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

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

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

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

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

Despite of high sequence identity (67%) of the deduced amino acid of *C. cephalonica* HP19 (CcHP19) cDNA with *Choristoneura fumiferana* GST (CfGST) cDNA, the affinity purified cytosolic GST from *C. cephalonica* had no enhancing effect on the 20E dependent ACP activity when compared with purified or recombinant HP19. Further, the haemolymph as well as the purified HP19 had negligible GST enzymatic activity. These studies together...
suggest that HP19 is not a GST enzyme and that GST function is not required for ecdysone mediated stimulation of ACP activity.

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

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

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

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

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

- Present study suggest that 20E induces the hexamerin uptake in fat body, possibly by the post-translational modification of receptor i.e., phosphorylation. Incubation of fat bodies kept in culture with \([\gamma^{32}\text{P}]\) ATP revealed phosphorylation of 120 kDa hexamerin receptor in presence of 20E suggesting that the receptor phosphorylation is a cell surface event due to which increase in hexamerin uptake occurs by the LLI larval fat body culture when the
medium is supplemented with 20E and ATP. This also suggest that phosphorylation of hexamerin receptor under the influence of 20E is a pre-requisite for hexamerin uptake. Inhibition of hexamerin receptor phosphorylation by tyrphostin (AG 879), a receptor tyrosine kinase inhibitor, suggests it as an autophosphorylation due to the intrinsic tyrosine kinase activity of the receptor.

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

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

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

- The study also indicates the short half-life of HP19 because once it is in combination with hormone and the target tissue *i.e.*, fat body, the effect on 20E regulated actions such as ACP activity stimulation or inhibitory effect on kinases and receptor phosphorylation is seen only for a limited time, after which there is recovery in the event. However, the shelf-life of HP19 is relatively long. Furthermore, HP19 is effective in extremely low concentration to mediate all the above mentioned 20E dependent actions. However, HP19 is not autophosphorylated to regulate these actions.
Present study clearly suggests that HP19 in combination with 20E regulates the hexamerin uptake (see Model 1 and 2). It further indicates the nongenomic regulation of few of the selected 20E dependent actions that is mediated by HP19 (see Model 3). However, these data give no convincing clue that regulatory role of HP19 on ecdysteroid action is either due to the kinase inhibitory or phosphatase activating effect (see Model 4).