Chapter II

Biochemical and biophysical properties of vitellogenin
INTRODUCTION

Vitellogenins have long been known to contain domains that are homologous parts of apolipoprotein B-100(apo B) of human low-density lipoprotein (LDL). Given the clear recognition of mammalian serum lipoproteins by the vitellogenin receptor of *S. serrata*, the hypothesis was further expanded to study the similarity between mammalian apo B, LDL and VLDL to the crab vitellogenin. Most of the homology identification studied so far were based on comparison and sequence alignment of amino acids of vitellogenin, apo B, apolipophorin, von Willebrand factor and lipoprotein lipase obtained from protein databases. However, an establishment of a kinship between these proteins has not yet been investigated at the level of immunological cross-identity. As a step towards this approach, in the present study, the immunological reactivity of Vg of *S. serrata* to antibodies to serum lipoproteins of mammals were investigated.

Other interrelated features that has been identified regarding vitellogenin especially in crustaceans and teleosts are its instability, sensitivity to proteolysis and proteinase-like characteristics (Komatsu and Hayashi, 1994, Komatsu et al., 1996; Silversand et al., 1993; Norberg and Haux, 1985). These properties were investigated in the present study as a comparative account between vitellogenin and lipovitellin of *S. serrata*. 
OBJECTIVES AND EXPERIMENTAL APPROACHES

1) Establishment of an identity between crab vitellogenin and mammalian LDL, VLDL and apolipoprotein B:
This was done by challenging crab Vg separately with anti-human LDL, VLDL and apo B antibodies and identifying cross-reactivity by Western blotting.

2) Demonstration of a protease activity by vitellogenin:
The ability of Vg to degrade complex polypeptides was determined using electrophoretic analysis.

3) Study of the effect of denaturants:
Urea-mediated unfolding of Vg and Lv was investigated by UV spectral studies.
MATERIALS AND METHODS

2.1 PURIFICATION OF VITELLOGENIN

This was carried out by the method described in section 1.3 (Chapter I).

2.2 PURIFICATION OF LIPOVITELLIN

Ovaries from vitellogenic female *S. serrata* were excised out, rinsed in Tris buffered saline (TBS) containing 20 mM Tris, 130 mM NaCl and 5 mM EDTA at pH 7.4 and blotted on a Whatman No.1 paper. 100 mg of ovary was weighed and homogenised in 1 ml TBS using a teflon homogeniser. The homogenate was then centrifuged at 4,500 g for 20 min at 4°C using a refrigerated centrifuge (Kubota KR-1500). The lipid cap of the ovarian extract was removed and the supernatant recovered. The centrifugation was repeated two more times and supernatant stored at -40°C. Similar to vitellogenin purification, lipovitellin was purified from the ovarian extract by a two-step process comprising of chromatography and preparative electrophoresis. The ovarian extract obtained from centrifugation was subjected to chromatography on Sephadex G-200 (Sigma, St. Louis, USA) gel filtration column (55 x 1.5 cm) which was previously equilibrated in TBS containing 0.2% sodium azide. Elution was performed in the same buffer and 100 fractions of 1 ml each were collected at a flow rate set at 1 ml/min. Absorbance at 280 nm was measured in all the fractions. Lipovitellin being a high molecular weight protein eluted immediately after the void volume, similar to Vg. The chromatographic peaks
containing Lv were pooled and concentrated using a Speed-vac concentrator (Savant, USA) at 4°C. The dried proteins were redissolved in 0.5 ml of Tris-HCl (0.05 M) pH 6.8 and applied separately to a 7.5% preparative native polyacrylamide gel. A vertical slice of the preparative gel was sliced and stained with Coomassie blue to localize the proteins. The corresponding region in the remainder of the lanes were cut and proteins were eluted separately from gel fragments in 3 ml of 0.05 M ammonium acetate buffer, pH 6.8 at 4°C. The eluates containing Lv were established for purity in a native polyacrylamide gel (7.5%).

2.3 PREPARATION OF RABBIT ANTISERA FOR LIPOVITELLIN

The purified lipovitellin (150 µg in 500 µl of PBS) was emulsified with equal volume of Freund’s complete adjuvant and adult male New Zealand rabbits were immunised with 150 µg protein/rabbit. Each rabbit was immunised by the subcutaneous route at multiple sites. Subsequent injections (100 µg in 500 µl PBS emulsified in equal volume of Freund’s incomplete adjuvant) were given after an interval of 3-4 weeks. Then rabbits were bled from the ear vein and serum was obtained by allowing it to stand at 37°C for 30 min. The clot formed was separated from the sides of the test tube by swirling it gently and the sample was allowed to stand at 4°C overnight. It was then centrifuged at 1,000 g for 10 min. After removal of the clot, the serum was centrifuged at 1,000 g and the clear supernatant stored at -20°C in 1 ml aliquots.
2.4 DOT BLOTTING USING ANTILIPOVITELLIN ANTISERA

The efficacy of the antilipovitellin rabbit antisera to bind lipovitellin and vitellogenin was tested by dot blotting technique. Different concentrations (5, 10, 15 and 20 μg) of Lv and Vg were dot blotted onto nitrocellulose membrane. The protein blots were allowed to dry at 30°C for 1/2 h. The membrane was then blocked at the unbound sites by treating with 5% milk (prepared from Nestle skimmed milk powder in TBS) and kept shaking on a rocker for 1 h at room temperature. This was followed by washes with TBS containing 0.05% Tween 20. Antiserum to lipovitellin (50 μg protein) was added in 2.5% milk in TBS and the membrane was incubated for 1 h at room temperature. The membrane was then exposed to secondary antibody (anti-rabbit IgG-horseradish peroxidase conjugate from Sigma, St. Louis, USA) which was used at a dilution of 1:20,000 for 1 h at room temperature. After washing thrice in TBS, the blot was developed using diaminobenzidine (0.75 mg of DAB, Sigma, St. Louis, USA) in 10 ml TBS in the presence of 0.3% hydrogen peroxide. Appearance of brown colour indicated binding of the antibody to the protein on the nitrocellulose membrane.

2.5 NATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS

Purified Vg and Lv protein were tested for purity by subjecting to native polyacrylamide gel electrophoresis (7.5%) following the method of Davis (1964). Tris-glycine buffer pH 8.6 was used as the electrode buffer. The protein samples (100 μg of Vg and Lv each and 150 μg of hemolymph and ovarian
extract) were all prepared in 10 mM Tris-HCl (pH 6.8). The samples were electrophoresed at 60V until the marker dye (bromophenol blue) reached the bottom of the stacking gel and then was increased to 150V. The run was stopped when the dye front reached the bottom of the separating gel. The gel was transferred to 7% TCA to fix the protein bands after which it was incubated in 0.2% Coomassie brilliant blue R250 staining solution overnight at room temperature. After removing from the stain, the gel was incubated in destaining solution containing methanol, distilled water and acetic acid (at the ratio of 5:5:1). Destaining was performed till the background of the gel appeared colourless and protein bands were clearly visible. The gel was stored in distilled H$_2$O.

2.6 WESTERN BLOTTING USING ANTISERA TO Vg, LDL, VLDL OR APO B

After electrophoresis in a native 7.5% polyacrylamide gel, the proteins (purified Vg, Lv and crude samples of hemolymp and ovary) were transferred to nitrocellulose membrane using a Hoeffer semidry blotter according to the procedure of Bjerrum and Schafer-Nielsen (1986). After the transfer was over, the bands were visualized by staining with Ponceau S (Salinovich and Monteralo, 1986). Four lanes of Vg and Lv each were also subjected to native electrophoresis (7.5%) and transferred to nitrocellulose membrane and the transfer was confirmed with Ponceau S staining. Four strips of nitrocellulose containing Vg and Lv each were cut and all were incubated for 1h at room temperature in blocking buffer (5% dry milk powder in TBS). This was followed
by two washes in TBS (containing 0.05% Tween 20). The membrane containing 
Vg and Lv along with crude extracts and one of the strips containing Vg and 
Lv were then incubated in antiserum to lipovitellin (50 µg protein) in 2.5% 
milk in TBS and incubated for 1 h at room temperature. The remaining three 
strips of nitrocellulose membrane containing Vg and Lv were incubated with 
either anti LDL, anti VLDL or anti apoB antibodies from Sigma, St. Louis, 
USA (50 µg protein each made in 2.5% milk in TBS) for 1 h at room 
temperature. The membranes were then washed in TBS three times to remove 
unbound antibodies and exposed to secondary antibody (anti-rabbit IgG-horse 
radish peroxidase conjugate from Sigma, St. Louis, USA) at a dilution of 
1:20,000. The incubation was carried out for 1 h at room temperature on a 
rocker platform. The blot was developed using the substrate diaminobenzidine 
(DAB from Sigma, St. Louis, USA) which was made in TBS (0.75 mg in 10 ml 
TBS) in the presence of 0.3%, H₂O₂. Appearance of brown bands indicated 
binding of the antibody to the transferred protein on the nitrocellulose 
membrane.

2.7 DOT BLOTTING OF LDL, VLDL AND HDL USING ANTI CRAB 
LIPOVITELLIN ANTIBODIES

LDL, VLDL and HDL fractions were obtained from rat plasma by 
potassium bromide ultracentrifugation as described in section 1.5 (Chapter I). 
LDL, VLDL and HDL separated at different densities (LDL d < 1.06 g/ml; 
VLDL d < 1.006 g/ml and HDL d < 1.19 g/ml) were collected from a potassium 
bromide density gradient, dialysed against PBS and stored at -70°C until use.
Each of these lipoproteins, along with Vg from S. serrata were dot blotted on a nitrocellulose paper and tested for their ability to bind anti crab Lv antibodies (50 μg protein). The blot was developed with DAB to visualize the binding of the antibody.

2.8 IMMUNOTURBIDIMETRIC ASSAY FOR APOB

Vitellogenin and lipovitellin were also analysed for the presence of apolipoprotein B by using an immunoturbidimetric assay of Albers and Marcovina (1992). Vg and Lv (50 μg protein each made in 250 μl TBS) were incubated in 100 μg in 250 μl TBS of antihuman apolipoprotein B goat antiserum provided in κ assay kit for apoB (Kamiya Biomedical Co., USA) at 37°C for 10 min. The immune complexes formed (which increases with increasing concentration of apoB) were measured at 600 nm using a UV-vis spectrophotometer (Hitachi, Japan). The concentrations of apoB in Vg and Lv were obtained from the standard plot obtained on performing the immunoturbidimetric assay with known concentrations of apoB.

2.9 NATIVE GEL ELECTROPHORESIS FOR DETERMINING PROTEOLYTIC PRODUCTS OF VITELLOGENIN

Polyacrylamide gel electrophoresis (7.5%) under non-denaturing, non-reducing conditions was carried out as described by Davis (1964) for determining the proteolyzed polypeptides of Vg. Purified Vg and Lv (150 μg each dissolved in 100 μl of 10 mM Tris-HCl pH 7.0) were incubated at 24°C for
12 hr with or without 1mM phenyl methyl sulfonyl fluoride (PMSF) and then subjected to electrophoresis as described previously. To determine the optimum pH for the protease activity of Vg, purified Vg and Lv (150 µg each) were dissolved in 100 µl of buffers of different pH (pH 4, 40 mM Tris-maleate buffer, pH 6, 80 mM Tris maleate buffer, pH 8, 25 mM Tris HCl buffer) for 12 h at 24°C and electrophoresis was carried out in non-denaturing condition as described.

2.10 PROTEINASE ACTIVITY OF VITELLOGENIN DETERMINED IN A CASEIN GEL

To detect proteinase activity of Vg on a casein gel, PAGE was carried out according to the method of Heussen and Dowdle (1980). Acrylamide solution containing 0.2% casein (Sigma, USA) was used to prepare 7.5% acrylamide gels without SDS. Samples (Vg, 150 µg) were prepared without boiling and electrophoresis was done under non-reduced conditions. After electrophoresis, the gel was incubated in 20 mM Tris-HCl (pH 7.0) containing 3 mM CaCl₂ at 37°C for 20 h. After incubation, the gel was immersed in acetic acid-methanol-water (1:4:9) and stained with 0.25% Coomassie Brilliant Blue R250 (Sigma, USA). Proteolyzed regions on the gel were detected as clear bands which did not take up the stain.
2.11 UV SPECTRAL STUDIES FOR UREA UNFOLDING

To monitor the unfolding of the proteins Vg and Lv in the presence of different concentrations of urea, spectral patterns in the UV regions were studied as described by Jaenicke and Rudolph (1989). The proteins (Vg and Lv, 150 µg each) were dissolved in 1 ml of 0.2 M phosphate buffer pH 7.0 containing either 2, 4, 6 or 8 M urea (Sigma, USA). The control samples included the protein incubated in phosphate buffer without urea. The samples were incubated at room temperature for 1 h on a rocking platform. The UV absorbance spectrum between 250 to 350 nm to detect absorbance of aromatic residues was studied using a UV-VIS spectrophotometer (Hitachi, Japan). In all the concentrations of urea, an appropriate control containing only urea in phosphate buffer and not protein was used to nullify interference from urea.
RESULTS

PURIFICATION OF VITELLOGENIN AND LIPOVITELLIN

Vitellogenin purification was carried out in the steps mentioned previously in Chapter I by gel filtration and preparative gel electrophoresis. Lipovitellin was purified from the vitellogenic oocytes of the crab by gel filtration in a Sephadex G-200 column (Sigma, USA) which resulted in the resolution of two major peaks (Fig.1). The first peak eluted at an elution volume of 30 ml whereas the second peak eluted at 61 ml. Further purification of the lipovitellin peak using preparative gel electrophoresis (7.5%) resulted in a single band in a native PAGE. Figure 2 shows purified Vg, Lv and crude hemolymph and ovarian proteins run in a 7.5% polyacrylamide gel electrophoresis under non-reducing conditions.

Next the reactivity of rabbit antilipovitellin antisera to purified Lv and Vg was tested by dot blotting using varying concentrations (5, 10, 15 and 20 μg) of the two proteins. It was observed (Fig.3) that both Vg (row1) and Lv (row2) reacted efficiently with antiLv antibodies. Control samples of BSA (row3) was non-reactive. Western blot using antiLv antibodies (Fig.4) showed that Vg and Lv in the crude and purified protein reacted.
Fig.1  Gel filtration chromatography of ovarian proteins of *S. serrata* on Sephadex G-200. Ovarian extract (100 mg/ml) in TBS was subjected to chromatography on a Sephadex G-200 column of length 55 x 1.5 cm, at a flow rate of 1 ml/min. 100 fractions of 1 ml were collected and absorbance at 280 nm was measured in each fraction. Two fractions eluted one at 30<sup>th</sup> fraction and another at 60<sup>th</sup> fraction. The first peak (indicated by arrow) contained Lv and was subjected to further purification.
Fig.2 Native PAGE on 7.5% polyacrylamide gel of purified Vg (100 µg, lane 2) and Lv (100 µg, lane 4) along with hemolymph (150 µg protein, lane 1) and ovarian extract (150 µg protein, lane 3). Vg and Lv obtained after purification by gel filtration chromatography and electrophoresis is observed as a single band (lane 2, lane 4). Arrow indicates Vg and Lv in all the four lanes.
Fig.3  Dot blot analysis of purified Vg and Lv using rabbit anti lipovitellin antibodies (dilution 1:2000) varying concentration (5,10,15 and 20 µg) of Vg (row1) and Lv (row2) were blotted. Control samples contained BSA (row3). Vg and Lv reacted well with antivitellin antibodies whereas control samples containing BSA did not react.

Fig.4  Western blot analysis of purified Vg and Lv and comparison with crude samples. Hemolymph proteins and ovarian protein extract (150 µg each made in TBS) were loaded in lane 1 and 3 respectively. Purified vitellogenin and lipovitellin (100 µg each) were loaded in lane 2 and 4 respectively and run in a 7.5% polyacrylamide gel without SDS under non-reducing conditions. After PAGE the proteins were blotted to nitrocellulose and probed with anti-Lv antibodies. In the crude samples only Vg and Lv bands reacted with anti Lv antibodies. Purified Vg and Lv reacted with anti Lv antibodies.
WESTERN BLOTTING OF VITELLOGENIN AND LIPOVITELLIN USING ANTI LIPOVITELLIN ANTIBODIES

First, the reactivity of Vg and Lv to rabbit anti Lv antibodies (50 µg) were tested by performing a Western blot. Both Vg and Lv appeared as dark bands (Fig.5).

WESTERN BLOTTING OF VITELLOGENIN AND LIPOVITELLIN USING ANTI LDL ANTIBODIES

In order to establish a relatedness between crab Vg and Lv and the mammalian lipoprotein, LDL, Vg and Lv fractionated on a 7.5% polyacrylamide gel was blotted to nitrocellulose and challenged with rabbit anti LDL antibodies (50 µg) followed by incubation with anti-rabbit peroxidase labeled secondary antibodies. Bands corresponding to Vg and Lv appeared (Fig.6) but with a lesser intensity than that observed in the Western blot using anti-Lv antibodies.

IMMUNOLOGICAL REACTIVITY OF Vg AND Lv WITH ANTI VLDL ANTIBODES

Next, the cross reactivity of Vg and Lv to antibodies to the mammalian lipid carrying protein, very low density lipoprotein (VLDL) was tested. It was observed that Vg and Lv reacted with anti VLDL antibodies with lesser intensity as that observed with anti LDL antibodies (Fig.7). Taken together
Fig.5  Western blotting of Vg (lane 1) and Lv (lane 2) using anti-lipovitellin antibodies (50 µg). Vg and Lv reacted strongly with anti Lv antibodies.

Fig.6  Western blot analysis of Vg (lane 1) and Lv (lane 2) using rabbit anti LDL antibodies (50 µg). Vg and Lv of crab reacted to a lesser degree with anti LDL antibodies.
these results suggest an immunological relatedness between Vg and Lv of crab and mammalian serum lipoproteins LDL and VLDL.

CROSS REACTIVITY WITH ANTI APOLIPOPROTEIN B ANTIBODIES

To characterize further the immunological identity between Vg and mammalian atherogenic lipoproteins, purified Vg and Lv of crab were assayed for apoB using immunoturbidimetric analysis. As apoB is the principal protein component of both LDL and VLDL it was assumed that the crossreactivity between anti-LDL and VLDL to Vg is based on apoB recognition. It has been shown in earlier reports that there are amino acid sequence similarities between Vg and apoB (Byrne et al., 1987; Baker, 1988; Babin et al., 1999). In the immunoturbidimetric assays it was observed that Vg and Lv possessed apoB activity (Table 1). In addition, anti-apoB antibodies reacted well as observed in the Western blot (Fig.8). These observations further substantiate the hypothesis that apoB is the common protein component of Vg, LDL and VLDL through which an immunological identity is established.

Table 1: Level of apolipoprotein B in vitellogenin and lipovitellin estimated by immunoturbidimetric assay

<table>
<thead>
<tr>
<th>Vitellogenin (µg)</th>
<th>ApoB (µg)</th>
<th>Lipovitellin (µg)</th>
<th>ApoB (µg)</th>
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<tr>
<td>500</td>
<td>230 ± 5</td>
<td>500</td>
<td>300 ± 11</td>
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<tr>
<td>1000</td>
<td>476 ± 7</td>
<td>1000</td>
<td>530 ± 7</td>
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<tr>
<td>1500</td>
<td>900 ± 10</td>
<td>1500</td>
<td>930 ± 10</td>
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Each value represents ±SEM with triplicate determinations. P < 0.1 (Student's t-test).
REACTIVITY OF RAT LDL, VLDL WITH ANTI CRAB Lv ANTIBODIES

To confirm the relatedness of these lipoproteins, LDL and VLDL from rat were dot blotted and challenged with anti crab Lv antibodies. Rat HDL was also included in the blot. It was observed that LDL and VLDL reacted with anti LV antibodies whereas HDL was not able to recognize these antibodies (Fig.9). These results suggest that lipovitellin of crab has epitopes that are also present in rat LDL and VLDL but are not found in HDL.

IDENTIFICATION OF A PROTEASE ACTIVITY BY VITELLOGENIN

Next, the nature of stability of the lipoproteins Vg and Lv were studied. First, purified Vg and Lv were kept at 24°C in Tris buffer for 12 hr and then subjected to 7.5% native polyacrylamide gel electrophoresis in order to determine if there was degradation of the native proteins. It was observed (Fig.10, Lane 1) that Vg partially degraded to release three more polypeptides of smaller molecular weights from the native protein suggesting an ability of Vg to proteolytically digest itself. Lipovitellin, however, remained intact in its native form (Fig.10, Lane 3). However, when the incubations were performed in the presence of protease inhibitor, phenylmethyl sulfonyl fluoride, both Vg and Lv remained stable (Fig.10, Lane 2 and 4).

Purified Vg and Lv were then incubated at different pH ranging from 4-8 at 24°C for 12 h. Figure 11 shows that whereas Lv remained stable at all pH
Fig. 7  Cross reactivity of Vg (lane 1) and Lv (lane 2) with anti VLDL antibodies (50 μg) by Western blot. Vg and Lv interacted weakly with anti VLDL antibodies.

Fig. 8  Western blot analysis of Vg (lane 1) and Lv (lane 2) using rabbit anti apoB antibodies to identify crossreactivity. The reaction of Vg and Lv with anti apoB antibodies was strong compared to anti LDL and VLDL antibodies.

Fig. 9  Dot blot analysis of crab Vg (1) and rat LDL (2) VLDL (3) and HDL (4) using anti crab Vg antibodies (dilution 1:2000). Anti Vg antibodies is seen to react well with Vg, LDL and VLDL but there is no reaction with HDL.
(Lane 4-6), Vg underwent cleavage only at an alkaline pH of 8 but was intact at pH 4 and 6 (Lane 1-3).

To demonstrate further the proteinase activity of Vg, purified Vg (lyophilized and stored at -40°C) was subjected to a 7.5% native polyacrylamide gel which was impregnated with the protein casein. After incubating the gel in buffer of pH 8.3 at 37°C for 12 h, it was stained with Coomassie blue. The area of gel surrounding Vg appeared as clear regions that did not take up the Coomassie blue stain (Fig.12) the remaining portion of gel stained blue due to the presence of casein.

Taken together, these observations suggest that Vg purified from the hemolymph has an intrinsic protease activity with which it undergoes partial autolysis and is able to degrade complex polypeptides like casein. Furthermore, the alkaline pH optima and inhibition by PMSF suggests that this protease is of the serine-protease type.

**EFFECT OF UREA ON THE CONFORMATIONAL STABILITY OF Vg AND Lv**

As the previous experiments suggested a difference in the stability patterns of Vg and Lv, conformational changes of these two proteins in the presence of the destabilizer urea were studied. For monitoring the changes, spectral changes at the UV range between 250-350 nm was studied at different (2,4,6 and 8M) urea concentrations. As the protein unfolds progressively with
Fig. 10  Auto-proteolysis of vitellogenin.
150 μg of Vg and Lv were run on 7.5% native PAGE. All samples were incubated at 24°C for 12 h. Lane 1-proteolyzed Vg showing 3 proteolysed bands of Vg. Lane 2-Intact Vg in the presence of inhibitor PMSF; Lane 3-Intact Lv without PMSF; Lane 4-Intact Lv with PMSF. Arrow indicates Vg and Lv bands.

Fig. 11  Effect of pH on proteolysis.
150 μg of protein was loaded in all lanes and subjected to 7.5% native PAGE. Lane 1-Vg incubated at pH 4.0, 24°C, 12 h; lane 2-Vg incubated at pH 6.0, 24°C, 12 h; lane 3-Vg incubated at pH 8.0, 24°C, 12 h. Vg had autoproteolytic activity at this pH as seen by the appearance of three new bands. Lanes 4,5,6-Lv incubated at pH 4,6,8 respectively at 24°C, 12 h did not undergo degradation.
Fig.12 Protease activity of purified Vg. 100 µg of Vg was applied to a 7.5% native gel containing 0.2% casein (lane 1 and lane 2). After electrophoresis, the gel was incubated, washed and stained as described in Materials and Methods. The clear patches (negative staining with Coomassie blue) indicate areas proteolysed by Vg.
increasing concentrations of urea, the hydrophobic core containing aromatic residues which absorb between 250-280 nm are exposed. It was observed that at 2 M urea, Vg did not undergo any change in the UV spectrum (Fig.13b), when compared to the native form (Fig.13a). At 4 M urea a small peak appeared for Vg at 274 nm which corresponds to the absorbance value of tryptophan (Fig.13c). When Vg was treated with 6M and 8M urea spectral analysis showed that the peak at 274 nm increased reaching a maximum absorbance at 8M (Fig.14a,b). In the case of lipovitellin it was seen that the spectral pattern underwent negligible changes at 0-8 M urea (Fig.15a-e). These observations demonstrate clearly that Vg is easily disrupted by urea and the tryptophan residues become exposed due to urea mediated unfolding. Lipovitellin, on the other hand, appears to be more resistant to the action of urea. These results further substantiate the notion that vitellogenin is a relatively labile lipoprotein when compared to its ovarian product lipovitellin.
Fig.13  UV absorbance spectra of vitellogenin.
Vitellogenin was incubated without urea (a) or with 2M (b) or 4M (c) urea for 1h. Arrow indicates peak at 274 nm.
Fig. 14  UV absorbance spectra of unfolded vitellogenin. Vitellogenin was incubated for 1 h in 6M (a) or 8M (b) urea. Arrow indicates peak at 274 nm.
Fig. 15  UV absorbance spectra of lipovitellin.  
Lipovitellin was incubated without urea (a) or with 2M (b),  
4M (c), 6M (d) or 8M (e) urea.
DISCUSSION

The multiplicity of ligand recognition by crab Vg receptor led to the hypothesis that perhaps there are regions of similarity between the different ligands (Vg, LDL and VLDL) that VgR recognizes. This prompted an investigation into the immunological relatedness between Vg and apolipoprotein B, the major protein component of LDL and VLDL.

The relationship between vitellogenins and the apolipoprotein B family was confirmed by phylogenetic and statistical approaches in cyclorhaphan insects (Hagedorn et al., 1998). Vitellogenins from Drosophila and Caenorhabditis elegans were also found to be homologous to human apoB and human von Willebrand factor by alignment of amino acid sequences (Baker, 1988a,b). Molecular modelling and mutagenesis have revealed that there are conserved structural motifs between invertebrate Vg and the major atherogenic lipoproteins such as apoB, microsomal triglyceride transfer protein (MTP) and protein disulfide isomerase (PDI) (Mann et al., 1999). All these reports confirm the homology of Vg to mammalian lipid transport proteins through sequence analysis and phylogenetic approaches. However, very few investigations have studied the identical nature of these lipoproteins at the immunological level. In view of this, in the current study the crossreactivity of antibodies between the crab and mammalian lipoproteins was used as a parameter to determine evolutionary relationship between crab Vg and related serum proteins from mammals. These studies revealed
that in a Western blot, anti apolipoprotein B antibodies reacted well to Vg of crab establishing the immunological identity of these lipoproteins. Immunoturbidimetric analysis also demonstrated the presence of apoB moiety in Vg purified from the crab. It is possible that the regions of similarity in LDL, VLDL and Vg are in the apoB moiety present in LDL and VLDL which is also probably present in crab Vg. The non-recognition of HDL, which does not contain apoB, by antiLv antibodies also suggest that apoB is vital for antibody recognition by anti crab Lv antibodies. The high sensitivity of Vg to anti apoB antibodies lends further support to this hypothesis. It has been proposed that paralogues retain common biochemical activities and acquire new function in the course of evolution through gene arrangements (Doolittle 1995, Henikoff et al., 1997). Vg is specifically devoted to the massive deposition of yolk reserves inside the oocytes. Cathepsin D catalyzes the intraoocytic fragmentation of both apoB and Vg in birds (Retzek et al., 1992; Elkin et al., 1995) which are internalized by binding and endocytosis by the same oocyte-specific receptor that belongs to the LDL receptor superfamily (Bujo et al., 1994). Apo B are used mainly in the stabilization and transport of endogenous and exogenous lipids and liposoluble substances towards different somatic tissues. Therefore the observations in the current study further supports the concept that the mammalian atherogenic proteins emerged from an ancestral protein - Vg - designed to play a crucial role in the intracellular and extracellular transfer of lipids and liposoluble substances.
In addition to the aforementioned investigations, studies on the stability of purified crab Vg and Lv were also performed in the present work. Vitellogenin after being internalized into the oocytes is acted upon by proteases, mainly cathepsin, to be rearranged into lipovitellin. In the oocytes lipovitellin derived from Vg is further lipidated to form a stable complex which forms yolk granules that are utilized during embryogenesis. Differences in the composition of lipid, carbohydrate and phosphorus between the vertebrates and invertebrates possibly reflect differences in the nutritional demands of the diverse embryos (Byrne et al., 1989). Vitellogenin in teleosts is highly sensitive to degradation (Selman and Wallace, 1983; Tyler and Sumpter 1990; Goodwin et al., 1992). In the absence of protease inhibitors Vg was observed to degrade into minor fragments which were reactive to anti Vg antibodies (Silversand et al., 1993). Interestingly, although Vg is sensitive to degradation it was observed to resist oxidation and also protect other serum lipoproteins from oxidation (Ando and Yanagida, 1999). The proteinase activity of Vg had been reported in sand crayfish (Komatsu and Hayashi, 1994) and eel (Komatsu et al., 1996). Vg in the subunit form was susceptible to degradation possibly by a proteinase activity of a Vg subunit itself. The present study confirms the previous reports by demonstrating a potential protease activity of Vg. As observed (Fig.12), purified Vg was able to digest casein in a casein impregnated gel. Furthermore, purified Vg in the absence of protease inhibitors underwent fragmentation within hours of purification, whereas Lv
was sturdy under the same conditions and remained as a single band in native PAGE of Vg and Lv.

The stability of Vg and Lv in the presence of denaturants were also investigated. Thus the extent of unfolding of Vg and Lv in the presence of urea was studied. Proteins that are configurationally stable requires a higher concentration of urea to unfold when compared to a labile protein. Urea, a chaotropic agent, unfolds complex polypeptides by disrupting its quarternary and tertiary structures so that the hydrophobic residues are exposed (Jaenicke and Rudolph, 1989). In this study, the absorbance spectra between 250-350 nm was monitored to study the degree of unfolding as the aromatic residues tyrosine, tryptophan and phenylalanine would be exposed to a larger extent with increase in unfolding. It was observed that Lv was a remarkably stable protein because even at 8 M urea (a concentration at which most proteins unfold) there were no significant spectral changes. In the case of Vg, even at 4 M urea a small peak, which increased with increasing urea concentrations, appeared at 274 nm which corresponds to tryptophan absorbance. Therefore, Vg completely unfolded at 8 M urea suggesting its extreme susceptibility to denaturants. The difference in stability of these proteins is probably due to the degree of lipid binding in these proteins. It was observed that in human apolipoprotein E, lipid binding decreases protease sensitivity (Wilson et al., 1991). On lipid binding it is possible that the protein undergoes conformational changes. In the case of the crab, the internal milieu of the oocyte has a remarkably high lipid
content upto 30% which possibly renders the Lv to be highly lipidated. This lipid cage formed around Lv probably resists the actions of denaturants and proteases making it a more stable form than its precursor Vg. As Lv has a longer storage life so as to last upto embryo nourishment, the present study suggests that Lv has achieved a stable conformation to perform its functions without losing its structural integrity.