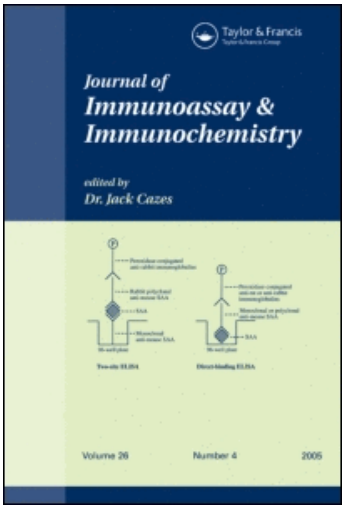

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Enzyme-Linked Immunosorbent Assay (ELISA) for Rainbow Trout (*Oncorhynchus mykiss*) Vittellogenin

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ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) VITELLOGENIN.

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ABSTRACT.

1. A specific and simple enzyme-linked immunoassay for rainbow trout *(Onchorynchus mykiss)* vitellogenin (Vtg) is described. This assay is performed using a rabbit antiserum for Vtg purified from trout plasma.

2. This assay is based upon the competition between soluble Vtg and Vtg adsorbed on microtiter plates, for the rabbit anti-Vtg antibody binding sites.

3. The adsorbed Vtg-antibody complexes are revealed through the peroxidase-antiperoxidase antibody, which is colored by o-phenylendiamin. This assay can be performed in a day and a night.

4. Under our conditions, 90-20% of binding gave a sensibility range of 33-1473 ng/ml. With almost a 50% binding yeld (335 ng/ml) the intra-assay coefficient of variation(CV) was 5.2% (n=26) and the inter-assay CV was 12.5% (n=5).

5. There was low immunological cross-reactivity with sera from other salmonids and with ovary extracts. Extracts of liver from oestrogenized male rainbow trout yielded displacements parallel to the vitellogenin standard and to mature female serum or oestrogenized male serum.

6. This enzyme immunoassay is simple and easy to use. Its great specificity allows its use only for the rainbow trout species.

KEY WORDS: vitellogenin, enzyme-linked immunosorbent assay, sex determination, fish, rainbow trout.

INTRODUCTION

Vitellogenin (Vtg), a phospholipoglycoprotein, is synthesized by the female fish liver and incorporated into oocytes by endocytosis after being carried to the ovaries through the blood (1, for review). Serum Vtg levels are directly related to the female reproductive stage. Thus, Vtg detection can be used to identify sex of fish when no sexual dimorphism occurs, and the measurement of its level in blood gives an index of female sexual maturity. The determination of Vtg levels was performed by means of indirect measurements, such as the measurement of protein-bound phosphorus or calcium (2-4), or with direct measurements using immunological recognition such as immunoagglutination (5), immunoelectrophoresis (6), or radio-immunoassays (RIA). But these methods had some disadvantages. Indirect methods turned out to be non-specific for Vtg. Immunoagglutination and immunoelectrophoresis were specific for Vtg but their sensitivity was low. RIAs have been widely developed in fish (1, for review, 7) and particularly in Salmonids (8-12). These assays are specific and sensitive; nevertheless, they require expensive equipment to be performed successfully. Enzyme-linked immunosorbent assays (ELISA) get around these difficulties. In the teleost species, ELISAs for Vtg have been described for sole (13), brown trout (14), catfish (15), striped bass (16), sea bass (17) and the Japanese sardine (18).

The complexity of the Vtg molecule induces considerable variation in the immunological determinants, which decreases the recognition in an heterologous system. Therefore, it is necessary to set up an assay for each species. To allow for an early determination of the sex of the animals and to follow the maturation of females, we chose to perform an ELISA for Vtg of rainbow trout (*Oncorhynchus mykiss)* in blood serum. A competitive method has been chosen because such a technique can be used in unspecialized laboratories, when field determination of sex or sexual stages are needed for practical purposes. We have tried to widen it to include the liver and ovary in order

to find out whether an earlier detection of Vtg in liver is possible and whether vitellus proteins can be used as a source of Vtg for purification.

MATERIAL AND METHODS.

Vtg Purification.

Vtg synthesis has been induced in male rainbow trout by injections of 2.5 mg/ kg of oestradiol-17ß (E2), twice a week for 2 weeks. E2 was dissolved in a small volume of ethanol diluted in a 150 mM NaCl solution and then injected intraperitoneally. A blood sample was taken, set aside for clotting purposes and centrifuged, then the serum was stored at -20°C until utilization.

Vtg was purified according to Tyler and Sumpter (7). The first step consisted in performing a gel filtration chromatography (Sepharose 6B, Pharmacia), in a 100mM bicarbonate buffer. The vitellogenic fractions were identified with immunodiffusion (19) involving an antiserum anti-rainbow trout Vtg which had been prepared for immunoagglutinations (5). Then, these fractions were concentrated on Amicon membranes (PM 50.000), and ammonium bicarbonate concentration was adjusted to 50 mM. The second step consisted in a ion exchange chromatography (DEAE Trisacryl, IBF). The elution was performed with an ammonium bicarbonate gradient (range: 50 to 200 mM). Vtg was stored in a concentrated solution (over 1 mg/ml) at -20°C.

Antibodies.

Specific antibodies were raised in rabbits (New Zealand, INRA, Jouy en Josas) by subcutaneous injections of 100 µg of purified Vtg every 2 weeks for 4 months. The first three injections were carried out with Freund's complete adjuvant (V/V), and the other five with Freund's incomplete adjuvant. The antiserum titer was followed through out the

immunization process by immunodiffusion. After the animals were slaughtered, the blood was centrifuged and the antiserum stored at -20° C.

The ELISA Procedure.

In this method, a competition for the antibody was established between Vtg coated on microplate wells and free Vtg. The assay was performed in a 96-well Greiner microtitration plate. After the adjustment phase, which will be described in next part, the assay procedure was performed as follows:

Washing: After each step of the assay, plates were washed 5 times with a Tris-HCL buffer (10 mM, pH 7.5) containing 150 mM NaCl, 0.1% Tween 20 and 5 mg/l of gentamycine sulphate (GIBCO)(TBS-T).

Coating: 150µl of a 100 ng /ml Vtg solution were incubated for three hours at 37°C in a sodium carbonate buffer (50 mM, pH 9.6) containing 5 mg/l of gentamycine.

Saturation: In order to reduce the background, the remaining free sites were blocked with pig serum diluted at 2% in TBS-T (TBS-T-SP) for 30 min. at 37°C.

Specific antibody incubation: Each well was filled with 75 µl of standards (purified Vtg, range: 3.9 to 4000 ng/ml) or sample and 75µl of specific diluted antibody (final dilution of $1:10^5$). Incubation lasted for 16 hours at 20°C. In order to determine the non-specific binding, four wells per plate were incubated without specific antibodies.

Secondary antibody incubation: The antigen-antibody complexes coupled onto the wells were incubated with 150 µl of the secondary antibody (sheep-anti-rabbit) diluted at $1: 4\ 10^3$ in TBS-T-SP for 2 hours at 37°C.

Peroxydase-anti peroxydase (PAP, DAKO) incubation: The PAP was diluted at $1: 5\ 10^3$ in TBS-T-SP and 150 µl were added in each well. It was allowed to incubate for 1 hour at 37°C.

Revelation: The peroxydase activity was revealed using a solution composed of 200µl of an o-phenylendiamin (0.5 g/l) and 30% oxygen peroxide (0.5 ml/l) solution in an

ammonium-acetate buffer (50 mM, pH 5) for 1 hour in total darkness. The reaction was halted by adding 50 µl of sulfuric acid 5M.

Measurement of optical density: Optical density was measured by a micro plate reader (Micro plate autoreader EL 311, Biotech Instrument) at 490 nm.

Statistical analysis.

The analysis of the competition curves was performed after linearisation through logit transformation, according to the following formula:

$$Y = a + bX$$

where $Y = \log [(B / B0) / 1- (B / B0)]$ and $X = \log (dose)$.

Students' t tests were used to compare the slope of each regression curve with the standard curve.

RESULTS

ELISA Validation.

Dilution test: Optimal dilutions for Vtg and for specific antibody were assayed by coating wells with serial dilutions of Vtg and incubating them with increasing dilutions of antibody (Fig. 1). A coating concentration of 100 ng/ml and a final antibody dilution of 10^5 were chosen in order to reach the highest optical density ($2 < Bo < 2.5$) after 1 hour of coloration development.

Time and temperature for coating: The most suitable conditions were obtained when coating was performed during 3 to 4 hours and at 37°C (Fig.2). After a 4-hours coating period, a decrease in the resulting optical density was noticed whatever the temperature .

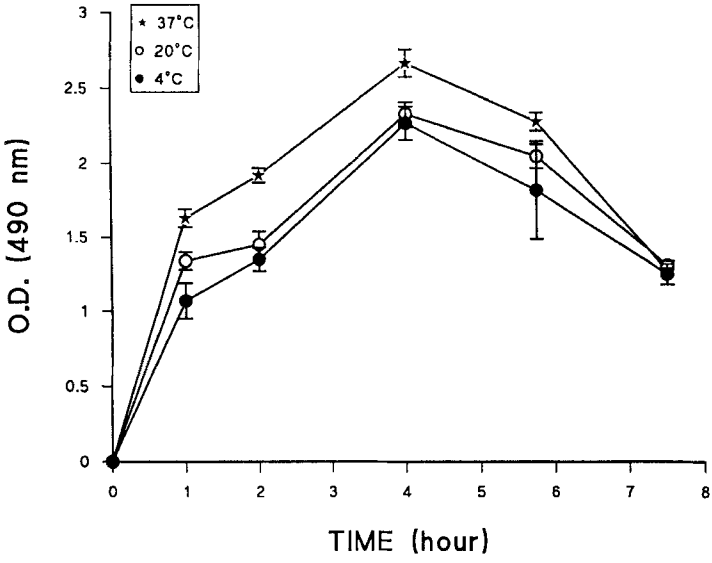

FIGURE 1. Determination of the time and temperature of coating. 15ng of Vtg were coated from 1 to 7.5 hours at 37°C, 20°C and 4°C.

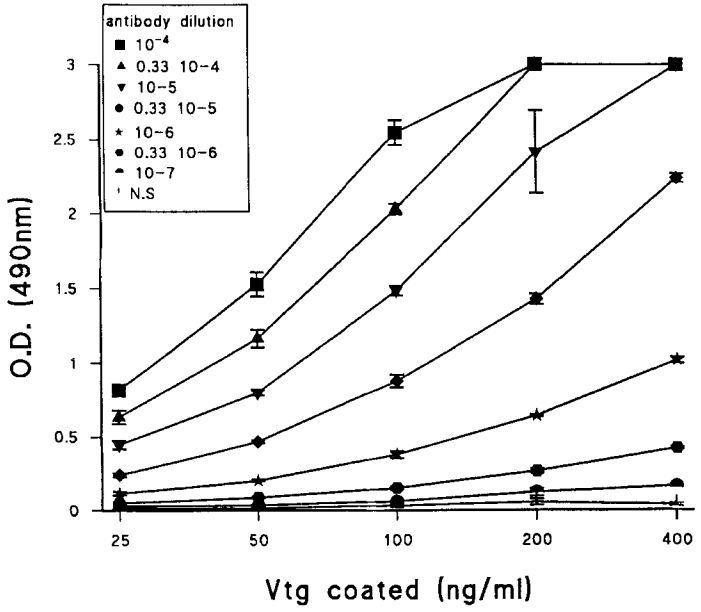

FIGURE 2. Determination of the concentration of coated Vtg and primary antibodies. B_0 evolution for increasing doses of coating with different anti-Vtg serum dilutions. All other steps were performed as described in Materials and Methods.

Assay Characteristics (Fig.3).

The assay detection limit (first dose of Vtg significantly different from $B_{0)}$ was 9.4 ng/ml. The usable range of the standard curve varied from 90% to 20% binding corresponding respectively to 33 ng /ml (± 10 ng / ml) and 1473 ng / ml (± 128 ng / ml) of Vtg. The sensitivity could be improved 3-fold by incubating the sample with specific antibody for 24 hours at 20°C before the competition involving coated Vtg began. For 50% of binding the coefficient of variation was 5.2% (n=26) within assays and 12.5% (n=5) between assays..

Specificity.

The parallelism between the standard curve and that obtained with increasing dilutions of serum of either vitellogenic, post ovulated or immature females, or oestrogenized males was checked (Fig 4a). There was no significant difference between these curves slopes, even for immature fish. Similarly, curves obtained with the cytosolic fraction of liver had no significant difference with the standard ones. However, the slope of the curve obtained with ovary extracts was significantly different for higher dilutions (Fig 4b).

Sera from various species of salmonids were then compared with purified Vtg and with rainbow trout serum (Fig.5). The displacement curves obtained either with oestrogenized male brown trout (*Salmo trutta*) serum or oestrogenized male brook char (*Salvelinus fontinalis*) serum turned out to be different from the standard curve. Nevertheless, they show a cross reactivity with the specific antibody amounting to 53% and 80%. The curves obtained with female Chinook salmon (*Oncorhynchus tshawytscha*) and oestrogenized male Atlantic salmon (*Salmo salar*) were partly parallel to the standard curves with respectively a 60% and 34% cross reactivity ratio.

We tested the specificity of the reaction obtained with sera from immature fish. In order to assess possible aspecific binding , we compared it with the binding obtained with

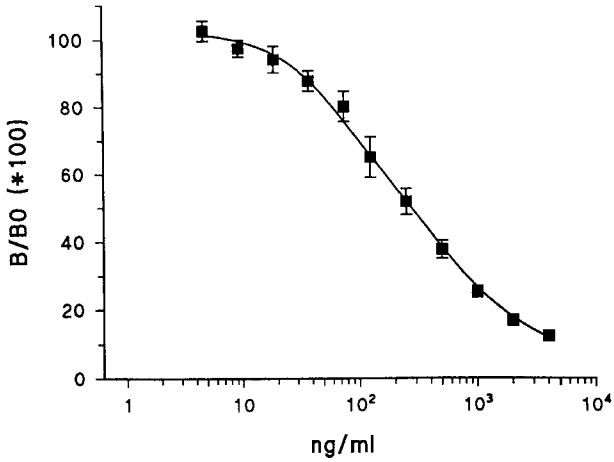

FIGURE 3. Standard curve obtained by serial dilutions of standard Vtg from 4000 to 3.9 ng/ml under Materials and Methods conditions. Each point is the average of 5 values.

ram serum, supposedly Vtg-free. Similar curves were obtained. In order to assess the specificity of the antibody, male serum and oestrogenized male serum were submitted to gel filtration chromatography (Fig.6). The screening showed a very low amount of Vtg in untreated male. However, a high peak of Vtg was detected in corresponding fractions of treated male serum. This demonstrates that the displacement observed in immature fish resulted from aspecific binding to a serum compound. To avoid such aspecific binding, we have diluted samples more than 16-fold.

DISCUSSION

The results presented here show that our assay is specific to rainbow trout Vtg. Nevertheless, an aspecific binding phenomenon occurred with serum compounds when serum dilutions were low (below 1:16). These compounds also exist in ram serum, which is supposed to be Vtg-free, as well as in male and immature trout. This might therefore be an aspecific serum effect which can easily be avoided by increasing serum dilution.

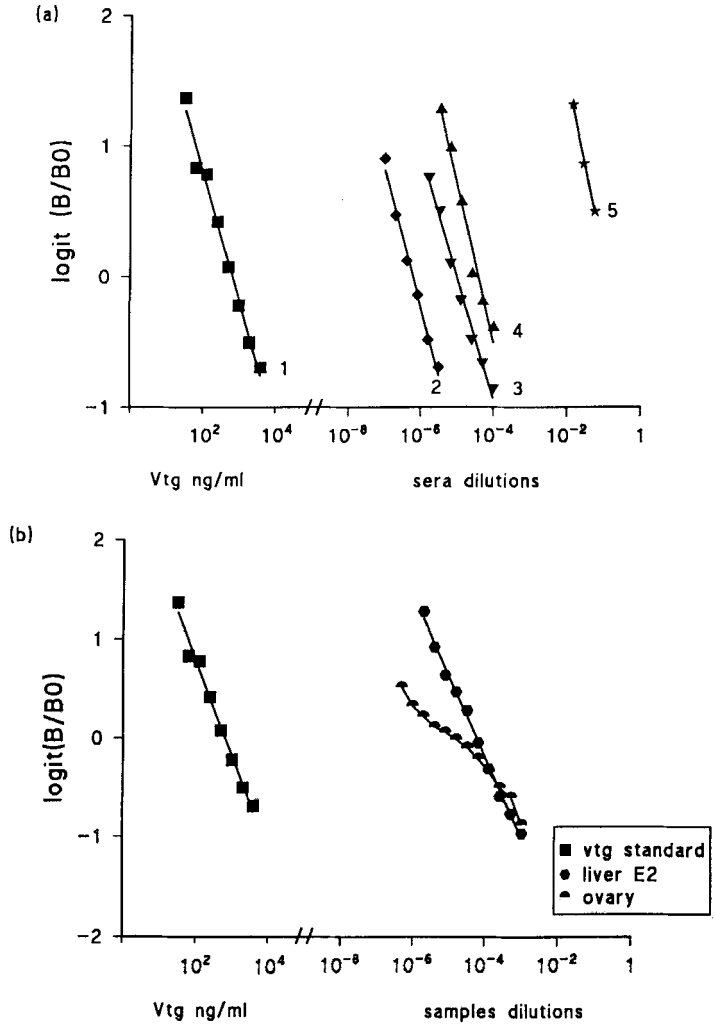

FIGURE 4. Parallelism between standard cuves and serum or tissue extracts dilutions from rainbow trout. (a) Displacement curves obtained with standard Vtg(1), serum from rainbow trout at the end of the vitellogenesis (2), from oestrogenised male (3), from post ovulated (4) and immature (5) females. (b) Displacement cuves obtained with the same standard Vtg and extracts of liver from oestrogenised male and ovary extracts. There is no parallelism between standard curve and displacement curve obtained with ovary extracts.

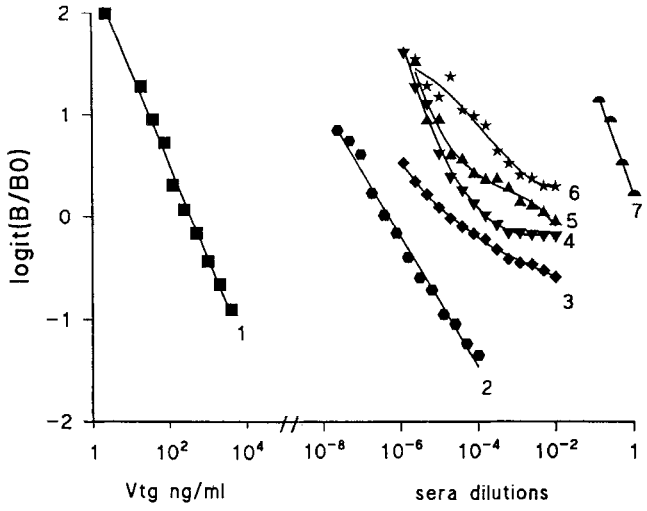

FIGURE 5. Displacement curves obtained with serial dilutions of sera from different salmonids. A ram serum (7) was used as control. (1) standard Vtg, (2) male oestrogenised rainbow trout,(3) male oestrogenised brook char, (4) mature female chinook salmon, (5) male oestrogenised brown trout, (6) male oestrogenised Atlantic salmon.

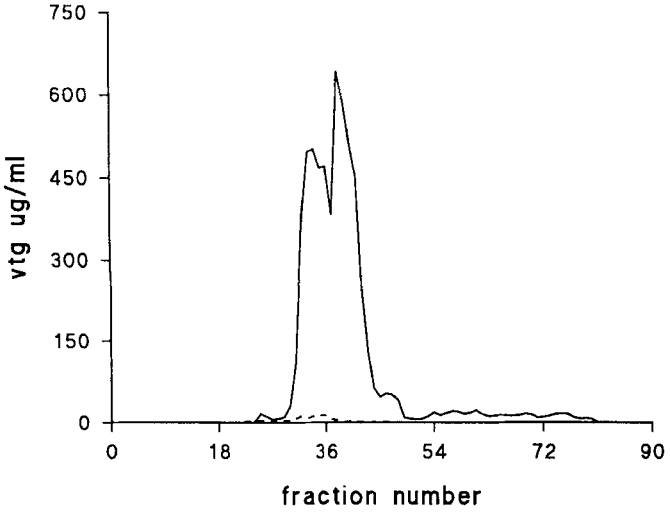

FIGURE 6. Vtg determination after gel filtration chromatography (sepharose 6B) of sera from oestrogenised male rainbow trout (continuous line) and untraited male (dotted line). Fractions were collected at 10ml/tube. Vtg in untraited serum was in the limit of detection of the assay.

The characteristics of the assay are similar to those described by other authors who have experimented with Vtg assays (13-15) for sensitivity and variability both within and among assays. We can also compare these characteristics to those obtained with RIAs described for salmonids (9-12). The processing time of these assays varies from 6 hours to 4 days for ELISA and from 1 to 4 days for RIA.

Several authors have measured the concentration of circulating Vtg among female salmonids. As regards rainbow trout, concentrations increase from 0.1 mg/ml to 12.9 mg/ml (20, 21) during the year preceding the first spawning. These levels remain within the same range observed for other salmonids (9, 12, 14).Therefore, the sensitivity of our assay allows the detection of Vtg at the very beginning of vitellogenesis, more than one year before sexual reproduction first takes place.

We analyzed parallelism between the standard curve and those obtained with the sera sampled from animals at various physiological stages: an oestrogenized male, and females at the end of vitellogenesis or post-ovulated. Our results show that the assay can be used for the assessment of the reproductive status of females. Even so, the curves obtained using liver extract are similar to the standard curve. We can, therefore, use this assay for the measurement of hepatic Vtg. This is no longer true for the intraovarian Vtg, the curves of which are significantly different from the standard curve. Vtg-derived proteins stocked in the ovary are immunologically different from the Vtg, so, this assay cannot be used to quantify the lipovitellin as some authors have done in ovary extracts (9, 13), or in egg protein fractions (10). Egg proteins cannot be used as a source of Vtg directly available for purification as was done by Idler et al (8).

The cross-reactions study with sera from other species shows that there are immunological homologies within Vtg, but also that they are quite different. This highlights the necessity of setting up a homologous assay for each species with the purpose of achieving quantitative determinations. However, this assay could be used for qualitative determinations among salmonids other than rainbow trout

In conclusion, the ELISA method we described is an easy and quick method to run (taking less than 24 hours) for rainbow trout Vtg detection. Its user-friendliness is all the more obvious since this competitive ELISA does not require very expensive material or technique. Its high sensitivity and reliability allows one to detect Vtg in serum or in the liver at any physiological stage, and to determine the sex of salmonids one year earlier than external criteria would normally allow for.

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