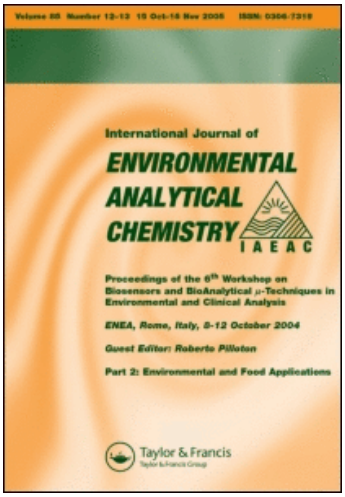

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Development of a Sensitive Luminometric Immunoassay for Determining Baseline Seasonal Changes in Serum Vitellogenin Levels in Male Flounder (*Pleuronectes Yokohamae*)

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DEVELOPMENT OF A SENSITIVE LUMINOMETRIC IMMUNOASSAY FOR DETERMINING BASELINE SEASONAL CHANGES IN SERUM VITELLOGENIN LEVELS IN MALE FLOUNDER (*PLEURONECTES YOKOHAMAE*)

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Vitellogenin is a sensitive biomarker used to study the effects of artificial estrogens in aquatic environments. We developed and optimized a luminometric immunoassay that was able to detect trace amounts of vitellogenin in the serum of male flounder collected from an uncontaminated reference site. The lowest measurable concentration of vitellogenin in the serum was approximately 0.08 ng/ml with purified protein diluted in buffer. The reproducibility of the vitellogenin measurements was determined by the analysis of triplicate samples and found to be about 5.3%, based on serum samples containing vitellogenin at 2.0 ng/ml. This method was successfully applied to samples collected from an uncontaminated reference site for the monitoring of baseline levels of serum vitellogenin in male flounder.

Keywords: Immunoassay; luminometry; vitellogenin; flounder; baseline level; environmental estrogen

INTRODUCTION

The disruption of hormonal and reproductive systems in wildlife by chemicals has become a major topic of scientific research[1]. There are many endocrine-dis-

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rupting chemicals which act as environmental estrogens[2–3]. Low levels of xenoestrogens (for example, ethynylestradiol, nonylphenols...) in the aquatic environment can disturb reproduction and developmental processes in fish and other animals. Vitellogenin (VTG), a precursor protein for egg yolk, has been used as a sensitive biomarker in studies of synthetic estrogenic compounds in aquatic environments[4–5]. The main female reproductive hormone, estradiol-17ß (E_2), induces VTG synthesis in the livers of oviparous female fish [6]. VTG is naturally present at very low concentrations in the sera of juvenile or male fish. However, male fish can also synthesize VTG in their livers when stimulated by estrogen [7]. The effects of environmental estrogens on fish species can therefore be assessed by examining the levels of VTG in the sera of male fish.

Elevated serum VTG concentrations have been reported in wild male carp, *Cyprinus carpio*[8–9], and caged male rainbow trout, *Oncorhynchus mykiss*, exposed to sewage effluent[5]. Until recently, research on the effects of environmental estrogens has been almost entirely limited to freshwater species. Although concentrations of contaminants may be lower in coastal areas than in rivers because of a diluting effect, it is obviously important to monitor the impact of environmental estrogens on marine wildlife. In marine environments, elevated serum VTG concentrations have been reported in male flounder, *Platichthys flesus*, captured in estuaries that receive effluent from sewage treatment plants[10–12]. In our previous paper, we observed seasonal changes in serum VTG levels in wild male flounder, *Pleuronectes yokohamae*, collected from Tokyo Bay, which receives a large amount of industrial effluent and domestic sewage effluent. We compared these results with those from male flounder collected from a reference site off the coast of Hokkaido[13–14]. Throughout the year we found elevated serum VTG levels in the male flounder collected from Tokyo Bay, but there were no significant increases in VTG levels in the fish from Hokkaido over the same time period[14].

There have been several reports on the presence of the natural low levels of vitellogenin in male fish[15–18]. The presence of VTG in the blood of male fish may be a consequence of the presence of physiologically active levels of estrogen. Estrogen has been found in the blood of wild male brown bullheads, *Ictalurus nebulosus*, at levels as high as 800 pg/ml, or 10%-30% of the levels normally seen in mature females[19]. To determine the role of environmental estrogens in such a result, it is necessary to examine the serum levels of VTG in males of the species captured at an uncontaminated reference site; for the same reason it is also important to have seasonal information on baseline changes in VTG levels at a reference site. In our earlier study, we measured VTG levels in the sera of *Pleuronectes yokohamae* by a sandwich enzyme-linked immunosorbent assay (ELISA) with fluorometric determination[13]. However, this method proved inef-

ficient for measuring trace levels of VTG in field samples from the uncontaminated reference site. The serum VTG values in male flounder at the reference site (about 50 ng/ml) were consistently low (close to the detection limit); we considered that this indicated that the fluorometric enzyme immunoassay was not sensitive enough to detect seasonal changes in VTG levels in these fish[14].

The objective of this study was to develop and optimize the sensitivity of a luminometric immunoassay for determining baseline serum vitellogenin levels in male flounder (*Pleuronectes yokohamae*). We were able to apply this luminometric method successfully to the monitoring of seasonal changes in baseline VTG levels in samples obtained from a reference site, located off Shiriuchi in Hokkaido, where sewage and wastewater inputs to the marine environment were considered negligible.

MATERIALS AND METHODS

Collection and storage of samples

Between September 1997 and September 1998, *P. yokohamae* were caught at the reference site, which was located off Shiriuchi, in the Strait of Tsugaru, Hokkaido. Blood was withdrawn from the caudal vein with a syringe and transferred to a sterilized tube containing aprotinin solution prepared from bovine lung (Takara Co. Ltd, Kyoto, Japan). The final concentration of aprotinin in each blood sample was 0.84 sigma trypsin inhibitor units. Each blood sample was kept at 4°C overnight, then centrifuged at 2000 rpm for 10 min to separate out the serum. The serum was stored at –40°C until analysis.

Preparation of purified VTG and antiserum

Flounder VTG for use as a standard was isolated according to the methods described for the purification of VTG in barfin flounder (*Verasper moseri*)[20]. A Superose 6 column was used to separate out the main peak, which had a molecular weight of about 480 kDa; this peak was collected as purified VTG.

In salmonid fishes, VTG is cleaved into 3 egg-yolk-protein components: lipovitellin (Lv), phosvitin and ß′-component[6]. A antisera against VTG, when used for immunological measurement of serum VTG, react with both VTG and the free-β′-component in serum, while a-Lv reacts only with VTG[21]. Accordingly, a-Lv should be used for accurate immunological measurement of serum VTG concentrations. Flounder Lv was purified according to the method described by

Matsubara and Sawano[20]. The precipitate of egg extracts by ammonium sulfate at 50% saturation was passed through a hydroxyapatite column. The fraction containing Lv was subjected to column chromatography on Superose 6. The main peak, with a molecular weight of about 380 kDa, was collected as purified Lv.

VTG levels in the serum samples were measured with polyvalent antiserum against Lv (a-Lv)[21]. $F(ab')_2$ was prepared from the antiserum (a-Lv) and biotinylated $F(ab')_2$ was carried out as described previously[22]. To remove the antibodies that react with common serum proteins, a-Lv was absorbed on to the male serum at a ratio of 1:3 (v/v). The absorbed a-Lv was characterized by immunoelectrophoresis, as described before[23].

Luminometric enzyme immunoassay of serum VTG

In this assay, 96-well ELISA microtiter plates (FluoroNunc 437591, Nalge Nunc International, Tokyo, Japan) were used for the immunoreaction.

Capture antibody

Each well was coated with 50 µl of the capture antibody, IgG (a-Lv), which was dissolved in sodium carbonate buffer (8.4 g $NaHCO_3$ + 10.6 g Na_2CO_3 per litre, pH 9.5), at a concentration of 25 µg/ml, and then incubated overnight (for 16 h) at 4°C.

Blocking of non-specific sites

After incubation, each well was washed 3 times with 200 µl of phosphate buffer saline (PBS) containing 0.05% Tween (0.8% NaCl, 0.02% KCl, 0.29% Na_2HPO_4-$12H_2O$, 0.02% KH_2PO_4). Non-specific binding sites were blocked with 200 µl of PBS containing 1% bovine serum albumin (PBS-BSA) (Bovine Albumin Fraction V, 82–045, ICN Biomedicals Inc., Aurora, OH, USA) for 1 h at room temperature.

Incubation of standards or samples

After each well had been washed again as described above, 50 µl of the VTG standard solution (0.01 to 1000 ng/ml) or the serum sample, serially diluted with PBS-1% BSA, were added and incubated for 2 h at room temperature.

Incubation with $F(ab)'_2$

After washing as described above, each well received 50 µl of the detection antibody, biotinylated $F(ab)'_2$, diluted in PBS at a concentration of 10 µg/ml and incubated for 2 h at room temperature.

Incubation with streptavidin – alkaline phosphatase conjugates

After each well had been washed as described above, streptavidin – 50 µl (0.2 U/ml) alkaline phosphatase conjugates (62–343, ICN Biomedicals Inc., Aurora, OH, USA) were added and incubated for 1 h at room temperature.

Immunoluminescence reaction

Each well was washed 3 times with 200 µl of PBS – 0.05% Tween. Each well then received 50 µl of substrate (LuciGLO chemiluminescent phosphatase substrate kit, Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) and was then incubated for 10 min at 25°C. The luminescence intensity of each well was measured with a microplate luminometer (LumiCount, Packard BioScience Company, Tokyo, Japan).

RESULTS AND DISCUSSION

Specificity of antiserum

On immunoelectrophoresis, the a-Lv reacted only with serum from vitellogenic females, not with serum from males (Figure 1). The a-Lv produced one distinct precipitin arc when it was reacted against purified VTG.

Optimization of ELISA

We examined the effects of incubation time on the ELISA reaction (Figure 2). Different concentrations of purified VTG (0.15, 2.42 and 38.7 ng/ml of serum) were used. The reaction of the capture antibody reached a plateau after 16 h at 4°C. We examined the effects of incubation time on the reaction of the detection antibody between 0.5 and 6 h at room temperature. This step reaction proceeded rapidly for the first 2 h. Also, we examined the effects of the incubation time on the reaction of streptavidin – alkaline phosphatase between 0.25 and 2 h at room temperature. By 1 h at room temperature this reaction had nearly reached a plateau.

We also examined the effects of concentration of antibody and reagent on the ELISA reaction at 3 concentrations of VTG (0.15, 2.42 and 38.7 ng/ml of serum) (Figure 3). The reaction of the capture antibody reached a plateau beyond 25 µg/ml at 4°C. When we examined the step reaction of the detection antibody between 0 and 50 µg/ml at room temperature, we found that it proceeded rapidly between 0 and 10 µg/ml. When we examined the reaction of streptavidin – alka-

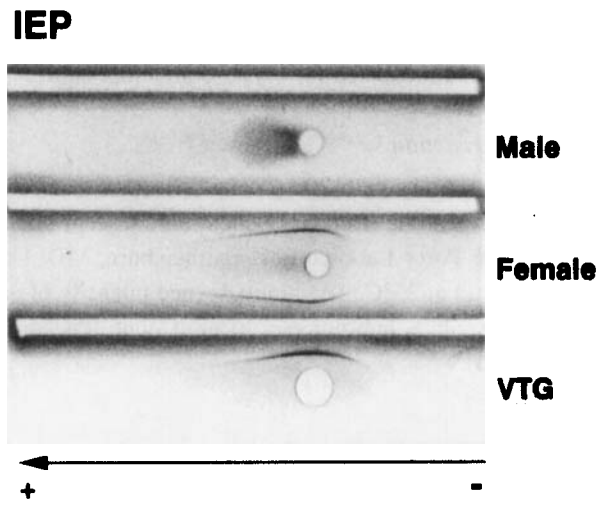

FIGURE 1 Immunoelectrophoresis patterns of serum samples and purified vitellogenin of flounder, using antiserum against purified lipovitellin. Male: serum from male; Female: serum from vitellogenic female; VTG: purified vitellogenin; IEP: immunoelectrophoresis

line phosphatase between 0.01 and 0.5 U/ml at room temperature, we found that it had nearly plateaued by 0.2 U/ml.

Assay of standard VTG

Typical sigmoidal standard curves were obtained for the luminescence intensity of purified VTG (Figure 4). We diluted VTG in buffer and in serum for standard curves and we did not find any difference between standard curves made in buffer and serum. These curves showed high sensitivity at serum vitellogenin levels of 0.02–0.08 ng/ml (Figure 4a) and wide linearity from levels of 0.1–10 ng/ml (Figure 4b). For validation of the ELISA, serum samples from male and female flounder were serially diluted from 20 to 10 000 times. Results of the ELISA validation tests showed a parallel decrease in luminescence intensity with sample dilution. The lowest measurable concentration of serum VTG was approximately 0.08 ng/ml with purified protein diluted in buffer. However, because of interference, serum samples had to be diluted at least 20 times, giving a sensitivity of about 1.6 ng VTG/ml of serum. This was about 10 times more sensitive than the fluorometric method we had used in the previous study[13]. This level of sensitivity was sufficient to detect baseline levels of VTG in the

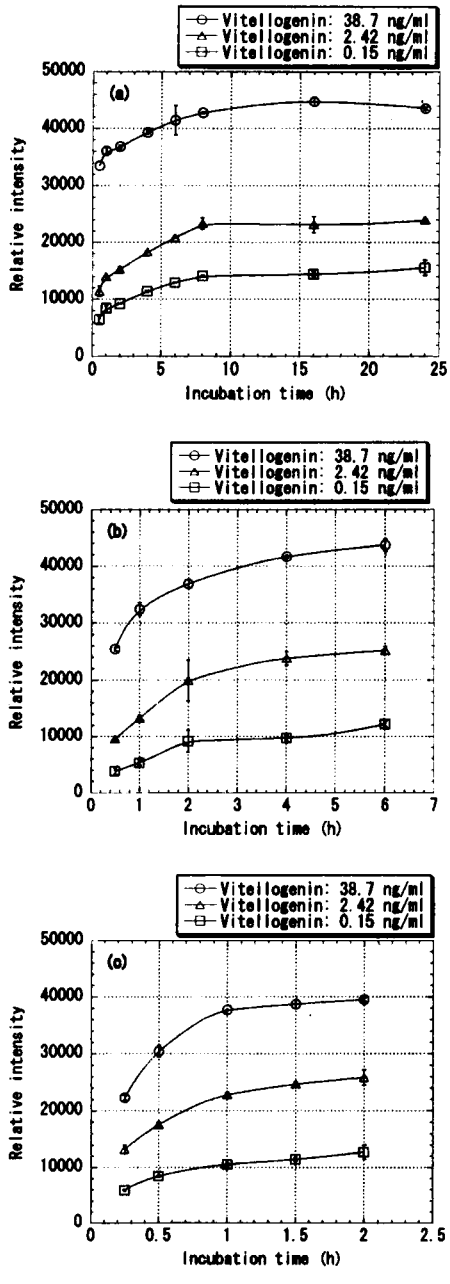

FIGURE 2 Effects of incubation time on the reactions of capture antibody (a), detection antibody (b) and streptavidin – alkaline phosphatase (c). Incubation temperatures: a, 4°C; b, room temperature; c, room temperature

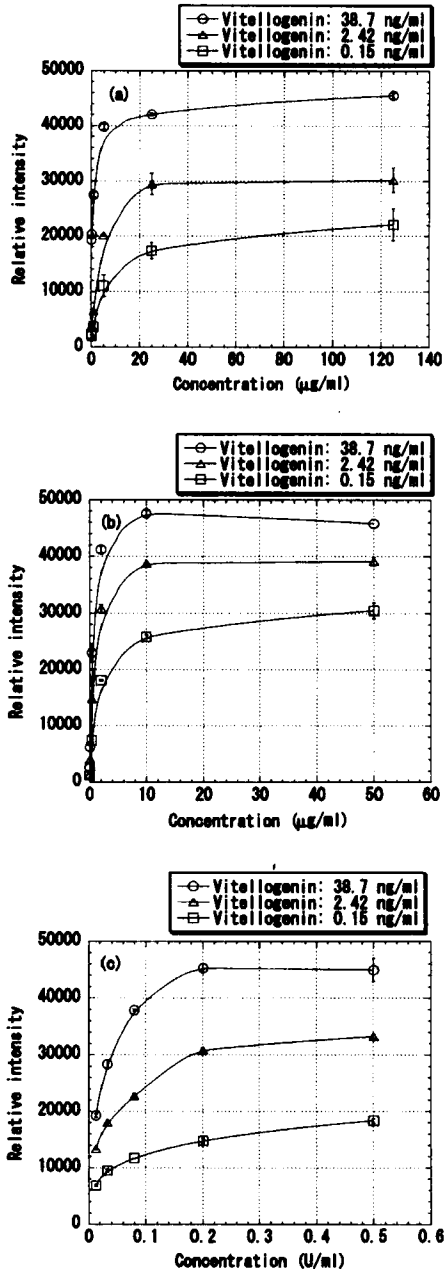

FIGURE 3 Effects of concentration on the reactions of capture antibody (a), detection antibody (b) and streptavidin – alkaline phosphatase (c). Incubation temperatures: a, 4°C; b, room temperature; c, room temperature

sera of male flounder taken from the reference site. We compared the concentrations of serum VTG in wild male flounder, as measured by luminometry and fluorometry (Table I). With luminometry, the intra- and inter-assay coefficient of variations were about 3.3% and 5.3%, respectively, taken on samples from males with 2.0 ng VTG/ml of serum, as determined by the analysis of triplicate samples. The analysis of serum VTG in 90 samples using the protocol described above required about 1.5 days.

TABLE I Serum vitellogenin levels (ng/ml) in wild male flounder at uncontaminated reference sites, as measured by luminometry and fluorometry[a]

Vitellogenin measured by Luminometry	*Fluorometry*
4.48 ± 0.21	< 20
3.09 ± 0.55	< 20
4.10 ± 0.52	< 20
3.73 ± 0.38	< 20
1.77 ± 0.22	< 20
7.34 ± 1.12	< 20
8.70 ± 0.96	< 20
61.6 ± 6.4	68 ± 7.5
33 ± 2.8	31 ± 1.8

[a] The details of this method were described in our previous report[13].

Seasonal variation in serum VTG levels

We applied the luminometric method to gather seasonal information on changes in baseline levels of VTG in male flounder collected from the reference site (Figure 5). The timings of sexual maturation and spawning of flounder off Shiriuchi in Hokkaido have been estimated from seasonal changes in the gonadosomatic index (GSI)[14]. The spawning season of flounder off Shiriuchi in Hokkaido occurs from the beginning of February to the end of March. E_2 and testosterone concentrations (n = 16) in the sera of male *P. yokohamae* collected from Hokkaido in winter have been measured at 10.6–258 pg/ml (mean 77.0) and <250–4198 pg/ml (mean 1047), respectively[14]. E_2 and testosterone concentrations (n = 19) in the sera of female *P. yokohamae* collected from Hokkaido in winter have been measured at <1.4–2660 pg/ml (mean 1110) and <250–2740 pg/ml (mean 627), respectively[14]. Both E_2 and testosterone in the sera of male and female fish were high during winter, when VTG levels in female fish and the

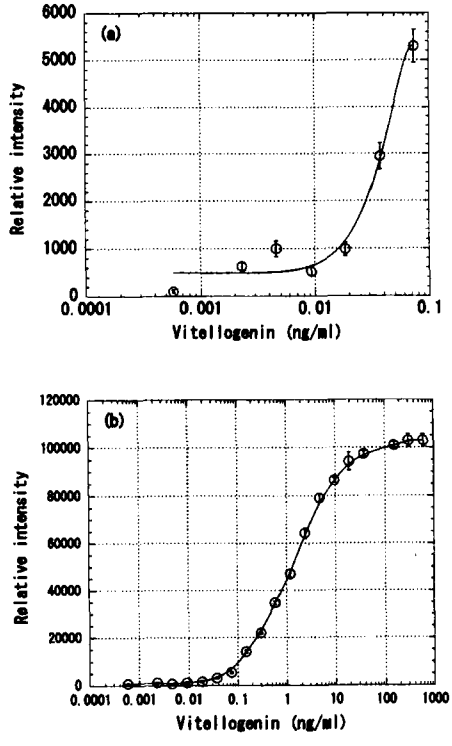

FIGURE 4 Standard curves for the luminescence intensity of purified vitellogenin from flounder. a: high-sensitivity range; b: wide-linearity range

GSI of both sexes were also high. Estrogen has been found in the blood of wild males at levels as high as 10% of those normally seen in mature females in winter. The detectable levels of VTG in the blood of wild male flounder at the reference site in winter may be a consequence of the presence of physiologically active levels of estrogen. Perhaps this species expresses VTG in both sexes in response to "natural" circulating E_2.

CONCLUSION

We have reported the development and optimization of a sensitive luminometric immunoassay for the determination of baseline serum vitellogenin levels in male flounder (*Pleuronectes yokohamae*). The development of a very sensitive method for the determination of very low levels of plasma VTG in male fish

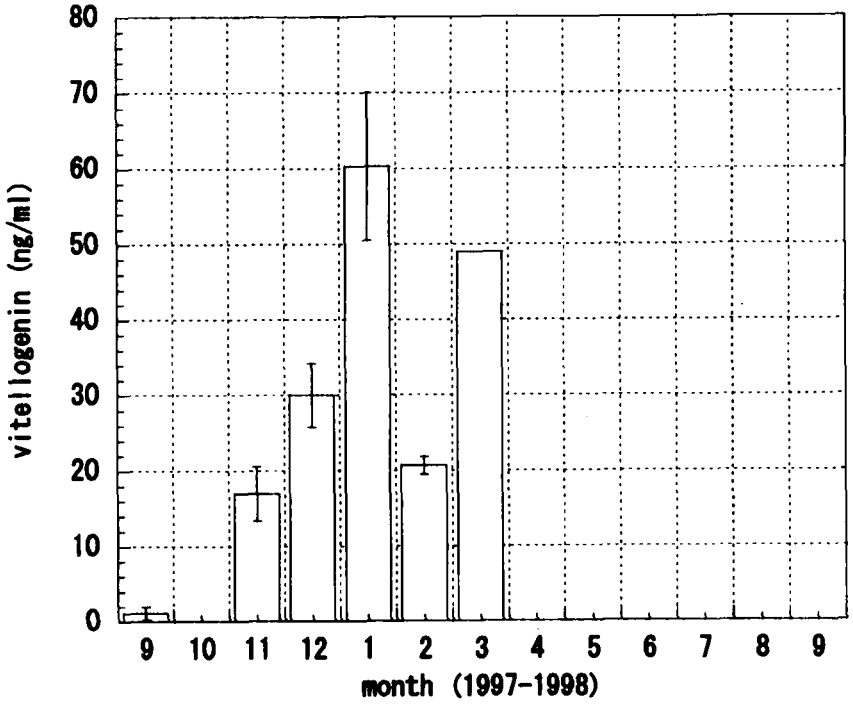

FIGURE 5 Seasonal changes of serum vitellogenin levels in serum samples taken from male flounder caught at the reference site off Shiriuchi, Hokkaido

allows, a) detection of trace amounts of estrogenic compounds and mainly, b) analysis of highly diluted serum samples, which can avoid a critical problem of the ELISA technique, the unspecific interference with serum compounds at high concentrations. This method can be applied successfully to monitor seasonal changes in baseline VTG levels in serum samples obtained from reference sites. Such results will be useful in interpreting the effects of environmental estrogens in ecosystems.

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