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Plasma Vitellogenin Levels during the Annual Reproductive Cycle of the Female Rainbow Trout (*Oncorhynchus mykiss*): Establishment and Validation of an ELISA

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ABSTRACT. Rainbow trout, *Oncorhynchus mykiss*, vitellogenin (Vtg) was purified from plasma of E_2-treated male by direct anion exchange chromatography and some of its biochemical characteristics were studied. Our results demonstrated that, under SDS-PAGE conditions, rainbow trout Vtg was composed of two molecular forms of 390 and 176 kDa representing, respectively, the dimeric form and the monomeric form of the molecule. The purified Vtg was used to raise a polyclonal antibody for Vtg (anti-Vtg). Using this anti-Vtg, a competitive enzyme-linked immunosorbent assay (ELISA) was developed for the quantification of rainbow trout Vtg. The practical sensitivity range of this ELISA was 20–320 ng/ml (80–20% of binding) and the detection limit was 9 ng/ml. The intra- and the inter-assay coefficients of variation (at 50% of binding) were estimated at 1.8% (n = 10) and 3.9% (n = 13), respectively. This ELISA was validated by detecting changes in Vtg levels in rainbow trout at different physiological stages, as well as in 2-year-old female rainbow trout throughout the reproductive cycle. comp biochem physiol 117B;1:75–84, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Enzyme-linked immunosorbent assay (ELISA), fish, rainbow trout, reproductive cycle, vitellogenin, vitellogenesis

INTRODUCTION

In teleosts, as in other oviparous vertebrates, vitellogenesis is a crucial period in the female reproductive cycle, characterized by pronounced growth of oocytes. Much of this growth results from the uptake of specific proteins, predominantly a hepatically-derived yolk protein precursor, vitellogenin (16,31). This glycolipophosphoprotein of high molecular weight is synthesized by the liver under estrogenic control and transported to the ovaries by the bloodstream where it is selectively and progressively sequestered into the growing oocytes (19,28). Once sequestered, vitellogenin (Vtg) is proteolytically processed as yolk proteins to constitute the main nutritional reserves of the developing embryo and larva (16,31).

Because Vtg levels represent a direct indicator of the female reproductive stage, various methods have been developed in teleostean for their quantification. For example, phosphoprotein phosphorus (4), total protein content (5), calcium (17), and alkali-labile phosphoprotein (21) have been used as indirect indicators of Vtg levels. However, these methods turned out to be non-specific for Vtg and failed to quantify Vtg adequately. For this reason, other methods based on immunological recognition have been developed for the direct determination of Vtg levels. Radial-immunodiffusion (5), immunoagglutination (10), or immunoelectrophoresis (13) proved to be specific, but their sensitivity was low. Although more specific and sensitive, radio-immunoassays (RIA) (25,26,29) required the use of radioisotopes to label Vtg, as well as expensive equipment in order to be performed successfully. The enzyme-linked immunosorbent assay (ELISA) method gets around these disadvantages and has been developed, in the last decade, for the quantification of Vtg plasma levels in various teleostean species (8,12,14,18). The great sensitivity and reliability of this method, its rapidity, and the lack of any radioac-

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tivity handling, prompted us to develop an ELISA for rainbow trout Vtg.

MATERIALS AND METHODS

Fish sampled

SPECIFIC GROUP. To validate our ELISA, plasma samples from rainbow trout at various physiological stages were collected. Male rainbow trout, *Oncorhynchus mykiss*, weighing between 2 and 3 kg were used as controls. Neomales weighing around 1.6 kg were obtained from genetic females which had been sexually reversed by precocious ingestion of methyl testosterone in the diet between the ages of 20 to 60 days. Triploid females weighed between 2.6 and 3.1 kg; immature females less than 100 g, and juvenile females around 360 g. Fish were anesthetized in 2-phenoxyethanol (1 : 3000) and the blood was collected from the caudal sinus into heparinized syringes. Blood samples were immediately centrifuged (2000 **g**, 10 min, 4°C), and the plasma was collected, frozen immediately on dry ice, and kept frozen at −20°C.

ANNUAL CYCLE. One hundred and eighty two-year-old adult female rainbow trout (winter strain spawning), with an initial weight around 1.5 kg, were reared under a natural-simulated photoperiod. The tanks were continuously supplied with mountain spring water at constant temperature (8.5°C), and the fish were fed twice daily with a pelleted diet for broodstock (Trouwit, Trouw, France). Twice a month until the fish spawned, plasma samples were collected on individually marked fish (n = 7), whereas the gonads and the liver were obtained from sacrificed fish randomly selected (n = 10). The gonadosomatic index (GSI) and the hepatosomatic index (HSI) were calculated as the ratio between the organ weight and the body weight. The mean oocyte diameter was obtained from 20 oocytes measured under binocular microscope.

Vitellogenin Purification and Characterization

HORMONAL INDUCTION OF VITELLOGENIN SYNTHESIS. Twenty male rainbow trout were used for the hormonal induction of Vtg synthesis. The control group (n = 10) was injected intraperitonally with a pure cocoa butter solution, whereas the hormone-treated group (n = 10) was injected, with 17-β estradiol dissolved in cocoa butter (3 mg E_2/kg of body weight, diluted in ethanol : cocoa butter, 1 : 9). After two weeks, fish were anesthetized and plasmas were collected.

PURIFICATION. The whole procedure was performed at 4°C in order to prevent the proteolytical breakdown of the Vtg. Two ml of plasma from an E_2-treated male were applied onto a DEAE-cellulose Biogel column (Bio-Rad). The sample was eluted at a flow rate of 36 ml/hr with a 100 mM Trizma base, 2 mM $CaCl_2$, 1 mM PMSF (pH 7.8) starting-buffer for 2 hr to elute non-adsorbed material. Adsorbed proteins were eluted with a linear gradient (0–200 mM) of NaCl in the starting-buffer for 4 hr. Absorbance of the eluted fractions was measured at 280 nm. Eluted fractions containing Vtg were identified on SDS-PAGE (7.5%), pooled, and concentrated using an Amicon cell (membrane pore size, 100 kDa) to the desired protein concentration. This latter parameter was determined by the Bradford method (2) using ovalbumin as the standard protein.

ELECTROPHORESIS AND IMMUNOLOGICAL PROCEDURES.

Gel Electrophoresis. Both native- and SDS-PAGE (0.1% SDS) were performed according to Laemmli (9), in discontinous gels, which included a 4.0% stacking gel and a 7.5% separating gel (SDS conditions) or a separating gel with a 4–16% linear gradient of acrylamide (native conditions). The protein components were stained with Coomassie Brilliant Blue R250.

Electroelution. Protein bands identified as Vtg were excised from a Coomassie-blue stained SDS-PAGE and were electroeluted according to the procedure described by Harrington (6).

Antibodies. For the preparation of anti-Vtg antibody, 100 μg of purified Vtg were emulsified in Freund's complete adjuvant and injected subcutaneously into rabbits according to the following schedule: four injections spaced by a week, followed by 15 resting days and two booster injections spaced by 15 days. At the end of the immunization, blood was collected by puncture of the ear vein, allowed to clot at 4°C for 24 hr, and the serum was collected. To remove the antibodies which react to common serum proteins, anti-Vtg was absorbed overnight at 4°C with male plasma (1 : 2 in volume). After centrifugation (2000 **g**, 5 min, 4°C), this antibody was stored at −20°C in glycerol (1 : 1). The antibody directed against the vitelline envelope proteins (anti-VEP) was kindly provided by Dr S.J. Hyllner (7). To remove any non-specific reactions, this anti-VEP was absorbed with both purified Vtg and male plasma protein (1 : 1 : 1) according to the same procedure described for the anti-Vtg.

Radial Immunodiffusion. The specificity of the anti-Vtg obtained was assessed by radial immunodiffusion in 1.5% agarose gel according to Ouchterlony (20).

Western Blotting. Proteins were transferred from gels (native- or SDS-PAGE) onto a PVDF membrane (0.45 μm) as described by Towbin *et al.* (27). Transfer was performed for 3 hr (0.8 mA/cm^2) at 4°C in a Tris buffer 25 mM (pH 8.3) containing 1.44% of glycine, 20% of methanol and 0.5% of SDS. The membrane was saturated for 1 hr at 37°C in a Tris-saline buffer (TBS = 20 mM Tris, 150 mM NaCl, pH 8) containing 5% of normal pig serum (NPS). After this saturation step, the membrane was incubated with the anti-Vtg (1 : 600 in TBS-NPS) for 2 hr at 37°C and rinsed 3 × 10 min with TTBS (TTBS = TBS + 0.05% Tween-20).

The antigen-antibody complexes were detected by incubating the membrane with the second antibody solution (horseradish peroxidase-conjugated goat-anti-rabbit IgG, 1 : 500 in TBS-NPS) for 1.5 hr at 37°C, followed by three washes. The revelation of the peroxydase activity was performed using an α-chloronaphthol solution (ammonium citrate 50 mM, pH 5 + 0.01% α-chloronaphthol + 0.04% H_2O_2 (30% w/v)). The whole procedure was performed at room temperature.

Non-competitive ELISA. Samples from eluted chromatographic fractions were diluted at least 1 : 2 in a coating buffer (see ELISA procedure), and directly incubated on microtitration plates overnight at 4°C. After a saturation step of 30 min, both anti-Vtg (1 : 200,000) and anti-VEP (1 : 20,000) were directly incubated for 3 hr. The antigen-antibody complexes were revealed according to the same procedure used in the competitive ELISA assay.

PREPARATION OF EGG YOLK EXTRACT. Egg yolk extract from vitellogenic oocytes was prepared according to Babin (1).

Fish Sampled

This assay was based on that described by Nuñez Rodriguez *et al.* (18) for sole *Solea vulgaris* Vtg. In this method, a competition for the anti-Vtg binding sites was established between the Vtg coated onto the microplate and the free Vtg contained in samples or in standard preparation.

Immobilization of Antigen on Solid Phase. The Vtg coating was performed on microtiter plates (Nunc Maxisorp Immuno II) with 200 μl/well of a coating buffer (50 mM sodium carbonate buffer, pH 9.6) containing 100 ng/ml of purified Vtg. In order to determine the non-specific binding, four wells were incubated with 200 μl/well of male plasma diluted in the same buffer at the same protein concentration (100 ng/ml). The plates were then covered and incubated at 4°C overnight (16 hr).

Saturation Step. The liquid content of the wells was discarded by inverting the plates. To saturate non-specific binding sites, plates were incubated for 30 min at 37°C with 200 μl/well of a PBS-t buffer (10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.05% Tween-20) containing 2% normal pig serum (NPS). After this, the liquid content of the wells was discarded and three successive washes of 1 min each were performed using PBS-t.

Specific Antiserum Incubation. 100 μl/well of the anti-Vtg (1 : 100,000, in the PBS-t-NPS) were added and the plates were incubated for 3 hr at 37°C with 100 μl/well of serial dilutions (factor 2) of samples or standard Vtg (from 2500 ng/ml to 5 ng/ml, in the PBS-t-NPS), followed by three washing cycles. The final concentration of the standard Vtg ranged from 1250 to 2.5 ng/ml and the anti-Vtg dilution was 1 : 200,000.

Secondary Antiserum Incubation. Each well received 200 μl of swine anti-rabbit IgG (Dako) diluted in the PBS-t-NPS (1 : 2500). Incubation was performed for 45 min at 37°C and the plates were then washed.

Peroxidase-Anti-Peroxidase (PAP) Complex Incubation. 200 μl/well of the PAP complex (Dako) diluted in the PBS-t-NPS (1 : 5000) were added. The plates were incubated for 30 min at 37°C and then washed.

Revelation. The peroxidase activity was revealed with 200 μl/well of an orthophenylenediamine (OPD) solution (20 ml of 0.1M phosphate − 0.44M citrate buffer, pH 5.0 + 10 mg OPD + 10 μl H_2O_2 (30% w/v)). The reaction was stopped after 30 min at 20°C in the dark, by adding 50 μl/well of 4 N H_2SO_4. Absorbances were measured 15 min later at 492 nm with a TITERTEK EIA microplate reader.

Expression of the Results and Statistical Analysis. Absorbance measurements were expressed by a non-linear model [Bi/Bo = f (log (dose or dilution))] where Bi represented the binding of sample or standard Vtg, and Bo, the maximum binding. The analysis of the competition curves was performed after linearization through a Logit-Log transformation [Logit = −ln [(Bo-Bi)/(Bi-NSB) = f (log (dose or dilution))] where NSB represented the non-specific binding. The parallelism between regression curves was tested by ANOVA as described by Snedecor and Cochran (24).

RESULTS

Purification of Vitellogenin

CHROMATOGRAPHIC PURIFICATION. The plasma protein concentration increased markedly after E_2 treatment from 48.2 mg/ml to 161 mg/ml. Figure 1a shows the elution profiles when plasma from the untreated male and the E_2-treated male were subjected to DEAE chromatography. When plasma from the E_2-treated male was chromatographed, a large and apparently homogenous peak (peak B) appeared around 75 mM of NaCl. It was immediately suspected to contain Vtg, since it was absent from the untreated male. When an aliquot of each eluted fraction was assayed in the non-competitive ELISA for Vtg (Fig. 1b), one major peak was observed. This corresponded very well to peak B (Fig. 1a), suggesting that peak B contained most of the Vtg eluted from the DEAE column. Small positive reactions were also observed with peak A and peak D, corresponding, respectively, to the void volume and to the final washing of the column with a high NaCl concentration (500 mM). No positive reaction with the anti-Vtg was observed in the fractions obtained when untreated-male plasma was chromatographed (Fig. 1b). When an aliquot of each eluted fraction was assayed in the non-competitive ELISA for VEP (Fig. 1c), most of the vitelline envelope proteins (VEP) were eluted in the void volume (peak A). Several other heterogenous positive reactions were also ob-

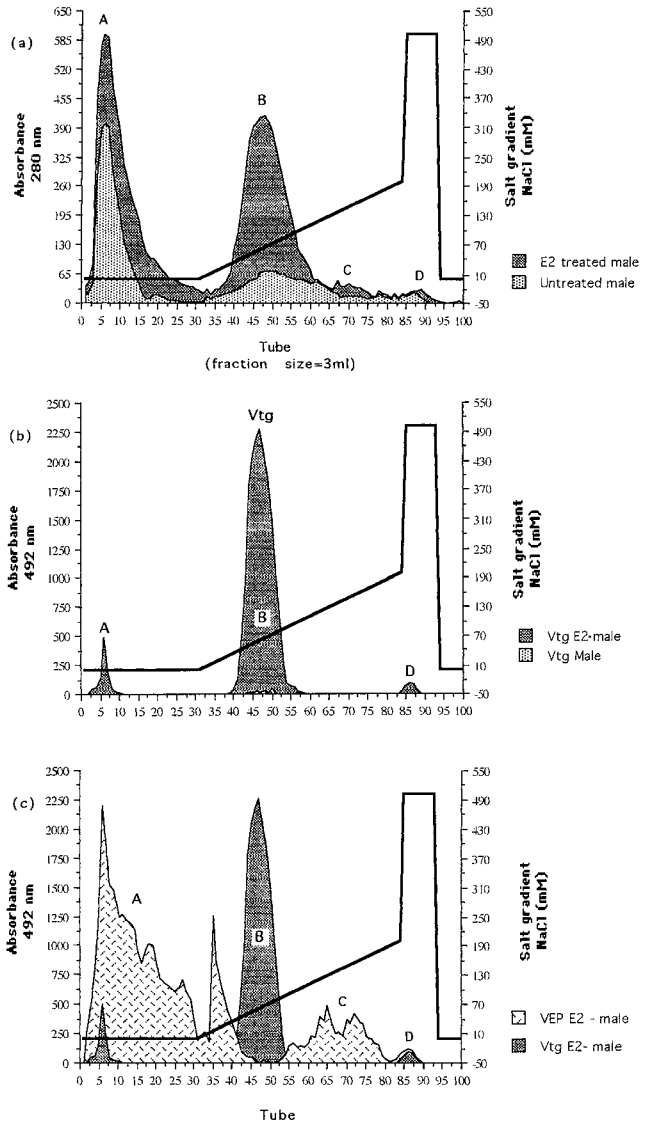

FIG. 1. Purification of rainbow trout Vtg. (a) Direct anion-exchange chromatography of E_2-treated-male plasma compared to plasma from an untreated-male. The Vtg was eluted with a linear NaCl gradient from 0 to 200 mM in which the Vtg eluted at a salt concentration of 75 mM. (b) The non-competitive ELISA performed using the antibody directed against Vtg showed, in E_2-treated male eluted fractions, the appearance of a symmetrical vitellogenic peak B, eluting at the 47th fraction. The absence of immunodetection of Vtg in untreated male eluted fractions demonstrated that the purified molecule was inducible by E_2. (c) The non-competitive ELISA performed using the antibody directed against vitelline envelope proteins (VEP) showed, in E_2-treated male eluted fractions, that VEP eluted on both sides of the vitellogenic peak (peak B).

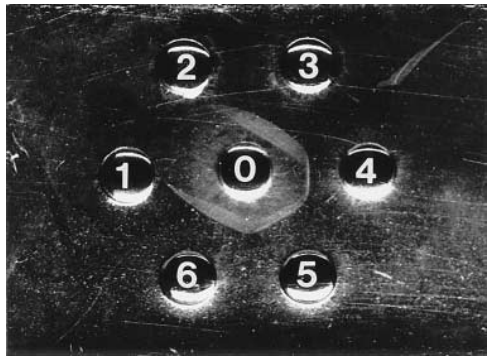

FIG. 2. Specificity of the anti-Vtg antibody. Immunodiffusion was performed in 1.5% agarose gel. The anti-Vtg was located in the central well (0) surrounded by wells containing untreated-male plasma (1), immature-female plasma (2), purified Vtg (3), vitellogenic-female plasma (4), E_2-treated-male plasma (5), and egg yolk extract (6). Samples were allowed to react for up to 24 hr at 4°C.

served, which corresponded, approximately, to VEP eluting when the concentration of NaCl reached 40 mM, 150 mM (peak C), and 500 mM (peak D). No positive reaction with the anti-VEP was detected in the vitellogenic peak B (Fig. 1c), hence these fractions were pooled, concentrated, and subsequently served as the immunogen and as the standard in the competitive ELISA.

ANTI-VITELLOGENIN SPECIFICITY. When radial immunodiffusion (Fig. 2) was performed to assess the anti-Vtg specificity, a continuous precipitation arc was observed between purified Vtg, vitellogenic female plasma, yolk extract, E_2-treated-male plasma, and anti-Vtg, but was not detected with male nor with immature female plasma.

VITELLOGENIN CHARACTERISTICS. Under native-PAGE conditions, Vtg appeared as a single protein band both in E_2-treated male and in the Vtg purified solution obtained after chromatography (Fig. 3a and 3b). Under SDS-PAGE conditions (Fig. 4a), Vtg appeared as two major protein bands of 390 kDa (protein I) and 176 kDa (protein II). To ensure that these proteins were indeed Vtg, both 390 and 176 kDa protein bands, suspected to be Vtg subunits, were individually electroeluted and tested on SDS-PAGE, both with and without a reducing agent (Fig. 4a). The addition of SDS and β-mercaptoethanol produced the breakdown of the native form of Vtg into several bands. The comparison of the SDS-PAGE (Fig. 4a) and the corresponding Western blot performed using the anti-Vtg (Fig. 4b), showed that protein II (176 kDa) was the Vtg monomer and protein I (390 kDa), the dimeric form or a rearranged electrophoretic form. The specific anti-Vtg also cross-reacted with several protein bands ranging from 120 to 80 kDa (Fig. 4b). These proteins were identified as Vtg breakdown products, since their intensity greatly increased with the use of β-mercapto-

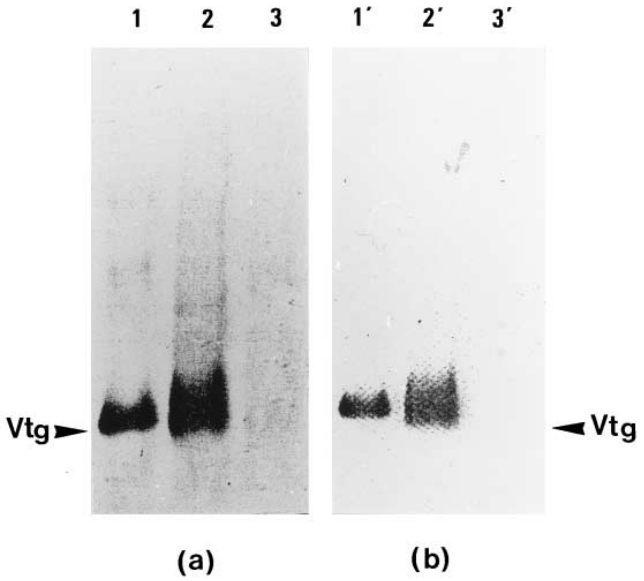

FIG. 3. Vtg identification on native-PAGE. (a) Native PAGE of purified Vtg (lane 1), E_2-treated-male plasma (lane 2) and untreated-male plasma (lane 3), stained with Coomassie blue. The arrowheads indicate the Vtg, which appears as a single focused band. (b) Corresponding Western blot using the antibody against rainbow trout Vtg (1:600). Vtg was present in the purified Vtg solution (lane 1′) and in E_2-treated male plasma (lane 2′), but was clearly absent from untreated-male plasma (lane 3′).

ethanol (Fig. 4a and 4b). No positive reaction with the anti-Vtg was detected with the untreated-male plasma (Fig. 4b).

ELISA Validation

ELISA CHARACTERISTICS. Working conditions in order to obtain a maximum value absorbance (Bo) near 1.5, which was in the range of the linear response given by our microplate EIA reader, were determined. Under these conditions, the interaction between the anti-Vtg and male plasma (non-specific binding, NSB) was less than 2.5% of the Bo, demonstrating that male plasma proteins did not interact significantly in our assay. The routine calibration curve ranged from 1250 ng/ml to 5 ng/ml (Fig. 5), with the most reliable part between 320 and 20 ng/ml (20 and 80% of maximum binding). Parallelism of 13 different standard curves was assessed by covariance analysis using the F-test on mean squares. No statistical differences were observed between the different regression lines (mean slope = −0.97; r = 99.5). The sensitivity of the assay, defined as the smallest amount of Vtg that produced a response significantly different from Bo, was estimated around 9 ng/ml, which corresponded to 92% of binding (n = 19). At 50% of binding, this value was 78 ng/ml (n = 19). This assay detection limit could be improved almost 3-fold by preincubating the sample and the standard Vtg with the specific anti-Vtg for 16 hr at 4°C. The inter-assay and intra-assay coefficients of variation obtained at 50% binding were 3.86% (n = 13) and 1.75% (n = 10), respectively.

ELISA SPECIFICITY. The degrees of parallelism between the standard competition curve, obtained with serial dilutions of the purified Vtg, and that obtained with increasing serial dilutions of various antigens are presented in Fig. 5. No competition curve was observed with the control male plasma, except for the lowest plasma dilutions (less than 1:30). This was attributed to a non-specific plasma effect, since this binding phenomenon also occurred with pig serum, which is Vtg free. To avoid such non-specific binding, all plasma samples were diluted more than 1:50. Competition curves were obtained with the Vtg standard preparation and with serial dilutions of plasma from a neomale, a triploid, an immature, a juvenile, a previtellogenic- and a vitellogenic-female, as well as with plasma from an E_2-treated male. The parallelism between these curves was assessed by F-test, after a Logit-Log transformation (results not shown). No statistical differences were observed between the slopes. A competition curve was also obtained with egg yolk, but this showed a different behavior compared to all the others, and parallelism was not retained.

VITELLOGENIN MEASUREMENTS.

In Rainbow Trout at Various Physiological Stages (Fig. 6). This ELISA was physiologically validated by measuring Vtg levels in plasma samples from rainbow trout at different physiological stages. Our data showed that plasma from untreated males did not contain Vtg, whereas neomales and triploid females, thought to be Vtg-free, had 1.40 and 5.20 μg of Vtg/ml, respectively. Immature females had a plasma Vtg level of 65 μg/ml, whereas juvenile females entering their sexual maturity for the first time had 420 μg/ml. Two-year-old adult females at previtellogenic and vitellogenic stages were also tested, and were found to have, respectively, 25 and 1000 times more Vtg than immature females. The plasma Vtg concentration of E_2-treated males was comparable with the physiological levels observed at the end of vitellogenesis in females (81 mg/ml and 60 mg/ml, respectively).

In the Monitoring of Rainbow Trout Vitellogenesis (Fig. 7). Seasonal changes in Vtg levels throughout the reproductive cycle of two-year-old female rainbow trout together with seasonal variations of morphometric parameters (GSI, HSI, and oocyte diameter) and the electrophoretical visualization of Vtg appearance in plasma, are shown in Fig. 7. From the seasonal profile of GSI (Fig. 7a), the reproductive cycle was divided into different physiological phases: a very slow development phase from February to May during which GSI remained at a minimum (around 0.5%), a slow development phase from May to mid-September during which GSI slowly but steadily increased from 0.5% to 1.3%, and a rapid development phase from mid-September to November during which GSI greatly rose, reaching a maximum of 18% in

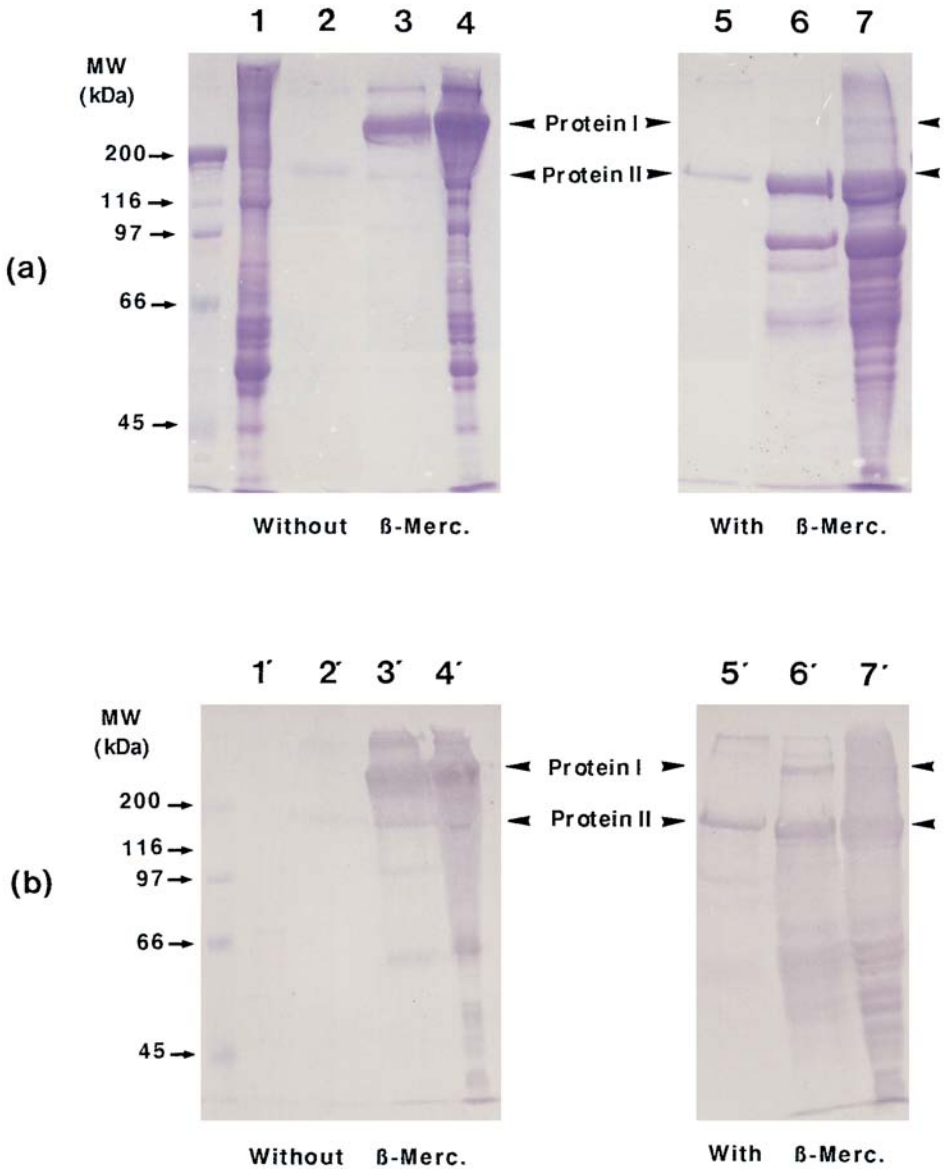

FIG. 4. Molecular weight determination of Vtg subunits. (a) SDS-PAGE (0.1% SDS) in 7.5% gel, of untreated male plasma without β-mercaptoethanol (lane 1), E_2-treated-male plasma without β-merc. (lane 4) and with β-merc. (lane 7), as well as of the electroeluted 176 kDa (protein II) and 390 kDa (protein I) Vtg bands without β-mer. (respectively, lanes 2 and 3), and with β-merc. (respectively, lanes 5 and 6). (b) Corresponding Western blot using the antibody directed against rainbow trout Vtg. The lanes 1′ to 7′ are identified as in Fig. 4a. The molecular weight of the standard proteins is indicated on the left: myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase-β (97.4 kDa), BSA (66.2 kDa) and ovalbumin (45 kDa).

late-November when fish entered the spawning period. During the post-ovulatory phase in December, GSI decreased abruptly to basal values. The lowest Vtg levels (less than 1 mg/ml) were observed in March (Fig. 7c) and the first significant increase in plasma Vtg levels in early April whereas GSI and HSI exhibited minimal values (0.5% and 1.2%, respectively). After a slow but gradual increase from 2.4 mg/ml in May to 10 mg/ml in July, Vtg levels rapidly increased from 10 to 35 mg/ml in September and then again, reaching a maximal value of 60 mg/ml in early November, two weeks before spawning. This rapid increase in Vtg levels was well correlated with a rise in HSI (Fig. 7a), in GSI (Fig. 7a) and in oocyte diameter (Fig. 7c). Vtg levels remained high (around 50 mg/ml) until spawning, in late November, and started to gradually decrease just after this. However, elevated Vtg levels (around 20 mg/ml) were still registered in December, one month after spawning. The seasonal changes in Vtg levels (Fig. 7c) were also greatly correlated with the seasonal appearance, on a Coomassie Blue–stained SDS-PAGE (Fig. 7b), of the two protein bands identified as Vtg (proteins I and II).

DISCUSSION

Purification and Characterization of Vitellogenin

PURIFICATION OF VITELLOGENIN. In the present study, E_2 was used because it is known to be the most potent estrogen for the induction of Vtg synthesis both in male and in female fish (11,30). In male rainbow trout, we observed that E_2 treatment caused an increase of the plasma protein concentration (48.2 mg/ml in untreated males, 161 mg/ml in E_2-treated males). This increase coincided with the massive

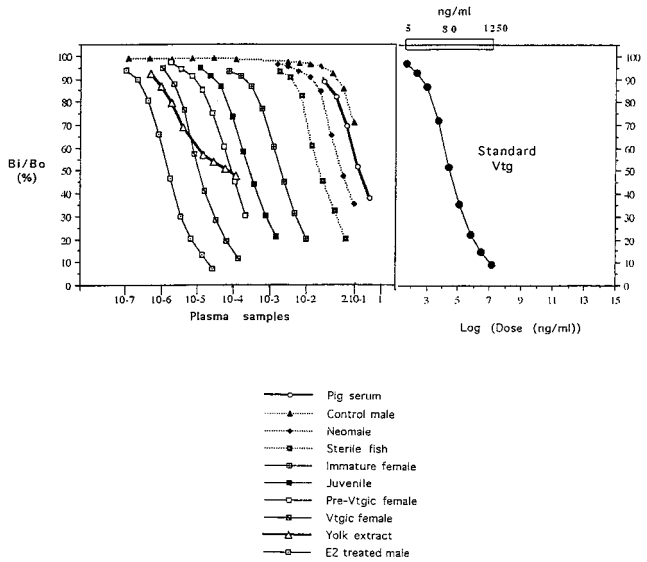

FIG. 5. Competition curves. Competition curves [Log (dose) = f [(Bi-NSB)/(Bo-NSB)] × 100, %] obtained with serial dilutions of various antigens were compared to the competition curve obtained with serial dilutions of the standard Vtg. No competition curve was observed with the untreated male plasma nor the pig serum whereas a great parallelism was observed in most biological samples tested compared to the standard curve (except for egg yolk).

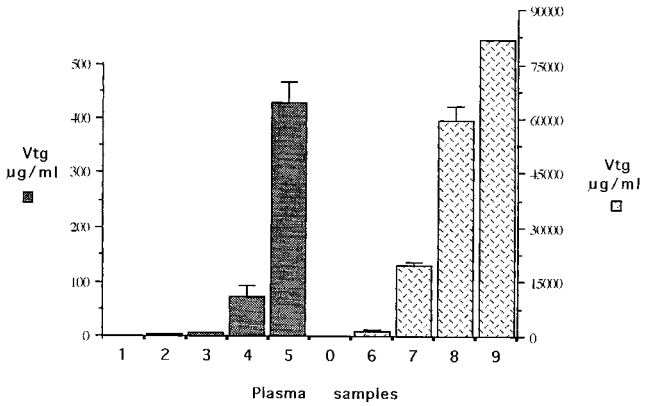

FIG. 6. Determination of Vtg levels in rainbow trout at various physiological stages. The Vtg plasma levels (µg/ml) varied from nearly undetectable to high. The following values were measured: 1- control males (0.38, n = 19), 2- neomales (1.45, n = 5), 3- triploid females (5.24, n = 11), 4- immature females (64.8, n = 45), 5- juvenile females (423, n = 7), 6- previtellogenic females (1440, n = 7). The highest Vtg levels were measured in 7- vitellogenic females (59600, n = 7), and in 8- E_2-treated males (81600, n = 3).

appearance in the blood of Vtg, as evidenced by anion-exchange gel chromatograms (Fig. 1a), non-competitive ELISA (Fig. 1b), native PAGE (Fig. 3) and SDS-PAGE (Fig. 4). Another group of E_2-inducible proteins that did not cross-react with the anti-Vtg but that strongly cross-reacted with the anti-VEP (Fig. 1c) was also detected in the E_2-treated male plasma. These proteins were identified as vitelline envelope proteins (VEP), which are known to be synthesized by the liver under the control of E_2 (7). Hence, care was taken with the purification of Vtg, the aim being to obtain Vtg free of any other plasma proteins. In this study, Vtg was isolated by one-step DEAE anion exchange chromatography from E_2-treated male plasma. A similar procedure has already been successfully used for the purification of Vtg from the rainbow trout (19,23) and from other teleost species (23). This procedure had obvious advantages, such as a great reproducibility, a good recovery (more than 50%) and a short processing time, which is of importance due to the lability of teleost Vtg (23). Moreover, this DEAE chromatography was sufficient to completely separate Vtg from other plasma elements known to be E_2-inducible (7), such as VEP (Fig. 1c), as well as to maintain Vtg integrity and antigenicity (Fig. 4).

MOLECULAR CHARACTERISTICS OF RAINBOW TROUT Vtg. Depending on the method used for its estimation, the molecular weight of native rainbow trout Vtg may vary from 342 to 535 kDa (16,31). In the present study, SDS-PAGE was used for the estimation of the molecular weight of rainbow trout Vtg because the addition of 0.1% of SDS to the electrophoretical procedure causes the dissociation of the Vtg into subunits. A similar procedure had already been used for the estimation of the molecular weight of Vtg in rainbow trout (1) as well as in other teleost species (15,23). A β-mercaptoethanol reduction, coupled with a specific electroelution of both protein I and II (identified as Vtg protein bands) and a Western blot performed with the anti-Vtg, enabled us to find the relationship linking these proteins (Fig. 4). We observed that the native rainbow trout Vtg was dissociated into a dimeric form (protein I, 393 ± 16 kDa, n = 7) and a monomeric form (protein II, 176 ± 4 kDa, n = 8) together with some breakdown products of lower molecular weight ranging from 120 to 80 kDa. The molecular weight of the monomeric form of rainbow trout Vtg (176 kDa) is similar to that previously reported for the same fish (1,23) and other teleost species (8,15,23).

ELISA Characteristics

The present results show that our ELISA fulfills the criteria classically retained for an immunoenzymatic method and for the quantification of Vtg levels.

A STRONG SPECIFICITY FOR VITELLOGENIN. The specificity of the assay was demonstrated by the fact that the anti-Vtg did not react with any other protein in the plasma different to Vtg as attested by the absence of any cross-reaction with control male plasma (Fig. 5). Nevertheless, a non-specific binding phenomenon was observed with male plasma constituents when plasma dilutions were less than 1:50. This interference was also found with pig serum, which is Vtg free, and was attributed to a non-specific

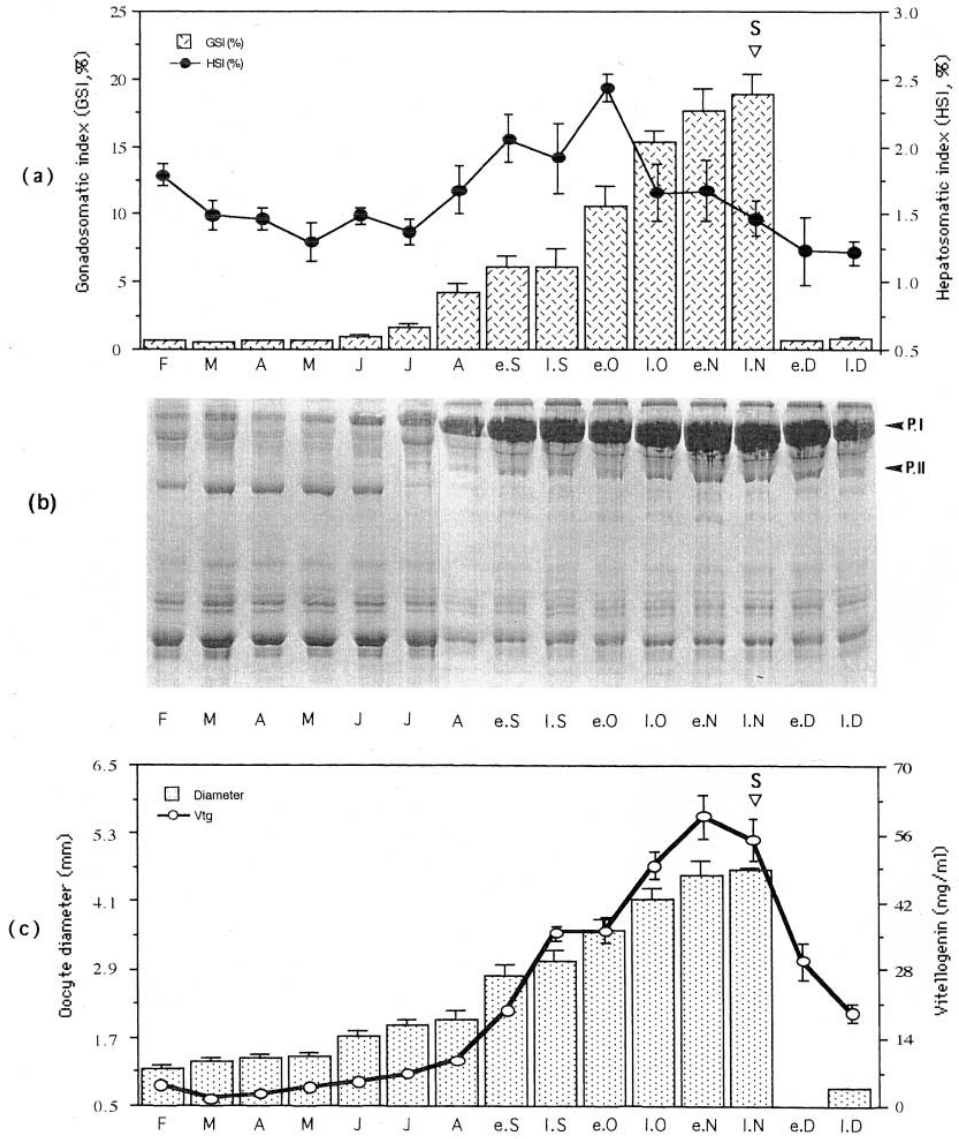

FIG. 7. Seasonal profile of Vtg in 2-year-old female rainbow trout. (a) Seasonal variations of the gonadosomatic index (GSI, Gonads weight/Body weight × 100, %) and the hepatosomatic index (HSI, liver weight/body weight × 100, %). The spawning period is indicated by an "S." The abbreviations e. and l. before the calendar months mean, respectively, early and late in the month. (b) Visualization on a Coomassie Blue–stained SDS-PAGE (7.5%) of the seasonal appearance in plasma, of the Vtg. Blood samples were regularly collected from the same marked female throughout the reproductive cycle. The same volume of plasma sample (20 μl) at the same dilution (1 : 30) was loaded on the gel in all wells. On the right, arrowheads indicate the position of the dimeric form (protein I, 390 kDa) and the monomeric form (protein II, 176 kDa) of the rainbow trout Vtg. (c) Seasonal profile of Vtg plasma levels and seasonal variations of the oocyte diameter. The spawning period is indicated by an "S."

plasma effect which can be avoided by diluting all the plasma samples, at least 50-fold. An excellent parallelism between the standard competition curve and those obtained with plasma samples from rainbow trout at various physiological stages was observed. This demonstrated that the anti-Vtg similarly recognized the purified Vtg and the native Vtg present in plasma samples. This was not true for the egg yolk sample, the competition curve of which was significantly different from the standard curve, suggesting a partial similarity with Vtg (45–85% of binding) and also a lack of parallelism. This anormal competition curve might be explained by the fact that when Vtg enters the growing oocytes, it is proteolyticaly cleavaged into yolk proteins (31), and that the yolk proteins are immunologically different from native Vtg. Consequently, in contrast to some other ELISA and RIA (14,18,25), this ELISA cannot be used to quantify lipovitelline in egg yolk extracts.

A HIGH REPRODUCIBILITY AND A GREAT SENSITIVITY. The low values of the intra- (1.8%) and the inter- (3.9%) assay coefficients of variation reflected the stability of the assay conditions and allowed for the comparison of Vtg levels both within and among assays. The sensitivity of the assay, defined as the concentration of Vtg that gave 92% of binding, was 9 ng/ml. Nevertheless, the sensitivity could be increased if necessary (at least 3-fold) by preincubating the samples. These assay characteristics were similar to those reported by other authors for Vtg ELISA developed in other teleost species (8,12,14,18) and for Vtg RIA developed in rainbow trout (26), as well as in other teleost species (25,29).

Such a high sensitivity enabled us to detect the presence of Vtg in neomales as well as in triploid and immature females often thought to be Vtg-free (Fig. 6). The presence of Vtg in neomales was easily explained by an incomplete

sex reversal. Indeed, several vitellogenic oocytes were observed on the testis surface in most mature neomales. In the case of triploid females, another hypothesis was advanced, notably the possible induction of Vtg synthesis by exogenous steroids. Indeed, in male Siberian sturgeon *Acipenser baeri*, it has been demonstrated that Vtg synthesis could be induced by the presence of phytoestrogens in the diet (22). Whereas the anti-Vtg did not cross-react with the immature female plasma in radial immunodiffusion (Fig. 2), a clear competition curve was observed in ELISA, and significant Vtg levels (65 μg/ml) were measured. This apparent difference is probably accounted for by the greater sensitivity of the ELISA compared to radial immunodiffusion, and emphasizes the fact that sensitive techniques, such as RIA and ELISA, should be used when low concentrations of Vtg are likely to be found, such as in immature females (65 μg/ml), and in juvenile female trout one year or more before their first ovulation (1 μg/ml; (3)). Concerning E_2-treated male plasma, Vtg levels were measured at around 80 mg/ml and were much higher than the levels usually measured in female plasma at the end of vitellogenesis (60 mg/ml). In both cases, Vtg tended to represent more than 50% of the total protein content of the plasma.

Purification and Characterization of Vitellogenin

The seasonal profile of Vtg was determined by ELISA in 2-year-old female rainbow trout reared under fish farming conditions (Fig. 7). Our data showed that during the period of previtellogenesis (February–May), the slight increase in Vtg levels (from 1 mg/ml to 3 mg/ml) was not followed by an increase in the gonadosomatic index (GSI), which remained at a minimum (around 0.5%). In contrast, the periods of endogenous vitellogenesis (May–July), now called the cortical alveolus stage (32), and exogenous vitellogenesis (July–early November), now called vitellogenesis (32), were characterized by a great correlation between the Vtg levels and the GSI (linear regression, $y = 3.1x + 1.18$, $r = 0.99$), demonstrating that the development of the ovaries was mainly related to the massive incorporation of Vtg into the growing oocytes (19). This great correlation stopped in early November, two weeks before spawning, while Vtg levels were maximal (60 mg/ml). In late November, when GSI reached its maximum value (18% of body weight), females entered the spawning period whereas Vtg levels still remained elevated (around 50 mg/ml). The relatively high levels of Vtg (30–50 mg/ml) measured during the post-ovulatory period, in December, was probably due to the clearance of the remaining Vtg from the blood, but could also be explained by the presence of ovarian yolk proteins massively released into the bloodstream in the course of the follicular atresia (1).

This seasonal profile of Vtg was similar to that previously reported in rainbow trout by van Bohemen *et al.* (30). The quantitative differences observed between both methods with respect to the levels of Vtg during the vitellogenesis period, 13 mg/ml (30) and 60 mg/ml in this study, could be explained by the fact that van Bohemen *et al.* used a densitometric method after gel electrophoresis, which gave estimations of Vtg levels rather than absolute values. The seasonal profile of Vtg we found in rainbow trout was also similar to that reported in the literature for other teleost species. For example, in *Dicentrarchus labrax* (14), the maximum levels of Vtg (around 3 mg/ml) were reached two months before spawning, in *Solea vulgaris* (18), the maximum levels of Vtg (3 mg/ml) appeared just before spawning, and in *Salmo salar* (25) the maximum levels of Vtg (around 20 mg/ml) were observed three months before spawning. The differences between species with respect to the levels of plasma Vtg and its seasonal profile, might be a consequence of different reproductive strategies, duration of the vitellogenic process and size reached by the oocytes during the growing phase.

CONCLUSION

Direct DEAE chromatography allowed us to obtain, in a short processing time, a rainbow trout Vtg free of any E_2-induced proteins such as vitelline envelope proteins. We demonstrated that rainbow trout Vtg exhibited a dimeric form (protein I, 390 kDa) and a monomeric form (protein II, 176 kDa) in female plasma. The ELISA we developed during this study to quantify Vtg plasma levels has several major advantages over the previously published methods. Unlike the RIA, it does not require the use of radiolabelled Vtg, but involves stable compounds which can usually be stored over a very long period of time. Moreover, it is easy to use, rapid, highly specific, highly sensitive and highly reproducible. Additionally, this ELISA should be of considerable interest in aquaculture in the early determination of sex, the quantification of Vtg in plasma at any physiological stage, as well as for investigations on the reproductive physiology of the rainbow trout.

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