Results - Chapter I

Identification, isolation and characterization of HP19 from C. cephalonica

Part of this work has been published in-

**Background**

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

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

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

When insect larvae are ligated behind the first pair of prolegs, *i.e.*, behind the hormone producing glands, the posterior part of the animal is known to be relatively free from endogenous ecdysteroids (Priester *et al.*, 1979; Burmester and Scheller, 1997a; Dutta-Gupta and Ashok, 1998). Figure 1 shows the effect of thorax-ligation (Fig. 1a) and injection of exogenous 20E (Fig. 1b) on the fat body ACP activity in LLI larvae. The ACP activity
declined gradually from 6 to 72 h after ligation. As the ACP activity in the fat body was significantly lower after 24 h of ligation, this time period was used for all hormone manipulation studies. Hormone injections of 80 nM 20E, i.e., the physiological concentration (Dutta-Gupta and Ashok, 1998) to 24 h post-ligated LLI larvae caused a significant increase in the ACP activity in fat bodies after 24 h, compared to the solvent treated larvae (Fig. 1b). The increase was also observed when 20E was injected to 48 h post-ligated LLI larvae for 24 h as compared to the solvent injection for 24 h to 48 h post-ligated larvae.

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

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

After loading total haemolymph protein on a sephadex G-50 column, we eluted several fractions (Fig. 3a) and checked their ability to mediate the 20E stimulated ACP activity. We found an active protein fraction with a molecular mass of approximately 22 kDa, calculated from the elution profile (Fig. 3a, inset), or 19 kDa, calculated from the mobility on a SDS-PAGE by the appearance of new protein band in the active fraction elute (Fig 3b, arrow). In another fractionation study, the total haemolymph protein was fractionated using specific molecular weight cut-off membrane filters of sizes 30 and 10 kDa (Fig. 4). Three fractions having masses above 100 kDa, 30-100 kDa and below 30 kDa were collected and the studies with them revealed that only the filtrate from 10 kDa cut off filter gave a protein fraction that mediated the 20E stimulated ACP activity of fat body cultures and an average increase from 0.7 to 1.2 n moles of PNP release/h/µg protein was consistently observed (Fig. 5a). These results clearly suggested that the active protein fraction present in the haemolymph, is a low mass protein of <30 kDa. On the basis of these results, the active
haemolymph protein fraction was purified by a strategy in which the total haemolymph protein was subjected to 60% salt precipitation, followed by fractionation using 30 and 10 kDa cut-off filters and finally the elution of active protein fraction by gel filtration chromatography (Fig. 4). The protein yield in the filtrate obtained from the 10 kDa cut off filter was insufficient to proceed for further purification. Therefore, the filtrate from the 30 kDa cut-off filters in which the HP19 is contained was used for gel filtration. The protein fraction eluted from the sephadex column that mediated the 20E dependent ACP activity (Fig. 6a), resulted in a contaminant free pure polypeptide band of 19 kDa (Fig. 6b). Hence, the active haemolymph protein was named as “HP19”. Starting with 50 mg total haemolymph protein, we obtained a 98.5-fold purification with 0.05% yield (Table 1).

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

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

The western blots show the specificity of HP19 antibody both on denatured (Fig. 9b) and non-denatured (Fig. 9c) PAGE. The specificity of the antibody was found to be high without much non-specific cross-reaction even when the total haemolymph protein per
sample was fairly high \textit{i.e.}, 40 and 80 µg per lane (Fig. 9b, lanes 3 & 4). A single protein band of 19 kDa detected in SDS-PAGE (Fig. 6b), denatured immunoblotting (Fig. 9b) and non-denatured immunoblotting (Fig. 9c) studies suggested a monomeric structure of HP19.

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

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

Co-culturing of different larval tissues with fat body demonstrated that HGLFB is the only HP19 synthesizing tissue. A stimulation of the ACP activity of fat body by 20E was only observed when it was co-cultured with HGLFB (Fig. 12a). The haemolymph used in all experiments was cell-free and therefore cannot be the site of HP19 synthesis. Western analysis of proteins from different tissues also revealed the presence of HP19 only in HGLFB (Fig. 12c, lane 2) and total larval body protein (Fig. 12c, lane 5). However, the apparent mass of HP19 in this tissue was approximately 5 kDa higher than the HP19 present in haemolymph (Fig. 12c, lane 1). The faint band of HP19 in the total larval body homogenate in the western blot (Fig. 12c, lane 5) is mainly due to the lower concentration of HP19 protein present in sample. The tissue specificity of HP19 biosynthesis was further confirmed by immunohistochemical staining of different tissue sections using HP19 antibody (Fig. 13). These studies once again showed that the HP19 is localized only in HGLFB (Fig. 13b and c) and not in tissues like visceral fat body (Fig. 13d), peripheral fat body (Fig. 13e), gut (Fig.
13f), carcass (Fig. 13g), Malpighian tubule (Fig. 13h) and salivary gland (Fig. 13i). In situ immunodetection of HP19 in larvae again showed the presence of HP19 only in the HGLFB (Fig. 14, arrow head inside the dotted circle).

**Developmental regulation of HP19**

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

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

We further confirmed whether a similar protein is present in other insects belonging to lepidoptera and diptera. The results presented in figure 17 suggest that apart from *C. cephalonica*, the haemolymph of other lepidopteran insects like *Bombyx mori, Spodoptera litura, Achaea janata, Galleria mellonela* and *Papilio demoleus* was able to stimulate the 20E dependent ACP activity in *C. cephalonica* fat body cultures. However, the stimulation in ACP activity was insignificant with *Calliphora vicina*, a dipteran insect as compared to the stimulation by the haemolymph of all other lepidopteran insect species. Western analysis of the haemolymph protein from these insect species with anti-HP19 IgG fraction further
indicated the presence of HP19 like protein in lepidopteran insects (Fig. 17c). However, similar protein was not detected in the haemolymph of *C. vicina* (Fig. 17c) despite of the relatively low degree of stimulation in ACP activity (Fig. 17a). The western blot also indicated that in these species, the protein is present in multiple forms or subunits as compared to the HP19 of *C. cephalonica*.

A detailed analysis of HP19 like protein in *B. mori* suggested that like *C. cephalonica*, only the haemolymph from LLI larval stage mediated the 20E dependent ACP activity stimulation (Fig. 18). Attempt on the characterization of the active haemolymph protein fraction (Fig. 19) resulted in appearance of approximately 26 kDa protein indicating that possibly this protein is responsible for the 20E dependent ACP activity stimulation (Fig. 19b, arrow head). These studies suggest that HP19 like protein is not species specific at least for the lepidopteran insect but is definitely stage specific.
Fig. 1: Identification of a factor(s) in the haemolymph that enhances the ACP activity in fat bodies of *C. cephalonica* larvae (LLI):-

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

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

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

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

At the end of the incubation, the fat bodies were removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenized and assayed for ACP activity. Each value is the mean ± S.D. of four independent determinations and for each assay, fat body from 2-3 insects was pooled. For (a) * is significantly different over † and all other values (p<0.05), for (b) † is significantly different from previous control values (p<0.05) and for (d) * is significantly different from all other values.
Fig. 2: Identification of the haemolymph factor(s) as protein:-

Effect of different pretreated *C. cephalonica* haemolymph (hae) in mediating the 20E dependent ACP activity of fat bodies kept in culture. The haemolymph (10 µl) was treated with heat (10 min, 100°C), acid (1 µl, 10 N HCl), alkali (1 µl, 1 M NaOH), absolute ethanol (100 µl) or V₈ protease (1 µg, 15 min, 0°C) prior to its addition to the fat bodies kept in culture in the presence of 80 nM 20E (in 10 µl 0.05% ethanol). The control contained equal volume of 0.05% ethanol. At the end of the incubation, the fat bodies were removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenized and assayed for ACP activity. Each value is the mean ± S.D. of four independent determinations. * Significantly different over all other values (p<0.05).
Fig. 3: Fractionation of haemolymph proteins by gel filtration chromatography and the identification of active haemolymph fraction as low mass protein:-

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

(b) Protein profile (12% SDS-PAGE) of major haemolymph fractions along with the active fraction (lane 5) that had the ability to mediate the 20E dependent ACP activity. Lanes- protein marker in kDa (1), crude haemolymph (2), fraction 20 (3), fraction 26 (4), fraction 33- the active fraction (5) and fraction 35 (6). Note the active fraction (arrow) calculated from the mobility on SDS-PAGE revealed the appearance of a 19 kDa protein (lane 5).
Fig. 4: Flow chart for the fractionation and purification of haemolymph protein that had the ability of mediating the 20E dependent stimulation of fat body ACP activity.
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Fig. 5: Fractionation using specific molecular weight cut-off membrane fractionators for partial purification of active haemolymph fraction that had the ability to mediate 20E dependent ACP activity:

(a) Effect of different haemolymph fractions obtained using 30 and 10 kDa fractionator on the ACP activity in fat bodies kept in culture in the presence of 20E. The fat bodies from two 24 h post-ligated LLI larvae were incubated with 80 nM 20E and 10 µl of fractionated haemolymph for 4 h. At the end of the incubation, the fat bodies were removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenized and assayed for ACP activity. Each value is the mean ± S.D. of 4 independent determinations. *Significantly different from all other values (p<0.05).

(b) SDS-PAGE (12%) showing the protein profile of various haemolymph fractions. Lanes- crude haemolymph (1), proteins retained in retentate of 30 kDa filter (2), proteins in the filtrate of 30 kDa filter (3), proteins retained in retentate of 10 kDa filter (4), proteins in the filtrate of 10 kDa filter (5) and protein marker in kDa (M). The amount of protein loaded in lanes 1-4 was 10 µg and lane 5 was 3 µg.
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Fig. 6: Purification of a haemolymph protein enhancing the 20E dependent ACP activity in larval fat bodies kept in culture. Identification of active haemolymph fraction as 19 kDa protein hence it was termed as HP19:-

(a) Elution profile of haemolymph proteins on sephadex G 50 matrix in terms of their ability to mediate the ACP activity of LLI larval fat bodies kept in culture in presence of 20E. The elution was carried using 10 mM Tris-HCl (pH 7.4).

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

The % yield was calculated at each step on the basis of the amount of protein recovered at that particular step with reference to the amount of protein present in the crude preparation. Fold purification was calculated indirectly by comparing the specific activity of the step, for which fold purification to be calculated to that of the specific activity of previous step. The specific activity was calculated on the basis of the ability of pooled fractions to mediate the 20E dependent ACP activity.

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total protein content (mg)</th>
<th>% Yield</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemocyte free total haemolymph protein (crude)</td>
<td>50</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation (60% supernatant)</td>
<td>36.72</td>
<td>73.44</td>
<td>1.28</td>
</tr>
<tr>
<td>Amicon fractionation (30 kDa) filtrate</td>
<td>6.72</td>
<td>13.44</td>
<td>5.12</td>
</tr>
<tr>
<td>Amicon fractionation (10 kDa) filtrate</td>
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<td>2.80</td>
<td>17.28</td>
</tr>
<tr>
<td>Sephadex G-50 eluates</td>
<td>0.023</td>
<td>0.046</td>
<td>98.45</td>
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</tbody>
</table>
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(a) The fat bodies from two 24 h post-ligated LLI larvae were incubated with 80 nM 20E together with different concentrations of purified HP19 for 4 h at 25°C. At the end of the incubation, the fat bodies were removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenized and assayed for ACP activity. Each value is the mean ± S.D. of 4 independent determinations. Note that the concentration of 40 ng was effective enough to mediate the 20E dependent stimulation of fat body ACP activity and also the physiological concentration of 20E (80 nM) was sufficient and optimal for HP19 assisted stimulation.

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

Fig. 7: Effect of different concentrations of HP19 and 20E on fat body ACP activity:-
(a) The fat bodies from two 24 h post-ligated LLI larvae were incubated with 80 nM 20E together with different concentrations of purified HP19 for 4 h at 25°C.
Fig. 8: Electroelution of HP19:-

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

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

(d) Shows the electroeluted HP19 used for antibody production. Lanes- (M) protein marker in kDa, (1) total haemolymph protein (10 µg) and (2) electroeluted HP19 (3 µg).
Fig. 9. Production and specificity of polyclonal antibody raised against HP19:-

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

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

(c) Western blot showing the specificity for HP19 (arrow) in non-denatured PAGE. Lane 1- 20 µg of total haemolymph protein was loaded.
Fig. 10: Functional test of HP19 antibody to check its specificity against HP19 and to confirm it as an antibody against HP19:-

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

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

<table>
<thead>
<tr>
<th>Condition</th>
<th>n moles of PNP released / h / µg fat body protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0</td>
</tr>
<tr>
<td>1:10</td>
<td>0.5</td>
</tr>
<tr>
<td>1:100</td>
<td>1.0</td>
</tr>
<tr>
<td>1:200</td>
<td>1.5</td>
</tr>
<tr>
<td>1:500</td>
<td>2.0</td>
</tr>
<tr>
<td>1:1000</td>
<td>2.5</td>
</tr>
<tr>
<td>1:10000</td>
<td>3.0</td>
</tr>
<tr>
<td>Ligated (24 h)</td>
<td>0.0</td>
</tr>
<tr>
<td>20E</td>
<td>0.5</td>
</tr>
<tr>
<td>20E + HP19</td>
<td>1.0</td>
</tr>
<tr>
<td>20E + immuno-complex (HP19 - αHP19)</td>
<td>1.5</td>
</tr>
<tr>
<td>20E + immuno-supernatant (HP19 - αHP19)</td>
<td>2.0</td>
</tr>
</tbody>
</table>
Fig. 12: Tissue-specific biosynthesis of HP19 by the hind gut associated lobular fat body (HGLFB):

(a) ACP activity in fat bodies from 24 h post-ligated LLI larvae co-cultured with different tissues dissected from 24 h post-ligated larvae in presence of 80 nM 20E for 4 h. At the end of the incubation, the fat bodies were removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenized and assayed for ACP activity. Each value is the mean ± S.D. of four independent determinations. For each determination the fat bodies from two larvae were used. *Significantly different from all other values (p <0.05).

(b) SDS-PAGE and (c) western blot of proteins from different tissues demonstrating the presence of HP19 only in the haemolymph and HGLFB. The protein on blot was probed with HP19 antibody. Lanes- haemolymph (1), HGLFB (2), visceral fat body (3), perivisceral fat body (4), total larval body (5), salivary gland (6), carcass (7) and gut + Malpighian tubule (8).
Fig. 13: Immunohistochemical localization of HP19 in HGLFB:

The tissue sections were immunostained using anti-rabbit IgG fraction as described in materials and methods. The control slide was processed with pre-immune rabbit serum to check the specificity of antibody (a). Note the presence of HP19 in HGLFB in low (b) and high magnification (c). Other tissues like visceral fat body (d), peripheral fat body (e), gut (f), carcass (g), Malpighian tubule (h) and salivary gland (i) did not show any cross-reactivity. Scale bar for (b) 10 µm = 0.15 cm and for (a and c to i) 10 µm = 0.45 cm.
Fig. 14: *In situ* immunodetection of HP19 in *C. cephalonica* LLI larvae:-

The LLI larvae were cut open through the dorsal surface and the tissue inside the dissected larvae were fixed and processed for detection of HP19, using anti-HP19 IgG fraction after tissue permeabilisation as described in materials and methods. The immunocrossreactivity was detected in the HGLFB (arrow head inside dotted circle). One set of control larvae were processed using pre-immune serum.
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Fig. 15: Developmental regulation of HP19:

Effect of 20E on ACP activity of fat bodies kept in culture in the presence of haemolymph from different developmental stages of C. cephalonica. Ten µl of fractionated haemolymph from ELI, MLI, LLI larval and prepupal (PP) stages, was added to the fat bodies kept in culture, incubated for 4 h with 80 nM 20E and assayed for ACP activity. Each value is the mean ± S.D. of four independent determinations. For each study, the fat bodies were dissected from two 24 h post-ligated LLI larvae. *Significantly different from all other values (p <0.05).
Fig. 16: Developmental regulation of HP19:-

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

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

For (c) and (e), 20 µg of total protein from final (Vth) instar developmental stages viz., ELI (lane 1), MLI (lane 2), LLI (lane 3) and PP (lane 4) was loaded in each lane and probed with anti-HP19 IgG fraction for detection of HP19 while in (d) 8 µg protein was loaded in each lane. Note the presence of highest concentration of HP19 in LLI stage both in haemolymph (b) as well as HGLFB (d and e) on western blots.
(a) Effect of 20E on the ACP activity of fat bodies of *C. cephalonica* kept in culture in the presence of haemolymph from different species of lepidoptera and a diptera. The fat bodies from two 24 h post-ligated *C. cephalonica* LLI larvae, kept in culture were incubated with 80 nM 20E and 10 µl of 1:20 diluted haemolymph from different species for 4 h. To the control cultures equal volume of insect Ringer was added with or without 20E. At the end of the incubation, the fat bodies were removed, rinsed, homogenized and assayed for ACP activity. Each value is the mean ± S.D. of four independent determinations. Note that haemolymph from all lepidopteran insects could mediate the 20E dependent action to stimulate ACP activity. However, the degree of mediation by LLI larval haemolymph of a dipteran insect (*Calliphora vicina*) was fairly low when compared with the haemolymph of all other lepidopteran species. * Significantly different over controls and over the mediation effect by *C. vicina* haemolymph (p<0.05).

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

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

The haemolymph sample was prepared from penultimate (IVth) instar larvae, different days of final (Vth) instar larvae and prepupae (PP) and used to check its ability to mediate the 20E dependent stimulation of ACP activity of *C. cephalonica* fat bodies kept in culture. Note that only the haemolymph from LLI stage could mediate 20E dependent ACP activation. Each value is mean ± S.D. of four independent determinations. *Significantly different from all other values (p <0.05).
Fig. 19: Partial characterization of HP19 like protein in silkworm, *Bombyx mori*:-

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

Cloning, sequencing and molecular characterization of HP19

Part of this work has been published in-

Background-

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

Comparison of HP19 with other regulatory conserved proteins-

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

cDNA cloning and sequence analysis of HP19-

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

library, prepared from RNA of HGLFB of LLI larvae was immunoscreened with anti-HP19 IgG fraction. The detailed cloning strategy is represented as a flow chart in figure 22. After three rounds of immunoscreening, 10 positive cDNA clones were picked for detailed examination (Fig. 23). The restriction analysis revealed 6 of the 10 clones to be of identical size (Fig. 24). Initial sequencing study with these identical size clones demonstrated significant sequence similarity among them. Furthermore, they showed homology with invertebrate glutathione S-transferases (GSTs). One of these clones was sub-cloned and totally sequenced (Fig. 25, GenBank accession AY369240). This HP19 cDNA was 634 nucleotides long, with an open reading frame of 585 bp, which encodes a protein of 195 amino acids. The calculated molecular mass of the translated unmodified protein was 22.95 kDa, which is close to the mass of HP19 detected in HGLFB, the tissue that synthesizes the protein (Fig. 12c, lane 2). The polypeptide comprises of 12.3% basic (9 arg, 1 his and 14 lys) and 13.3% acidic (10 asp and 16 glu) residues, but no cys residue. The estimated isoelectric point (pl) is 5.36. The cDNA sequence begins with the methionine start codon at position 1 and translation stop codon at 586. A 3’ untranslated sequence containing a polyadenylation signal AATAAA is located at 588 nucleotide followed by a poly (A)_29 tract. The polyadenylation signal overlapped the translation stop codon by one base. Comparison of the C. cephalonica HP19 (CcHP19) cDNA with the sequences in the GenBank, showed 67% identity with Choristoneura fumiferana GST (CfGST) (Feng et al., 1999). Similarity of HP19 cDNA with other invertebrate GSTs was found to be less than 38%. The comparison of the amino acid sequence of CcHP19 with the 4 best matching invertebrate GSTs is shown in figure 26. Percentage identity of CcHP19 cDNA with other GSTs is shown in figure 27. These analyses revealed that except CfGST, the identity with all other GSTs is 35% or less. Although, the CcHP19 cDNA sequence revealed 67% identity with CfGST, affinity purified GST from C. cephalonica had no enhancing effect on the 20E dependent ACP activity when compared with the purified or recombinant HP19 (Fig 29a). Furthermore, the haemolymph as well as the purified HP19 had negligible GST activity (Fig. 29b). Figure 28 shows the expression of recombinant CcHP19 in E. coli XL1 blue strain that allows regulated expression (refer Clontech protocol PT3003-1; Wood et al., 1985). Analysis of ACP activity in different tissues (Fig. 30) suggests that haemolymph also has negligible ACP activity. Hence the increase in ACP activity is not due to the sequestration of the enzyme from the haemolymph but is mainly due to the stimulation. Figure 31 shows the identification of GST in various tissues from the LLI larval stage of C. cephalonica using antibody against the GST of C. fumiferana (Fig. 31a) with which the CcHP19 showed highest sequence identity i.e.,
67% (Feng et al., 1999), and the purification of cytosolic GST by affinity chromatography (Fig. 31b, lane 6). Although the CfGST antibody cross-reacted with a protein in haemolymph as well as HGLFB of C. cephalonica whose molecular range is comparable to the mass of HP19 (Fig. 31a), however, the affinity purified C. cephalonica cytosolic GST could not replace the function of HP19 on 20E actions on ACP activity stimulation (Fig. 29). The molecular mass of purified GST from C. cephalonica was found to be approximately 24.5 kDa.

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

Southern analysis of genomic DNA of the total larval body digested with EcoRI or HinfI, and probed with HP19-cDNA revealed HP19 as a single copy gene (Fig. 32a). Northern hybridization with total RNA isolated from whole body as well as different tissues displayed the tissue specific expression of HP19 gene in HGLFB (Fig. 32b). The 0.66 kb HP19 signal obtained in northern analysis indicates a mass of about 24 kDa which corroborates with the mass of HP19 synthesized in the HGLFB detected on western blot (Fig. 12c, lane 2).
Fig. 20: Comparison of HP19 with 14-3-3 protein:-

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

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

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

(b) Comparison of HP19 with ubiquitin protein using polyclonal antibody against ubiquitin. Lanes (1) and (2) are the direct western blot analysis of rat brain and total haemolymph proteins from LLI larvae of *C. cephalonica*, whereas lanes 4 and 5 are the immunoprecipitated HP19 (using anti-HP19 IgG fraction), reprobed either with HP19 antibody (lane 4) or with polyclonal antibody against ubiquitin (lane 5). Lane 3- shows the immunoprecipitated HP19 (arrow) using anti-HP19 IgG fraction. The other two bands represent the heavy and light chain of IgG. Note, though ubiquitin antibody identified a 19 kDa band (arrow) in larval haemolymph in direct western blot analysis (lane 2), but fails to cross-react with immunoprecipitated HP19 (lane 5). The ubiquitin shows cross-reaction with rat brain sample (lane 1).
Fig. 22: Cloning strategy for cDNA cloning of HP19 gene.
Fig. 23: Immunoscreening of HGLFB cDNA expression library using anti-HP19 IgG fraction for selection of HP19 encoding cDNA:

(a-c) are photographs of the immunoscreening blots after 1st, 2nd and 3rd round respectively. Here only one of the blots, is shown for each round of screening. After 3 rounds, 10 positives were picked and used for further analysis.
Fig. 24: Restriction analysis (double digest) of all the positives obtained after third round of immunoscreening of HGLFB-cDNA expression library:

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

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

The translation start signal at 1 and stop codon at 586 are in bold letters. The putative polyadenylation signal is underlined. Two putative N-glycosylation sites are shaded. The GenBank accession number for the sequence is AY369240.
Fig. 26: Alignment of the deduced amino acid sequence of *C. cephalonica* HP19 (CcHP19) with GST sequences of other insects (BLAST search):-

*Choristoneura fumiferana*- CfGST (AF128867); *Blattella germanica*-BgGST (U92412); *Manduca sexta*-MsGST (L32092) and *Musca domestica*- MdGST (U02616). The identical amino acid positions are shaded and gaps are indicated by dashes. CcHP19 showed 67% identity with CfGST, 35% with BgGST, 32% with MsGST and 31% with MdGST.
Fig. 27: Phylogenetic tree and percentage identity of *C. cephalonica* HP19 (CcHP19) with some of the invertebrate GSTs:

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

![Image](image1)

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

![Image](image2)

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

(a) Effect of affinity purified cytosolic *C. cephalonica* GST on 20E dependent fat body ACP activity. Note that the presence of either recombinant HP19 or HP19 purified from haemolymph mediated the 20E stimulated enzyme activity, whereas presence of GST did not have any effect. The purified GST (40 ng), CcHP19 (1 µg) or purified HP19 (40 ng) were added to the fat bodies kept in culture in the presence of 80 nM 20E and incubated for 4 h at 25°C. The control contained equal volume of 0.05% ethanol. At the end of the incubation, the fat bodies were removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenized and assayed for ACP activity. Each value is the mean ± S.D. of four independent determinations. * Significantly different over all other values (p<0.05).

(b) GST activity in different larval tissues and in purified HP19. Note that both haemolymph as well as purified HP19 have negligible GST activity. * Significantly different over all other values (p<0.05).
Fig. 30: ACP activity in different LLI stage larval tissues:

Note that the activity is negligible in haemolymph and significantly low in HGLFB as compared to the activity in visceral fat body. * Significantly different over all other values (p<0.05).
Fig. 31: Studies on glutathione S-transferase:-
(a) Western analysis of glutathione S-transferase (GST) in different larval tissues using antibody against GST of *C. fumiferana*.
(b) Purification of the cytosolic GSTs from *C. cephalonica* using glutathione CL-agarose affinity chromatography (for details refer materials and methods). Lanes- (M) protein marker in kDa, (1) crude homogenate, (2) 10,000 g 45 min supernatant, (3) 100,000 g 1 h supernatant (cytosolic fractions), (4) 100,000 g 1 h pellet (microsomal fractions), (5) DEAE eluate (active pooled fraction) and (6) affinity eluate (active fraction).
Fig. 32: Characterization of HP19 at molecular level:-

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

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

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

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

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

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

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

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

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

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

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

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

The results obtained from morphological and histological studies also suggest that HP19 plays a role in hexamerin sequestration, which is a 20E dependent process. The whole mount preparation of fat body of controls (Fig. 37a-e) and HP19 antibody injected larvae (Fig. 37f-j) show a clear difference in the morphology which is more pronounced in 10 and 14 days post-injected larvae. Histological study reveals the presence of large number of darkly stained granules in the fat body of both control and HP19 antibody injected insects till 7 day post-injection (Fig. 37k-m and p-r). There was further decline in the number of cytoplasmic granules in both 10 days post-injected experimental and control insects. However, granules number increased significantly in controls (Fig. 37o) when compared with experimental insects after 14 day of injection (Fig. 37t) and it is mainly due to the sequestration of hexamerins from haemolymph. Immunohistochemical study using hexamerin antibody (Fig. 38, lane 2) further substantiate the histological findings, where intense immunostaining is seen only in controls after 10 and 14 days post-injection (Fig. 37x and y) and which is more or less absent in HP19 antibody injected insect fat body (Fig. 37c’ and d’). Figure 38 shows the specific antibody raised against the purified hexamerins of \textit{C. cephalonica}. The hexamerins were purified (Fig. 38a) by passing the total haemolymph proteins of \textit{C. cephalonica} using gel filtration column sephadex G50-150 (Fig. 38a, lane 2) and ion exchange column, DEAE sephacel (Fig. 38a, lane 3). The purified hexamerins were used for antibody production. Western analysis (Fig. 38c) revealed that the antibody was highly specific for hexamerins and it identified the hexamerin both in haemolymph (lane 1) and in fat body (lane 2).
Fig. 33: Effect of anti-HP19 IgG fraction injection to final (Vth) instar larvae (a) of *C. cephalonica*:

The antibody injected larvae were allowed to grow in culture room along with the control groups (for details refer materials and methods chapter). Note the abnormal development of antibody injected larvae (i-m) as compared to the control larvae (b-h). Each arrow head in the control set indicate the gradual and normal development of the last (Vth) instar larvae into a healthy adult.
Fig. 34. Effect of anti-HP19 IgG fraction injection to final instar larvae (See Fig. 33a) of *C. cephalonica*:

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

Controls
(Normal i.e., uninjected, wounded and pre-immune serum injected larvae)

- 10% mortality.
- Normal silk secretion
- Reduction in body and head capsule size from 8 days onward in all controls.
- Pupation after 13-15 days, except in insect Ringer control where the pupation was little delayed, pupa however was well developed.
- Emergence of well developed adults in all types of controls after 21-23

Experimental
(HP 19 antibody injected larvae)

- 15% mortality.
- Reduced silk secretion
- Delayed reduction in body and head capsule size, seen after 11 days of injection.
- Pupation normally seen after 13-15 days but abnormally developed i.e. non viable larval-pupal intermediate.
- Delayed metamorphosis and almost all adults abnormally developed, i.e. nonviable pupal-adult intermediate.

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

Table 2. Morphological and behavioral changes upon anti-HP19 IgG fraction injection to final (Vth) instar *C. cephalonica* larvae.
Fig. 35. Changes in the ACP activity in *C. cephalonica* larvae after different days of anti-HP19 IgG fraction injection:

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

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

Note the decline in hexamerin concentration (arrow) in haemolymph (a, lane 5) and appearance of it in the fat body protein (c, lane 5) in control after 14 days of post-injection. These changes however were absent both in haemolymph (b) and fat body (d) of the antibody injected larvae. Lanes (1) 0 day i.e., the day of injection and (2-5) 4, 7, 10 and 14 days post-injected.
Fig. 37. Shows the morphological (a-j), histological (k-t) and immunohistochemical (u-d') changes in control (C) as well as in the HP19 antibody injected larvae (E).--

The changes are quite evident with respect to hexamerin sequestration and the HP19 antibody injected larvae showed more or less no sequestration and very little immuno-staining (carried out using hexamerin antibody) was detected in these insects (d') when compared with controls (y).
Fig. 38: Purification of hexamerins and production of polyclonal antibody against hexamerins:-

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

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

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

Effect of HP19 on 20-hydroxyecdysone regulated actions

Part of this work has been published in-


**Background**

Extensive studies from other as well as our laboratory have revealed that in addition to the increase in lysosomal activity in the whole body as well as in the fat body, 20E also regulates a wide variety of functions in insects. It is reported to stimulate the synthesis of various proteins in different tissues during the postembryonic development of the lepidopteran insects (Ray et al., 1987a, b; Sridevi et al., 1988a, 1989; Ismail and Dutta-Gupta 1990a; Dutta-Gupta et al., 1996; Shanavas et al., 1996). The uptake or sequestration of storage proteins (hexamerins) by the fat body (KiranKumar et al., 1997) as well as the male accessory reproductive glands (Ismail and Dutta-Gupta, 1990c, 1991; Dutta-Gupta and Ismail, 1992; Ismail et al., 1993) was also shown to be regulated by ecdysteroids.

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

The post-translational modifications of proteins by reversible protein phosphorylation are well known to regulate several cellular actions (Graves and Krebs, 1999; Davies et al., 2000). These phosphorylation events are often suggested to be regulated by hormones. The ecdysteroids are also known to stimulate phosphorylation of few fat body proteins with their significance being discussed (Itoh et al., 1985; Sass, 1988). In the present study an attempt is made to understand the role of 20E on the phosphorylation of few fat body proteins of *C. cephalonica* and effect of HP19 on them. In this endeavor three protein, namely hexamerin receptor, tyrosine kinase and CaM kinase II were identified as potential markers. This section of thesis deals with the extensive study carried on these proteins.
**Phosphorylation of 120 and 60 kDa protein induced by 20E-**

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

**Phosphorylation of 48 kDa protein in silk worm Bombyx mori-**

The phosphorylation of fat body proteins of *C. cephalonica* revealed phosphorylation of 4 proteins of masses 32, 48, 60 and 120 kDa (Fig. 39b). Of these, 48 kDa protein has been earlier shown to undergo phosphorylation under the influence of juvenile hormones I (JHI) in *B. mori* CNS (Shanavas *et al.*, 1998). Protein phosphorylation studies were extended to *B. mori*, basically to find if the phosphorylation profile of proteins in different tissues is comparable with *C. cephalonica*. It was interesting to note that the same protein was phosphorylated in the fat body of *C. cephalonica* (Fig. 39b, lane 3, arrow). In the present study, the phosphorylation of 48 kDa protein was found to be dependent on a tyrosine kinase as the monoclonal antibody against phosphotyrosine identified the phosphorylated 48 kDa protein (Fig. 41a, lane 1). Further genistein, a broad-spectrum tyrosine kinase inhibitor, inhibited the phosphorylation (Fig. 41b, lane 2) as compared to control (Fig. 41b, lane 1). This 48 kDa protein phosphorylation was found to occur in all the insect tissue investigated (Fig. 41c) with significant phosphorylation in CNS (lane 1) and salivary gland (2). Because the phosphorylation of this protein was shown to be regulated by JHI (Shanavas, 1997) and
not by 20E, hence, it was not considered worthwhile to extend the study further on 48 kDa protein as the present study is focused on the regulation of ecdysteroid dependent actions.

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

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

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

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

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

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

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

Earlier studies from our laboratory reported that a 120 kDa polypeptide- (i) is located in the cell membrane of fat bodies, (ii) binds to *C. cephalonica* hexamerin and (iii) is
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responsible for the hexamerin sequestration. Additional experiments were carried out to unambiguously identify the phosphorylated 120 kDa as the hexamerin receptor. The fat body proteins were first phosphorylated either in absence or in presence of 20E, transferred to nitrocellulose membrane followed by incubation with biotinylated hexamerin. The results revealed that the biotinylated hexamerin selectively bound to 120 kDa protein (Fig. 46b, lanes 1 & 2), hence it could be considered as the hexamerin receptor. This ligand blot, when autoradiographed, showed that the same 120 kDa protein was highly phosphorylated in presence of 20E (Fig. 46c, lane 2).

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

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

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

The 20E induced phosphorylation of the 120 kDa protein was found to be calcium (Fig. 39b, lane 4) as well as protein kinase C independent (Fig. 39b, lane 5). Hence, the
possibility of tyrosine kinase mediation was checked. Tyrosine phosphorylation was studied by using anti-phosphotyrosine antibodies (Donaldson and Cohen, 1992). Immunoblot analysis of the phosphorylated protein with anti-phosphotyrosine antibody showed cross reactivity of the antibody with the 120 kDa receptor. As compared to 20E untreated (Fig. 48a, lane 1), the 20E treated phosphorylated 120 kDa protein showed a higher cross-reactivity (Fig. 48a, lane 2) which confirmed the above assumption, i.e., tyrosine kinase mediates the 120 kDa protein phosphorylation, and is induced by 20E. Genistein, an inhibitor of tyrosine kinase, partly inhibited the 20E induced phosphorylation of the 120 kDa protein (Fig. 48b, lane 3) when compared with the 20E treated sample (Fig. 48b, lane 2).

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

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

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

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

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

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

**Developmental and hormonal regulation of fat body tyrosine kinase activity**

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

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

ATP is known to be cell impermeable but even then the phosphate moiety from the ATP was transferred to the hexamerin receptor, in the fat bodies kept in culture and the receptor was phosphorylated (Fig. 54). Therefore, it is assumed that the phosphorylation event is probably a cell surface phenomenon and occurs due to the intrinsic kinase activity of the receptor. The results presented in figure 57 provide evidence to this assumption. When the fat bodies kept in culture was incubated along with [γ32P] ATP, the receptor was found to be phosphorylated under the influence of 20E (Fig. 57a). Immunohistochemical staining of fat body sections using monoclonal antibody against the phosphotyrosine residue, further suggested the presence of protein phosphorylated at tyrosine residue (Fig. 57c and d). Further, the use of series of synthetic receptor tyrosine kinase inhibitors, tyrphostins (Ohmichi et al., 1993) shows (Fig. 57e-i) that one of the inhibitor AG 879, significantly
inhibited the phosphorylation of the receptor at 100 µM concentration (Fig. 57i). Though the inhibitor concentration required for inhibition was fairly high but it does suggest that probably the receptor undergoes autophosphorylation, due to the intrinsic tyrosine kinase activity.

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

The back phosphorylation experiment result presented in figure 58 shows that the 19 kDa haemolymph protein, HP19 from *C. cephalonica* inhibits the 20E stimulated phosphorylation of hexamerin receptor. Back phosphorylation experiment, where the fat body tissue was cultured in absence or presence of 20E with or without HP19 / genistein in order to allow the sites of phosphorylation to be occupied by the endogenous ATP present in the fat body cells prior to the *in vitro* phosphorylation experiment using [γ32P] ATP was carried out. In such case (Fig. 58a and b), the incorporation of [γ32P] in 120 kDa hexamerin receptor was low in presence of 20E (lane 2) as compared to control (lane 1). The incorporation of [γ32P] in presence of 20E and HP19 (lane 4) was higher as compared to 20E alone (lane 2) or HP19 alone (lane 5). It is interesting to note that the autophosphorylation of 60 kDa CaM kinase II (Shanavas *et al.*, 1998) was also inhibited by HP19 in presence of 20E, hence there is higher incorporation of [γ32P] (Fig. 58, lane 4). Furthermore, a broad spectrum tyrosine kinase inhibitor, genistein had no inhibitory effect on the 60 kDa protein, and the sites of this fat body protein were occupied by endogenous ATP, hence no [γ32P] incorporation was seen after the *in vitro* phosphorylation reaction (lane 3) and the results were comparable with 20E treatment (lane 2). In case of back phosphorylation studies, the pattern of tyrosine kinase activity in these tissues (Fig. 58c), prior to the *in vitro* phosphorylation reaction clearly showed a higher activity in 20E treated fat body, which in turn might be responsible for the phosphorylation of sites leading to low incorporation of [γ32P] in 120 kDa receptor during the *in vitro* phosphorylation reaction (Fig. 58 a and b). The results of figure 59 clearly reveal that HP19 not only inhibits the phosphorylation in intact fat body tissue but also in the fat body homogenate preparation. These results together indicate that HP19 blocks the 20E regulated hexamerin receptor phosphorylation at the larval stages of development in *C. cephalonica*. 

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Fig. 39: *In vitro* incorporation of \( \gamma^{32}P \) ATP into the fat body proteins of *C. cephalonica* under different phosphorylating conditions:-(a) 10% SDS-PAGE and (b) Autoradiograph. Lanes- 1 mM CaCl\(_2\) (1), 1 mM CaCl\(_2\) + 80 nM 20E (2), 1 mM EGTA (3), 1 mM EGTA + 80 nM 20E (4) and 1 mM CaCl\(_2\) + 100 \( \mu \)M phosphatidylserine + 10 \( \mu \)M diolein (5). The phosphorylation of the 60 kDa (arrow) and 120 kDa (arrow) bands was stimulated in the presence of 20E. Lane M- protein marker (kDa).
Fig. 40: Effect of different time incubations on the *in vitro* incorporation of $\gamma^{32}$P ATP into the fat body proteins of *C. cephalonica*:

(a) 10% SDS-PAGE and (b) Autoradiograph. The *in vitro* phosphorylation reaction was initiated by addition of $\gamma^{32}$P ATP for different time periods, 10 sec (lane 1), 30 sec (lane 2), 1 min (lane 3), 2 min (lane 4) and 5 min (lane 5). After the desired time period, the reaction was stopped with addition of 3 X SDS sample buffer followed by immersion in boiling water bath for 2 min. Equal amount of protein was used for 10% SDS-PAGE and subsequently processed for autoradiography.
Fig. 41: Phosphorylation of 48 kDa protein in the CNS of silkworm *Bombyx mori*:

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

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

(c) Autoradiograph showing the phosphorylation of 48 kDa protein (arrow) in different tissues of *B. mori*. An equal amount of protein (10 µg) was loaded on each lane. Lanes- CNS (1), salivary gland (2), fat body (3) and epidermis (4).
Fig. 42: Lineweaver-Burk and Michaelis-Menten plots of the phosphorylation of a peptide substrate pGLU$^4$- myelin basic protein fragment 4-14 (MBP$_{4-14}$) by endogenous protein kinase C (PKC) of C. cephalonica fat body. Initial rates were measured under standard condition for 15 min by filter paper assay. The ATP (a) and synthetic peptide substrate (b) concentration were varied as indicated.
Fig. 43: Endogenous fat body protein kinase C (PKC) activity in *C. cephalonica*:

Changes in the fat body PKC activity during different developmental stages (a), upon ligation of LLI larvae for different time periods (b) and *in vivo* effect of 20E (hormone injection) to 24 h and 48 h post-ligated LLI larvae (c). The values are mean ± S.D. of four independent determinations.
Fig. 44: Calcium/calmodulin dependent protein kinase II (CaM kinase II) activity in fat body of C. cephalonica and its regulation:-

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

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

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

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

Each value is the mean ± S.D. of four independent determinations. * Significantly different over † (p <0.05).
Fig. 45. Autoradiograph and densitogram showing the effects of thorax-ligation (a) and 20E (b) on the in vitro phosphorylation of the 120 kDa protein:

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

(b) Effect of 20E on in vitro phosphorylation of the 120 kDa protein in fat body homogenate prepared from 24 h post-ligated LLI larvae. Lanes- unligated (1), 24 h ligated i.e., untreated control (2), 20E treated (3 to 5) viz., 40 nM (3), 80 nM (4) and 150 nM (5) 20E. For this study fat body was dissected from 24 h post-ligated LLI larvae, homogenized and used. Note the enhanced phosphorylation of the 120 kDa protein in presence of 20E. Quantitation of the phosphorylated protein was done using UNSCAN-IT gel software.
Fig. 46: Demonstration of phosphorylated 120 kDa protein as hexamerin receptor:–
SDS-PAGE (a), ligand blot (b) and autoradiograph of ligand blot (c). Fat body proteins isolated from 24 h post-ligated larvae were first in vitro phosphorylated either in absence or in presence of 20E, resolved on 10% SDS-PAGE, blotted on to nitrocellulose membrane and probed with biotinylated hexamerin. The membrane was subsequently processed for autoradiography. Lanes- protein marker in kDa (M), phosphorylated fat body proteins in absence of 20E (1) and in presence of 80 nM 20E (2).
Fig. 47: Independence of tissue integrity for the phosphorylation of 120 kDa hexamerin receptor in *C. cephalonica*:-

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

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

(d) Autoradiograph showing the effect of 20E on the phosphorylation of 120 kDa protein in intact fat body tissue of 24 h post-ligated LLI larvae by back phosphorylation study. The fat bodies kept in culture were incubated with 80 nM 20E (in 0.05% ethanol) for 4 h at 25°C. The control contained equal volume of 0.05% ethanol. At the end of incubation, the fat bodies were removed from the culture medium, rinsed, homogenized and subjected to *in vitro* phosphorylation using [$\gamma^{32}$P] ATP.
Fig. 48: Evidence of tyrosine kinase mediation for the phosphorylation of 120 kDa hexamerin receptor:

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

(b) Autoradiograph showing the effect of genistein on the phosphorylation of the 120 kDa hexamerin receptor. The fat body homogenate from 24 h post-ligated LLI larvae was first incubated either with 80mM 20E (lane 2) or with 80 mM 20E + 40 μM genistein (lane 3) for 5 min at 30°C followed by in vitro phosphorylation for 1 min with [γ³²P] ATP. Lane 1- control.
Fig. 49: Identification of hexamerin receptor and its phosphorylation in other hexamerin sequestering tissue MARG:-

Uptake of hexamerins (a), identification of 120 kDa hexamerin receptor by ligand blotting (c) and its phosphorylation (d) in fat body (lane 1) and MARG (lane 2) of *C. cephalonica*. The uptake of $[^{35}\text{S}]$ methionine labeled hexamerins (a) was carried by incubating the fat bodies kept in culture and MARG tissue (from freshly eclosed males) for 4-6 h (for details see materials and methods). Membrane proteins from these tissues were resolved on SDS-PAGE (b) and probed with biotinylated hexamerins for ligand blotting (c). To check for the phosphorylation, the proteins were *in vitro* phosphorylated and processed for autoradiography (d).
Fig. 50: Autoradiograph showing lack of phosphorylation of 120 kDa protein (arrow) in non-hexamerin sequestering tissues:-

Different insect tissues from LLI larval stage was homogenized and used for \textit{in vitro} phosphorylation reaction as described in materials and methods. Lanes- fat body (1), epidermis and central nervous system (2), gut and Malpighian tubule (3) and haemolymph (4). Note the presence of a phosphorylated hexamerin receptor in fat body (lane 1) of \textit{C. cephalonica}. Presence of a faint band in lane 2 is mostly due to the contaminating fat body, which is normally associated with epidermis.
Western analysis of hexamerins in the haemolymph and the fat body of different developmental stages of *C. cephalonica*. Note the relatively high rate of synthesis, release and accumulation of hexamerins at LLI as compared to ELI larval stage. These hexamerins are sequestered by the prepupal and pupal fat body. (a and b) Proteins were separated on SDS-PAGE transferred to nitrocellulose membrane and stained using Ponceau S stain. (c and d). Immunostaining was carried out using antibodies generated against hexamerins.
Fig. 52: [$^{35}$S] methionine labeling (bio-labeling) of hexamerins from larval forms of C. cephalonica:

The LLI larvae were injected with 10 µCi [$^{35}$S] methionine and allowed to grow in normal culture condition. After 16 h the haemolymph was collected and hexamerins were partially purified by passing the crude haemolymph fractions through sephadex G-25 column in order to remove unincorporated [$^{35}$S] methionine, salts and other low mass proteins. The radiolabeled proteins were resolved on 7.5% SDS-PAGE and incorporation was visualized by fluorography. Note a predominant labeling of hexamerins in both crude (lane 1) as well as sephadex G-25 eluate (lane 2).
Fig. 53: Evidence of ecdysteroid dependent activation of hexamerin receptor for the uptake of hexamerins in *C. cephalonica*:

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

(b) The autoradiograph shows the *in vitro* phosphorylation of the hexamerin receptor in the fat body of the above-mentioned developmental stages. For this study, the fat body homogenates prepared from the control (−20E *i.e.*, sample number 1, 3 and 5) in the above experiment were used.
Fig. 54: Effect of hexamerin receptor phosphorylation on the uptake of hexamerins by LLI larval fat bodies kept in culture:

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

(b) Corresponding fluorograph showing similar hexamerin uptake pattern as (a).
Fig. 55: Lineweaver-Burk and Michaelis-Menten plots of the phosphorylation of a synthetic peptide substrate (A-7433, Sigma) by endogenous tyrosine kinase of *C. cephalonica* fat body. Initial rates were measured under standard condition for 2 min by filter paper assay. The ATP (a) and synthetic peptide substrate (b) concentration were varied as indicated.
Fig. 56: Endogenous fat body tyrosine kinase activity of fat body of *C. cephalonica*:-

Changes in the activity of fat body tyrosine kinase of *C. cephalonica* during different developmental stages (a), upon ligation of LLI larvae for different time periods (b) and stimulation in the activity by 20E in vivo (by injection to 24 h post- ligated LLI larvae) as well as in the fat bodies kept in culture (c). * A significant increase in enzyme activity was seen upon 24 h under in vivo and 4 h under in vitro hormone treatment. The values represent mean ± S.D. of four independent determinations.
Fig. 57: Possible identification of receptor tyrosine kinase mediated phosphorylation of hexamerin receptor:

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

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

(e-i) Effect of tyrphostins, synthetic tyrosine kinase inhibitor on the phosphorylation of hexamerin receptor. The fat body homogenates were incubated with different concentration of these inhibitors for 10 min at 30°C, prior to in vitro phosphorylation.
Fig. 58: Effect of HP19 on the 20E dependent phosphorylation of 120 kDa hexamerin receptor and autophosphorylation of 60 kDa CaM kinase II in fat body of *C. cephalonica*:

Autoradiograph (a), the corresponding densitogram (b) and the endogenous tyrosine kinase activity in the fat body (c) from the back phosphorylation of fat body proteins. The fat body tissue from 24 h post-ligated LLI larvae was rinsed thoroughly in TC-100 insect culture medium. The tissue was then incubated for 4 h in absence (lane 1) or presence of 20E (lane 2) and 20E with 40 µM genistein (lane 3) or 40 ng HP19 (lane 4) or HP19 alone (lane 5). The incorporation of $[^{32}\text{P}]$ ATP was found to be relatively high in control (−20E), genistein and HP19 along with 20E treated fat body tissue as compared to the 20E alone or HP19 alone treated fat body. The endogenous tyrosine kinase activity in these tissue as expected show reverse pattern compare to (a) and (b). The quantitation of the phosphorylated protein band intensity was done using UN-SCAN-IT gel software. For (c) the values are mean ± S.D of three independent determinations.
Fig. 59: Inhibitory effect of HP19 on the 20E dependent stimulation of endogenous tyrosine kinase activity in fat body homogenate:

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

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

Part of this work has been published in-

Background-

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

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

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

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

**Characterization of C. cephalonica ACP (CcACP) cDNA**

After establishing the HP19 mediated nongenomic effect of ecdysteroid on ACP activity, effort was made in the present study to understand this phenomenon at RNA level. For this, the approach was taken to clone the ACP of *C. cephalonica*. Incidentally while screening for the hexamerin cDNA from the fat body expression library using polyclonal antibody against *C. cephalonica* hexamerins (Nagamanju et al., 2003), one of the false positive showed sequence homology with phosphatidic acid phosphatase (PAP). This clone was *in vitro* transcribed to get RNA probe that was used for hybridization screening of the fat body cDNA library and thus one clean positive was picked (Fig. 62a) and used for second round of screening. After two rounds of screening two positive clones were picked (Fig. 62b). Restriction analysis of these clones suggested it to be of identical size (Fig. 63, lanes 2 & 5). One of the clones was subcloned and partially sequenced (GenBank Accession AF317884). The partial cDNA sequence of *C. cephalonica* PAP (Fig. 64) showed ~38% sequence identity with *Drosophila melanogaster* phosphatidic acid phosphatase (PAP), wumen and tunen gene.
(Fig. 65). When this clone was used for Southern hybridization, it showed that CePAP is a product of multiple copy gene (Fig. 66a, lanes 1 & 2). Expression of this gene in various larval tissues of *C. cephalonica* (Fig. 66b) matched with the activity profile of ACP (Fig. 66c). The highest level of expression (Fig. 66b, lane 2) as well as activity (Fig. 66c) was obtained in the visceral fat body. The total larval body also showed high level of expression (Fig. 66b, lane 1) as well as the ACP activity compared to other tissues. This is because of the presence of visceral fat body in this preparation that constitutes a large fraction of total insect body (Keeley, 1985). The results presented in figure 67a show the gradual increase in the expression level of ACP gene from ELI larvae to prepupal stage and this is consistent with the activity profile seen in the fat bodies from these developmental stages (Fig. 67b).

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

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

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

In the earlier results section (Chapter IV), it was shown that 20E stimulates the phosphorylation of *C. cephalonica* hexamerin receptor present in fat body cells as well as other tissues and it is partly mediated by a tyrosine kinase. This phosphorylation occurs in homogenate, membrane as well as in intact tissue and is a pre-requisite for hexamerin uptake during the prepupal and pupal stages. Further, this phosphorylation is unaffected by inhibitors of transcription and translation. The study was extended to understand the rapid nongenomic regulation of receptor phosphorylation by 20E and role of HP19 in this process. The results in figure 69a show that the addition of HP19 either in absence (lane 1) or in presence (lane 2) of
20E inhibited the basal (lane 3) as well as 20E stimulated (lane 4) phosphorylation of the 120 kDa hexamerin receptor. For this experiment, the fat body homogenate was incubated with 20E and / or HP19 for 30 sec prior to the in vitro phosphorylation. This rapid inhibition was also seen whether the homogenate was incubated simultaneously along with 20E and HP19 (Fig. 69b) or initially incubated with 20E for different time periods followed by fixed time incubation with HP19 (Fig. 69c) or vice versa (Fig. 69d). However, with longer incubation periods, there was no significant inhibition by HP19 in the phosphorylation status of receptor protein (Fig. 69b-d). This study indicates that HP19 rapidly blocks the 20E regulated hexamerin receptor phosphorylation in larval fat body.

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

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

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

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

Regulation of HP19-

Present study indicated a multiple role of HP19 on few of the 20E dependent actions. The HP19 mediated the 20E stimulation of ACP activity and inhibited the 20E induced
phosphorylation of hexamerin receptor, activity of tyrosine kinase and CaM kinase II activity as well as its autophosphorylation. Present study suggests that HP19 is either a kinase inhibitor or a phosphatase activator, however, at the moment it is not very clear. Earlier studies from our laboratory do suggest that the regulatory action of HP19 like protein might be due the possession of protease like property (Vasanthi, 1999). The low concentrations of the protein required to regulate the 20E dependent actions also suggest probable regulatory nature of the protein. In the present study effort was also made to understand the relative stability of HP19 (shelf life) in the total haemolymph as well as in the partially purified fraction. It was found to be modestly stable for a period of upto 30 days upon proper storage condition (–4°C). However, stability in terms of its interaction with hormone to regulate various actions remains to be tested. Comparisons with other ubiquitously distributed regulatory molecules like 14-3-3, stathmin and ubiquitin, which have known multiple roles in cellular functions, HP19 did not show any immunological similarity effect at western level (Figs. 20 and 21 in chapter II of results section). In order to understand whether HP19 was itself phosphorylated to regulate these 20E dependent actions, in vitro phosphorylation of haemolymph proteins were carried out (Fig. 72). The results indicated that there is no phosphorylation of HP19 in absence or presence of calcium either in HGLFB (Fig. 72b), a tissue that synthesizes this protein or in the haemolymph (Fig. 72a) where it is released.
Fig. 60: Nongenomic regulation of fat body ACP activity by 20E in presence of HP19:-

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

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

All the values are mean ± S.D. of four independent determinations.
Fig. 61: Effect of protein biosynthesis inhibitors on HP 19 action:-

The fat bodies from two 24 h post-ligated LLI larvae were cultured initially with 10 µCi of [\(^{35}\)S] methionine for 2 h followed by an additional incubation with 80 nM 20E / 40 ng HP19 / 1 mM actinomycin D / 1 mM cycloheximide for 2 and 4 h. The 20E stimulated protein synthesis is inhibited by the inhibitors (a). ACP activity is stimulated by 20E in presence of HP19 and is not blocked by inhibitors (b). The values are mean ± S.D. of four independent determinations.
Fig. 62: Hybridization screening of fat body cDNA expression library using RNA probe to pick cDNA encoding ACP:-

(a and b) are respectively the hybridization screened blots of 1st and 2nd round of screening. While immunoscreening the _C. cephalonica_ fat body expression library for hexamerin encoding genes using hexamerin antibody (Nagamanju _et al._, 2003), one of the false positive upon sequencing showed homology with phosphatidic acid phosphatase (PAP). This PAP clone was used as the primary bait to clone ACP of _C. cephalonica_ after _in vitro_ transcription. The RNA probe from PAP cDNA clone was then used for hybridization screening of fat body cDNA expression library. Note that in (a) there was one clean positive after 1st round of screening which was used for 2nd round of screening (b) and after two rounds of screening, 2 positive clones were picked and used for further analysis.
Fig. 63: Restriction analysis (double digest) of the positives obtained by hybridization screening of HGLFB-cDNA expression library:-

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

Fig. 64: The partial cDNA nucleotide sequence of *C. cephalonica* ACP phosphatase (CcACP):

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The GenBank accession number for the sequence is AF317884.
Fig. 65: Alignment of the deduced amino acid sequence of *C. cephalonica* ACP (CcACP) with ACP sequences (PAP) of other insects (BLAST search):-

*A. melanogaster* tunen- DmTun (AF236058); *D. melanogaster* wunen- DmWun (AF145595); *H. sapiens* type 2 phosphatidic acid phosphatase 2a2- HsPap (AF014403) and *Cavia porcellus* phosphatidic acid phosphatase 2a2- CpPap (AF088284). The identical amino acid positions are shaded and gaps are indicated by dashes. CcACP showed 38% identity with DmTun and DmWun, 36% with HsPap and 33% CpPap.
Fig. 66: Characterization of ACP of *C. cephalonica*:

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

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

(c) ACP activity in different LLI larval stage tissues. Note that the activity profile is comparable to the northern profile (b) though activity was negligible in haemolymph and significantly low in HGLFB as compared to the activity in visceral fat body. The results presented are mean ± S.D. of four independent determinations. * Significantly different over all other values (p<0.05).
Fig. 67: Differential activity (a) and northern expression profile (b) of ACP at different developmental stages of last (Vth) instar larvae:

Equal quantity of total RNA from various developmental stages was probed with CcACP partial cDNA. Note the parallel increase in enzyme activity and transcript level from early-last instar to prepupal stage. For (a), the results presented are mean ± S.D of four independent determinations.
Fig. 68: Nongenomic action of 20E on ACP activity mediated by HP19: evidence at transcript level:-

Northern expression, the corresponding densitogram (a) and activity profile of ACP (b) in the fat bodies kept in culture that was incubated with 20E alone or along with HP19. The fat bodies from six 24 h post-ligated LLI larvae were cultured as described in materials and methods with 20E (80 nM in 0.05% ethanol) or 20E + HP19 (40 ng) for 4 h. The control contained equal volume of 0.05% ethanol. The fat body from unligated larvae was used directly for the assay. At the end of the incubation, the fat bodies were removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenized and part of the homogenate was used for RNA isolation and rest for the ACP activity assay. For northern study, equal quantity of total RNA from various tissues was probed with CcACP partial cDNA. For activity study, each value is the mean ± S.D. of four independent determinations. * Significantly different over all other values (p<0.05). Note that equal quantity of total RNA from different tissues (represented as ribosomal RNA) showed no significant change in transcript level though there was significant increase in the activity when the fat bodies kept in culture were incubated with 20E and HP19.
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Fig. 69: Nongenomic regulation of 120 kDa hexamerin receptor phosphorylation by 20E in presence of HP19:

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

(b-d) Rapid inhibition of 20E induced phosphorylation of hexamerin receptor by HP19. The 20E induced phosphorylation of hexamerin receptor was rapidly inhibited by HP19 (within 0-5 min) in either of the conditions i.e., (b) when 20E and HP19 were added simultaneously to fat body homogenate and incubated for different time periods or (c) when the fat body homogenate was preincubated with 20E for different time periods followed by incubation with HP19 for 15 min or (d)-vice-versa. After the incubation the fat body homogenates were subjected to in vitro phosphorylation and processed for electrophoresis followed by autoradiography.
Fig. 70: Nongenomic regulation of fat body tyrosine kinase activity by 20E in presence of HP19:

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

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

All the values are mean ± S.D. of four independent determinations.
Fig. 71: Evidence of nongenomic regulation of fat body CaM kinase II of *C. cephalonica*:

(a) Shows the effect of 20E and/or HP19 on CaM kinase II activity in fat body tissues kept in culture. The fat bodies from two 24 h post-ligated LLI larvae were cultured as described in materials and methods with 20E (80 nM in 0.05% ethanol) or HP19 (40 ng) or 20E + HP19 (40 ng) for 4 h. The control contained equal volume of 0.05% ethanol. At the end of the incubation, the fat bodies were removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenized and used for CaM kinase II assay.

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

Note both in fat bodies kept in culture and in homogenate, HP19 inhibited the activity. The inhibition in activity by HP19 was more pronounced in combination with 20E. Each value is the mean ± S.D. of four independent determinations. * Significantly different from 20E + HP19 treated group (p<0.05).
Fig. 72: Phosphorylation of total haemolymph and HGLFB proteins:

The total haemolymph (a) as well as HGLFB (b) proteins from *C. cephalonica* were subjected to *in vitro* phosphorylation as described in materials and methods in absence or presence of Ca$^{2+}$ (0.1 mM CaCl$_2$). The phosphorylated proteins were resolved on 12% SDS-PAGE, transferred onto nitrocellulose membrane, probed with anti-HP19 IgG fraction, processed for western analysis for the detection of HP19 in these tissues. Finally, the processed blots were autoradiographed to check the autophosphorylation of HP19. Note in both the tissues, HP19 (arrow) remained unphosphorylated in absence or presence of Ca$^{2+}$.