DEVELOPMENT AND VALIDATION OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY TO MEASURE VITELLOGENIN IN THE ZEBRAFISH (DANIO RERIO)

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Abstract—In this study, an enzyme-linked immunosorbent assay (ELISA) was developed to quantify vitellogenin (Vtg) in zebrafish (Danio rerio). Zebrafish Vtg (zf-Vtg) was purified from whole-body homogenates of estradiol-exposed zebrafish, and polyclonal antibodies against zf-Vtg were raised. Using purified zf-Vtg as a standard and anti–zf-Vtg antibodies (DR-264), a competitive ELISA method was set up and validated. The working range of the assay is from 1 to 30 ng/ml (20–80% binding), and the detection limit is 0.4 ng/ml for purified zf-Vtg. In whole-body homogenates samples, the practical detection limit is higher than that for purified Vtg (40 ng/ml) due to matrix effect. The intra- and interassay variations were 4.7% and 14%, respectively, at 50% binding (n = 36). Its usefulness to detect changes in Vtg concentration in other cyprinid fish was also tested. In addition, the assay was used to assess Vtg induction in male zebrafish exposed to 17β-estradiol (E2). Exposure of male zebrafish to 0.1, 1, 10, and 100 μg/L of E2 for 7 d led to a Vtg induction from the lowest concentration. The results show the suitability of the developed ELISA to quantify Vtg inductions in zebrafish, the cross-reactivity of DR264 antibodies with commonly used cyprinids, and the potential of zf-Vtg induction as a sensitive biochemical endpoint that could be used to detect estrogenic properties of chemical substances.

Keywords—Estrogens Vitellogenin Enzyme-linked immunosorbent assay Zebrafish Cyprinids

INTRODUCTION

In recent years, it has been shown that man-made chemicals and natural substances present in the environment are able to disturb the normal physiology and endocrinology of organisms [1]. These substances, termed endocrine-disrupting chemicals, have been defined as “exogenous substances that cause adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function” [2]. An important class of these substances are those acting as estrogens (xeno-estrogens). They have the capability to bind to the estrogen receptor as agonist and to initiate responses similar to those of the endogenous estrogen compounds [3]. Although most of the xeno-estrogen compounds are weakly estrogenic, exposure of organisms to some of these substances can cause reproductive impairment in the laboratory [4]. The Organization for Economic Cooperation and Development (OECD) recommends developing and validating new and improved guidelines for assessment of endocrine disruption in both mammalian and nonmammalian species [5]. For the latter, fish should represent a valuable model to assess endocrine-disrupting effects in oviparous species. Among the endpoints that are recommended, such as gonadal histology, the uses of relevant biochemical markers of endocrine disruption have to be considered.

Vitellogenin (Vtg) is an egg yolk precursor protein synthesized in the liver under the control of E2 by an estrogen receptor–dependent mechanism. Vitellogenin is released into the blood stream and transported to the ovaries, where it is incorporated into the growing oocytes and enzymatically cleaved into phosvitin and lipovitellin. Vitellogenin synthesis is normally limited to the reproductive female of oviparous vertebrates. Male and juvenile organisms possess the Vtg gene, which remains inactive under normal conditions when little E2 is produced but can be induced by exposure to (xeno-)estrogens. Because Vtg synthesis appears to be estrogen-specific, the induction of Vtg in male or immature fish can serve as a sensitive and reliable biomarker for exposure to estrogen agonists in oviparous vertebrates [6,7].

Small fish species, such as zebrafish (Danio rerio), present numerous advantageous advantages for laboratory testing methods. First, the zebrafish is a member of an ecologically important family of fish (Cyprinidae). Second, the zebrafish reaches its sexual maturity rapidly (90 d), and it can easily reproduce under laboratory conditions. Moreover, the spawning period occurs at any time of the year, and the number of eggs laid per female is generally high. Furthermore, it is a recommended and widely used fish species in many standard ecotoxicological OECD guidelines.

Use of the zebrafish as a test species to identify estrogenic substances requires development of a specific and sensitive method to measure Vtg induction. Immunoassays, such as the enzyme-linked immunosorbent assay (ELISA), have been shown to be sensitive and specific methods to quantify Vtg in various fish species [8], and the aim of the present study was to develop and validate a highly sensitive ELISA for the quantification of zebrafish Vtg (zf-Vtg). Development of such an assay requires, however, a substantial amount of purified Vtg to produce specific antibodies for use as standard and to coat the microtiter plate [7]. For larger fish species, such as rainbow trout and carp, Vtg is usually purified from plasma, but because of the small size of the zebrafish, large plasma samples are not easy to obtain. Consequently, plasma cannot be used to
purify zF-Vtg in large quantities, and instead, whole-body homogenate is used for purification of Vtg from this species. The circulating Vtg is most commonly measured in the plasma, but in zebrafish, it is much more convenient to measure Vtg in whole-body homogenate. Whole-body homogenates have been successfully used for determination of the amounts of sex-steroid hormones in larvae of Japanese flounder [9] and for measurement of Vtg induction during early life stages of fathead minnow exposed to E2 [7].

In the first part of this work, Vtg was purified from whole-body homogenate of estradiol-exposed zebrafish and partially characterized. Specific polyclonal antibodies were raised against the zf-Vtg, and a competitive ELISA was set up and validated to quantify zf-Vtg in whole-body homogenate. Second, the ELISA was used to quantify zf-Vtg induction in adult male zebrafish exposed to graded concentrations of the natural steroid hormone E2 to determine whether the zf-Vtg ELISA can be used to assess the estrogenic potency of a chemical substance as well as to assess the sensitivity of this species to exogenous estrogens. Finally, this study assessed the applicability of the zf-Vtg ELISA for use in detecting changes in Vtg concentrations in other fish species widely used in ecotoxicological work (roach, fathead minnow, Japanese medaka, rainbow trout, three-spined stickleback).

**MATERIALS AND METHODS**

**Origins of different fish species**

Adult male and female zebrafish (Cyprinidae, *D. rerio*) were obtained from a commercial fish farm (BioInternational, Nantes, France). Adult male and female Japanese medaka (Adrianichthyidae, *Oryzias latipes*) were kindly provided by Muriel Mambrini (INRA, Jouy en Josas, France). Adult male and female three-spined stickleback (Gasterosteidae, *Gasterosteus aculeatus*) were sampled from a wild population in the springtime and held in the laboratory for at least two weeks before the exposure took place. Adult male and female roach (Cyprinidae, *Rutilus rutilus*) were obtained from a commercial fish farm (Moulin de Rogny, Aisne, France) and were held in tanks supplied with dechlorinated city water. Rainbow trout (* Oncorhynchus mykiss*) came from an experimental fish farm (INRA, Gournay sur Aronde, Oise, France) or were obtained from a commercial fish farm (Bognày Fiskeoppdrett, Hamre, Norway). Carp were imported from a commercial fish farm in Singapore. Plasma samples from control male and estrogenized female fathead minnow (*Pimelophales promelas*) were kindly provided by Robert Bringolf and Robert Summerfelt (Iowa State University, Ames, IA, USA).

**Vitellogenin induction in different fish species**

In small fish (zebrafish, Japanese medaka, and stickleback), Vtg synthesis was induced by aqueous exposure to E2. For each species, fish of mixed sex were exposed in separate 5-L glass aquaria to 100 µg/L of E2 (Sigma, St. Louis, MO, USA) for one week. A control group for each species was exposed to solvent alone (0.01% w/v final) after E2 was initially dissolved in ethanol. The water was renewed every day. After one week of exposure, the fish were weighed, sexed, and homogenized.

In roach, rainbow trout, and carp, Vtg synthesis was induced by intraperitoneal injection of E2 (2 or 10 mg/kg) dissolved in soybean oil (Sigma). Control fish were not injected.

**Exposure of zebrafish to graded concentrations of E2**

Exposure took place in 5-L aquaria filled with ISO tap water (International Organization for Standardization NF EN ISO 7346; pH 7.6 ± 0.3; dissolved oxygen, 510 ± 10 mg/L; conductivity, 650 ± 40 µS/cm [mean ± SEM]). Before the water was renewed, the fish were fed with Tetramin® (Tetrawerke, Melle, Germany). Groups of 10 adult male zebrafish were exposed to E2 at a nominal concentration of 0.1, 1, 10, and 100 µg/L for 7 d. Stock solutions of E2 were prepared by dissolving an appropriate amount of E2 in absolute ethanol and were stored at −20°C. To reach the nominal concentrations, 0.4 ml of stock solution was added to 4 L of water. Ethanol alone was added in the control so that the concentration of solvent was the same in all groups. Contaminated water was renewed each day. At the end of the exposure period, fish were killed, weighed, and measured (full length). Tests were removed and weighed to determine the gonadosomatic index (GSI). For the Vtg quantification, body homogenates were made according to the protocol described below.

**Fish sampling**

**Blood sampling.** For the large fish species (rainbow trout, carp, and roach), blood plasma samples were collected one week after E2 injection into chilled, heparinized syringes and immediately transferred to vials containing the protease inhibitor aprotonin (2 trypsin inhibitor units/ml), aliquoted, and stored at −80°C. Control plasma samples were prepared from noninduced juvenile or male fish.

**Whole-body homogenization procedure.** For Vtg measurement by ELISA, the small fish species (zebrafish, stickleback, and Japanese medaka) were homogenized in ELISA buffer (phosphate buffer saline [PBS], 1% w/v bovine serum albumin [BSA], and 1 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.3) in a ratio of 1:2 (w:v). After centrifugation of the homogenates (3,000 g, 15 min, 4°C), the supernatants were withdrawn, aliquoted, and stored at −80°C until use.

For zf-Vtg purification, zebrafish were homogenized in 0.1 M Tris-HCl, 1 mM PMSF (pH 8.5, 4°C) with a ratio of 1:4 (wt/volume buffer). Because the ovaries of maturing females are a source of degradation products of the Vtg (i.e., lipovitellin and phosvitin), the ovaries were removed before homogenization. The whole-body homogenates were centrifuged at 10,000 g for 15 to 20 min at 4°C. The supernatant was subsequently withdrawn and filtered through a 0.22-µm filter.

**Zebrafish-Vtg purification**

Zebrafish-Vtg was purified from whole-body homogenate of E2-induced fish according to a previously described method [10]. This method has been shown to be effective in purifying fish Vtg from plasma of various estrogenized fish species, including other Cyprinidae. However, due to the different nature and complexity of the biological matrix (i.e., whole-body homogenate instead of plasma), some modifications were introduced in the general protocol.

All the chromatographic steps were performed at room temperature using an ÄKTA Purifier 10 liquid chromatography system (Amersham Pharmacia Biotech, Uppsala, Sweden). One milliliter of sample was applied onto a Resource Q column of 1 ml (Amersham Pharmacia Biotech) equilibrated with five column volumes of 0.1 M Tris-HCl, 1 mM PMSF (pH 8.5). The bound proteins were separated by a 10-column-volumes linear gradient of 0 to 0.6 M NaCl at a flow rate of 2.5 ml/
min. Fractions of 1 ml were automatically collected on a fraction collector. The column was washed with three volumes of 1 M NaCl at the same flow rate. Before the gel permeation chromatography, the fractions containing Vtg were concentrated by ultrafiltration (3,000 g, 4°C; Amicon Centrifu lus 100; Millipore, Bedford, MA, USA). Two-hundred microliters of ultrafiltered sample were then applied onto a gel permeation column (Superdex 200 HR 10/30; Amersham Pharmacia Biotech) equilibrated with carbonate-bicarbonate buffer (pH 9.6). The proteins were eluted using an isocratic elution with 1.5 column volumes at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected. Total protein was determined using a Dc Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) with BSA (Sigma) as standard.

**Characterization of zf-Vtg**

*Gel electrophoresis.* Samples from control and E2-treated fish and chromatographic fractions were analyzed by native and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 0.1% SDS) performed according to the method described by Laemmli [11] in a discontinuous gel with a 4% stacking gel and a 7.5% resolving gel of acrylamide. For SDS-PAGE analysis, the samples were diluted in sample buffer (0.5 M Tris-HCl [pH 6.8], 10% glycerol, 0.1% SDS, and 0.02% bromophenol blue). When β-mercaptoethanol was included in sample buffer (5%, v/v), the tested sample was heated at 95°C for 5 min. For native PAGE analysis (without SDS and β-mercaptoethanol), samples were diluted in 0.5 M Tris-HCl (pH 6.8) and 10% glycerol and were not heated.

Protein molecular weight markers were included in the electrophoresis (Kaleidoscope Prestained Standards; Bio-Rad Laboratories; or high-molecular-weight electrophoresis calibration kit; Amersham Pharmacia Biotech). After electrophoresis, the protein bands were stained with Coomassie brilliant blue R250. The logarithm of molecular mass markers was plotted against electrophoretic mobility, and linear regression was used to calculate the relative molecular mass of the samples.

*Preparation of antibodies.* Polyclonal antibodies against zf-Vtg were raised in rabbits. Two rabbits (New Zealand white × black) were immunized by subcutaneous injections of 150 μg of purified zf-Vtg emulsified in Freund's complete adjuvant. Two booster injections of 150 μg of zf-Vtg in Freund's incomplete adjuvant were given four and eight weeks after the first injection. The rabbits were bled through the ear vein 10 to 13 d after the last booster dose. The blood was allowed to clot for 1 h at room temperature, and the antiserum was collected after centrifugation. The IgG fraction of the antiserum was isolated by affinity chromatography on a HiTrap Protein G column (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The purified IgG fraction was subsequently passed over a PD-10 column (Amersham Pharmacia Biotech) for buffer exchange and diluted to an appropriate concentration in the following buffer: 0.1 M Na-phosphate (pH 7.4), 0.15 M NaCl, 0.1% Na-azide, and 1% BSA. The final antibody preparation, designated DR-264, was stored at 4°C.

**Characterization of antibodies.** The specificity of the DR-264 antibodies was tested in Western blot analysis using purified Vtg and plasma or whole-body homogenate samples from zebrafish, common carp, fathead minnow, roach, medaka, rainbow trout, and stickleback. Samples containing purified Vtg (0.5 μg per well), plasma (10 μl of a 1:200 dilution per well), or whole-body homogenate (10 μl of a 1:100 dilution per well) were separated by SDS-PAGE in 7.5% polyacrylamide gels [11]. Western blotting was performed essentially as described previously [12]. Briefly, the separated proteins were electrotransferred to a nitrocellulose membrane, and the membrane was blocked, washed, and incubated with DR-264 (1:500 dilution) overnight at room temperature, then washed and incubated with peroxidase-labeled secondary antibody (goat anti-rabbit IgG, horseradish peroxidase–conjugate, 1: 3,000 dilution; Bio-Rad Laboratories) for 3 h at room temperature, washed, and developed (10 min) using 4-chloronapthol/H2O2 as substrate.

**Zebrafish-Vtg competitive ELISA**

The method described below is based on a competition for the anti-Vtg antibodies between the Vtg in the plasma sample and the Vtg adsorbed on the microtiter plate. The assay was calibrated using zf-Vtg standards. During the method development, optimal concentrations of the reagents were determined. The established zf-Vtg ELISA procedure is described below.

*Coating.* Microtiter plates (96-well Nunc Maxisorp; Nunc, Roskilde, Denmark) were coated with 100 μl per well of purified zf-Vtg solution at a concentration of 100 ng/ml in 0.05 M carbonate/bicarbonate buffer (pH 9.6). To determine the nonspecific binding, 100 μl of coating buffer containing no zf-Vtg were added to two wells. The plates were sealed and incubated for 16 h at 4°C.

**Preincubation of standards and whole-body homogenate samples with primary antibody.** The standard of zf-Vtg was serially diluted by a factor of two in PBS, 1% BSA from 125 to 0.1 ng/ml. Similarly, whole-body homogenate samples were serially diluted from 1:10 to 1:100,000. Standard solutions of zf-Vtg or whole-body homogenate dilutions (150 μl) were mixed (1:1) with primary antibody solution (final dilution of 1:500 in PBS, 1% BSA). Similarly, whole-body homogenate samples were serially diluted from 1:10 to 1:100,000. To determine the absorbance at maximal binding (B0), samples containing only antibody (no Vtg) were included in each assay. All the zf-Vtg standard and sample solutions were mixed thoroughly, placed at room temperature for 30 min, and subsequently incubated overnight at 4°C.

**Blocking.** The ELISA plates were washed three times with 200 μl of PBS, 0.05% Tween 20 (PBS-T) and blocked with 200 μl of PBS, 2% BSA for 1 h at 37°C to avoid nonspecific binding and, hence, to reduce the background signal.

**Incubation with primary antibody solution.** After a washing step as described above, the sample-antibody solutions from the preincubation step were mixed thoroughly, and 100 μl of each preincubated sample were transferred to the coated and blocked plate. All the samples were added in duplicate. The plates were then incubated for 2 h at room temperature.

**Incubation with secondary antibody solution.** Plates were washed three times with PBS-T, and each well received 100 μl of secondary antibody (horseradish peroxidase goat anti-rabbit IgG) diluted 1:2,000 in PBS and 1% BSA. Incubation was performed for 2 h at 37°C.

**Revelation.** Plates were washed five times with PBS-T, and the peroxidase activity was revealed by adding 100 μl of tetramethyl benzidine enzyme substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). The enzyme reaction was stopped after 30 min by addition of 50 μl of 1 M phosphoric acid (H3PO4), and the absorbance was read at 450 nm using a microplate reader.

**Calculation of results.** The percentage binding (B/B0) in
each standard or sample dilution was calculated using the following relationship:

\[ \frac{B_i}{B_0} = \left( \frac{\text{standard or sample absorbance} - \text{NSB}}{\text{maximal binding absorbance} - \text{NSB}} \right) \times 100 \]

The \( B_i/B_0 \) standard data were fitted using least-squares method in a four-parameters logistic model according to the following equation:

\[ \frac{B_i}{B_0} = \frac{(A - D)}{1 + \left( \frac{\text{concentration}}{\text{EC50}} \right)^a} + D \]

where \( A = \) maximal binding value, \( B = \) slope of the curve, \( \text{EC50} = \text{zf-Vtg concentration leading to 50\% inhibition of the maximum binding (B0)}, \) and \( D = \) maximal inhibition value.

Validation of the zf-Vtg ELISA method

Parallelism between standard and dilution curves of various fish species samples. Plasma or whole-body homogenate samples from control males, E2-exposed males, and vitellogenic females of the different fish species were serially diluted in PBS, 1% BSA and assayed to assess the parallelism between the competition curves obtained with the plasma or homogenate samples and the zf-Vtg standard curves.

Reproducibility. To assess within-series precision and reproducibility of the ELISA method, zf-Vtg standard curves were performed over a period of 9 d. Standard curves were made in quadruplicates and were fitted with a four-parameters logistic model. The zf-Vtg concentrations at 90%, 80%, 50%, and 20% binding were calculated from each modeled curve and analyzed according to the method described by Caporal-Gautier et al. [13,14] using a one-way analysis of variance (with \( n - 1 = 35 \) and \( K - 1 = 8 \) as the degree of freedom). Intra- and interassay coefficients of variation (CV) expressed as a percentage were calculated for the zf-Vtg concentration at 90, 80, 50, and 20% binding using the following relationships:

\[ \text{CV intraassay} = \frac{\text{intra group variance}^2}{\text{general mean}} \times 100 \]

\[ \text{CV interassay} = \frac{\text{total variance}^2}{\text{general mean}} \times 100 \]

Detection limit. The detection limit of the zf-Vtg ELISA assay was defined as the value three standard deviations below the mean of the \( B_0 \) values (\( n = 48; \) mean value zero standard = 3 \( \pm \) standard deviation).

Matrix effect

To determine to what extent the whole-body homogenate could interfere with the Vtg quantification, 0.1, 0.2, 0.5, 1, 2, 4, 8, 16, 30, 60, and 125 ng/ml of purified zf-Vtg were added to a whole-body homogenate of a nonexposed male fish diluted 1:10, 1:50, and 1:100. The obtained curves were compared to a normal standard curve performed in PBS, 1% BSA.

Applicability of the zf-Vtg ELISA in other fish species

The plasma (or whole-body homogenates) from control male and E2-induced cyprinid fish (carp, fathead minnow, and roach) and noncyprinid fish (Japanese medaka, stickleback, and rainbow trout) were serially diluted and assayed as described above for the zebrafish whole-body homogenates. The dilution curves were then compared with the curve of the zf-Vtg standard.

Statistical analysis

Vitellogenin data were log-transformed to conform to the normality test and to homogeneity of variance (Levene’s test). The 95% confidence interval (95% CI) was calculated on the Log-transformed data using the critical values of Student’s \( t \)-distributions. One-way analysis of variance was performed on GSI, weight, length, and \( K \) factor and on the log-transformed Vtg data, followed by Dunnett’s test for postcomparisons of groups.

RESULTS

Zebrafish-Vtg purification and characterization

The elution profiles after anion-exchange chromatography of control males and of males exposed to 100 \( \mu \)g/L of E2 are shown in Figure 1A. In whole-body homogenate of E2-exposed fish, a large peak was eluted at a conductivity of 28 \( \mu \)S/cm, corresponding to a salt concentration of 0.35 M NaCl. This
peak was not observed in whole-body homogenate of control fish when applied to the same column; thus, it was identified as the Vtg peak. To ensure a maximal purity of the protein, Vtg fractions were subjected to gel permeation chromatography to remove trace contaminants and the salt (desalting). In this step, the elution profile was characterized by one symmetric peak eluted at an apparent molecular mass of \(375 \times 10^3\) relative molecular mass (Fig. 1B).

Figure 2A and B shows the electrophoretic pattern under nondenaturing and nonreducing conditions of proteins from whole-body homogenate of control and induced zebrafish and purified zf-Vtg. Two bands of high molecular weight (I and II) appeared after the 7-d treatment with 100 \(\mu\)g/L of \(E_2\). The two bands were not observed in control fish (Fig. 2A), and they are assumed to be two Vtg isoforms. Similarly, when the fraction from the gel permeation chromatography was subjected to native electrophoresis, only two bands were stained (Fig. 2B). Gel electrophoresis analysis revealed that the two bands had the same relative mobility as bands I and II observed in whole-body homogenate of estrogenized fish. Bands I and II migrated at a position of 520,000 and 440,000 kDa, respectively. Because in native PAGE the separation principle is not directly linked to molecular mass, estimations of the molecular mass are given as an indication only. When the same solution was subjected to gel electrophoresis under denaturing (SDS) and reducing (\(\beta\)-mercaptoethanol) conditions, several protein bands were stained, probably corresponding to different zf-Vtg monomers as well as to degradation products of the protein (Fig. 2C). The relative molecular masses of the three major bands were 150, 130, and 120 kDa, respectively. Additional faint bands were detected with molecular masses of 178, 103, 99, 79, and 60 kDa. The purified solution was used as antigen for rabbit immunization and as standard in the ELISA method under development.

**Antibody production and characterization**

Immunization of two rabbits with purified zf-Vtg resulted in production of a high titer of antibodies in both rabbits after the first booster injection. A second booster injection resulted in an increased titer of the antiserum of one rabbit, and this antiserum was used for further studies. After purification of the IgG fraction of the antiserum, the antibodies (DR-264) were initially characterized by Western blot analysis using purified Vtg and plasma samples or whole-body homogenates from a variety of fish species (Fig. 3). The results showed that DR-264 binds zf-Vtg both in purified form and in whole-body homogenate samples from \(E_2\)-induced zebrafish. The antibody also showed a strong cross-reaction with purified carp Vtg and with Vtg in plasma samples from carp, fathead minnow, and roach. In contrast, only a very weak cross-reaction was observed with Vtg from whole-body homogenate samples of medaka (only observed when high amounts of whole-body homogenate were loaded on the gel; data not shown). No significant cross-reaction with Vtg was observed in samples from rainbow trout or stickleback. No significant nonspecific bind-
Fig. 4. Determination of optimal concentration of zebrafish vitellogenin (zf-Vtg) and primary antibody (DR-264) for the development of the zf-Vtg enzyme-linked immunosorbent assay. Abs = absorbance.

Fig. 5. Cross-reaction of whole-body homogenates from mature female, immature female, control male, and two estrogenized male zebrafish (one at 0.1 and one at 100 μg/L of 17β-estradiol [E2]) in the zebrafish vitellogenin (zf-Vtg) enzyme-linked immunosorbent assay. B/B0 = percentage binding.

Fig. 6. Comparison of curves obtained using zebrafish vitellogenin (zf-Vtg) standards in enzyme-linked immunosorbent assay buffer and in whole-body homogenate of male diluted 1:10, 1:50, and 1:100. In the routine assay, zebrafish samples were diluted 1:100 to avoid the matrix effect. B/B0 = percentage binding.

Table 1. Characteristics of the zebrafish vitellogenin (zf-Vtg) enzyme-linked immunosorbent assay (ELISA)∗

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∗For routine measurement, the ELISA assay with a combination of 100 ng/ml of purified Vtg and antibody dilution of 1:500 was selected.

CV = coefficient of variation.

Development and validation of ELISA

Figure 4 presents the results of a criss-cross serial dilution test in which concentrations of both zf-Vtg and primary antibody are varied. Several combinations of coating concentrations and primary antibody dilutions that yielded an optimal absorbance (between 1.5 and 2.0) were selected, and standard curves were generated to evaluate the performance of each. Based on the results of this experiment (data not shown), the 100 ng/ml-1:500 and the 200 ng/ml-1:1,000 combinations were retained, because optimal absorbance at 0 ng/ml of zf-Vtg (B0) and zf-Vtg concentrations at 80%, 50%, and 20% binding were satisfactory for both combinations. The ELISA precision (intra- and interassay variation) as well as the detection limit of each assay were then further analyzed; the results of these experiments are summarized in Table 1.

Both assays are characterized by a low detection limit: 0.3 and 0.4 ng/ml, respectively, when calculated as B0 minus three standard deviations. The combination A has a broader working range (B/B0: 20–80%, 1–110 ng/ml) compared to combination B, but because the intra- and interassay coefficients of variation were significantly lower and consistent across the standard curve for the combination B (100 ng/ml-1:500), combination B was selected for the routine assay.

Parallelism between zf-Vtg standard curve and zebrafish whole-body homogenates samples

Figure 5 shows the competition curves obtained by serially diluting whole-body homogenates of females and estrogenized males. A very good parallelism was observed between the competition curves and the zf-Vtg standard curve. In contrast, no significant reaction was detected with whole-body homogenate of control males. However, binding of the zf-Vtg coated on the microplate to the antibody was inhibited in the presence of very low dilutions of male zebrafish homogenates.

Matrix effect

Figure 6 shows the results obtained when purified Vtg was added to a whole-body homogenate of a nonexposed male fish containing no detectable Vtg. For the lowest dilutions (1:10 and 1:50), we observed a clear inhibition of the binding of zf-Vtg coated on the microplate and a lack of parallelism between proteins other than Vtg was observed in control samples from the fish species tested.
the standard addition curve and the standard curve. For 100-fold diluted homogenate, the percentages of binding were very close to those observed for the standard curve, except at very low concentrations of Vtg. The slight discrepancy of the data occurred at concentrations close to the detection limit of the assay. In 1:100 diluted homogenate, the zf-Vtg concentration leading to 80, 50, and 20% inhibitions were 0.2, 4.3, and 41 ng/ml, respectively (i.e., very close to the values observed for a standard curve) (Table 1). Hence, to avoid the matrix effect, a dilution of at least 1:100 was chosen for the routine assay. Consequently, the detection limit for Vtg in whole-body homogenate samples was 40 ng/ml.

Cross-reaction of plasma or whole-body homogenate samples from other fish species in zf-Vtg ELISA

Noncyprinid fish species. The cross-reaction of samples from rainbow trout, stickleback, and Japanese medaka in the zf-Vtg ELISA are shown in Figure 7. Plasma from control male and female rainbow trout was unable to displace zf-Vtg at any dilution, with the percentage of binding always being more than 95%. Due to the absence of cross-reaction in the zf-Vtg ELISA, the concentration of plasma Vtg in female trout was undetectable, whereas the concentration determined by a rainbow trout ELISA [15] was 25 mg/ml. A solution of purified stickleback Vtg showed a limited cross-reaction. The estimated concentration was 7.8 µg/ml as assessed by the zf-Vtg ELISA, whereas the protein concentration of the purified solution determined using the DC Protein Assay kit gave a concentration of 1.7 mg/ml (a more than 230-fold difference). Similarly, the Japanese medaka Vtg cross-reacted very weakly: Inhibition was observed in both males and females from the control and exposed groups, and always at low dilutions (1:10). It is very likely that this effect was largely due to nonspecific binding, because at this dilution, a matrix effect was observed with the whole-body homogenate of zebrafish.

Cyprinid fish species. No cross-reaction was observed with plasma from control male carp or control male fathead minnow; Vtg concentrations were less than the detection limit of the assay (data not shown). Binding of the antibodies to the zf-Vtg coated on the microplate was inhibited in the presence of plasma from control male roach at low dilutions: The concentration measured was 550 ng/ml. Dilutions of plasma from E₂-exposed carp (sex unknown) and from E₂-exposed female fathead minnow and female roach had binding curves similar to that of the zf-Vtg standard (Fig. 8), in contrast to the E₂-induced male roach binding curves, which were not parallel to the zf-Vtg standard curves. In exposed carp and fathead minnow, the concentrations were 195 and 25 µg/ml, respectively. In different control female roach, the concentrations were 4.6, 15, and 56 µg/ml (Fig. 8 shows the binding curve for one female containing 4.6 µg/ml). After one intraperitoneal E₂ injection of 2 mg/kg, the Vtg levels reached 63 and 13 µg/ml in males and 76 µg/ml in a female. However, one can note that the Vtg concentration determined for male roach could not be completely accurate because of the lack of full parallelism of the observed curves for male roach.

Zebrafish semistatic exposure to E₂

To validate the usefulness of the developed ELISA for quantification of Vtg-inductions in zebrafish, groups of 10 adult male zebrafish were exposed to E₂ at various concentrations. Exposure to E₂ did not produce mortality in adult male zebrafish, suggesting that neither the solvent nor the tested concentrations of E₂ had an acute toxicity effect in our test conditions. Figure 9 shows the whole-body homogenate concentrations of Vtg measured by the zf-Vtg ELISA after 7 d of exposure. Exposure to E₂ lead to a significant Vtg induction in males (analysis of variance, p < 0.0001). In the control group, the measured Vtg concentrations were, for some fish, less than the practical detection limit of the assay (i.e., <40 ng/ml) and, for other fish, up to 560 ng/ml. The geometric mean for Vtg in whole-body homogenate in nonexposed males was 132 ng/ml (95% CI, 72–161 ng/ml): Exposure to E₂ led to high Vtg inductions. Measured mean concentrations in the
Different letters are significantly different (p < 0.001).

group exposed to 0.1 μg/L of E2 were 40.5 μg/ml (95% CI, 30–125 μg/ml). From 1 μg/L of E3, Vtg concentrations reached a plateau and were more than 2 mg/ml, corresponding to a more than 18,000-fold increase in Vtg.

To characterize these induction levels, Vtg concentrations were also measured in unexposed females. Depending on their state of maturity, the Vtg concentrations (mean ± standard deviation) varied as follows: 3.97 ± 2.7 μg/ml (GSI = 3.6 ± 1.6%, n = 3), 60.1 ± 25 μg/ml (GSI = 10.0 ± 2.4%, n = 12), and 442.5 ± 180 μg/ml (GSI = 15 ± 4%, n = 10).

DISCUSSION

In this article, we report the establishment and validation of an immunoassay to detect and quantify Vtg in the zebrafish (D. rerio). Different methods have been used to quantify Vtg induction. These include measurement of the alkaline-labile protein–bound phosphorus [16,17] and gel electrophoresis [18,19], the main advantage of which is that they are independent of the fish species. However, these methods are generally less sensitive compared to immunochemical methods and, consequently, are more suitable for detection of high Vtg induction. Moreover, alkaline-labile phosphate levels can be subject to confounding factors, such as dephosphorylation of the protein due to prolonged storage and hemolysis of the plasma sample [20]. Very specific and sensitive methods to detect changes in Vtg mRNA, such as Northern blot analysis and reverse transcriptase–polymerase chain reaction, have been developed [21–23]. However, dose–effect relationships are difficult to establish, because Vtg mRNA can be stabilized (half-life increase). Hence, the level of Vtg mRNA may not necessarily increase even though a significant increase of the protein may be obtained [22]. Immunochemical methods, such as radioimmunoassay (RIA) [24,25] and, more recently, ELISA [7,20,26], have been set up to measure the circulating Vtg in different fish species. These methods appear to be very sensitive and specific, allowing the quantification of low amounts of Vtg (ng/ml). With small fish species, such as zebrafish, methodological difficulties related to their small size (e.g., for induction and purification of the Vtg) had to be overcome in the development of such antibody-based assays.

Induction, purification, and characterization of the zf-Vtg

Exposure to E2 by intraperitoneal injection is widely used to induce synthesis of Vtg in larger fish, such as trout and carp. In small fish species, this type of exposure is more delicate to perform, because the volume of carrier in which E2 is dissolved must be very low (microliters) so that the fish do not suffer from the injections. We thus chose to expose fish to high concentrations of E2 via the water for a short period of time, leading to a massive induction of Vtg in male zebrafish, as demonstrated by anion-exchange chromatography as well as by gel electrophoresis of whole-body homogenate of control and E2-exposed male zebrafish (Figs. 1 and 2A).

On the other hand, adult zebrafish are too small to obtain enough blood samples to purify Vtg in large amounts. To avoid these problems, some authors have used egg yolk proteins as a source of antigenic material to develop polyclonal anti-zebrafish egg yolk antibodies, which were subsequently used to detect Vtg in fish exposed to chemicals [27,28]. The main advantage of using egg yolk proteins is that they can be easily obtained from ovaries in the large quantities necessary for immunization and standard production [28]. However, it may give antibodies that react to epitopes that are not identical to those found in the circulating Vtg, resulting in a less accurate quantification of Vtg. To avoid these potential problems, whole-body homogenate was used as a source of nonproteolytically cleaved Vtg for immunization and standard production. Use of this biological matrix as a source of Vtg requires centrifugation and filtration of the supernatant to eliminate debris and particles that could interfere with the first chromatographic step. Moreover, due to the high viscosity of the whole-body homogenate compared to plasma samples, it is necessary to dilute the sample before injection onto the Resource Q column. Centrifugation at 10,000 g, filtration through a 0.22-μm filter, and dilution until complete clarification occurs allow the use of a high-resolution column at a high flow rate. Under these conditions, the combination of anion-exchange chromatography and gel permeation chromatography gave, in a short time, up to 3,000 μg/ml of purified zf-Vtg solution (Fig. 2B).

In the E2-exposed fish as well as in the purified Vtg solution, two bands (I and II) were observed (Fig. 2A and B). These two proteins are likely to be two isoforms of the circulating zf-Vtg, probably produced from different zf-Vtg genes [29]. It has been demonstrated that more than one form of Vtg exists in other fish species, such as in Oreochromis aureus [30], O. mossambicus [31], and Epinephelus malabaricus [32]. The presence of a Vtg variant in the fathead minnow (P. promelas) has also been suggested [20]. When a purified solution of zv-Vtg was subjected to SDS-PAGE, numerous protein bands were stained that corresponded to monomers from the two different Vtg molecules and to breakdown products of the native proteins (Fig. 2C). Similar results have been observed for the purified solution of Vtg from different teleost fish species [10,20,26,33,34].

Validation of zf-Vtg ELISA

The developed ELISA is a competitive binding assay in which antibodies raised against zf-Vtg are preimmubated together with samples (or standards) overnight at 4°C. Non-equilibrium assays have been previously developed to measure Vtg in teleost fish and have been proved to enhance the sensitivity of ELISA [7,35]. The zf-Vtg ELISA developed in the present study has a detection limit of 0.4 ng/ml and is comparable to RIAs for Vtg [25,36,37] and to very sensitive ELISAs developed for carp [7] and rainbow trout [34]. The high sensitivity of the assay was an important criterion in the
method development, because our objective was to use it to detect weak estrogenic effect in chemical-exposed fish. However, the sensitivity of the zf-Vtg ELISA was reduced due to the matrix effect at high concentration of whole-body homogenate. A start dilution of whole-body homogenate samples of adult fish of 1:100 is required to avoid this nonspecific interaction (Fig. 6), giving a practical detection limit of 40 ng/ml in the whole-body homogenate. Matrix effect at low dilutions has been previously reported for Vtg ELISA [15,33–35,38], whereas RIA appears to be less sensitive to matrix effect, allowing accurate quantification of Vtg in the range of 1 to 100 ng/ml (J.P. Sumpter, personal communication). The working range as well as the intra- and interassay coefficients of variation of the assay were satisfactory and comparable to those of other ELISAs for teleost Vtg [7,15,33–35,38].

For E2-exposed males and females, full binding curves parallel to the standard curves were observed, showing that the ELISA is suitable for quantification of Vtg in whole-body homogenate. Using the zf-Vtg ELISA, E2-exposed male fish and females were easily distinguished from control males, and immature females were easily distinguished from both control males and sexually mature females (Fig. 5).

Zebrafish-Vtg concentrations in control, female, and E2-exposed male fish

In the present study, detectable levels of Vtg in some non-exposed male zebrafish have been measured. The cause of this low expression is uncertain. Expression of low levels of circulating Vtg could be due to an exposure to phyto-estrogens through the diet or due to the presence of weak estrogenic compounds, as suggested by Tyler et al. [7]. On the other hand, the low expression of Vtg in male zebrafish could be a response to the natural circulating E2. Very recent studies have shown seasonal change of serum Vtg levels in the male flounder (Pleuronectes yokohamae) [39], as occur in a salmonid fish species (Oncorhynchus clarkii) [40]. Regarding this latter fish species, those authors reported Vtg concentrations in some male fish up to 8 μg/ml two months before the breeding season. Clearly, further studies should be conducted to determine the cause of this low expression of Vtg in untreated male zebrafish.

Vitellogenin induction in male fish exposed to E2 was dose-dependent and occurred from the lowest concentration of E2 (100 ng/L) after only 7 d of exposure. For the three highest doses of E2, Vtg induction reached a plateau. In the fathead minnow, it has been shown that the vitellogenic response in male fish exposed to E2 exhibited a dose-dependent response, with a plateau by hormone dose from 10⁻⁹ M (as measured by alkaline-labile phosphate levels) [16] or 10⁻⁸ M after 7 d of exposure (as measured by dot blot analysis) [20]. The maximal measured levels were more than fourfold higher than those reported for mature females. Although our study was not designed to determine precise changes in Vtg concentrations during the reproductive cycle of females, the concentration of Vtg in females are comparable to those reported for maturing female zebrafish as well as for other female cyprinids [8,41].

Significant Vtg inductions have been measured after 7 d of exposure to 75 ng/L of E2 in juvenile rainbow trout [42], during early life stages of fathead minnows exposed to 50 ng/L of E2 for 30 d [7], and in male roach exposed to 100 ng/L of E2 for 21 d [41]. The vitellogenic response in zebrafish is smaller than that observed for juvenile male trout. After one week of exposure at 100 ng/L of E2, Vtg levels were approximately 40 μg/ml in zebrafish, compared to more than 1 mg/ml in plasma of juvenile rainbow trout [42]. Similar results were observed for trout and roach exposed to 100 ng/L of E2 (i.e., the Vtg levels reported in roach were 30-fold less than those in male trout) [42]. It is very likely that these differences in the magnitude of vitellogenic response between salmonid and cyprinid fish species are due to physiological considerations, because female salmonids are able to produce Vtg up to 60 mg/ml [33] whereas Vtg concentrations measured in mature female cyprinids fish peak at approximately 1 mg/ml [25]. In the present study, evidence for Vtg induction in male zebrafish exposed to 100 ng/L for only 7 d strongly suggest that zebrafish is a very sensitive fish species and that measurement of Vtg can be very useful to assess the estrogenic activity of chemical substances.

Applicability of zf-Vtg ELISA in other fish species

The results obtained by Western blot analysis (Fig. 3) and ELISA (Fig. 8) showed that the zf-Vtg antibodies are very specific to the Vtg from the cyprinid family. Full binding curves were observed for females and exposed males, whereas no or only weak cross-reaction occurred in control male cyprinid fish. No or very weak cross-reactivity was detected in noncyprinid fish. It is very likely that the DR-264 antibodies recognized an epitope of the Vtg molecule conserved throughout the cyprinid family. Previous studies have shown that the Vtg molecule is highly conserved within a family but differs between families [8,12,37]. Our results show that the DR-264 antibodies and the zf-Vtg ELISA can be used to detect and measure changes in Vtg concentration, at least in the three cyprinid fish species tested in the present study.

CONCLUSION

The purification protocol previously developed for large fish [10] has been successfully applied to purify Vtg from whole-body homogenate of a small fish species, the zebrafish (D. rerio). This allowed production of specific polyclonal antibodies and development of a very sensitive ELISA. The ELISA allowed determination of accurate concentrations of Vtg from whole-body homogenate of male and female zebrafish. Moreover, the ELISA can be used to measure changes in Vtg concentration from a variety of cyprinid fish commonly used in ecotoxicological work. The induction of Vtg as measured in male zebrafish exposed at a low concentration of E2 (100 ng/L) after a short period of time (7 d) suggests that the zebrafish is a sensitive species to exogenous estrogen and a suitable species to assess the impact of endocrine-disrupting chemicals in fish.

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