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Sea bass (*Dicentrarchus labrax* L.) vitellogenin. I—Induction, purification and partial characterization

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In sea bass (males and non-vitellogenic females) estradiol (E_2) treatment caused the appearance in the bloodstream of a new protein that was identified as the vitellogenin (VTG) molecule for this teleost species. The molecular weight of the sea bass VTG (445 kDa) and its amino acid composition was similar to that of other teleosts. VTG was purified by double-step chromatography and then used to obtain specific antibodies. The localization of VTG in the liver and in the ovary was carried out by immunocytochemistry. The effects of the E_2 treatment on the synthetic activity of the liver were studied throughout the modifications observed at the histological and biochemical levels.

Key words: *Dicentrarchus labrax*; Vitellogenin.

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Introduction

Vitellogenin (VTG), a lipophosphoglycoprotein, is known to be the yolk precursor protein in oviparous vertebrates. VTG is synthesized in the liver under the stimulation of estrogens, released into the bloodstream and selectively incorporated by the growing oocytes via receptor-mediated endocytosis. In the oocytes, the proteolytic cleavage of VTG gives rise to the yolk proteins, lipovitellin and phosvitin (Wallace, 1985; Wallace and Selman, 1990).

The synthesis of VTG has been artificially induced using gonadotropic hormones (Burzawa-Gerard and Dumas-Vidal, 1991), androgens (Hori *et al.*, 1979; Le Menn, 1979) and estrogens (Sundararaj and Nath, 1981). Van Bohemen *et al.* (1982) found that estradiol was the most potent estrogen for the induction of VTG synthesis in *Oncorhynchus mykiss*. Since then, estradiol has been normally used for the induction of VTG synthesis in many fish species (Mommsen and Walsh, 1988). Treatment of animals with estradiol is associated with the stimulation of the synthetic activity of the liver, as observed at biochemical (Emmersen and Emmersen, 1976; Ng *et al.*, 1984; Haux and Norberg, 1985; Korsgaard *et al.*, 1986; Korsgaard, 1990) and histological levels (Selman and Wallace, 1983; Tam *et al.*, 1983; Ng *et al.*, 1984). In hepatocytes, estradiol causes the expression of the VTG genes, the accumulation into the cytoplasm of the VTG mRNAs and the synthesis of the primary VTG peptide (Wahli *et al.*, 1981). Carbohydrates, lipids, phosphorus and different ions such as calcium and iron, are successively added to the core of this primary peptide, resulting in the final form of the circulating VTG (Tata and Smith, 1979). Although the structure of the VTG is widely conserved, differences are observed between species, probably due to differences in the translocation processes (Ding *et al.*, 1989).

VTG has been purified and characterized in several species of fish including teleosts (Hara and Hirai, 1978; Hara *et al.*, 1980, 1983; De Vlaming *et al.*, 1980; So *et al.*, 1985; Hamazaki *et al.*, 1987b; Fremont and Riazi, 1988; Bradley and Grizzle, 1989; Takemura *et al.*, 1991;

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Murakami *et al.*, 1991; Sullivan *et al.*, 1991; Kishida *et al.*, 1992; Goodwin *et al.*, 1992), elasmobranchs (Craik, 1978; Perez and Callard, 1992) and cyclostomes (Yu *et al.*, 1991). In recent years, sea bass has gained a growing importance in European aquaculture and, as a consequence, several studies related to the environmental and hormonal control of its reproductive cycle have been performed (Carrillo *et al.*, 1989; Prat *et al.*, 1990). The vitellogenic process of sea bass has been studied at histological, ultrastructural and electrophoretical levels (Mayer *et al.*, 1988; Zanuy *et al.*, 1989; Mayer *et al.*, 1990; Mosconi *et al.*, 1992; Carnevali *et al.*, 1992) but to date, no attempt has been made to identify, characterize and purify its VTG in order to accomplish a more dynamic study of the process of vitellogenesis in this species.

The aim of this study was, (1) the purification and characterization of the VTG molecule in this species and (2) to observe the changes produced in liver and plasma by the E_2 treatment. With this study we want to have an appropriate background for further studies on the vitellogenic processes in this economically important species.

Materials and Methods

Hormone treatment

Adult male and female sea bass (between 1.5–2.5 kg of body weight), reared in our laboratory (east coast of Spain, 40°N 0°E) in natural conditions of temperature and photoperiod, were distributed, at the beginning of June, into four homogenous groups: three experimental groups treated with different doses of estradiol (0.5, 2 and 5 mg E_2/kg body wt) and one control group injected with the vehicle (ethanol diluted 1:9 in NaCl 0.9%). Animals received six intraperitoneal injections, given every 2 days.

At the end of the experiment, blood was collected in heparinized tubes containing PMSF (1 mM), centrifuged (1500 *g* for 20 min at 4°C) and plasma stored at −20°C. Plasma was collected and stored in the presence of PMSF in order to prevent the proteolytical breakdown of the VTG. Liver and gonads were taken up and either frozen (−20°C) or fixed for biochemical or histological studies. RNA and DNA contents of the liver were determined according to Buckley (1979). The levels of total proteins in liver and plasma were measured by the method of Lowry *et al.* (1951). VTG plasma levels were measured by ELISA (Mañanós *et al.*, 1994).

Vitellogenin purification

Plasma of E_2-treated animals, stored at −20°C with PMSF (1 mM) was centrifuged (1500 *g* for 10 min at 4°C) and 1 ml applied on to a 6B-Sepharose column (850 × 16 mm). The sample was eluted with Tris–HCl buffer 100 mM (pH 7.8), containing PMSF 1 mM, at a flow rate of 60 ml/hr and fractions were collected every 6 min. Fractions containing VTG were pooled and concentrated to a final vol of 5 ml using a Sartorius ultrafiltration system. Immediately after, this solution was applied on to a DEAE-Sepharose column (350 × 16 mm) and eluted with a linear gradient (150–300 mM) of Tris–HCl buffer (pH 7.8), PMSF 1 mM, at a flow rate of 36 ml/hr. Fractions of 3 ml were collected and their absorbance read at 280 nm. The whole procedure was performed at 4°C. Molecular weight determinations were carried out both by gel filtration on 6B-Sepharose using thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa) and aldolase (158 kDa) as standard proteins, and by SDS electrophoresis using phosphorylase-*b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa) as standard proteins.

Immunization procedure

The VTG preparation (1 mg VTG/ml) was mixed with complete Freund's adjuvant (1:1) and injected subcutaneously in rabbits. Injections of 1 ml (for each rabbit) of the mixed solution were given every week for 4 weeks; after 15 days of "resting period" the injections were repeated every 2 weeks until no further increase in the antibody titer could be detected (four injections after the "resting period"). Then, blood was collected, allowed to clot at 4°C for 48 hr and serum stored at −20°C.

Electrophoresis and immunoblotting

Native and SDS–PAGE (0.1% SDS) were performed in discontinuous gels with 4.5% stacking gel and resolving gel with a linear gradient of acrylamide 4–16% (Laemmli, 1970). Gels were stained for proteins with Coomassie Brilliant Blue R-250.

For immunoblotting, proteins were transferred on to a nitrocellulose paper (NTC). Transfer was performed for 2 hr (50 mA) at 4°C in Tris–HCl buffer 25 mM (containing 14.4 g/l of glycine), pH 8.3. After this, the paper was saturated for 2 hr in Tris–saline buffer, 10 mM, pH 7.6 (TBS), containing 5% swine serum and washed three times (10 min) with TBS. After the saturation step, the paper was incubated with the antibody solution (diluted 1:3000 in TBS) overnight. The antigen–antibody complexes were detected by the peroxidase–anti-peroxidase (PAP) method: secondary antibody incubation (goat–anti-rabbit diluted 1:1000) for 45 min, followed by PAP incubation (diluted 1:500) for

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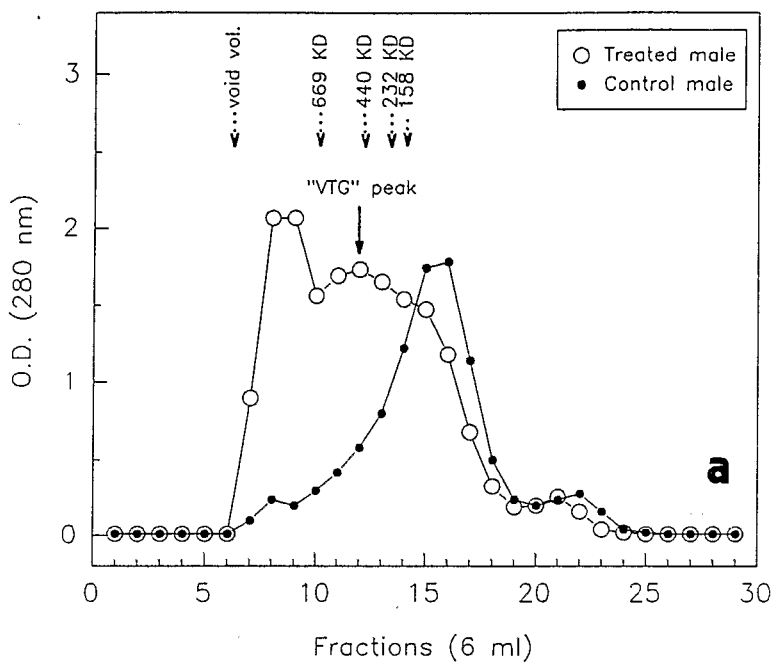
Histology and immunocytochemistry

Tissues were fixed in Bouin–Hollande for 48 hr and embedded in paraffin. Sections (7 μm) were stained with hematoxylin and eosin for histological observation. The quantification of the nuclear size was performed by image analysis. Immunocytochemistry was performed by the PAP method as described for immunoblotting.

Amino acid analysis

For analysis of amino acid composition, the VTG preparation (1 mg VTG/ml) was hydrolyzed with HCl 6-N in an evacuated tube at 110°C for 21 hr. The residue was stored at 4°C in the presence of HCl 0.025 M. Analysis was performed by high-pressure liquid chromatography (HPLC).



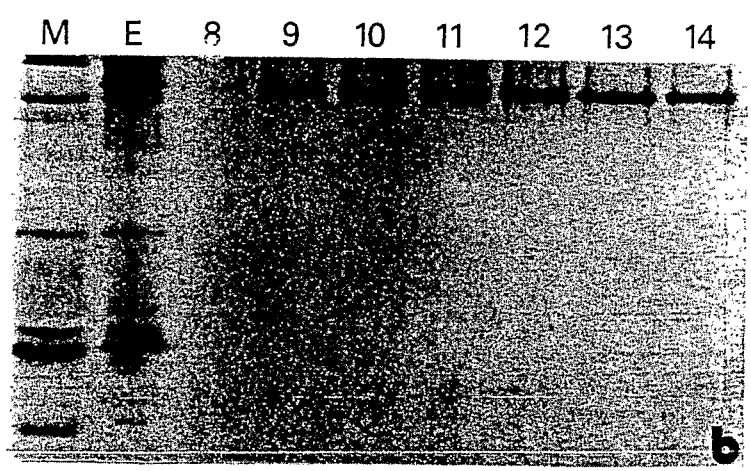


Fig. 1. Purification of sea bass vitellogenin, step 1. (a) First chromatography on gel filtration of plasma from control and E_2-treated males. Sample (1.5 ml of plasma) was eluted in a 6B-Sepharose column (850 × 16 mm) with 100 mM Tris–HCl buffer (pH 7.8), containing 1 mM PMSF, and fractions (6 ml) collected every 6 min. (b) Electrophoresis of the initial plasmas (control male in lane M and E_2-treated male in lane E) and the eluted fractions 8–14 from the gel-filtration chromatography. The electrophoresis was performed in native conditions on 6% polyacrylamide gels; bands were stained for proteins with Coomassie Brilliant Blue R-250.

Results

Purification and partial characterization of the vitellogenin molecule

Sea bass VTG was purified from plasma of E_2-treated animals using a double chromatography method, a gel filtration on 6B-Sepharose followed by ion-exchange on DEAE–Sepharose. Figure 1(a) shows the elution profile on gel filtration of plasmas from control and E_2-treated males. The E_2 treatment produced an increase of the peak eluted just after the void volume and the appearance of a peak centered around fraction 12. Electrophoresis of the eluted fractions, together with the initial plasmas, is shown in Fig. 1(b). Plasma from the E_2-treated males presented one protein that was absent in the control males. This E_2-induced protein was suspected to be the VTG molecule. This protein was eluted in the first fractions of the gel filtration (fractions 9–14 of Fig. 1a) but it was not completely resolved from other plasma proteins. Fractions containing this protein were pooled and applied to the second chromatography step.

Figure 2 shows the elution profile on DEAE–Sepharose of the pooled fractions of the gel filtration. These proteins were resolved in three peaks, two minor peaks (centered in fractions 24 and 44, respectively) and one major peak eluted at 250 mM of the Tris–HCl gradient. Pooled fractions containing the major peak were eluted in electrophoresis as shown in Fig. 2 (inside). We observed that the major peak contained only the E_2-induced protein; we strongly suspected that this band was the VTG molecule. Therefore, from now this protein will be referred to as "VTG". The homogeneity of the single peak corresponding to "VTG" in the DEAE–Sepharose and the existence of only one band when this peak was run in electrophoresis, showed that the solution of "VTG" was free of other contaminants and with no detectable signs of degradation.

Figure 3(a) shows that the purified "VTG", that appears in the male plasma after the E_2-treatment, is present in the plasma of natural vitellogenic females. Immunoblotting of this gel (Fig. 3b), using antibodies raised against the purified solution of "VTG" (AbVTG), showed that the "VTG" band was immunostained both in the plasma of E_2-treated males and in the plasma of natural vitellogenic females, whereas no reaction was observed with the control of male plasma. Therefore, "VTG" is a female-specific protein and is induced in males by the estradiol treatment.

The addition of SDS to the electrophoretic procedure (Fig. 4) produced the breakdown of the circulating form of "VTG" in one major

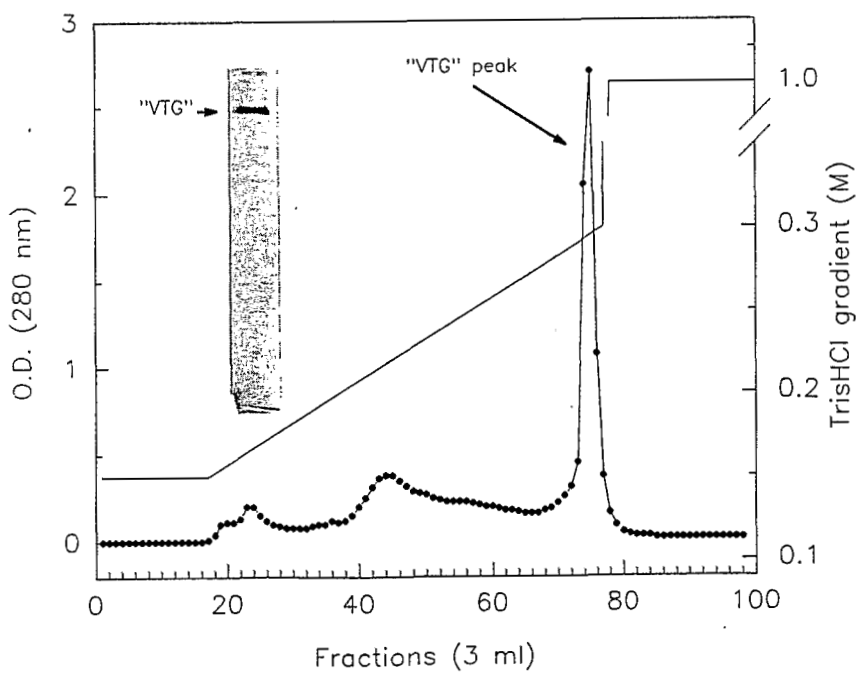


Fig. 2. Purification of sea bass vitellogenin, step 2. Second chromatography on DEAE–Sepharose. Pooled fractions from the gel-filtration chromatography (9–14) were concentrated (to a final volume of 5 ml) and eluted on a DEAE–Sepharose column (350 × 16 mm) with a 150–300 mM gradient of Tris–HCl buffer (pH 7.8), 1 mM PMSF. Inside is shown the elution in electrophoresis of the pooled solution of the fractions containing the "VTG" peak. The electrophoresis was performed in native conditions in 4–16% polyacrylamide gels and stained for proteins with Coomassie Brilliant Blue R-250.

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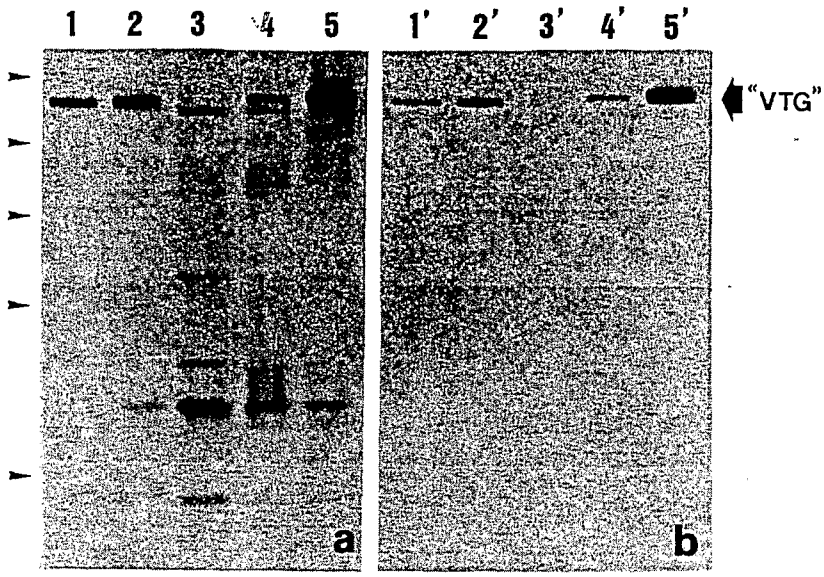

Fig. 3. (a) Native PAGE (4–16% gel) and (b) immunoblotting of the purified "VTG" preparation (lane 1 and 1'), the pooled solution of "VTG" after the gel filtration chromatography (lane 2 and 2'), control male plasma (lane 3 and 3'), vitellogenic female plasma (lane 4 and 4') and E_2-treated male plasma (lane 5 and 5'). Gels were stained for proteins with Coomassie Brilliant Blue R-250. Immunoblotting was performed with the AbVTG (diluted 1/3000 in Tris–saline buffer, 10 mM, pH 7.6) and complexes detected by the PAP method after transfer of the proteins to a nitrocellulose paper. On the left, arrowheads indicate the position of the molecular weight markers (from top to bottom, 669, 440, 232, 140 and 67 kDa).

band of 180 kDa, probably corresponding to the "VTG" monomer, and four minor bands with molecular weights of 113, 97, 86 and 73 kDa (Fig. 4, lane 1). The "VTG" monomer was highly visible in the plasma of E_2-treated males and more slightly in the plasma of natural vitellogenic females, but was absent in the plasma of control males (Fig. 4, lanes 4, 2 and 3, respectively). The presence of "VTG" in the female plasma is concomitant with the vitellogenic process and is present in plasma over the spawning period, as observed when comparing plasma from females at different reproductive stages (Fig. 4, lanes 5, 6 and 7). On the other hand, it can be observed that the "VTG" monomer is absent in an egg extract (Fig. 4, lane 8) which contained one major band of 88 kDa, and two groups of bands of around 70 and 25 kDa. Nevertheless, Fig. 5(b) showed, by immunocytochemistry,

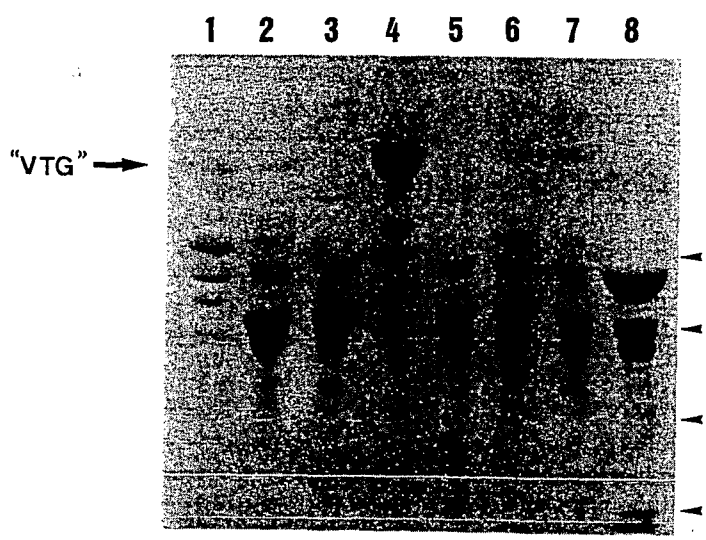

Fig. 4. SDS–PAGE (0.1% SDS) in 4–16% gel, of the purified "VTG" preparation (lane 1), vitellogenic female plasma (lane 2), control male plasma (lane 3), E_2-treated male plasma (lane 4), previtellogenic female plasma (lane 5), vitellogenic female plasma (lane 6), ovulated female plasma (lane 7) and egg extract (lane 8). Gels were stained for proteins with Coomassie Brilliant Blue R-250. On the right, arrowheads indicate the position of the molecular weight markers (from top to bottom, 94, 67, 43 and 30 kDa).

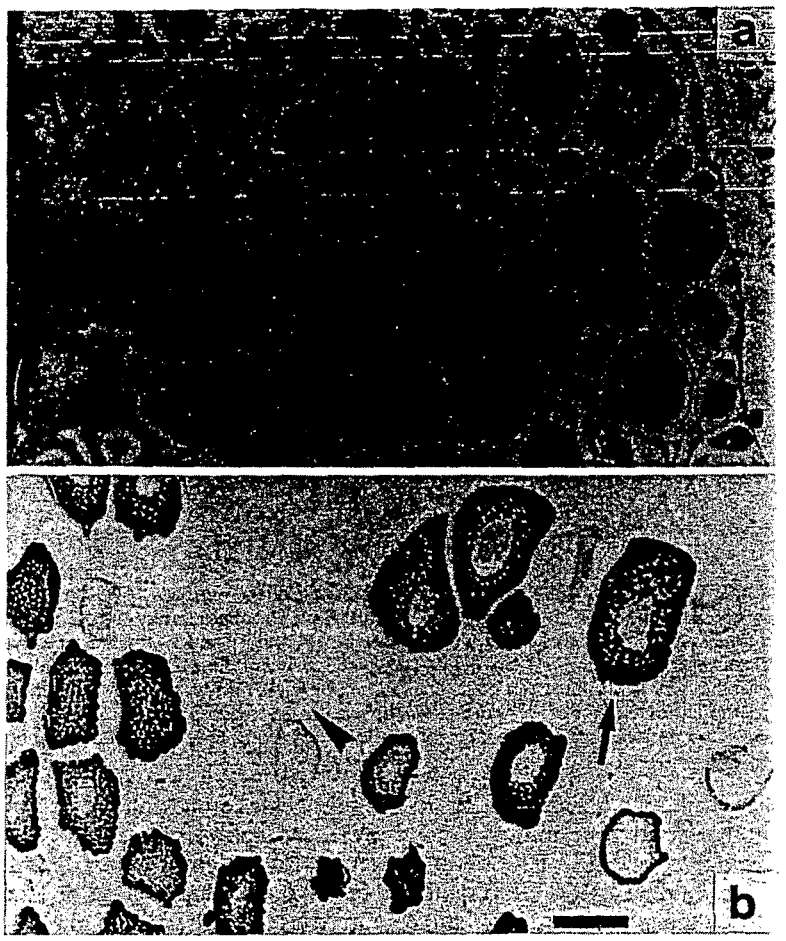

Fig. 5. Ovary from a vitellogenic female, stained with hematoxylin and eosin in (a) and immunostained using the AbVTG in (b). Strong immunoreaction was detected in the growing oocytes (arrow) while no reaction was shown in the previtellogenic and immature ones (arrowhead). AbVTG was diluted 1:3000 in Tris–saline buffer, 10 mM, pH 7.6 and the reaction detected by the PAP method. Scale bar = 600 μm.

that AbVTG recognized antigenic "VTG" sites in the growing oocytes, indicating that these egg yolk proteins were derived, at least in part, from the "VTG" molecule. Immature and previtellogenic oocytes remained unstained.

The molecular weight of the circulating form of "VTG" was estimated to be around 445 kDa by gel filtration. The amino acid composition of sea bass "VTG" is shown in Table 1. Alanine and glutamic acid are the most concentrated amino acids while methionine and histidine are the least concentrated.

Effects of the estradiol treatment

The induction of VTG synthesis by E_2 treatment produced an increase in the activity of protein synthesis in the liver, as reflected by changes in its composition and structure. Table 2 summarizes some biochemical changes which occurred in the liver after the E_2 treatment. The content of total proteins in the liver was slightly increased in the treated groups

Table 1. Amino acid composition of sea bass VTG compared with that of VTG from other teleost species

Amino acid	Amino acid residue (mol.%) Sea bass	Goldfish*	Medaka†	Rainbow trout‡
Asp	6.3	6.5	8.0	8.4
Thr	4.9	5.5	4.7	5.0
Ser	8.4	6.9	10.3	7.5
Glu	11.1	11.9	10.6	11.5
Pro	6.0	5.5	4.2	5.2
Gly	4.8	4.6	4.4	4.2
Ala	13.2	12.8	10.4	11.7
Val	7.2	6.9	6.8	7.1
Met	0.9	2.0	2.4	2.6
Ile	6.3	6.6	5.7	5.5
Leu	10.2	10.8	9.7	9.5
Tyr	3.1	2.6	3.6	3.0
Phe	3.6	2.9	3.4	4.0
His	1.5	2.3	2.4	2.1
Lys	6.9	7.0	7.6	7.1
Arg	5.4	4.9	5.0	4.5
	99.8	99.7	99.2	98.84

*Data from De Vlaming *et al.* (1980).
†Data from Hamazaki *et al.* (1987b).
‡Data from Hara and Hirai (1978).

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Table 2. Biochemical modifications induced by estradiol treatment in the liver of sea bass

Group	Total proteins (mg/g liver)	RNA (mg/g liver)	DNA (mg/g liver)	RNA/DNA	Prot/DNA
Control	96.81 ± 9.13 (4)[a]	17.31 ± 1.08 (7)[a]	3.08 ± 0.16 (6)[a]	5.99 ± 0.26 (6)[a]	30.37 ± 3.62 (4)[a]
0.5 mg E_2/kg	111.49 ± 4.06 (6)[a]	23.09 ± 0.20 (6)[b]	2.99 ± 0.14 (7)[a]	7.98 ± 0.49 (6)[a]	37.05 ± 1.34 (6)[a]
2 mg E_2/kg	112.87 ± 4.71 (5)[a]	22.96 ± 0.77 (5)[b]	2.60 ± 0.17 (5)[a]	9.05 ± 0.73 (5)[b]	44.05 ± 2.53 (5)[b]
5 mg E_2/kg	118.56 ± 8.48 (4)[a]	24.66 ± 2.16 (4)[b]	2.78 ± 0.25 (4)[a]	9.21 ± 1.28 (4)[b]	42.95 ± 0.87 (4)[b]

Differences for every parameter between the groups are expressed with different letters ($P < 0.05$).

with respect to the controls (not significantly different); when expressed in relation to DNA, this increase was significantly different ($P < 0.05$) for the groups injected with 2 and 5 mg E_2/kg. The content of liver RNA was significantly ($P < 0.05$) increased in all treated groups with respect to the controls; nevertheless, when it was expressed in relation to DNA only the groups injected with the higher doses (2 and 5 mg E_2/kg) maintained this difference. DNA content of the liver was not affected by the E_2 treatment.

Table 3 summarizes some changes in the structure of the liver associated with the E_2 treatment. The hepatosomatic index (HSI) and the nuclear size of the hepatocytes were increased in all treated groups with respect to the controls but this increment was significantly different ($P < 0.05$) only for the groups injected with 2 and 5 mg E_2/kg. The hypertrophy of the hepatocytes (assessed by the number of nuclei/area unit) was only observed in the two groups that received the highest doses of E_2.

Figure 6 shows histological sections of liver from control and E_2-treated animals. The hepatocytes of the control group (Fig. 6a) were smaller and more vacuolized than those of the treated ones (Fig. 6b). On the other hand, liver slices were immunostained with the AbVTG for the localization of VTG into the hepatocytes. No reaction was observed in the liver of the control group (Fig. 6c) whereas in the liver of the E_2-treated animals a positive immunostaining was detected in the cytoplasm of some hepatocytes (Fig. 6d).

In plasma, the E_2 treatment caused a significant increase in the content of both VTG and total proteins (Fig. 7). Before the E_2 injections, the plasma VTG levels were undetected in males and very low (around 100 μg/ml) in females. These levels remained unchanged in the control group throughout the experiment. Two days after the first injection a slight, but not significant ($P < 0.05$), increase in the VTG levels was observed in the three treated groups, when compared to the controls. Six days after the first injection, the VTG levels were significantly increased in all treated groups with respect to the controls and reached a maximum of around 50 mg/ml after the fourth injection. Total proteins were significantly increased in all the treated groups with respect to the controls at day 8 after the beginning of the experiment.

Discussion

In this study, E_2 was used for the induction of VTG synthesis in male and female sea bass, because it is known to be the most potent estrogen for the induction of VTG synthesis in fish (Sundararaj and Nath, 1981; van Bohemen *et al.*, 1982).

Several methods have been developed for the purification of amphibian VTG, ultracentrifugation (Redshaw and Follett, 1971), precipitation with dimethylformamide (Ansari *et al.*, 1971), selective precipitation with Mg^{2+}-EDTA (Wiley *et al.*, 1979) and chromatography (Wallace, 1970; Wiley *et al.*, 1979). In fish, the purification of VTG has normally been performed by chromatography, because the other methods have failed to completely isolate VTG from other plasma components (Wiley *et al.*, 1979; de Vlaming *et al.*, 1980). In this study, sea bass VTG was purified from plasma of E_2-treated animals using a double chromatography method. Similar procedures have been successfully used for the purification of VTG in *Anguilla japonica* (Hara *et al.*, 1980), *Salmo*

Table 3. Structural modifications induced by estradiol treatment in the liver of sea bass

Group	HSI	Nuclei/area* (* = 13.2 μm²)	(μm²)
Control	0.87 ± 0.09 (7)[a]	117 ± 10 (7)[a]	14.95 ± 0.20 (7)[a]
0.5 mg E_2/kg	1.25 ± 0.07 (7)[a]	86 ± 5 (7)[b]	15.05 ± 0.16 (7)[a]
2 mg E_2/kg	2.38 ± 0.12 (7)[b]	58 ± 3 (7)[c]	18.53 ± 0.62 (7)[b]
5 mg E_2/kg	2.18 ± 0.17 (6)[b]	57 ± 3 (7)[c]	20.20 ± 0.56 (7)[b]

Differences for every parameter between the groups are expressed with different letters ($P < 0.05$).

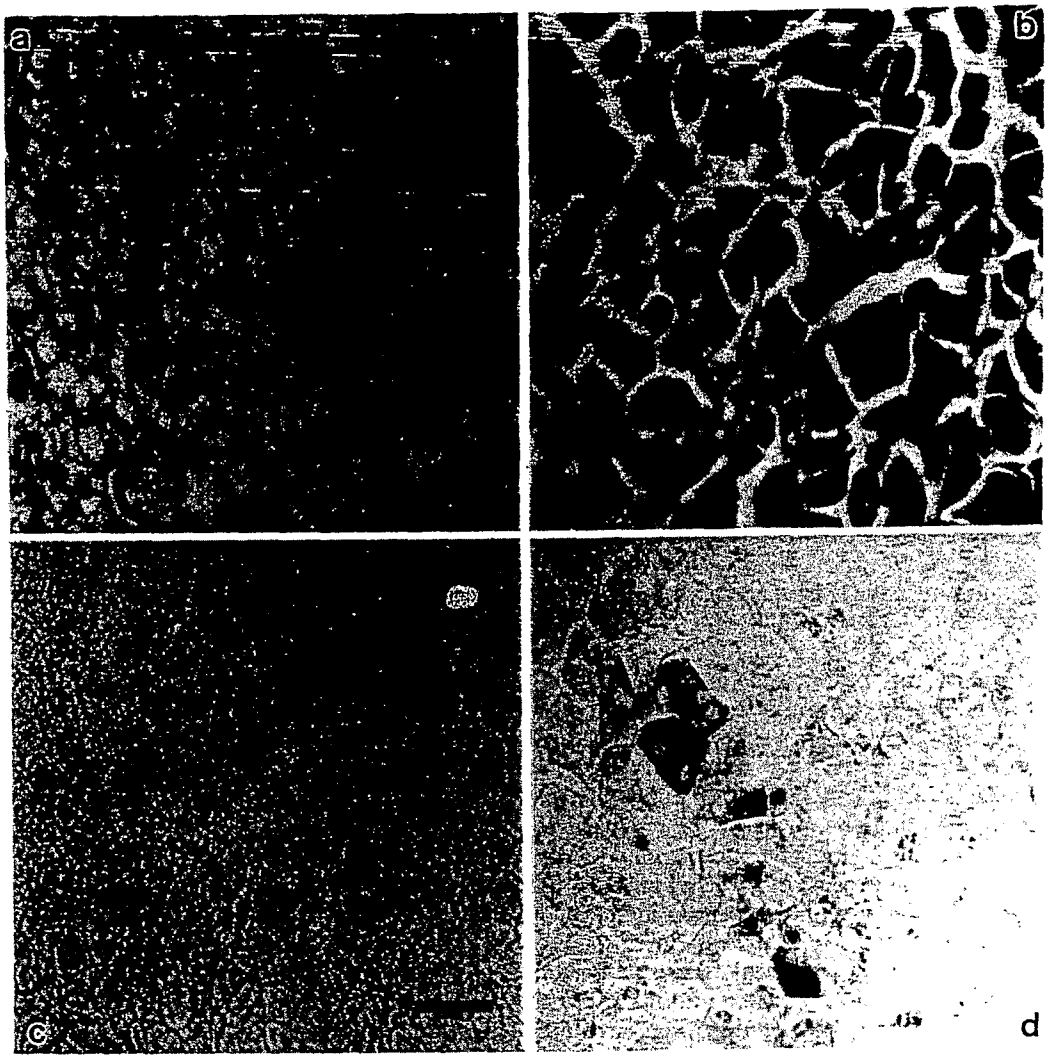

Fig. 6. Histology and immunocytochemistry of the liver from control and E_2-treated males. (a) and (b): histological sections of liver from control and E_2-treated animals, respectively. Sections were stained with hematoxylin and eosin. (c) and (d): immunocytochemical staining of liver from control and E_2-treated animals, respectively, using the AbVTG (diluted 1:3000 in Tris–saline buffer, 10 mM, *p*H 7.6). Complexes were detected by the PAP method. Immunoreaction was detected in a part of the hepatocyte population (arrowhead). Scare bar = 40 μm.

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salar (So *et al.*, 1985), *Oryzias latipes* (Hamazaki *et al.*, 1987b), *Cyprinus carpio* (Tyler and Sumpter, 1990) and *Anguilla anguilla* (Burzawa-Gerard and Dumas-Vidal, 1991). In sea bass, this methodology is necessary to separate the VTG molecule from the other plasma components. Indeed, the first chromatography on gel filtration was insufficient to completely remove VTG from other plasma elements, as shown in Fig. 1.

In order to avoid the proteolysis of the sea bass VTG, plasma was stored in the presence of PMSF which was added as well to the buffers used in the purification procedure. The isolated sea bass VTG was eluted in electrophoresis as a single band (Fig. 2, inside) and in gel filtration as a single and symmetrical peak (not shown), indicating that no detectable signs of degradation were produced after sampling and storage and throughout the purification procedure and further freezing of the solution. In addition, the purity of the VTG preparation was tested using the AbVTG and when an immunoblot was performed (Fig. 3b) only the "VTG" band was recognized by the AbVTG both in the plasma of natural vitellogenic females and in the plasma of E_2-treated males while no reaction was detected with the control male plasma.

The protein isolated in this study has characteristics typically associated with the VTG molecules; i.e. (1) being E_2-inducible, (2) being a female-specific protein and (3) being the yolk precursor protein.

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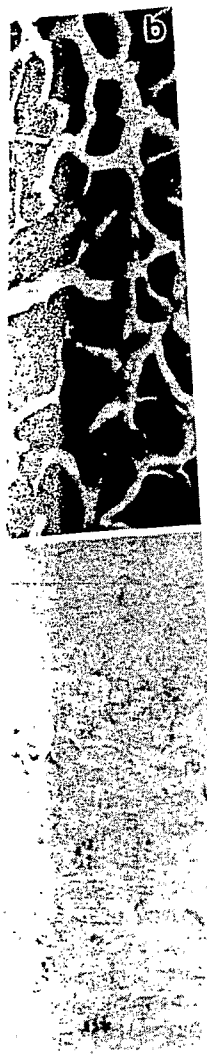

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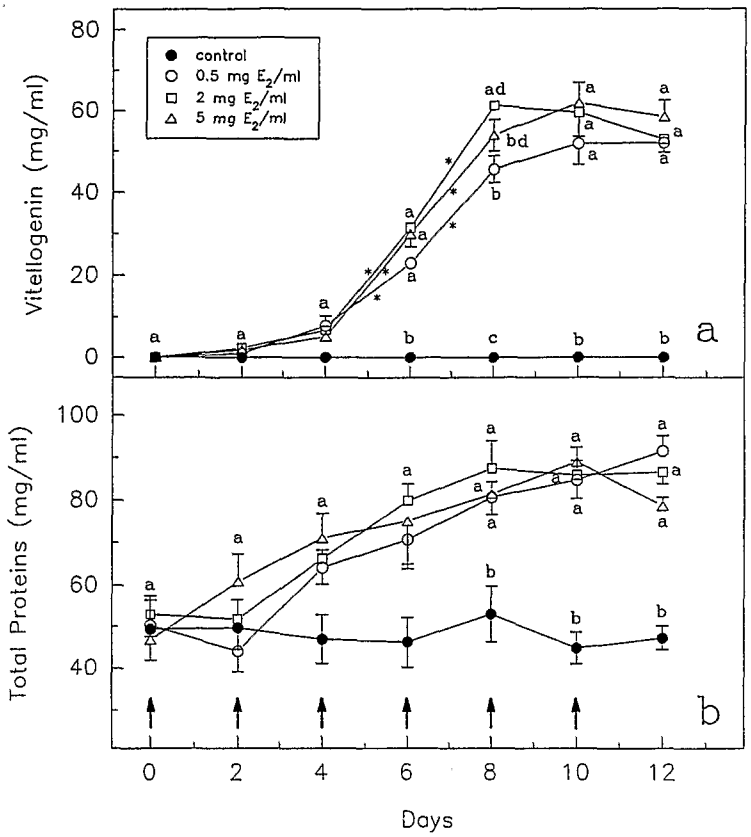

Fig. 7. Plasma levels of (a) vitellogenin and (b) total proteins, during the estradiol treatment. The VTG levels were measured by ELISA and the levels of total proteins by Lowry *et al.* (1951). At the bottom, arrows indicate the time of estradiol injections. Different letters represent significant differences ($P < 0.05$) between the groups for the same sampling (when there is only one letter for the four groups in a sampling point, it means that there are no differences between them); (*) over the lines represents differences ($P < 0.05$) from the previous sampling for the same group; $N = 7$ for each sampling.

Firstly, the E_2 inductivity of sea bass VTG was observed by gel filtration and electrophoresis by comparing plasma from treated and untreated males (Fig. 1). In sea bass, no other protein was observed to be induced by the E_2 treatment whereas, in the teleost *Oryzias latipes*, Hamazaki *et al.* (1987a) found that another protein different to VTG was induced by treatment with estradiol. This protein, a glycoprotein synthesized by the liver, was found to be a component of the egg envelope (Hamazaki *et al.*, 1987c). Other E_2-inducible proteins different to VTG were also found in amphibians (Mitchell *et al.*, 1985; Holland and Wangh, 1987), but the nature and function of these proteins remain unclear. On the other hand, it is well known that VTG is synthesized in the liver under the influence of estradiol and in accordance with that, sea bass VTG was localized in the liver tissue of the E_2-treated animals using the AbVTG (Fig. 6). Thus, considering that previously discussed, this new protein purified from the plasma of E_2-treated sea bass, can be considered as the probable VTG.

Secondly, this protein is female-specific as shown by its presence in the plasma of natural vitellogenic females and its absence in the plasma of non-treated males and previtellogenic females (SDS–PAGE, Fig. 4). On the other hand, the AbVTG did not react with any of the bands of the male plasma, whereas a strong reaction was found with the band identified as VTG in the vitellogenic female plasma (Fig. 3b). This methodology has been widely used in other teleost species (Hara *et al.*, 1983; Bradley and Grizzle, 1989) and it has proved to be a good tool for the characterization of VTG as a female-specific protein. Notwithstanding, Ding *et al.* (1989) and Pelissero *et al.* (1991) detected the presence of VTG in the male plasma of *Oreochromis aureus* and *Acipenser baeri*, respectively. Although the significance of the presence of VTG in males of tilapia remains unclear, in the Siberian sturgeon it has been demonstrated to be induced by the presence of phytoestrogens in the diet (Pelissero *et al.*, 1991).

Thirdly, the protein isolated in this study was found to be related to the yolk proteins, as

shown by immunocytochemistry of the ovary using the AbVTG (Fig. 5). VTG antigenic sites were localized in the cytoplasm of the growing oocytes. The same procedure was used by Ollevier and Covens (1983) for the localization of VTG antigenic sites in the ovary of *Gasterosteus aculeatus*. As shown by electrophoresis (Fig. 4) neither the circulating form of VTG (445 kDa) nor the VTG monomer (180 kDa) was present in the egg extract, which probably corresponds to a rapid breakdown of VTG into the growing oocytes (Wallace and Selman, 1985; Greeley *et al.*, 1986).

The molecular weight of the sea bass VTG was found to be around 445 kDa as determined by gel filtration. Nevertheless, care should be taken with this value, because this method was reported to be not the most appropriate one for the estimation of the molecular weight of proteins containing highly charged groups in its structure (Norberg and Haux, 1985). In native PAGE, the native form of sea bass VTG was eluted at a position corresponding to a mol. wt of around 510 kDa. SDS electrophoresis was reported to be the best method for the estimations of the mol. wt, nevertheless, in sea bass, the addition of SDS to the electrophoretic procedure (non-denaturing conditions) caused the dissociation of the native VTG in its monomer (180 kDa) together with some breakdown products of lower mol. wt. The mol. wt of the native form of sea bass VTG is encompassed in the range of the mol. wt reported for VTGs from other fish species, e.g. 380 kDa for *Carassius auratus* (de Vlaming *et al.*, 1980), 440 kDa for *Salmo trutta* (Norberg and Haux, 1985), 550 kDa for *Heteropneustes fossilis* (Nath and Sundararaj, 1981) and 640 kDa for *Morone saxatilis* (Sullivan *et al.*, 1991). This heterogeneousness in the mol. wt of fish VTGs could be partially due to the method employed in its estimation since, for the same species, *Oncorhynchus mykiss*, the mol. wt of VTG has been found to vary from 342 to 535 kDa, depending on the method used for its estimation (Mommsen and Walsh, 1988). The mol. wt of the monomeric form of sea bass VTG (180 kDa) is similar to that reported for other teleost species, 170 kDa for *Morone saxatilis* VTG (Kishida *et al.*, 1992), 175 kDa in *Oncorhynchus mykiss* (Babin, 1987) and 185 kDa in *Scophthalmus maximus* (Silversand and Haux, 1989). Finally, the amino acid composition of sea bass VTG was very similar to that reported for VTGs from other teleost species (Table 1).

The induction of VTG synthesis in sea bass produced modifications both in liver and plasma. The HSI and the RNA contents of the liver in the treated groups were increased with respect to the controls, whereas proteins and DNA remain unchanged (Table 2). The increment of the HSI and the contents of liver RNA are normally associated with the estradiol treatment (Haux and Norberg, 1985; Korsgaard, 1990) but liver proteins are reported to remain unchanged in some species (Korsgaard *et al.*, 1986; Korsgaard, 1990) probably due to the rapid secretion of the newly synthesized proteins into the bloodstream (Ng and Idler, 1983). The increase in the liver size (HSI) of the E_2-treated animals was due, at least in part, to the hypertrophy of the hepatocytes, as shown by the decrease in the number of nuclei/area unit (Table 3). The existence of hyperplasia could not be assessed by the methodology used in this study. Hypertrophy of the liver, after treatment with estradiol, was observed in *Oncorhynchus mykiss* (Haux and Norberg, 1985) and *Epinephelus akaara* (Tam *et al.*, 1983). Nevertheless, Korsgaard *et al.* (1986) demonstrated that the increase of the liver size induced by estradiol in *Salmo salar* was produced by hyperplasia rather than hypertrophy of the hepatocytes. In this study, sea bass VTG was localized in the liver of E_2-treated animals by immunocytochemistry using the AbVTG. As observed by Pacoli *et al.* (1991) in *Ictalurus punctatus*, immunoreaction was only detected in some hepatocytes.

Treatment with estradiol caused, in sea bass, the increment of the content of total proteins in plasma, similarly to that observed in *Oncorhynchus mykiss* (van Bohemen *et al.*, 1982), and the massive appearance of VTG in the blood (maximum levels around 50 mg VTG/ml in the treated animals). The lack of a dose-response on the VTG levels for the different groups can be explained by the fact that the three doses of estradiol assayed could cause the maximum response, thus minor doses should be tested in order to assess this point and to obtain lower responses. The VTG titer of 50 mg/ml was much higher than the levels found in natural vitellogenic females (around 3 mg/ml) (Mañanós *et al.*, 1994). As observed in the flounder, the treatment of natural vitellogenic females with estradiol causes an increase in the VTG levels found during vitellogenesis (Emmersen and Emmersen, 1976).

In conclusion, the protein isolated in this study can be considered as the VTG molecule for sea bass, since it fits all the characteristics typically associated with the VTG molecules. On the other hand, the procedure used for the purification of sea bass VTG in this study provided a pure solution of VTG as attested by electrophoretic and immunological studies, and could be applied to the purification of VTG from other fish species.

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