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Ultrastructure, Solubilization and Protein Composition of Eggshell (Chorion) of *Gesonula punctifrons* (Stal, 1861) (Orthoptera: Acrididae)

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ABSTRACT

Developmental changes in ultrastructure and polypeptide composition of chorion of *Gesonula punctifrons* (Orthoptera: Acrididae) have been studied by Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE). Along with morphogenesis and maturation of chorion pentagonal and hexagonal architecture and 'cap' like structure appeared in the surface of the eggshell. Transmission Electron Microscopic studies revealed presence of protein fiber deposition and their intercalation with polysaccharides. SDS-PAGE studies showed presence of five polypeptide bands in all the developmental stages and suggested possibilities of aggregate formation among different polypeptide components.

Key words: *Gesonula punctifons,* eggshell, SEM, TEM, SDS-PAGE.

INTRODUCTION

In insects eggshell or chorion covers the mature oocyte and is composed of proteinaceous materials that are synthesized and secreted by the follicular epithelium at the end of vitellogenesis (Margaritis, 1985). Numerous studies have described the variation in chorionic architecture of insects for its use as a taxonomic tool (Hinton, 1981). Earlier light microscopic studies on orthoptera chorion were done by Slifer (1937), Roonwal (1936), Hartley (1961) and Katiyar (1960) undertook a light microscopic study of eggshell surfaces of 10 Indian acridids. But Hinton and Service (1969) pointed out that photographs of eggshell surface taken by light microscope were not sufficient to give proper impression of surface sculpture and therefore use of Scanning Electron Microscope was essential. In these respect few species of Acrididae has been studied so far. Ganguly *et al*. (2008) for the first time studied the surface sculpturing of two Indian acridids using Scanning Electron Microscope. Shyam Roy and Ghosh (2010), described the surface morphology of chorion of *Oxya hyla hyla* with developmental changes. But the morphogenesis of the surface structure of Orthopteran eggshell is absent while that has been well studied in case of muga silkworm *Antheraea assama* (Dey *et al*. 2003). Therefore study of morphogenesis of surface sculpturing

of eggshell of acridids remained a defined field of enquiry. Apart from that the protein components of the chorion layer, their synthesis including genetic regulation of that process have been investigated for a number of insects including *Drosophila*, silkmoth *A. polyphemous* and *Aedes* (Gelinas and Kafatos, 1973; Margaritis *et al*., 1980; Li and Li, 2006). In this respect one dimensional and two dimensional Poly Acrylamide gel electrophoresis have produced significant information. Multiple protein have been found in the eggshell of different insects 22 in *Drosophila*, 182 in silkmoth, 3 in *Aedes* and 2 in *Rhodnius* (Fakhouri *et al*., 2006; Regier *et al*., 1980; Li and Li, 2006; Bouts *et al*., 2007). In this regard no work has yet been done with Orthopteran eggshell while only Furneaux (1970) studied the amino acid composition of eggshell of house cricket *Acheta* (Orthoptera). In this premise here we describe the surface architecture and its morphogenesis in chorion of *Gesonula punctifrons* (Acrididae) and its protein components as revealed by Scanning Electron Microscopy, Transmission Electron Microscopy and Electrophoretic studies.

MATERIALS AND METHODS

Tissue preparation:

G. punctifrons (paddy grasshopper) were collected from the paddy fields in and around Agartala city (Latitude 23° 50' N and Longitude 91° 25' E). Mature eggs were dissected out and collected from the mature ovarian follicle and oviduct and after laying eggs were collected just after laying of the eggs before pod formation. These eggs were cleaned with brush in 100mM Tris-HCl (pH-8).

Scanning Electron Microscopy (SEM):

For SEM studies the eggs were fixed in Karnovsky's fixative (16% Paraformaldehyde Solution, 50% Glutaraldehyde EM Grade, 0.2M Sodium Phosphate Buffer, Distilled Water) for four hours and thoroughly washed in 100mM phosphate buffer (pH 7.2) with three changes. Dehydration was carried out in graded acetone (30, 50, 70, 80, 90, 95 and 100%) all steps were done for 15 minutes and 2 changes in each at 4°C (Mazzini and Gaino, 1985). The acetone dehydrated eggs were subjected to Tetra Methyl Silane for drying. Then the eggs were mounted on copper stubs. The coating was done by Gold-Palladium in coat ion sputter JFC-1100 and the coating was 35nm thick. Finally the eggs were observed under a JEOL JSM 6360 Scanning Electron Microscope and photographs were taken.

Transmission Electron Microscopy (TEM):

For TEM eggs were fixed in 4% glutareldehyde for four hours then washed in 100mM 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 by ultramicrotome of RMC. Sections were stained with uranyl acetate and led citrate (Ma *et al*. 2002) and examined by JEOL 2100 Transmission Electron Microscope.

Solubilization:

For solubilization of the chorion ripe eggs collected from follicle, oviduct and after laying were cut by a sharp blade for removing of yolk materials. Then those were thoroughly washed in 100mM Tris-HCl (pH 8) containing 1% SDS.

The collected empty eggshells were treated with different solubilizing solutions developed and used by earlier researchers (presented in Table. 1) but failed to produce satisfactory result with *Gesonula* chorion. Finally the chorion was solubilized in a solution developed by the authors (400mM Tris-HCl, pH 8.4, 4% β-mercaptoethanol, 6M Urea, 1% SDS). That solution produced satisfactory result and after centrifugation at 5,000 rpm for 5 minutes the resulting supernatant was used for electrophoretic analysis (Harris and Angal, 1989). SDS-PAGE analysis of the resulting supernatant was made at 15% acrylamide concentration. The 15% SDS-PAGE was done by following the method developed by Laemmli (1970) with some modifications.

Table. 1. Different methods followed for chorion dissolution of different insect.

Chorion of Insect	Earlier Researchers	Solubilizing Buffer with working temperature and pH
Gryllus mitratus	Kawasaki *et al*. 1971	8M Urea, 10mM DTT, 30mM EDTA, 0.2M Tris-HCl, pH 8.6 at 20^0 C.
Silkmoth	Regier *et al*. 1980	8M Urea, 0.36M Tris-HCl and 0.03M DTT to a concentration of 7mg/ml, pH 8.4 at 20^0C.
Aedes aegypti	Li *et al*. 2005	20 mM Sodium Phosphate, 1% CHAPS, 2M Urea, 0.15M KCl, 2mM PMSF and 2mM EDTA-Na_2, pH 7 at 20^0C.
Drosophila sp.	Fakhouri *et al*. 2006	8M Urea, 5% CHAPS, 40mM Tris-base, protease inhibitor at 20^0C.
Rhodnius prolixus	Bouts *et al*. 2007	8M Urea, 0.36 M Tris-HCl 0.03M DTT and 0.1 M PMSF, pH 8.4 at 20^0C.

RESULT AND DISCUSSION

Egg morphology:

Ultrastructural evaluation of the follicle stage egg revealed that the chorion had some small spicule-like (S) structures and some circular foldings (CF) (Fig. 1.). In these stage 'cap' like structure was not present. This feature was different with the findings of *O. hyla hyla* (Shyam Roy and Ghosh, 2010) where 'cap' started to form from the follicle stage. In the oviduct stage of chorion maturation, the surface area of the chorion was plain without any sculpture (Fig. 2d). but the 'cap' like architecture was present in this stage that was less developed (Fig. 2b.). In this stage the anterior pole had some peculiar structure with pentagonal architecture (PA) which covered the whole end surface of the pole (Fig. 2c.). Some longitudinal ridges (R) joined the two poles of the egg (Fig. 2a.). The egg surface of *G. punctifrons* showed a sculptured structure in chorion of after laying stage. The ultrastructural evaluation by SEM showed that the whole surface was covered by pentagonal (PA) and hexagonal architecture (HA) (Fig. 3). These structures started from the anterior pole and runs towards the middle area (Fig. 3a-c). Numerous fold like structures (N) were found within the area of the pentagonal and hexagonal architectures and might be that these structures were deposited with time (Fig. 3d.). These architectures were specific for after laying

stage only. Ganguly *et al*. (2008) have presented similar findings in two species of Indian acridids. An exceptional 'cap' like structure (C) has been seen to be present at the posterior pole of the egg (Fig. 3b.). Such 'cap' has also been identified in *O. hyla hyla* at the same pole (Shyam Roy and Ghosh, 2010). Yu and Crities (1988) have also shown presence of same 'cap' like structure in Nematoda eggshell and suggested that the structure was responsible for fertilization in that pole. The complexity of the surface structure and the 'cap' increased with time of maturity of chorion (Fig. 1b., 2b., 3b). From these observations it can be concluded that the complexity of the structure increased with maturity of the chorion and therefore degree of complexity from follicle cell stage to after laying stage increased gradually.

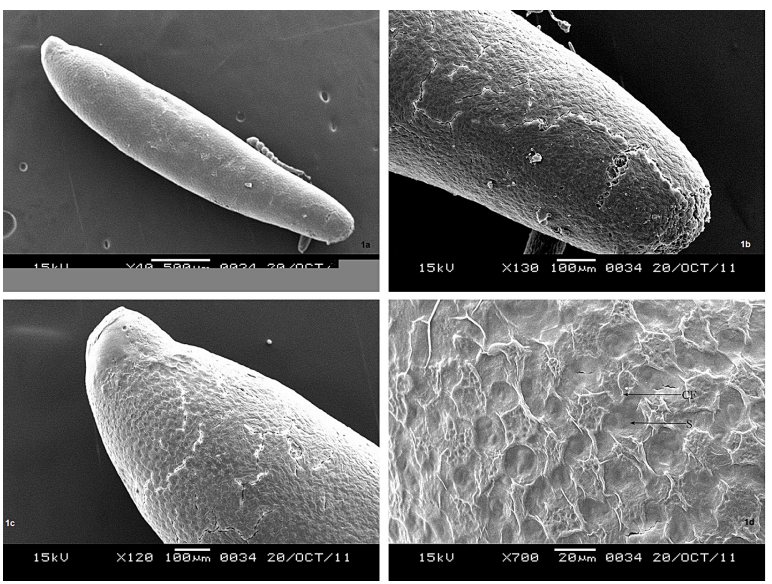

Fig. 1. Follicle stage of chorion. Circular foldings (CF), spicules (S). 1a. Whole zone, 1b. Posterior pole, 1c. Anterior pole, 1d. Middle zone

TEM study of chorion:

TEM studies of the developing chorion in various stages were made. In the follicle stage, egg chorion has five distinct layers were visualized (Fig. 4.), the vitelline membrane (VM, ca. 0.09µm), innermost chorionic layer (ICL, ca. 0.09µm), air layer (AL, ca. 0.12µm) and the outer chorionic layer (C, ca. 1.18µm-2.35µm). Outer side of the chorionic layer was covered by follicle cell layer (F, ca. 3.3 µm-3.65 µm). In this stage chorion was partly formed and remained attached with the follicle cells. The air layer has shown to be present in *Drosophila* (King *et al*., 1956) and in *Lygus* (Ma *et al*., 2002). In the present study, wax layer was absent (Irles and Piulachs, 2011).

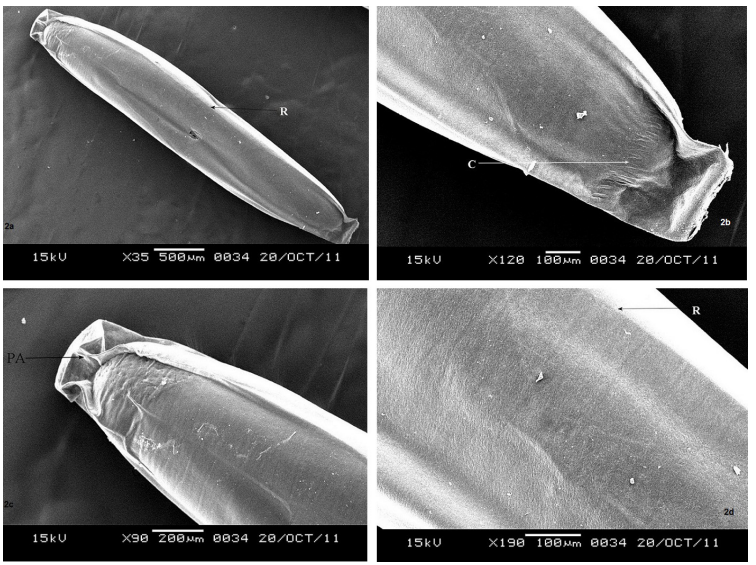

Fig. 2. Oviduct stage of chorion. 'cap' like structure (C), longitudinal ridges (R), pentagonal architecture (PA). 2a. Whole egg, 2b. Posterior pole, 2c. Anterior pole, 2d. Middle zone

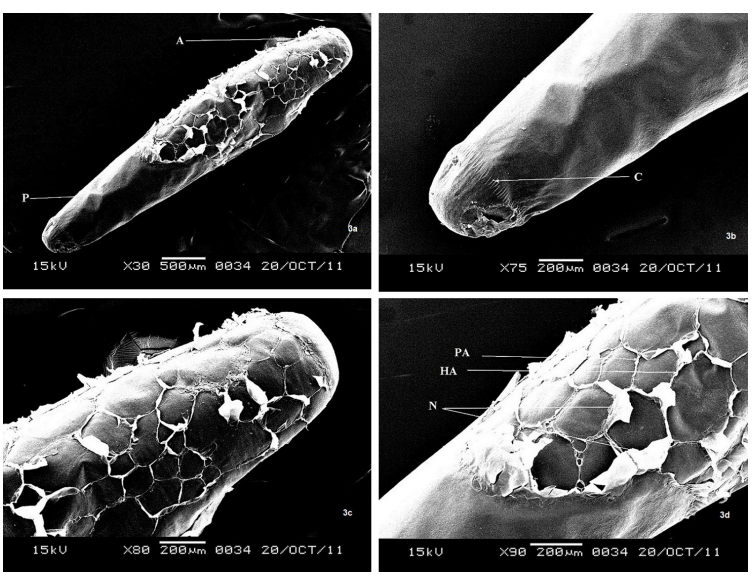

Fig. 3. After laying stage of chorion. Anterior pole (A), posterior pole (P),'cap' like structure (C), pentagonal architecture (PA), hexagonal architecture (HA), numerous fold like structures (N). 3a. Whole egg, 3b. Posterior pole, 3c. Anterior pole, 3d. Middle zone.

In the oviduct stage egg, four layers were present, the vitelline membrane (VM, ca. 0.03 µm), innermost chorionic layer (ICL, ca. 0.05 µm), air layer (AL, ca. 0.08µm) and the outer chorionic layer (C, ca. 0.76µm) (Fig. 5.). The outer chorionic layer was detached from follicle cell layer and became more developed and rigid with the presence of thread like structure of protein deposition that were about 0.03µm-0.05 µm thick and 0.39µm-1.05µm long. Droplets of polysaccharides (p) were also visible. As the chorionic structure was protein in nature, the post translational modifications were going which results into the formation of the rigid and tightly bound thread like structures in the chorionic layer. In the after laying stage four layers of chorion were present, the vitelline membrane (VM, ca. 0.21 µm), innermost chorionic layer (ICL, ca. 0.14 µm), air layer (AL, ca. 0.07µm) and the outer chorionic layer (C, ca. 2.5µm) (Fig. 6). The modification of the thread like structures of proteins (t) had occurred and that might be due to intercalation with droplets of polysaccharides (p). Ma *et al.* (2002) has shown presence of such phenomena in development of *Lygus* chorion. The dimensions of these proteinaceous structures were about 0.71µm- 1.07µm long and 0.03µm-0.05 µm wide. The space between these protein fibers were also observed.

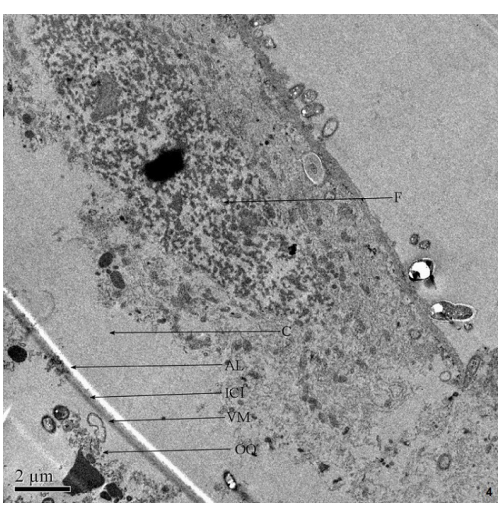

Fig. 4. Follicle stage of chorion with five layers of chorion. Vitelline membrane (vm or VM), inter chorionic layer (icl or ICL), air layer (al or AL), outer chorionic layer (ch or C), follicle cell layer (f or F), and the oocyte (oo or OO). Scale bar=2µm.

Protein profile:

The follicle stage egg chorion and the oviduct stage chorion was dissolved completely by the solution as designed (materials and methods) but the matured chorion remained partially undissolved. This experiment revealed several polypeptide bands in all the three stages of maturation. The electrophoresis pattern of molecular weight markers and the proteins of solubilized chorions of three stages are presented in Fig. 7. The molecular weights of polypeptides in three developmental stages

showed a wide range of variation. In the follicle stage chorion 17 polypeptides, in the oviduct stage chorion 6 polypeptides and in the after laying stage 6 polypeptides have been revealed (Table. 1).The molecular weights of the follicle stage chorion ranged from 18.2 kDa to 97.7kDa. In the oviduct and after laying stage chorion the range was between 18.2kDa to 50kDa. From all these polypeptides 5 polypeptides were commonly available in all the three stages and their molecular weights are 18.2 kDa, 31.6 kDa, 34.7kDa, 41.7kDa and 50 kDa respectively (Table. 2.). From these observations it appeared that during morphogenesis protein aggregation occurred and the final structure became more complex which has also been seen by SEM and TEM studies. The process led to insoluble proteinaceous structure and thus the number of polypeptide bands differed in every stages of maturation. Although similar studies have been made by earlier researchers in insects of different orders by one dimensional and two dimensional poly Acrylamide gel electrophoresis, this is the first report on Orthopteran eggshell. Murugan and Prasanth Jacob (1988) studied protein composition of haemolymph of *G*. *punctifrons*. Furneaux (1970) studied the amino acid composition of eggshell of house cricket *Acheta* (Orthoptera). In *Drosophila* 22, in silkmoth 182, in *Aedes* 3 and in *Rhodnius* 2 protein components have been shown to present in eggshell (Fakhouri *et al*., 2006; Regier *et al*., 1980; Li and Li, 2006; Bouts *et al*., 2007). Earlier in Orthoptera Mazzini (1978) studied the chorion of *Tettigonia viridissima* by SEM and the amino acid composition of the eggshell. Thus from the results it may be inferred that in Orthoptera, specially in grasshopper, there is a wide diversity of polypeptides and the final structure emerged as a result of aggregation or bonding of several polypeptides. This investigation pursue information about the timming of the assembly of the eggshell of the matured ovariole and its hardening by relating the presence of proteins and will able to correlate the synthesis of specific proteins with chorionic ultrasturucture of *G. punctifrons*.

Table. 2. Molecular weight and Rm values of polypeptides of eggshells of *Gesonula punctifrons* in different stages of growth.

Rm values			logMW			Molecular weight		
Follicle stage	**Oviduct stage**	**After laying stage**	**Follicle stage**	**Oviduct stage**	**After laying stage**	**Follicle stage**	**Oviduct stage**	**After laying stage**
0.1	-	-	4.99	-	-	97.7 KD	-	-
0.17	-	-	4.94	-	-	87.1 KD	-	-
0.18	-	-	4.92	-	-	83.2 KD	-	-
0.19	-	-	4.91	-	-	81.3 KD	-	-
0.20	-	-	4.90	-	-	79.4 KD	-	-
0.21	-	-	4.89	-	-	77.6 KD	-	-
0.22	-	-	4.88	-	-	75.9KD	-	-
0.25	-	-	4.86	-	-	72.4KD	-	-
0.28	-	-	4.82	-	-	66.1KD	-	-
0.37	-	-	4.74	-	-	55KD	-	-
0.39	-	-	4.72	-	-	52.5KD	-	-
0.42	0.42	0.42	4.7	4.7	4.7	50KD	50KD	50KD *
-	-	0.44	-	-	4.68	-	-	47.9KD
0.53	0.53	0.53	4.62	4.62	4.62	41.7KD	41.7KD	41.7KD*
0.56	0.56	-	4.56	4.56	-	36.3KD	36.3KD	-
0.61	0.61	0.61	4.54	4.54	4.54	34.7KD	34.7KD	34.7KD*
0.64	0.64	0.64	4.50	4.50	4.50	31.6KD	31.6KD	31.6KD*
0.94	0.94	0.94	4.26	4.26	4.26	18.2KD	18.2KD	18.2KD*

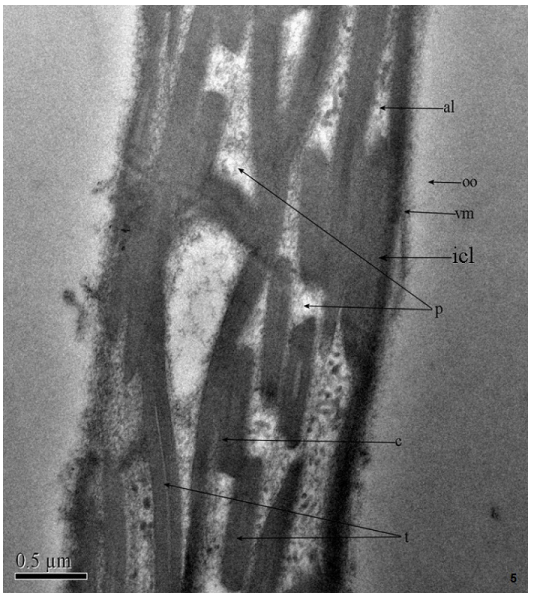

Fig. 5. Oviduct stage of chorion maturation. Outer chorionic layer (ch or C), vitelline membrane (vm or VM), inter chorionic layer (icl or ICL), Air layer (al or AL), oocyte (oo or OO), thread like structure (t), polysaccharide droplets (p) . Scale bar= 0.5µm.

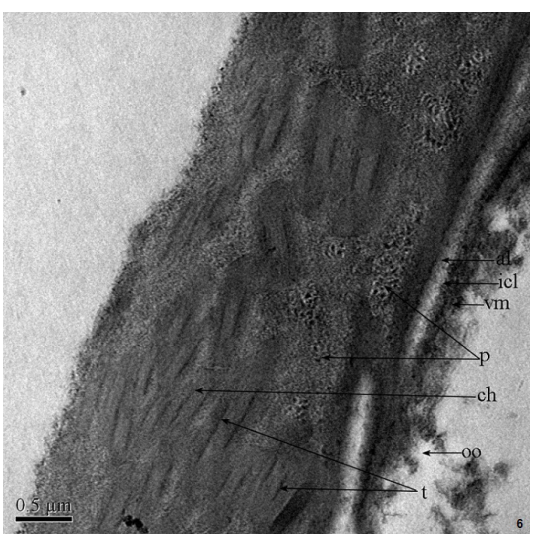

Fig. 6. After laying stage of chorion maturation. Outer chorionic layer (ch or C), inter chorionic layer (icl or ICL), vitelline membrane (vm or VM), Air layer (al or AL), oocyte (oo or OO), thread like structures (t), polysaccharide droplets (p). Scale bar= 0.5µm.

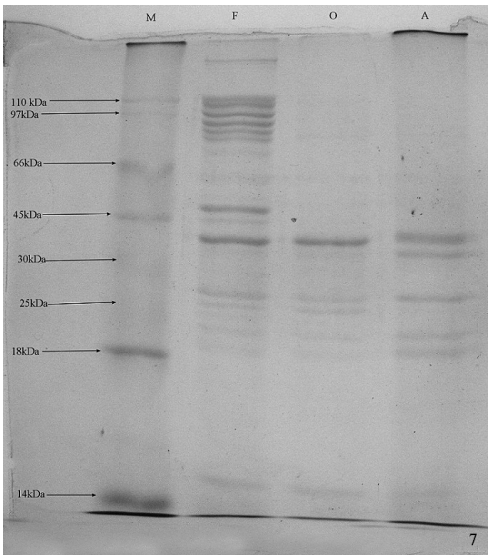

Fig. 7. SDS-PAGE Electrophorogram of solubilized chorion proteins of three different stages of chorion (F-follicle stage, O-oviduct stage, A-After laying stage) of *Gesonula punctifrons*. M- Marker proteins.

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