Materials and Methods

CHEMICALS

MSF, Acrylamide, bis-acrylamide, nylon membrane (0.4 μm pore size), Tween-20 and 4-Chloro-1-naphthol were purchased from Sigma Chemicals, USA. Leupeptin and Pepstatin A were purchased from Fluka, Switzerland. Protein molecular weight standard markers were purchased from Genei, Bangalore. Primary antibody for the vitellogenin protein of M. sexta and H. virescens were obtained as a generous gift from Prof. John H. Law, Center for Insect Science, University of Arizona, Tucson, U.S.A and Dr. Sonny B Ramaswamy, Department of Entomology, Kansas State University, U.S.A, respectively. Primary antibody to the vitellogenin receptor protein of the mosquito, A. aegypti was obtained as a generous gift from Dr. A. Raikhel, Department of Entomology, Michigan State University, U.S.A. Secondary antibody (Horse radish peroxidase labeled Goat-anti-rabbit IgG) was purchased from Genei chemicals, Bangalore, India. Gold labeled secondary antibody and fluorescein isothiocyanate (FITC) labeled secondary antibody were a generous gift from Dr. Jae-In-Park, School of Forest Resource, College of agriculture, Korea. Tris base, Tris HCl, NaCl, Sodium azide, ammonium persulphate, EDTA, Glycine, Sodium Dodecyl Sulphate, Silver nitrate, Coomassie brilliant blue R-250, Coomassie brilliant blue G-250, Periodic acid/ Schiff (PAS) stain, Oil Red ‘O’ stain, Methyl Green stain and other chemicals were purchased from SRL chemicals, India. Sephadex G-200 was purchased from Amersham
Pharmacia Biotech, Sweden. The other chemicals used were of analytical grade from Qualigens fine chemicals, Ranbaxy, Himedia, Spectrochem, and SD fine chemicals, India.

**INSTRUMENTS**

**a) Electronic balance**

All weighing were carried out in a top loading mono-pan electronic balance (Sartorius Basic, Germany) with an accuracy of 0.1 mg.

**b) Spectrophotometer**

Spectra-2000 (UV-Vis, Hitachi, Japan) was used for quantification of proteins.

**c) Refrigerated Centrifuge**

Refrigerated Centrifuge SIGMA –3K30 (German make) was used for centrifugation of protein samples.

**d) Ultracentrifuge**

Beckmann SER 92E 2422 (Rotor: NVT 65, CLASS HR) was used in the purification of vitellogenin protein by density gradient ultracentrifugation and Beckmann LE-80 (Rotor: Ti-80) was used for oocyte membrane protein isolation.

**e) Electron Microscope**

The immunogold labeled ovarian tissue sections were examined with a FEI MORGAGNI 250 transmission electron microscope.
f) Fluorescent Microscope

Nikon Trinocular Fluorescence Microscope, model E 200 was used for visualizing the fluorescent labeled ovarian tissue sections.

g) Scanning Densitometer

Hoefer GS 300 Transmittance/ Reflectance densitometer with windows 3.1 software package and PHOTO-CAPT MW, version 99.04 software packages were used for quantitative determination of proteins.

h) Ultraflow freezer

Nuaire –85° C ultraflow freezer was used to store the protein samples.

i) Ice Making Machine

Icematic F90 Compact Electronic ice making machine was used to make ice for sample collection and processing.

j) Digital Shaking bath

Digital Shaking bath with temperature control was used for destaining gels and for incubating nitrocellulose paper during western blot analysis.

k) Lyophilizer

Martin Christ Lyophilizer, model Alpha 1-2 LD was used for freeze-drying the samples.
l) Blotting apparatus

BIORAD POWERPAC 200 and Trans-Blot SD semidry transfer unit were used to transfer the polypeptides from the gel to the nitrocellulose membrane.

m) Electrophoretic Apparatus

Broviga (Balaji Scientific Services, Chennai) electrophoretic unit (vertical model) was used for separation and analysis of the proteins.

n) Power Pack

The Bio-Rad model 1000/500 constant voltage/ constant current was used for all the separation techniques.

o) Gel documentation Unit

Hero lab instrument RH-2 (German make) was used for documentation of the polyacrylamide gels.

p) Microwave oven

Samsung microwave oven (Bio ceramic) was used for antigen retrieval in the slides being processed for fluorescent microscopy.

q) Milli-Q water

Millipore Academic (U.S.A) was used for obtaining pyrogen-free ultrapure water.
**Spodoptera litura** - the experimental animal

The larvae of *S. litura* were reared at 25° C under a 16h light and 8 h dark photo-regime. The rearing was standardized on castor leaves, chick pea semi-synthetic diet (Santharam and Balasubramanium, 1980) and a diet of soy bean and red kidney bean, formulated by us.

**Life cycle of Spodoptera litura**

The life cycle of the insect ranges from 23 – 37 days. However, various intrinsic and extrinsic factors such as larval phase, population density, prevailing weather conditions, the plant - growth stage at which the leaves were obtained and leaf quality determine the caterpillar’s rate of feeding, development, and moth’s fecundity and life span (Salama et al., 1971). The life cycle of this holometabolous insect comprises of the egg, larval, pupal and adult stages as shown in Fig. 1.

**Eggs**

The eggs of these species are normally laid in an irregular fussy manner on the ventral side of the host plants leaves. The female moth covers the egg masses with brown hairs giving them a “Felt – like” appearance. The eggs hatch in 3 – 4 day’s period. It was observed that the cluster of eggs adheres strongly to the surface. If not removed in a day’s time, there is a greater possibility of damage to the egg during the process is great. In addition, it is also noted that neonates feed on the unhatched eggs initially.
Fig.1 Represents the lifecycle of the cotton pest *Spodoptera litura*, on the castor leaves. The duration of each of the metamorphic stage in the life cycle has been indicated on the dotted lines.
Life cycle of Spodoptera litura

Fig 1
**Larva**

On hatching, the neonates are 2 – 3 mm long with white bodies and black heads and are very difficult to detect visually. As the caterpillars start feeding, they are translucent green with a dark thorax. The larvae are nocturnal and during the day they can be found at the base of plants or under pots. The feeding activity of the young caterpillars causes “windows” in the leaves. The larvae that emerge from eggs laid on upper branches of the plant or on glass house structures or hanging pots, can reach the plants below by “parachuting” down on silken threads, that they spin. The larvae undergo five molts before they pupate. Thus, there are six instars in the larval stage. Each instar may span to a period of 2-3 days but the VI instar lasts for 4-6 days. It was observed that the caterpillar after the molt, feeds on the molted skin. They develop characteristic marking on their backs, soon after emergence. The pattern of these marking are variable, a bright yellow stripe along the length of the dorsal surface is seen which is characteristic of *S. litura* larvae. The older larvae can completely defoliate plants if present in large numbers. Stems, buds, flowers and fruits may also be damaged. The larval period ranges from 19-20 days. The caterpillar burrows into the soil below the plant for several centimeters and there it pupates without a cocoon. As it does so, it produces a quantity of fluid, and drowns in this when it pupates in captivity in an empty glass jar. It will pupate successfully if 0.5 cms of sand is provided.

**Pupa**

The pupa is 15-20 mm long, red brown with characteristic markings at the tip, which helps sex differentiation. The pupal stage is about 7-9 days in
winter, but is prolonged in summer. The female moths emerge in a day or two prior to the male moth.

**Adult**

The adult moth is brown with a complex pattern of cream streaks criss-crossing the forewing. The hind wings are silvery white. It has a wing-span of about 4 cms. The adult moth has a proboscis, which helps them feed on the nectar from flowers. The female moth attracts the male by secretion of pheromones. An adult female lays about 400-700 eggs. Effective egg laying is usually on the fourth day after adult eclosion. Oviposition occurs in installments and extends from fourth to seventh day and then recedes. The female moth is characteristic in having tufts of anal hairs. The male moths have a blue-gray band running from the apex to the inner margin of each forewing.

**Rearing on castor leaves**

The rearing room was provided with adequate supply of water, washing facilities and sufficient ventilation. The room was used only for rearing. Before the commencement of rearing, the trays, buckets and cages were disinfected with 2% formalin, washed with distilled water and dried. All the rearing materials used were disinfected regularly to avoid acquisition of pathogens.

The egg masses with greenish tinge (healthy colony) were collected from the field, surface sterilized with 10% formalin for 1 minute and washed thoroughly in running water to remove any traces of formalin, completely.
The eggs were stored in a box after drying under shade. The eggs hatched in 3-4 days. The hatched larvae were released in trays with fresh castor leaves (Fig.2a). The tray was covered with clean muslin cloth. Insects were routinely provided with fresh castor leaves once daily for the early two instars, twice daily for the advanced third instars and fourth instars and thrice daily for the fourth, fifth and sixth instar. Fecal matters and uneaten leaves were removed every day before supplying fresh leaves to the experimental larvae. The entire larval period range from 19 – 20 days. When the larvae pupate (Fig.2b), they were transferred to a tray with sterile soil. After a day, the pupae were washed with water. The floating pupae were discarded. The pupae were sexed at this stage. The healthy and normal pupae were shifted onto sterile cotton in a petridish and placed in a cage. The pupal period lasted from 7 – 9 days. The emerged adults (Fig.2c) were introduced into adult emergence cage (Fig.2d). 10% sucrose solution was provided for the adult to feed. The adults were then allowed in the oviposition basket at the rate of 8 – 10 per basket. Conical flask with either castor or nerium leaves were provided. The egg masses from each basket were removed every day with a soft bristled brush, disinfected with formalin and the cycle of rearing was repeated (Rabindra et al., 1999).

**Preparation of semi-synthetic diet**

The semi-synthetic feed was prepared following the method of Santharam (Manual for Biocontrol programme). The diet formulated by us contained a combination of Red kidney beans (Rajma) and Soyabeans in the ratio of 99:1 as a protein supplement, in the place of chick pea.
Fig.2 Represents the rearing of *Spodoptera litura* on castor leaves and semi-synthetic diets

(a) The larvae of *Spodoptera litura*, feeding on the castor leaf. (b) The determination of the length of the pharate pupae, pupae and female adult moth (c) emerged from the larvae that were fed on castor leaves, taken as one of the parameters to choose a desirable diet. (d) The cage used for rearing the adult moth (e) The neonates of *Spodoptera litura* fed on the semi-synthetic diet containing chick pea as a protein supplement in the feed preparation. (f) The larvae of *Spodoptera litura* fed on the semi-synthetic diet, containing a combination of red kidney beans (Rajma) and soyabean at a ratio of 99:1 as the protein supplement in the feed.
<table>
<thead>
<tr>
<th>S. No.</th>
<th>Ingredients</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>CHICK PEA SEEDS</td>
<td>100 g</td>
</tr>
<tr>
<td>2.</td>
<td>AGAR AGAR</td>
<td>12.8 g</td>
</tr>
<tr>
<td>3.</td>
<td>METHYL β HYDROXY BENZOATE</td>
<td>2.0 g</td>
</tr>
<tr>
<td>4.</td>
<td>SORBIC ACID</td>
<td>1.0 g</td>
</tr>
<tr>
<td>5.</td>
<td>WESSON'S SALT (OPTIONAL)</td>
<td>7.2 g</td>
</tr>
<tr>
<td>6.</td>
<td>CARBENDAZIN</td>
<td>0.5 g</td>
</tr>
<tr>
<td>7.</td>
<td>FORMALDEHYDE-40%</td>
<td>1.0 ML</td>
</tr>
<tr>
<td>8.</td>
<td>YEAST (BREWER'S DRY)</td>
<td>30 g</td>
</tr>
<tr>
<td>9.</td>
<td>VITAMIN (ABDEC)</td>
<td>2 ML</td>
</tr>
<tr>
<td>10.</td>
<td>ASCORBIC ACID</td>
<td>3.2 g</td>
</tr>
<tr>
<td>11.</td>
<td>STREPTOMYCIN SULFATE</td>
<td>0.04 g</td>
</tr>
<tr>
<td>12.</td>
<td>DISTILLED WATER</td>
<td>800 ML</td>
</tr>
</tbody>
</table>

The chick pea were soaked over-night in 400ml of distilled water. It is cooked and blended into a fine paste along with the other ingredients, excluding ascorbic acid, streptomycin sulfate, vitamin drops (because they are heat susceptible) and agar agar.

Agar agar was melted in the rest of the distilled water and added to the chickpea paste and blended in the mixie. The diet mix was cooled to 60°C. The rest of the ingredients were added.

While still warm, the diet was dispensed into sterile rearing containers (trays for neonates and 3 ml vials for mid instar larvae) placed in laminar flow
hood. The trays were closed and the vials were plugged with sterile cotton. This feed was used for rearing, after it solidified.

For rearing early instar larvae, the diet was dispensed in plastic trays (Fig.2e) of size 30 x 20 x 6 cm to a depth of about 1.0 cm (approximately 150 g of diet/tray). For rearing late instar (from 4th instar onwards) the diet can be dispensed into heat-sterilized antibiotic vials of 3-5ml capacity (Fig.2f) at the rate of 3 g/vial. This prevented the spreading of infection and cannibalism.

Sample collection and extraction of proteins

Haemolymph was bled into an ice-chilled 1.5 ml tube containing a few crystals of phenylthiourea (to prevent tyrosinase activity) by cutting the prolegs of larvae or the anterior part of the ventral abdomen of pupae or the region at the proximity to the wings of adult moth with scissors. After removal of haemocytes and debris by centrifugation at 5000 rpm for 5 min at 4°C, the sample was stored at −85°C. Fatbody was dissected in ice-cold Tris buffer, pH 7.2 (10mM EDTA, 50mM Tris HCl, 0.4M NaCl, 1mM PMSF). It was then homogenized in approximately about 2 vol. of Tris and centrifuged at 8000 rpm for 10 min. The supernatant was stored at −85°C until use. The ovaries were dissected out from the pupae and adult moths, homogenized in PBS, pH 7.2 (50 mM phosphate, 400 mM NaCl, 1 mM PMSF) and centrifuged at 10,000 rpm for 10 min to remove the debris. The whole animal and larval samples were subjected to ice cold 10% TCA precipitation, followed by addition of 0.5% Sodium carbonate, after a spin at 2000 rpm for 15 minutes.
Bradford's assay for protein estimation

The total protein estimation was carried out according to the method of Bradford (1976) with bovine serum albumin as standard. To 1 to 5 μl of the protein extract (depending on tissue), 2.5 ml of the Bradford reagent (100 mg Coomassie brilliant blue G-250 in 50 ml of 95% ethanol and 100 ml of 85% orthophosphoric acid, made up to 1000 ml) was added and mixed well. The OD was measured at 595 nm after 2 min.

Electrophoresis

Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) of the samples was performed at room temperature according to the method of Laemmli (1970). A 7% linear resolving gel and 3% stacking gel were used to separate the proteins. A sandwich was made with two glass plates separated by spacer strips (1.5 mm thickness). The resolving gel mix was poured into the space between the glass plates. A seam of distilled water was layered on to the gel to exclude oxygen from inhibiting polymerization and to ensure a uniform flat gel surface. Presence of a sharp interface between the polymerized gel and the overlay was an indication of complete polymerization. After decanting the water layered on the surface, 3% stacking gel was prepared and poured over the separating gel and Teflon comb (1.5 mm thickness) was inserted to form wells. Care was taken not to trap any air bubbles while casting the gel. After polymerization, the Teflon comb was removed. After removing the basal strip, the glass plates with polymerized gel were fixed to the electrophoretic apparatus. The resolving gel buffer with pH of 8.8, contained 1.5M Tris base and 10% SDS and the stacking gel buffer with pH of 6.8, contained 0.5M Tris base and 10% SDS. Protein samples were mixed with equal
volume of sample buffer of pH 6.8 (60 mM Tris-Cl, 5% SDS, 1% β-Mercaptoethanol, 0.002% bromophenol blue and 6% glycerol) and kept in boiling water bath for a few seconds and then loaded on to each well.

The resolving gel was prepared as follows:

<table>
<thead>
<tr>
<th>CHEMICALS</th>
<th>RESOLVING GEL (7%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide stock containing 0.8% bisacrylamide</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Lower Tris (pH 8.8)</td>
<td>1.6 ml</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>3.2 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>40 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

The polypeptide marker for the SDS-PAGE was in the order of increasing migration: Myosin 205; Galactosidase-121; Bovine serum albumin-82; Ova albumin 50; Carbonic anhydrase-34; Soybean trypsin inhibitor-28; and Lysozyme-19, respectively. Native 7% polyacrylamide gel was executed in similar way but SDS and β-Mercaptoethanol were omitted. Electrophoresis was carried out for 3 h at a constant voltage of 30V in the region of stacking gel and 90V in the region of resolving gel. The running buffer (electrode solution) contained 0.025M Tris, 0.185M glycine and 0.1% SDS (3 gms of Tris, 14.4 gms of glycine and 1 g SDS in 1 litre of distilled water). After electrophoresis, the gels were stained with the desired stain.
The stacking gel consisted of the following composition:

<table>
<thead>
<tr>
<th>CHEMICALS</th>
<th>STACKING GEL (2%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide stock containing 0.8% bisacrylamide</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>Upper Tris (pH 6.8)</td>
<td>0.45 ml</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>0.72 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>14 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>4 µl</td>
</tr>
</tbody>
</table>

The molecular weight marker used for the native gel was in the order of increasing migration: Urease hexamer-545; Urease trimer-272; Bovine serum albumin dimer-132; Bovine serum albumin monomer-66; lactalbumin-14.2. Molecular weights were interpolated from linear regressions of protein standards, using Rf vs. log transformed molecular weights (Rosenberg, 1996).

**Coomassie blue staining and destaining**

After electrophoresis, the gel were stained with staining solution containing 0.2% Coomassie blue R-250 in 50% methanol and 7% acetic acid for 6 hours (1g Coomassie blue R-250, 250 ml of methanol, 35 ml acetic acid made up to 500 ml). The gels were later destained with 7.5% acetic acid and 30% methanol.

**Silver Staining**

The proteins on gels were stained by silver staining according to Merril et al. (1984). The proteins on the gels were fixed overnight in the fixative containing 50% ethanol, 12% acetic acid and 0.1% formaldehyde. The gel was
then washed twice with 50% ethanol and treated for 1 min with sodium thiosulphate, the excess of which was removed by washing twice in water. The gel was then immersed in 0.2% silver nitrate containing 0.05% formaldehyde for 20 minutes. Reduction of silver nitrate to metallic silver and enhancement of colour was performed in a developer solution. The reaction was arrested after brown protein bands appeared against yellow background.

**Periodic acid/ Schiff (PAS) staining**

PAS staining was carried out, following the method of Zacharius *et al.* (1969). Appearance of pink bands showed the presence of glycoproteins. This was performed to detect the glycoproteic nature of vitellogenin.

**Oil Red ‘O’ staining**

Oil Red ‘O’ staining was performed following the method of Collins *et al.* (1995) to determine the lipoproteic nature of vitellogenin. A clear reddish brown band appeared in the region where lipoprotein was present.

**Methyl Green staining**

Methyl Green staining, following the method of Cutting and Roth (1973) was carried out to detect the phosphoproteic nature of vitellogenin. A bluish green band appeared in the region where phosphoprotein resolved.

**Western Blotting**

Western blot analysis of the desired proteins ware carried out according to Towbin *et al.* (1979). The Proteins extracted were separated on 7% SDS-PAGE. Immediately after the separation of the proteins, the gels were soaked in
Towbin's transfer buffer of pH 8.5 for 30 min. Simultaneously, a nylon membrane of the same dimension as the gel, was soaked and made wet from beneath in the transfer buffer. The gel and the NC membrane were then sandwiched between two extra-thick filter papers.

The order from the anode (bottom) was as follows

Pre-wet filter paper, pre-wet membrane, equilibrated gel and again, Pre-wet filter paper followed by the cathode (top). Care was taken to avoid the trapping of air bubbles. The setup was placed in the Trans Blot Semi Dry Transfer Cell. The transfer was carried out for 30 minutes at 20V. After transfer, the nitrocellulose membrane was recovered from the assembly and air-dried. Non-specific binding of the antibody to the proteins was blocked by incubating the membrane in blocking solution containing 3% milk powder and 0.005% Tween-20 in transfer buffer saline (TBS) of pH 7.2 for 3 h with gentle shaking in a platform shaker. The membrane was then washed twice in TBS-Tween-20 for 10 min each, with gentle shaking. The proteins were probed with primary antibody (1: 500 dilution) for the target proteins. For Vg, polyclonal antiserum of *M. sexta*, was used as the primary antibody and for the vitellogenin receptor, Rabbit anti-mouse vitellogenin receptor polyclonal antibody was used. The excess antibody was removed by washing twice with TBS-Tween-20 for 10 min each, with gentle shaking. The primary antibody was again probed with secondary antibody, goat anti-rabbit IgG, tagged to the marker enzyme Hydrogen peroxidase. The incubation was carried out for 2-3 hours with gentle agitation. The membrane was then washed twice in TBS-Tween-20 and twice again with TBS, each for 10 min. The proteins were
detected by the colour developed on addition of the chromogenic substrate solution. The purple coloured bands were visualized and photographed.

**Densitometric scanning**

The quantity of the Vg (190 kDa) protein in the haemolymph, fat body, ovary and eggs were measured using Hoefer GS 300 Transmittance/Reflectance densitometer and the peak areas were marked.

**Direct Antigen Coating - Enzyme Linked Immuno Sorbent Assay (DAC-ELISA)**

DAC-ELISA was carried out following the method of Perlmann and Perlmann. (1994). The 64 wells titre plate was coated with a predetermined volume of the haemolymph, fat body, ovary (obtained from the pupa and the adult moth on the different days and different time intervals) containing equal concentration of the total proteins and incubated overnight in the fridge. The wells were washed thrice at 3 min interval with washing buffer (PBS-T, pH 7.2; PBS with 0.05% Tween 20). Blocking solution (1% BSA in PBS-T) was added to the wells and incubated at 37 °C for 1 h followed by washes as mentioned above. The primary antibody (raised against the Vg protein of *H. virescens*) was added at 1: 1000 dilutions and incubated at 37 °C for 1.30 hrs. After repeating the washing step, the secondary antibody, conjugated to alkaline phosphatase was added at a dilution of 1: 10,000 and incubated at 37 °C for 1.30 hrs. The substrate (p-nitro phenyl phosphate) was added and the colour development was allowed in the dark. The reaction was stopped by the addition of 3M NaOH. The male haemolymph sample of *S. litura* was used as a negative control.
Purification of Vitellogenin from adult female haemolymph

Haemolymph from Day 1 to Day 4 adult females were used for purification by using the method described by Osir et al. (1986) and Engelmann and Mala (2000), with some modifications. Haemolymph was collected by injecting PBS (pH 7.2) into the abdomen, and then draining the diluted haemolymph into an ice-chilled 1.5 ml tube containing a few crystals of phenylthiourea. The haemocytes and debris were removed by centrifugation at 5000 rpm for 5 min at 4°C and then subjected to the purification steps as given below.

i) Preparative Ultracentrifugation

Following the removal of hemocytes, potassium bromide was added to the haemolymph to form a 44% solution. The solution was placed in quick Seal centrifuge tube (Beckman). This was over layered with phosphate buffered saline, and centrifuged (50,000 rpm, 2h, 4°C) in a Beckman VTI-65 vertical rotor to form a density gradient. Eventually, all proteins float or sink to the zone that corresponds to their density. Having a lower density, the yellow colored lipoprotein floats higher than non-lipoproteins. The separated proteins were fractionated and desalted by dialysis.

ii) Gel Permeation Chromatography

Gel Permeation Chromatography was carried out according to the method of Rosenberg (1996). The subphase obtained was applied to a Sephadex G-200 (fine grade) column (1 cm X 120 cm) equilibrated with PBS (pH 7.2). The homogeneity of the placed column and its void volume (Vo) were determined by eluting the marker, Blue Dextran-2000. The elution volume of
Blue Dextran-2000 was taken as the void volume of the column. The effluent of the column was collected manually with approximately 1 ml per tube and the absorbance of the individual chromatographic fractions (the column effluent) were monitored at 280 nm fractions containing partially purified Vg were pooled and lyophilized.

**Preparation and solubilization of Ovarian Membranes**

Ovarian membranes were prepared from the vitellogenic ovaries of the adult moth. (Dhadialla et al., 1992; Sappington et al., 1995; Tao et al., 1996; Warrier and Subramonium, 2002). All procedures were performed at 4°C. After centrifugation of the ovary homogenate for 15 min at 10,000 x g, the pellet was resuspended in 20 ml of the same buffer and washed four times to completely remove yolk proteins. The resulting pellet was resuspended in 15 ml of the same buffer, homogenized and then centrifuged at 500 x g for 5 min. The resulting supernatant was filtered through 100-μm nylon mesh and ultracentrifuged at 1,00,000 x g for 1 h. The membrane pellet was resuspended in 1.5 ml of the same buffer by repetitive aspiration through a 22-gauge hypodermic needle and designated as the crude ovarian membrane preparation. The crude membrane preparation was stored at -80°C for up to 1 month for later use. The crude membrane preparation was mixed with an equal volume of binding buffer containing 2.4% n-octyl-β-D-glucopyranoside and then stirred for 15 min on ice. The resulting extract was centrifuged for 15 min at 10,000 x g to remove insoluble materials. The supernatant was filtered through a 0.45-μm disposable syringe filter, held on ice, and then used for receptor binding assay.
**Ligand Binding Assay**

The ligand binding assay was performed by a modification of dot blot (Rosenberg, 1996; Hiramatsu *et al.*, 2002; Persaud *et al.*, 2003). The oocyte membrane extract, dissolved in the binding buffer (BB- 20mM Tris-HCl, 2mM CaCl$_2$, 150mM NaCl, pH 7.0) with 0.2% trition X-100, in the required concentration, were immobilized on the pre-conditioned PVDF membrane and allowed to air dry. The membrane was immersed in the blocking solution containing 5% nonfat skim milk and incubated at 25°C for 1hrs and then washed thrice in wash buffer, containing trition X-100, for 5 minutes each. Following this, the membrane was immersed in the binding buffer containing 5% nonfat skim milk and Vg in required concentrations, for 5 hrs at 25°C. After washing, the incubation was carried out in the binding buffer with the primary antibody (1:500), which was the anti-rabbit vitellogenin antibody (raised against Vg of *M. sexta*), for 4 hrs at 25°C. After this, the incubation was with fluorescein isothiocyanate labeled secondary antibody (1:500), for 3hrs at 25°C. The binding was visualized under UV light and the results were documented for densitometric analysis.

(i) For optimizing the ideal Ca$^{2+}$ concentration, for the Vitellogenin and vitellogenin receptor interactions, 1.5 mM, 2 mM and 2.5 mM Ca$^{2+}$ concentration were used in the BB, at VgR concentration of 6µg/ml and Vg concentration of 3µg/ml.

(ii) Analysis of binding activity at constant Vg concentration (3µg/ml) was carried out at varied membrane concentrations of 4µg/ml, 6µg/ml, 8µg/ml and 10µg/ml of binding buffer. The male haemolymph (8µl/ml)
was used as the negative control and purified Vg (5\(\mu\)g/ml) as positive control.

(iii) Concentration dependent binding of Vg to its receptor was investigated at a constant membrane concentration of 6\(\mu\)g/ml and increasing Vg concentration of 2\(\mu\)g/ml, 2.5\(\mu\)g/ml, 3\(\mu\)g/ml and 3.5\(\mu\)g/ml. purified Vg at a concentration of 5\(\mu\)g/ml was used as the positive control.

(iv) To determine the optimal amount for maximum inhibition of binding of Vg to its receptor in the ovarian membrane extract (6\(\mu\)g/ml), four different concentrations of suramin (4 mM, 4.5 mM, 5mM and 5.5mM in the binding buffer) was added along with the Vg (3\(\mu\)g/ml).

**Fluorescent Microscopy**

**Fixation**

The ovary (naive tissue) samples collected from the adult moth, on random days were fixed in buffered formalin for 48 hours and the tissues were washed overnight by placing it under running tap water. Samples were further processed for the immunoflourescent detection following the method of Robinson *et al.* (2001)

**Dehydration**

The washed tissues were dehydrated in the following percentage of alcohol for 1 hour each: 30%, 50%, 70%, 90% and 100%

**Cleaning**

The tissues were placed in xylene: alcohol (1:1) and then xylene for half an hour, each. Xylene was used to remove any traces of water in the tissues.
Cold infiltration

The tissues were placed in xylene and paraffin wax was added to it and left overnight.

Hot infiltration

The tissues were transferred to embryo cups containing paraffin wax in liquid state in the hot air oven and after 20 minutes, the tissues were transferred to another embryo cup containing fresh wax. This was repeated thrice. The tissues were then embedded in a boat form and were left undisturbed overnight. The excess amount of wax present in the boat was removed by trimming. The trimmed tissues were fixed in a wooden piece and sectioning was carried out using a microtome to a size of 3 μm and fixed on a clean glass slide.

Deparaffinization

The slides fixed with tissues were placed in a couplin jar filled with Xylene, for half an hour. This step was performed twice to deparaffinize the wax present in the slides.

Dehydration

The slides were placed in a couplin jar with 70% alcohol for half an hour followed by 100% alcohol. The slides were then washed with PBS buffer (pH 7.4).
**Antigen Retrieval**

The slides were placed in a coupling jar with Tris-HCl buffer (pH 10) in microwave oven for 5 minutes followed by washing with PBS buffer (pH 7.4).

**Immuno detection**

The Primary antibody (rabbit anti vitellogenin polyclonal antibody raised against Vg of *M. sexta*) was added in the dilution ratio of 1:1000 and the slides were incubated for 1 hour at room temperature. The slides were then washed with PBS buffer (pH 7.4). Secondary antibody conjugated with FITC was added in the dilution ratio of 1:1000 and the slides were incubated for 1 hour at room temperature in dark and the washed with PBS buffer. Propidium iodide (10mg/ml PBS) was added to the slides and incubated for 15 minutes in dark, washed with PBS and placed in 70% and 100% alcohol and then xylene, each for 15 minutes. After repeating the step again, the slides were mounted with cover slip using DPX mountant and examined under fluorescence microscope with blue filter.

**Immunogold Electron Microscopy : Transmission Electron Microscopy**

The adult ovary and egg of *S. litura* were prepared for ultra structural immunocytochemistry by fixation in a mixture of 2% glutaraldehyde and 2% Para formaldehyde in 0.1M phosphate buffer (pH 7.4) for 30 minutes at room temperature. Samples were then prepared for LR white gold embedding by the method of Barber *et al.* (1991). Thin sections were cut and mounted on uncoated nickel grids. The grids were floated on a drop of filtered 1% fish
gelatin for twenty minutes to reduce nonspecific binding. The sections were then transferred without washing to a drop of primary antibody (Rabbit antivitellogenin antibody raised against Vg of *H. virescens* / Rabbit antivitellogenin receptor antibody raised against VgR of *A. aegypti*) for 2 hours at room temperature. Control grids were floated on non-immune serum. After washing with jets of filtered phosphate buffered saline, each grid was floated on a drop of protein A-gold (40nm gold particles) diluted 1:20 with filtered Phosphate buffer for 1 hour at room temperature. Washing was performed using jets of filtered phosphate buffer followed by floating on drop of phosphate buffer. Final washing was carried out with jets of filtered distilled water and stained with uranyl acetate and lead citrate. The sections were examined with a FEI MORGAGNI 250 transmission electron microscope (AIIMS, New Delhi).