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Purification and Partial Characterization of Vitellin of *Oxya hyla hyla* Serville, 1831 (Orthoptera: Acrididae)

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ABSTRACT

Insect female specific sex limited protein vitellin has been identified and purified from the mature oocyte of *Oxya hyla hyla* (Orthoptera: Acrididae), a paddy field grasshopper of Tripura. The homogeneity of the DEAE- cellulose column purified protein has been tested by electrophoresis and HPLC studies. HPLC and fluorescent spectroscopy studies have revealed that the protein is quite stable and could tolerate treatment with 6M Urea. SDS-PAGE analysis has shown that it is composed of seven subunits and the molecular mass of the native protein is 459KDa. Further fluorescent spectroscopic studies have shown that the protein is rich in tryptophan while presence of tyrosine is little or nil.

Key words: *Oxya hyla hyla,* vitellin, purification, HPLC, fluorescent spectra.

INTRODUCTION

In most oviparous animals, oogenesis is characterized by an accumulation of abundant quantity of yolk proteins in the growing oocyte which serves the nutritive requirements of the growing embryo. In most of the animals the major yolk protein is Vitellin (Kim *et al*., 1988). Vitellin is a high molecular mass lipoglycoprotein generally composed of multiple subunits of unequal size and constitute more than 50% of the buffer soluble proteins in the egg (Lensky and Skolnik, 1980). In the oocytes of most insects this yolk protein deposition is a process of receptor mediated endocytosis which requires both maternal donation of serum vitellogenin and the competence of the oocyte to receptor mediated endocytosis (Raikhel, 1986).Several criteria have been attributed to define vitellins. They are usually considered to be sex limited proteins present in haemolymph and oocyte of ovogenic female of a species (Hagedorn and Kunkel, 1979). Vitellins of eight different insect orders was first purified and basis of on the subunit composition had been divided in three groups (Harnish and White, 1982). Among Orthoptera *Locusta migratoria* received wide attention and their vitellogenesis and vitellin had been studied in great details (Gellisen *et al*., 1976; Chinzei *et al*., 1981). Properties of *Locusta* vitellin including its solubility and isoelectric pH has also been reported. It was suggested by various workers that internal cleavage of vitellogenin

polypeptide chain occurred in the oocyte to produce vitellin (Shinoda *et al*., 1996) In recent years differential uptake of yolk protein components and their deposition in the different zones of Locust ovariole has also been demonstrated (Peel and Akam, 2007). But still considerable gap of knowledge exists regarding the biochemical and biophysical properties of vitellin of any Indian Insect species in general and Orthoptera in particular. In view of that the present study reports purification and partial characterization of vitellin of an Indian grasshopper *Oxya hyla hyla* (Acrididae) which is a commonly a paddy pest.

MATERIAL AND METHODS

Insect source and rearing condition

Both mature and immature male and female *Oxya hyla hyla* (more than 100) were collected from the paddy fields adjacent to the Agartala city. This population was maintained on paddy leaves at optimal rearing condition 26 ^{0}C and RH 62-70%. Water was provided *ad libitum*. Regular washing of the cages were done. The ripe oocytes were collected from the mature females and haemolymph of both male and vitellogenic female were collected by cutting the thoracic legs.

Chemicals and Equipments

All chemicals, protease inhibitor and DEAE- cellulose were obtained from Sisco Research Laboratories and other fine chemicals were obtained from Sigma. Plastocraft cold centrifuge, Chemito UV- 2600, Hitachi F 4500, Water's HPLC and BioRad Fraction Collector were used for spectrophotometric, spectro flurometric and chromatographic purposes. All the glass goods including the chromatographic columns were made of Borosil.

Preparation of oocyte protein

Terminal oocytes taken from the rest of the ovary were homogenized in a mortar and pestle in 50 mM Tris – HCl buffer (pH-7.2) containing 0.2M NaCl and 1µm PMSF. The homogenate was centrifuged at 10,000 RPM at 8 ^{0} C for 20 minutes. The filtered supernatant was used as a reference material for purification and other studies.

Collection of heamolymph

The haemolymph oozing out of the cut coxa of the hind legs of both male and female *Oxya* were collected by capillary tube. The haemolymph mixed with equal volume of 50mM Tris–HCl buffer (pH- 7.2) containing 0.2% Triton X 100 was centrifuged at 10,000 RPM at 8^{0}C for 20 minutes at 8^{0}C. The cell free supernatant was collected that served as the source of male and female plasma proteins.

Native PAGE and SDS- PAGE

Native PAGE analysis was performed using a vertical slab gel electrophoresis system at 5% acrylamide concentration (Davis, 1964). The protein sample was added

with 0.2 % Triton X-100 for solubilizing that in absence of NaCl within the separating gel. Sodium dodecyl sulphate (SDS)–PAGE was performed to determine the subunit composition of purified protein at 10% acrylamide concentration in reduced condition. Before electrophoresis the protein sample including the marker were reduced at100^{0}C for 5 minutes in sample buffer containing marcaptoethanol and SDS (Weber and Osborn, 1969). Molecular weight markers 30-250 KDa were taken as standard for comparing the relative mobility and log molecular weight of proteins. Comassie brilliant blue R-250 staining was performed.

Identification of vitellin and its purification

For identification of vitellin, male plasma, vitellogenic female plasma and oocyte extract were parallelly electrophoresed in native electrophoretic system. Vitellin being female specific, the parallel bands present only in the female heamolymph and oocyte were identified as vitellin and taken as reference for other studies. For purification of vitellin supernatant of the egg homogenate was dialyzed against 50mM Tris – HCl buffer (pH 7.2) for 24 hrs at 8 ^{0} C. The precipitate obtained in the dialysis bag was centrifuged and pellet was resuspended in 50mM Tris – HCl buffer (pH 7.5) containing 0.2M NaCl which produced a clear solution. This solution was applied on a DEAE – cellulose column equilibrated by Tris HCl (50 mM, pH 8.1). The column was washed with the same buffer till the unbound part completely came out of the column as evidenced by very low absorbance at 80nm. The bound protein was eluted with a linear 0-0.5 M NaCl linear gradient containing buffer and 3ml fractions were collected. Absorbance of the fractions were taken at 280nm and plotted. The peak fractions were pooled and rechromatographed with the same column material using same buffer and gradient. Purity of the peak fractions were monitored by polyacrylamide gel electrophoresis (native PAGE).

HPLC

Purity and molecular weight of vitellin was determined with Water's protein pack 300 size exclusion (SE) - HPLC column. The column was equilibrated with 0.01M $NaPO_4$buffer, pH-7.0 containing 0.5M NaCl at a flow rate of 1.0ml/minute. The elution profile was followed at 280nm. The column was calibrated with the molecular weight markers: Blue Dextran; BSA (66.4 KDa); Myoglobin (18.8 KDa,) and Human Insulin (7.0KDa,). The calibration relating log molecular weight verses retention time showed linear dependency (R^2= 0.987). All samples were passed through Spartan (0.22µm) filters before application to HPLC. For analyzing the conformational stability, the purified molecule was equilibrated in 8M urea solution for 10 minutes and again passed through the column to judge the stability of the molecule.

Fluorescence Spectroscopy

0.1mg/ml of the protein solution in 20mM Na-PO4, pH 7.5 was used for studying emission spectrum from 300 to 500nm after excitation at 280nm (Tyrosine and Tryptophan) or 295nm (Tryptophan only) wave length. Excitation and emission slit widths were maintained at 5nm in a Hitachi F4500 spectroflurimeter. The instrument

was attached to a constant temperature water bath (Poly science, USA) set at 25±0.5^{0}C. A 3ml quartz cuvette was used.

RESULTS AND DISCUSSION

Identification of vitellin by native-PAGE analysis

Male hemolymph, female haemolymph and mature oocytes extracted in 50mM Tris-HCl (pH 7.2), 0.1M NaCl and 0.2% Triton X- 100 was analyzed parallely by native-PAGE. The comparison of the protein bands present in the male haemolymph, female haemolymph and oocyte extract revealed that in male haemolymph six major bands and in the female haemolymph seven major bands were present. In ripe oocyte only one band was present which had similar Rm with a band of female haemolymph. This clear distinct band was present in vitellogenic female haemolymph and oocyte extract but absent in male haemolymph. These bands (Rm= 0.3) indicated, female specific protein bands and of vitellogenin and vitellin (Fig.1). In number of insect species including Hemiptera, Lepidoptera and Orthopteran, such results have been obtained (Chen *et al*.,1978; Shinoda *et al*., 1996). Hagedorn and Kunkel (1979) have already discussed such identical Rm values of vitellogenin and vitellin. Apart from that their isoelectric points and immuno reactivity have also been reported to be identical.

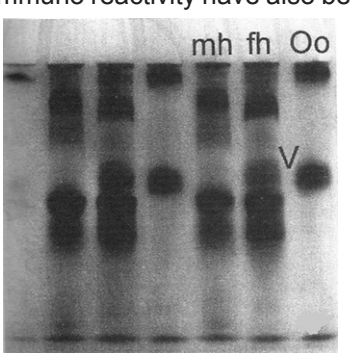

Fig. 1. Electrophorogram of male haemolymph (mh), female haemoly,ph (fh), and oocyte (Oo) extract. Female haemolymph and oocyte extract shows a clear band of vitellin (V).

Purification and characterization of vitellin

Ripe oocytes were homogenized in 50mM Tris-HCl, pH 7.5 containing 0.2M NaCl and 0.1M PMSF. The homogenate was centrifuged at 10,000 RPM for 15minutes at 4^0 C. The supernatant was taken and dialyzed against 50mM Tris-HCl, pH 7.5.When turbidity appeared the solution was again centrifuged and the pellet was taken. That pellet was solubilized in same buffer and applied on a DEAE- cellulose anion exchange column pre-equilibrated in the same buffer. The column was eluted with 50mM Tris-HCl, pH 7.5 at a flow rate of 20ml/ hour and each 2ml fraction was collected. The optical density of each fraction was monitored at 280nm (the λmax of protein). First fifty fractions eluted most of the unbound proteins and amount of

protein present in the fractions were very low (Fig. 2). When unbound protein was completely removed the whole setup was connected with a gradient maker with two tanks one containing the 50mM Tris-HCl, pH 7.2 and other containing Tris-HCl buffer having 0.5M NaCl. The linear gradient elution process was continued for about five hours and after that the protein content of each fraction was measured by studying the absorbance at 280nm (Fig. 2). Hundred fractions were collected and it was observed that fraction eighty five to hundred contained high amount of protein and produced a sharp peak. These fractions were pooled and lyophilized. The lyophilized and concentrated fractions were tested for presence of vitellin by native-PAGE analysis. The Rm values of the purified peaks and previously identified vitellin were compared. It was observed by native PAGE that the lyophilized fraction had the same Rm value of the pre-identified vitellin (Fig. 3). From this finding it became apparent that was pure vitellin. From construction of the elution profile it was clear that this vitellin part was eluted at gradient 0.25M NaCl concentration. Previously in *Locusta migratoria* such elution of vitellin has been observed to be done at 0.25M KCl and in case of Hemiptera this elution took place at 0.25-0.35 M NaCl gradient (Shinoda *et al*., 1996). So it appeared that molecular composition of *Oxya* vitellin had similarity with molecular properties with those vitellins. Finally that was concentrated to 1mg/ml and was used for native-PAGE, HPLC and SDS-PAGE analysis for studying purity of vitellin and its subunit composition. The native-PAGE result is shown in Fig. 3. which indicated that vitellin was in electrophoretically pure state. For judging the purity of that fraction it was solubilized in the same buffer containing 0.5M NaCl and was injected in Water's protein Pac 300-SE column. It was observed that the protein solution repeatedly gave a single peak with a minor hump (Fig. 4). It indicated that the protein was in pure state and the minor hump perhaps was due to some kind of association. The retention time was 5.65 minutes. The sharp peak indicated high state purity of vitellin and also suggested of very high molecular weight. In case of cockroach (Imboden *et al*., 1987) similar kind of observation have been reported. So the results clearly suggested that vitellin was in state of purity and was suitable for further molecular analysis.

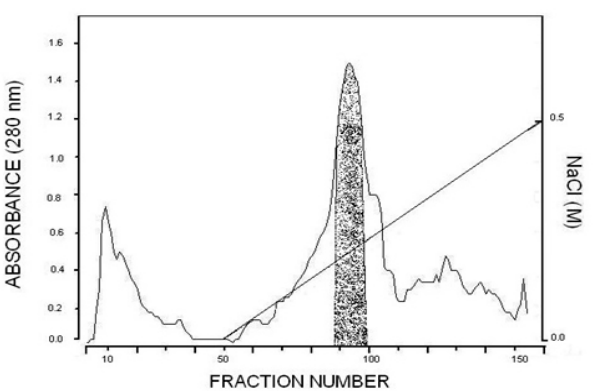

Fig. 2. DEAE-Cellulose ion exchange chromatography profile of ripe oocyte extract.

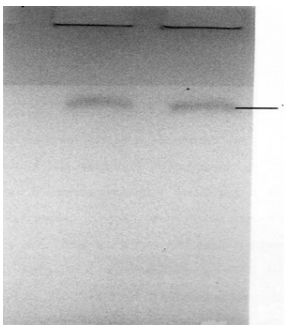

Fig. 3. Profile of Native- PAGE analysis of purified vitellin.

Subunit composition of purified vitellin and its molecular weight

For determining the molecular weight of vitellin the purified and lyophilized protein was subjected to SDS-PAGE analysis in a completely denaturing condition with 9% gel concentration. Along with electrophoresis of pure vitellin molecular weight markers were also applied. It was observed that when Rm values of the protein markers were plotted against logarithm of their molecular weights that produced a reasonable good straight line. The result showed that vitellin was composed of seven subunits and the molecular weight of the subunits were100KDa, 89KDa, 79KDa, 70KDa, 50KDa, 40KDa and 31KDa respectively (Fig. 5). Thus the total molecular mass of Native vitellin was 459KDa. Regarding the member of subunits in *Oxya* it can be concluded that in the native condition also it contained seven subunits and it is in conformity with that of some other Acrididae as Garma and Gill (2002) have reviewed the problem and have shown that in case of acrididae the number of polypeptides varied from 3 to 7. Depending on the molecular weight of polypeptides they have suggested a two gene model. So, it might be possible that in case of *Oxya* also the same model works.

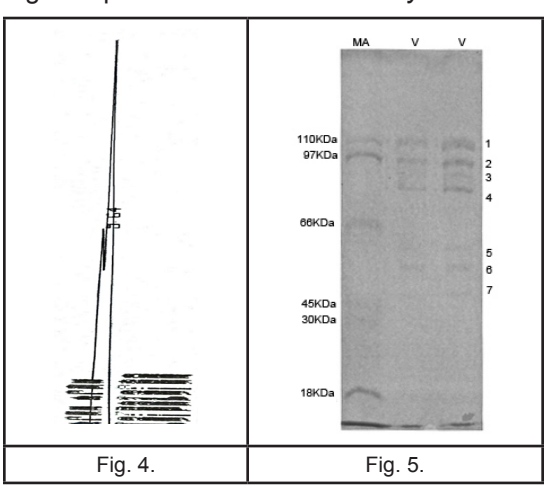

Fig. 4. Fig. 5.

Fig. 4. HPLC profile (abs. at 280nm) of purified vitellin. Fig. 5. SDS-PAGE profile of vitellin.

Denaturation and Spectroscopic Studies

To understand the stability of vitellin under denaturing condition the purified vitellin was kept in 8M Urea solution for 10 minutes and after equilibration the solution was again subjected to HPLC analysis. It was observed (Fig. 6) that again a single sharp pick came with 6.48 retention time without any minor pick. This indicated that the native vitellin was stable under denaturing condition with Urea. When absorption and fluorescent emission spectra of vitellin was taken it was observed that the molecule had strong absorption at 280nm (λmax ex: 280nm) and emission at 331.4nm (λmax, em: 331.4nm). In case of insect such study is reported for the first time while in shrimp that has been reported to be 329nm (Garcian-Orozco *et al*., 2002). The result of emission study is presented in Fig. 7. To understand the stability of the native molecule within buffer the emission spectra was taken at 10minutes intervals. The result showed that only after 10minutes slight increase in emission occurred and at 20 and 30 minutes interval no change took place. This suggested that the molecule was sufficiently stable. To understand the content of aromatic amino acids (tryptophan and tyrosine) vitellin solution was subjected to excitation at 280nm and 295nm separately. Interestingly when the excitation wavelength was changed from 280nm to 295nm there was 60% quenching of emission without altering the EM_{max} 331.4nm (Fig. 8).This clearly indicated that tyrosine content of the protein was either very low or nil and Tryptophan content was higher. But earlier works by hydrolysis method (Chinzei *et al*.,1981) indicated that tyrosine was present in *Locusta* vitellin while tryptophan was absent or in low percentage. But the present study suggested that Oxya vitellin had different aminoacid composition from that of *Locusta*. The result of HPLC with urea indicated that the subunits of native vitellin were not easily dissociable and that indicated some kind of disulphide bridge might had hold the subunits together.

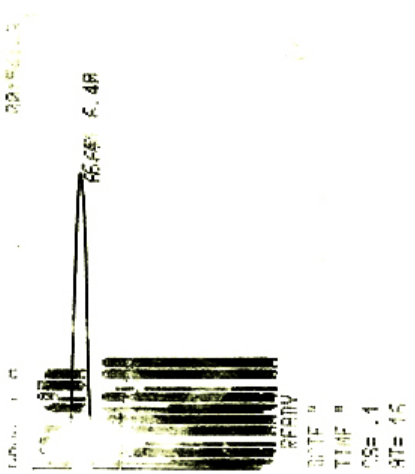

Fig. 6. HPLC profile of purified vitellin.

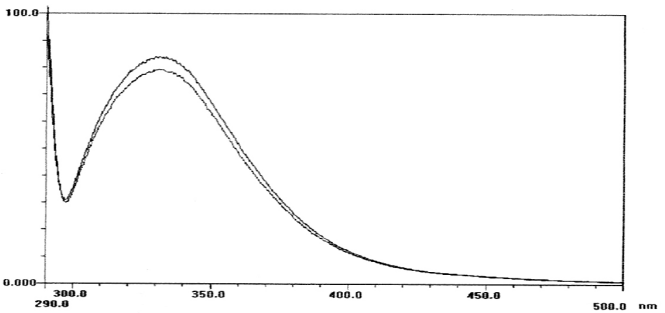

Fig. 7. Fluroscent emission spectra of purified vitellin.

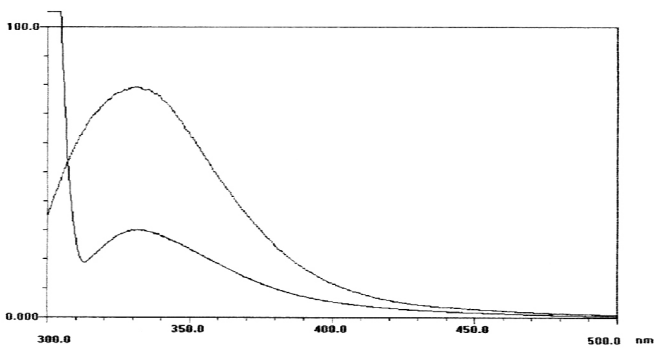

Fig. 8. Emission spectra of vitellin by excitation at 280nm (upper) and 295nm (lower).

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