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Two-step purification method of vitellogenin from three teleost fish species: rainbow trout (*Oncorhynchus mykiss*), gudgeon (*Gobio gobio*) and chub (*Leuciscus cephalus*)

François Brion[a,b], Françoise Rogerieux[a], Patrice Noury[b], Bernard Migeon[b], Patrick Flammarion[b], Eric Thybaud[a], Jean-Marc Porcher[a,*]

[a]*INERIS, Ecotoxicological Risk Assessment Unit, Verneuil-en-Halatte, France*
[b]*CEMAGREF, Laboratory of Ecotoxicology, Lyon, France*

Abstract

A two-step purification protocol was developed to purify rainbow trout (*Oncorhynchus mykiss*) vitellogenin (Vtg) and was successfully applied to Vtg of chub (*Leuciscus cephalus*) and gudgeon (*Gobio gobio*). Capture and intermediate purification were performed by anion-exchange chromatography on a Resource Q column and a polishing step was performed by gel permeation chromatography on Superdex 200 column. This method is a rapid two-step purification procedure that gave a pure solution of Vtg as assessed by silver staining electrophoresis and immunochemical characterisation.

Keywords: Purification; *Oncorhynchus mykiss*; *Gobio gobio*; *Leuciscus cephalus*; Vitellogenin

1. Introduction

Vitellogenin (Vtg), a dimeric glycolipophosphoprotein of high molecular mass, is an egg yolk precursor protein synthesised by the female fish liver under estrogen stimulation. Vtg is incorporated into the growing oocytes after being carried to the ovaries in the blood and is degraded into the yolk proteins lipovitellin and phosvitin. Vtg detection has been used to identify sex when no sexual dimorphism occurs [1] and to give an index of female sexual maturity [2]. More recently, Vtg has been proposed as a biomarker for xenobiotics estrogens [3]. These substances, present in the aquatic environment, are able to mimic the action of the natural steroid hormone 17β-estradiol (E2) and to disturb normal physiology and endocrinology of living organisms. Vtg synthesis is normally limited to reproductive female fish but, in male or immature fish, expression of the protein can be induced by exposure to xenoestrogens. For example, it has been shown that exposure of fish to sewage effluent, containing estrogenic substances, induce Vtg synthesis in male rainbow trout [3,4]. In order to assess the potential estrogenicity of chemical substances and their effects on reproduction, it is important to accurately measure the plasma Vtg levels. Different enzyme-linked immunoassay (ELISA) methods have been developed for various teleost fish species [5–8]. These assays are specific and sensitive, but due to the complexity of the Vtg molecule, there is considerable

*Corresponding author. INERIS-BP 2, Parc Technologique ALATA, F-60550 Verneuil en Halatte, France. Tel.: +33-344-556-584; fax: +33-344-556-605.
E-mail address: jean-marc.porcher@ineris.fr (J.-M. Porcher)

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variation in the immunological determinants. Therefore it is necessary to set up an assay for each species, which requires a significant amount of purified Vtg of the corresponding species in order to use it as a standard in the ELISA method.

In the present study, vitellogenin from estradiol-treated rainbow trout (*Oncorhynchus mykiss*) plasma was isolated using Resource Q anion-exchange chromatography and Superdex 200 gel permeation chromatography columns connected to a high-performance liquid chromatography (HPLC) system. The effect of buffer ion hydrogen concentration was investigated using Hitrap Q as anion exchanger. The two-step chromatographic method developed to purify rainbow trout vitellogenin was then tested to two cyprinid fish species of ecotoxicological interest: gudgeon (*Gobio gobio*) and chub (*Leuciscus cephalus*).

2. Materials and methods

2.1. Fish

A total of 20 juvenile rainbow trout, with an average mass of 25.6 g, were obtained from an experimental fish farm (INRA, Gournay sur Aronde, Oise, France). Adult male ($n=20$) and female ($n=20$) gudgeons, with an average mass of 11.4 g, were purchased from a commercial fish farm (Les Clozioux, Brinon sur Sauldre, Cher, France). Chub ($n=35$, average mass=45 g) were captured by electrofishing in a low polluted area (Drôme river, France). All fish were maintained in laboratory aquaria until Vtg induction.

2.2. Source of vitellogenin

Vtg synthesis was induced in fish by intraperitoneal (i.p.) injections of 2 mg/kg of estradiol (E2) (Sigma, St. Louis, MO, USA) dissolved in ethanol and diluted in peanut oil (Sigma) (ethanol–peanut oil, 1:9). Non-induced fish received ethanol–peanut oil only. Rainbow trout and chub were subjected to i.p. injections, once a week during two weeks. Due to occurrences of mortality, gudgeon received only one i.p. injection of E2 at the same dose. After treatment, blood samples (≈1 ml) were collected from the caudal sinus with heparinized syringes. The blood was centrifuged in heparinized tubes containing the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF, Sigma) at a final concentration of 1 m*M*. Centrifugation was performed at 3000 *g*, 4°C during 15 min. Plasma was then collected and stored at −80°C until purification.

2.3. Purification of the Vtg

Ion exchange and gel permeation chromatographies were performed using an Äkta purifier 10 liquid chromatography system (Amersham Pharmacia Biotech, Uppsala, Sweden). The system consists of a separation unit including: Pump P-901 (0–25 MPa) equipped with two pump modules A/B; mixer chamber M-925 of a mixer volume of 0.6 ml and two-way switching valve SV-903 connected to the pumps; a UV monitor UV-900 allows the simultaneous recording of up to three wavelengths; monitor pH/C 900 for the on-line measurement of pH and conductivity; on-line filter; injection valve INV-907 and outlet valve PV-908. The system was controlled by a personal computer running Unicorn software. For fractionation, the system was connected to a Frac-901 fraction collector.

During anion-exchange and gel permeation chromatography, absorbances at 280 and 254 nm (mAU), conductivity (mS/cm), pH, temperature, flow-rate (ml/min) and pressure (MPa) were continuously recorded.

All the solutions were filtered through a 0.22-μm filter (Millipore, France) and degassed. The chromatographic procedures were carried out at room temperature.

2.4. Sample preparation and clarification: gel permeation chromatography

Samples of estradiol-treated fish were thawed at 4°C and pooled. Plasma was loaded with a sample loop of 1 ml on a 5-ml Hitrap desalting column (Amersham Pharmacia Biotech). The column was equilibrated with 0.1 *M* Tris–HCl, 1 m*M* PMSF, pH 8.5 during two column volumes. The buffer used was that of the binding buffer used in the capture step (see Section 2.5). Elution of the sample was performed with an isocratic elution of 1.5 column

volumes at a flow-rate of 5 ml/min. The diluted sample was then kept in ice to reduce protease activity. This procedure was repeated five times to obtain 10 ml of clarified sample.

2.5. Capture and intermediate purification step: anion-exchange chromatography

2.5.1. pH scouting

In the method development, optimum pH of the binding buffer was first determined. A strong anion exchanger (5 ml Hitrap Q Sepharose fast flow column, Amersham Pharmacia Biotech) was used for pH scouting. The tested range of pH varied from 8.5 to 7.0. For each run, the column was equilibrated with two column volumes and 250 μl of a clarified sample (see Section 2.4) was loaded. Elution of the bound proteins was performed by increasing ionic strength with a linear NaCl gradient (0.0–0.5 *M*) at 5 ml/min, 10 column volumes. Fractions of 1 ml were automatically collected. Vtg detection was assessed in unbound and eluted fractions using a specific ELISA method (see Section 2.7.3). The selected pH was the one giving the best resolution of the Vtg peak (see Results).

2.5.2. Routine procedure

After pH optimisation, a 1-ml Resource Q column (Amersham Pharmacia Biotech) was used for capture and intermediate purification in the routine purification procedure. The sample loop of 10 ml (Superloop 10 ml, Amersham Pharmacia Biotech) was fully loaded with clarified sample. The column was equilibrated with five volumes of starting buffer (0.1 *M* Tris–HCl, 1 m*M* PMSF, pH 8.5) and a sample volume of 1 ml was automatically applied to the column. Unbound proteins were eluted with two column volumes of starting buffer. Bound proteins were separated with a linear NaCl gradient (starting buffer+0–0.5 *M* NaCl) performed on 10 column volumes at a flow-rate of 4 ml/min. Fractions of 1 ml were collected. To ensure that no protein remained on the column, it was washed with three volumes of starting buffer with 1 *M* NaCl. The procedure was repeated until complete injection of the sample loaded in the superloop.

Fractions containing Vtg were then pooled and concentrated by ultrafiltration (3000 *g*, 4°C) using an Amicon cell with a 100 000 molecular mass cut-off membrane (Centriplus 100, Amicon).

2.6. Polishing step: gel permeation chromatography

The gel permeation column (Superdex 200 HR 10/30 25 ml, Amersham Pharmacia Biotech) was equilibrated with two column volumes of 50 m*M* carbonate–bicarbonate, pH 9.6 at a flow-rate of 1 ml/min. Ultrafiltered sample was then applied to the gel permeation chromatography column using a sample loop of 100 μl, i.e., sample size represents less than 1% of the total gel bed volume (V_t). Proteins were eluted isocratically on 1.5 column volumes at a flow-rate of 0.2 ml/min and fractions of 0.5 ml were collected. The column was calibrated for molecular mass determination with ribonuclease (43 000 rel. mol. mass), aldolase (158 000 rel. mol. mass), ferritin (440 000 rel. mol. mass), thyroglobulin (669 000 rel. mol. mass) (Low and High Molecular Weight Gel filtration Calibration Kit, Amersham Pharmacia Biotech). The void volume (V_0) was determined using Blue Dextran. The elution volumes (V_e) for the proteins markers and vitellogenins were determined by measuring the volume of the eluent from the point of application to the centre of the elution peak. Estimated distribution coefficients (K_{av}) were calculated using the following equation: $K_{av} = (V_e - V_0)/(V_t - V_0)$. The logarithms of the molecular mass markers were plotted against their K_{av} and equation of the linear regression was used to calculate the relative molecular mass of the vitellogenins.

2.7. Analytical procedures: electrophoresis and immunological detection of the Vtg

2.7.1. Gel electrophoresis

Plasma samples from control and estradiol treated-fish and chromatographic fractions were analysed by Native and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (0.1% SDS) performed according to Laemmli [9], in discontinuous gel with a 4% stacking gel and a 7.5% resolving gel of acrylamide or in 4–15% linear gradient gel (Ready gel, BioRad, Hercules, CA, USA). For SDS–PAGE analysis, samples were diluted in sample buffer (0.5 *M* Tris–HCl, pH 6.8, 10% glycerol, 0.1%

SDS, 0.02% Bromophenol Blue). When β-mercaptoethanol was added in sample buffer (5%, v/v), the tested sample was heated at 95°C for 5 min. For native PAGE analysis, samples were diluted in 0.5 *M* Tris–HCl, pH 6.8, 10% glycerol and not heated.

Proteins markers were included to electrophoresis (Kaleidoscope Prestained standards, Bio-Rad, Hercules, CA, USA or High Molecular Weight Electrophoresis Calibration Kit, Pharmacia Biotech). 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. After electrophoresis, the protein bands were stained with Coomassie brilliant Blue R250 or with silver stain kit (QuickSilver stain Kit, Amersham). Silver staining is at least 100-fold more sensitive than Coomassie Brilliant Blue with a detection limit of 2–5 ng protein/band [10]. For this reason, this method was used to assess the purity of the Vtg.

2.7.2. Western blotting

Proteins were transferred onto nitro-cellulose membrane (0.45 μm) using Mini trans Blot cell (Bio-Rad). Transfer was carried out at 100 V for 1 h at 4°C with 25 m*M* Tris base, 192 m*M* glycine, pH 8.3 as transfer buffer. For proteins separated by SDS–PAGE, 20% methanol was added to the transfer buffer. The membrane was saturated overnight at 4°C with phosphate buffered saline (PBS), 2% bovine serum albumin (BSA). After the saturation step, the membrane was rinsed 3×10 min with PBS-T20 (0.05%) and incubated with anti-Vtg (1:5000 in PBS, 2% non-fat dry milk) for 1 h at room temperature. After three washes, the membrane was incubated for 1 h with the second antibody solution (horseradish peroxidase-conjugated goat anti mouse IgG, Bio-Rad) diluted 1:3000 in PBS, 2% non-fat dry milk. The antigen–antibody complexes were immunodetected using enhanced chemiluminescence (ECL, Amersham Life Sciences). The bound horseradish peroxidase catalyses an enhanced chemiluminescent reaction producing light with a maximal emission at 428 nm which is captured on an autoradiographic film (Hyperfilm, Amersham Life Sciences).

2.7.3. Non competitive ELISA

In order to identify fractions containing Vtg, a non-competitive ELISA was used. It was established and validated using plasma of induced and non-induced juvenile rainbow trout. Chromatographic fractions were diluted in 0.05 *M* carbonate–bicarbonate, pH 9.6 at a final concentration of 50 μg/ml and coated on a microtiter plate (Nunc Maxisorp, Nunc, Roskilde, Denmark) during 16 h at 4°C. Plates were then washed three times with 200 μl of PBS-T20 (0.05%). To avoid non-specific binding, plates were blocked with 200 μl of PBS, 2% BSA during 1 h at room temperature. The blocked plates were then washed three times with PBS-T20. One hundred microliters of primary antibodies (1:25 000 in PBS, 1% BSA) was added to the wells. The primary antibodies used were monoclonal mouse anti-salmon Vtg (Biosense Labs., Bergen, Norway). Incubation with primary antibodies occurred during 1 h at 37°C. After a washing step, each well received 100 μl of secondary antibody (horseradish peroxidase goat anti-mouse or anti-rabbit IgG, Bio-Rad) diluted 1:3000 in PBS, 1% BSA. Incubation was performed during 1 h at room temperature. The perodixase activity was revealed with 100 μl/well of TMB (TMB EIA Substrate Kit, Bio-Rad). The reaction was stopped after 5 min with 0.5 *M* H_2SO_4 and the plate was read at 450 nm with a microplate reader (EL 340, BioTek Instruments, UK).

2.7.4. Plasma protein concentration

Total plasma protein was determined according to the method of Bradford [11] using BSA as a standard (Sigma) on a multiparameter analyser (Cobas Fara, Roche, Switzerland).

3. Results

3.1. Induction of Vtg synthesis

In juvenile rainbow trout, total plasma proteins increased from 41.4±14.0 mg/ml in the control group (n=10) to 87±18.9 mg/ml in the E2-treated group (n=10). SDS–PAGE of plasma from estrogen-treated and control fish confirmed that the increase was due to appearance of a new band of high

molecular mass. Western Blot analysis showed that this band strongly cross-react with the anti-salmon Vtg antibodies whereas no positive reaction occurred in control (Fig. 1). Consistent with the immunoblot analysis, indirect ELISA confirmed the vitellogenin induction in juvenile rainbow trout.

3.2. Sample preparation and purification of the Vtg

Clarification and conditioning of plasma sample were achieved using Sephadex G25 gel. Samples were clarified, diluted 1:2 and rapidly transferred into the buffer used in the first purification step, i.e., the pH (8.5) and the ionic strength (2.2 mS/cm) of the eluted sample were those of the binding buffer used in anion-exchange chromatography.

In order to optimise binding conditions of the rainbow trout vitellogenin (RT-Vtg), the pH value was chosen after pH scouting. Fig. 2 shows the elution profile when plasma from E2-treated rainbow trout was loaded to Hitrap Q equilibrated at different pH. In this experiment, each eluted fraction was assayed using a non-competitive RT-Vtg ELISA protocol to detect the Vtg peak. At the lowest pH, Vtg was eluted with other contaminant proteins whereas for the highest pH we observed a well-defined Vtg peak separated from most of the other proteins. RT-Vtg peak appeared as the last component eluted from the column at a salt concentration of 0.35 *M* of NaCl. The use of pH 8.5 was chosen in order to obtain adequate resolution of the RT-Vtg peak.

In order to further optimise this purification step, SOURCE media (Amersham Pharmacia Biotech) was selected to speed up purification and increase resolution. As shown in Fig. 3, Resource Q allowed one to decreased separation time of the Vtg from most bulk contaminants and also to reduce buffer consumption. Concentrating effect of the anion-exchange chromatography was also maximised since Vtg peak was eluted in 1 ml fraction. As a result, capture and intermediate purification steps of the RT-Vtg were combined in a one-step purification.

Plasma from other fish species were tested to

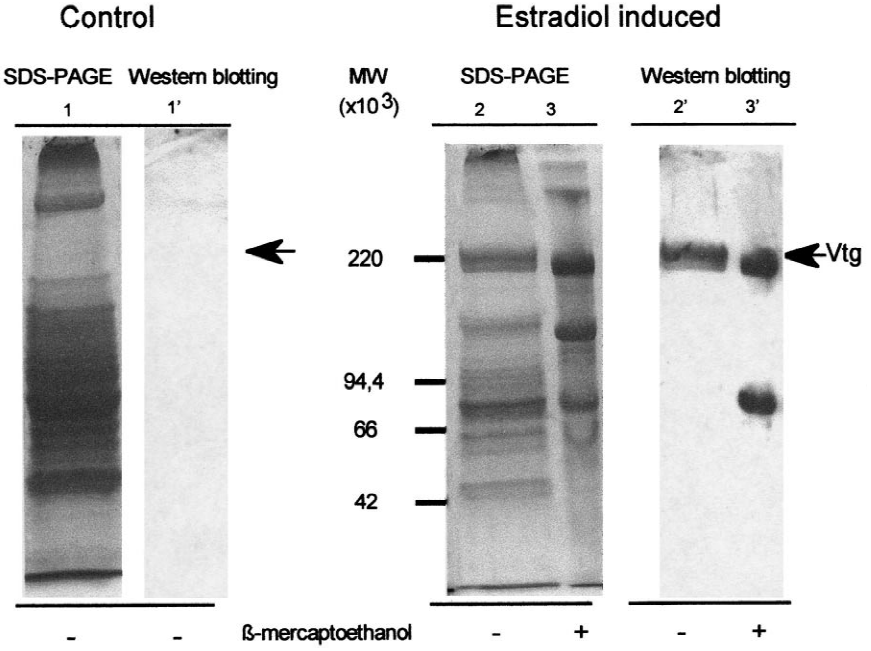

Fig. 1. SDS–PAGE and corresponding Western blotting of control and estradiol-treated plasma of rainbow trout. Gels were stained with Coomassie Blue R250. Non-induced plasma: lines 1 and 1′; estradiol-induced plasma: without β-mercaptoethanol (lines 2 and 2′) with β-mercaptoethanol (lines 3 and 3′). MW refers to the molecular masses of the standard proteins.

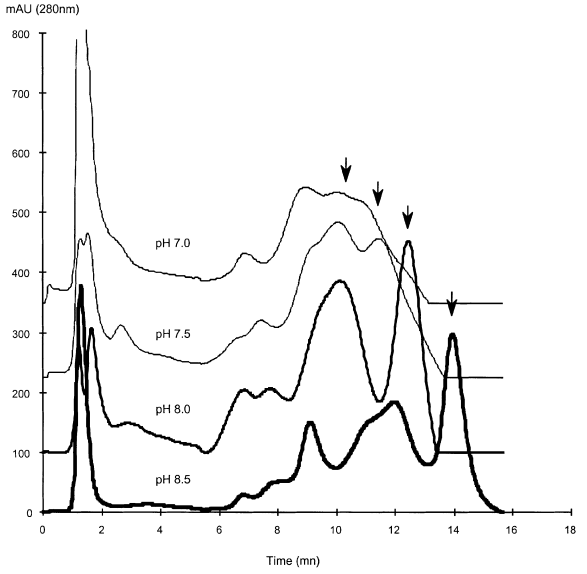

Fig. 2. Anion-exchange chromatography: effect of binding buffer pH variations on the elution profile of the rainbow trout vitellogenin. Arrows indicates elution positions of vitellogenin detected by ELISA. For clarity, a shift in the chromatogram baselines was introduced (150 mAU). Column: Hitrap Q; eluent buffer: 0.1 *M* Tris–HCl, 1 m*M* PMSF, 0.0–0.5 *M* NaCl; flow-rate: 5 ml/min. Absorbance at 280 nm was measured.

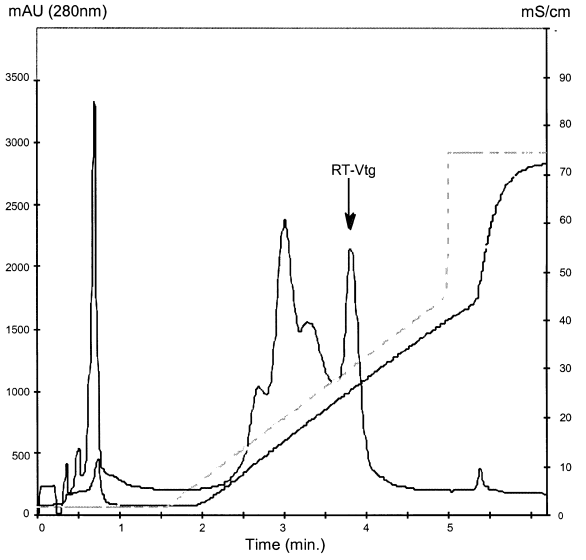

Fig. 3. Elution profiles of plasma proteins from estradiol-treated rainbow trout in capture and intermediate purification step. Column: Resource Q; elution buffer: 0.1 *M* Tris–HCl, pH 8.5, 0.0–0.5 *M* NaCl; flow-rate: 4 ml/min; absorbance: 280 nm. The conductivity profile shows the gradient used for the elution (theoretical gradient: dotted line; measured gradient: solid line).

check if the conditions developed for the rainbow trout (pH, ionic strength, flow-rate, gradient slope) were suitable for the two cyprinid species. For E2-treated plasma, we observed a large peak (approximately 25% of the total peak area) which was identified as the Vtg peak since it was absent from the untreated male plasma of the two species (Fig. 4). As for RT-Vtg, G-Vtg and Ch-Vtg were the last proteins to be eluted at a salt concentration close to that of the RT-Vtg (0.37 *M* and 0.35 *M* of NaCl, respectively).

Fractions containing Vtg were loaded onto a high-resolution gel permeation chromatography column. Superdex 200 HR prep. grade gel permeation media was selected since the molecular mass of most fish teleost Vtg is within the optimal separation range for this medium. The main goals of this final purification step were (i) to remove trace contaminants or breakdown product of the Vtg and (ii) to transfer and collect purified Vtg in a buffer suitable for its future use. Therefore the eluent buffer was the coating buffer used in the competitive ELISA. Fractions

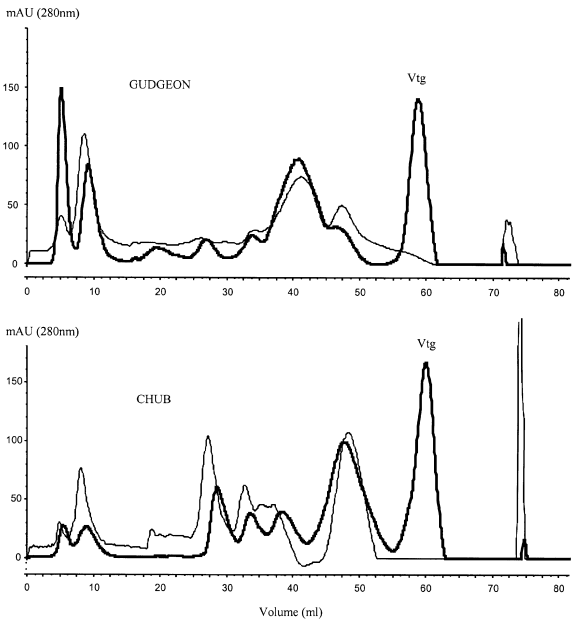

Fig. 4. Elution profiles of proteins from control (thin line) and estradiol-treated fish (bold line) on Hitrap Q column. Binding buffer: 0.1 *M* Tris–HCl, 1 m*M* PMSF, pH 8.5; gradient 0.00–0.5 *M* NaCl.

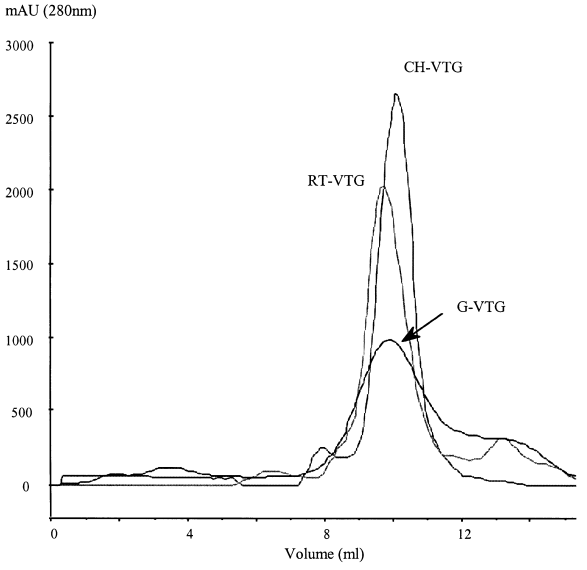

Fig. 5. Gel permeation chromatography on Superdex 200 HR 10/30 column. Polishing step of vitellogenin fractions from anion-exchange chromatography. Eluent buffer: 0.05 *M* carbonate–bicarbonate, pH 9.6; flow-rate: 0.2 ml/min.

containing Vtg collected in anion-exchange chromatography were concentrated by ultrafiltration to reduce sample volume loaded to the gel. As shown in Fig. 5, fractions containing Vtg were eluted in one main and symmetric peak excepted for gudgeon. Therefore, fractions containing gudgeon vitellogenin eluted from 8 to 10 ml were collected and the others were not to avoid contaminant in the final product.

3.3. Electrophoresis and Western blotting

Under native electrophoretic conditions (without SDS or β-mercaptoethanol), solutions obtained after the two-step chromatography appeared as a single band protein (Figs. 6 and 7) with no detectable signs of degradation product. Electrophoresis of purified RT-Vtg under SDS–PAGE denaturing conditions produced the breakdown of the native form of Vtg into several protein bands: one major band with an apparent molecular mass of 240 000, probably corresponding to the Vtg monomer and three minor bands with apparent molecular masses of around 170 000,

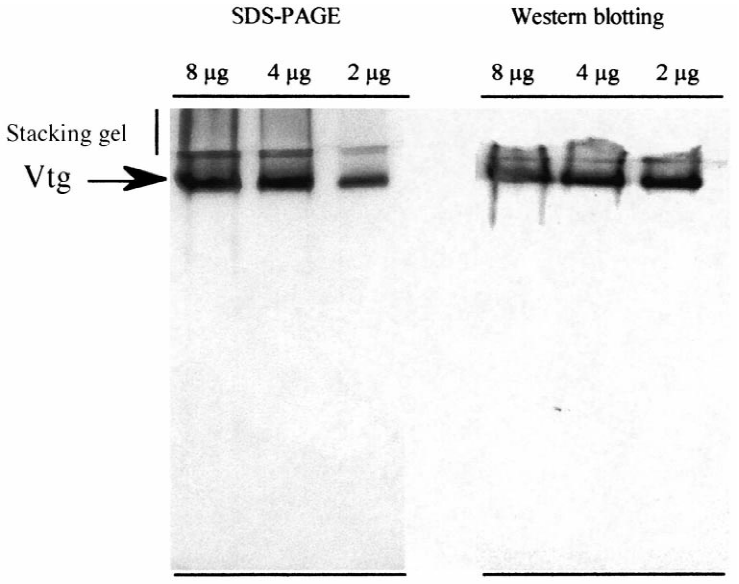

Fig. 6. Native polyacrylamide gel electrophoresis of purified rainbow trout vitellogenin and corresponding Western blotting. Electrophoresis was performed with a 4% stacking gel and 7.5% (w/v) resolving gel of acrylamide and silver stained. Immunoblotting was performed with BN-5 anti-salmon Vtg antibody; complexes were immunodetected by enhanced chemiluminescent method after transfer of proteins to a nitrocellulose paper.

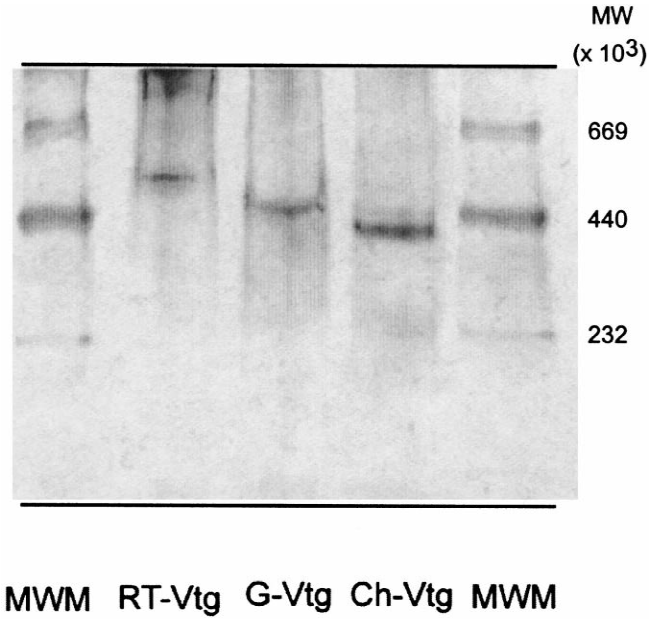

Fig. 7. Native polyacrylamide gel electrophoresis patterns of vitellogenins obtained after anion-exchange chromatography and gel permeation chromatography on a 4–15% linear gradient gel electrophoresis. Gel was silver stained. RT-Vtg, rainbow trout; G-Vtg, Gudgeon; Ch-Vtg, Chub; MWM, molecular mass markers; MW refers to the molecular masses of the standard proteins.

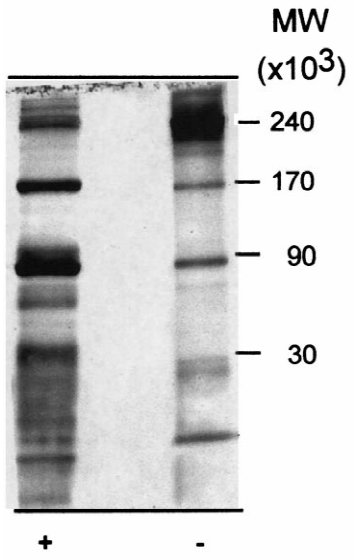

Fig. 8. SDS–PAGE (0.1% SDS) patterns of purified rainbow trout vitellogenin on a discontinuous 4–7.5% gel electrophoresis. (+) With β-mercaptoethanol, (−) without β-mercaptoethanol. Gel was silver stained. MW refers to the molecular masses of the standard proteins.

90 000 and 30 000. SDS–PAGE of purified rainbow trout vitellogenin (Fig. 8) under reducing and denaturing condition increased the intensity of the molecular mass 170 000 and 90 000 bands and the number of degradation products.

4. Discussion

Several methods have been developed for the purification of oviparous vertebrates. Selective precipitations with Mg^{2+}-EDTA have been used to purify Vtg [12]. This represents a rapid and a simple method that does not require specific equipment. However, Vtg concentration is critical for the precipitation process, i.e., Vtg precipitation is not completed or does not occur when plasma has low titre of Vtg [13]. The precipitation with $MgCl_2$ and EDTA has often been associated with a chromatographic method to obtain pure and undegraded Vtg [12,13]. In fish, Vtg has usually been purified by two or one chromatographic methods based on size and/or charge of the target molecule. In most procedures, the first step consisted in performing a gel filtration

with gel permeation column, followed by anion-exchange chromatography. This double chromatography protocol has been used to purify Vtg in *Anguilla japonica* [14], *Cyprinus carpio* [15], *Dicentrarchus labrax* [7], *Oncorhynchus mykiss* [1], *Zoarces viviparus* [16]. In other studies Vtg has been purified by a one-step anion-exchange chromatography [17,18].

In this study, Vtg has been purified from E2-treated fish using a two-step purification protocol: anion-exchange chromatography followed by gel permeation chromatography. Conditioning of plasma sample for the first purification step was achieved using gel filtration on a Superdex G 25 column.

Anion-exchange chromatography was chosen as the first chromatographic step due to the high protein-binding capacity of this method. The elution profile of the rainbow trout vitellogenin is affected by pH since it determines charge on both the protein and ion exchanger. The running pH 8.5, which provides the best resolution of vitellogenin, was selected after scouting. When this parameter was fully optimised, concentrating effect of the anion-exchange chromatography was maximised by the use of the Resource Q column. As a result, capture and intermediate purification steps of the RT-Vtg were combined in a one-step purification. Other parameters can influence the separations results. It has been shown that the use of a more complex gradient with Mono Q anion-exchange chromatography column can improve the separation [17]. Temperature can also influence the elution profile and was attributed to proteolysis [17]. To avoid proteolysis of Vtg, plasma samples were stored in the presence of the serine protease inhibitor PMSF. PMSF was added both in plasma and in purification buffer. PMSF and aprotinin are usually used in purification protocols and are effective in preventing vitellogenin proteolysis of rainbow trout [20] or sea bass [7]. The procedure we developed seems more convenient to avoid the proteolysis of the Vtg molecule since Vtg is separated from most other contaminant proteins in a very short time. In contrast, it is likely that, when gel permeation chromatography is used as first purification step, vitellogenin is more subjected to proteolysis.

In gel permeation chromatography, the fractions from anion-exchange chromatography containing rainbow trout and chub Vtg were eluted in one main peak indicating that no detectable signs of degradation were produced throughout the purification procedure. However, for gudgeon, degradation seemed not to be completely eliminated even if great precautions were taken. This is probably due to the fact that gudgeon Vtg is more labile than the other two. Other studies have shown that sea trout or goldfish vitellogenins are more susceptible to degradation than other teleost Vtg such as rainbow trout [13,19].

The final purification step was performed by a gel permeation chromatography that had the advantages to separate degradation product from the native form (i.e., dimeric form) of the Vtg and to store the final product in the desired buffer.

The relative molecular masses of the rainbow trout, gudgeon and chub Vtg were found to be around 442 000, 435 000 and 424 000, respectively as determined by gel permeation chromatography. These results are in accordance to those obtained by gel permeation chromatography from rainbow trout (i.e., $M_r \approx 500\,000$ [20] or 440 000 [13]) and from other fish species: M_r 300 000 for *Morone saxatilis* [6], 390 000 for *Cyprinus carpio* [15], 440 000 for *Salmo trutta* [13], and 445 000 for *Dicentrarchus labrax* [7].

Since in native PAGE the separation principle is not linearily linked to molecular mass, estimation of relative molecular mass provided may be questionable [17]. On native polyacrylamide gel electrophoresis, the native forms of trout, gudgeon and chub vitellogenins were eluted at a position above M_r 400 000. Depending on the method used (ultracentrifugation, gel permeation chromatography or gradient gel electrophoresis) for its estimation, the relative molecular mass of the native (i.e., dimeric form) rainbow trout Vtg may varied from 342 000 to 535 000 [21]. We can also notice that the relative molecular mass might be underestimated in gel permeation chromatography due to a possible interaction of the lipid content of the vitellogenin and the gel matrix. In SDS–PAGE, the relative molecular mass for the rainbow trout vitellogenin was estimated to be 240 000 for the monomeric form.

As a conclusion, the procedure used in the purification of the rainbow trout vitellogenin was successfully applied to other fish species. Using a two-step

purification protocol, pure solution of Vtg, as shown by electrophoretic studies, was obtained. Combination of anion-exchange and gel permeation chromatographies had obvious advantages, such as reproducibility and a short processing time. The procedure was efficient to obtain purified Vtg of three teleost species completely separated from other plasma elements. Moreover, integrity and antigenicity of purified Vtg were maintained.

The purification of the vitellogenin was the first step of the development of a quantitative ELISA method. Using purified RT-Vtg as a standard, a specific and sensitive quantitative ELISA was developed to assess the estrogenicity of chemical compounds under laboratory conditions. Similar ELISA methods for chub and gudgeon Vtg are under development and will be used under laboratory or field study conditions.

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