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Development of enzyme-linked immunosorbent assays for two forms of vitellogenin in Japanese common goby (*Acanthogobius flavimanus*)

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Abstract

Two vitellogenins (Vgs) were detected in serum from estradiol-17β (E_2)-injected Japanese common goby (*Acanthogobius flavimanus*). Vitellogenins with molecular masses of 530 kDa (Vg-530) and 320 kDa (Vg-320) were purified, and used to raise specific antisera in rabbits. Sandwich enzyme-linked immunosorbent assays (ELISAs) for Vg-530 and Vg-320 were developed using the antisera and the isolated Vgs. The sensitivity ranges of these ELISAs were 1.25–160 ng/ml for Vg-530 and 0.26–66 ng/ml for Vg-320, and very low cross-reactivity was found with the alternate Vg in each assay. Treatment of male gobys with E_2 by injection and immersion induced both Vgs in sera in a dose-dependent manner. The mean concentrations of the Vgs increased from 10 ng/L E_2 exposure for three weeks. Serum concentrations of the two Vgs in field-collected maturing females increased in accordance with increment of E_2 level and ovarian development, and the mean concentrations of Vg-530 were higher than those of Vg-320 in maturing female. These results indicate that the sandwich ELISAs for Vg-530 and Vg-320 developed in the present study is useful as an assay system for surveys of estrogenic activity in coastal areas of Japan.

1. Introduction

It is established that estrogenic substances, termed "environmental estrogens," are widely present in aquatic environment. These include both natural substances (E_2, estrone, phytoestrogens, etc.), as well as artificial substances (17α-ethynylestradiol, alkylphenolic chemicals, polychlorinated biphenyls, etc.). These compounds can be detected at significant concentrations in the aquatic environment, such that they have the potential to perturb reproductive and developmental processes in fish and other vertebrates (review see Sumpter, 1995). In rivers in the United Kingdom (UK), widespread occurrence of abnormal intersexuality in roach (*Rutilus rutilus*) (Jobling et al., 1998) and guggen (*Gobio gobio*) (Van Aerle et al., 2001) have been reported. Furthermore, a relationship has been established between those sexual perturbations and exposure to environmental estrogens in sewage treatment plant effluents (Jobling et al., 1998). Such sexual perturbation in freshwater fish has also been observed in the USA (Goodbred et al., 1997). Recently, feminization in male flounder (*Platichthys flesus*) in some estuarine and marine waters in the UK has been observed and is manifested by abnormally high vitellogenin (Vg) concentrations in male serum and the existence of ovotestis (Matthiessen et al., 1998; Allen et al., 1999). Influences of environmental estrogens have also been suggested in male flounder (*Pleuronectes yokohamae*) in Tokyo Bay, Japan (Hashimoto et al., 2000).

Based on the presence of estrogenic compounds and the probability of perturbation of reproduction, rapid

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survey methodology in the coastal environment of Japan was deed to be necessary in order to elucidate estrogenic impacts in coastal waters. The occurrence of hormonally active substances in the aquatic environment has been an issue of concern for Japanese government regulators, scientists, and the public, and the development of sensitive and rapid assays for detecting hormonally active substances is now an area of high research priority.

It is well established that serum Vg of male fish can be used as a sensitive biomarker of endocrine disruption via environmental estrogens (e.g., Harries et al., 1996; Purdom et al., 1994). The Vg is egg yolk precursor glycophospholipoprotein of high molecular weight ($\geqslant$ 205 kDa) (see review Specker and Sullivan, 1994). In female fish, Vg is synthesized in the liver where its synthesis is under estrogenic control. It is then secreted into the bloodstream, where it is transported to the ovary and incorporated into the growing oocytes by receptor-mediated endocytosis; it is then processed into smaller yolk proteins (for reviews see Mommsen and Walsh, 1988; Ng and Idler, 1983; Selman and Wallace, 1989; Wallace, 1985). Those yolk proteins are classified as lipovitellin (Lv), phosvitin (Ng and Idler, 1983), and β′-component (Campbell and Idler, 1980; Hara and Hirai, 1978; Markert and Vanstone, 1971; Matsubara and Sawano, 1995). Recent research has verified the presence of two vitellogenins (Vgs) in at least some fish species (review see Hiramatsu et al., 2003). The Vg concentration in female serum commonly serves as a useful indicator of sexual maturation because its rise correlates with oocyte growth, however males and immature fish also have the potential to synthesize Vg if exposed to exogenous estrogen. Therefore, the Vg in male serum has been proposed as an excellent biomaker for environmental estrogens as it is absent in males not exposed to environmental estrogens (Sumpter and Jobling, 1995). For measuring Vg concentration in fish, various methods such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and single radial immunodiffusion have been developed (Specker and Sullivan, 1994). We have selected the sandwich ELISA for the measurement of Vg in the Japanese common goby (*Acanthogobius flavimanus*) because of its high sensitivity and needless for radioisotopic reagents, making it superior for routine screening of serum samples.

For the survey of environmental estrogens in coastal areas of Japan, we used the Japanese common goby since they are distributed throughout Japan, are easy to catch, and can be held in the laboratory. Moreover, we found that Japanese common goby has two biochemically and immunologically distinct Vgs as has been shown in other species, which provides an advantage for surveys of environmental estrogens by assessing the levels of both Vg to ensure the induction of Vg.

2. Materials and methods

2.1. Samples

Japanese common goby *Acanthogobius flavimanus* (total length 118–232 mm) were caught by rod-and-line in Kami-iso Bay, Hokkaido in September 1997, 1998, and 1999. For purification of Vgs, a total of 20 fish in the sexually immature phase were anesthetized with methyl *p*-aminobenzoate, weighed, and injected intramuscularly with estradiol-17β (E_2). The dose of E_2 was 1 mg dissolved in 1 ml propylene glycol per 1 kg body weight. Fish were then kept for 6 days in a 1-kl aquarium with running seawater. Blood samples were taken from the dorsal vessel with a syringe. For inhibition of serine proteases, 10 mM phenylmethylsulfonyl fluoride (PMSF) and 10% aprotinin solution (from bovine lung; Sigma, St. Louis, MO, USA) in 0.9% NaCl were previously contained to the syringe at a ratio of 10% v/v of blood. The blood was transferred to a sterilized tube and allowed to clot at 4 °C followed by centrifugation at 5000*g* to separate the serum. The sera were collected and pooled, and a part of the pooled serum was mixed immediately with equal volume of sodium dodecyl sulfate (SDS) sample solution (33.3% glycerol, 5% SDS, and 16.6% 2-mercaptoethanol). These samples were stored at −80 °C until used.

2.2. Estradiol-17β experimental exposures

2.2.1. Treatment by injection

A total of 115 fish were divided in seven groups, and kept in 1-kl aquariums with running seawater at a temperature of 17 °C. Each group of fish were anesthetized with methyl *p*-aminobenzoate, weighed, and injected intramuscularly with 0, 0.1, 1, 10, 25, 100, or 1000 μg/kg E_2 dissolved in propylene glycol by 100 μl precision syringe (Gastight syringe, Hamilton, Reno, NV, USA), on day 0, 3, and 6. Nine days after first injection, blood samples were collected and treated as described above.

2.2.2. Treatment by immersion

A total of 68 fish were divided in five groups and kept in 80 L aquaria with running seawater at a temperature of 17 °C. After acclimation for 3 days, fish were exposed to E_2 at nominal concentrations of 0, 1, 10, 100, 1000 ng/L, respectively. E_2 was first dissolved in ethanol and then added to a glass mixing vessel by peristaltic pump, at a rate of 1.5 ml/h, where they were mixed with seawater flowing into the aquarium at a rate of 30 L/h. The actual concentrations of E_2 in the aquaria of 100 and 1000 ng/L were monitored throughout the experiment by time resolved fluorescent immunoassay as described bellow. Before the exposure, blood samples of four male fish were taken as an initial Vg control. After three

weeks of exposure, blood samples were collected as described above.

2.3. Maturing field-collected females

Adult females of the Japanese common goby were captured in Kasiwazaki, Niigata in October, November, December 1999, October, December 2000, and January 2001. The spawning season of the Japanese common goby starts from February or March in the middle part of Honshu (Dotu and Mito, 1955). The fish were anesthetized, measured for body length, weight, and gonad weight, and then serum samples collected as described above. Gonadosomatic index (GSI) was calculated as gonad weight × 100/body weight. Ovaries were fixed with Bouin's solution at room temperature.

Ovaries containing vitellogenic oocytes were received from Hayama Marine Science Laboratory, Kajima Technical Research Institute. The mature females were captured in Sagami Bay. A piece of ovary (about 0.1 g) was dissected by scissors in sterilized cold 0.9% NaCl, then homogenized in the same saline at a concentration of 5% w/w, followed by centrifugation at 15,000*g* for 10 min. The supernatant was used as the oocyte homogenate.

2.4. Chromatography

Gel chromatography was performed using a prepacked column of Superose 6 HR 10/30 (Amersham Pharmacia Biotech, Buckinghamshire, England) as described by Matsubara and Sawano (1995).

Hydroxylapatite column chromatography (15× 40 mm, Bio-Rad) was performed as follows. The column was equilibrated with 100 mM potassium phosphate (PP), pH7.3, containing PMSF. After 500 μl of E_2-injected fish serum was loaded, the flow-through protein was eluted with the same buffer. The bound protein was dissociated with 500 mM PP, pH 7.3. The flow rate was 1.0 ml per minute and 1 ml of fractions were collected.

Anion exchange chromatography on Mono-Q HR 5/5 column (5 × 50 mm, Amersham Pharmacia Biotech) was performed as follows. The column was equilibrated with 20 mM Tris–HCl, pH 8.0, containing 150 mM NaCl, and proteins were eluted with a linear NaCl gradient (150–500 mM) at a flow rate of 1.0 ml per minute. Fractions containing the desired protein peaks were collected, and concentrated with a concentrator (Ms. BTAURY-KN, Atto, Tokyo, Japan). Protein concentration of the purified proteins were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

2.5. Electrophoresis and Western blotting

SDS–polyacrylamide gel electrophoresis (PAGE) was performed on precast acrylamide gels with a gradient of total acrylamide concentrations from 5–20% (Atto). The gels were stained with Coomassie brilliant blue R-250 (CBB). Estimation of molecular masses of protein bands by SDS–PAGE was performed using molecular weight markers (SigmaMarker Wide Range: Sigma).

Semidry transblotting after SDS–PAGE was performed using a polyvinylidene difluoride (PVDF) membrane (Immobilon-P: Millipore, Bedford, MA, USA). Western blotting was carried out using specific antibodies as the primary antibodies at a 1:10,000 dilution and goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) at a 1:20,000 dilution as the secondary antibody. Immunoreactive proteins were detected by applying nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate in 100 mM Tris–HCl, pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$.

2.6. Preparation of antibodies

Specific antisera to Vg-530 (a-Vg-530) and Vg-320 (a-Vg-320) were raised in rabbits. The antisera were previously absorbed with immature Japanese common goby serum at ratios of 6:1 in volumes to improve the specificity. The mixture of the antiserum and the immature fish serum were incubated for overnight at 4 °C, following centrifugation at 15,000*g* for 10 min, then the supernatant was collected. Purification of IgG of the absorbed a-Vg-530 and a-Vg-320, and their $F(ab')_2$ were carried out by the method described in Ishikawa et al. (1983) with minor modification. The IgG was purified with ammonium sulfate precipitation and DE52 (Watman International, Kent, UK) ion-exchange chromatography. The purified each IgG was digested with pepsin (P-6887, Sigma), then the $F(ab')_2$ was collected with gel chromatography using Superose 12 HR 10/30 (Amersham Pharmacia Biotech).

2.7. Enzyme-linked immunosorbent assay

The 96-well polystyrene microtiter plate (Corning, USA) was coated with 100 μl/well of a-Vg-530 IgG or a-Vg-320 IgG diluted in sodium bicarbonate buffer, pH 9.6, containing 0.02% NaN_3 then incubated overnight at 4 °C. The non-specific binding sites were saturated by incubating the plates with 250 μl of 1% BSA and 5% skim milk in the same buffer for 1 h at room temperature. After two successive washes with 10 mM PBS, pH 7.3, containing 0.05% Tween 20 (PBST), 100 μl of serially diluted Vg standard solution (1.25–160 ng/ml of purified Vg-530 or 0.26–66 ng/ml of purified Vg-320 serially diluted with PBS containing 0.5% BSA) or individual serum samples (diluted 1:100 to 1:1,000,000 in PBS containing 0.5% BSA) were introduced to the wells and incubated for 1.5 h at room temperature.

Each well was then washed five times with PBST, and received 100 μl of biotin labeled antibody (a-Vg-530

$F(ab')_2$ or a-Vg-320 $F(ab')_2$ diluted 1:3000 in PBS–0.5% BSA. The plates were maintained for 1.5 h at room temperature. After the plate was washed as above, each well was received avidine–HRP (Dako A/S, Denmark) diluted 1:5000 with PBST–1% BSA. The plates were maintained for 1 h at room temperature, then washed five times with PBST. For coloration, each well received 150 μl of enzyme substrate solution prepared as follows: 13.2 mg of 3,3′,5,5′-tetramethylbenzidine (Dojin Chemical, Kumamoto, Japan) dissolved in 1 ml of *N*,*N*-dimethylformamide added to 100 ml of 0.1 M acetate buffer, pH 5.5. Color development was started by adding 50 μl of 0.033% of hydrogen peroxide, then incubating for 20 min at room temperature. The reaction was stopped by adding 50 μl of 2 N sulfuric acid. The colorimetric data of absorbance at 450 nm is measured using MTP-100 microplate reader (Corona, Ibaraki, Japan).

2.8. Measurement of E_2 concentration of seawater, male fish, and field-collected female

The E_2 concentrations of seawater, male fish sera of E_2 exposure experiment, and field-collected female sera were measured using time resolved fluorescent immunoassay (DELFIA Estradiol Kit, Wallac Oy, Finland) using the time-resolved fluorometer (DELFIA 1234 Fluorometer, Wallac Oy, Finland). The seawater in the aquarium of 100 and 1000 ng/L E_2 exposure were collected every two days, removed sediments by centrifugation at 10,000*g* for 10 min, diluted three times by distilled water for measurement. The sera of the male fish that were exposed to E_2, and the field-collected females were measured as follows. The serum of 5 μl of each individual of the same treatment group or that collected in the same month was mixed together, and measurement were repeated three times.

2.9. Light microscopy observations

To check the developmental stage of ovary, we chose eight fish that showed the average level of GSI in each month. The ovaries, which were fixed in the Bouin's solution for one day, were subsequently dehydrated in ethanol, then embedded in paraffin. The samples were sectioned at 5-μm thickness, stained with hematoxylin-eosin, and examined by light microscopy. The developmental stages of oocytes were classified as described by Sato et al. (1987).

2.10. Statistical analysis

We used the Jonckheere–Terpstra test (Hollander and Wolfe, 1973) to test the relationship between the E_2 dose and Vg concentration. The null hypothesis is that the Vg concentrations in the samples with different doses have come from the same underlying population distribution. The one-sided alternative hypothesis is that the distribution of Vg concentration increases with a higher dose of E_2. After the Jonckheere–Terpstra test, we conducted post-hoc tests to examine which treatments are different form other treatments. (one-tailed Mann–Whitney's *U*-tests with Bonferroni adjustment; Sokal and Rohlf, 1997). For the Bonferroni method, we used an adjusted significant level of $\alpha' = \alpha/k$ where $\alpha = 0.05$ and k is the number of post-hoc tests. We conducted the post-hoc tests between the control and each treatment.

3. Results

3.1. Identification of the Japanese common goby Vgs

Vg protein is thought to be easily degraded by proteolysis during purification (Buerano et al., 1995; Ng and Idler, 1983; Norberg and Haux, 1985; Selman and Wallace, 1983). In order to know the intact molecular masses of Vg and major yolk protein by SDS–PAGE, E_2 treated fish serum (E_2S) and vitellogenic oocyte homogenate were immediately mixed with SDS sample solution after separation of serum and homogenizing oocyte, respectively. Fig. 1 shows the SDS–PAGE patterns of the male fish serum, E_2S, and the oocyte homogenate. When male fish serum and E_2S was compared, the major protein bands of 178 and 127 kDa were newly induced by E_2 injection (lane 2). In the oocyte homogenate, the 157, 127, and 122 kDa proteins were observed as main protein bands (lane 3). From

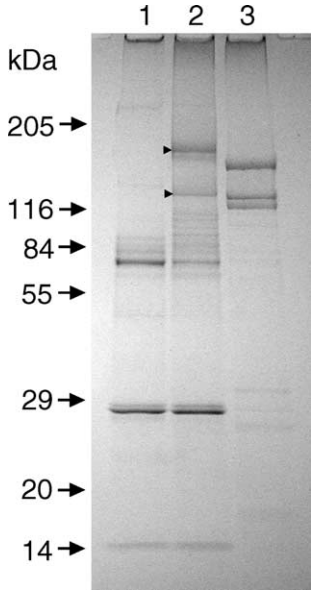

Fig. 1. SDS–PAGE patterns of male fish (lane 1), E_2 treated fish serum (lane 2), and the oocyte homogenate (lane 3). Numbers at the left are MW standards. The arrowheads at the left of lane 2 indicate the major protein bands of 178 and 127 kDa which newly induced by E_2 injection.

these results, at least two or three major bands were verified in E_2S and oocyte homogenate, respectively, even though proteolytic degradation was inhibited.

To separate proteins which contained these major protein bands, the combination of gel filtration chromatography and SDS–PAGE were performed. Fig. 2 shows the elution profiles of E_2S after Superose 6 gel chromatography (A) and the SDS–PAGE of each fraction (B). Comparing the chromatograph of male fish serum (data not shown) to that of E_2S, injection of E_2 induced major UV-absorbing peaks that corresponded to molecular masses of 530 kDa and a shoulder at approx. 320 kDa (Fig. 2A). The SDS–PAGE analysis shows that the peak of 530 kDa migrated as a major 110 kDa protein band, three smaller bands of approx. molecular masses of 95, 80, and 70 kDa, and a faint band of 178 kDa (Fig. 2B, fraction 9). On the other hand, the 320 kDa fraction migrated as 127 kDa protein band (fractions 12 and 13). The molecular masses of protein bands were correctly estimated by diluted peak fraction (data not shown). The absence of the 110 kDa protein band in the SDS–PAGE pattern of E_2S (Fig. 1, lane 2) suggesting that the 110 kDa protein band was derived from 178 kDa protein band by proteolytic cleavage during the gel filtration chromatography.

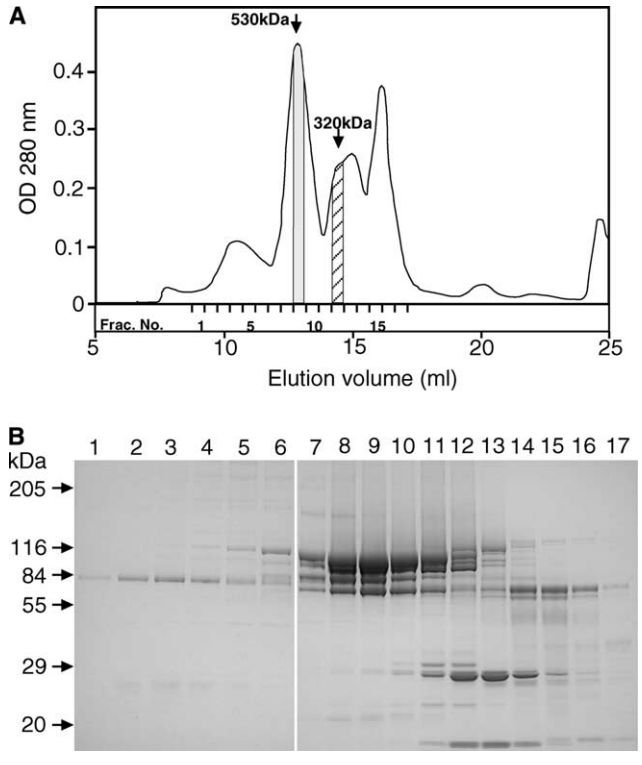

Fig. 2. Elution profile of E_2 treated fish serum after Superose 6 gel chromatography (A), E_2 treated fish serum after Superose 6 gel chromatography and 5–20% gradient SDS–PAGE of the chromatographic fractions (B). Numbers on the electrophoreogram correspond to the fraction number shown in (A). Numbers at the left are MW standards.

Elution profile of the oocyte homogenate after Superose 6 gel chromatography was shown in Fig. 3A. A major UV-absorbing peak corresponding to a molecular mass of 320 kDa and an obvious shoulder with 480 kDa were observed in the chromatogram. When the fractions were further analyzed by SDS–PAGE (Fig. 3B), the 480 kDa shoulder migrated as a major 122 kDa protein band and a faint 157 kDa band (fractions 4 and 5). Meanwhile, the 320 kDa peak was mainly consisted of a 127 kDa protein band (fraction 7). These results show that E_2 injection induced two serum proteins of 530 and 320 kDa, which seemed to be Vgs of Japanese common goby, and they likely to represent the two major yolk proteins of 480 and 320 kDa following incorporated into vitellogenic oocyte.

3.2. Purification of the Japanese common goby Vgs

The E_2 induced 530 and 320 kDa serum proteins, which were temporary designated as Vg-530 and Vg-320, were purified by combinations of chromatographies. We used the oocyte homogenate instead of E_2S for the purification of Vg-320, since the Vg-320 had no

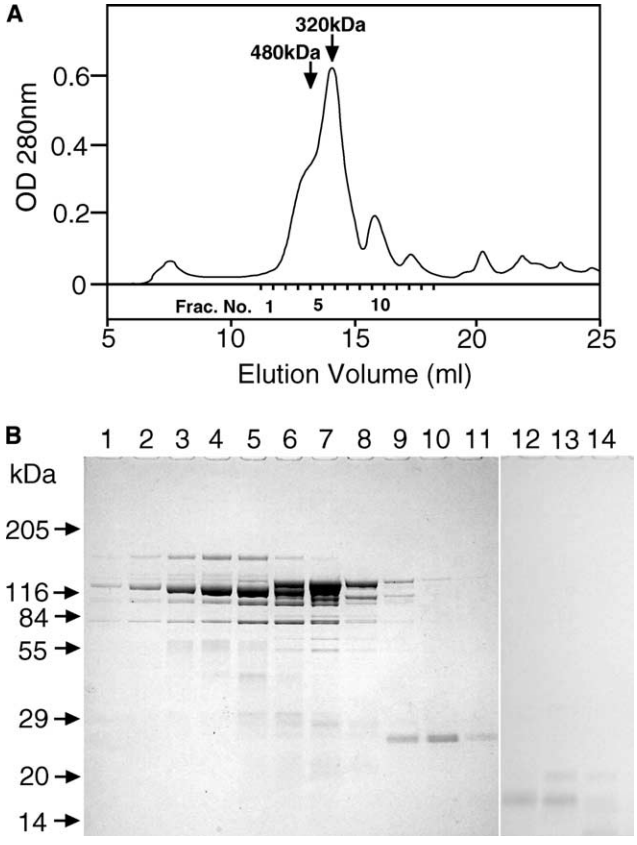

Fig. 3. Elution profiles of the oocyte homogenate after Superose 6 gel chromatography (A), and 5–20% gradient SDS–PAGE of the chromatographic fraction (B). Numbers on the electrophoreogram are the fraction number shown in (A). Numbers at the left of the electrophoreogram are MW standards.

change in both native and polypeptide molecular masses though the accumulation process into oocyte. Furthermore, the proportional ratio of the Vg-320 versus Vg-530 was low in the E_2S when compared. Two step chromatographies using hydroxylapatite column and Superose 6 column were employed for purification of the Vg-530 from the E_2S. A single and symmetrical peak was observed in the last step of Superose 6 column chromatography (data not shown), and the peak fraction was collected as the purified Vg-530.

Four step chromatographies were carried out for purification of the Vg-320 from oocyte homogenate. The oocyte homogenate was first applied to a hydroxylapatite column chromatography, then the flow-through fraction was applied to the Superose 6 gel filtration chromatography. The 320 kDa peak fraction was further applied to Mono Q anion exchange chromatography, and flow-through fraction was collected. The obtained preparation showed a single and symmetrical peak at a position of 320 kDa in Superose 6 column chromatography. The peak fraction was collected as the purified Vg-320. These purified Vgs were used for preparation of specific antisera (a-Vg-530 and a-Vg-320).

Western blot analysis was performed to examine specificity of these antisera and relationships between the Vgs and the major yolk proteins in vitellogenic oocytes. As shown in Fig. 4, the a-Vg-530 strongly reacted to 178 kDa band and weakly reacted to some smaller bands in E_2S. On the other hands, the a-Vg-320 strongly reacted to 127 kDa band and weakly reacted to a smaller band. Meanwhile, in Western blots of oocyte homogenate, both antisera were mainly reacted to 157 and 127 kDa bands (data not shown) suggested that both Vg-530 and Vg-320 were E_2 inducible yolk precursor proteins. Thus, they were considered to be two forms of Vgs of Japanese common goby.

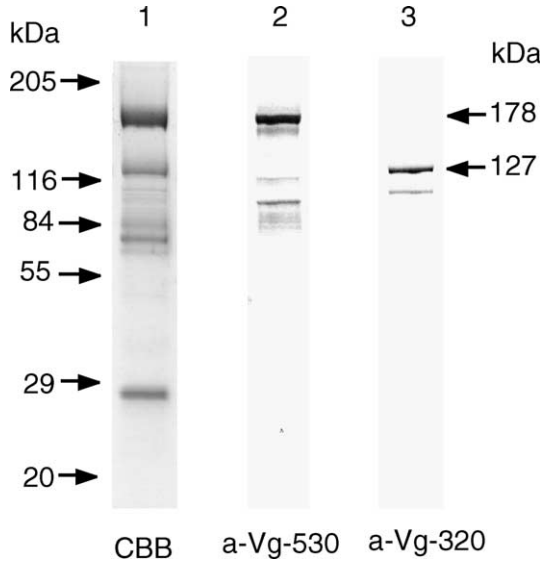

Fig. 4. SDS–PAGE (CBB staining, lane 1) and Western blots (lanes 2 and 3) of E_2 treated fish serum. The a-Vg-530 IgG was used for lane 2, and the a-Vg-320 IgG was used for lane 3. Moleculer mass values on the right of the lane 3 and the left of the lane 1 indicate migration positions of marker proteins.

3.3. Establishment of ELISA

Sandwich enzyme-linked immunosorbent assays (ELISAs) were developed using the antisera and the isolated Vgs. The sensitivity ranges of the ELISAs were 1.25–160 ng/ml for Vg-530 (Fig. 5A) and 0.26–66 ng/ml for Vg-320 (Fig. 5B) in the condition used in the present study. Very low cross-reactivity ($< 0.5\%$) was found with the alternate Vg in each assay (Fig. 5). Precision was measured by intra- ($n = 8$) and inter-assay ($n = 4$) coefficients of variation (CV). The CV of the intra- and inter-assay in the ELISA for Vg-530 were 5.7 and 13.3%, respectively, and those in the ELISA for Vg-320 were 3.5 and 3.3%, respectively.

Specificity of these ELISAs were evaluated by measuring E_2S and male serum diluent which were serially diluted from 1:200 to 1:6,400,000 with PBS containing 0.5% BSA. These absorbance curves of the diluted E_2S showed full parallelism with standard curves in both Vg-530 and Vg-320. These ELISAs did not show any cross-reactivity with the immature male serum (Fig. 6).

3.4. Dose response of E_2-induced vitellogenin synthesis

To examine the dose response of E_2-induced Vg synthesis, E_2-injection experiment was performed. Both

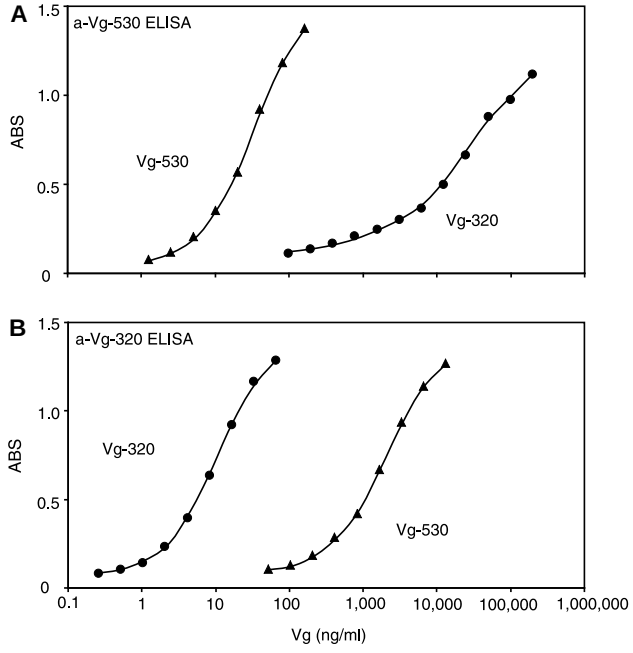

Fig. 5. Standard curves of Vg-530 and Vg-320 on ELISAs for Vg-530 (A) and Vg-320 (B). Each point represents the mean of duplicate determinations.

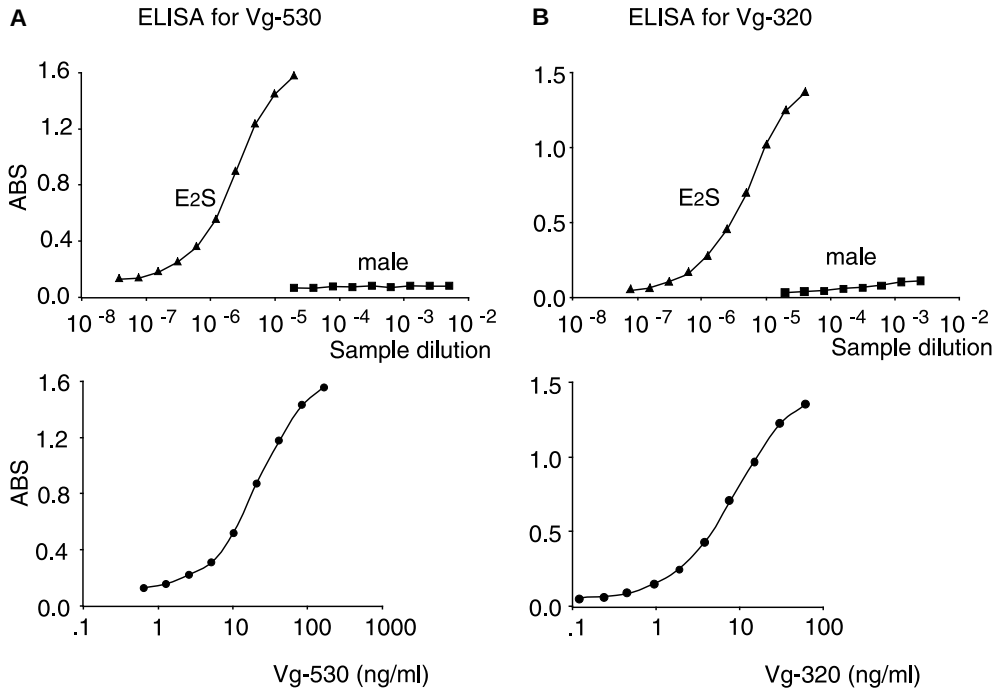

Fig. 6. The dilution curves of E_2 treated fish serum (E_2S), male serum, and Vg-530 on the ELISA for Vg-530 (A), and those of E_2S, male serum, and Vg-320 on the ELISA for Vg-320 (B). Each point represents the mean of duplicate determinations.

Vg concentrations significantly increased with higher dose of E_2 injection (Jonckheere–Terpstra test, $t^* = 6.876$, $P < 0.0001$ in Vg-530 and $t^* = 7.407$, $P < 0.0001$ in Vg-320; Fig. 7A). There were no significant difference between the control and 0.1, 1 μg/kg treatments. In the 10 μg/kg treatment, the concentration of Vg-530 increased, but there was no significant difference between the control (post hoc tests; $P = 0.2049$). In the 25, 100, 1000 μg/kg treatments, both concentration of Vg-530 and Vg-320 were significantly higher than the control (post hoc tests; Vg-530: $P = 0.0004$, 0.0006, and 0.0006, respectively, Vg-320: $P = 0.0003$, 0.0004, and 0.0004, respectively).

Moreover, we exposed male Japanese common goby to various E_2 concentrations of their rearing water for examining the sensitivity of Vg synthesis. The actual E_2 concentrations (expressed as mean ± SEM) of higher two exposure groups (100, 1000 ng/L) were 75.2 ± 8.6 ng/L, and 634 ± 155 ng/L, respectively. During the treatments, serum Vg concentrations of the fish significantly increased in a higher dose of E_2 (Jonckheere–Terpstra test, $t^* = 5.321$, $P < 0.0001$ in Vg-530 and $t^* = 6.322$, $P < 0.0001$ in Vg-320; Fig. 7B). There were no significant differences between the initial control and 0 (control), 1 ng/L treatments. In the 10 ng/L treatment, serum concentrations of Vg-530 and Vg-320 of some fish

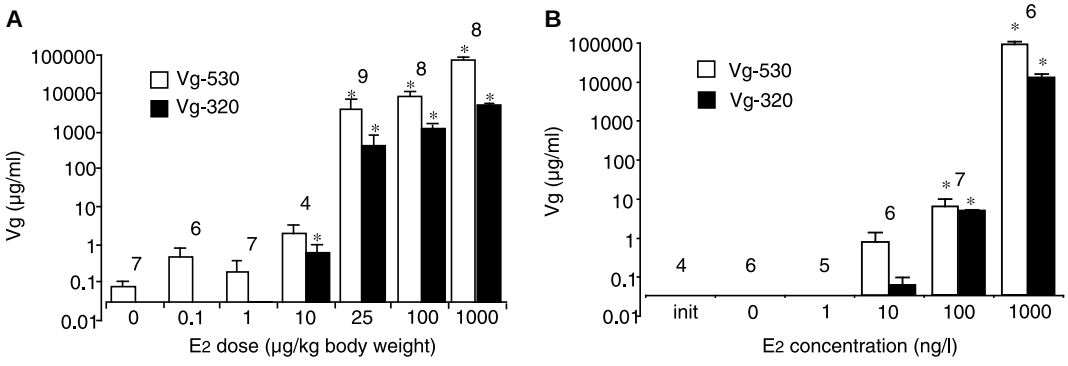

Fig. 7. Changes in serum concentrations (mean ± SEM) of Vg-530 and Vg-320 in male fish of E_2 treated by injection (A) and immersion (B). The initial control (init) also indicates in Fig. 7B. An asterisk (*) indicate that the values are significantly different from that of the control at $P < 0.05$. The numbers on the top of the column indicate the numbers of samples. Mean concentrations of Vg-530 were significantly higher than those of Vg-320 in these experiments ($P = 0.02$).

clearly increased, but there were no significant differences between the control group (post hoc tests; $P = 0.0293$ in Vg-530 and $P = 0.3445$ in Vg-320). In the 100, 1000 ng/L treatments, both concentration of Vg-530 and Vg-320 were significantly higher than the control (post hoc tests; Vg-530: $P = 0.0026$, and 0.0011, respectively, Vg-320: $P = 0.0017$, and 0.0014, respectively). The E_2 concentrations of the pooled sera of each group (0–1000 ng/L) were 86.1, 153.3, 403.0, 2353.6, and 2560.5 pg/ml, respectively.

In both injection and water exposure experiments, mean concentrations of Vg-530 were significantly higher than those of Vg-320 in these experiments (binomial test; $s+ = 10$, $s- = 0$, two-tailed, $P = 0.002$).

3.5. Concentrations of Vgs and E_2 in maturing females

GSI, serum concentrations of the E_2, and levels of Vg-530 and Vg-320 were measured in maturing females collected in October to January 1999, 2000, and 2001 (Fig. 8). The GSI was very low (0.4%) in October. It gradually increased through November, and then rapidly increased from December (0.9%) to January (1.9%). Almost all oocytes in the ovary were at peri-nucleolus stage in October. In November, oocytes at yolk vesicle stage were observed in the ovaries of two of eight individuals. The variant stages of oocytes observed in ovaries of two individuals were at the peri-nucleolus stage, two were at yolk vesicle stage, and four were at early to mid yolk stage in December. In January, early to mid yolk stage oocytes were observed in seven of eight females, and one was at yolk vesicle stage. From these observations of oocyte development over time, we can conclude that vitellogenesis in Japanese common goby is initiated in December. Serum E_2 concentrations gradually increased from 0.4 ng/ml in October through 0.5 ng/ml in November then rapidly increased from 2.0 ng/ml in December to 6.7 ng/ml in January. This rapid increase of E_2 concentration was concomitant with the rapid increase in GSI (Fig. 8A). The concentrations of both Vgs were very low (<1 μg/ml) in October and November (Fig. 8C). From November to December, Vg concentrations rapidly increased from 0.6 ± 0.3 to 758 ± 280 μg/ml in Vg-530 and from 0.7 ± 0.3 to 149 ± 50 μg/ml in Vg-320, and then they were maintained at high levels (Vg-530: 882 ± 142 μg/ml, Vg-320: 196 ± 38 μg/ml) in January.

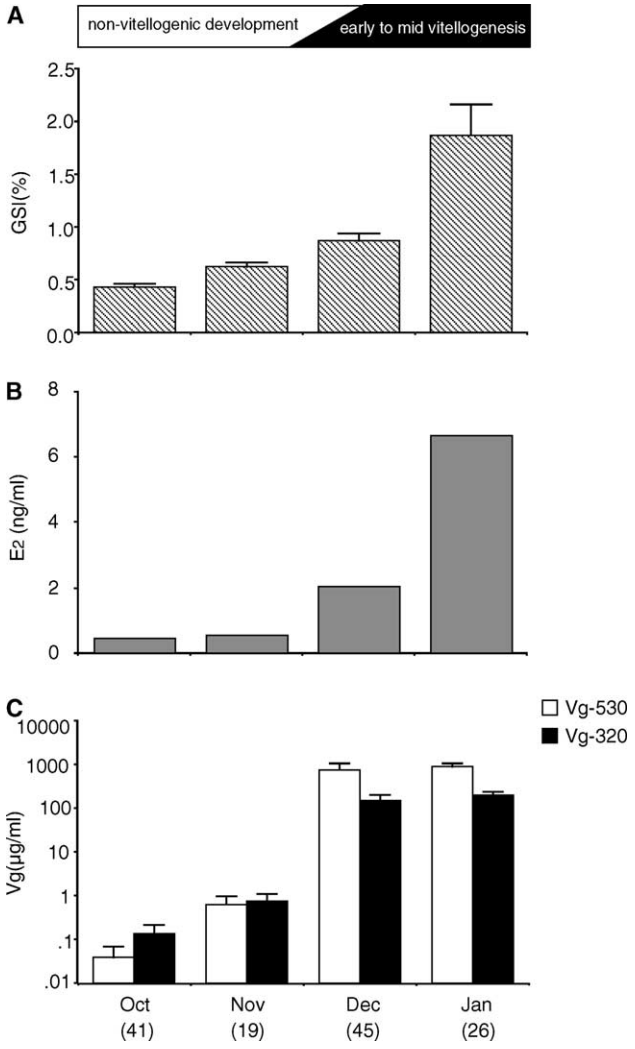

Fig. 8. Changes in GSI (A), serum concentrations of E_2 (B), and Vg-530 and Vg-320 (C) of maturing female Japanese common goby captured in October to January at Niigata, Japan. The spawning season starts from February or March. Each column represents the mean, and vertical bars represent the SEM. The numbers under the *x*-axis indicate the numbers of samples.

4. Discussion

In the present study, we found two distinct forms of Vg (Vg-530 and Vg-320) in sera from mature females and E_2-treated immature Japanese common goby. The molecular masses of the intact Vgs of the Japanese common goby were 530 and 320 kDa in their native form, and 178 and 127 kDa, respectively in their denatured and reduced state (SDS–PAGE). Recently, it has been shown by protein and mRNA analyses that some fish possess two Vgs (see review Hiramatsu et al., 2003). Two Vgs were detected by protein analysis in tilapia species, *Oreochromis aureus* (Ding et al., 1989), *Oreochromis mossambicus* (Kishida and Specker, 1993; Takemura and Kim, 2001), *Oreochromis niloticus* (Buerano et al., 1995), and barfin flounder *Verasper moseri* (Matsubara et al., 1999). In barfin flounder, the molecular masses of the two Vgs were very similar to each other in their native condition (Vg A: 530 kDa and Vg B: 550 kDa), and in SDS–PAGE (Vg A: 168 and Vg B: 175 kDa, Matsubara et al., 1999). Two Vgs exhibiting similar molecular masses were also reported in mummichog *Fundulus heteroclitus* (LaFleur et al., 1995a,b) and haddock *Melanogrammus aeglefinus* by mRNA

analysis (Reith et al., 2001). Meanwhile, in tilapia species, the molecular masses of the two Vgs are very different from each other in both their native form (*O. aureus*, 500 and 300 kDa; Ding et al., 1989) and their denatured and reduced form in SDS–PAGE (*O. aureus*, 180 and 130 kDa; Ding et al., 1989; *O. mossanbicus*, 200 and 130 kDa; Kishida and Specker, 1993; 210 and 140 kDa; Takemura and Kim, 2001; and *O. niloticus*, 185 and 120 kDa; Buerano et al., 1995) as in Japanese common goby.

The molecular mass of Vg-530 in Japanese common goby was similar to the molecular mass of the typical native Vg in other fish such as flounder (Emmersen and Petersen, 1976), turbot *Scophthalmus maximus* (Silversand and Haux, 1989), coho salmon *Oncorhychus kisutch* (Hara et al., 1993), and rainbow trout *Oncorhynchus mykiss* (Shibata et al., 1993). However, the molecular mass of the Vg-320 was quite different from these other fish Vgs. Kishida and Specker (1993) first reported the existence of a smaller Vg in *O. mossanbicus*, and showed its unique characteristic of very low phosphorus content. Recently, complete sequence of mRNA of the smaller Vg was reported in zebrafish *Danio rerio* (Wang et al., 2000). The sequence of this Vg contained complete open reading frames encoding 1233 amino acid residues, and the predicted Vg possessed no polyserine Pv domain. Our recent analysis showed that the Vg-320 mRNA of Japanese common goby contained complete open reading frame encoding 1238 amino acid residues, and the predicted amino acid sequence of the Vg had a similarity to the Pv-less type Vg of the zebrafish (Ohkubo et al. unpublished data). Considering the phylogenic difference between the zebrafish and Japanese common goby, the Pv-less Vg may widely present at least from cypriniformes fish to perciforms fish.

The focus of our research was to establish immunological assay systems of Vg for the survey of exposure to environmental estrogens. It is a great advantage to find the two different E_2-inducible proteins (Vg-530 and Vg-320) because they provide a dual measure of the effects of exposure to environmental estrogens since both are E_2-inducible proteins. In addition, the Vg-320 showed unique characteristic that the Vg does not change molecular mass following incorporation into the oocyte. The Vg-320 is probably not processed after uptake into the oocyte and is thus the only Vg in vertebrate to date that shows no change in its molecular mass during vitellogenesis.

The occurrence of an artifactual proteolytic degradation of Vg through the purification process has been reported in several fish (Buerano et al., 1995; Ng and Idler, 1983; Norberg and Haux, 1985; Selman and Wallace, 1983). Inaba et al. (1997) characterized trypsin-like serine protease, which degrades Vgs during the purification in the plasma of tilapia. In spite of a presence of serine protease inhibitors, the purified goby Vg-530 yielded mainly a 110 kDa protein band, and three small bands of approx. molecular masses of 95, 80, and 70 kDa, instead of 178 kDa band as observed by SDS–PAGE. Although the molecular mass of the denatured and reduced protein changes following SDS–PAGE, the native molecular mass of Vg-530 showed no change after purification. Therefore, the proteolytic degradation of Vg-530 is thought to be due to "nicking," and thus the purified Vg-530 has almost the same molecular mass as the intact Vg-530. On the other hand, Vg-320 showed up on SDS–PAGE as mainly a 127 kDa protein band and that seemed not to degrade through the purification procedure. The purities of the Vg-530 and Vg-320 were also evaluated by western blotting using specific antisera to Vg-530 (a-Vg-530) and Vg-320 (a-Vg-320) obtained by immunization with purified preparations of Vg-530 and Vg-320, respectively. The a-Vg-530 IgG and a-Vg-320 IgG strongly reacted to their target proteins at 178 and 127 kDa, respectively, and showed little cross-reactivity in each other. Thus, high purities of the both Vg preparation were also supported and these antisera were verified to be useful for establishing the ELISAs.

ELISAs for two forms of Vg have been reported in tilapia (Kishida and Specker, 1993; Takemura and Kim, 2001), and our research now provides a similar assay for Japanese common goby. A highly sensitive ELISA is necessary to detect the presence of Vgs in male fish that exposed environmental estrogen in seawaters of Japan, because the Vg concentrations of field-collected male fish were relatively low (range: 25–2200 ng/ml in Tokyo Bay, Hashimoto et al., 2000) when compared to fish in freshwater that are affected by sewage effluent (Jobling et al., 1998). The sensitivity ranges of the ELISAs established in the present study were 1.25–160 ng/ml for Vg-530 and 0.26–66 ng/ml for Vg-320. These sensitivity ranges are similar to those reported for Vg ELISAs of other spices (e.g. Lomax et al., 1998; Parks et al., 1999; Takemura and Kim, 2001). The parallelism observed between each dilution curve of the purified Vg and E_2S in both ELISAs demonstrates precision and accuracy of our ELISAs to specifically measure the Vgs following the E_2 exposure. In addition, the ELISAs show little cross-reactivity to non-treated male serum, thus, they are useful to measure low concentrations of the Vgs in the male fish for survey of estrogenic activity in seawater.

Using the Vg-ELISAs, we measured serum levels of the two Vgs in field-collected females of Japanese common goby at different maturational stages to know whether the two Vgs were produced in females in accordance with their sexual maturation. The two Vg concentrations showed a good correlation with elevation of serum E_2 concentration and gonadal development. It is clear that production of both Vgs in maturing females depends on endogenous estrogenic stimulation. The

highest serum concentration of Vg-530 was about 1 mg/ml and was observed in the maturing females in January, at which time the ovary is comprised of primarily to mid-vitellogenic oocytes. Although it was not clear whether the concentration was at a maximum, the value was similar to the peak concentrations of Vg in other perciform marine fish including sea bass *Dicentrarchus labrax* (3 mg/ml; Mananos et al., 1994) and gilthead seabream *Sparus aurata* (1.5 mg/ml; Mosconi et al., 1998). In contrast, the concentrations of Vg-320 in the serum of maturing females (about 200 μg/ml) were about five times lower than the concentrations of Vg-530 in January. The same situation was observed when male fish were injected or exposed to E_2. The difference between the concentrations of the Vg-530 and Vg-320 was striking following high E_2 exposure. A similar situation was observed in tilapia where the concentration of the small Vg (VTG-130) was lower than the large Vg (VTG-200) in the serum from E_2 injected fish. Furthermore, the level of VTG-130 reached a plateau on the 7th day after E_2 injection whereas the level of VTG-200 continued to increased until the 14th day after injection (Kishida and Specker, 1993). It seems likely that there is a possibility of an unequal activation of the two Vg genes as a result of E_2 stimulation in both Japanese common goby and tilapia. Further investigation is necessary to clarify the mechanism of synthesis regulation of the two Vg in Japanese common goby.

Serum E_2 concentrations of the maturing females, which had ovaries at the late peri-nucleolus oocyte stage (November) and at the primary yolk stage (December) were 0.5 and 2.0 ng/ml, respectively. These E_2 concentrations correspond to the values at the onset of vitellogenesis of field-collected female Japanese common goby. The result of E_2 exposure in this study revealed that some individual started Vg synthesis in the 10 ng/L E_2 treatment while the Vg concentration was significantly elevated in 100 ng/L E_2 treatment. Similar results have been reported in rainbow trout and roach (*R. rutilus*) where the Vg concentrations significantly increased from 100 ng/L E_2 treatment (Routledge et al., 1998). The serum E_2 concentration that induces Vg synthesis in situ is much higher than the effective concentration of exposure. This discrepancy in the E_2 levels between maturing field-collected female sera and the surrounding experimental water suggests the involvement of an mechanism of accumulation of E_2 from the environment to the circulation. Actually, the E_2 concentration of fish sera which were exposed E_2 in this study were much higher than the surrounding water (10 ng/L: 0.4 ng/ml, 100 ng/L: 2.4 ng/ml). Thus, it is likely that a mechanism of bioaccumulation of E_2 in serum play a significant role in the Vg production in male fish receiving an E_2 exposure. Further investigation is necessary in order to elucidate whether other environmental estrogens (other than E_2) have such an accumulation mechanism.

In conclusion, our results demonstrate that the Vg ELISAs established in the present study are adequate to measure widely from ng/ml level to mg/ml level. Based on this study, routine measurements of Vgs from Japanese common goby can now be made throughout Japan's coastal regions.

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