

# Characterization of a Heat-Stable Fraction of Lipovitellin and Development of an Immunoassay for Vitellogenin and Yolk Protein in Winter Flounder (*Pleuronectes americanus*)

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**ABSTRACT** An enzyme-linked immunoabsorbent assay was developed for detection and quantification of the yolk protein lipovitellin (Lv) and its plasma precursor, vitellogenin (Vg), in winter flounder (*Pleuronectes americanus*). Native Lv was found to be a mixture of heat-stable and heat-labile molecules in mature, ovulated eggs. A heat-stable Lv fraction was purified from extracts of unfertilized eggs by brief heat treatment and gel permeation chromatography on Bio-Gel A-1.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of heat-stable Lv revealed a single polypeptide of 94 kD, while native Lv also possessed several smaller polypeptides, suggesting that heat-labile Lv contains proteolytic cleavages of the 94-kD polypeptide which destabilize its structure. The Stokes radius of the native protein on Bio-Gel A-1.5 was estimated at 4.50 nm, while the Stokes radii of heat-stable and heat-labile Lv were 4.26 nm and 5.17 nm, respectively. Heat-stable Lv was used to produce a rabbit polyclonal antiserum which reacted with a single 175-kD polypeptide in Western blots of vitellogenic female winter flounder serum, but did not react with any component of male serum. Ouchterlony double diffusion using this antiserum demonstrated immunological identity of Lv, heat-stable Lv, and Vg. The anti-Lv antiserum was used to construct an homologous ELISA with a linear response between 25 and 300 ng/ml. This assay was used to characterize a Bio-Gel A-1.5 column profile of serum from an estradiol-treated male winter flounder, and a single peak, with Stokes radius of 6.70 nm, was identified as Vg. Winter flounder Vg was confirmed to be a dimer, while Lv from mature eggs was found to be a monomer of a lower molecular weight polypeptide. *J. Exp. Zool.* 278:156-166, 1997. © 1997 Wiley-Liss, Inc.

Developing embryos and prefeeding larvae of oviparous animals are dependent on yolk reserves for nutrients essential for growth and development. The major yolk proteins in these animals are derived from vitellogenin (Vg), a large glycopospholipoprotein which bears a striking degree of structural and functional homology across a wide range of vertebrate and invertebrate species (Byrne et al., '89). In fishes, as in other vertebrates, Vg is synthesized and secreted by the liver in response to 17 $\beta$ -estradiol (reviewed by Ng and Idler, '83; Mommsen and Walsh, '88; and Sprecker and Sullivan, '94). It is rapidly and specifically taken up by receptor-mediated endocytosis from the blood into maturing oocytes (Selman and Wallace, '82, '83; Tyler et al., '88a,b, '90a,b, '91; Kanungo et al., '90; Nagler et al., '92, '93), where it is proteolytically cleaved and stored as the yolk

proteins lipovitellin (Lv) and phosvitin (Tyler et al., '88a,b). In many fishes, these proteins undergo additional proteolytic processing during oocyte maturation, often yielding a complex array of polypeptides as observed on electrophoretic gels of mature egg extracts (Wallace and Begovac, '85; Wallace and Selman, '85; Greeley et al., '86; Carnevali et al., '92, '93). Such additional processing appears to be linked to oocyte hydration (Craik and Harvey, '84; Wallace and Selman, '85; Greeley

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et al., '86), and is accompanied by a sudden decrease in oocyte phosphoprotein levels and a concomitant rise in the concentration of inorganic phosphate (Craik, '82; Craik and Harvey, '84).

A multitude of approaches have been developed as indirect and direct indicators of Vg levels in fishes. Such techniques include measurement of serum calcium, alkali-labile phosphorus, and phosphoprotein phosphorus, as well as Vg-specific quantitative immunoassays (Mommensen and Walsh, '88; Specker and Sullivan, '94). Recently, many investigators have employed the enzyme-linked immunosorbant assay (ELISA) as a sensitive, convenient and rapid method for quantifying Vg (for review see Specker and Sullivan, '94). However, purification of Vg, usually the first step for immunoassay development, is often difficult in teleosts, frequently yielding preparations which contain partially degraded or aggregated Vg (Kanungo et al., '90; Tao et al., '93; Silversand et al., '93; Specker and Sullivan, '94). Some investigators have circumvented this difficulty by raising antibodies against purified yolk proteins for immunodetection of Vg (Idler et al., '79; Campbell and Idler, '80; Chen, '82; Hara et al., '83; Perez and Callard, '93; Okumura et al., '95).

Winter flounder (*Pleuronectes americanus*) have come to be regarded as a sentinel species for determining the presence and biological impact of marine pollutants (Gronlund et al., '91; Hughes and Hebert, '91; Nelson et al., '91; Tay et al., '92). This fish is a demersal species, often lying nearly buried in soft sediments (Bigelow and Schroeder, '53), and is thus closely exposed to pollutants which concentrate in the sediment. Early life stages are particularly vulnerable, as this species reproduces in some of the most polluted estuaries along the east coast of the United States (Hughes and Hebert, '91). Recent work has begun to focus on the impact of pollutants on winter flounder embryos and larvae (Black et al., '88; Nelson et al., '91; Pereira et al., '92), but little is known about the effects of xenobiotics on flounder yolk synthesis, uptake into oocytes, and utilization by the developing animal. A study by Pereira et al. ('92) showed that winter flounder from polluted sites in Long Island Sound have elevated levels of serum alkali-labile phosphate and lower levels of total ovarian lipid relative to animals captured from cleaner sites, suggesting that the pathway by which Vg is produced and transported to oocytes is perturbed in the fish exposed to xenobiotics. The same study also demonstrated that winter flounder which were captured from

polluted sites in Boston Harbor and had developed liver tumors showed a decrease in serum alkali-labile phosphate. However, no data regarding specific levels of serum Vg or oocyte yolk proteins are available.

In the present study, we describe a heat-stable fraction of Lv from mature, unfertilized eggs of winter flounder, which allows a novel approach to Lv purification. The purified Lv is used to develop an immunoassay to detect, quantify, and further characterize Lv and Vg. Such an assay will be useful in elucidating the normal mechanisms of yolk metabolism in winter flounder, as well as determining the effects of environmental pollutants on this system.

## MATERIALS AND METHODS

### *Animals, eggs and serum*

Adult *P. americanus* were collected off the coast near Milford, Connecticut, and in New Haven Harbor by otter trawl in February and March 1992 and March 1994. The animals were maintained at the National Marine Fisheries Service Milford Laboratory, Milford, Connecticut, in flowing, ambient sea water (0–4°C) for up to two weeks. Eggs were stripped from ripe females, transported on ice to the University of Massachusetts, Amherst, and frozen at –20°C until use. Blood was collected by caudal vein puncture at the Milford Laboratory in February 1994 in 3 ml vacutainer tubes (Becton Dickinson, Rutherford, NJ), and aprotinin (Sigma, St. Louis, MO) was immediately added to a final concentration of approximately 1 TIU per ml blood. The blood was transported to the University of Massachusetts on ice, and allowed to clot at 4°C overnight. Remaining blood cells were removed by centrifugation, and the serum was drop-frozen in liquid nitrogen and stored at –80°C until use.

Estradiol-treated male winter flounder were given intramuscular injections of estradiol (in peanut oil) at approximately 40 µg per g body weight on days 1, 3, 5, 8, 10, 12, and 15, and bled on day 15 (April 1995). Aprotinin was immediately added to the blood to a final concentration of approximately 0.6 TIU per ml blood. The serum was prepared and stored as described above.

### *Preparation of yolk extract and Lv*

Yolk proteins were extracted from unfertilized eggs by homogenization in ice-cold column buffer (0.4 M KCl, 0.05 M KH<sub>2</sub>PO<sub>4</sub>, pH 7.2), supplemented with 2 mM phenylmethylsulfonyl fluoride (PMSF) immediately before homogenization, at 4

ml buffer per gram eggs. The homogenate was centrifuged at  $3,500 \times g$  for 15 minutes at  $4^{\circ}\text{C}$ , and the supernatant was added to a Bio-Gel A-1.5 column ( $85 \times 1.5$  cm; Bio-Rad, Melville, NY) in column buffer, using a flow rate of 10 ml per hour at  $4^{\circ}\text{C}$ . Peak fractions were pooled and concentrated with a vacuum ultrafiltration unit (Millipore, Bedford, MA; MW cutoff = 30 kD).

Lv purified by heat denaturation of egg extract was prepared as above, except that the egg extract was heated at  $85^{\circ}\text{C}$  for 7 minutes and the resulting precipitate removed by centrifugation at  $3,500 \times g$  for 15 minutes at  $4^{\circ}\text{C}$ . The supernatant was then added to the Bio-Gel A-1.5 column as described above.

### ***Protein determination***

Protein concentration was determined by the Bradford assay, modified for use on microtiter plates (Redinbaugh and Campbell, '85). The standard for this assay was Lv prepared by gel permeation chromatography of whole egg extract. The Lv column peak was dialyzed against 1 mM PMSF lyophilized, and stored in aliquots of 5 mg/ml in phosphate-buffered saline (PBS) at  $-80^{\circ}\text{C}$ .

### ***Gel permeation chromatography calibration***

Ten mg each of two or three protein standards of known Stokes radius were mixed together from 10 mg per ml stock solutions and fortified with 5 mg blue dextran (Pharmacia, Uppsala, Sweden; void volume marker, MW = 2,000,000) and 7 mg potassium hydrogen phthalate (Fisher, Orangeburg, NY; total volume marker, MW = 204). Each calibration mixture was applied to a gel permeation column (1 cm  $\times$  1.6 cm, Bio-Gel A-1.5). The column was eluted at  $4^{\circ}\text{C}$  for approximately 20 hours using a constant flow rate of 10 ml per hour, regulated by a metering pump (Milton Roy Minipump). The column partition coefficients of the standards were determined as  $K_{av} = (V_e - V_o) / (V_t - V_o)$ , where  $V_e$  is the elution volume of the standard,  $V_o$  is the void volume, and  $V_t$  is the total volume. The protein standards (Sigma) were carbonic anhydrase (2.01 nm), ovalbumin (2.83 nm), bovine serum albumin (3.62 nm), alcohol dehydrogenase (4.55 nm) and thyroglobulin (8.6 nm). The native and heat-treated egg extracts were both dialyzed at  $4^{\circ}\text{C}$  against column buffer containing 1 mM PMSF before application to the column in order to remove low molecular weight material which otherwise obscured the total volume peak.

### ***Polyclonal antiserum preparation***

A polyclonal antiserum against heat-stable Lv was prepared according to Kunkel ('88). In brief, a male New Zealand white rabbit was subcutaneously injected with 125  $\mu\text{g}$  heat-stable Lv in a 1:1 emulsion of Freund's complete adjuvant with PBS (0.15 M NaCl, 0.10 M  $\text{NaH}_2\text{PO}_4$ , 0.05% EDTA, pH 7.0). The rabbit was boosted after a month with a subcutaneous injection of 125  $\mu\text{g}$  heat-stable Lv in a 1:1 emulsion of Freund's incomplete adjuvant and PBS, and, after another month, with five daily intravenous injections of 50  $\mu\text{g}$  heat-stable Lv in PBS. Blood was collected from the central auricular artery 1 week after the last booster injection, following anesthetization and vasodilation by droperidol-fentanyl injection (Tillman and Norman, '83). The blood was allowed to clot for approximately 1 hour at  $37^{\circ}\text{C}$ , then was incubated at  $4^{\circ}\text{C}$  overnight to allow maximal clot contraction. The remaining blood cells were then removed from the serum by centrifugation, and 0.2% sodium azide was added.

### ***Polyacrylamide gel electrophoresis and Western blot***

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemli ('70), using mini-gels (100  $\times$  80  $\times$  0.75 mm) consisting of 4% and 10% acrylamide for the stacking and separating gels, respectively (10.2:1 acrylamide:bis), and cast in a Bio-Rad (Melville, NY) Mini-Protean II cell. The samples were heated in a boiling water bath for 4 minutes in Laemli sample buffer. Molecular weight standards were obtained from Bio-Rad (Broad Range Standards). Proteins were resolved at constant voltage (150 V), and the gels stained with 0.1% Coomassie Blue R-250 (Fisher, Orangeburg, NY) in 40% methanol, 10% acetic acid. Destaining was in 40% methanol, 10% acetic acid.

Western blot analysis was performed on proteins separated by SDS-PAGE as described above. The polypeptides were electrophoretically transferred to a nitrocellulose membrane (Micron Separations, Inc.) overnight at  $4^{\circ}\text{C}$  using a Bio-Rad Trans Blot Cell. The blot was blocked with 3% gelatin in Tris buffer (0.02 M Tris, 0.5 M NaCl, pH 7.5), incubated with rabbit anti-Lv antiserum (1:10,000) as primary antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, 1:2,500) as secondary antibody, and was developed with 0.5% (w/v) 4-chloro-1-naphthol (Bio-Rad) and 0.015% (v/v) hydrogen peroxide in Tris buffer/methanol 6:1. Each antibody incubation was followed by three washes in Tris buffer.

### *Immunoassays*

Two-dimensional double-diffusion was modified from the method of Ouchterlony ('49) for use on microscope slides (Kunkel and Lawler, '74). The gel was incubated for 43 hours at 4°C in a humid chamber (to prevent gel desiccation) while precipitin lines were allowed to form.

ELISA was performed by dot blotting 100 µl aliquots of the protein solutions (in PBS) onto a nitrocellulose membrane (Bio-Rad Dot Blot apparatus). The dot blots were washed twice with 100 µl PBS, and the nitrocellulose membrane was removed from the blotting apparatus. The membrane was blocked with 1% gelatin in PBS, and incubated overnight in anti-Lv antiserum (1:4,000 in blocking solution) at room temperature. Following three washes in PBS, the membrane was incubated in horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3,000 in blocking solution). After three additional washes in PBS, the membrane was developed with 4-chloro-1-naphthol and hydrogen peroxide for 15–30 minutes as described above for Western blots, substituting PBS for Tris buffer. The color reaction was quantified by densitometry as follows. The developed nitrocellulose membrane was imaged with a video camera (MTI CCD71), using a Matrox PIP-640 frame buffer board to grab the image. Densitometric integration of individual dots was performed with custom software which used a ring six pixels wide about each dot to define the background. Lv standards for this assay were prepared by gel permeation chromatography of unheated egg extracts, as described above.

### *Affinity purification of anti-lipovitellin antiserum*

A portion of the anti-Lv antiserum was affinity-purified against the 94-kD Lv polypeptide by a modification of the method of Olmsted ('81). In this technique, the 94-kD Lv polypeptide was resolved in whole unfertilized egg extract by SDS-PAGE as described above, except that a preparative comb was used in the stacking gel, allowing one sample to be placed across most of the width of the gel, with one additional well for standards. Proteins were then electrophoretically transferred to a nitrocellulose membrane as previously described. The membrane was equilibrated in PBS, blocked with gelatin (3% in PBS), allowed to air dry, and cut into strips 6 mm wide (running the length of the gel). One of the strips was developed as a Western blot (as described above) in order to determine the location of the 94-kD polypeptide.

A second 6-mm strip, adjacent to the first, was incubated with the anti-lipovitellin antiserum (diluted 1:50 in PBS containing 1% gelatin). The strip was then allowed to air dry, and the section containing the 94-kD polypeptide (as determined against the developed strip) was cut. This section was further cut into four pieces. These small pieces of nitrocellulose membrane were placed in a microcentrifuge tube and washed three times in PBS. The bound antibody was then eluted with three 1 ml washes (3 minutes each) of ice-cold elution buffer (0.2 M glycine-HCl, pH 2.5); each wash was immediately mixed with 83 µl of 0.4 M Tris-HCl, pH 8.0. The washes were then pooled and mixed with an additional 2.5 ml of 0.4 M Tris-HCl, pH 8.0, yielding a pH of about 7.5.

A third 6-mm strip of the original blot was incubated in a solution containing the pooled eluates (5.2 ml) plus 2.6 ml of 3% gelatin in Tris buffer; this yielded a solution containing 1% gelatin, 0.55 M NaCl, 0.12 M Tris, 0.073 M glycine, pH 7.5. The strip was then washed three times in Tris buffer, and incubated with secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase; Bio-Rad) diluted 1:500 in Tris buffer containing 1% gelatin. Following three washes in Tris buffer, the strip was developed with 3-chloro-1-naphthol (Bio-Rad) and hydrogen peroxide as described for Western blots.

## RESULTS

Extracts of winter flounder ovulated, unfertilized eggs were examined by SDS-PAGE (Fig. 1). One 94-kD polypeptide, presumed to be Lv based on its size and abundance, was predominant, though bands of 60 kD, 24 kD and several faint bands were also observed. Gel permeation chromatography of egg extract on Bio-Gel A-1.5 yielded one absorbance peak between the void and total volume peaks (Fig. 2). When this peak was examined by SDS-PAGE, however, a heterogeneous population of polypeptides nearly identical to those found in whole extract was observed (Fig. 1). A slight shoulder present on the left side of the Lv peak was found to be enriched in these smaller polypeptides relative to the Lv peak itself (not shown).

The 94-kD polypeptide was soluble, relative to these smaller yolk polypeptides, in high salt (0.4 M KCl) extraction buffer following a brief heat treatment at 85°C (Fig. 1). This property was used to purify the 94-kD polypeptide from egg extract. Following heat treatment, the soluble extract fraction was further purified by gel permeation chro-

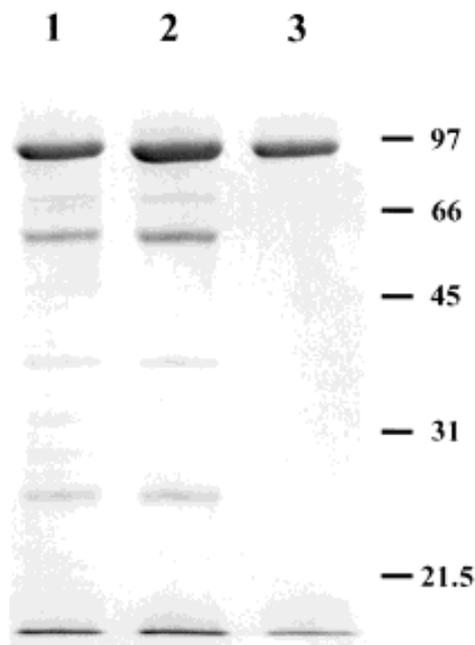


Fig. 1. Purification of winter flounder Lv. SDS-PAGE of: **lane 1**, total egg extract (7  $\mu$ g); **lane 2**, gel permeation peak of egg extract (7  $\mu$ g); **lane 3**, gel permeation peak of heat-treated egg extract (4  $\mu$ g). Molecular weight markers are indicated at right.

matography, yielding a Lv preparation which was nearly homogeneous on SDS-PAGE (Fig. 1). (We refer to this Lv fraction as “heat-stable” to indicate its greater stability than the “heat-labile” fraction, which precipitated under these heating conditions.).

The gel permeation profile of heat-stable extract showed a slight shift in the Lv peak towards the total volume peak, relative to untreated egg extract, and complete absence of a shoulder to the left of the peak (Fig. 2). In order to resolve the heat-labile peak from the larger Lv peak, we overlaid the Bio-Gel A-1.5 profiles of native and heat-stable Lv and, by subtraction of the heat-stable Lv profile from that of native Lv, obtained an image of the Lv peak which comprises the shoulder (Fig. 2).

Native and heat-stable extracts were run on a gel permeation column against standards, and the Stokes radii were determined to be 4.50 nm and 4.26 nm for native and heat-stable Lv, respectively (Fig. 3). The heat-labile peak determined in Figure 2, though asymmetric, has a larger mean Stokes radius estimated at 5.17 nm (Fig. 3).

Heat-stable LV was used to produce a rabbit polyclonal antiserum. This antiserum was characterized by Ouchterlony double diffusion, in

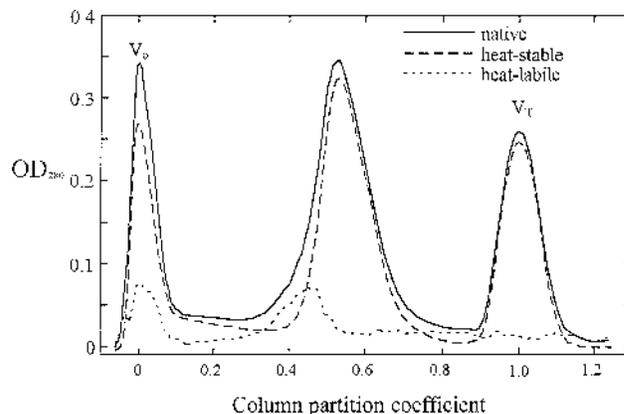


Fig. 2. Bio-Gel A-1.5 gel permeation chromatography of native and heat-stable egg extracts. The smooth elution profiles of the native (n) and heat-stable (hs) extracts were estimated by cubic spline interpolation of the observed profiles within the MatLab for Windows software environment. The heat-labile (hl) curve was then obtained as  $hl = n - (1.12)hs$ . The coefficient, 1.12, was adjusted to extinguish the main peak of the native Lv profile leaving the residue of the heat-labile shoulder.  $V_0$  = void volume peak,  $V_T$  = total volume peak.

which heat-stable Lv showed complete identity with a component of whole egg extract and of vitellogenic female winter flounder serum (Fig. 4). No precipitin line was observed with male serum.

A Western blot utilizing this antiserum also showed no reaction with male serum (Fig. 5). The antiserum did, however, recognize one polypeptide of 175 kD in vitellogenic female serum. Numer-

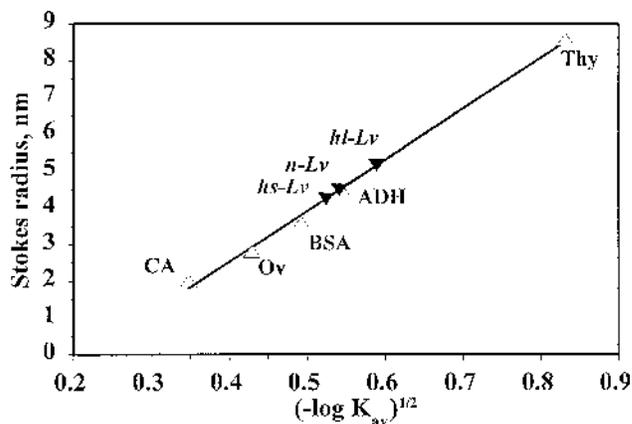


Fig. 3. Stokes radius determination of native (n-LV), heat-stable (hs-LV), and heat-labile (hl-LV) Lv (filled triangles), obtained by plotting Stokes radius versus the square root of the negative log of the column partition coefficient,  $K_{av}$ , of calibration standards. The standards (open triangles) were: CA, carbonic anhydrase (2.01 nm); Ov, ovalbumin (2.83 nm); BSA, bovine serum albumin (3.62 nm); ADH, alcohol dehydrogenase (4.55 nm); and Thy, thyroglobulin (8.6 nm). Note that native Lv overlaps the alcohol dehydrogenase standard.

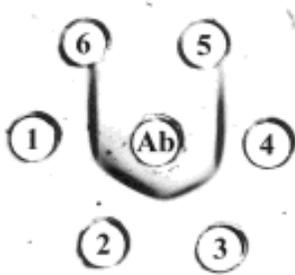


Fig. 4. Ouchterlony double immunodiffusion of Vg and Lv using anti-Lv antiserum (Ab). 1, gel permeation peak of heat-stable extract (3  $\mu$ g); 2, whole egg extract (10  $\mu$ g); 3 and 4, vitellogenic female serum (two different individuals, 16  $\mu$ g and 17  $\mu$ g, respectively); 5 and 6, male serum (two different individuals, 13  $\mu$ g and 16  $\mu$ g, respectively).

ous minor bands smaller than the 94-kD polypeptide in whole egg extract also cross-reacted with the antiserum. Small amounts of antiserum affinity purified against the 94-kD polypeptide cross-reacted with the same minor bands of native Lv (not shown), indicating that these minor bands are immunologically related to the heat-stable Lv major polypeptide, and are not reacting with antibodies specific to contaminants present in the immunizing Lv preparation.

The anti-Lv antiserum was used to construct an homologous ELISA for quantification of winter flounder Vg and Lv. In this assay, protein solutions were dot blotted onto nitrocellulose, developed using horseradish peroxidase-conju-

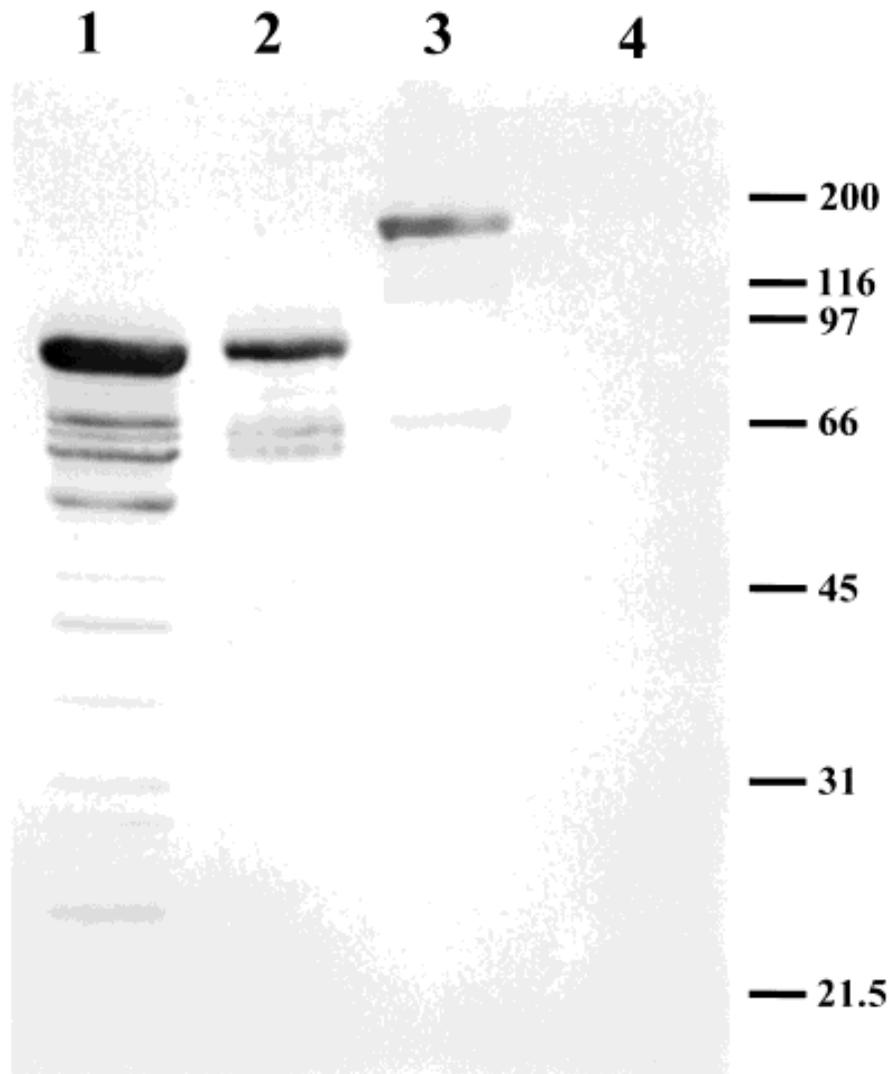


Fig. 5. Western blot of winter flounder egg extracts and sera with anti-Lv antiserum. **Lane 1:** whole egg extract (7  $\mu$ g); **lane 2,** gel permeation peak of heat-stable extract (3  $\mu$ g);

**lane 3,** vitellogenic female serum (12  $\mu$ g); **lane 4,** male serum (12  $\mu$ g). Molecular weight standards are indicated at right.

TABLE 1. Sample arrangement for dot blot ELISA<sup>1</sup>

2	3	1	4	7	8	6	1
15	10	8	9	3	16	10	5
16	11	13	6	11	4	9	1
14	12	5	7	12	2	15	1
1	15	14	16	10	1	11	1
6	9	12	3	9	7	16	2
4	13	7	10	13	14	3	8
8	5	2	11	5	6	4	1
							5

<sup>1</sup>Four replicates of sixteen samples are shown as arranged on a nitrocellulose membrane (adapted from design SR40 of Bose et al., '54).

gated secondary antibody and 4-chloro-1-naphthol; the color development was then quantified by densitometry. In order to compensate for differences in light intensity across the nitrocellulose membrane during densitometry, the samples were blotted in quadruplicate according to a design adapted from Bose et al. ('54; Table 1). This pattern successfully compensates for position-related changes in the relative absorbance of individual samples (not shown). The ELISA standard curve depicted in Figure 6 is linear, with an  $r^2$  of 0.990 for Lv between the amounts of 2.5 and 30 ng per 100  $\mu$ l sample. This figure is representative of six experiments, though some variation in color intensity occurred among individual experiments. For this reason, at least four Lv standards were included on each membrane.

The ELISA was used to identify Vg in a gel permeation chromatography profile of serum from an estradiol-injected male winter flounder (Fig. 7). Two peaks were observed between the void volume and total volume peaks, one of which coincided with the single peak detected in ELISA analysis of the eluted fractions. In addition, the

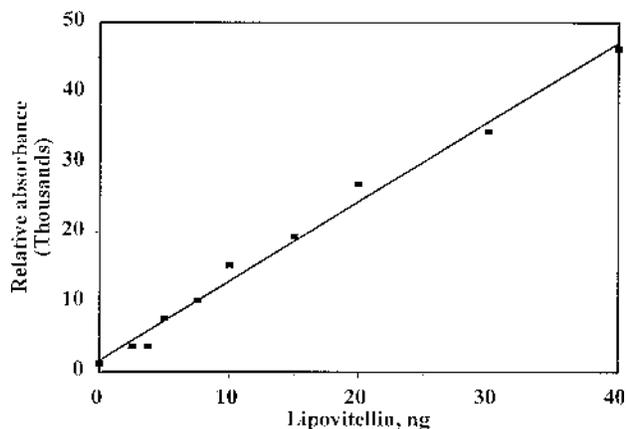


Fig. 6. ELISA standard curve for winter flounder Lv. Each point represents the mean of four samples.

anti-Lv antiserum bound a small amount of material associated with the void volume peak, indicating that a slight degree of Vg aggregation occurred during sample storage and processing. The Stokes radius of the Vg peak was determined against standards, and found to be 6.70 nm. This large size suggests that the 175 kD Vg polypeptide is present as a homodimer in serum.

## DISCUSSION

In extracts of ovulated, unfertilized eggs, we found that lipovitellin is present as a mixture of heat-stable and heat-labile molecules. The relative heat stability of the 94-kD Lv polypeptide allows a rapid and convenient approach for yolk protein purification. Since Lv comprises the vast majority of protein in mature flounder eggs (Fig. 1), mature eggs provide an abundant and easily accessible supply of Lv for immunoassay development and standardization. Using the purified heat-stable Lv, we developed such an immunoassay for detection and quantification of Vg and Lv in serum and in unfertilized eggs, respectively, of winter flounder. This approach circumvents many of the difficulties associated with Vg purification and storage which other investigators have encountered during immunoassay development (Tao et al., '93; Silversand et al., '93; Specker and Sullivan, '94). This relative ease of purifying and storing Lv rather than Vg may reflect on the functions of these molecules, since Vg serves only to transport yolk material from the liver to developing oocytes, while Lv exists as a longer-term storage molecule within the yolk.

In extracts of mature, ovulated eggs examined by SDS-PAGE (Fig. 1), three major yolk polypeptides were observed, of 94 kD, 60 kD and 24 kD, along with several minor bands. The 94 kD protein subunit was predominant, and probably corresponds to a reported 94.4 kD Vg-derived polypeptide in oocytes from the coelom of midvitellogenic winter flounder ovaries (Nagler and Idler, '90). The predominant Vg-related polypeptide in such oocyte preparations, however, is of 101.4 kD (Nagler and Idler, '90). No band of this size was present in ovulated egg preparations (Fig. 1), suggesting that the 101.4-kD polypeptide is proteolytically processed to a 94-kD form in the course of oocyte maturation. Such processing events have been reported in the maturing oocytes of several other fishes (Wallace and Begovac, '85; Wallace and Selman, '85; Greeley et al., '86; Carnevali et al., '92).

A yolk protein not originating from Vg has also

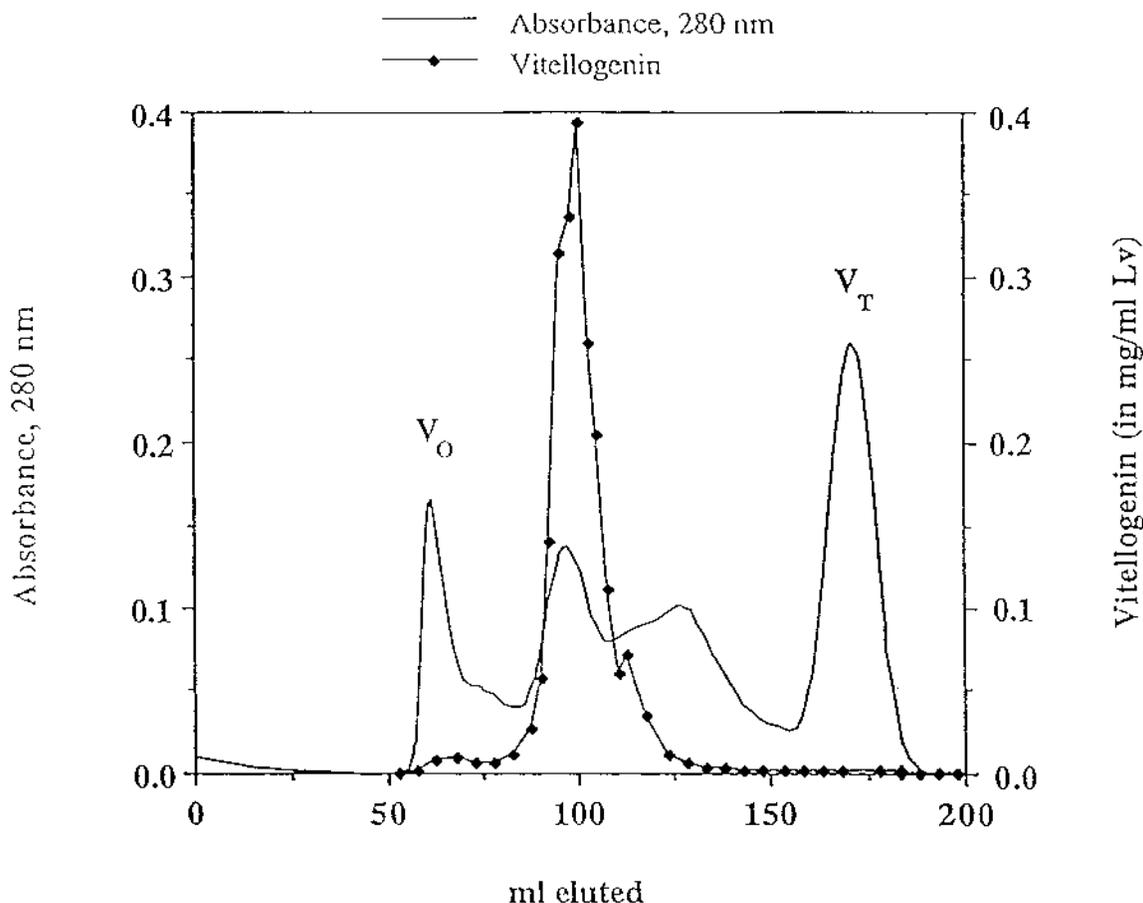


Fig. 7. Elution profile of estradiol-treated male winter flounder serum (15 mg total protein) on Bio-Gel A-1.5.  $V_O$ , void volume peak;  $V_T$ , total volume peak. Left axis, optical

density at 280 nm; right axis, column fractions (2.5 ml) probed for Vg by ELISA using anti-Lv antiserum. The right axis unites (mg/ml) are standardized for Lv.

been described in winter flounder midvitellogenic oocytes. This polypeptide, which runs at 68.7 kD on SDS-PAGE (Nagler and Idler, '90), derives from a serum glycolipophosphoprotein of 1,170 kD native molecular mass (Nagler and Idler, '87), termed VHDL II (Nagler and Idler, '90). Although, in terms of mass, Vg and VHDL II are incorporated into oocytes at approximately equal levels, the yolk protein derived from VHDL II appears to be present in oocytes in smaller quantity than the Vg-derived proteins (Nagler and Idler, '90). In mature, ovulated eggs, we did not observe this 68.7-kD polypeptide (Fig. 1). Although such a band might be obscured by a 67-kD polypeptide present in egg extracts, which is not present in midvitellogenic oocytes (Nagler and Idler, '90), it is clear that, in ovulated eggs, the vast majority of extracted protein is derived from Vg, as demonstrated by Western blots using anti-Lv antiserum (Fig. 5).

Gel permeation chromatography of untreated egg extracts yielded one absorbance peak between the void volume and total volume peaks (Fig. 2). This peak consisted of multiple polypeptides, similar to whole egg extract, with the 94-kD band predominant (Fig. 1). A shoulder present to the left of this peak was enriched in polypeptides smaller than 94 kD (not shown). Heat-stable extract, however, lacked both the shoulder on the absorbance peak (Fig. 2) and these smaller polypeptides (Fig. 1). Nonetheless, the polyclonal antiserum raised against heat-stable Lv reacts with these smaller bands in Western blots of native egg extract (Fig. 5), even following affinity purification of the antiserum against the 94-kD polypeptide (not shown). Taken together, these data suggest that the polypeptides smaller than 94 kD observed in SDS-PAGE of native extracts are the products of proteolytic nicking of the 94-kD Lv polypeptide.

The proteolytic cleavage of Lv in native extracts

is not an artifact produced by freezing the eggs for storage, since extracts of unfrozen eggs produce an identical electrophoretic profile on SDS-PAGE (not shown). These results, however, are consistent with the complex electrophoretic patterns observed in extracts of mature eggs of other fishes, where yolk proteins have undergone proteolytic processing in the course of final oocyte maturation (Wallace and Begovac, '85; Wallace and Selman, '85; Greeley et al., '86; Carnevali et al., '92, '93). Since, as we observed in winter flounder eggs, the nicked Lv is less stable than the intact 94-kD Lv in vitro, it is possible that such proteolytic activity may render a portion of the stored Lv more accessible to degradation and utilization by the developing flounder.

Proteins which have undergone proteolytic nicking often appear larger than the native molecule upon gel permeation chromatography, perhaps because the nicked ends of such polypeptides extend outward from the native conformation of the molecule. In order to determine if this explanation is consistent with the apparent size of the Lv molecules in this shoulder, we overlaid the Bio-Gel A-1.5 profiles of native and heat-stable Lv and, by subtraction of the heat-stable Lv profile from that of native Lv, obtained an image of the Lv peak which comprises the shoulder (Fig. 2). This minor peak, though asymmetric, has a mean Stokes radius estimated at 5.17 nm, which is substantially larger than the Stokes radius of the heat-stable Lv, 4.26 nm. This substantial shift in Stokes radius suggests that if the change in molecular conformation of nicked Lv is due solely to the extension of cleaved ends of the polypeptide away from the native configuration of Lv, such extension must be considerable, increasing the longest axis of the protein by approximately 50%. Thus, it is more likely that the nicked ends of the Lv polypeptide facilitate dimerization of these molecules. Since Lv has been described as an elliptical molecule in other vertebrates (reviewed by Banaszak et al., '91), its dimerization along the long axis of the molecule could yield an increase in Stokes radius consistent with the shift we observed.

The polyclonal antiserum raised against heat-stable Lv reacts strongly with the native protein in egg extract, as demonstrated by Ouchterlony double diffusion (Fig. 4). As anticipated, this antiserum also binds Vg, the plasma precursor of Lv (Figs. 4 and 5). Such recognition is specific, as demonstrated by Western blotting, where the antiserum reacts with a female-specific serum polypeptide of approximately 175 kD, but does not

bind any component of male serum (Fig. 5). This 175-kD polypeptide corresponds to a female-specific serum polypeptide of 180 kD from winter flounder previously reported to be Vg (Nagler and Idler, '87).

The ELISA constructed using this antiserum produced a linear standard curve between LV concentrations of 25 and 300 ng/ml. Although this assay was developed for Lv, the specificity of the polyclonal antiserum for Vg in winter flounder serum makes it suitable for Vg quantification as well. This specificity was used to characterize the gel permeation profile of serum from an estradiol-treated male winter flounder. The Stokes radius of the Vg peak identified by ELISA was determined against standards to be 6.70 nm, which is consistent with a dimer of the 175-kD polypeptide observed on SDS-PAGE.

Conversion of Lv from a dimer in immature oocytes to a monomer in mature, ovulated eggs has recently been implicated in barfin flounder (Matsubara and Sawano, '95) and in Japanese flounder (Matsubara et al., '95). While it is unknown whether Lv is a monomer or dimer in winter flounder oocytes prior to maturation, our results suggest that Vg is a dimer prior to its uptake into oocytes, and that processing of this protein eventually yields predominantly monomeric Lv in the mature egg.

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