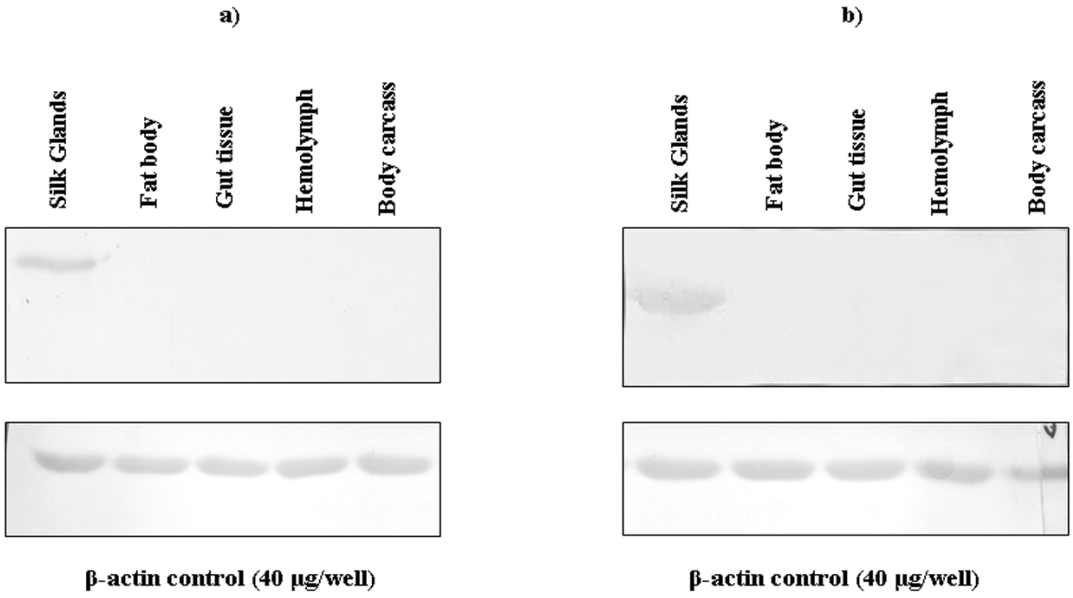

Fig. 27. Immunoblots showing the tissue distribution profile of **a)** L-fibroin and **b)** P25 proteins along with the β-actin control.

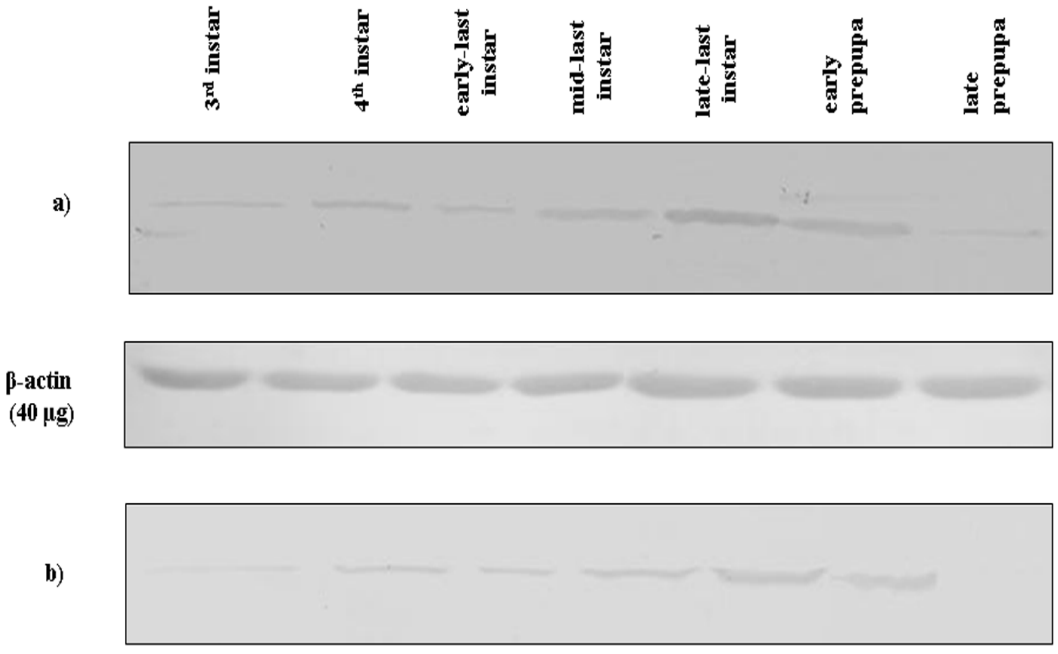

Fig. 28. Immunoblots showing the developmental profile of **a)** L-fibroin and **b)** P25 proteins from 3rd larval instar to late-prepupal stage along with β-actin control

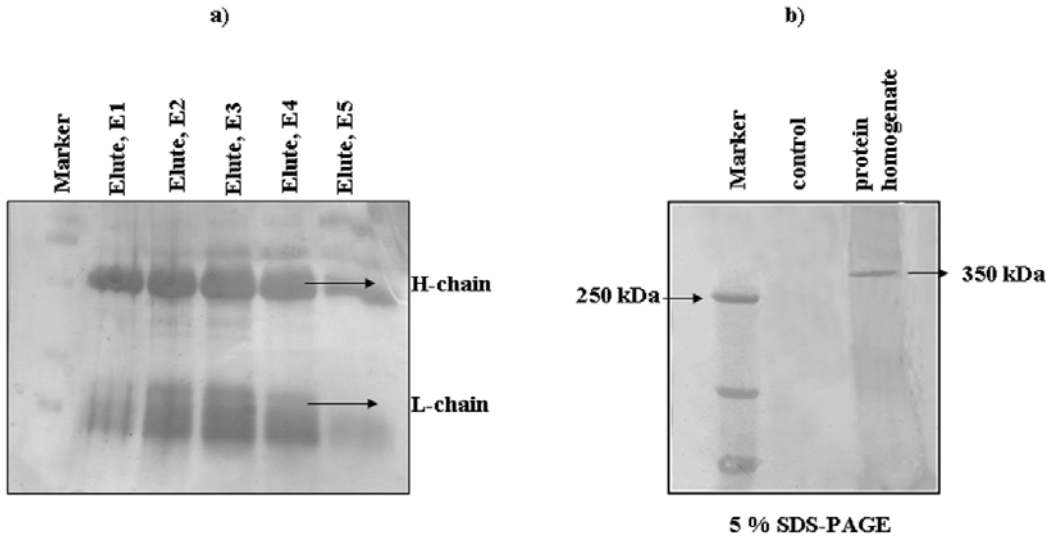

Fig. 29. a) SDS-PAGE purification profile of serum IgG fraction generated against synthetic KLH conjugated polyalanine peptide **b)** Western blot analysis of H-fibroin using anti-rabbit polyalanine IgG. Note the presence of a high molecular weight (350 kDa) protein detected in the salivary gland homogenate which is absent in control (gut homogenate).

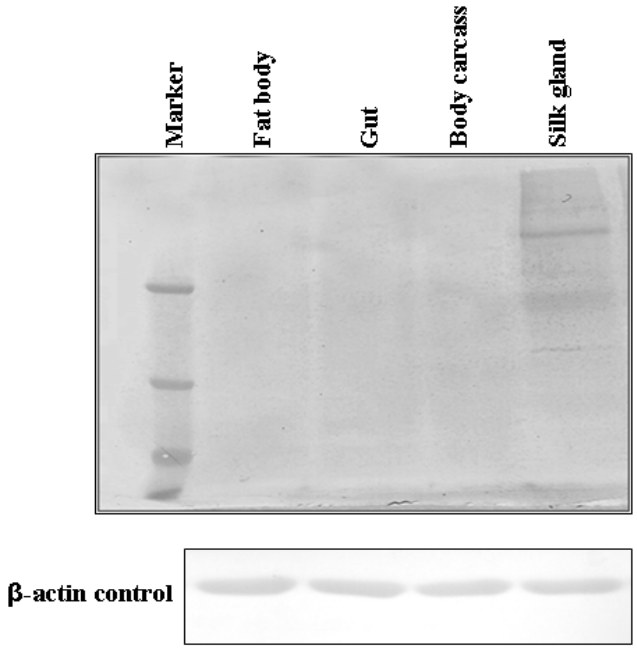

Fig. 30. Western blot showing the tissue distribution profile of H-fibroin protein along with the β-actin control using anti-rabbit polyalanine antibody

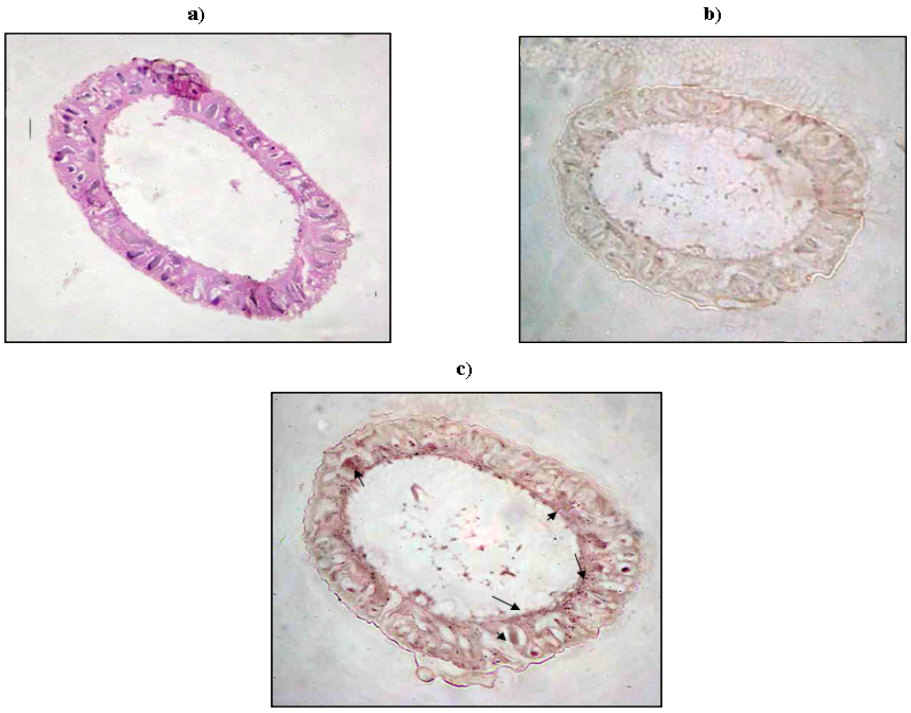

Fig. 31. Immunocytochemical localization of H-fibroin in *C. cephalonica* silk glands (saggital section). **a)** H & E stained section **b)** control pre-immune serum treated section and **c)** immunoreactivity with the anti-rabbit polyalanine antibody. The silk gland lumen and the cytoplasm of the polyploidy cells show strong cross reactivity with the antibody.

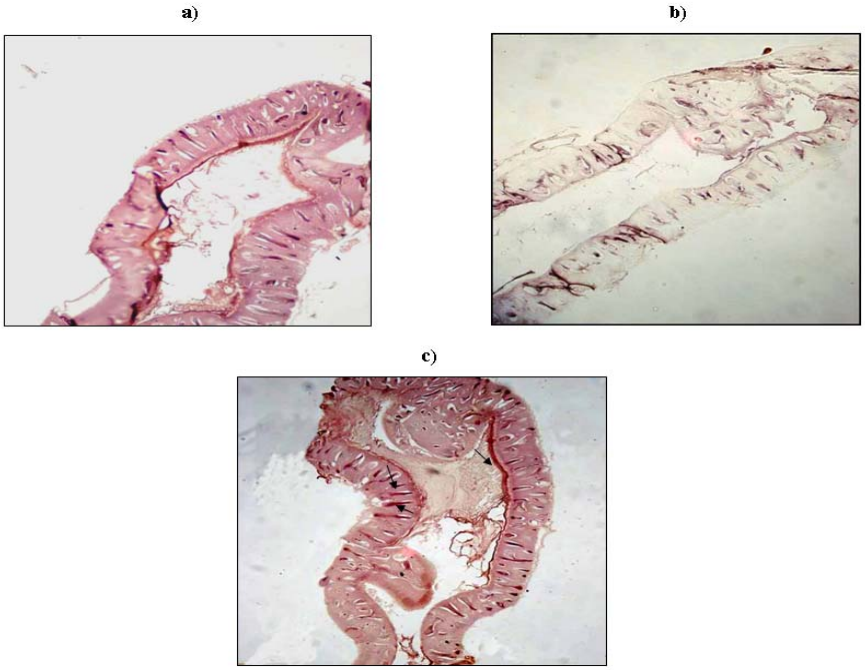

Fig. 32. Immunocytochemical localization of H-fibroin in *C. cephalonica* silk glands (cross section). **a)** H & E stained section **b)** control pre-immune serum treated section and **c)** immunoreactivity with the anti-rabbit polyalanine antibody. The silk gland lumen and the cytoplasm of the polyploidy cells show strong cross reactivity with the antibody.

Fig. 33. Developmental profile of H-fibroin protein from 3rd instar larvae to late-prepupal stage along with β-actin control (lower panel).

The finding that the mature L-fibroin protein of *C. cephalonica* whose deduced sequence shows presence of 251 amino acid residues with a predicted M_r of 25.4 kDa, is in agreement with the molecular weight of L-fibroin protein detected using the anti-L-fibroin antibody. Similar molecular weight of L-fibroins of *B. mori* (Kimura et al., 1985; Yamaguchi et al., 1989) and *G. melonella* (Zurovec et al., 1995) were reported earlier. The mature P25 protein of *C. cephalonica* would contain 202 amino acid residues with a deduced M_r of 23 kDa. However, the molecular weight of P25 protein detected in the present study using anti-P25 antibody is found to be approximately 28 kDa. This difference in the molecular weight is most likely associated with the possibility of glycosylation as the cDNA sequence shows the presence of two potent glycosylation sites. Various levels of glycosylation of P25 protein were also reported in insects, *B. mori* and *G. mellonella* and the molecular weights of P25 ranged between 27-30 kDa, in them (Tanaka et al., 1993; Zurovec et al., 1998 a; Tanaka and Mizuno, 2001).

In the present study several attempts were made to clone full length cDNA of H-fibroin of *C. cephalonica*. However, this was not possible because of several repeat sequences. Therefore, a different strategy was used for generation of polyclonal. The partial fragment of H-fibroin cloned in the present study, has several polyalanine repeats in its deduced amino acid sequence. These polyalanine motifs are approximately of 6-10 residues and were also found in the H-fibroin proteins of *G. mellonella* and *P. interpunctella* (Sehnal and Zurovec, 2004) which are phylogenetically related to *C. cephalonica*. Based on this a KLH-conjugated $(polyalanine)_{10}$ peptide was procured commercially. The KLH-conjugated peptide becomes highly immunogenic. The large size and distinct epitopes of KLH generate a substantial immune response and abundance of lysine residues couple the hapten (polyalanine peptide) and allows a high hapten : carrier protein ratio increasing the likelihood of generating hapten-specific antibodies. The molecular weight of H-fibroin in the salivary gland of *C. cephalonica* detected with the generated antibody is found to be approximately 350 kDa. This matched with the size of H-fibroin found in *B. mori* (350 kDa), *G. mellonella* (500 kDa) and *Antheraea* species (200-250 kDa) (Inoue et al., 2000; Hwang et al., 2001; Zurovec and Sehnal, 2002). It is well documented that silk proteins are synthesized in glandular polyploid cells with ramified nucleus and are secreted out into the lumen (Sehnal and Akai, 1990). The immunolocalization studies of H-fibroin protein in the silk glands of *C. cephalonica* also show intense immuno cross-reactivity of the anti-rabbit polyalanine antibody in the lumen as well as in cytosol of the polyploidy cells of salivary gland.

The detection of H-fibroin selectively in the silk glands further reiterates the specificity of the generated antibody. The tissue specific nature of these proteins as well their stage specific expression in *C. cephalonica* demonstrated that their translational profile

corroborates with that of the transcriptional pattern. The synchronous developmental regulation pattern seen for H-fibroin, L-fibroin and P25 at mRNA and protein level suggests that these three proteins are co-expressed in the silk glands of *C. cephalonica*.

RESULTS AND DISCUSSION

PART II

Effect of hormones (20E and JH analog, methoprene) on H-fibroin, L-fibroin and P25 gene expression during last-instar larval development

Two insect hormones namely, 20-hydroxyecdysone (20E) and juvenile hormone (JH) regulate large number of physiological as well as metabolic processes that occur in an insect life cycle. However, the regulation of silk genes in insects by these hormones, both independently as well as synergistically need to be evaluated in detail. In the present work, an attempt was made to study the effect of 20E and a JH analog, methoprene, on the expression of H-fibroin, L-fibroin and P25 genes using organ culture.

Initially, we checked the viability of silk glands in insect culture medium through various time points. Later, different concentrations of 20E and methoprene were used to monitor the expression of silk protein genes. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] staining showed that the silk glands are viable in the culture medium till 24 h (Fig. 34). Hence, a time period of ½ h to 24 h were chosen for hormone treatment studies.

For the present study, level of 20E in the hemolymph was estimated by enzyme immunoassay. During ELI, LLI larval and EPP stages the concentration was found to be 0.008 pg/µl, 0.4 pg/µl and 5.7 pg/µl respectively (Fig. 35) and these were considered as physiological. For further studies the effect of physiological as well as higher concentrations of 20E on the expression of H-fibroin, L-fibroin and P25 genes was analyzed. The transcript level of ethanol treated glands, which were cultured through the same time points were used as external control. The results clearly show that at the physiological concentration of 20E expression of all the three genes remained unaltered at both LLI and ELI larval stages when compared with the respective controls (Figs. 36-41). However, when higher concentrations of 20E were used (i.e. 0.4 pg/µl in case of ELI stage silk glands and 4 pg/µl and 5.7 pg/µl in case of LLI stage silk glands) the transcript

level declined significantly (Figs. 36-41). This study clearly shows that physiological dose of 20E does not alter the normal expression of these genes, whereas treatment with higher doses causes a significant decline in the expression of all the three silk genes.

For evaluating the effect of JH, its analogue methoprene was selected and concentrations similar to that of 20E were used. The expression of H-fibroin, L-fibroin and P25 remained unaltered at various concentrations of methoprene used (0.4 pg/ µl or 4 pg/ µl or 0.4 ng/µl) during the last-instar larval development (Figs. 42-44). However, addition of 20E to salivary gland cultures at the concentration (5.7 pg/µl) that declined the expression of the silk genes (Figs. 36-41) followed by methoprene treatment (0.4 pg/ µl or 4 pg/ µl or 0.4 ng/µl), retrieved back the declined gene expression to normal level as seen during the LLI stage (Figs. 45-47). This study suggests that methoprene counteracts the 20E dependent inhibition of the expression of these genes during late-last instar larval development and prevents the decline in expression which is seen after 20E (5.7 pg/µl) treatment.

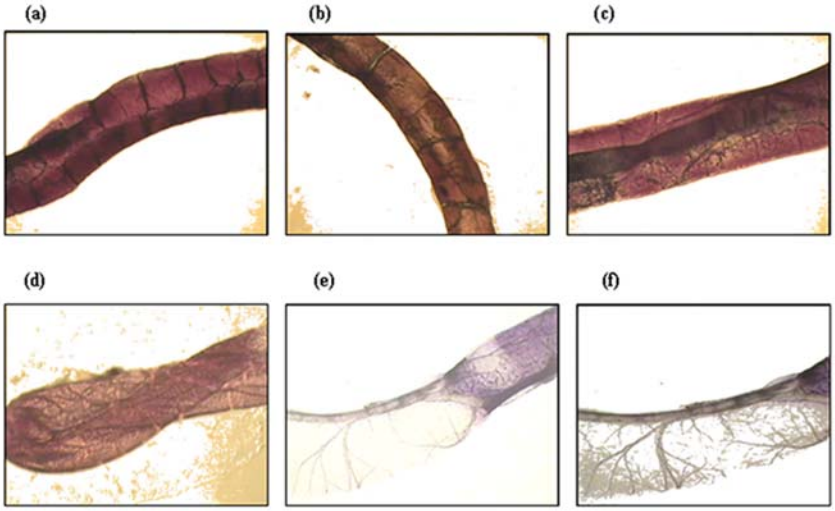

Fig. 34. Micrographs showing the MTT stained cultured silk glands of *C. cephalonica*. (a) Positive control: freshly excised salivary gland (b) salivary gland cultured for 3 h, (c) 6 h, (d) 12 h, (e) 24 h and (f) negative control: heat killed salivary gland. All the images were taken at 40 X magnification.

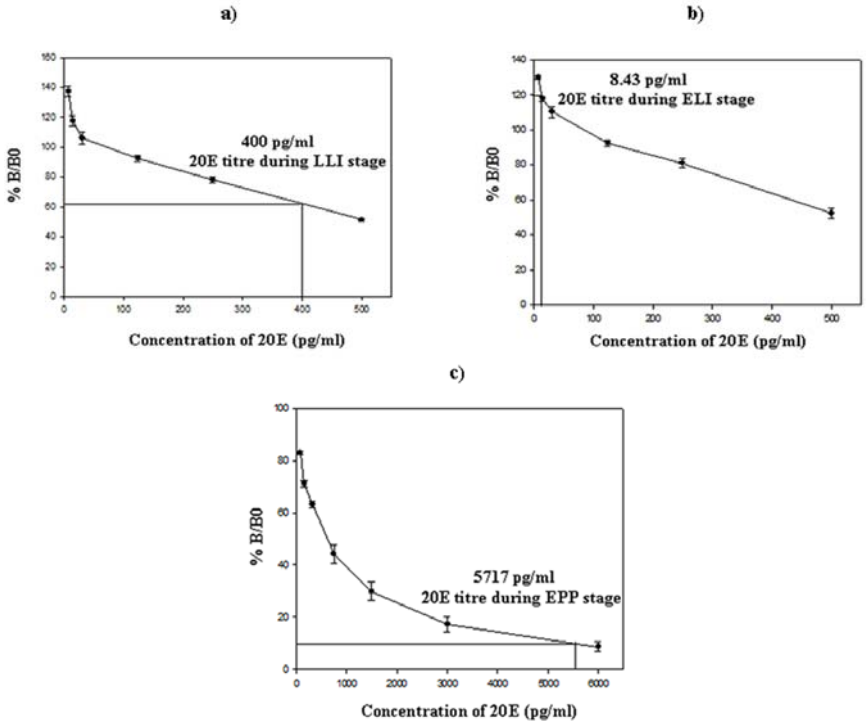

Fig. 35. Enzymeimmunoassay (EIA) as standard curve of 20E. X-axis represents the concentration of 20E in pg/ml. Y-axis represents the $\%B/B_0$ (% sample or standard bound/Maximum bound). The experiment was done in triplicates and the data is expressed as ± S.E.M., n=3. The $\%B/B_0$ value of the test sample was extrapolated onto the X-axis and the haemolymph levels of 20E during the a) LLI larval stage b) ELI larval stage and c) EPP stage was calculated.

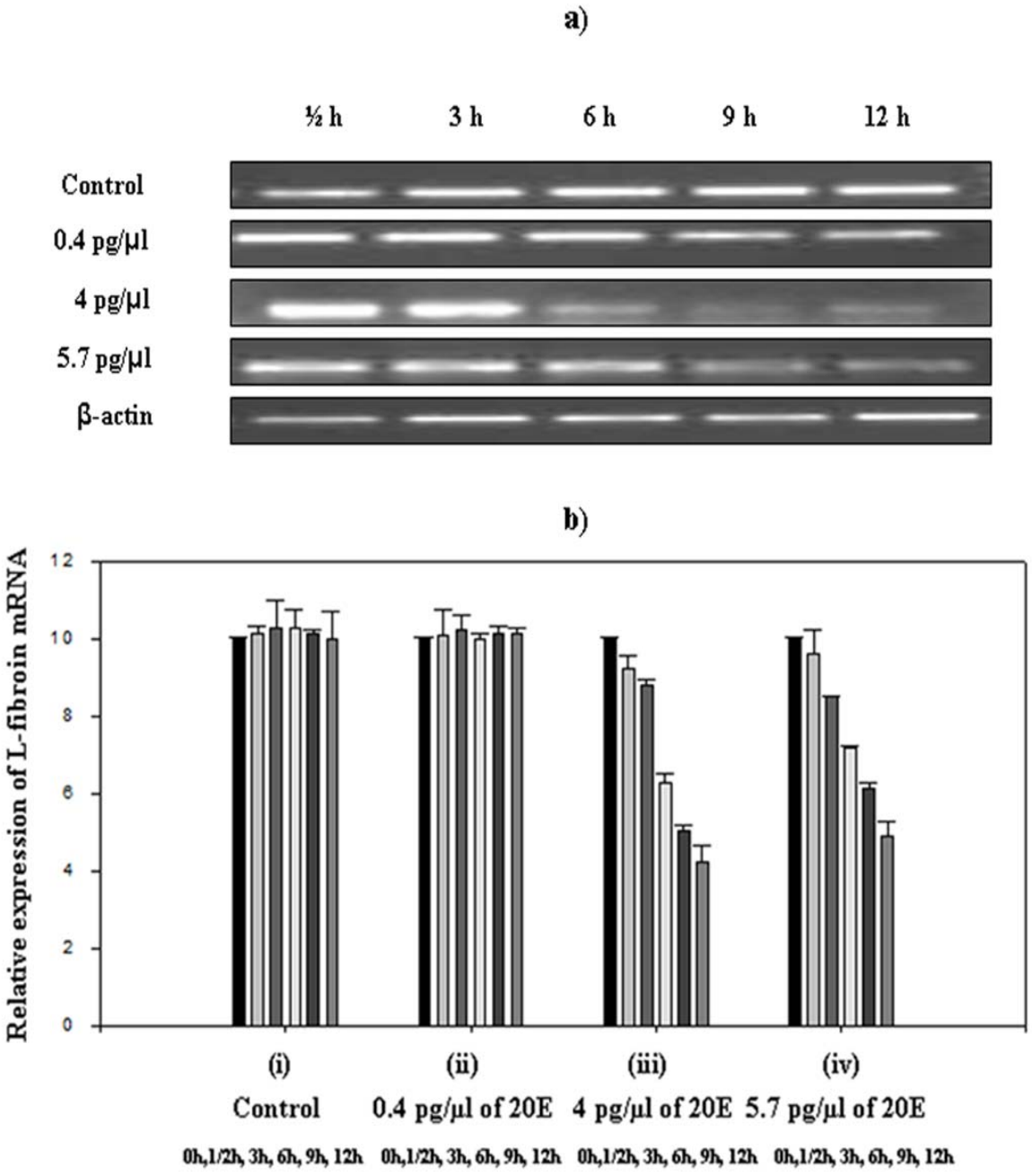

Fig. 36. Effect of 20E on L-fibroin expression of LLI larval silk glands a) Semi-quantitative PCR showing the expression at time points ½ , 3 , 6 , 9 and 12 h in control (ethanol treated) and hormone treated glands (0.4 pg/µl, 4 pg/µl and 5.7 pg/µl of 20E). β-actin was used as internal control. b) Real-time PCR (n=3) showing the expression at 0 , ½ , 3 , 6, 9 and 12 h time points in i) control (ethanol treated) and hormone treated glands ii) 0.4 pg/µl of 20E, iii) 4 pg/µl of 20E and iv) 5.7 pg/µl of 20E. L-fibroin expression is reported as fold change relative to 0 h control.

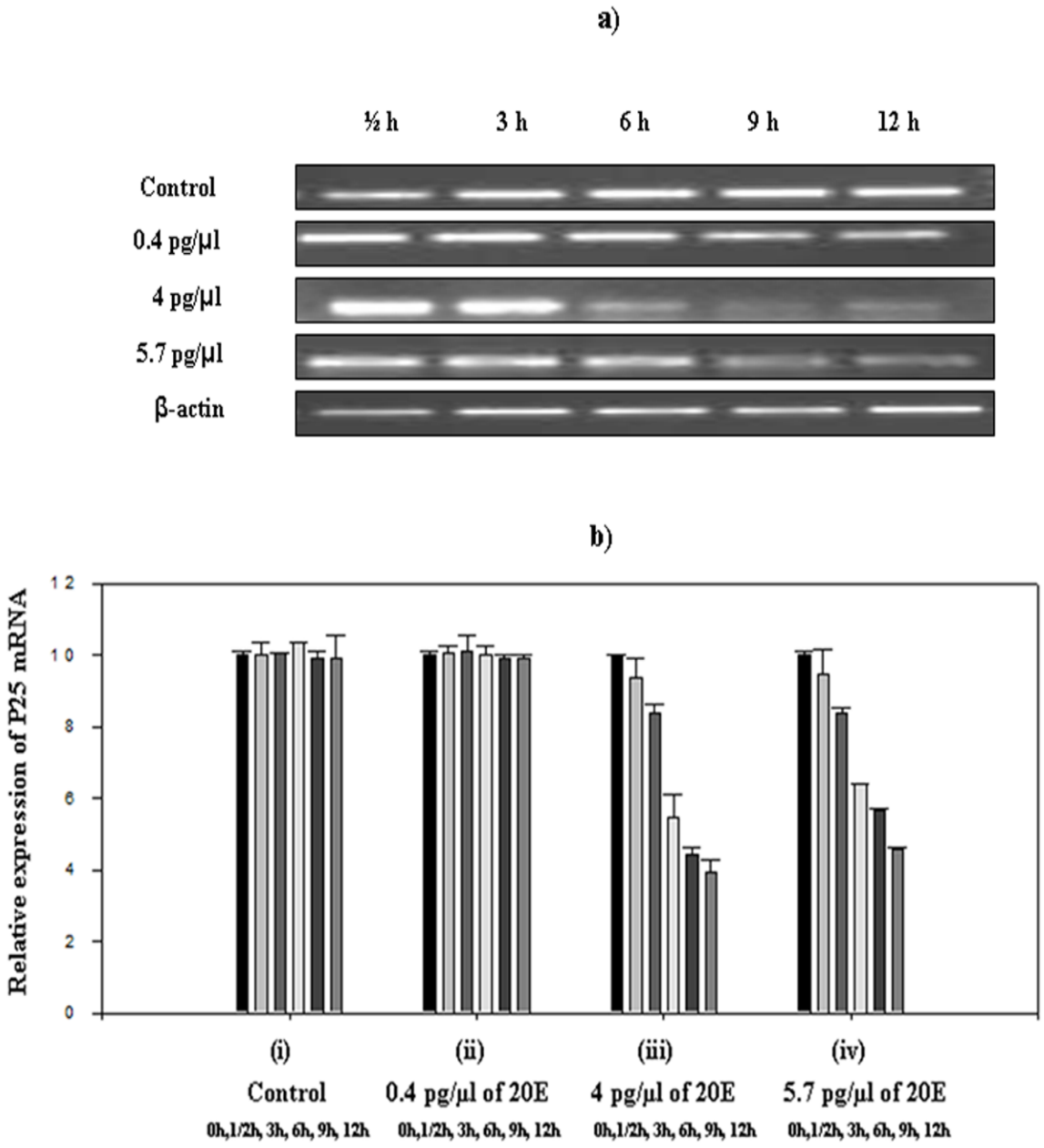

Fig. 37. Effect of 20E on P25 expression of LLI larval silk glands a) Semi-quantitative PCR showing the expression at time points ½ , 3, 6, 9 and 12 h in control (ethanol treated) and hormone treated glands (0.4 pg/µl, 4 pg/µl and 5.7 pg/µl of 20E). β-actin was used as internal control. b) Real-time PCR (n=3) showing the expression at 0, ½, 3, 6, 9 and 12 h time points in i) control (ethanol treated) and hormone treated glands ii) 0.4 pg/µl of 20E, iii) 4 pg/µl of 20E and iv) 5.7 pg/µl of 20E. P25 expression is reported as fold change relative to 0 h control.

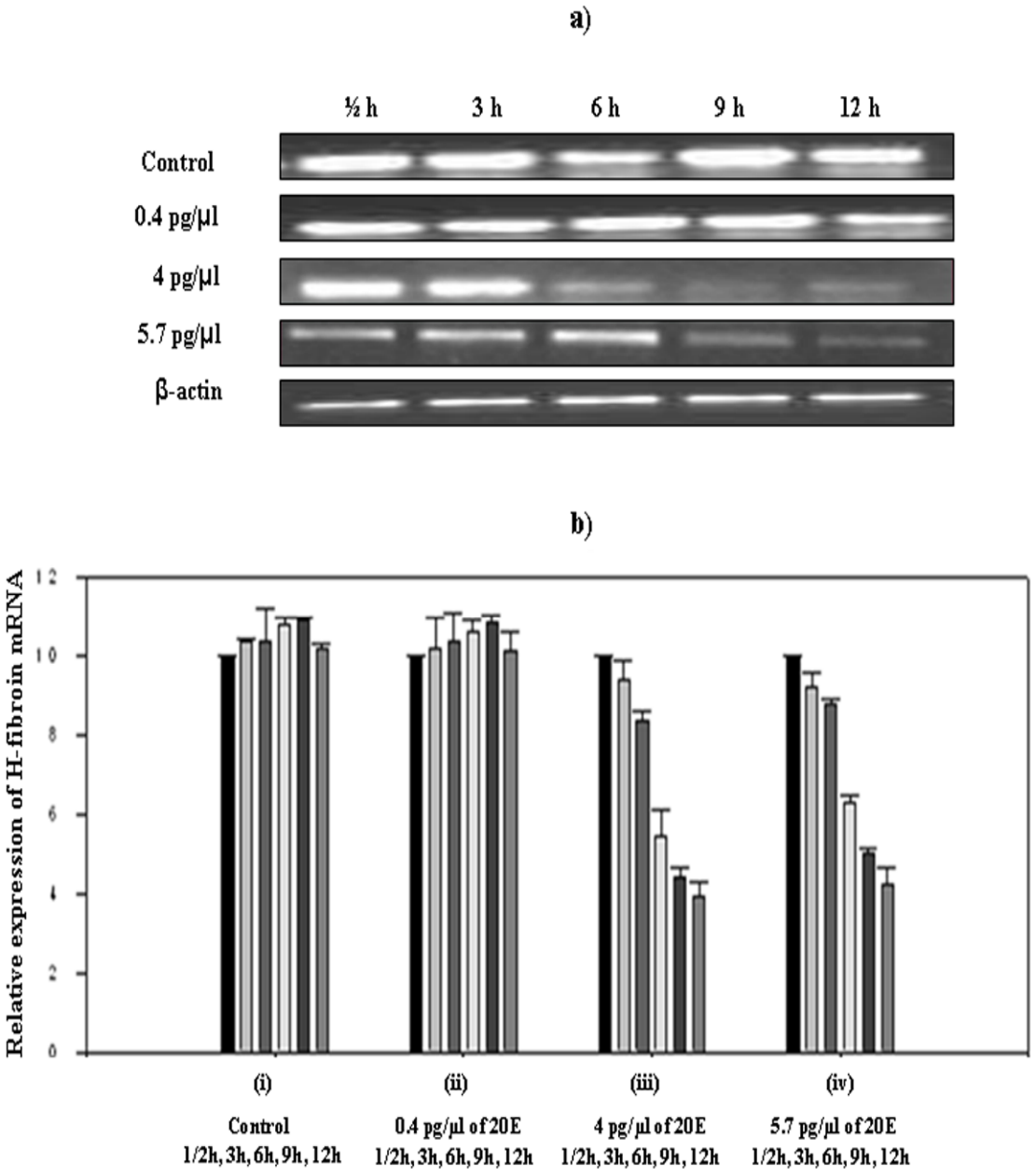

Fig. 38. Effect of 20E on H-fibroin expression of LLI larval silk glands a) Semi-quantitative PCR showing the expression at time points ½ , 3, 6, 9 and 12 h in control (ethanol treated) and hormone treated glands (0.4 pg/µl, 4 pg/µl and 5.7 pg/µl of 20E). β-actin was used as internal control. b) Real-time PCR (n=3) showing the expression at 0, ½, 3, 6, 9 and 12 h time points in i) control (ethanol treated) and hormone treated glands ii) 0.4 pg/µl of 20E, iii) 4 pg/µl of 20E and iv) 5.7 pg/µl of 20E. H-fibroin expression is reported as fold change relative to 0 h control.

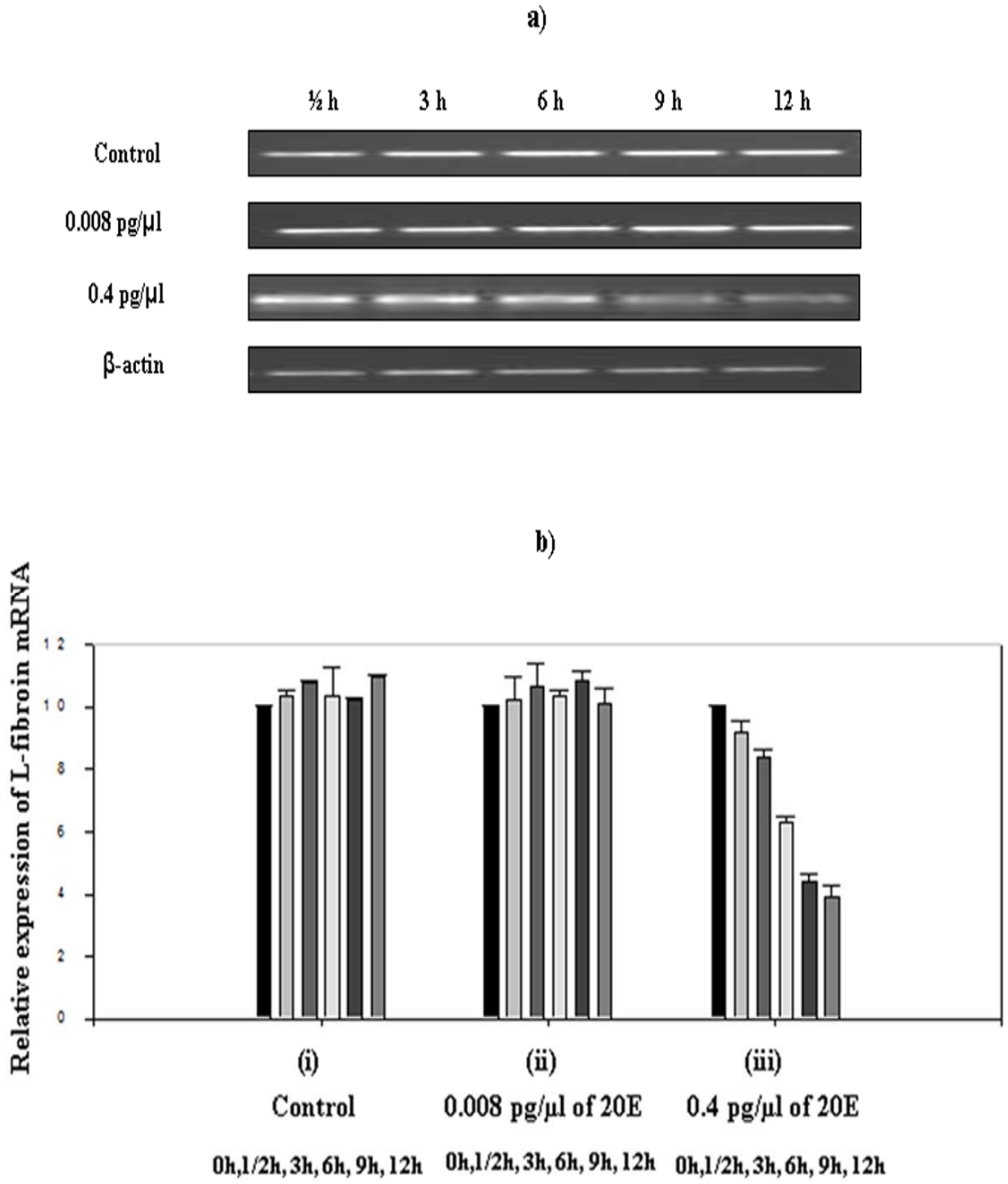

Fig. 39. Effect of 20E on L-fibroin expression of ELI larval silk glands a) Semi-quantitative PCR showing the expression at time points ½ , 3 , 6 , 9 and 12 h in control (ethanol treated) and hormone treated glands (0.008 pg/µl and 0.4 pg/µl). β-actin was used as internal control. b) Real-time PCR (n=3) showing the expression at 0 , ½ , 3 , 6, 9 and 12 h time points in i) control (ethanol treated) and hormone treated glands ii) 0.008 pg/µl of 20E and iii) 0.4 pg/µl of 20E. L-fibroin expression is reported as fold change relative to 0 h control.

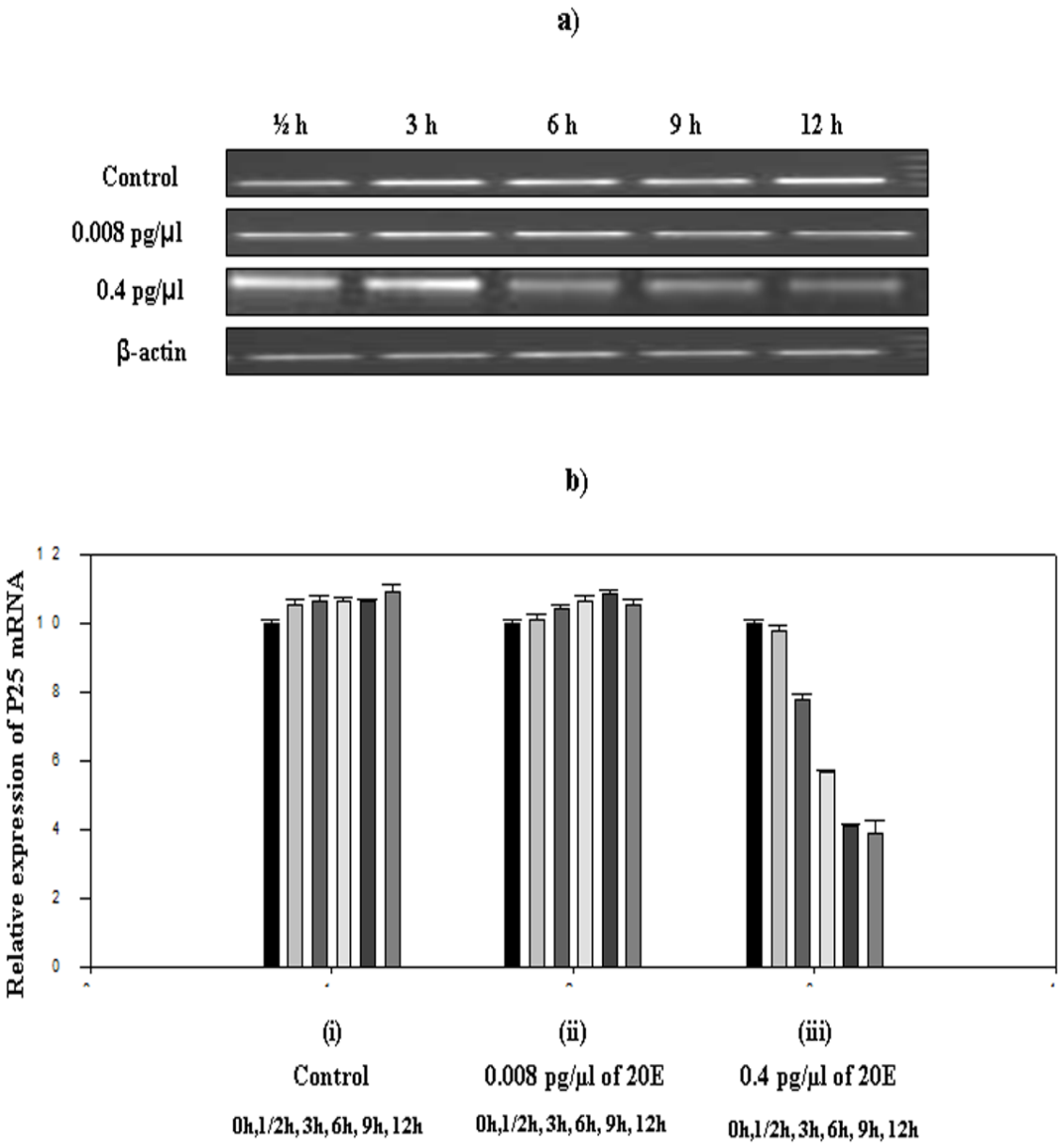

Fig. 40. Effect of 20E on P25 expression of ELI larval silk glands a) Semi-quantitative PCR showing the expression at time points ½ , 3 , 6 , 9 and 12 h in control (ethanol treated) and hormone treated glands (0.008 pg/µl and 0.4 pg/µl). β-actin was used as internal control. b) Real-time PCR (n=3) showing the expression at 0 , ½ , 3 , 6, 9 and 12 h time points in i) control (ethanol treated) and hormone treated glands ii) 0.008 pg/µl of 20E and iii) 0.4 pg/µl of 20E. P25 expression is reported as fold change relative to 0 h control.

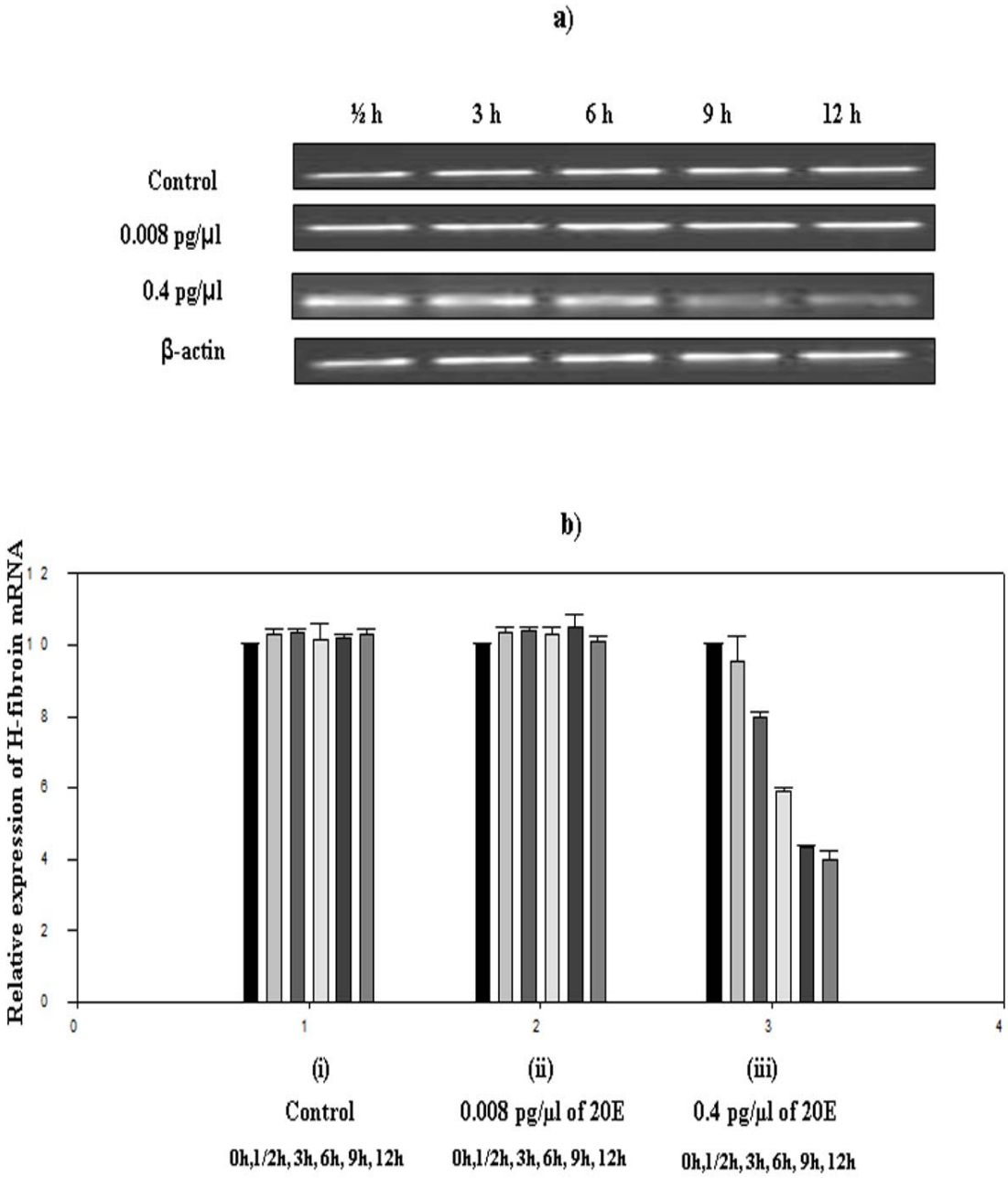

Fig. 41. Effect of 20E on H-fibroin expression of ELI larval silk glands a) Semi-quantitative PCR showing the expression at time points ½ , 3 , 6 , 9 and 12 h in control (ethanol treated) and hormone treated glands (0.008 pg/µl and 0.4 pg/µl). β-actin was used as internal control. b) Real-time PCR (n=3) showing the expression at 0 , ½ , 3 , 6, 9 and 12 h time points in i) control (ethanol treated) and hormone treated glands ii) 0.008 pg/µl of 20E and iii) 0.4 pg/µl of 20E. H-fibroin expression is reported as fold change relative to 0 h control.

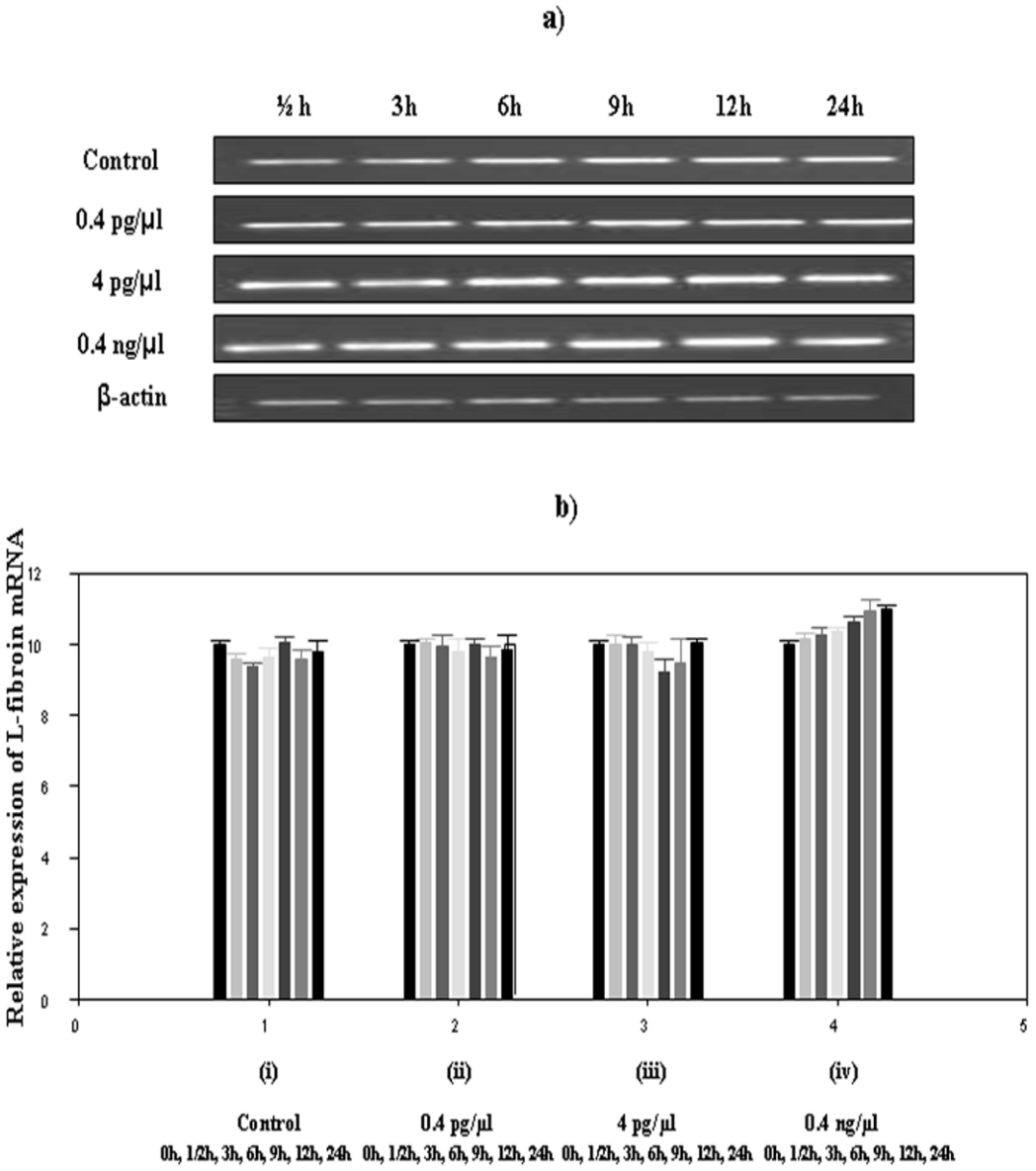

Fig. 42. Effect of methoprene on L-fibroin expression of LLI larval silk glands a) Semi-quantitative PCR showing the expression at time points ½ , 3 , 6 , 9 and 12 and 24 h in control (acetone treated) and hormone analog treated glands (0.4 pg/µl, 4 pg/µl and 5.7 pg/µl of methoprene). β-actin was used as internal control. b) Real-time PCR (n=3) showing the expression at 0 , ½ , 3 , 6, 9 and 12 and 24 h time points in i) control (acetone treated) and hormone analog treated glands ii) 0.4 pg/µl of methoprene, iii) 4 pg/µl of methoprene and iv) 0.4 ng/µl of methoprene. L-fibroin expression is reported as fold change relative to 0 h control.

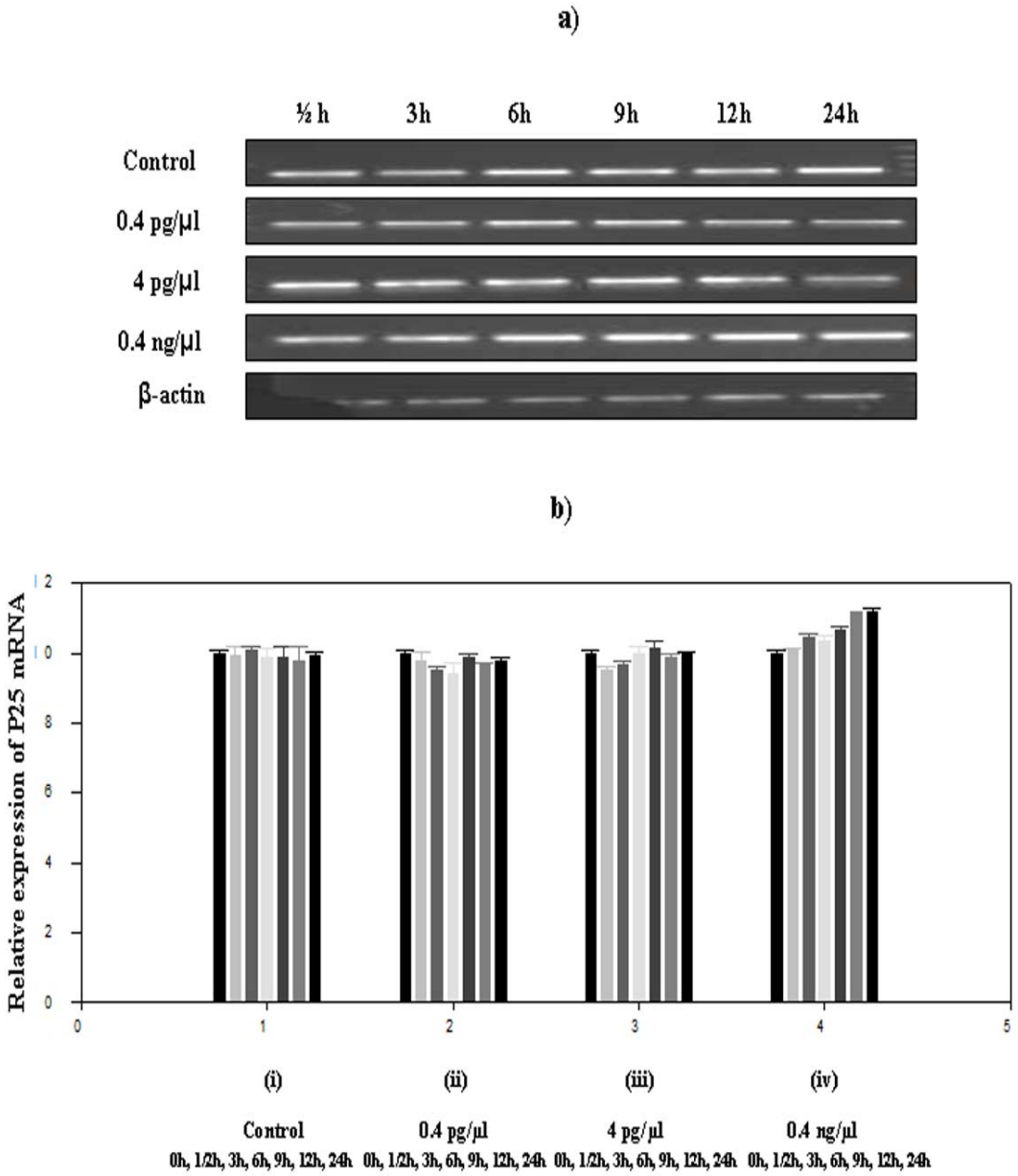

Fig. 43. Effect of methoprene on P-fibroin expression of LLI larval silk glands a) Semi-quantitative PCR showing the expression at time points 0, ½ , 3 , 6 , 9 and 12 and 24 h in control (acetone treated) and hormone analog treated glands (0.4 pg/µl, 4 pg/µl and 5.7 pg/µl of methoprene). β-actin was used as internal control. b) Real-time PCR (n=3) showing the expression at ½ , 3 , 6, 9 and 12 and 24 h time points in i) control (acetone treated) and hormone analog treated glands ii) 0.4 pg/µl of methoprene, iii) 4 pg/µl of methoprene and iv) 0.4 ng/µl of methoprene. P-fibroin expression is reported as fold change relative to 0 h control.

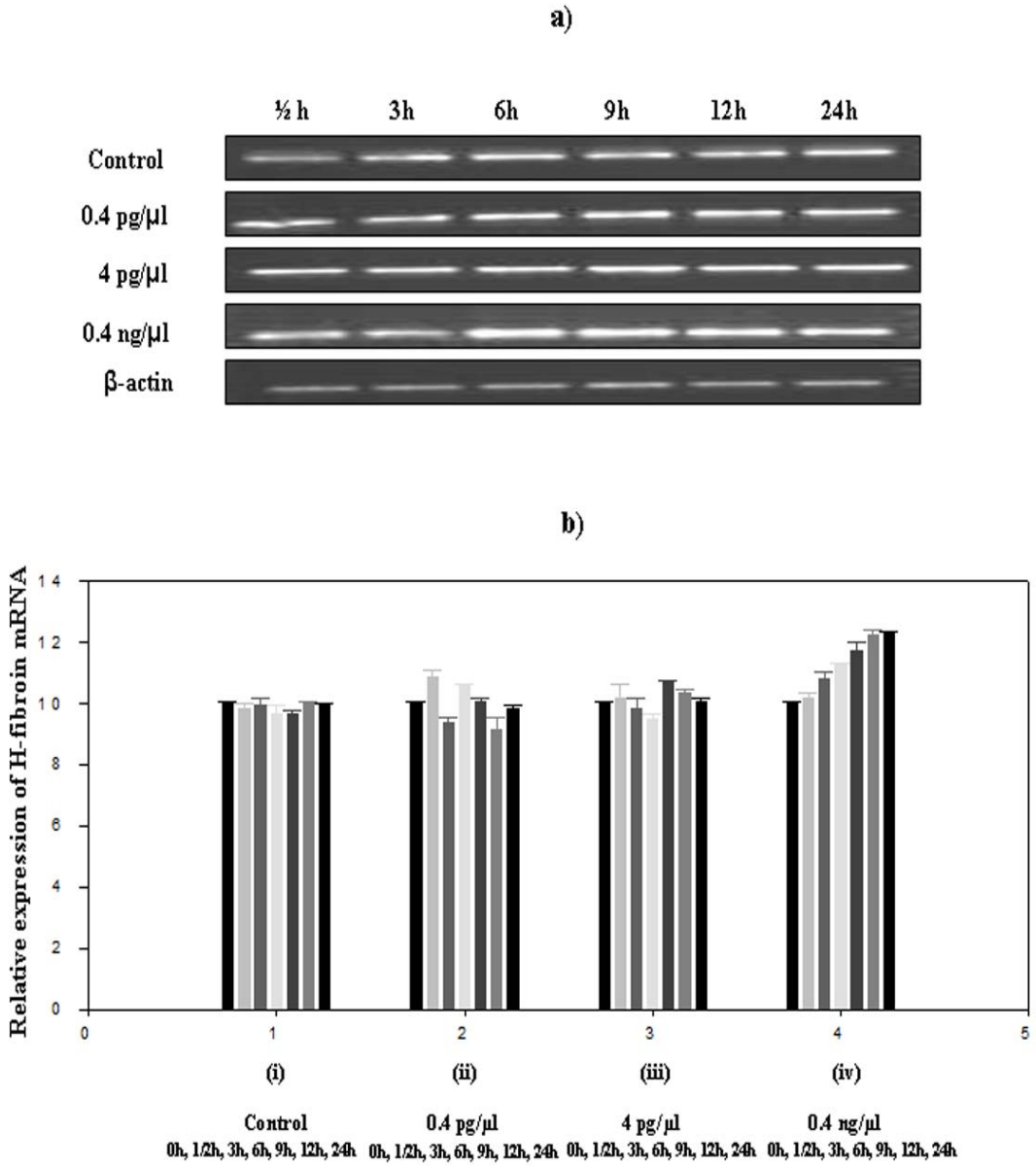

Fig. 44. Effect of methoprene on H-fibroin expression of LLI larval silk glands a) Semi-quantitative PCR showing the expression at time points ½ , 3 , 6 , 9 and 12 and 24 h in control (acetone treated) and hormone analog treated glands (0.4 pg/µl, 4 pg/µl and 5.7 pg/µl of methoprene). β-actin was used as internal control. b) Real-time PCR (n=3) showing the expression at 0 , ½ , 3 , 6, 9 and 12 and 24 h time points in i) control (acetone treated) and hormone analog treated glands ii) 0.4 pg/µl of methoprene, iii) 4 pg/µl of methoprene and iv) 0.4 ng/µl of methoprene. H-fibroin expression is reported as fold change relative to 0 h control.

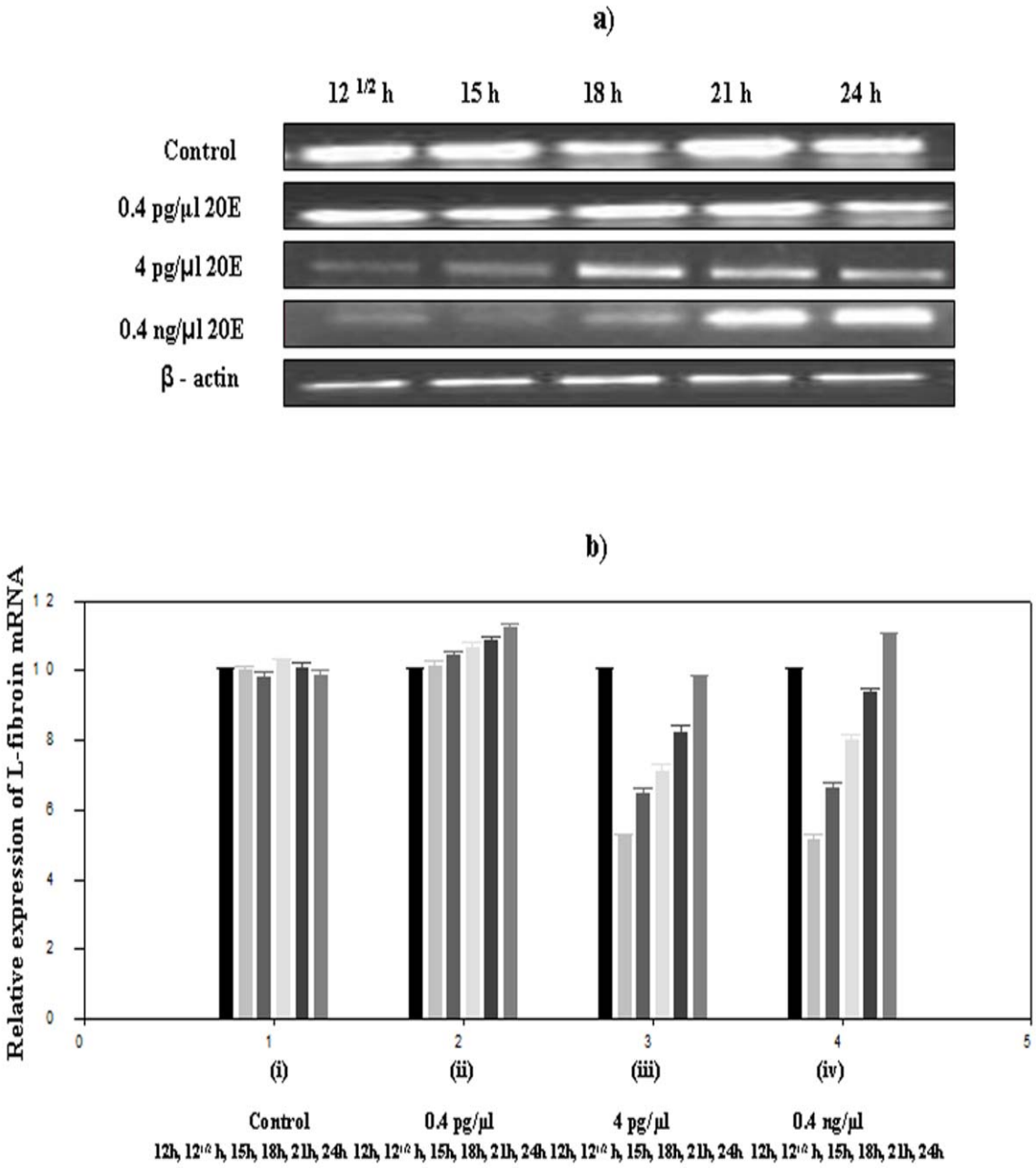

Fig. 45. Effect of methoprene on L-fibroin expression of LLI larval silk glands after treatment with 5.7 pg/µl of 20E a) Semi-quantitative PCR showing the expression at time points $12^{1/2}$, 15, 18, 21 and 24 h in control (acetone treated) and hormone analog treated glands (0.4 pg/µl, 4 pg/µl and 5.7 pg/µl of methoprene). β-actin was used as internal control. b) Real-time PCR (n=3) showing the expression at $12^{1/2}$, 15, 18, 21 and 24 h time points in i) control (acetone treated) and hormone analog treated glands ii) 0.4 pg/µl of methoprene, iii) 4 pg/µl of methoprene and iv) 0.4 ng/µl of methoprene. L-fibroin expression is reported as fold change relative to 12 h control.

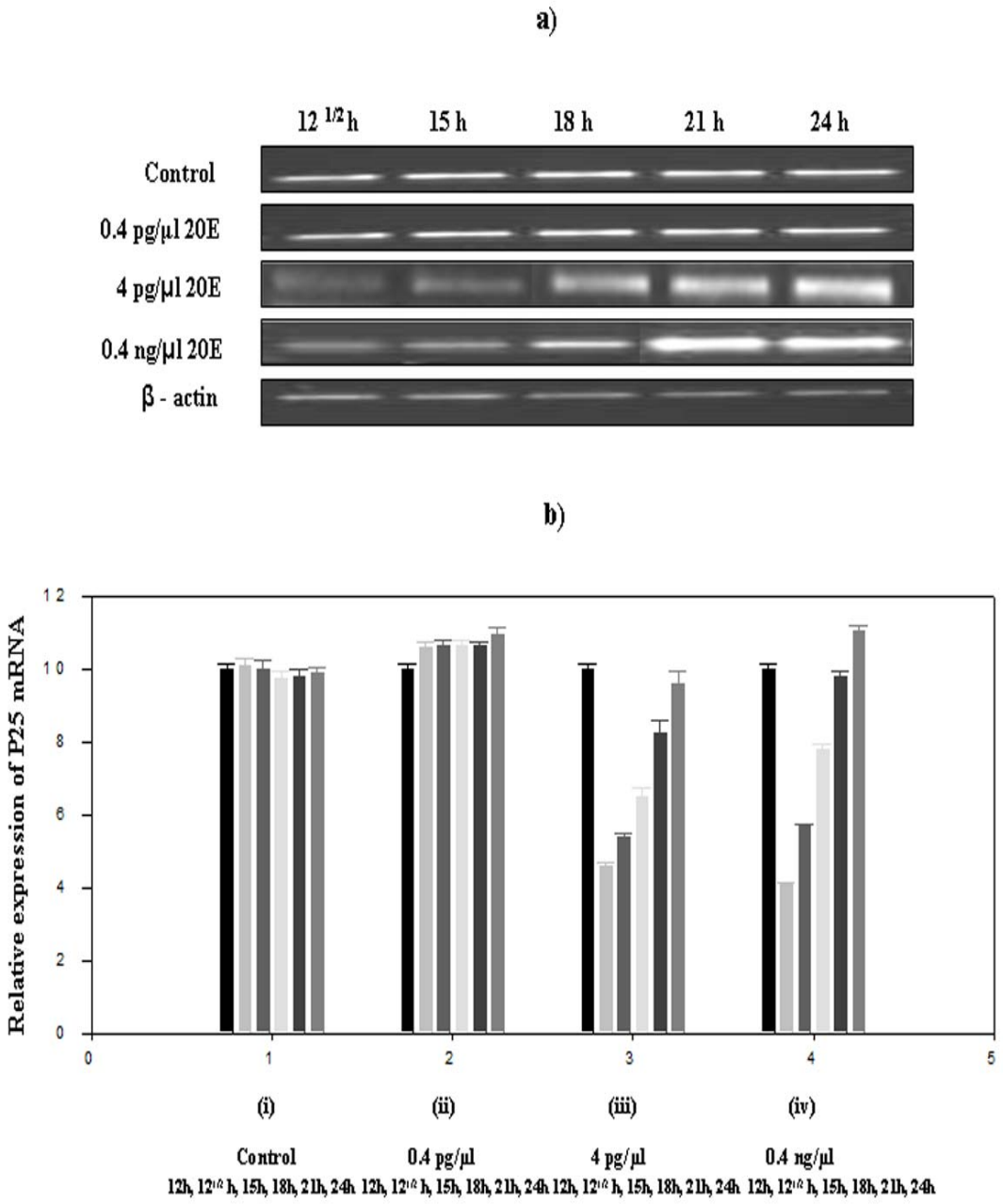

Fig. 46. Effect of methoprene on P25 expression of LLI larval silk glands after treatment with 5.7 pg/ µl of 20E a) Semi-quantitative PCR showing the expression at time points $12^{1/2}$, 15, 18, 21 and 24 h in control (acetone treated) and hormone analog treated glands (0.4 pg/µl, 4 pg/µl and 5.7 pg/µl of methoprene). β-actin was used as internal control. b) Real-time PCR (n=3) showing the expression at $12^{1/2}$, 15, 18, 21 and 24 h time points in i) control (acetone treated) and hormone analog treated glands ii) 0.4 pg/µl of methoprene, iii) 4 pg/µl of methoprene and iv) 0.4 ng/µl of methoprene. P25 expression is reported as fold change relative to 12 h control.

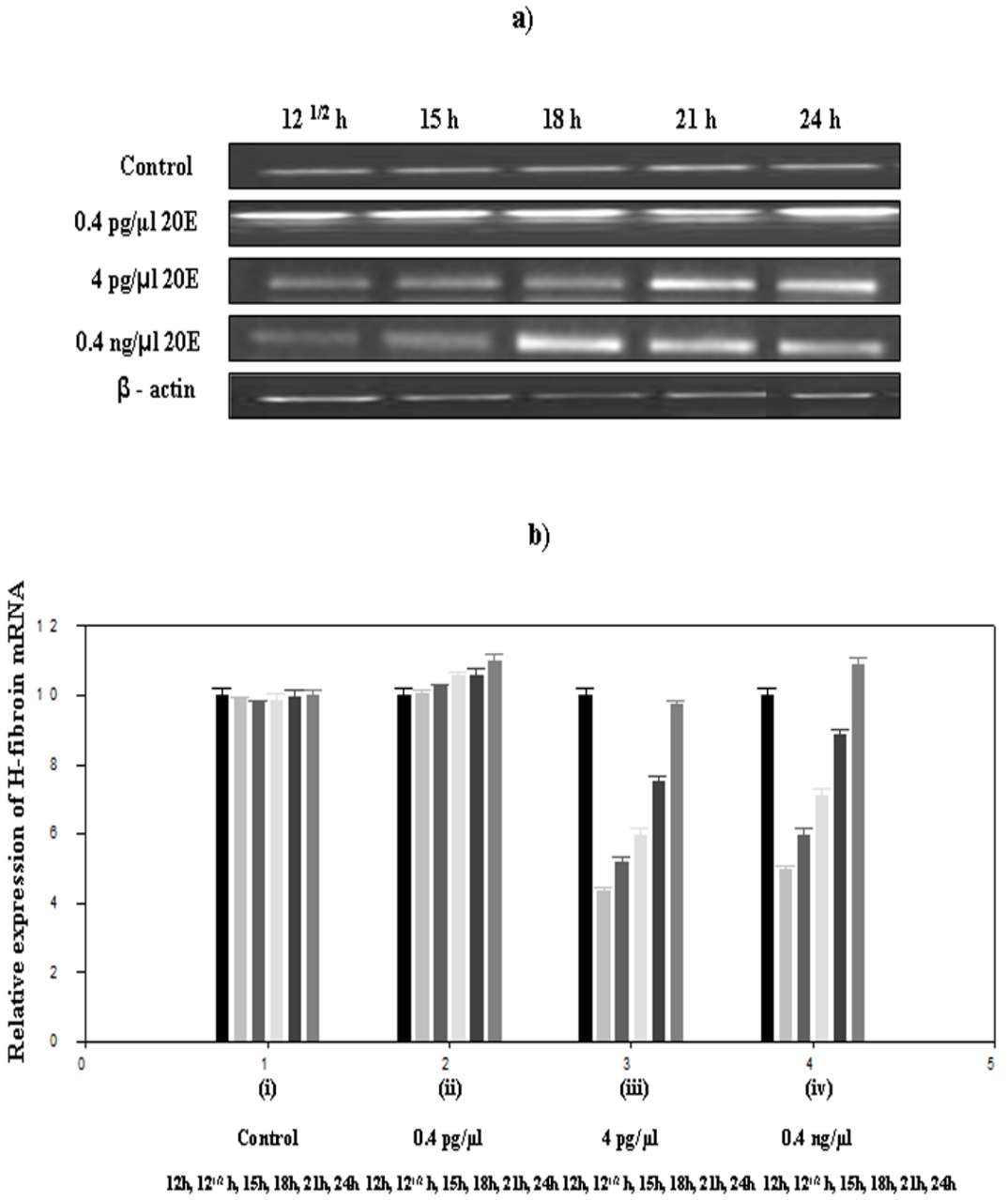

Fig. 47. Effect of methoprene on H-fibroin expression of LLI larval silk glands after treatment with 5.7 pg/µl of 20E a) Semi-quantitative PCR showing the expression at time points $12^{1/2}$, 15, 18, 21 and 24 h in control (acetone treated) and hormone analog treated glands (0.4 pg/µl, 4 pg/µl and 5.7 pg/µl of methoprene). β-actin was used as internal control. b) Real-time PCR (n=3) showing the expression at $12^{1/2}$, 15, 18, 21 and 24 h time points in i) control (acetone treated) and hormone analog treated glands ii) 0.4 pg/µl of methoprene, iii) 4 pg/µl of methoprene and iv) 0.4 ng/µl of methoprene. H-fibroin expression is reported as fold change relative to 12 h control.

In *B. mori*, the development and function of silk glands were shown to be hormonally regulated (Sehnal and Akai, 1990). Maximum silk fibroin synthesis was observed in the last larval instar (Suzuki and Suzuki, 1974). It was also shown in *G. mellonella* that silk glands readily respond to hormones during this stage (Sehnal et al., 1983). In the present study, the developmental profile of all the three genes of *C. cephalonica* depicts the highest expression during the last instar larval development. Based on this parameter, the last instar larvae were chosen to study the effect of 20E and methoprene, a JH analog.

Earlier *in vivo* studies on the participation of ecdysteroids in the silk gland regulation, performed on intact larvae, showed their effect on various parameters like total RNA synthesis, quality of the silk produced, cytological changes and the ultra structure of the secretory cells (Shigematsu and Moriyama, 1970; Grzelak et al., 1993; Grzelak, 1995). The *in vitro* effect(s) of 20E were shown either on isolated nuclei or cultured silk glands (Maekawa et al., 1984; Kodrik and Sehnal, 1994). However, none of these conclusively showed the effect of 20E on the expression of silk genes at transcriptional level. On the other hand contradicting studies reported the effect of various hormones including ecdysteroids in the regulation of expression of silk protein genes. It was also found that low titers of ecdysteroids are necessary for proper silk gland function (Sehnal and Akai, 1990). In *C. cephalonica*, treatment with physiological concentration of 20E (0.4 pg/µl) during LLI larval stage, did not alter the expression of any of the genes, when compared with that of ethanol treated control glands. Further, at this stage, these genes were shown to attain maximum expression. Therefore, it was not clear whether low dose of 20E is required for normal expression. Therefore, we monitored the effect of 20E on the expression these genes in ELI larval silk glands. The haemolymph level of 20E during ELI larval stage (0.008 pg/µl) too did not alter the rate of transcription at this stage,

suggesting that a physiological dose of 20E is probably required for normal expression of the silk genes. Previous studies on *B. mori* revealed that high concentrations of ecdysteroid cause silk gland degeneration during the pupal molt (Shigematsu and Moriyama, 1970). Similar phenomenon was also reported in the case of *G. mellonella* too (Sehnal and Michalik, 1984; Kodrik and Sehnal, 1994).

The pupal molt in lepidopteran insect is associated with high titers of ecdysteroids in various species (Calvez et al., 1976; Plantevin et al., 1984). The studies on developmental pattern of silk genes in *C. cephalonica*, show that their expression declined during the pupal stage. Therefore, on treatment of LLI larval silk glands with higher dosage of 20E (4 pg/µl and 5.7 pg/ul seen during EPP stage), the expression of these genes decreased significantly. Such a prominent effect of 20E could be most likely mediated by ecdysone receptors present in the salivary glands cells (Elmogy et al., 2004; Sun and Song, 2006). Treatment of ELI larval silk gland with 0.4 pg/µl of 20E (the physiological concentration of 20E found during the succeeding LLI larval stage) reconfirmed, the declined expression of all the three genes in presence of higher concentration of 20E. This finding suggests that high concentrations of 20E have an inhibitory effect on silk gene expression. Present study further revealed that 20E regulates the gene expression in a dose-dependant manner during the last-instar larval development.

Methoprene is a JH analog which mimics the action of naturally occurring JHs. Hence, in our study methoprene was used in place of natural juvenile hormone to study the effects of JH application. JH action during larval development, at least in lepidopteran insects as well as other holometabolous insects is to maintain the '*status quo*', probably to allow proper larval molting and to prevent premature metamorphosis (Zhou and Riddiford, 2002).

At the end of larval development, circulating JH gets degraded, enabling ecdysteroids to trigger metamorphosis. JH reappears in many adult insects to control both oogenesis and male accessory gland function (Wyatt and Davey, 1996). These juvenile hormone effects reported cannot be directly extrapolated for the changes that occur in silk glands when the hormone causes a prolongation of the last larval instar.

The effect of JH on silk glands depends on the time of application. In case of *B. mori*, topical application of JH at the beginning of the fifth (last) larval instar increased the silk production (Akai and Kobayashi, 1971; Garel, 1983; Sehnal and Akai, 1990). In later fifth larval instar, silk gland development was only delayed and restoration of enhanced duration of larval life took place without significant increase in the silk production (Daillie, 1979). An overall response, independent of the developmental stage, was protection of silk glands from degeneration in *G. mellonella* i.e. the hormone restored the function of silk glands at transcriptional level (Grzelak et al., 1982; Sehnal et al., 1983). The induction of degeneration by ecdysteroids in the absence of JH was also demonstrated both under *in situ* and *in vitro* conditions (Chinzei, 1975; Tripoulas and Samols, 1986). Methoprene was applied to LLI larval silk glands of *C. cephalonica* when probably the natural hormones level is fairly low or absent. Therefore hormone treatment at various concentrations (0.4 pg/ µl, 4 pg/ µl, 0.4 ng/ µl), irrespective of the dose, probably resembled a persistent high level of juvenoid and retained the silk gene expression in the LLI larval stage. Hence, the expression of H-fibroin, L-fibroin and P25 genes were observed similar to that of respective control. The transcript level of acetone treated glands that were cultured through the same time points was used as external control. This result suggests that JH keeps *'status quo'* expression of these three genes and probably prevents the decline in expression that is normally found during the EPP and LPP stages.

Further, at this stage all the three silk genes were shown to attain maximum expression. Therefore, to eliminate any discrepancy whether JH actually had any effect on the expression, silk glands were treated with EPP stage hemolymph concentration of 20E (5.7 pg/ul) for 12 h that inhibited the expression of these genes, was followed by treatment with various concentrations of methoprene (0.4 pg/ µl, 4 pg/ µl, 0.4 ng/ µl). In all the cases, the expression of the three genes was retrieved back to the normal levels. Kodrik and Sehnal (1994) reported that lower concentrations of 20E stimulated and higher concentration of 20E inhibited silk gland RNA synthesis in *G. mellonella*. Both these effects were abolished with physiological (1ng/ml) and higher doses of JH II or juvenoid. Present study further confirms that methoprene counteracts the action of 20E and maintains the '*status quo*' expression of these genes during late-last instar development of a lepidopteran insect.

RESULTS AND DISCUSSION

PART III

20E regulation of H-fibroin gene, its role in insect development and a probable target for control

Part II of our study reveals that silk genes of *C. cephalonica* are under hormonal control at transcriptional level. This regulation by hormones (20E and JH analog, methoprene) is most likely mediated by hormone receptors which upon interaction with ligands bind to regulatory elements present in the upstream region of these genes. Therefore, we adopted genome walking strategy to construct genomic libraries and to clone the upstream regions of H-fibroin, L-fibroin and P25 genes. We were unable to find any regulatory regions in the short upstream fragments that were cloned from L-fibroin and P25. However, we were successful in identifying an ecdysone response element in the upstream region of H-fibroin. Based on this finding, further studies were carried out to investigate the mechanism of hormone regulation of H-fibroin gene.

A PCR product of 590 bp was amplified from the *EcoRV* library and the 5' flanking region of H-fibroin was cloned. The upstream analysis revealed a TATA sequence at -59 relative to the ATG sequence. An ecdysone response element (ERE) was found at -65 position that overlaps with the TATA box. At -254 position, a silk gland specific factor binding site (SGF-1) was located (Fig. 48). A ten base A+T region that consists of TAAT motif in the core was found to be the binding site for posterior silk gland specific (SGF2) and ubiquitous (SGF-3 and SGF-4) factors. At -322 nucleotide, *Drosophila* homeodomain proteins, EVE (*even-skipped*) and ZEN (*zerknullt*) binding sites were detected. Upstream region of H-fibroin of *B. mori* was also cloned to investigate the presence of ERE sequence (Fig. 49).

Luciferase assay is extensively used for the analysis of functional activity of various regulatory regions in the upstream regions of variety of genes. To perform luciferase assay, PCR based progressive deletion constructs were cloned into pGL2 basic vector

(Fig. 50a). Four different fragments were generated from the PCR amplified upstream region and cloned. 1) 79 bp fragment that contained no promoter element sequences, 2) 104 bp fragment that contained TATA box sequence, 3) 127 bp fragment that contained both TATA box and ERE sequence and 4) 351 bp fragment that contained TATA box, ERE and SGF-1 binding sequence were cloned (Fig. 50b).

The purpose of luciferase assay was to find out the functional activity of the putative ERE identified in the *C. cephalonica* H-fibroin upstream region. The deletion constructs that were cloned, were used for transfection. Transfected EcR-293 cell lines were induced by Ponasterone A. The ecdysteroid analog Ponasterone A being lipophilic permeates into the cells easily. The concentration of 4 pg/µl of 20E was shown to decrease the H-fibroin gene expression significantly (Fig. 38), therefore, similar dose of Ponasterone A was used for induction. The high luciferase activity seen in ERE containing construct implicates that upon induction with synthetic 20E analog, RXR acts as a pairing partner for EcR in the absence of USP and then the dimmer probably binds to the ERE region and regulates the H-fibroin gene expression. Transient transfection studies reveal that the 341 bp PCR construct that consists of TATA box flanked by ERE and SGF1 shows maximum luciferase activity. The 127 bp construct even after the deletion of SGF-1 binding sequence also show a fairly high activity, while the construct containing only the TATA box shows a moderate promoter activity (Fig. 51). These studies clearly show the presence of a functional ERE in the H-fibroin region. The finding also suggests that 20E effect on H-fibroin expression in *C. cephalonica* is probably mediated by ecdysone receptors (EcR) present in the silk gland cells.

The presence of EcR in the nuclear extract was confirmed by immunoblot using a heterologous antibody generated against *Chironomus* (Fig. 52a). The nuclear extract prepared from 20E induced salivary glands demonstrated a complex formation with [γ^{32}P] dATP labeled oligomeric sequence containing ERE motif, while no complex was observed in the absence of 20E induction. The shift in the band is observed, when probed with EcR specific antibody further confirms the binding. Further, the mutated oligonucleotide sequence does not show any binding, in the presence of 20E (Fig. 52b).

Increasing doses of RH-5849 and RH-5992 were added to the glands and the expression of H-fibroin gene was monitored. The H-fibroin expression decreases significantly upon treatment with 2 ng/ µl of RH-5849 *in vitro*. This decline is more prominent in the cultured glands that received RH-5992 at a concentration of 1.5 ng/µl (Fig. 53).

When the larvae were fed with similar concentrations of the above insecticides, the development was affected and they metamorphosed abnormally. There was a defect in the slippage of head capsule (Fig. 54) and many insects died during pupal stage due to defective cocoon formation (Fig. 55). Those which survived metamorphosed into defective/underdeveloped adults.

Differences are also seen between the ultrastructure of the silk fibers of control and insecticide fed insects. The mean diameter of a single fiber of RH-5849 fed insects is 1.19 µm and that of RH-5992 fed insects is only 876 nm. A remarkable decrease in the diameter of the fiber is also visualized when compared to the control that shows a mean fiber diameter of 2.16 µm (Fig. 56). The fibers from insects treated with either RH-5849 or

RH-5992 are found to be devoid of sticky coating (bleb like structures on the fiber) when compared to control (Fig. 57).

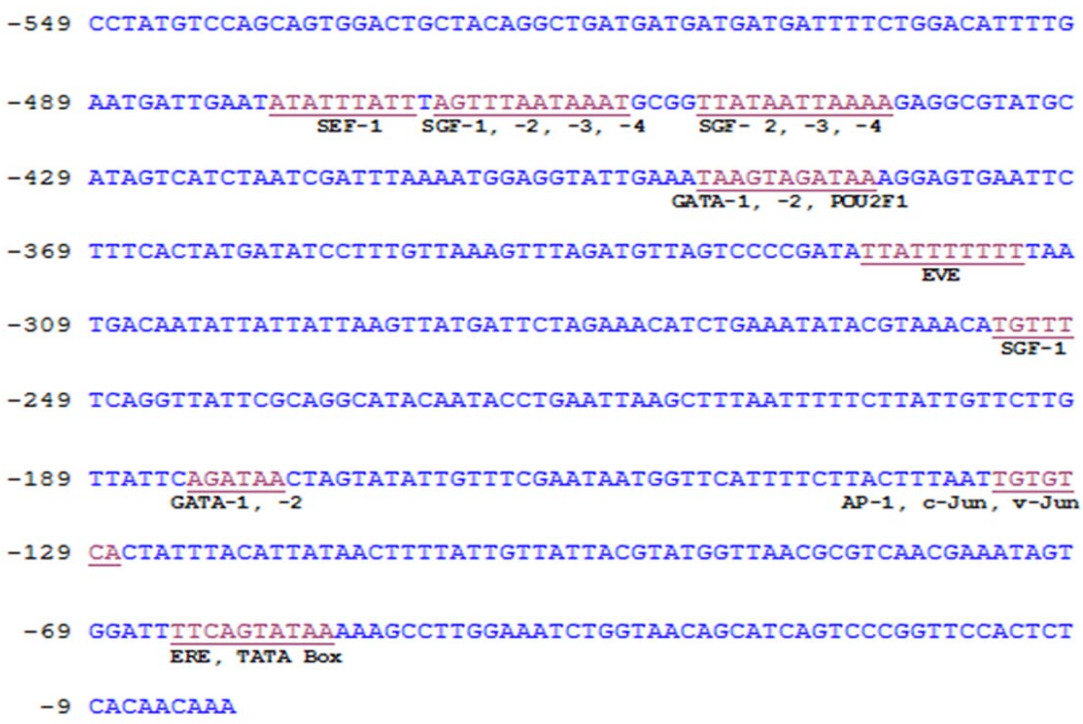

Fig. 48. Sequence of the 5' upstream region of *C. cephalonica* H-fibroin gene (GenBank accession no. GQ901977). The potential binding sites for specific transcription factors are underlined in red.

Fig. 49. Sequence of the 5' upstream region of the *B. mori* H-fibroin gene. The potential binding sites for specific transcription factors are underlined in red.

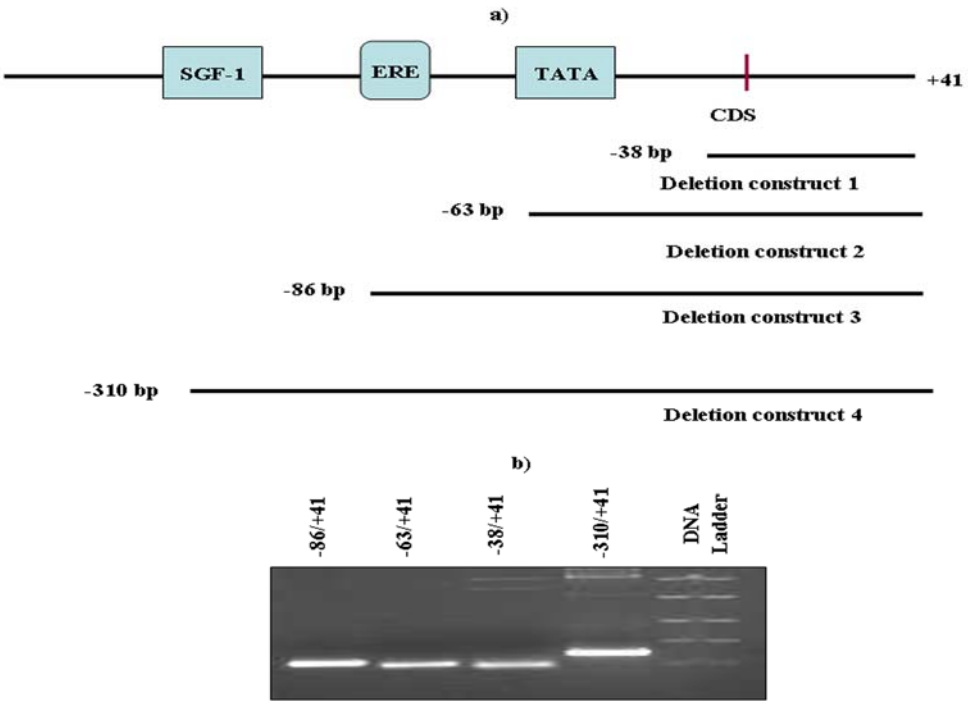

Fig. 50. a) Schematic representation of cloning of deletion constructs, CDS stands for coding sequence **b)** Cloning of deletion constructs. Lane 1: -86/+41, lane 2: -63/+41, lane 3: -38/+41, lane 4: -310/+41 amplified construct respectively and lane 5: DNA ladder.

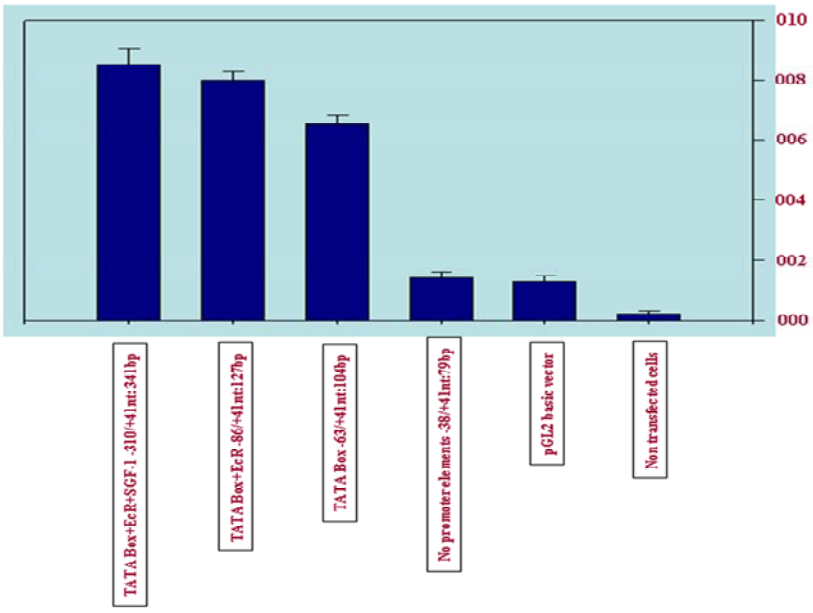

Fig. 51. Functional analysis of different H-fibroin promoter constructs co-transfected with pRL-TK into EcR293 cells. Luciferase activity is presented as relative to the activities measured for *Renilla* luciferase.

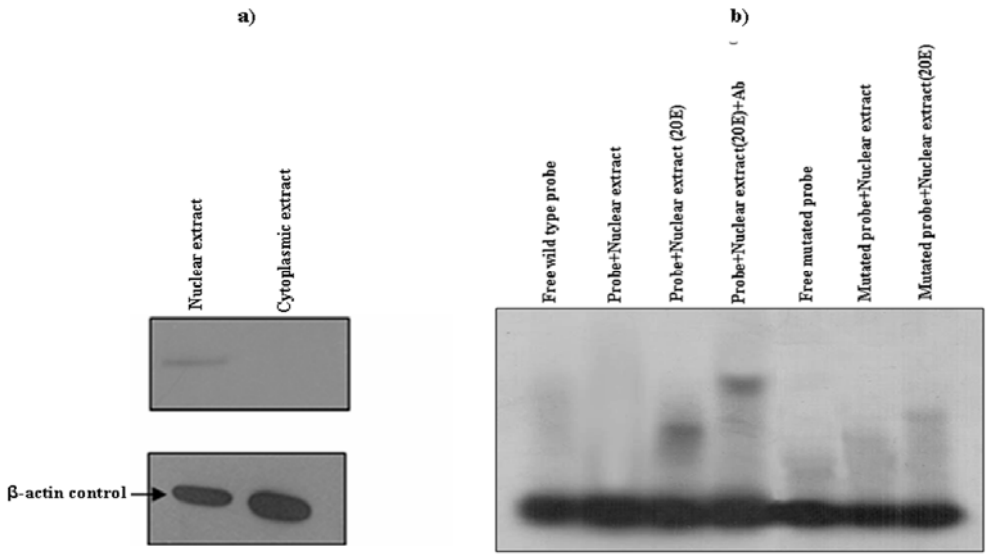

Fig. 52. a) Nuclear and cytosolic extracts (40 µg protein) each prepared from the larval salivary glands were immunoblotted against anti-Ch*ironomus* ecdysone antibody. β-actin was used as loading control. **b)** Gel shift assay showing the binding of EcR protein to ERE oligomer in the presence of 20E.

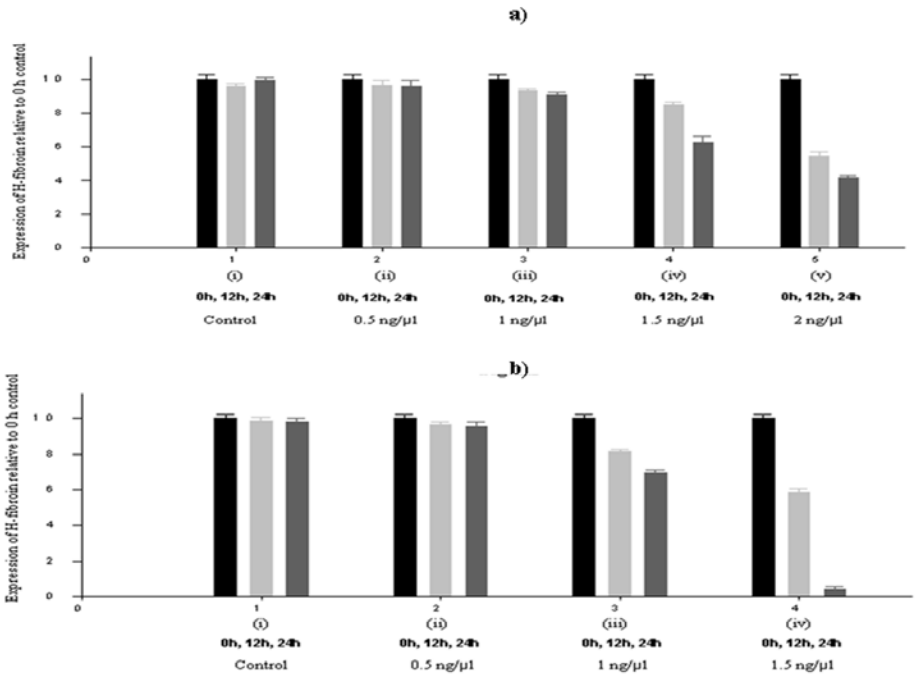

Fig. 53. Effect of RH-5849 and RH-5992 on H-fibroin gene expression using organ culture studies. **a)** Relative expression of H-fibroin gene upon treatment with increasing concentrations of RH-5849 (i) control (ethanol treated), (ii) 0.5 ng/µl (iii) 1 ng/µl (iv) 1.5 ng/µl and (v) 2 ng/µl **b)** Relative expression of H-fibroin gene upon treatment with increasing concentrations of RH-5992 (i) control (ethanol treated), (ii) 0.5 ng/µl (iii) 1 ng/µl (iv) 1.5 ng/µl.

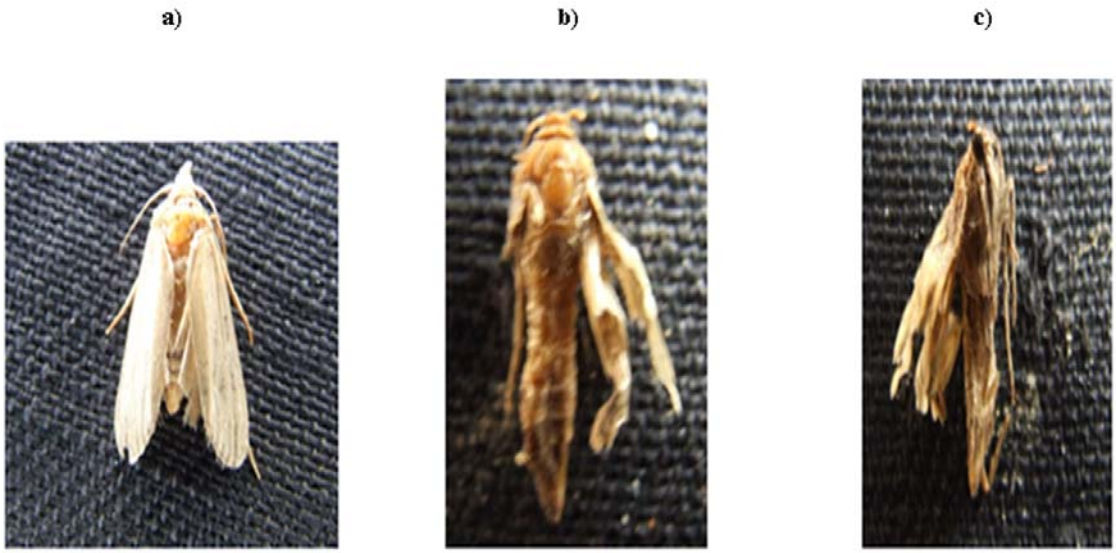

Fig. 54. Effect of RH-5849 and RH-5992 on metamorphosis and adult development **a)** normal adult **b)** defective head capsule slippage and defective adult formation **c)** adult with stunted growth

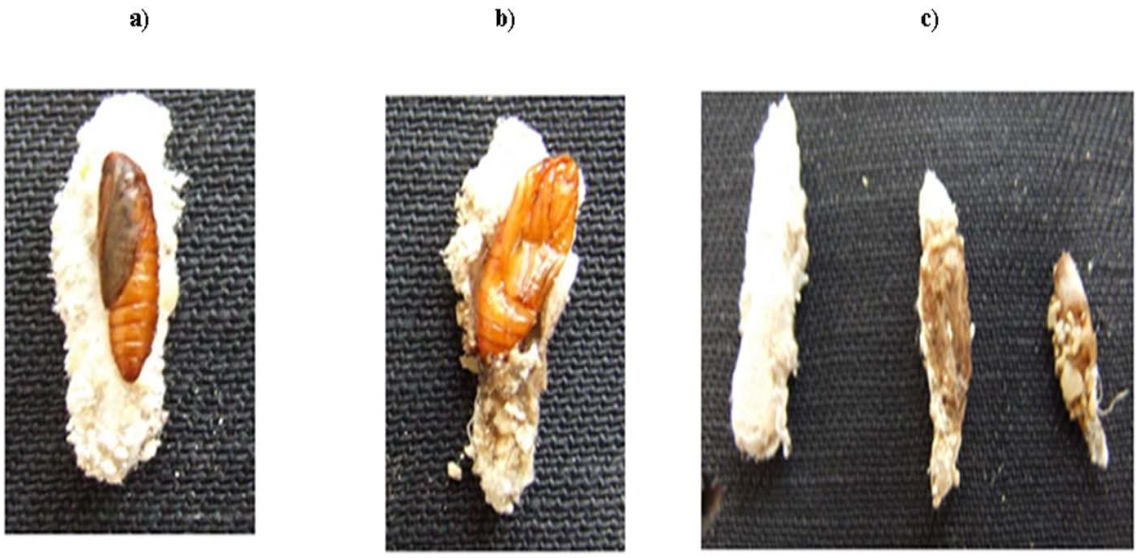

Fig. 55. Effect of RH-5849 and RH-5992 on larval-pupal metamorphosis **a)** normal pupa **b)** defective pupa **c)** defective cocoon with reduced quantity of silk fibers.

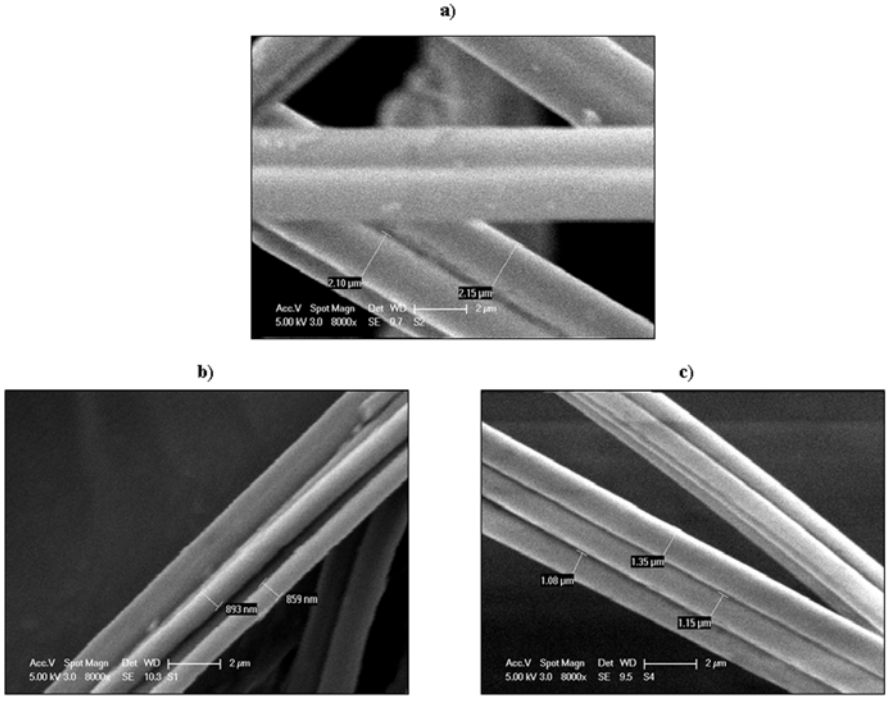

Fig. 56. Effect of RH-5849 and RH-5992 on ultrastructure of silk fibers. Change in diameter. Silk fibers from **a)** control insects **b)** RH-5849 fed insects **c)** RH-5992 fed insects. Please note the reduction in fiber diameter.

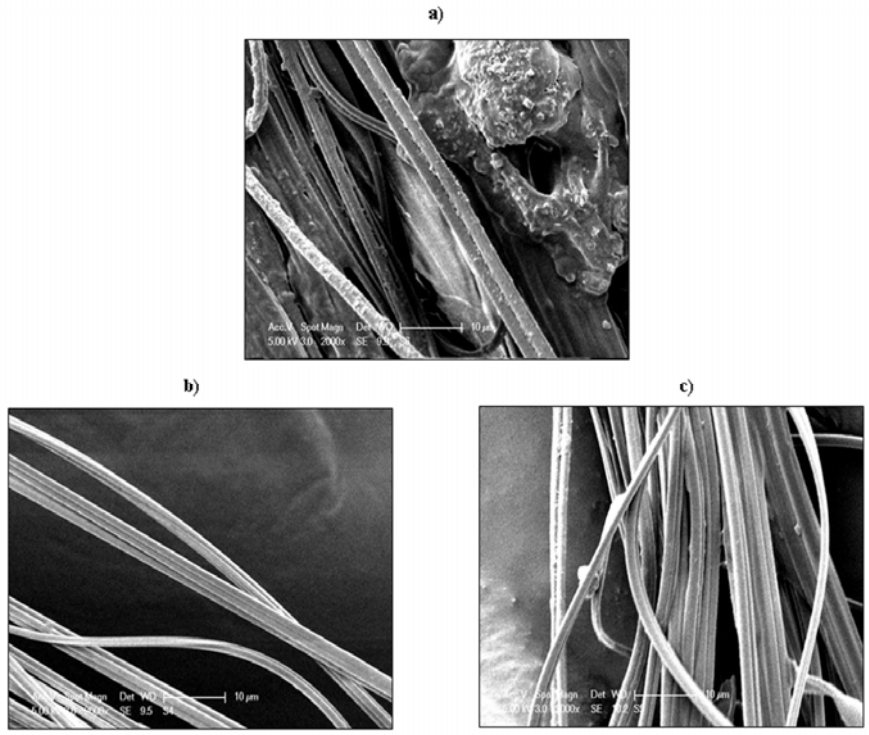

Fig. 57. Effect of RH-5849 and RH-5992 on ultrastructure of silk fibers. Sticky coating of silk fibers is shown in **a)** controls while absent in **b)** RH-5849 fed insects **c)** RH-5992 fed insects.

The principal biologically active steroid hormone in insects is 20E (Gilbert et al., 1980). The action of 20E is mediated by a heterodimer of two nuclear receptors, ecdysone receptor (EcR) and ultraspiracle (USP) (Yao et al., 1993). Ecdysteroids are known to specifically promote EcR association with USP, the insect homologue of retinoid X receptor (RXR), and controls transcription of genes containing ecdysone response elements (EREs) (Riddihough and Pelham, 1987; Oro et al., 1990; Luo et al., 1991). Extensive *in vitro* and *in vivo* analyses have led to the identification of various EREs, which are invariantly composed of direct or inverted repeats of the consensus sequence (G/T)NTCANTNN(A/C)(A/C) (Antoniewski, 1993).

Previously, EcR was identified and characterized in *D. melanogaster* salivary glands and the photo-affinity labeling studies showed that 20E gets covalently linked to the receptor protein (Schaltmann and Pongs, 1982). The EcR gene of *D. melanogaster* encodes three protein isoforms namely, EcRA, EcRB1 and EcRB2, which differ only in their transactivation domain and exhibit differential transcriptional capabilities (Talbot et al., 1993). The developmental expression of EcR was analyzed in *B. mori* and was shown that the anterior silk gland (ASG) has high levels of EcR. Further, EcRA was found to be present during the intermolt period, while EcRB1 appeared at the time of the molts. A small increase in ecdysone induced the expression of the EcR gene (Kamimura et al., 1997). In a different study, it was demonstrated that *B. mori* ASG undergoes degeneration, through programmed cell death in response to a metamorphic increase in hemolymph ecdysteroid titre at the end of last larval instar (Terashima et al., 2000). These reports suggest that the development of salivary gland in insects is 20E regulated.

Structural analyses of the 5' flanking region of the H-fibroin gene from several strains of *B. mori* (Suzuki and Adachi, 1984; Ueda et al., 1985) and a closely related species, *B. mandarina* (Kusuda et al., 1986) showed that the immediate 5' flanking sequence, which is found to be highly conserved, is probably important for its expression. The binding sites for SGF-1, -2, -3 and -4 factors and homeodomain proteins which are found in 5' flanking sequence of *B. mori* fibroin, are also seen in the H-fibroin upstream region of *C. cephalonica*. It was also predicted that SGF-1 might play an important role in the expression of silk protein genes, because it also binds to a similar site in sericin-1 gene. The regions for SGF-2, -3, and -4 contain ten-base pairs of A+T repeats. The TAAT motif in the core region was shown to be important for the binding of these three proteins and also *Drosophila* homeodomain proteins, EVE (*even-skipped*) and ZEN (*zerknullt*) (Hui et al., 1990). In the present study an analysis of the upstream region of the *B. mori* H-fibroin was carried out basically to compare it with that of *C. cephalonica*. It is worthwhile to mention that the ERE sequence overlapped with the TATA box is also present in the H-fibroin upstream region of *B. mori* (Tsujimoto and Suzuki, 1979). However, its functional activity has not been analyzed yet. The consensus sequence obtained from the comparison of various hormone response elements from various species was shown to be (G/T)NTCANTNN(A/C)(A/C) (Antoniewski, 1993). The identified sequence of ERE in the H-fibroin of *C. cephalonica*, TTTCAGTATAA is well in agreement with the earlier reported consensus sequence.

Upon 20E exposure, puffing responses of the polytene chromosomes in *D. melanogaster* salivary glands were observed. In this model, 20E binds with an ecdysone receptor protein and directly activates the transcription of a small set of early genes which then activate many late genes (Ashburner et al., 1974). The presence of EcR and its induction by

20E was also shown in *B. mori* silk glands (Kamimura et al., 1997). These studies clearly suggest that 20E regulates various processes in the salivary gland via EcR, which controls gene transcription by binding to specific sequences. It was previously shown in *Drosophila* that an ERE is found in the *Hsp*27 promoter (Riddihough and Pelham, 1987). Treatment with ecdysone, *in vitro*, enhanced the DNA binding capability of the receptor. The most extensively studied promoters are the four small heat shock protein genes of *Drosophila* which are known to be activated by ecdysone as well as heat shock (Ireland et al., 1982). These reports further strengthen our results that EcR binds to ERE sequence upon induction with 20E and henceforth regulates H-fibroin expression. Furthermore, in the absence of 20E there is no complex formation and hence signal is not detected. The mutated oligonucleotide does not bind to EcR even after induction by 20E further confirms that EcR binds specifically to ERE sequence in the upstream region of H-fibroin gene, only when it is induced by 20E.

Both RH-5849 and RH-5992 are bisacylhydrazine insecticides that exhibit their activity via interaction with the ecdyteroid receptor proteins. Upon treatment with these compounds, a state of ecdysteroid excess, called hyperecdysonism is induced and these insecticides bind to the ecdysteroid receptor complex with high affinity (Dhadialla et al., 1998). Moreover, the changes in EcR expression were also observed in RH-5992 treated larvae of *Cydia pomonella* (Sun et al., 2003). From the results obtained in the present study as well as earlier data available, we hypothesize that RH-5849 and RH-5992 mimic the 20E excessive state that might cause the down regulation of H-fibroin gene via EcR complex. Dhadialla et al., (1998) showed that insecticide intoxicated larvae prematurely slipped their old head capsules in an attempt to ecdyse. In *Manduca sexta*, RH-5849 halted feeding and resulted in premature lethal molt (Wing et al., 1998). By these

observations, one can conclude that the RH-5849 and RH-5992 treatment results in disruption of the normal larval-pupal-adult transition in these insects.

The sticky coating of the silk fiber is provided by sericin proteins that are secreted in the MSG (Gamo et al., 1977; Sinohara, 1979). In the present study we see the absence of the globular secretory protein, probably sericin in the silk produced by RH5849 and RH5992 fed insects. However, it remains to be investigated whether sericin expression is down regulated upon treatment with these hormone analogs and leads to decrease in the overall diameter of the silk fiber in *C. cephalonica*.

CONCLUSION

The sequences of L-fibroin, P25 and H-fibroin gene and their analyses in *C. cephalonica* infer the presence of H-L/P25 elementary unit type in this species. These are single copy genes that are specifically present as single transcripts in salivary glands. These developmentally regulated genes are highly expressed during the last instar larval development. The antibodies generated against the recombinant L-fibroin and P25 proteins expressed in bacteria and the commercially synthesized polyalanine peptide were used for characterization of these genes at translational level. The molecular weights of the L-fibroin, P25 and H-fibroin proteins were found to be 25 kDa, 28 kDa and 350 kDa respectively. The tissue specific nature and the synchronous developmental pattern of these proteins corroborates with that of their respective transcript levels suggesting that these three proteins are co-expressed. Further, it was shown that the expression of these silk genes is under direct control of 20E and juvenile hormone during the last instar larval development. 20E regulates the gene expression in a dose-dependent manner. Physiological concentration does not alter the normal expression, whereas higher concentrations have an inhibitory effect on these genes. Juvenile hormone counteracts the action of 20E and maintains the '*status quo*' state of these genes. Present study clearly indicates that an interplay between hormones is essential for the expression of silk genes in *C. cephalonica*. The last part of our study reveals that the regulation of H-fibroin gene is ecdysteroid dependent and EcR mediated. The various effects observed upon non-steroidal 20E analog treatment further signify the role of 20E mediated regulation of H-fibroin in insect development. More studies on such silk producing insect pests in this direction may provide an insight for their efficient control and management.

However, these studies further pave way for many more avenues to work on. The effect(s) of 20E as well as methoprene on H-fibroin, L-fibroin and P25 expression in the

earlier larval stages of this insect pest will give a more detailed understanding of their regulation. The upstream regions of both L-fibroin and P25 need to be cloned and analyzed further for the presence of any ecdysteroid regulatory elements that might control these genes. The mechanism of regulation of juvenile hormone on the silk genes of *C. cephalonica* needs to be investigated. The involvement of EcR in the 20E mediated H-fibroin gene regulation confirms its presence in the salivary gland cells. Hence, *C. cephalonica* EcR should be cloned and characterized and the effect of 20E and juvenile hormone on its expression could be monitored at various developmental stages. Also, the role of sericin proteins, if any, in silk fiber formation should be analyzed. The other interesting phenomenon noticed in the present study is the absence of sericin/globular proteins on the surface of silk fibers produced nonsteroidal 20E agonist treated insects. Hence it would be worthwhile to analyze the hormonal regulation of sericin genes in *C. cephalonica*.

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Light chain fibroin and P25 genes of *Corcyra cephalonica*: Molecular cloning, characterization, tissue-specific expression, synchronous developmental and 20-hydroxyecdysone regulation during the last instar larval development ☆

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ABSTRACT

The biologically active ecdysteroid hormone, 20-hydroxyecdysone (20E), regulates various processes like molting, metamorphosis and reproduction in insects. However, its role in expression of silk genes is obscure. The silk core in insects is generally constituted of a complex of three proteins namely, H-chain fibroin (H-fibroin), L-chain fibroin (L-fibroin) and P25. In the present study, we report full-length cDNA cloning and characterization of L-fibroin and P25 genes from rice moth, *Corcyra cephalonica*. Northern analyses demonstrated 1.1 and 1 kb transcripts of L-fibroin and P25 respectively. The tissue expression pattern shows the presence of these transcripts specifically in the salivary gland. These two genes are developmentally regulated at transcriptional level and their maximum expression is observed during the late-last instar larval stage. Semi-quantitative and real-time PCR studies revealed that 20E regulates the expression of these genes in a dose-dependant manner. This study further shows that physiological dose of 20E does not alter the normal expression of these two genes, whereas treatments with higher doses cause a significant decline in the expression. This study clearly suggests the role of 20E in the regulation of L-fibroin and P25 at molecular level.

1. Introduction

Among insects, lepidopteran larvae produce silk from a pair of salivary glands, each of which consists of silk-secreting posterior, middle and anterior regions (Sehnal and Akai, 1990). The posterior section gland (PSG) produces fibrous silk core that is typically composed of a large protein, designated as heavy chain fibroin (H-fibroin) and is associated with two small proteins, L-fibroin and P25 (also called fibrohexamerin). The middle section gland (MSG) secretes several sericins (ranging between 65 and 400 kDa), which provide a sticky coating on the fiber and also add several low molecular components with presumably protective functions to the silk (Zurovec et al., 1998a; Nirmala et al., 2001). For the silkworm *Bombyx mori*, it was shown that H-fibroin (~390 kDa) and L-fibroin (~25 kDa) molecules are linked by a disulphide bond and six such heterodimers are assembled with a single P25 molecule into a complex called elementary fibroin unit (Inoue et al., 2000). The site of disulphide linkage between H- and L-fibroins was identified as the 20th residue from the carboxyl terminus (Cys-20) of H-fibroin and Cys-172 of L-fibroin (Tanaka et al., 1999a). This H–L bonding was shown to be essential for the efficient secretion of fibroin (Takei et al., 1984, 1987). It was also predicted that P25 facilitates proper folding of H-fibroin by hydrophobic interactions (Tanaka et al., 1999b). In the pyralid moth *Galleria mellonella*, the larger H-fibroin (~500 kDa) is disulphide linked to the L-fibroin and non-covalently linked to P25 (Zurovec and Sehnal, 2002). The construction of the silk filament from the three protein components has been conserved for nearly 150 million years of lepidopteran evolution (Yonemura and Sehnal, 2006). An extreme deviation of the silk structure occurs in Saturniid silk moths, which possess dimers of modified H-fibroin and lack L-fibroin and P25 (Hwang et al., 2001; Tanaka and Mizuno, 2001). Recently, it was shown that the larvae of Trichoptera contain homologues of H-fibroin (>500 kDa) and L-fibroin (25 kDa) but not P25 (Yonemura et al., 2006). It remains intriguing that although the presence of these proteins are important for proper secretion, they are completely absent in few insect species. Hence, it's necessary to examine L-fibroin and P25 in many other insect species for better understanding.

The development as well as function of silk glands was shown to be under hormone control (Sehnal and Akai, 1990). Ecdysone is the principal steroid hormone in insects that is synthesized by the action of prothoracicotropic hormone (PTTH) on the prothoracic glands. The inactive form of ecdysone is released into the hemolymph which

☆ *Corcyra cephalonica* L-chain fibroin and P25 nucleotide sequences have been submitted to GenBank under the accession Nos. GQ901975 and GQ901976 respectively.

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is converted to active form, 20-hydroxyecdysone (20E) in the peripheral tissues (Gilbert et al., 1980; Riddiford et al., 2001). In *B. mori*, it was found that low titers of ecdysteroids are necessary for proper silk gland function. On the other hand, high concentrations of ecdysteroids caused silk gland regression during the larval molts or degeneration during the pupal molts (Shigematsu and Moriyama, 1970). The hormonal studies on *B. mori* were mainly intended to commercially exploit its silk (Miao et al., 2004). In case of *G. mellonella* too, similar results were obtained (Sehnal and Michalik, 1984). However, the effect of 20E on the expression of silk genes was not addressed at molecular level. Further, most of the earlier studies were performed on intact larvae and therefore, the effect(s) observed might not be truly physiological as the presence of endogenous hormones would interfere and the regulation of transcription of genes could only be seen as secondary effect (Grzelak and Kumaran, 1985).

We chose *Corcyra cephalonica* as our experimental model to study the regulation of 20E on L-fibroin and P25 gene expression during the last larval instar. *C. cephalonica* is a worldwide pest on stored cereal grains and legumes. Hence, this study would be the first instance to examine the role played by 20E in silk gene expression of a stored grain insect pest. Moreover, to our knowledge there are no studies on the independent effect of 20E on the expression of L-fibroin and P25. Further, the developmental regulation of these two genes was also not analyzed earlier. Hence, the present study reports cloning of full-length cDNA, molecular characterization and developmental regulation of L-fibroin and P25. Similarities and differences with the known corresponding sequences in *B. mori* and *G. mellonella* have been highlighted. Furthermore, we found that the effect of 20E on L-fibroin and P25 is dose-dependent and it regulates the transcription of both the genes. Taken together, in this paper we have made an attempt to provide the molecular structure of L-fibroin and P25 genes and discussed the role played by 20E in regulation of their expression during the last instar larval development.

2. Materials and methods

2.1. Experimental insects

C. cephalonica is commonly known as rice moth and belongs to the order Lepidoptera and family Galleridae. The larval forms were reared in culture troughs that contained coarsely crushed sorghum seeds. The cultures were maintained at 26 ± 1 °C, 60 ± 5% relative humidity (RH) and 14:10 h light:dark (LD) photoperiod. The larval development proceeds through five instars and is completed in about 45–50 days. The final (5th) larval instar was further classified based on their body weight and head capsule size into early-last (ELI), mid-last (MLI) and late-last instar (LLI) followed by wandering or early-prepupal (EPP) and late-prepupal (LPP) stages (Lakshmi and Dutta-Gupta, 1990). For the present study, the above mentioned stages were used.

2.2. Cloning of L-fibroin and P25

Total RNA was isolated from the silk glands using Trizol reagent (Sigma). Five micrograms of total RNA was used for cDNA preparation using Superscript™ III first strand synthesis system (Invitrogen). Degenerate primers were designed using LaserGene and GeneRunner softwares (versions 3.05). Partial clone of L-fibroin was obtained using, F1 [ACG AGC GCA TTW GCM GCY CC] as the forward primer and R1 [GCG GMT RTT GAA GGC RTT GTA CAG] as the reverse primer. Partial clone of P25 was obtained using, F2 [TTC AAC GCT WCK TAC ATM GAC] as the forward primer and R2 [GGC AGA GCR RYG ACG TCG G] as the reverse primer. The PCR products were cloned into p-GEM-T easy vector (Promega).

2.3. 5′ RACE and 3′ RACE (rapid amplification of cDNA ends)

For obtaining the full-length cDNAs of L-fibroin and P25, 5′ and 3′ RACE reactions were carried out using RNA-ligase mediated RACE system (Invitrogen). Preparation of 5′ and 3′ cDNA (RACE) templates and RT-PCR were done using gene specific primers. The primers designed for L-fibroin 5′ RACE and 3′ RACE were L1 [GCG GAT GTT GAA GGC GTT GTA CAT GCG T] and L2 [CTG TCT CCA GCT ACT TAA CGG AC]-3′ respectively. The primers designed for P25 5′ RACE and 3′ RACE were P1 [GCA CCC AAA GTC GCA AAT GAG AGA G] and P2 [GGC TGA CAC ATC AGT GCT GTC CGT AGA] respectively. All the PCR conditions were programmed as specified in the manufacturer's protocol. The amplified products were cloned into p-GEM-T easy vector (Promega).

2.4. Northern hybridization

Total RNA was isolated from different tissues. The quality and integrity of the RNA was checked using denaturing RNA electrophoresis. The RNA was transferred onto nylon membrane (Hybond+, Amersham Biosciences). Pre-hybridization was carried out for 3 h in Church buffer (0.5 M sodium phosphate buffer (pH 7.2), 1 mM EDTA, 7% SDS and 1% BSA) at 65 °C. [α^{32}P]d ATP labeled probes were prepared from the PCR amplified L-fibroin and P25 full-length cDNA clones using Hexalabel random primer labeling kit (MBI Fermentas). The radiolabelled probes were purified by passing the mixture through Sephadex G-25 column. Hybridization with the prepared probes was carried out for 16 h at 65 °C. After hybridization, first wash was given at room temperature with 5 × SSC for 5 min, second low stringent wash was done using 2 × SSC, 0.1% SDS for 10 min, third medium stringent wash was done with 1 × SSC, 0.1% SDS for 10 min and the final high stringent wash was performed using 0.1 × SSC, 0.1% SDS for 15 min. All the washes were carried at 65 °C. The blot was dried and was exposed to X-ray film. The signals were detected by autoradiography.

2.5. Southern hybridization

Genomic DNA was isolated and quantified as described elsewhere (Sambrook et al., 2001). Ten micrograms of the purified genomic DNA was digested with four different restriction enzymes i.e. *EcoRI*, *BamHI*, *HindIII* and *KpnI* (MBI Fermentas) and subjected to 0.8% agarose gel electrophoresis. The gel was soaked for 45 min in several volumes of 0.4 N NaOH and 1 M NaCl followed by neutralization for 30 min in 0.5 M Tris–HCl (pH 7.2) and 1 M NaCl. Digested DNA was transferred onto nylon membrane and was cross-linked by UV irradiation. The pre-hybridization and hybridization protocol used were same as in the case of northern experiments.

2.6. Organ culture of salivary glands

Salivary glands dissected under sterile condition in ice cold insect Ringer (130 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2$ and 1 mM PMSF) were rinsed in 100 µl of TC-100 insect culture medium (Sigma) with traces of streptomycin sulfate. This was followed by transfer of the gland to fresh 500 µl medium for 1 h as preconditioning prior to the required experimental set up. Based on the experiment, the tissue was incubated in the culture medium along with different concentrations of 20E for varying time periods in an incubator (95% relative humidity, 5% CO_2 and 25 °C). After incubation, the tissue was rinsed in ice cold Ringer and processed for RNA isolation. The choice of TC-100 medium for culture was due to less complex composition as compared to other insect media, contains no insect hemolymph (important for present study) and developed to support growth of lepidopteran cells in culture (Gardiner and Stockdale, 1975).

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2.7. MTT staining

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Himedia Chemicals) was used to measure cell viability. The MTT dye was dissolved (5 mg/ml) in PBS (pH 7.0). The salivary glands cultured in 500 µl of TC-100 medium were incubated in 50 µl of MTT solution in dark for 1 h and were observed under the microscope. Freshly excised salivary glands (0 h) were used as positive control.

2.8. Enzyme immunoassay

The protocol of the manufacturer was followed (Cayman Chemicals). Briefly, the 96-well plate that was precoated with mouse monoclonal anti-rabbit antibody was incubated with acetylcholine esterase linked to 20E (tracer), 20E antiserum and either standard or unknown sample. Washes were performed to remove all unbound reagents. The assay is based on the competition between 20E (in the hemolymph) and 20E tracer for a limited amount of 20E antiserum. The Ellman's reagent that contains the substrate for acetylcholine esterase was added to the wells. The product of this enzymatic reaction has a distinct yellow color and was measured at 412 nm. For the assay, hemolymph was collected from different larval stages and to that 5 volumes of diethyl ether was added. The upper ether layer that contained the hormone was transferred into a new tube and the extraction procedure was repeated twice. The combined ether extract was evaporated by heating at 30 °C under a gentle stream of nitrogen. The dried extract was dissolved in the appropriate buffer for estimation.

2.9. Semi-quantitative and Real-time PCR

Total RNA was isolated from control as well as hormone treated tissue. The purity and quantity of RNA was checked by using Nanodrop (ND-1000) spectrophotometer. Five micrograms of total RNA was reverse transcribed using Superscript III™ first strand synthesis system (Invitrogen). PCR reactions (20 µl) were performed using Power SYBR Green Master mix (Applied Biosystems). Reactions were set up using primer sets for L-fibroin (sense 5′-TAT TAC TCG TCG CGT CGA GCG C-3′ and antisense 5′-CAG CTC ACC GAG AGC TGC AAC-3′), P25 (sense 5′-TCC TTG TGC TAG CTG GCA CG-3′ and antisense 5′-TGA ACC GGG GGA TGG TGT ACT-3′) and *C. cephalonica β-actin* gene (sense 5′-GGT AGT AGA CAA TGG CTC CGG-3′ and antisense 5′-CCC AGT TAG TGA CGA TTC CGT G-3′). Semi-quantitative PCR products were visualized on 1% agarose gel. For real-time PCR, reactions were measured using Fast 7500 Real-time PCR system (Applied Biosystems). PCR conditions were optimized to generate >95% PCR efficiency. Dissociation curve analysis was performed after the last cycle to confirm amplification of a single product. The real-time results are presented as change in expression relative to control using target gene Ct values normalized to that of *β-actin* gene Ct values based on the $2^{(-\Delta\Delta C(T))}$ method (Livak and Schmittgen, 2001).

2.10. Statistical analysis

Data were expressed as mean ± SEM ($n = 3$). Significance between groups was analyzed by ANOVA followed by Student Newman–Keuls test using Sigmastat (version 3.1). Values were considered significant at $P < 0.05$.

3. Results

3.1. Cloning of full-length cDNA of L-fibroin and P25 genes

The PCR amplification of L-fibroin and P25 using degenerate primers yielded 550 and 400 bp fragments respectively. These cDNA sequences were used further for designing the primers, to obtain full-length cDNA. The 5′ and 3′ cDNA ends were cloned and sequenced. The coding sequence of *C. cephalonica* L-fibroin

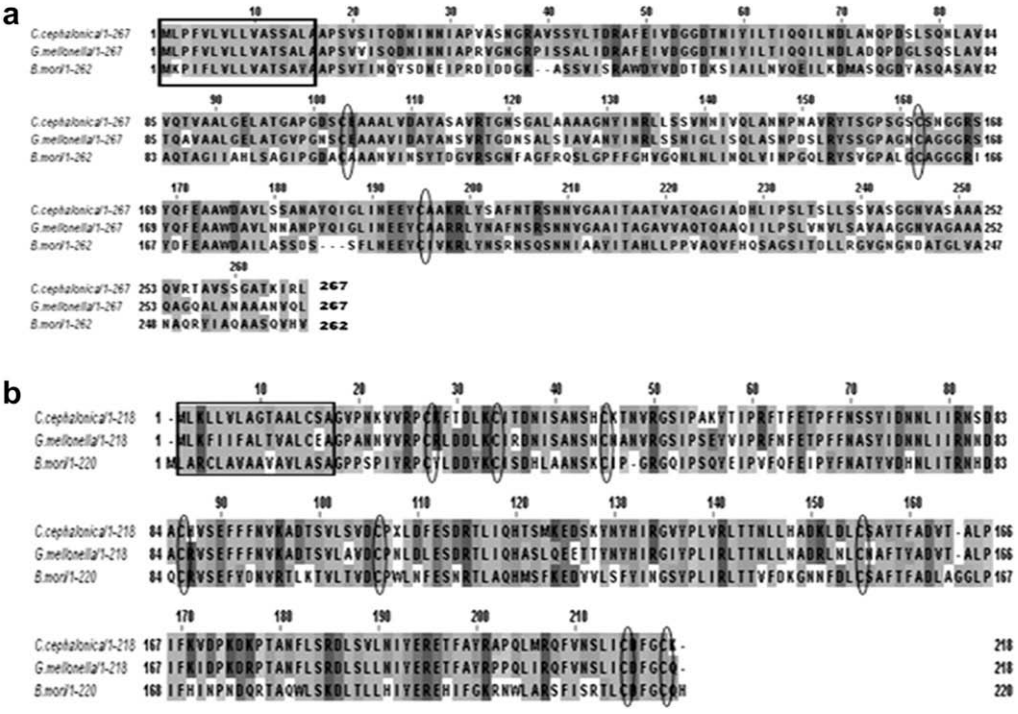

Fig. 1. (a) ClustalW multiple alignment of L-fibroin from *C. cephalonica* (GenBank Accession No. GQ901975) with its counterparts in *G. mellonella* and *B. mori* (GenBank Accession Nos. S77817 and X17291 respectively). All the three conserved cysteine residues are encircled and the conserved signal peptide sequence is in rectangle. (b) ClustalW multiple alignment of P25 from *C. cephalonica* (GenBank Accession No. GQ901976) with its counterparts in *G. mellonella* and *B. mori* (GenBank Accession Nos. AF009827 and X04226 respectively). All the eight conserved cysteine residues are encircled and the conserved signal peptide sequence is shown in rectangle.

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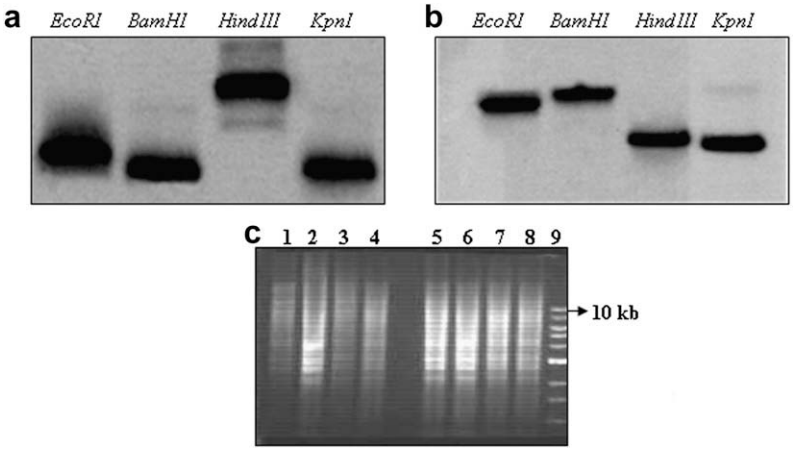

Fig. 2. Southern blot analysis of genomic DNA extracted from *C. cephalonica* salivary gland, digested with *EcoRI* (lane 1), *BamHI* (lane 2), *Hind III* (lane 3) and *KpnI* (lane 4) respectively and probed with plasmid containing full-length cDNA of (a) L-fibroin and (b) P25. (c) The profile of DNA digested with *EcoRI* (lanes 1 and 5), *BamHI* (lanes 2 and 6), *Hind III* (lanes 3 and 7) and *KpnI* (lanes 4 and 8) along with the 10 kb ladder (lane 9) is shown.

consists of 804 nucleotides and codes for a polypeptide of 267 amino acids. The 3′ UTR region consists of 280 nucleotide bases and the 5′ UTR region consists of 41 bases. The total mRNA of L-fibroin accounts for 1125 bases. The 3′ UTR region of L-fibroin contains one polyadenylation signal. The characteristic feature of three conserved cysteine residues, in all known L-fibroins is seen in this species too. *C. cephalonica* L-fibroin shows 71% homology with *G. mellonella* and 40% homology with *B. mori*. The N-terminal region shows a high degree of homology with both the insect species (Fig. 1a). The *pI* value for the deduced L-fibroin sequence is 4.8. L-fibroin nucleotide sequence has been submitted to GenBank (Accession No. GQ901975).

The coding sequence of *C. cephalonica* P25 consists of 657 nucleotides and the corresponding peptide consists of 218 amino acids. The 3′ UTR region is 330 bases long and the 5′ UTR region consists of 53 nucleotides. The total mRNA of P25 consists of 1040 bases. Two polyadenylation signal sites are present in the 3′ UTR region. The eight cysteine residues present in the coding sequence are well conserved among all the P25 sequences reported. *C. cephalonica* P25 shows 78% homology with *G. mellonella* and 50% homology with *B. mori* (Fig. 1b). The *pI* value of the deduced P25 sequence is 8.3. P25 nucleotide sequence has been submitted to GenBank (Accession No. GQ901976).

3.2. Southern and Northern analyses of L-fibroin and P25

Southern blot analyses of L-fibroin and P25 identified a single hybridizing band, suggest that both these genes exist as a single copy per haploid genome (Fig. 2a and b). Northern analyses show a single 1.1 kb transcript of L-fibroin in the salivary gland of *C. cephalonica*, the size of which matches exactly with the corresponding full-length cDNA sequence of 1125 nucleotides (Fig. 3a). In case of P25, a single 1 kb transcript was obtained which is expected from its full-length cDNA sequence (Fig. 3b). The intensity of the signal obtained shows that these genes are highly expressed in the salivary gland. The sizes of the transcripts were determined by *Rf* values plotted against log values of RNA ladder (MBI Fermentas). Further, it was shown that the transcripts for L-fibroin and P25 were selectively found in the salivary gland and were not detected in fat body, gut and the remaining body carcass (Fig. 3c and d).

3.3. Developmental pattern of L-fibroin and P25

Real-time study shows that both L-fibroin and P25 genes are developmentally regulated (Fig. 4a and b). The quantitative PCR analysis depicts that the expression of both the genes increased

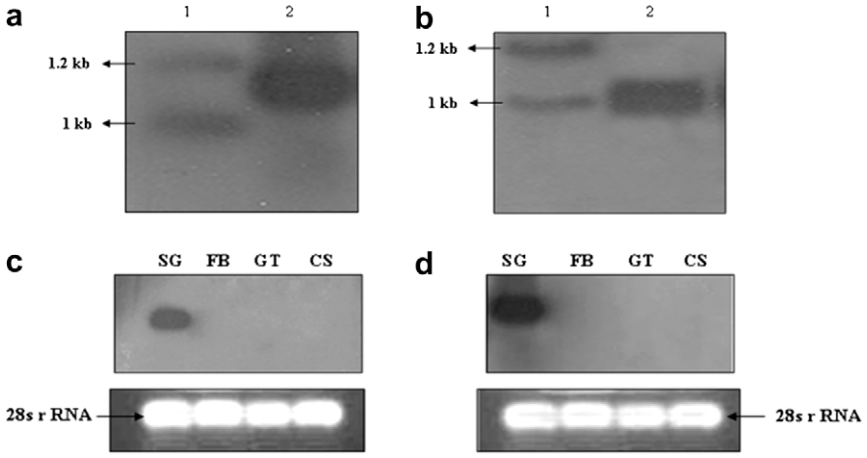

Fig. 3. Northern blots showing the transcript sizes of (a) L-fibroin (lane 1: marker and lane 2: ~1.1 kb transcript) (b) P25 (lane 1: marker and lane 2: ~1 kb transcript). Tissue-specific expression of (c) L-fibroin and (d) P25 is seen in the larval salivary glands (SG) of *C. cephalonica*. Please note that signal was not detected in the fat body (FB), gut (GT) and the remaining body carcass (CS). Equal loading of RNA (28S rRNA) is shown.

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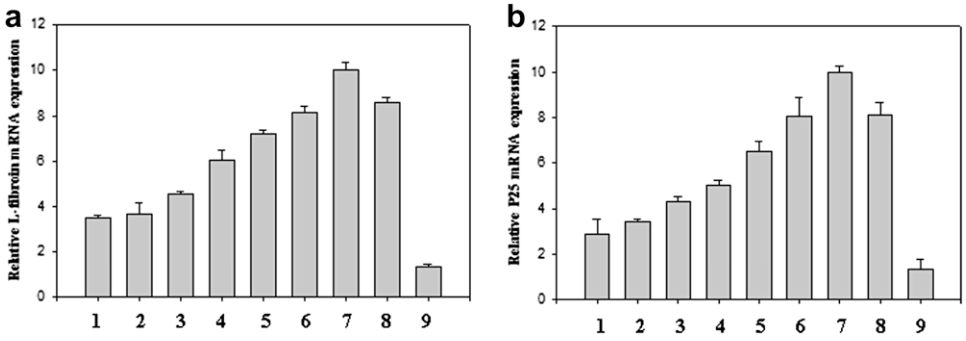

Fig. 4. Stage-dependent expression of (a) L-fibroin and (b) P25 in *C. cephalonica* salivary glands by real-time RT-PCR. L-fibroin and P25 expression is reported as fold change relative to late-last instar larvae (7). 1: 1st instar, 2: 2nd instar, 3: 3rd instar, 4: 4th instar, 5: early-last instar, 6: mid-last instar, 7: late-last instar larvae, 8: early-prepupal and 9: late-prepupal. Each value was expressed as mean ± SEM, $n = 3$.

gradually during larval development from 1st instar to 5th instar. Thereafter, the expression began to decline during the wandering early-prepupal (EPP) stage and was significantly low at late-prepupal (LPP) stage. The maximum expression was observed during the late-last instar larval stage.

3.4. Effect of 20E on L-fibroin and P25 mRNA expression

The MTT staining studies showed that the salivary glands were viable in the culture medium till 24 h (Supplementary Fig. 1). Hence the time points of ½, 3, 6, 9 and 12 h were chosen for hormone treatment studies. Hemolymph levels of 20E estimated by enzyme immunoassay method during ELI, LLI larval and EPP stages were found to be 0.008, 0.4 and 5.7 pg/μl respectively. These concentrations of 20E were considered as physiological (Supplementary Fig. 2). Further, we examined the effect of physiological as well as higher concentrations of 20E on the expression of L-fibroin and P25 genes using semi-quantitative and quantitative PCR. The transcript level of ethanol treated glands that were cultured through the same time points was used as external control. It was found that, at hemolymph levels of 20E the expression of L-fibroin and P25 was unaltered at both LLI (Figs. 5 and 6) and ELI larval stages (Figs. 7 and 8) when compared with the control. When higher concentrations of 20E were given (0.4 pg/μl in case of ELI stage silk glands and 4 and 5.7 pg/μl in case of LLI stage silk glands) there was a decline in the expression of both the genes (Figs. 5–8).

4. Discussion

4.1. Analyses of L-fibroin and P25 sequences

So far, three types of silk fibroin structures have been elucidated in insects. The H–L/P25 structure is well characterized in *B. mori* and *G. mellonella,* while in Saturniid moths the H–H fibroin

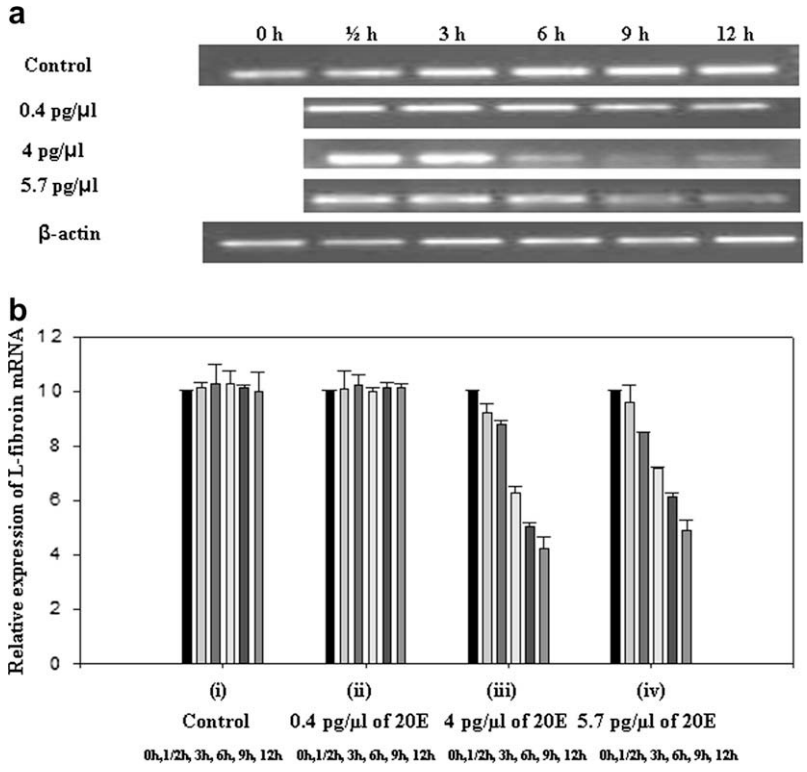

Fig. 5. Effect of 20E on L-fibroin expression of LLI larval silk glands. (a) Semi-quantitative PCR showing the expression at time points 0, ½, 3, 6, 9 and 12 h in control (ethanol treated) and hormone treated glands (0.4, 4 and 5.7 pg/μl of 20E). β-Actin was used as internal control. (b) Real-time PCR ($n = 3$) showing the expression at 0, ½, 3, 6, 9 and 12 h time points in (i) control (ethanol treated) and hormone treated (ii) 0.4 pg/μl of 20E, (iii) 4 pg/μl of 20E and (iv) 5.7 pg/μl of 20E. L-fibroin expression is reported as fold change relative to 0 h control.

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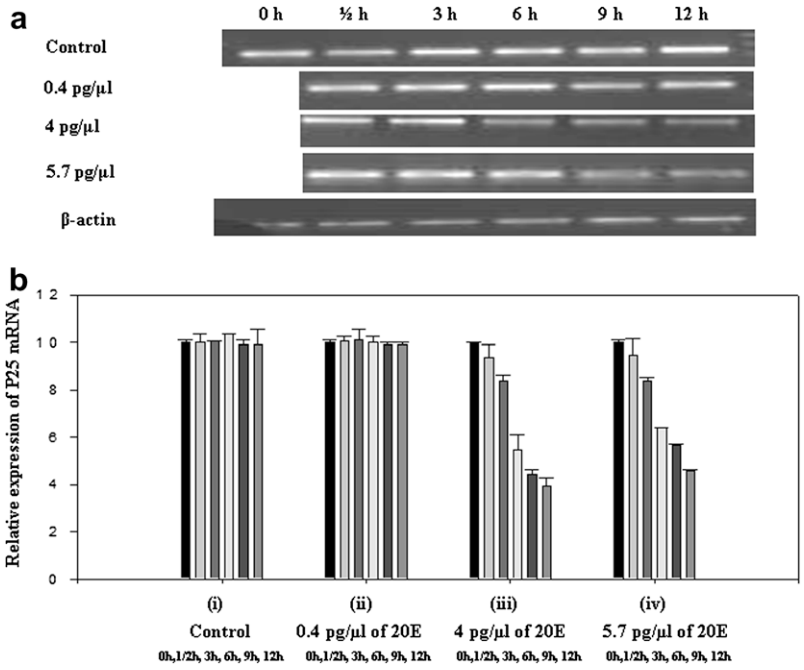

Fig. 6. Effect of 20E on P25 expression of LLI larval silk glands. (a) Semi-quantitative PCR showing the expression at time points 0, ½, 3, 6, 9 and 12 h in control (ethanol treated) and hormone treated glands (0.4, 4 and 5.7 pg/µl of 20E). β-Actin was used as internal control. (b) Real-time PCR ($n = 3$) showing the expression at 0, ½, 3, 6, 9 and 12 h time points in (i) control (ethanol treated) and hormone treated (ii) 0.4 pg/µl of 20E, (iii) 4 pg/µl of 20E and (iv) 5.7 pg/µl of 20E. P25 expression is reported as fold change relative to 0 h control.

structure is predominant (Sehnal and Zurovec, 2004). In case of Trichoptera larvae the silk core comprises of H-fibroin and L-fibroin, but not P25 (Yonemura et al., 2006). Our study confirms the presence of L-fibroin and P25 in *C. cephalonica*. Further, we have cloned a partial H-fibroin cDNA (unpublished result) from this species suggesting the presence of H–L/P25 elementary unit type (Inoue et al., 2000). For L-fibroin sequence, the maximum probability of signal peptide cleavage site exists between 16 and 17 amino acid residues. The presence of signal peptide cleavage site indicates that L-fibroin is a secretory protein. Hence, the mature L-fibroin protein containing 251 amino acid residues has a deduced M_r of 25.4 kDa, which is well in agreement with the molecular weight of L-fibroins of *B. mori* (Kimura et al., 1985; Yamaguchi et al., 1989) and *G. melonella* (Zurovec et al., 1995). The *pI* value of *C. cephalonica* L-fibroin suggests its acidic nature.

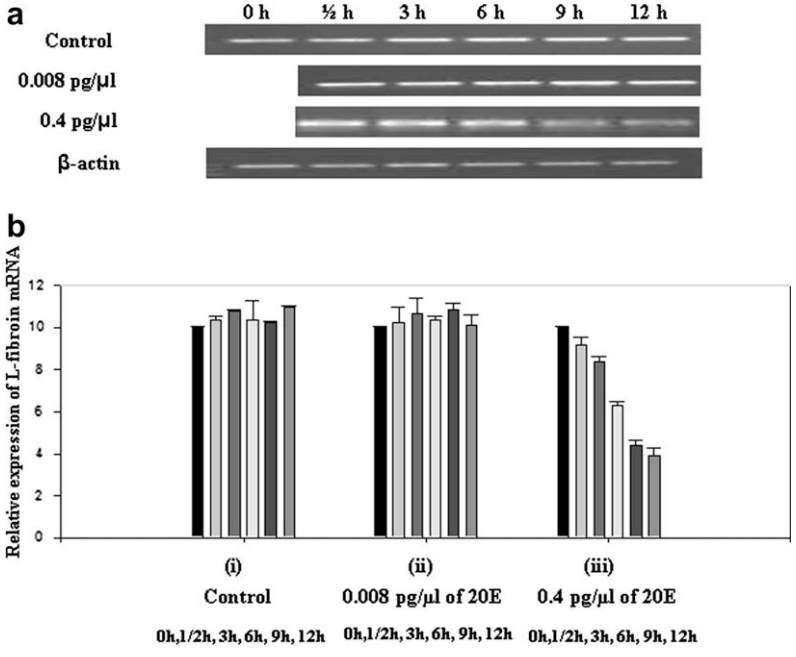

Fig. 7. Effect of 20E on L-fibroin expression of ELI larval silk glands. (a) Semi-quantitative PCR showing the expression at time points 0, ½, 3, 6, 9 and 12 h in control (ethanol treated) and hormone treated glands (0.008 and 0.4 pg/µl). β-Actin was used as internal control. (b) Real-time PCR ($n = 3$) showing the expression at 0, ½, 3, 6, 9 and 12 h time points in (i) control (ethanol treated) and hormone treated (ii) 0.008 pg/µl of 20E and (iii) 0.4 pg/µl of 20E. L-fibroin expression is reported as fold change relative to 0 h control.

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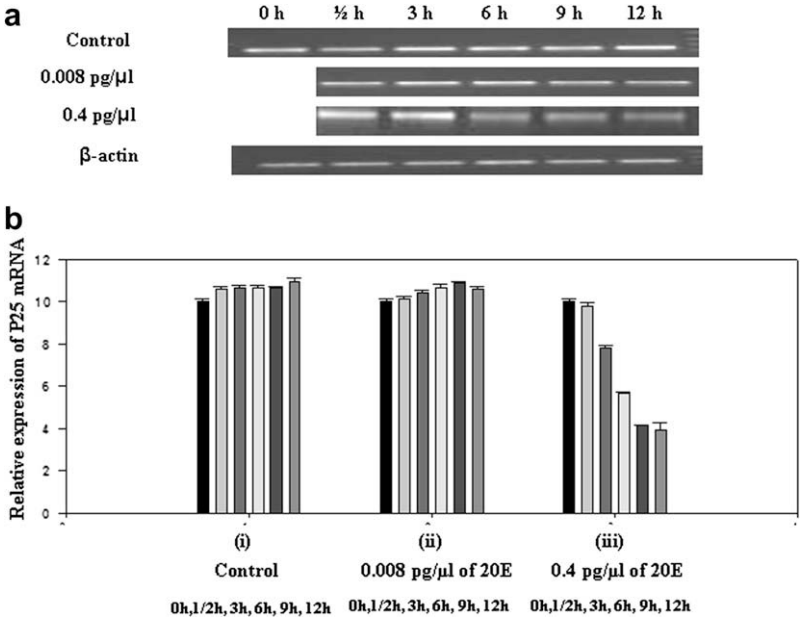

Fig. 8. Effect of 20E on P25 expression of ELI larval silk glands. (a) Semi-quantitative PCR showing the expression at time points 0, ½, 3, 6, 9 and 12 h in control (ethanol treated) and hormone treated glands (0.008 and 0.4 pg/µl). β-Actin was used as internal control. (b) Real-time PCR (n = 3) showing the expression at 0, ½, 3, 6, 9 and 12 h time points in (i) control (ethanol treated) and hormone treated (ii) 0.008 pg/µl of 20E and (iii) 0.4 pg/µl of 20E. P25 expression is reported as fold change relative to 0 h control.

The deduced M_r of P25 protein of *C. cephalonica* is similar to 25 kDa. In P25 sequence, the signal peptide cleavage site is present between 16 and 17 residues indicating that P25 too is a secretory protein. Thus, the mature P25 protein would contain 202 amino acid residues with a M_r similar to 23 kDa. One interesting feature observed in the P25 sequence is that it has two putative glycosylation sites. Earlier studies reveal differences in the number as well as the position of glycosylation sites among the insects (Inoue et al., 2003). The *B. mori* model, where a P25 consists of three separate sugar moieties bound to six different H-fibroin molecules might not be applicable to our insect model. The *pI* value of deduced P25 sequence of *C. cephalonica* was found to be 8.3, which is basic. The *pI* values vary among various insect species. In case of *B. mori* and *G. mellonella* it was found to be acidic, while in *Papilio xuthus* and *Dendrolimus spectabilis* it was basic in nature. Results obtained suggest that these differences exist among closely related insects too.

The conserved location of cysteines, several other residues and the patterns of charge distribution indicates that certain regions of the H-, L-fibroin and P25 are probably important for their mutual interactions (Yonemura and Sehnal, 2006). Further, the notably high conservation of signal sequences suggests that the use of certain sites of entry into the endoplasmic reticulum might be important for the linkage of these proteins (Inoue et al., 2003). The presence of purine bases at −3 position and AUG as the start codon in the sequences of L-fibroin and P25 shows the presence of Kozak sequence (Kozak, 1984) on their transcripts.

4.2. Northern and Southern analyses of L-fibroin and P25

A single 1.1 kb transcript of L-fibroin was shown to be expressed in *C. cephalonica*. The presence of only one polyadenylation site in *C. cephalonica* might account for the single transcript of L-fibroin. In case of *G. melonella*, L-fibroin was shown to contain multiple polyadenylation sites and therefore exhibit alternative splicing to form two different transcripts (Zurovec et al., 1995). The structure of the L-fibroin gene was elucidated only in *B. mori*. The gene consists of 13,472 nucleotides and its coding region is interrupted with six exons while L-fibroin exists as a single copy gene (Kikuchi et al., 1992). A single copy L-fibroin gene was also reported in *G. mellonella* (Zurovec et al., 1995). Southern hybridization analysis indicates that L-fibroin is present as single copy gene in *C. cephalonica* too.

The mRNA of P25 of *C. cephalonica* was found to be approximately 1 kb and it was shown to be expressed as a single transcript. A single P25 mRNA of about 1.3 kb was found in *B. mori* (Couble et al., 1985). The homologous mRNA of *G. mellonella* is around 2 kb due to the extension at the non translated 3′ end (Yang et al., 1998; Zurovec et al., 1998b). Although multiple transcripts for P25 have not been shown in any of the species, the number of polyadenylation site varies among different species. The P25 gene, present in a single copy, is nearly 3500 nucleotides long in *B. mori* (Couble et al., 1985). Our results corroborate with the earlier ones that P25 exists as a single copy per haploid genome in *C. cephalonica* too. Northern analyses data also confirm the tissue-specific expression of both L-fibroin and P25 genes. The expression was found to be specific to salivary gland.

4.3. Developmental regulation of L-fibroin and P25

Developmental expression of both the genes revealed a similar pattern. Maximum expression was seen during the LLI larval stage which declined towards the end of prepupal stage (LPP) where the insect stops feeding and commits to pupa formation. It was shown that Lepidopteran silk glands become secretory prior to hatching. During larval life these glands increase their secretory potential by growing in size and ploidy. Moreover, it was found that maximal growth of the silk glands occur in the last larval instar, especially in those species that use the silk primarily or exclusively for cocoon formation (Sehnal and Akai, 1990). These results strengthen our data showing a gradual increase in the expression from 1st larval instar to LLI larval stage and clearly suggest that the transcription of both the genes are developmentally regulated. Our study becomes the first report of developmental regulation of these genes at molecular level.

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4.4. 20E regulation of L-fibroin and P25

Maximum silk fibroin synthesis and greater cellular changes were observed in the last larval instar (Suzuki and Suzuki, 1974). Maximum expression of L-fibroin and P25 during the last instar larval development was observed in our study too. Based on this, last instar larvae were chosen to study the regulation of 20E on the transcription of these genes. The *in vivo* studies on the participation of ecdysteroids in the silk gland regulation, performed on intact larvae, showed the effect on various parameters like total RNA synthesis, quality of the silk produced, cytological changes and the ultra structure (Shigematsu and Moriyama, 1970; Grzelak et al., 1993; Grzelak, 1995; Yang et al., 1995). The *in vitro* effect(s) of 20E were shown either on isolated nuclei, cultured silk glands or ligated larvae (Maekawa et al., 1984; Kodrik and Sehnal, 1994). But none of these studies showed the action of 20E on the expression of L-fibroin and the P25 at transcriptional level.

Hormones including ecdysteroids participate in the regulation of expression of silk protein genes. It was also found that low titers of ecdysteroid are necessary for proper silk gland function (Sehnal and Akai, 1990). In *C. cephalonica*, treatment with physiological concentration of 20E (0.4 pg/μl) during LLI stage, did not alter the expression of both the genes when compared with that of ethanol treated control glands. Further at this stage both the genes were shown to attain maximum expression. Therefore, it was not clear whether low dose of 20E is required for normal expression. Therefore, we monitored the effect of 20E on the expression of both these genes in ELI larval silk glands. The hemolymph level of 20E during ELI stage (0.008 pg/μl) too did not alter the normal expression of both the genes at this stage. Studies in *B. mori* reveal that high concentrations of ecdysteroid cause silk gland degeneration during pupal molt (Shigematsu and Moriyama, 1970). Similar results were obtained in the case of *G. mellonella* too (Sehnal and Michalik, 1984; Kodrik and Sehnal, 1994). Moreover, it was shown that pupal molt is associated with high titers of ecdysteroids (Calvez et al., 1976; Plantevin et al., 1984). The developmental pattern of L-fibroin and P25 genes in *C. cephalonica* shows that their expression declined during pupal stage. Therefore, on treatment of LLI larval silk glands with higher dosage of 20E (4 and 5.7 pg/μl seen during EPP stage), the expression of these genes declined significantly. Such prominent effect of 20E is most likely mediated by ecdysone receptors present in the salivary glands cells (Elmogy et al., 2004; Sun and Song, 2006). Treatment of ELI larval silk gland with 0.4 pg/μl of 20E (the physiological concentration of 20E found during LLI larval stage) once again caused a decline in the expression of L-fibroin as well as P25 genes which reconfirms that the high concentration of 20E has an inhibitory effect on these genes. Present study revealed that 20E regulates L-fibroin and P25 expression in a dose-dependant manner.

In summary, full-length cDNAs of L-fibroin and P25 were cloned from salivary glands of *C. cephalonica*. Tissue specific nature of these two genes was demonstrated. Present study also shows that these two genes are developmentally regulated at transcriptional level. Further, we also show the independent action of 20E on the expression of L-fibroin and P25 at mRNA level, which was not reported earlier. Our study emphasizes the role of 20E in the regulation of L-fibroin and P25, which are important for proper secretion of silk from insects.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygcen.2010.02.007.

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