Development of a Microtiter Plate Enzyme-linked Immunosorbent Assay for 17α, 20β-21-trihydroxy-4-pregnen-3-one, a Teleost Gonadal Steroid

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A simple solid-phase microtiter plate enzyme-linked immunosorbent assay (ELISA) for 17α, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S), a teleost maturation inducing steroid, was developed using 20β-S-3-CMO-horseradish peroxidase as the label, and a homologous antiserum raised in rabbits. Second antibody coated to the plate was used to capture the antiserum with labeled or unlabeled 20β-S. Within three hours, a steep standard curve covering 1.5–192 pg/well was obtained. The sensitivity and precision of this ELISA was almost the same as those of ordinary steroid radioimmunoassays. This non-isotopic assay system also allows prompt measurement of 20β-S in catfish plasma with good specificity.

Key words: ELISA, steroid, teleost, 20β-S, catfish

Much information on the mechanisms of gonadal maturation in teleosts has been collected following the development of specific radioimmunoassays (RIAs) for gonadal steroids. However, RIAs have an essential problem, the use of radioisotopes. Moreover, some of the teleost gonadal steroids, such as 17α, 20β-dihydroxy-4-pregnen-3-one or 11-ketotestosterone, are commercially unavailable in a tritiated form; one must synthesize the labeled steroids from 3H-labeled precursors for specific RIAs. It is necessary, therefore, to develop appropriate non-isotopic assays for teleost gonadal steroids.

Since Van Weemen and Schuurs devised a hapten enzyme immunoassay for estradiol,1 many non-isotopic assays have been developed in various disciplines for the measurement of steroid hormones.2,3 Recently, the application of multiwell microtiter plates as the solid support has shown to be of great advantage in progesterone,4 testosterone,5,6 and estradiol7) enzyme-linked immunosorbent assays (ELISA). These assays, however, are only for common steroids, not for many teleost-specific ones.

17α, 20β, 21-Trihydroxy-4-pregnen-3-one (20β-hydroxylated compound S; abbreviated to 20β-S in the following) was purchased from Steraloids Inc. (Wilton, NH). Other steroids were obtained from Sigma Chemical Co. (St. Louis, MO). Horseradish peroxidase (type VI) was also purchased from Sigma. Affinity purified goat anti-rabbit IgG was obtained from Bio-Rad Laboratories (Richmond, CA). Other reagents were of reagent grade and were obtained from Wako Chemicals Co. (Tokyo).

Preparation of 3-Carboxymethyl oxime of 17α, 20β, 21-Trihydroxy-4-pregnen-3-one (20β-S-3-CMO)

Carboxylic acid derivatives of 20β-S were prepared following the method of Hosoda et al.13) with some modifications. About 25 mg of 17α, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S) was dissolved in a mixture of 100 μl chloroform and 300 μl methanol. Sodium acetate and carboxymethylxylamine hemichloroform and 300 μl methanol. Sodium acetate and carboxymethylxylamine hemihydrochloride, 12.5 mg each, were dissolved in a droplet of distilled water and several drops of methanol, and were added to the steroid solution at room temperature. The conversion of 20β-S into 20β-S-3-CMO was monitored by spotting an aliquot of the solution on a TLC plate (silica gel 60 F254, Merk, Darmstadt) and developing in a chloroform:methanol (5:1, v/v) system. After completion of the conversion, about 50 min after the start of the reaction, 3 ml of distilled water was added to stop the reaction. Extraction was performed twice by adding 15 ml ethyl acetate and shaking vigorously. The combined ethyl acetate phases were washed twice with distilled water. After evaporation of the solvent, about 30 mg of 20β-S-3-CMO was obtained.
Preparation of Active Ester of 20\ß-S-3-CMO

In order to make water-soluble steroid-protein conjugates, N-hydroxysuccinimide ester was prepared from 20\ß-S-3-CMO using a carbodiimide method.13) About 25 mg 20\ß-S-3-CMO, 20 mg N-hydroxysuccinimide, and 30 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl were mixed and dissolved into 250 \( \mu \)l dimethylformamide (DMF). This mixture was left for one hour at room temperature, and conversion of 20\ß-S-3-CMO into its N-hydroxysuccinimide ester (active ester) was monitored by TLC. After completion of the conversion, the reactant was extracted twice with ethyl acetate, and the pooled solvent was washed twice with distilled water. The ethyl acetate fraction was dehydrated with anhydrous sodium sulfate and evaporated under a gentle stream of nitrogen. Finally, about 31 mg of active ester of 20\ß-S-3-CMO was obtained.

BSA-steroid Conjugate

The active ester of 20\ß-S-3-CMO was conjugated to BSA using a 30:1 molar ratio of steroid to BSA. First, 50 mg BSA, 1.5 ml distilled water, and 25 \( \mu \)l \( \text{NaOH} \) were mixed, and 0.5 ml DMF was slowly added to the mixture. About 15 mg of active ester of 20\ß-S-3-CMO was dissolved into 300 \( \mu \)l DMF, then slowly added to the mixture while stirring. After 30 min of stirring, the reactant was dialyzed against running water overnight.

After the dialyzation, BSA-steroid conjugate was precipitated at pH 4.5, and centrifuged at 3000 rpm for 15 min. The precipitate was then dissolved in distilled water, adjusting the pH to 7.0 with 1 N \( \text{NaOH} \), and diluted to a concentration of 10 mg protein/ml and stored at -20°C.

Antibody Preparation

Antisera against 20\ß-S were raised in three female normal white rabbits by five monthly immunizations with the BSA-steroid conjugate, according to a conventional immunization procedure.14) Each rabbit received 2 mg of the immunogen emulsified in Freund's complete adjuvant in a conventional immunization procedure. After completion of the conversion, the reactant was extracted twice with ethyl acetate, and the pooled solvent was washed twice with distilled water. The ethyl acetate fraction was dehydrated with anhydrous sodium sulfate and evaporated under a gentle stream of nitrogen. Finally, about 31 mg of active ester of 20\ß-S-3-CMO was obtained.

Enzyme-steroid Conjugate

The active ester of 20\ß-S-3-CMO was conjugated to horseradish peroxidase (HRP) using a 1:1 molar ratio of steroid to enzyme. About 26 \( \mu \)g of the active ester dissolved in DMF was added to a small glass tube and the solvent was evaporated. Then 2 mg HRP dissolved in 400 \( \mu \)l of distilled water was added while shaking the tube vigorously with an electric mixer. The shaking was continued for 30 min at room temperature to complete the reaction. After purification by chromatography on a Sephadex G25, the conjugate fraction (about 800 \( \mu \)l) was diluted to 10 ml in assay buffer (x25), and stored in 50 \( \mu \)l aliquots at -80°C.

Stock Solutions

The following solutions gave optimum results in the present assay system: coating buffer, 0.05M carbonate buffer pH 8.4, containing 0.05% \( \text{NaNO}_2 \); washing solution, 0.85% \( \text{NaCl} \); blocking solution, 0.05M PBS containing 0.1% BSA, 3% sucrose, and 0.005% thimerosal; assay buffer, 0.05M borate buffer, pH 7.8, containing 0.1% BSA and 0.01% thimerosal; substrate solution, 0.2M citrate buffer, pH 4.5, containing 0.01% \( \text{H}_2\text{O}_2 \), added with 0.5% o-phenylenediamine immediately before use; stopping solution, 6N \( \text{H}_2\text{SO}_4 \). All the solutions except for the stopping solution were stored at 4°C.

Preparation of Second Antibody-coated Microtiter Plate

Flat-bottomed microtiter plates (MS-3596 F/H plate, Sumitomo Bakelite Co., Tokyo) were coated with 100 \( \mu \)l per well of goat anti-rabbit IgG (15 \( \mu \)g/ml in coating buffer). The plate was tightly covered with a plate seal and incubated at 4°C for 48 h. After removal of unbound antiserum by aspiration, the wells of the plate were washed three times with washing solution, inverted and dried on paper towels. Blocking solution was then added to each well (200 \( \mu \)l) and the plate was tightly sealed and incubated at 4°C for 24 h. Finally, the wells were emptied by inversion and dried on paper towels. The drying was completed by leaving the plates in a refrigerator for 24 h. The second antibody-coated plates could be stored in a refrigerator for at least six months.

ELISA Procedure

The wells of a second antibody-coated plate were loaded with 50 \( \mu \)l of standard or sample, 50 \( \mu \)l of diluted steroid-enzyme conjugate solution and anti-20\ß-S solution (all dissolved in assay buffer) in this sequence. Samples were applied in duplicate, and standards were applied in triplicate to each plate. The plate was incubated at 20°C for 2 h covered with a plate seal, then drained and washed three times with washing solution. Then 150 \( \mu \)l of substrate solution was added to each well, and the plate was sealed and incubated at 20°C for 40 min. Color development was stopped by adding 50 \( \mu \)l stopping solution to each well. The absorbance of each well was then measured at 492 nm with a microtiter plate analyzer (model 2550, Biorad Laboratories, Richmond, CA).

Samples

Two mature female walking catfish (B.W. 464 and 360 g) were injected with HCG (0.8 IU/g B.W.). Fourteen hours after the injection blood samples were taken from the caudal vasculature with a heparinized syringe. After centrifugation at 3,000 rpm for 20 min, steroids were extracted twice using ten times volume of diethyl ether from the plasma. The ether was evaporated with nitrogen, and the sample was reconstituted with assay buffer or 70% methanol.

Separation of 20\ß-S by HPLC

In order to check for possible interference by unknown substances in the plasma to the assay, 20\ß-S in the sample was measured before and after high performance liquid chromatography (HPLC). For HPLC, a 10 \( \mu \)l of plasma extract dissolved in 70% methanol was applied to a JASCO 880-870 system with a reverse-phase column (Japan Spectroscopic Co., Finepak SIL C18S; 4.6 mm x 150 mm). The eluant system was 70% methanol in water at a flow rate of 1.0 ml/min. The elution pattern of six steroids containing 20\ß-S is shown in Fig. 2. A 20\ß-S fraction was placed in a small tube and dried under nitrogen.
Then the residue was dissolved in an appropriate volume of assay buffer for an ordinary assay.

**Results**

A standard curve for 20β-S obtained from the present ELISA system (antiserum dilution, 1:100,000; labeled hormone dilution, 1:2,500) and competition curves for serial dilution of plasma extract from HCG treated female walking catfish are shown in Fig. 1. A steep standard curve covering 1.5-192 pg/well (30-3840 pg/ml) was obtained. The intra- and interassay coefficient of validation were 6.0% (N=4, duplicate) and 8.7% (N=4, duplicate) respectively, near 50% binding. Competition curves for plasma extract from catfish were almost parallel to the standard curve (Fig. 1). The sensitivity of this assay was 1.63 pg/well (32.5 pg/ml).

The antiserum S230 cross reacted to several C21 steroids including 17α, 20β-dioH-P, another teleost maturation inducing steroid, in less than 1% (Table 1). Around 1% of cross reaction was observed to testosterone, progesterone, and cortisol. Cross reaction to estradiol was negligible. The 50% displace dose was 0.28, 25, 28, 31, and 35 ng/ml for 20β-S, testosterone, progesterone, cortisol, 17α, 20β-dioH-P, 20β-S, respectively (Table 1). It was more than 100 ng/ml for other steroids.

HPLC separation of six steroids showed that 20β-S was clearly separable from other possible cross reactive steroids, i.e., testosterone, progesterone, cortisol, and 17α, 20β-dioH-P (Fig. 2). About 90.3% (mean of two samples) of 20β-S was collected after HPLC separation.

**Discussion**

The non-isotopic immunoassay for a teleost gonadal steroid reported here has several advantages to RIAs; it does not suffer from problems caused by the use of radioisotopes, and one cycle of the assay including measurement is concluded within 3 h. Incubation time can be prolonged overnight at 4°C, if necessary, without any significant change of results (results not shown). The sensitivity and precision of this ELISA were almost the same as those of ordinary steroid RIAs. These results show that the present ELISA system is a practical method for monitoring 20β-S levels especially in the laboratories where radioisotopes are unavailable.

More sensitive microtiter plate enzyme immunoassays for steroids have been developed using first antibody-coated plates. This method is, however, not so convenient when measuring several kinds of steroids because each assay requires specific plates coated with highly purified first antibody. On the other hand, plates coated with second antibody can be commonly used for various steroid ELISAs, and first antibody does not necessarily required to be purified. This second antibody method is suitable for teleost gonadal steroids; sometimes several kinds of steroids must be measured to monitor various maturation stages in teleost species. We have already developed ELISAs for testosterone, 11-ketotestosterone, 11β-hydrox-
yandrostenedione, estradiol-17β, 17α-hydroxyprogesterone, 17α, 20β-diol-P, 17α, 20α-diol-P, deoxycortisol, and cortisol based on the second antibody method. The antiserum S230 cross reacted relatively little to steroids other than 20β-S, and 20β-S levels measured by the ELISA did not differ significantly before and after HPLC separation. These results indicate that the present ELISA system is very specific to 20β-S, and that plasma 20β-S levels can be measured after simple extraction. In certain cases, such as plasma from a stressed fish, however, plasma cortisol levels should be checked; the antiserum cross reacted to cortisol by 0.9% and the levels sometimes increase over 100 ng/ml.16,17)

20β-S has been reported to be a maturation inducing steroid in Atlantic croaker9) and spotted seatrout.8) The present ELISA system previously revealed that plasma 20β-S increased during induced ovulation in walking catfish,11) and this is also the case in goldfish and in killifish (K. Asahina, unpublished data). This ELISA is convenient for practical use as well as for experimental use, and could be used to provide much information about the reproductive physiology of fish by applying it to a variety of species.

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References