Development and Application of an Effective Detection Method for Fish Plasma Vitellogenin Induced by Environmental Estrogens

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Vitellogenin is a protein induced by estrogens, including environmental chemicals with estrogenic activity. To measure the effects of environmental estrogens, we developed an effective and rapid one-step method of detecting and purifying fish plasma vitellogenin using a high-performance anion-exchange chromatography column, POROS-HQ. Vitellogenin in a plasma of estradiol-treated male fish (mummichog and red sea bream) was eluted as a single peak with a retention time of 10 minutes from the column, which gives an almost pure preparation as assessed by SDS-PAGE. The lowest detectable amount of vitellogenin was 2 μg per assay. The method was used to analyze the plasma vitellogenin level of aquacultured red sea breams caught in August, when the spawning season is over, and usually no vitellogenin is detected in either females or males, physiologically. However, the data showed that in addition to a few females, some male fish synthesized vitellogenin, suggesting that some chemicals or unknown factors with estrogenic activity have induced fish in the ocean to produce vitellogenin.

Key words: endocrine disrupters; environmental estrogens; red sea bream; vitellogenin; HPLC

Recently, it has been suggested that there is a potential risk from environmental chemicals with endocrine-disrupting activity, either hormone agonists or antagonists, that can alter the hormonal balance in animals and other people.1) Among such endocrine disrupters, environmental estrogens are chemicals that mimic estrogenic activities through binding to estrogen receptors, and may affect the health of various vertebrates.2-5) To date, several chemicals; such as bisphenol A and p-oyl-phenol, are accepted as candidates for such environmental estrogens.6-8)

To evaluate the causes and effects of environmental estrogen, we should not only accumulate the data measuring environmental estrogen levels but also by assaying for their effects on animals and humans on a world-wide scale. By measuring vitellogenin level in male fish plasma, we sought to find whether or not environmental estrogens have affected aquatic animals. Vitellogenin, the egg yolk protein precursor, is a glycoprophosphoprotein synthesized by the liver of most female oviparous animals, including fish, in response to estrogen and secreted into the blood.9-11) Generally, plasma vitellogenin levels in male fish are negligible. However, male livers also synthesize and secrete vitellogenin into the blood in response to estrogens injected in the fish or dissolved in water that the fish are exposed to. Thus, vitellogenin levels in male fish plasma should suggest the effect of chemicals with estrogenic activity. Because vitellogenin is a highly protease-sensitive protein,12-14) an addition of enough protease inhibitors for plasma preparation and the development of a rapid analytical method are important points in measuring plasma vitellogenin levels. In view of these points, in this study, we developed an effective and rapid one-step method for detection as well as purification of fish plasma vitellogenin using a high-performance anion-exchange chromatography column, POROS-HQ.

Materials and Methods

Materials. Estradiol-17β, phenylmethanesulfonyl fluoride (PMSF), aprotinin, and heparin were obtained from Sigma Chemicals. A marker protein mixture for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a protein staining kit (Quick-CBB) were from Pharmacia and Wako Pure Chem., respectively. A POROS-HQ column (4.6 × 100 mm) was purchased from PerSeptive Biosystems. An antiserum against the vitellogenin of the red-spotted grouper (Epinephelus aestivalis) was raised as described elsewhere (K. Soyano, manuscript in preparation). All other chemicals were reagent-grade compounds obtained from commercial sources. The three-year-old red sea breams (Pagrus major) (799 ± 48 g) used in this study were cultured in the Nagasaki area and captured in August 1997.

Estrogen treatment. Male mummichog (Fundulus heteroclitus) and male red sea bream with an average body weight of 10 g and 800 g, which were gift of Dr. Akira Shimizu (National Research Institute of Fisheries Science, Yokosuka) and obtained from a fish farm in Nagasaki, respectively, were acclimated for one week before the experiments. Mummichog or red sea bream were kept in 675-L basins containing aerated sea water. The water temperature was kept at 20–25°C. The sea water was renewed every 3 days. The photoperiod was 12 h light/12 h dark. No food was given during the

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experiment. Estradiol-17β was suspended in propyrene glycol at the concentration of 4 mg/ml. Vitellogenin synthesis was induced by intraperitoneal injections (3 times) every 3 days with 1 mg of estradiol-17β/kg of body weight. Control fish were not injected. Two days after the last estrogen treatment, blood was collected.

\textit{Plasma preparation.} Fish blood was taken from the caudal blood vessel with a cold and heparinized syringe and was immediately transferred into a centrifuge tube and mixed with 0.1 volume of saline containing 10,000 KIU/ml aprotinin, 0.1% PMSF, and 1 U/ml heparin. The blood was centrifuged and the resultant plasma was immediately used for further experiments, or stored at -80°C until use. All preparative procedures were done at 4°C.

\textit{Histological observation.} Immediately after the fish were killed, the gonadal tissues were cut into small pieces, which were fixed with Bouin’s solution for 24 h, dehydrated through a series of graded alcohol concentrations, and embedded in paraffin. The sections were cut to a thickness of 8 μm and were stained with Delafeld’s hematoxylin and eosin.

\textit{Separation of plasma protein by HPLC.} Proteins in a plasma sample were separated with an ion-exchanger (POROS-HQ column) connected to a high-performance liquid chromatography (HPLC) system (Jasco) consisting of a 801-SC gradient programmer, two 880-PU pumps, and an 875-UV UV monitor with flowcell and recorder (Chromatocorder 12) using two solutions, Solution A (20 mM Tris-HCl, pH 8.0) and Solution B (20 mM Tris-HCl, pH 8.0, containing 1.5 M NaCl). To avoid clogging up the column and to prevent proteolytic degradation during column operation, the plasma was diluted with two volumes of 20 mM Tris-HCl (pH 8.0) containing 10,000 KIU/ml aprotinin and filtered with a 0.22-μm filter. The treated plasma was put on HPLC operating with a gradient system (0-2 min; Solution A 100%, 2-3 min; linear gradient with 0-10% of Solution B, 3-4 min; 10% Solution B, 4-10 min; linear gradient with 10-80% of Solution B, 10-11 min; linear gradient with 80-100% of Solution B and 11-14 min; 100% Solution B) at a flow rate of 2.5 ml/min. Absorbance at 280 nm was monitored and each peak was collected. The collected samples were either used for further experiments or stored at -80°C until needed.

\textit{SDS-PAGE and Western blot.} SDS-PAGE was done by the method of Laemmli\textsuperscript{18} using a 6% polyacrylamide gel under reducing conditions. The separated proteins in the gel were stained with Coomassie blue using a Quick-CBB, and the vitellogenin in the gel was detected by Western blot analysis as described\textsuperscript{19} using anti-red spotted grouper vitellogenin-antiserum after being transferred onto a nitrocellulose filter (BioTrace NT, Gelman Sciences). This was followed by detection with an ECL Western blot detection system (Amersham). Phosphoproteins in the gel were stained as nitro-phosphomolybdate complexes by the method of Cutting and Roth\textsuperscript{17} after SDS in the gel was removed by washing with 10% sulfosalicylic acid.

\textit{Miscellaneous.} Protein was measured by the method of Bradford\textsuperscript{18} using a Protein Assay kit from Bio-Rad with bovine serum albumin as the standard. Amino-terminal amino acid sequence analyses were done on an automated Applied Biosystems model 492 protein sequencer with an on-line phenylthiohydantoin analyzer.

\textbf{Results}

\textit{Chromatography of fish plasma proteins on a POROS-HQ column} To develop a convenient method for analyzing and purifying fish plasma vitellogenin, we attempted to find a suitable HPLC column with respect to the rapidity and the accuracy of the analysis using plasma samples from mummichog and red sea bream, the vitellogenin of which was induced by estradiol-17β. An anion-exchange column, POROS-HQ, was found to be the most suitable, so far. Figures 1-A and 2-A show chromatograms of plasma proteins from male mummichog and red sea bream treated with estradiol-17β, respectively. Compared with chromatograms of control fish plasma proteins (no treatment with estradiol-17β), newly-appeared protein peaks (peak-5 for mummichog and peaks-4 and -5 for red sea bream) with the retention time of 10 minutes were detected, indicating that these proteins are vitellogenin induced by the effects of estradiol-17β. Analysis of these proteins by SDS-PAGE under reducing conditions showed that these proteins were almost pure, and their apparent molecular weights were estimated to be 170,000 for the peak-5 protein of mummichog and 160,000 for both the peaks-4 and -5 proteins of red sea bream, respectively (Figs. 1-B and 2-B).

\textit{Assessment of vitellogenin} The value of an apparent molecular weight (170,000) of an estradiol-17β-induced-protein in mummichog (peak-5 protein in Fig. 1-A) is in accordance with that obtained from the amino acid sequence of mummichog vitellogenin (1704 residues), which was deduced from its cDNA by LaFleur et al.\textsuperscript{20}. The secreted mummichog vitellogenin is N-terminal blocked.\textsuperscript{19} Similarly, the peak-5 protein was N-terminal blocked. To confirm the peak-5 protein is vitellogenin, its amino acid sequences of internal peptides were analyzed using tryptic peptides. As a result, three purified tryptic peptides from the peak-5 protein were in agreement with amino acid residues #70-84, #1634-1650, and #1662-1682 of mummichog vitellogenin (data not shown). Furthermore, a Western blot analysis using anti-red spotted grouper vitellogenin-antiserum (Fig. 1-C) also indicated that the peak-5 protein is vitellogenin. These data clearly show that the purified peak-5 protein is mummichog vitellogenin.

Similar to the case of mummichog, both proteins in peak-4 and peak-5 in Fig. 2-A were assessed to be red sea bream vitellogenin on the basis of molecular weight (Fig. 2-B, the reactivity against vitellogenin antibody (Fig. 2-C), and, in addition, staining significance with
phosphoprotein staining (Fig. 2-D). Although further experiments for measuring the phosphate content in proteins of peak-④ and peak-⑤ are required, it can be presumed that the appearance of two vitellogenin protein peaks may be caused by the deference of phosphorylation. As mentioned above, since both plasma proteins of red sea bream were vitellogenin induced by estrogen, it is reasonable to measure the level of both proteins for measuring estrogenic activity on red sea bream. Accordingly, a fraction containing peak-④ and peak-⑤ was used as a purified vitellogenin standard of red sea bream for the following experiments after measuring the protein concentration.

**Standard curve of vitellogenin measurement**

Samples of purified vitellogenins from estrogen-treated plasma of mummichog and red sea bream were put on a POROS-HQ column. Figure 3 shows the standard curve of the red sea bream vitellogenin versus absorbancy at 280 nm. The lower limit of detection was 2 µg per chromatography.

*Detection of vitellogenin in plasma from cultured red sea bream*

A total of 21 (7 males and 14 females) cultured red sea bream plasma were analyzed for vitellogenin by POROS-HQ column chromatography and we did histochemical observation of gonadal tissue. When 10-µl plasma samples from each were analyzed, a vitellogenin peak with the retention time in the vicinity of 10 minutes, which was confirmed by Western blot analysis (not shown), was detected in 10 (2 males and 8 females) out of 21 plasma samples. Figure 4 shows typical chromatograms of vitellogenin-positive male (Sample 1) and negative female (Sample 2) plasma samples. The amount of vitellogenin in this sample (Sample 1) was calculated to be 4.8 µg from the vitellogenin peak and the concentration of vitellogenin in the plasma was calculated to be 0.48 mg/ml. Histological observation indicated that the oocytes of plasma vitellogenin-positive female
were immature (perinucleolus stage oocytes). Furthermore, it was revealed that the plasma vitellogenin-positive male had immature testis and immature oocytes.

Discussion

Various different separation methods of vitellogenin have been developed, including ultracentrifugal separation, selective precipitation, DEAE-cellulose chromatography, and liquid chromatography using columns of TSK-G 4000 SW, TSK-DEAE 5-PWT, and Mono Q. These methods are usually time-consuming and thus inadequate for many plasma sample analyses done in the field. In this study, we have developed a more rapid and convenient detection and purification method by a high-performance liquid chromatography using a POROS-HQ column for fish plasma vitellogenin. This is an effective one-step method, which gives a pure preparation of vitellogenin in a short processing time, i.e., after collecting samples of fish plasma, it takes only 10 minutes to detect and purify vitellogenin. It should be noted that this method is advantageous for detecting plasma vitellogenin induced by estrogen as a chromatographic peak with the retention time of 10 minutes, because control male plasma without estrogen treatment gives no peaks with the retention time in the vicinity of 10 minutes. Judging from the stained profile of separated proteins by POROS-HQ column chromatography with Coomassie blue, anti-vitellogenin an-
tured red sea breams synthesize vitellogenin. Twenty-one plasma samples were prepared from red sea breams cultured for 3 years in the Nagasaki area and captured in August 1997. In August, the red sea bream are usually finished spawning. Thus in this season, in females, oocytes are immature (perinucleolus stage) and vitellogenin synthesis is negligible. Thus, vitellogenin should be not detected in the plasma of female or male red sea breams. However, it was found that 10 (2 males and 8 females) out of 21 cultured red sea breams synthesized vitellogenin. Furthermore, it was found that 2 plasma vitellogenin-positive male red sea breams were bisexual, morphologically. Although much more data are required, these data may indicate that some chemicals or unknown factors with estrogenic activity have affected these sea water fish. It is necessary to identify and measure such chemicals or factors systematically.

Fig. 3. Standard Curve of Vitellogenin Measurement of Red Sea Bream.

Samples of purified red sea bream vitellogenin (0.4 mg/ml) were chromatographed on a POROS-HQ column and the absorbance at 280 nm of the vitellogenin peak was plotted versus amounts of injected vitellogenin.

Fig. 4. Typical Elution Profiles after POROS-HQ Column Chromatography of Vitellogenin-Positive Male (Sample 1) and Vitellogenin-Negative Female (Sample 2) Plasma from Cultured Red Sea Breams.

Ten μl of plasma were analyzed by POROS-HQ column chromatography as described under Materials and Methods. An arrow indicates the elution position of vitellogenin.

References