INTRODUCTION

*Spodoptera litura* is a serious pest of tobacco, castor, banana, gram, groundnut, jowar, maize, tomato, potato and other crops etc (Moussa *et al.*, 1960). *Helicoverpa armigera* is a polyphagous, multi-generation noctuid pest, attacking several crops like cotton, pulse, oil seeds, millets and vegetables etc (Jayaraj *et al.*, 1990). *Eupterote mollifera* is commonly attacking the trees of *Moringa mollifera* and avimaram, *Holoptelea integrifolia* (Roxb.). The coconut pest, *Oryctes rhinoceros* attacking commonly on *Cocos nucifera* (Plate 1-4).

Control of the above polyphagous pests is being currently undertaken by cultural, mechanical and chemical methods. It is well known that these pests have developed resistance to the treatment of chemical pesticides. This crucial situation has warranted to develop new frontier areas to manage these pests. One such promising area currently received much attention is the regulation of molting and metamorphosis under the co-ordination of two important principal hormones namely, prothoracicotropic hormone (PTTH, a brain neurohormone) and ecdysone (molting hormone).

Knowledge on the precise developmental stages of insects is essential for physiological and endocrinological studies. Staging of insect development have been carried out by employing various criteria such as larval age, weight, spiracle development, head capsule slippage, gutpurge, wandering, nonfeeding (Williams, 1952;
PLATE 1 Culture of *Spodoptera litura* in natural condition

(a) Field

(b) 4th instar on feeding
PLATE 2 Collection place of *Helicoverpa armigera*

(a) Bendi field near Annur, Coimbatore district, 35 km north of Coimbatore

(b) *Helicoverpa armigera* 5th instar collected from bendi field
(a) Infected tree of Avimaram by *Eupterote mollifera*

(b) Grouping of *Eupterote mollifera* during nonfeeding stage
(c) *Eupterote mollifera* in Avi leaves

(d) Pharate pupal stage of *Eupterote mollifera*
PLATE 4

(a) *Oryctes rhinoceros* feeding stage

(b) *Oryctes rhinoceros* spining stage
Spiracle development in the penultimate instar has been considered as a useful sign to determine the molt of the larvae. Studies on this aspect has been restricted much except the studies on *Bombyx mori*. In the present study, a time-table has been developed based on the development of new spiracle, which is a visible characteristic for staging the larva. The spiracle index thus obtained has been correlated with the morphological and endocrinological events during molting and metamorphosis in the penultimate larva of *Spodoptera litura*.

The prothoracic gland (PG) was discovered as a granulated vessel by Lyonet (1762) and subsequently recognised as a gland by Toyama (1902), meanwhile, Kopec (1917, 1922) demonstrated by *in vivo* experiments that the insect brain produced a substance essential for insect metamorphosis. Ke (1930) termed it as the prothoracic gland. Subsequently, Hachlow (1931) identified the function of the gland in relation to insect development. Fukuda (1940, 1941) demonstrated that a substance secreted from the prothoracic gland into the haemolymph which elicited pupation. Further experiments of Piepho (1942) on wax moth, *Galleria mellonella* larvae confirmed that a substance released from the brain induced the prothoracic glands to secrete a molting factor. Williams (1947, 1952) provided definitive evidence for this brain prothoracic gland axis and termed as "brain hormone", now referred to as PTTH. Karlson and his colleagues...
(Butenandt and Karlson, 1954; Karlson et al., 1965; Hubner and Hoppe, 1965; Gilbert and King, 1973) characterized the chemical nature of the molting factor from extracts of whole insects of *Calliphora*. Two poly hydroxylated steroids having molting hormone activity were identified (20-hydroxyecdysone) using the *Calliphora* bioassay. It is generally believed that the changes in the molting hormone titre in the blood of numerous insects reflect the variations of prothoracic gland activity and modifications in the rate of hormone metabolism (Smith, 1985; Koolman and Karlson, 1985).

Prothoracic gland structure in various holometabolous (Mc Daniel *et al*., 1976; Glitho *et al*., 1979) and hemimetabolous insects has been reported (Smith and Nighout, 1982; Hirn *et al*., 1979). Further studies on the structure of the gland and changes in ecdysteroid titre have been reported in the insects, *Locusta migratoria* (last instar) (Hirn *et al*., 1979), *Manduca sexta* (5th instar) (Sedlak *et al*., 1983) and *Spodoptera littoralis* (last instar) (Zimowska *et al*., 1985) and in 6th instar larva of *Spodoptera litura* (Rabeeth, 1997).

Variations in the levels of molting hormone synthesised by the prothoracic gland in the haemolymph have been determined in various insects such as, *Drosophila melanogaster* (Borst *et al*., 1974; De Reggi *et al*., 1975), *Manduca sexta* (Bollenbacher *et al*., 1975), *Philosamia cynthia* (Calvez *et al*., 1976) and *Bombyx mori* (Calvez *et al*., 1976; Coulon and Feyerisen, 1977), *Pieris brassicae* (LaFont *et al*., 1977; Claret *et al*., 1977), *Tenebrio molitor* (Delbecque *et al*.,
1978), *Calpodes ethlius* (Dean *et al*., 1980) and *Lymantria dispar* (Kelly *et al*., 1992).

The prothoracicotropic hormone, a peptide neurohormone synthesized by specific neurosecretory cells in the insect's brain and released from its neurohaemal organ, the corpus allatum, into the haemolymph at specific times during insect development (Gilbert *et al*., 1981; Bollenbacher and Gilbert, 1981). PTTH, thus released inducts the prothoracic glands to synthesize and release the ecdysone which is then hydroxylated to become active hormone. The hydroxylated ecdysone together with the ecdysone has been reported to elicit molting process.

Timing of PTTH release from the brain to synthesize and release ecdysteroid from the prothoracic gland for the larval and pupal molt have been determined in various insects such as *Bombyx mori* (Fukuda, 1944), *Hyalophora cecropia* (Williams, 1952), *Galleria mellonella* (Beck, 1970), *Calpodes ethlius* (Locke, 1970), *Manduca sexta* (Truman and Riddiford, 1974), *Pieris brassicae* (LaFont *et al*., 1975), *Heliothis virescens* (Loeb and Hayes, 1980), *Lymantria dispar* (Kelly *et al*., 1992) and *Spodoptera litura* (Rabeeth, 1997).

Most of the studies carried out have been related to the relationship between ecdysteroid level and various developmental events during metamorphosis, (Hodgetts *et al*., 1977; LaFont *et al*., 1977; Delbecque *et al*., 1978; Weilgus *et al*., 1979). Information on the interaction of PTTH and ecdysone on the larval molting has not been

Extensive studies on the isolation of prothoracicotropic hormone by Japanese in the insects *Bombyx mori* (Ishizaki et al., 1983; Nagasawa et al., 1984; Matsuo et al., 1985) *Samia cynthia* (Mizoguchi et al., 1987) have been carried out. Subsequently research on this aspect have been initiated in *Manduca sexta* (Bollenbacher et al., 1984), *Lymantria dispar* (Masler et al., 1986) and *Periplaneta americana* (Richter, 1992). Such studies have revealed that PTTH exists in the family of neuropeptides with two molecular mass ranges in *Bombyx mori* (small ca 4 kDa and large, ca 22 kDa). The small forms have been recently named as 'bombyxins'. Further studies in *Manduca sexta* have also revealed the existence of two molecular forms of PTTH i.e. 7 kDa and 27 kDa. Recently, the presence of small and large PTTH have been identified in *Lymantria dispar* and the molecular mass include 4-6 kDa and 15 to 20 kDa for small and large PTTH respectively from both larval and pupal brains.

In the present study the information on the PTTH release, prothoracic gland development, release of ecdysteroid and its level by Radioimmunoassay have been carried out. Development of spiracle in the penultimate larva has been observed to carry out larval assays in relation to crude PTTH and ecdysone action on larval molt. In addition, prothoracicotropic hormone has been isolated by High performance liquid chromatography and the results are discussed in relation to PTTH of other insect species.
The success of insect pests as diverse and the largest group of animals in the earth mainly rests on various adaptive features. One such feature is the presence of protective covering namely, the cuticle. The cuticle is the supra molecular assembly of protein, chitin, phenolic compounds, lipid, minerals, water and other compounds (Hepburn, 1985). Chitin protein complex is the major structural component of the cuticle not only present in the arthropod but also present in the plant kingdom. It has reached highest concentration as well as extreme physiological relevance in arthropod cuticle (Jeuniaux, 1963; 1982; Neville, 1975; Hepburn, 1976; Muzzarelli, 1977; Locke and Smith, 1980; Spindler, 1983; Kramer et al., 1985). Although, the cuticle is playing important role in the protection of insects, they ought to undergo periodic shedding of the old cuticle during molting in order to maintain the growth of the body.

Molting is the event during which the old cuticle is cast off and new cuticle is synthesized. The initiation of molting is indicated by the first changes in the epidermal cells which divide or enlarge in size. Changes in the shape and size of the epidermal cells generate a tension on the surface of the cellular layer. This results in the separation of epidermal layer from the non-living molting cuticular structures. This process is termed as 'apolysis' and considered to be the first sign of molting (Chapman, 1985).

Cuticle degradation and synthesis are carried out by molting fluid, a liquid which lies between the new and old cuticle. The molting fluid contains several enzymes and are associated with degradation of
old cuticular structures as well as to synthesize new cuticle (Bade and Stinson, 1976).

Among the enzymes reported to occur in the molting fluid, chitinases play an important role in the degradation of old cuticular structures and supply of the basic precursor material namely N-acetylglucosamine to synthesize new cuticular chitin structures.

Chitinases are widely distributed in living organisms. These enzymes in bacteria appear to have a nutritional or scavenging role. They are the most important enzymes from the ecological point of view and are entrusted the task of removing and recycling the chitin, produced on the continents and especially in the oceans (Berkely, 1979). The chitinases of fungi on the otherhand probably function in the softening or lysis of cell walls that appears to be necessary for growth, for the initiation of hyphal branches, and for cell separation. In higher plants and animals, chitinases are suggested to act as defensive agents against fungal infections. Chitinases however, in arthropods are the enzymes involved in the break down of the chitin polymer in the cuticle during the time of molting.

During molting, the molting fluid accumulated in the molting space is known to contain chitinolytic, proteolytic and esterolytic enzymes (Hepburn, 1985; Riddiford, 1985; Koga et al., 1991). Ecdysone is suggested to induce the epidermal cells to produce these enzymic proteins in the molting space along with the degraded cuticular structures. The hormone induces several other hydrolytic enzymes
including acetylcholinesterase and β-galactosidase from an embryonic cell line of *Drosophila melanogaster* (Best - Belpomme and Courgeon, 1980). DOPA decarboxylase, a non-hydrolytic enzyme is also induced by ecdysteroid *in vivo* and *in vitro* in certain flies (Sekeris and Fragoulis, 1985).


Occurrence of chitinolytic enzymes have been localized in different tissues which include haemolymph, salivary gland, digestive midgut, silk gland, fat bodies and testes in addition to integument and molting fluid (Kramer *et al*., 1985).
Two types of chitinolytic enzymes have been identified to participate in the degradation of chitin biopolymer during molting of the insects (Bade and Stinson, 1976). Studies carried out in *Bombyx mori* (Kimura, 1973a, b; Nagamatsu et al., 1995). *Locusta migratoria* (Spindler, 1983), *Manduca sexta* (Kramer et al., 1985; Fukamizo and Kramer, 1985a, b) revealed the isoenzymic nature of the chitinolytic enzymes in the pupal haemolymph, molting fluid and pharate pupal cuticle. Two types of chitinolytic enzymes are 1. Exochitinases 2. Endochitinases. Though detailed studies have been carried out in *Manduca sexta* (Dziadik - Turner et al., 1981) and *Bombyx mori* (Kimura, 1973a, 1976a; Koga et al., 1987, 1992; Nagamatsu et al., 1995), studies in the other insect species are much neglected in spite of their central importance in the molting process. In the present study, exochitinase have been isolated from the pharate pupal cuticle of *Eupterote mollifera* and *Oryctes rhinoceros*. Exo-enzymes of cuticular source from these insects were partially purified by gel filtration and the purified proteins were localized in the SDS-PAGE. In addition, purified exochitinase proteins were fractionated in HPLC. The characteristics of the purified exochitinase have been determined.

The sclerotization of the cuticle involves three compounds namely, phenol, protein and an enzyme phenoloxidase. In the egg capsule of cockroach, it is observed that phenol oxidase oxidizes the phenolic compound to produce a highly reactive substance namely quinone which react with the cuticular proteins via amino groups results in the insolubility of cuticular proteins (Pryor, 1940). Since then
quinone tanning scheme of sclerotization became popular and widely accepted one.

The presence of phenoloxidase subsequently has been reported in various tissues of insects such as haemolymph, larval and pharate pupal cuticle, molting fluid, oothecal cuticle and the eggs of insects. Generally the haemolymph phenoloxidase is suggested to involve in the functions of insect immunity. Whereas, the cuticular and oothecal phenoloxidases are suggested to involve in the sclerotization process (for a review, Brunet, 1980; Andersen, 1990; Sugumaran, 1990).

Though haemolymph phenoloxidase has been proposed to involve in the cuticular sclerotization by earlier authors, reports of Ohnishi (1954) made it clear that cuticular and haemolymphal phenoloxidases are different entities based on their oxidation of phenolic substrates in Drosophila virilis. Moreover, Yamazaki (1969; 1972) has extracted the cuticular phenoloxidase using trypsin and reported that the laccase type of phenoloxidase may be involved in the cuticular stabilization.

Studies carried out in Bombyx mori (Yamazaki, 1972), Schistocerca gregaria (Andersen, 1979), Calliphora vicina (Barrett and Andersen, 1981), Calpodes ethilus (Barrett, 1984a), Oryctes rhinoceros (Logankumar, 1992) and Lymantria dispar (Thangaraj and Aruchami, 1992) have shown that the cuticular matrix is containing two types of phenoloxidases namely o-diphenoloxidase (tyrosinase,
phenoloxidase, o-diphenoloxidase, E.C.1.10.3.1) and laccase
(E.C.1.10.3.2).

The enzyme tyrosinase is characterized by its ability to oxidize o-diphenolic compounds to o-quinones and hydroxylate tyrosine and other monophenols to diphenols which can be used for further oxidation. This enzyme is very sensitive to inhibitors such as phenylthiourea, thiourea and sodium diethyldithiocarbamate. Cuticular tyrosinase shows many similarities to haemolymphal enzyme in their characteristics. LaiFook (1966) has identified histochemically the enzyme tyrosinase in the cuticle of Calpodes ethlius in an inactive proenzyme and shown that it is activated upon injury by means of proteolytic enzyme in the cuticle. It is interesting to note that recently, Logankumar et al. (1996) have isolated cuticular tyrosinase in proenzymic condition in the larva of Oryctes rhinoceros.

Based on the formation of quinone, an antifungal and antibacterial compound, this enzyme has been suggested as wound healing phenoloxidase upon injury (LaiFook, 1966; Hackman and Goldberg, 1967; Barrett and Andersen, 1981; Barrett, 1984 a, b; 1987 a, b; Logankumar, 1992). Though the cuticular tyrosinase is suggested to involve in the wound healing and defense functions, the role of tyrosinase in the larval, pharate pupal cuticle and molting fluid has not been.

The other cuticular enzyme, laccase has been identified in the cuticular matrix of several insects. The enzyme laccase separated
from the cuticle of *Bombyx mori* (Yamazaki, 1972), *Schistocerca gregaria* (Andersen, 1979), *Calliphora vicina* (Barrett and Andersen, 1981), *Lucilia cuprina* (Barrett, 1987a) has been suggested to differ from the enzyme, tyrosinase in several respects. This enzyme is capable of oxidizing a wide range of substrates including o- and p-diphenols and classified as laccase type (E.C.1.10.3.2). The enzyme exhibited activity in the acidic pH, thermostable, and inhibited more by inhibitors like sodium azide, sodium fluoride. The enzyme appears its activity in the prepupal stage and showed higher activity at the unsclerotized cuticle stage of puparium formation and suggested that laccase enzyme is destined to involve in sclerotization process of the cuticle (Barrett and Andersen, 1981).

The laccase identified in the prepuparium of *Drosophila*, unsclerotized cuticle of *Bombyx mori* (Yamazaki, 1972), *Schistocerca gregaria* (Andersen, 1979) and *Manduca sexta* (Morgan et al., 1990) are soluble only after trypsin digestion. It has been suggested that enzyme may be buried deeply in the cuticular matrix and trypsin treatment liberates the enzyme protein while extraction (Andersen, 1979). However, the enzyme laccase in the cuticle of *Calliphora vicina* (Barrett and Andersen, 1981), *Lucilia cuprina* (Barrett, 1987a) and *Helicoverpa armigera* (Rabeeth, 1997) could be obtained by extraction in the borate buffer and liberation of the enzyme from the cuticular matrix did not require any tryptic digestion.

Tyrosinase or laccase or both enzymes have been purified from the insect cuticles such as, *Bombyx mori* (Yamazaki, 1972),
Schistocerca gregaria (Andersen, 1978), Calliphora vicina (Barrett and Andersen, 1981), Lucilia cuprina (Barrett, 1987a), Sarcophaga bullata, Phormia regina, Lucilia sericata and Musca domestica (Barrett, 1987 b), Manduca sexta (Thomas et al., 1989) Spodoptera litura and Helicoverpa armigera (Rabeeth, 1997).

In the present study, phenoloxidases in the larval cuticle of Helicoverpa armigera and both larval and pharate pupal cuticle of Eupterote mollifera and Oryctes rhinoceros have been extracted and partially purified by gel filtration on Sephacryl S-400. Purified enzymes were localized in SDS-Polyacrylamide gel electrophoresis and their molecular weights were estimated. In situ inhibition of cuticular phenoloxidase has been noticed. In addition, the characteristics of the enzymes have been determined.