Materials and Methods
2.1. Insect rearing

The study was carried out with two species of grasshoppers, *Oxya hyla hyla* (Serville) and *Gesonula punctifrons* (Stal) occurring in the paddy fields of Agartala city (Latitude 23° 50’ N and Longitude 91° 25’ E). Season of abundance of these two grasshoppers depended on the availability of paddy leaves and were generally from March to October. The insects were collected manually by hand nets. After collection the insects were reared in the laboratory conditions in big plastic jars supplied with fresh paddy and grass leaves with water. In this condition the laying of eggs, incubation for 30-45 days and successful hatching and development were obtained from three to four generations. Female *Oxya* and *Gesonula* in different stages of maturity were collected from the stock culture and directly from the paddy fields and used for different experimental purposes.

2.2. Microscopic Methods

2.2.i. Tissue Processing

*Gesonula punctifrons* and *Oxya hyla hyla* (paddy grasshopper) collected from the paddy fields were dissected out and collected from the mature ovarian follicles, oviduct and after laying eggs were collected just after laying of the eggs before pod formation within rearing jars. These eggs were cleaned in 100mM Tris-HCl buffer (pH-8) with brush.
2.2.ii. Live analysis of eggshell

The ripe ovaries were dissected by needle and forceps and kept in Ringer’s solution (1.3g NaCl, 0.05g KCl, 0.06g CaCl₂ in 200ml of distilled water). For living cell analysis dissected ovaries taken in ringer’s solution were directly observed under LEICA Microscope (DM 1000) and subsequently photography were made.

2.2.iii. Histochemical Methods

For histochemical studies the dissected ovaries were placed in 4% Formaldehdy in a watchglass overnight for fixation. After fixation ovaries were transferred to distilled water and kept overnight for complete removal of fixative. Then ovaries were transferred in graded series of alcohol (30, 50, 70 and 90%) for one hour in each grade and in 100% for overnight to dehydrate. The dehydrated tissues were cleaned in xylene and then transferred in xylene-paraffin mixture (60°C) for overnight for diffusion of xylene. After that the tissues were transferred to full paraffin for two hours at 60°C. Embedding was done in molten paraffin and allowed the paraffin to get solid. The prepared blocks were sectioned at 5μm thickness by using a LEICA rotary microtome (LEICA RM 2125RT).
2.2.iv. Staining

Sectioned tissues were de-paraffinized with xylene and were stained by mercury-bromphenol blue and PAS methods (Pearse, 1975).

**Mercury-bromphenol blue staining for protein**

After re-hydration (100, 90, 70% and distilled water) slides were flooded with mercury-bromphenol blue solution (0.1%) and allowed to stand for 30 minutes. The stain was then poured off and the slides were immersed in 0.5% acetic acid for overnight. When the unbound stains were totally removed tissue sections were differentiated and dehydrated in n-butyl alcohol. These were followed by a cleaning in xylene for 3-5 minutes and were mounted in DPX. Stained sections were viewed under LEICA Microscope (DM 1000) and subsequently photographs were taken.

**Periodic acid – Schiff staining for carbohydrate**

The hydrated slides were dipped in 1% aqueous periodic acid solution for 10 minutes for oxidation and kept in running tap water for 30 minutes. After washing, the slides were transferred to Schiff’s reagent (De Tomasi, 1936) for 10 minutes and briefly washed in 1% potassium-metabisulphite solution for 2 minutes. Then the slides were washed in distilled water. The slides were dehydrated and mounted in DPX. Slides were viewed under LEICA Microscope (DM 1000) and subsequently photographs were taken.
2.2.v. Scanning electron microscopy

After dissection of the insects with ultra-fine forceps and needles, eggs from different stages of development i.e. from follicle stage, from oviduct stage and after laying stages were collected and washed in 100mM phosphate buffer (pH 7.2) with brush. After washing the eggs were kept in Karnovsky’s fixative (16% Paraformaldehyde Solution, 50% Glutaraldehyde EM Grade in 0.2M Sodium Phosphate Buffer) for four hours. After fixation the fixed eggs were washed in 100mM phosphate buffer (pH 7.2) with three changes. The fixed eggs were dehydrated by graded acetone (30, 50, 70, 80, 90, 95 and 100%) all steps were done for 15 minutes with 2 changes in each at 4°C (Mazzini and Gaino, 1985) and the final dehydration was done in Tetra Methyl Silane for 10 minutes with two changes at 4°C and after that was brought to room temperature (25-26°C) to dry. The eggs were mounted on aluminium stubs. On the mounted eggs coating was done in Fine Coat ION Sputter JFC-1100 (JEOL) with gold-palladium. The thickness of the coat was 50nm. After coating the subsequent photographs were taken in JEOL-6360 Scanning Electron Microscope.

2.2.vi. Transmission electron microscopy

For transmission electron microscopy eggs were fixed in 4% glutaraldehyde in 0.1M phosphate buffer (pH 7.2) for four hours and then washed in 0.1 M phosphate buffer (pH 7.2). The fixed eggs were dehydrated in graded series of ethanol (30, 50, 70, 90, 95 and 100%). The dehydrated eggs were passed through araldite CY212 embedding medium at 60°C for 24 h. Sections were made
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by ultramicrotome of RMC. Sections were stained with uranyl acetate and lead citrate (Ma et al., 2002). Finally their sections were examined by JEOL 2100 Transmission Electron Microscope and photography was made.

2.3. Biochemical Methods

2.3.i. Isolation of chorion and its solubilization

After collection of eggs through ultra-fine forceps, needles and brush, the eggs were cut by blade keeping them in a pre-chilled Petridish and washed in 100mM Tris-HCl (pH 8.2) with 1% SDS using brush. Washing was repeated for four times which ensured the complete removal of yolk materials from the eggshells. The collected empty eggshells were treated with different solubilizing solutions developed and used by earlier researchers (presented in table 1) for different insects of different orders Diptera, Hemiptera and Lepidoptera etc. But those solubilization buffer systems failed to produce satisfactory result with Oxya and Gesonula chorion. Finally through a process of trial and error the chorion was solubilized in a solution developed by the present worker (400mM Tris-HCl, pH 8.4, 4% β-mercaptoethanol, 6M Urea, 1% SDS and boiling in water bath for 7-10 minutes). This solution produced satisfactory result and most portions of the eggshell were solubilized. After solubilization the solution was centrifuged at 5,000 RPM for 5 minutes and the resulting supernatant was used for SDS-PAGE analysis.
Table: 1. Different methods followed by earlier researchers for chorion dissolution of different insect:

<table>
<thead>
<tr>
<th>Chorion of Insect</th>
<th>Earlier Researchers</th>
<th>Solubilizing Buffer with working temperature and pH</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gryllus mitratus</em> (Order: Orthoptera)</td>
<td>Kawasaki <em>et. al.</em>, 1971</td>
<td>8M Urea, 10mM DTT, 30mM EDTA, 0.2M Tris-HCl, pH 8.6 at 20°C.</td>
</tr>
<tr>
<td><em>Silkmoth</em> (Order: Lepidoptera)</td>
<td>Regier <em>et al.</em>, 1980</td>
<td>8M Urea, 0.36M Tris-HCl and 0.03M DTT to a concentration of 7mg/ml, pH 8.4 at 20°C.</td>
</tr>
<tr>
<td><em>Aedes aegypti</em> (Order: Diptera)</td>
<td>Li <em>et al.</em>, 2005</td>
<td>20 mM Sodium Phosphate, 1% CHAPS, 2M Urea, 0.15M KCl, 2mM PMSF and 2mM EDTA-Na2, pH 7 at 20°C.</td>
</tr>
<tr>
<td><em>Drosophila sp.</em> (Order: Diptera)</td>
<td>Fakhouri <em>et al.</em>, 2006</td>
<td>8M Urea, 5% CHAPS, 40mM Tris-base, protease inhibitor at 20°C.</td>
</tr>
<tr>
<td><em>Rhodnius prolixus</em> (Order: Hemiptera)</td>
<td>Bouts <em>et al.</em>, 2007</td>
<td>8M Urea, 0.36 M Tris-HCl 0.03M DTT and 0.1 M PMSF, pH 8.4 at 20°C.</td>
</tr>
</tbody>
</table>
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Estimation of protein

During the course of study of the eggshells at different stages of maturation the dissolved material was used for biochemical analysis. So it was essential to estimate the protein contents of the solution under soluble condition. For that purpose protein content of the solutions were estimated following the method of Lowry et al., (1951) by using the standard curve prepared against Bovine serum albumin.

2.3.ii. Electrophoresis method

Sodium dodecyl sulfate-Poly Acrylamide gel electrophoresis was performed in the presence of SDS for analyzing the polypeptide diversity following the method of Laemmli (1970). Electrophoresis was carried out with solubilized eggshells of the three different stages of chorion (follicle stage, oviduct stage and after laying stage). For this method first the protein solution was reduced and ligated with SDS by heating the protein samples in a water bath for 5 minutes along with equal volume of sample buffer (0.125 M Tris-HCl, pH 6, 4% SDS, 10% β-mercaptoethanol, 20% sucrose and 0.04% bromphenol blue). The resulting solution contained SDS-bound polypeptides in open form and this was applied on to the casted gel lane. Approximate molecular weight of the separated polypeptides were to be determined by this method as Sigma molecular weight marker having known molecular weight polypeptides which were loaded in the extreme left hand side of the gels. For performing SDS-PAGE two stage gel
system was employed. First for separating the resolving gel was casted containing 15% total acrylamide concentration in the separating gel buffer containing SDS (1.5M Tris-HCl, pH 8.8 and 0.1% w/v SDS). Above the separating gel stacking gel was laid in stacking gel buffer (0.5M Tris-HCl, pH 6.8, 0.1% w/v SDS). In this gel the total acrylamide concentration was 4%. The electrophoresis was performed with electrode buffer containing 0.025M Tris, 0.192 M glycine, pH 8.3 and 0.1% w/v SDS. The electrophoresis was conducted at 16 mA constant current for 2 hours. When the tracking dye reached the bottom electrophoresis was stopped. The separated gel was immersed in the staining solution (0.1% coomassie brilliant blue R-250, 41.7% methanol and 16.7% acetic acid in aqueous solution) for overnight and then destained by destainer (Methanol : Acetic acid : Water; 30 : 10 : 60). After complete destaining the Rm values of the known molecular weight markers and the analysed protein sample was calculated using the formula, distance travelled by the protein band divided by the distance travelled by the tracking dye. The Rm values of the known molecular weight proteins/polypeptides were plotted against their log molecular weight in a graph paper. This plotted straight line was used as the standard curve for estimating the molecular mass of the unknown polypeptides by using their Rm values. Using this standard curve the molecular mass of the polypeptide components of the different protein samples or the peptide subunits was determined. Photographs were taken with SONY DSC-W130 camera in transilluminator (Biotech).
2.3.iii. Atomic Absorption Spectroscopy

For Atomic Absorption Spectroscopy eggshells or chorions were cut with sharp blade and washed with brush in de-ionized distilled water to remove the yolk materials. The insoluble chorions were further washed in 95% and 100% ethanol followed by repetitive washes in de-ionized distilled water to remove the aldehyde contents. After air drying 0.5g chorion sample were treated with few drops of hydrogen peroxide, kept overnight and evaporated to dryness. After that 0.5ml of perchloric acid was added and heated, this process was repeated for 2-3times. After drying, 2-3ml of HCl was added to the sample and the solution was diluted with de-ionized distilled water (Dey et al., 2003). The analysis was carried out in Atomic Absorption Spectrophotometer, Perkin-Elmer, A Analyst 200. After that necessary calculations were made manually by computer.

2.3.iv. Spectroflurimetry

Chorions of *Oxya* and *Gesonula* were solubilized with 400mM Tris-HCl, pH 8.4, 4% β-mercaptoethanol, 6M Urea soluble in boiling water bath. In this process SDS was omitted because of its denaturing nature which may effect the spectroscopic observations. Soluble samples were diluted 100x with deionised distilled water and scanned at 280nm and 295nm for the abundance of protein (Tyrosine and Tryptophan) and Tryptophan. Millipore water served as a blank. Excitation and emission slit widths were maintained at 5nm in a Hitachi F4500 spectroflurimeter. The instrument was attached to a constant
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Temperature water bath (Poly science, USA) set at 25 ±0.5°C. A 3ml quartz cuvette was used for sample analysis.

2.3.v. Size exclusion HPLC

Oxya and Gesonula chorion solutions were made in 400mM Tris-HCl, pH 8.4, 4% β-mercaptoethanol, 6M Urea in boiling water bath. Oxya and Gesonula chorion solutions were diluted to 10x with deionized distilled water and applied for the detection of Mw using Waters Size-Exclusion (SE-) HPLC column (Protein - Pak 125, 78 x 300 mm, fractionation range, 5-80 kDa). The column was equilibrated with 20mM Na-phosphate, pH 7.5 containing 0.5 M NaCl at a flow rate of 1ml/min. Elution of the samples were followed both at 280 and 220 nm to include both proteins and peptides, if any, and the chromatograms were developed isocratically with the equilibrating solvent. A blank was run with 10 µl of water which served as a control where no spurious peak appeared. Test samples, either the protein (50 µl) or the reference Molecular weight markers were applied to the column. A calibration curve correlating log Molecular weight and elution volume (or elution time) of the marker proteins showed a linear dependency from where the apparent Mw of the unknown proteins were determined.
2.3.vi. Mass spectroscopy

In-gel digestion:

The major protein bands 55kDa from coomassie stained -gels were selected and marked a & b alphabetically. These bands were excised manually using a clean scalpel blade, sliced into 1 mm cube, placed in a 2 ml eppendorf tube and dehydrated in acetonitrile, which was then removed and further dried in vacuum(Shevchenko et al. 1996). 0.05 ml, 20mM DTT in 100mM NH₄HCO₃ was added to cover the gel pieces and the protein was reduced for 1 h at 60 °C. After cooling to room temperature, DTT solution was replaced by same volume of 55mMIodoacetamide (IAA) in100mM NH₄HCO₃ and incubated for 45 min at room temperature in dark. The IAA solution was removed and the gel pieces were rinsed thrice with 100mM NH₄HCO₃ and acetonitrile successively using vortex. The liquid phase was removed and gel pieces were completely dried in vacuum. The gel pieces were swollen in 0.025 ml digestion buffer containing 100mM NH₄HCO₃, 5mM CaCl₂ and 1μg of trypsin gold in ice bath for 1 h; after which another 0.025ml of digestion buffer was added and incubated overnight at 37 °C. Peptides were extracted with three changes of 50% acetonitrile/0.1% TFA which was then dried or concentrated using vacuum centrifugation.

Maldi-Tof Analysis:

All analysis was performed using a 4800 MALDI TOF/TOF (Applied Biosystems) operated in reflectron mode. Peptide mixture was desalted using C18 zip tip and analysed using a saturated solution of CHCA (α-cyano-4-hydroxycinnamic acid) in 50% acetonitrile/0.1% trifluoroacetic acid. The MS/MS
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The peak of the most intense tryptic peptide mass ion peak were searched against MSDB database of all sequences using Mascot (Matrix Science, Ltd., London, United Kingdom; http://www.matrixscience.com) search program with fixed and variable modifications; Carbamidomethyl (C) and Oxidation (M) respectively.

2.3.vii. Computational analysis of protein structure

In the present study Homology Modelling was made to build the three-dimensional protein structure from protein sequence. Homology modelling is currently the most accurate computational method to generate reliable structural models and is routinely used in many biological applications. Using experimentally determined structures of related protein sequence as template the three-dimensional protein structure was generated. SWISS-MODEL workspace, an integrated web-based modelling system, was used for this purpose.

The objective of the present piece of work was to elucidate the formation and characterization of the different components of chorion of Gesonula punctifrons and Oxya hyla hyla and all the various analyses were made to elucidate those objectives. In that way light microscopy, electron microscopy and all other methods including SDS-PAGE revealed that the eggshell was composed of different protein components. In that way MALDI analysis of a predominant protein band was undertaken to elucidate the mass and amino acid composition of that specific protein band belonging to the chorions of grasshoppers. Such analysis had already been undertaken by some workers earlier for other insect groups. The MALDI data generated the mass of different
fragments and on that basis the computerized version of MALDI predicted the probable sequence using of peptides by built in Mascot software using MSDB. The results predicted the probable source of protein which had maximum similarity with NCBI Accession Number. On the basis of that following the NCBI accession number the sequence and function of the whole protein was predicted. Such method had already been adopted by Li and Li (2006) for analysis of the chorion protein structure of *Aedes aegypti*. 