

Vitellogenin isolation, purification and antigenic cross-reactivity in three teleost species

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Running title: Vitellogenin characterisation in three teleosts

Abstract

Vitellogenin (vtg) was isolated from greenback flounder (*Rhombosolea tapirina*), rainbow trout, (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) plasma. The molecular weight of each native molecule, as determined by gel filtration, was 540, 383 and 557 kD respectively. With SDS-PAGE under reducing conditions, rainbow trout vtg dissociated into 1 major subunit (~154 kD), and Atlantic salmon and greenback flounder vtg dissociated into 3 major subunits (~ 159, 117, 86 kD and 155, 104, 79 kD respectively). Polyclonal antisera, produced against only the highest molecular weight subunit from each species following excision of these bands from reducing gels, were reactive with all subunits in Western blots. These antisera were found to be highly species-specific as determined by competition ELISA. No cross-reactivity was noted, even between the two salmonid species. These data suggest that excision of bands from gels is a simple procedure for the preparation of highly specific antisera, and that cross-species assays employing heterologous antisera are unlikely to be possible.

Key words: Vitellogenin, Atlantic salmon; Rainbow trout; greenback flounder; cross-reactivity

1. Introduction

Vitellogenin (vtg) is a high molecular weight (250 – 600 kD) glycopospholipoprotein synthesised in the liver in response to the steroid hormone 17- β estradiol (E_2)(reviewed in Specker and Sullivan, 1994). It is secreted into the bloodstream from where it is taken up by developing oocytes and cleaved into the two yolk proteins, lipovitellin and phosvitin (Tyler et al., 2000). Male fish also possess the vtg gene but expression is not normally found, due to the absence of substantial levels of circulating estrogens (Harries et al., 1997). However, in response to exogenous E_2 treatment (Yao and Crim, 1996) or in response to estrogen analogues in the environment (Harries et al., 1997; Metcalfe et al., 2001), vtg secretion can increase dramatically. Measurement of vtg is therefore useful in female fish to assess reproductive condition (Specker and Sullivan, 1994) and in male fish to screen for potential endocrine disrupting xenobiotics (Harries et al., 1997; Nilsen et al., 1998).

Vtg is considered to be an ancient protein for which, in most species studied, the genes belong to multigene families (Chen et al., 1997). Some regions, notably the E_2 receptor binding region, are highly conserved (Bidwell and Carson, 1995). Analysis of teleost vtg genes has identified from one (La Fleur et al., 1995) to several (Lee et al., 1994; Trichet et al., 2000; Wang et al., 2000) distinct vtg genes, which may give rise to different forms of vtg. Structurally, vtg, has most commonly been described as a dimeric molecule consisting of identical monomers (Mosconi et al., 1998). However, in some instances two vtg subunits, differing in molecular weight have been described (reviewed in Specker and Sullivan, 1994), and the presence of three to four subunits has been suggested in some early work (reviewed in Utarabhand and Bunlipatanon, 1996). These differences may be genetically based or be the result of post-translational modification.

Vtg is usually measured by radioimmunoassay, or by ELISA using a specific antibody raised against purified vtg. Because some regions of the gene are highly conserved it is reasonable to expect that antibodies may be reactive across species. Most commonly polyclonal antisera are prepared by inoculation of native vtg (Heppell and Sullivan, 1999; Mosconi et al., 1998; Nilsen et al 1998; Mourot and Lebail 1995) and monoclonal antibodies have also been developed (Nilsen et al., 1998; Heppell et al., 1995). However, the activity of these antibodies does not appear to follow a predictable pattern, as polyclonal antisera are not cross-reactive with all species which have been tested, despite being directed towards multiple epitopes (Yao and Crim, 1996; Mourot and Lebail 1995; Heppell et al., 1995), and certain monoclonals are cross-reactive, even though they recognise a single antigenic epitope (Nilsen et al., 1998; Heppell et al., 1995).

The aim of the present work was to further study this phenomenon with polyclonal antisera prepared against a highly purified antigen, with a view to clarifying whether cross-reactive epitopes are present under these conditions and hence whether a generic assay for vtg is readily achievable. Accordingly antigen was purified, by a simple process previously developed within our laboratory, and used to develop highly specific polyclonal antisera (Watts et al., 2001). Vtg was first extracted from plasma of E₂-treated males and then subjected to further purification by reduction on SDS-PAGE gels. Isolated protein bands were then excised from these gels and used as immunogens to raise antisera against vtg from two salmonids, rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*), and a flatfish, greenback flounder (*Rhombosolea tapirina*). It was considered that the phylogenetic closeness of the two salmonids would maximise the likelihood of cross-reactivity occurring

whereas the greenback flounder would test whether the relationship held across more phylogenetically separate taxa.

2. Materials and Methods

2.1 Samples

Male fish, held at the School of Aquaculture, University of Tasmania in re-circulating systems connected to 'Aquahort' temperature control systems, were used for the experiments. Holding temperatures were 12° C (greenback flounder), 15° C (rainbow trout) and 14° C (Atlantic salmon). Both rainbow trout and Atlantic salmon were three years old whereas greenback flounder were two years old. Rainbow trout were injected with either saline (one fish) or saline suspension of E₂ (four fish) at a dose of 5 mg kg⁻¹ body weight. Four greenback flounder and four Atlantic salmon, were each divided into two equal treatment groups and injected with either saline, or a saline suspension of E₂ at a dose of 2 mg kg⁻¹ body weight. Atlantic salmon were injected twice at an interval of one week, and rainbow trout and greenback flounder were injected three times at weekly intervals. Injection volume was 1ml kg⁻¹ body weight for all three species. All fish were bled one week after the last injection into heparinised syringes. Following centrifugation, the plasma fractions were treated with a saturated solution of the serine protease inhibitor phenylmethyl-sulphone (PMSF: Sigma) at a ratio of 2:1 v/v plasma PMSF, then frozen at -20° C for storage.

2.2 Isolation of vtg and molecular weight determination

One ml of each plasma: PMSF sample was diluted 1:1 with elution buffer (0.05 M Tris base, 0.15 M NaCl, pH 8.0) containing 5% glycerol, and applied to the top of a Sephacryl HR 300

(Amersham Pharmacia Biotech AB) 900 x 16 mm column. A molecular weight standards kit containing: blue dextran (2000 kD), thyroglobulin (669 kD), apoferritin (443 kD), alcohol dehydrogenase (150 kD), β -amylase (200 kD), albumin (66 kD) and carbonic anhydrase (29 kD) (Sigma) was used for calibration. The samples were eluted with elution buffer at a flow rate of 10 ml h⁻¹ and collected as 2 ml fractions. The absorbance of each fraction was measured at Abs₂₈₀. Molecular weights of peak fractions were calculated from the linear fit of the standard curve of log molecular weight of standards v V_e/V_o value, where V_e = elution volume and V_o = void volume determined by blue dextran.

Putative vtg fractions, identified by their presence in E₂ but not saline injected fish, were further purified and molecular weight of subunits determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in reducing gels immediately after elution from the gel filtration column. Fractions, at a concentration of 1.0 mg ml⁻¹ protein, were diluted 1:1 in 0.125 M Tris base, 4% SDS, 20% glycerol, 10% mercaptoethanol (sample buffer) and boiled for 4 min to reduce. Electrophoresis was carried out in 10% gels (Bollag et al., 1996) in a Mighty Small SE 250 vertical electrophoresis unit (Hoefer) for 1 h in 25 mM Tris base, 192 mM glycine, 0.1% SDS, pH 8.3. Wide Range Protein Standards (Mark 12 Novex) were included for molecular weight determination. The gels were fixed and stained with 0.025% Coomassie brilliant blue, 50% methanol, 10% acetic acid for 1 h, then destained in 50% methanol 10% acetic acid, and washed for up to 24 h in several changes of distilled water. The molecular weight of the reduced proteins was calculated from the 2nd order polynomial equation fitted to the curve of relative mobility (Rf) v log₁₀ molecular weight of the standard proteins.

2.3 Antibody production

Antigen was prepared from the highest molecular weight vtg protein band excised from reducing gels (Watts et al., 2001) for each fish. Briefly 100 µl of vtg, reduced as before, was loaded into preparative wells formed in four 5 % stacking gels over 10% SDS-PAGE resolving gels, electrophoresed, fixed and stained as previously described. Following extensive rinsing in distilled water, the high molecular weight bands located at ~ 155 kD (greenback flounder), ~154 kD (rainbow trout) and 159 kD (Atlantic salmon) were excised from the gels and liquefied in a glass-glass tissue grinder (Fischer) in 4 ml 0.008 M Na₂HPO₄, 0.002 M NaH₂PO₄, 0.15 M NaCl pH 7.4 (PBS).

Two sheep were inoculated subcutaneously, with vtg from each species, at multiple sites, with 1 ml of liquified, reduced vtg:gel mixture added to 1 ml adjuvant (Montanide ISA 50: Tall Bennett, Australia). Four inoculations were given in total, comprising an initial inoculation followed by a booster one month later and then two subsequent boosters at intervals of two weeks. A pre-immunisation bleed was taken from the sheep for use as a negative antibody control. All blood was allowed to clot at room temperature and placed at 4°C for clot retraction to take place. Serum was separated, aliquotted and stored at -20°C.

2.4 Antiserum reactivity

Western blots. Purified vtg from each fish species was diluted, reduced and electrophoresed as before. Following electrophoresis, gels were equilibrated by soaking for 10 min in 48 mM Tris, 39 mM glycine 20% methanol pH 9.2 (transfer buffer). After equilibration, antigens were transferred from gels to 0.45 µm nitrocellulose membrane (Biorad) in a Hoefer Semi-

Phor Semi-dry transfer unit at 0.8 mA cm^{-2} for 1.5 h (Biorad Bulletin 1721). Following transfer, immunoblots were blocked for 1 h in 20 mM Tris-HCl, 0.5 M NaCl, pH 7.2 (TBS) with 5% skim milk (TBSB). Antigen recognition was carried out for 1 h with sheep antisera at a dilution of 1:1000 in TBS with 3% skim milk (TBSD). Bound antibodies were detected with alkaline phosphatase anti-sheep conjugate (Sigma) diluted 1:30,000 in TBSD. The complex was visualised by development in BCIP/NBT substrate (Biorad kit). As negative antibody controls pre-immune sheep sera were used. Multimark Multi-Coloured Standards (Novex) were used for determination of transfer efficiency.

Determination of cross-reactivity. Optimal concentrations of antigen and antibody were determined by chequerboard titrations (Crowther, 1995). An antigen competition ELISA was developed to assess antigenic cross-reactivity as follows. Wells of microtitre plates (Greiner) were coated with 100 μl volumes containing $2.5 \mu\text{g ml}^{-1}$ of purified vtg antigen in 50 mM sodium carbonate (pH 9.6) (one plate per antigen). In separate plates (termed dilution plates), duplicate serial dilutions of every purified vtg were prepared in TBSD using 100 μl volumes. To each dilution plate 100 μl volumes per well of either rainbow trout, Atlantic salmon or greenback flounder vtg antiserum, were then added diluted in TBSD to give final concentrations of 1: 1000 (rainbow trout and Atlantic salmon vtg antisera), 1:100 (greenback flounder vtg antiserum), and dilutions from 40 to 40,000 ng ml^{-1} for each vtg. All plates were incubated overnight, with shaking, at 4°C . Following incubation, antigen coated plates were washed x4 (Immunowash: Biorad), with 300 μl volumes of TBS. After washing, unreacted sites on coated wells were blocked for 1 h at 37°C with 300 μl volumes of TBSB. Following blocking, wells were drained, then 100 μl volumes were transferred from each well of the corresponding dilution plate to wells on antigen coated, blocked plates. Incubation was carried out for 1 h at 37°C with shaking and then plates were washed x4 as before. The

vtg/antiserum complex was labelled with 100 µl per well of donkey anti-sheep antiserum conjugated with alkaline phosphatase (Sigma) diluted to 1:10,000 in TBSD. After a 1 h incubation at 37°C wells were washed as before and then 100 µl of 1mg ml⁻¹ para-nitrophenylphosphate in 0.01% MgCl₂ 10% diethanolamine buffer pH 9.8, were added to each well. Following incubation for 1 h at 37°C for greenback flounder and Atlantic salmon antiserum, or overnight at 4°C for rainbow trout antiserum, absorbance was read at A₄₀₅. Wells negative for primary antibody were used to assess non-specific binding (nsb), and maximum antibody binding was determined from wells with zero standard. Percent binding $[(A_{405}\text{standard}-\text{nsb})/(A_{405}\text{zero standard}-\text{nsb}) \times 100]$ was plotted against log₁₀ vtg concentration.

3. Results

3.1 Isolation of vtg and molecular weight determination

A major protein peak corresponding to the theoretical value for fish vtg (Nath, 1999) was identified by gel filtration in plasma from each of the E₂ inoculated fish and was absent from saline treated fish (fig 1 a, b and c). Preceding the putative vtg peak, there was a high molecular weight peak which, in rainbow trout plasma from E₂ inoculated fish, was significant. However, this peak was also present, but to a lesser extent, in plasma from saline inoculated fish; a similar result was seen with Atlantic salmon, although the peaks were quite small. In flounder however, this peak may have been E₂ inducible, as it was not present in the saline inoculated fish. However, the molecular weight of this peak (either in the native, or reduced state, fig 2 lane 8) is not consistent with what would be expected for vtg (Nath, 1999).

Under reducing SDS-PAGE the putative vtg resolved into various subunits (fig 2). For rainbow trout one dominant, and several minor high molecular weight bands were present; for Atlantic salmon and greenback flounder putative vtg three distinct high molecular weight bands were present. A high molecular weight subunit approximately 152-157 kD was common to each species, and in rainbow trout this was the dominant band. Atlantic salmon and rainbow trout plasma shared a similar molecular weight protein of between 110-117 kD and greenback flounder and rainbow trout shared a band at ~102 – 103 kD. A common band at between 76 – 87 kD was also present in each species (Table 1).

3.2 Reactivity of antisera

In Western blots of 10 % reducing gels three major high molecular weight vtg bands were reactive with the corresponding antiserum for each species (Table 1). These bands corresponded to the three highest molecular weight bands demonstrated by Coomassie brilliant blue protein stain in reducing SDS-PAGE. Other, lower molecular weight proteins, which were present in rainbow trout and Atlantic salmon vtg, were slightly reactive with homologous antiserum and were therefore most likely attributable to vtg breakdown products.

By competition ELISA, each antiserum was highly reactive with its specific antigen (fig 3 a, b and c). A slight cross-reactivity was noted between antiserum to Atlantic salmon vtg and purified rainbow trout vtg (fig 3b) but neither of the other two antisera was reactive with any purified vtg other than that to which it had been produced (fig 3a and c).

4. Discussion

Gel filtration demonstrated that plasma from male fish from all three species that had been inoculated with E₂ contained a high level of protein with a molecular weight close to that described for vtg in other species (fig 1) (Nath, 1999; Yao and Crim, 1996). This peak was absent from saline-injected fish indicating that this protein fraction was E₂-inducible and therefore reasonably identifiable as putative vtg. The molecular weight estimates obtained for these native vtgs are within the range compared with other gel filtration estimates for teleosts (reviewed in Specker and Sullivan, 1994). However, the value we obtained for rainbow trout vtg (340 kD) is low compared with the literature value (550 kD) (Specker and Sullivan, 1994). This may be explained either by the potential lack of precision of the molecular weight estimation method or by the extremely labile nature of the native vtg molecule, which has been shown to be susceptible to proteolysis during isolation (Silversand et al., 1993). It is possible that some degradation could have occurred during the isolation procedure.

In the present study, purified vtg dissociated into different subunits under SDS-PAGE in reducing conditions (fig 2), suggesting a different structure for vtg in each species. Three major molecular weight subunits were found for both greenback flounder and Atlantic salmon vtg. In most species vtg is composed of monomers or dimers (Matsubara et al., 1999; Specker and Sullivan, 1994), but vtg of goldfish (*Carassius aurata*) and Japanese eel (*Anguilla japonica*) also appear to be composed of from three to four subunits (reviewed in Utarabhand and Bunlipatanon, 1996). Only one dominant subunit was found in rainbow trout, in agreement with a previous description of vtg for this species (Silversand et al., 1993), but several minor bands were present which may also be attributable to vtg.

Immunostains with homologous antisera to the Atlantic salmon and greenback flounder vtgs in the present study, showed distinct reactivity with three major subunits of each vtg corresponding to the three dominant bands demonstrated by protein stain (Table 1). Three high molecular weight bands were also strongly reactive to rainbow trout vtg antiserum. As the antisera had been produced against only the high molecular weight band it would strongly suggest that all three bands are vtg subunits and that the minor bands were also likely to be attributable to vtg.

In cross-reaction (fig 3) the present study has shown that the antisera produced following inoculation with subunits were highly species-specific. A slight degree of cross-reactivity was only noted between rainbow trout vtg with antiserum to Atlantic salmon vtg. Antisera to rainbow trout and greenback flounder vtg were only reactive with their respective antigens. In general, polyclonal antibodies recognise cross-reactive epitopes between related species (Nilsen et al., 1998). For example anti-rainbow trout vtg antiserum was 53% reactive with estrogenised serum from brown trout (*Salmo trutta*), 80% reactive with estrogenised brook charr (*Salvelinus fontinalis*) serum, 60% reactive with estrogenised Chinook salmon (*Oncorhynchus tshawytscha*) serum and 34% reactive with estrogenised Atlantic salmon serum (Mourot and LeBail, 1995). This relationship has also been demonstrated with monoclonal antibodies (Nilsen et al., 1998). Lack of cross-reactivity between unrelated species has previously been demonstrated (Yao and Crim, 1996) but, in contrast, some studies have shown vtg cross-reactivity between non-related teleosts (Heppell et al., 1995; Heppell and Sullivan 1999), even with monoclonal antibodies. Sequencing studies of teleost vtg cDNA have shown that regions possessing high homology are present (Korte et al., 1999; Wang et al., 2000) and multiple sequence alignment has enabled conserved sequences to be identified (Folmar et al., 1995). Conservation of particular genetic regions is reflected in

amino acid sequencing studies which have verified that teleost vtg, in those species so far studied, is similar in primary structure (Folmar et al., 1995; Heppell and Sullivan, 1999). This would be expected to result in similar antigenicity between vtgs, but this does not appear to be the case, and conserved epitopes may not necessarily be highly antigenic (Heppell et al. 1995). Despite areas of peptide homology demonstrated by sequencing studies, vtg is likely to undergo extensive post-translational modification which affects tertiary structure and hence epitope availability. Our studies did not demonstrate any appreciable cross-reactivity, even between closely related salmonid species. This result may well have been generated by the technique for antibody production, which effectively produced a highly specific antiserum but may have reduced the availability of shared epitopes. Secondary purification via reducing SDS-PAGE may have resulted in loss of some structural epitopes and exposure of immunologically distinct epitopes, as during this procedure the molecule undergoes changes in shape. It is therefore difficult to make any assumptions regarding phylogeny from studies using antigenicity, as cross-reactivity depends on epitope availability, which is often unpredictable and subject to change.

In conclusion, antigen preparation by inoculation of protein bands excised from reducing gels effectively produced highly specific antisera to vtg in the three species examined, and no appreciable cross-reactivity was noted, despite the fact that two vtgs were from closely related salmonids. Although only the major high molecular weight band was excised in each case, the antibody it produced reacted mainly with three major bands from each purified vtg in Western blots of reducing gels. In view of the fact that vtg is readily degradable and breaks into smaller proteins on storage (Silversand et al., 1993), and many assays are carried out on previously frozen plasma, the use of antisera to vtg subunits may maximise epitope

availability. Antisera to whole vtg may be less reactive with stored vtg as conformational epitopes may be lost on storage.

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Captions to figures

Figure 1a, b, c: Gel filtration elution profile of plasma from a) rainbow trout, b) Atlantic salmon and c) greenback flounder, inoculated with E₂ (E2) and saline (S). Molecular weights of major peaks are indicated (kD). Putative vtg peak is arrowed. * Value derived by extrapolation of standard curve.

Figure 2: SDS-PAGE analysis of elution peak fractions in 10% reducing gels stained with Coomassie brilliant blue. Lanes 1, 4 and 7 are molecular weight standards; lanes 2, 5 and 8 are peak 1; lanes 3, 6 and 9 are putative vtg peaks.

Figure 3 a, b and c: Reactivity of vtg antisera with each purified vtg. a) Reaction with antiserum to rainbow trout vtg. b) Reaction with antiserum to Atlantic salmon vtg. c) Reaction with antiserum to greenback flounder vtg.

Table 1: Approx. molecular weight (kD) of subunits of vtg peak fraction, derived from 10% SDS-PAGE reducing gels. Major bands are highlighted. Reaction in Western blot (WB) is indicated as: +++ strong reactivity, ++ moderate reactivity, +weak reactivity.

Flounder	Flounder	Trout	Trout	Salmon	Salmon
kD	(WB)	kD	(WB)	KD	(WB)
153.4	++	156.7	+++	152.4	+++
		117.0	+++	110.9	++
102.1	++	103.3	+++		
		98.4	+		
76.6	++	87.4	+	76.4	+++
				62.7	+
				55.8	+