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Rockfish (*Sebastes schlegeli*) Vitellogenin: Purification, Characterization and Development of Sandwich ELISA System

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Vitellogenin (VTG) was purified from serum of estradiol-17β-treated rockfish (*Sebastes schlegeli*) by precipitation with EDTA-$Mg^{2+}$and ammonium sulfate and two step chromatography (anion exchange chromatography and gel permeation chromatography) was performed on FPLC system. Rockfish VTG (rfVTG) was characterized and its properties were determined. The monomers have apparent, molecular mass of about 188 kDa as indicated by SDS-PAGE. Amino acid composition analysis of rfVTG was similar to VTG from other oviparous teleosts. Cysteine and lysine were present at relatively high level. Leucine was present at relatively lower level than in other species. The N-terminal amino acid sequence was evaluated to identify rfVTG. Western blot analysis using an antibody against the purified VTG showed that the antibody reacted with both plasma of estradiol-17β treated male and purified VTG, whereas there was no reaction with male serum of the control. An ELISA was developed using monoclonal and polyclonal antibodies against rfVTG. The assay range was 3.2 ng/mL and 1,000 ng/mL and the value of the intra and inter assay variations were within 9.7% and 11.2%, respectively. Recovery rate was 96.8%. The sandwich ELISA could be useful for the detection of VTG and could be good for screening of estrogenic compounds.

Key words: ELISA, Rockfish, *Sebastes schlegeli*, Vitellogenin (VTG), Monoclonal antibody, Polyclonal antibody,

Introduction

Several environmental chemicals are found to affect the endocrine system in wildlife. Some of them can modulate or mimic the action of sex steroid hormones. Environmental chemicals have bioactivity similar to the endogenous estradiol-17β (E_2), which is known to influence development, sexual maturation and reproduction of fishes. Vitellogenin (VTG), a female specific glycolipophosphoprotein of high molecular weight, is synthesized in the liver of mature female fish in response to stimulation of E_2, and released into the bloodstream. VTGs are transported to the ovary where they are taken up by the oocyte during vitellogenesis period (Ng and Idler, 1983). The VTG sequestered in oocytes are proteolytically cleaved into the predominantly smaller yolk proteins, lipovitellin (s) and phosvitin(s) (Selman and Wallce, 1983, 1989; Tyler et al., 1990; Kanungo et al., 1990; Wallace and Selman, 1985). These reserves are believed to serve as the main nutrition sources for the developing embryo (Fyhn and Serigstad, 1987; Rønnestad and Fyhn, 1993; Rnnestad et al., 1993; Finn et al., 1995; Matsubara et al., 1999). Knowledge on biochemical characteristics of VTGs is required for the improvement of information on reproductive physiology and nutritional role in the embriogenic development.

Since exposure of male fish to estrogenic compound triggers VTGs production in considerable amount, VTGs could be used as a biomarker to detect functional esterogenicity of compounds. Thus, VTG has been extensively isolated and partially characterized from several oviparous species of teleost: goldfish (*Carassius auratus*) (De Vlaming et al., 1980), eel (*Anguilla japonica*) (Hara et al., 1980), tilapias (*Oreochromis aureus* and *O. mossambicus*) (Ding September 2004)

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carp (*Cyprinus carpio*) (Tyler and Sumpter, 1990), striped bass (*Morone saxatillis*) (Tao et al., 1993), Sea bass (*Dicentrarchus labrax*) (Mananose et al., 1994), gilthead seabream (*Sparus aurata*) (Mosconi et al., 1998), grouper (*Epinephelus malabaricuc*) (Utarabhand and Bunlipatanon, 1996) and fathead minnow (*Pimephales promelas*) (Parks et al., 1999). Methods for the assessment of VTG in various species have been developed, including arctic charr (*Salvelinus alpinus*) (Johnsen et al., 1999), brown trout (*Salmo trutta*) (Sherry et al., 1999), sole (*Solea vulgaris*) (Rodriguez et al., 1989), japanese medaka (*Oryzias latipes*) (Nishi et al., 2002), rainbow trout (*Oncorhyncus mykiss*) (Bon et al., 1997).

Normally, VTGs are measured using Radioimmunoassay (RIA) and enzyme-linked immunoassay (ELISA). However, ELISAs have been increasingly used in the last few years as it do not require radioactive labeling which is deleterious to VTG. Most of these ELISAs were measured using immunoassay based on polyclonal antibodies. However, Andrew et al. (1992) indicated that polyclonal antibodies could have failed in the detection of small quantities of non-specific antibodies that react to proteins other than VTG. Recent studies, have established VTG production as a good indicator of estrogenic effects in freshwater species. But there are limited studies on marine species (Daniel et al., 1998), though xenoestrogens are commonly found in marine environment.

The rockfish (*Sebastes schlegeli*) is common in coastal waters of Korea and is commercially important. This species was categorized as viviparous fish, because development of the late embryonic stage dependents maternal nutrition as energy source, in addition to that stored in yolk accumulated in oocyte during vitellogenesis (Boehlert et al., 1986). Viviparous teleosts establish the maternal-embrionic trophic relationship and have reproductive mechanisms, which are lacking in oviparous teleosts, including internal fertilization and embryogenesis in the ovary. Therefore, it is interesting to characterize the VTG that may serve various physiological and nutritional roles for oogenesis and embryogenesis. However, investigation on VTG in viviparous teleosts has been carried out only in *Zoarces viviparus* (Korsgaard and Petersen, 1979,1998), *Cymatogaster aggregata* (de Vlaming et al., 1983) and *S. taczanowskii* (Takemura et al., 1991).

In the present study, we purified and characterized the rfVTG. Then compared with VTGs of other oviparous and viviparous teleosts. We developed a sandwich ELISA system for quantification of plasma VTG using monoclonal and polyclonal antibodies against rfVTG. This assay will not only be utilized in the assessment of reproductive stages in females but also will provide information on the suitability of it as a tool for xenoestrogen' effects.

Materials and Methods

Hormone treatment and blood sampling

Male rockfish weighting 200-400 g were obtained from the National Fisheries Research and Development Institute (NFRDI) and fishes were acclimated for 2 weeks in the laboratory. VTG synthesis in male fish was induced by three intraperitoneal injections every 10 days with 2 mg estradiol-17β (Sigma) dissolved in ethanol per kilogram of body weight. After treatment, blood was collected from the caudal sinus with heparinized syringes. The blood was transported in a heparinized tube containing serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF, Sigma) at final concentration of 1 mM, and centrifuged at 4,000 rpm, 4℃ during 15 min. Plasma was then collected and stored at -80℃ until purification.

Vitellogenin purification

VTG was prepared using double precipitation by EDTA-Mg^{2+} (Wiley et al., 1979). Two chromatographic procedures were performed on an anion-exchanger Mono Q HR 5/5 column (Pharmacia LKB) in 20 mM Tris-HCl (pH 8.0, 1 mM PMSF) with a flow rate of 1 mL/min. Protein was eluted in a linear segment sodium chloride gradient (0-0.5 M). The VTG-enriched fractions were separated on a Superdex 200 HR 10/30 column (Pharmacia LKB) in 20 mM Tris-HCl (pH 8.0, 1 mM PMSF, 0.15 M NaCl) with a flow rate of 0.5 mL/min. The elute was collected in 5 mL fractions and absorbance at 280 nm was monitored.

Electrophoresis and determination of molecular weight

Fractions containing purified VTG were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. SDS-PAGE was performed according to the methods of Laemmli (1970). After electrophoresis, gel was fixed and stained with 0.2% Coomassie brilliant blue R-250. Molecular mass marker (Sigma) included myosin (200 kDa), *Escherichia coli* β-galactosidase (116 kDa),

rabbit muscle phosphorylase b (97 kDa), bovine serum albumin (65 kDa), and hen egg white ovalbumin (43 kDa). The molecular weight of VTG was determined by gelfiltration chromatography. As standard proteins carbonic anhydrase bovine erythrocyte (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), sweet potato β-amylase (200 kDa), horse spleen aproferritin (443 kDa), bovine thyroglobulin (669 kDa), blue dextran (2,000 kDa) were used. These proteins were separately layered onto a superdex 200 HR 10/30 column (Pharmacia LKB) and eluted in 20 mM Tris-HCl buffer (pH 8.0) with a flow rate of 0.5 mL/min.

Amino acid compositional analysis

For analysis of amino acid composition, the purified VTG was hydrolyzed with 6 M HCl in an evacuated tube at 110℃ for 24 hr. After hydrolysis, sample dried and dissolved in 1.4 mM NaHAc containing 0.1% TFA and 6% CH_3CN. Analysis were performed with a Hewlett packard 1100 series at the Korea Basic Science Institute (KBSI).

N-terminal amino acid sequence analysis

The purified VTG of rockfish was subjected to 10% SDS-PAGE. For N-terminal amino acid sequencing, the protein was electroblotted onto PVDF (polyvinylidene difluoride) membrane at 50 volt for 5 hr at 4℃. Protein sample on PVDF membrane were detected in Coomassie blue staining solution. The band was excised from the membrane and sample was carried out at the KBSI.

Protein assay

The total protein concentration was determined using the Bradford Assay kit (Bio Rad Co.). Samples were diluted 1/100 and bovine serum albumin (BSA) was used as standard. Absorbance was measured at 450 nm.

Polyclonal antibody production

A sample of the purified rfVTG (identified by N-terminal sequence) was mixed with Freund's complete adjuvant (1:1) and injected (i.p.) into New Zealand white rabbit. Three successive injections were performed with two weeks intervals with same dose of VTG in Freund's incomplete adjuvant (1:1). Blood was collected 4 days after the last injection and centrifugation at 4,000 rpm at 4℃ for 10 min. The supernatant antisera were stored at -80℃ until use.

Monoclonal antibody production

The production procedure published by Harlow and Lane was modified (1988). Balb/C mice were injected with purified rfVTG (50 μg mouse) emulsified in Freund's complete adjuvant (1:1). Mice were injected two times at intervals with 30 μg of VTG in Freund's incomplete adjuvant (1:1). Three days before fusion of the spleen cell with myeloma cell line (sp2/0-Ag14), mice boosted with 50 μg of VTG. Spleen cell were fused with myeloma cell in the presence of 50% polyethylene glycol using a ration of 1:7. The fused cells were cultured in RPMI 1640 and HAT (0.1 mM hypoxantine aminopterin and 0.016 mM thymidine) to select for hybridoma cell supplemented with 15% fetal bovine serum in 37℃, 5% CO_2. Hybridomas proceeding reactive media were cloned in 96 well plates by limiting dilution. A single VTG specific clone then injected to Balb/C mice for a higher antibody titer. Monoclonal antibody collected from ascites fluids after centrifuge and precipitated the antibodyammonium sulfate complex. These complex were dialyzed in 0.01M PBS buffer and stored at 4℃ in 0.01% sodium azide until using.

Evaluation by western blotting

The specificity of the antibody was examined by western blot analysis of plasma proteins separated by SDS-PAGE. Separated proteins were transferred onto PVDF nitrocellulose membrane. Non-specific binding sites on the membrane were blocked with 0.5% goat IgG in TBST (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.05% Tween 20) for 2 hr at 37℃. Antibody of rfVTG was diluted 1:5000 in TBST and the membrane was incubated for 1 h at room temperature. After washing, membrane was incubated with secondary antibody (goat anti-mouse IgG biotin conjugate) for 30 min at room temperature. The blots were developed using ABC kit (Vector, Laboratories, Burilingame, CA) for 30 min. Substrate solution containing 0.02% 3,3'-Diaminobenzidine tablets (Sigma) and reaction was stopped with distilled water.

Sandwich ELISA procedure

Monoclonal antibody (First antibody I) coating

The coating was performed in 96-well microtiter plates (Nunc F96 Mixisorp TM Immunoplate) were coated in 100 μL of carbonate buffer, pH 9.6, containing monoclonal antibody. The monoclonal antibody was diluted in carbonate buffer (1:2000) and then the plates incubated for 16 hr at 4℃. The content of the well was discarded by inverting the plates, and three successive washes of 5 min with PBST

(0.01 M phosphate buffer pH 7.4, 0.15 M NaCl and 0.05% Tween 20). The nonspecific binding sites were saturated by incubating the plates at 37℃ for 1 hr with 150 μL PBS containing 0.3% BSA. Followed by a three-wash cycle with PBST.

Antigen incubation

200 μL of the VTG standard solution (7.8-1000 ng mL^{-1}) serially diluted into PBS containing 0.3% BSA, 0.1% Tween 20 and plasma samples diluted and pipetted into the wells. the plates incubated for 4 hr at 37℃. Followed by a three-wash cycle with PBST.

Polyclonal antibody (First antibodyII) incubation

The polyclonal antibody was diluted in PBST (1:3000) and then the plates incubated for 4 hr at 37℃, followed by a three-wash cycle with PBST.

Second antibody incubation

Each well received 200 μL of goat anti-mouse IgG biotin conjugate goat (Sigma; diluted 1:100,000 in PBST). The plate was incubated for 45 min at 37℃ and then washed.

Avidin/biotin horseradish peroxidase H reagent complex (ABC) incubation

ABC (Elite Kit, Vector Lab, CA) was distributed in the well, and the plate was incubated for 30 min at 37℃ and then washed.

Visualized of the reaction

Each well received 200 μL of following solution prepared immediately before use 20 mL of citrate-phosphate buffer (pH 5.2), containing tetramethylbenzidine (TMB) enzyme substrate (Sigma). The reaction stopped after 20 min. with 0.5 M H_2SO_4 and absorbance of the reaction was determined at 450 nm with a microplate reader (Biology microstation).

Results

rfVTG purification

VTG was purified from plasma of the E_2-treated male by anion exchange chromatography with mono Q column and by gel-filtration chromatography with HR 10/30 column. In the anion exchange chromatography, VTG, selectively precipitated by EDTA-Mg^{2+} eluted with approximately 0.25 M NaCl at a retention time of 26 min. There were two peaks observed, the first sub band and the second main band, predominated in the plasma of E_2-treated male rockfish, but was absent in the plasma of untreated male (Fig. 1A, B). In order to purify the main protein, the probable VTG peak was pooled and subjected to the gel-filtration chromatography (Fig. 2A) and was confirmed in SDS-PAGE. The protein band was stained by coomassie blue (Fig. 2) and showed one protein band with an apparent molecular weight of but absent in the normal male plasma. The band suggests to the VTG of rockfish and corresponds to the VTG monomer. The molecular weight of rfVTG was estimated by the gel-filtration with superdex HR 10/30 column (Fig. 3), the molecular weight was estimated by the interpolation between standards and was about 188 kDa.

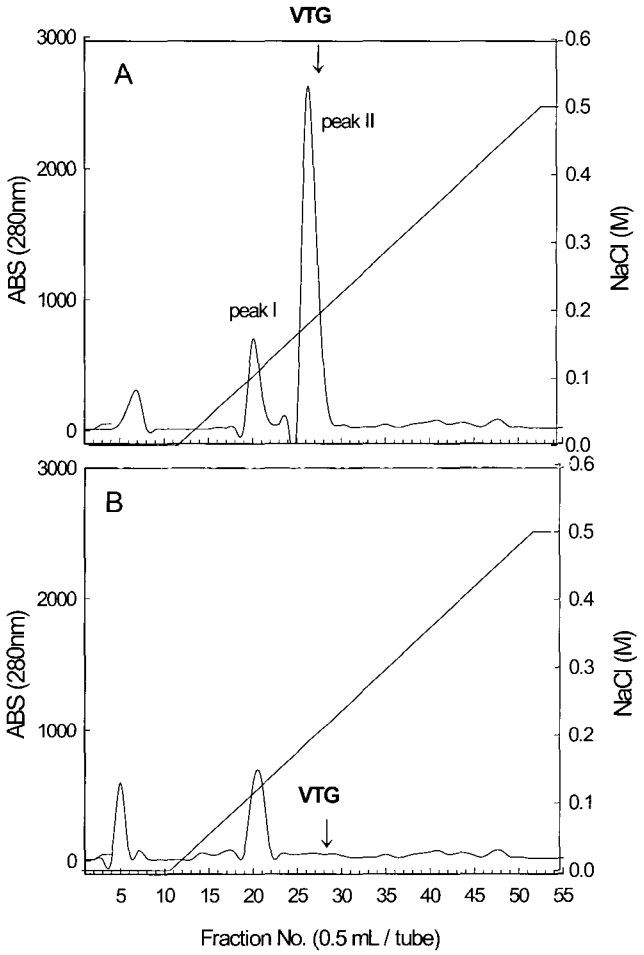

Fig. 1. Purification of rockfish (*Sebastes schlegeli*) VTG using FPLC techniques. Serum extraction of rockfish was applied to Mono Q HR anion-exchange chromatography column. (A) elution profile of E_2 treated male serum. (B) elution profile of non-E_2 treated male serum.

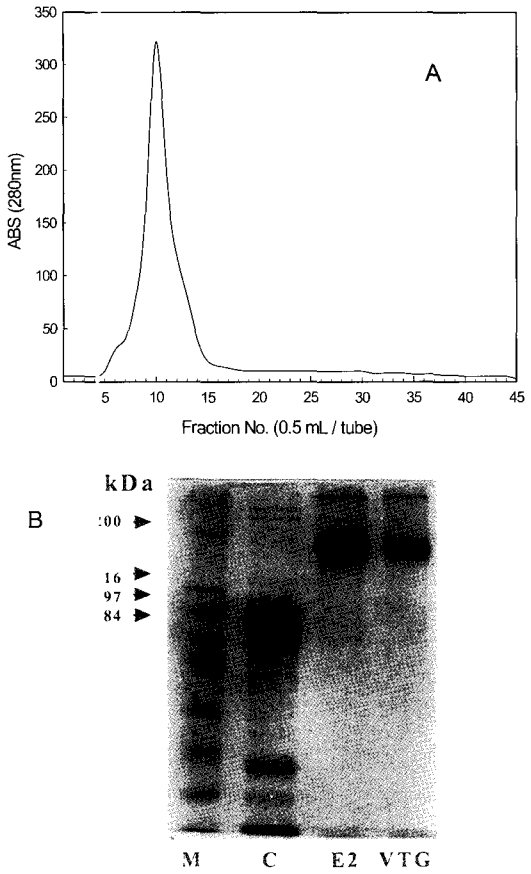

Fig. 2. (A) The VTG-enriched fraction was applied to Superdex 200 HR gel permeation column chromatography. (B) Identification of purified VTG in rockfish (*Sebastes schlegeli*) using 8% SDS-PAGE. M, molecular weight marker; C, E_2-untreated male serum; E_2, treated male serum; VTG, purified rfVTG.

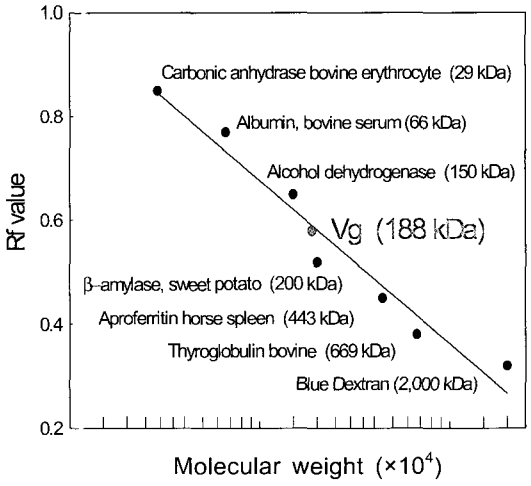

Fig. 3. Molecular weight determination of rockfish (*Sebastes schlegeli*) VTG by Superdex 200 HR gel permeation column chromatography.

N-terminal amino acid sequence analysis and amino acid composition

Amino acid sequence of probable rfVTG was evaluated to further confirm identification of the protein. We were able to obtain N-terminal sequence of 20. The amino acid sequence was arranged and fitted with N-terminal sequence position between amino acids, and compared with known proteins in National center for biotechnology information (NCBI) data bank (Table 1). The identification of amino acid revealed 68% to 88% similarity to the other species. The rfVTG showed identity of 88% with the mummichog. Purified rfVTG was subjected to amino acid analyses for further characterization. The amino acid composition of rfVTG was compared to VTG from six other oviparous teleosts (Table 2). Non-polar amino acid of the rfVTG was predominant (52%) and showed similarity with other species. However there was little variation on proportion of few amino acids. The rfVTG consisted of lower proportion of tyrosine (0.90%) and glutamine (7.31%) but higher proportion of cysteine (2.94%) than in the other species. The lowest proportion of amino acids in the rockfish was tyrosine (0.9%) and the highest proportion of amino acids was alanine (12.7%).

Antiserum production and evaluation by western blotting

Western blot analysis using the antisera against VTG indicated the specificity of antisera. In western blot analysis, the VTG band of 188 kDa was immunostained both in the plasma of E2-treated male and the purified VTG, whereas there was no reaction in the plasma of normal male fish (Fig. 4).

ELISA validation

The sandwich ELISA for rockfish was established using twp specific antibodies, a monoclonal antibody that gave high sensitivity was used for microtiterplate coating and a polyclonal antibody was used for high binding. The optimal assay concentrations were obtained with a coating monoclonal antibody dilution of 1:2000 and polyclonal antibody dilution of 1:3000 (Fig. 5). In these concentrations, the sample range of dilution was $1:10^4$. The specificity of the antibody tested by ELISA using serial dilutions of plasma from control male. No significant reaction was detected with the male plasma, but the ELISA system was showed the parallelism between standard curve and mature female serum (Fig. 6) The intra-assay CV

Table 1. Comparison of the N-terminal amino acid sequences of rockfish (*Sebastes schlegeli*)

Fish	*												2	0	dentities (%)
Rockfish[a]	V	Q	V	N	F	A	P	E	F	V	T	G	K	T	Y	V	Y	K	Y	E	-
Mumminchog[b]		G	Q	N	F	A	P	E	F	A	A	G	K	T	Y	V	Y	K	Y	E	88 (15/17)
Haddock[c]	-	-	V	N	F	A	P	D	F	A	S	S	K	T	Y	V	Y	K	Y	E	77 (14/18)
Rainbow trout[d]	-	-	V	N	F	A	P	D	F	A	A	S	K	T	Y	V	Y	K	Y	E	77 (14/18)
Fathead minnow[e]	Q	Q	I	N	L	V	P	E	F	A	P	D	K	T	Y	V	Y	K	Y	E	68 (13/19)

a, The present study' Genbank No.: b, (UO7055) c, (AF284035) d, (AJ011691) e, (AF130454).

Table 2. Comparison of the amino acid compositions in the rockfish (*Sebastes schlegeli*) with other species

Amino acid	Percentage of total amino acids						
	Rockfish*	Striped bass[a]	Ocean pout[b]	Cod[b]	Fathead minnow[c]	Carp[d]	Rainbow trout[d]
aspartic acid	5.17	7.6	8.0	8.79	7.1	6.7	9.2
threonine	6.01	5.2	4.69	5.14	4.5	5.4	6.2
serine	8.45	7.2	6.88	6.09	3.0	7.6	7.3
proline	5.01	4.3	4.13	4.13	6.2	5.9	4.6
glutamic acid	7.31	8.3	11.9	11.4	12.6	11.8	11.1
glycine	5.87	4.2	2.80	3.02	4.6	5.1	5.1
alanine	12.7	11.9	7.57	7.79	15.3	12.6	11.5
cysteine	2.94	1.0	1.32	1.69	0	0.12	1.0
valine	6.87	7.8	6.83	6.76	8.3	6.3	6.6
methionine	2.16	2.8	3.38	2.48	0.5	1.9	2.0
isoleucine	6.23	7.0	5.60	6.58	7.3	5.4	5.0
leucine	5.97	10.8	9.61	10.99	12.3	10.5	9.5
tyrosine	0.90	3.4	3.58	4.21	0.1	2.8	2.9
phenylalanine	4.31	3.4	4.41	3.97	3.3	2.8	4.0
lysine	9.89	7.3	8.74	8.71	8.3	6.3	7.1
histidine	2.06	3.1	2.59	2.67	2.2	3.4	2.9
arginine	5.25	4.9	6.54	5.08	4.3	5.0	4.5

The present study. a, Tyler and Sumpter (1990); b, Zuxa and Laurence (1996); c, Louise et al., (1999); d, Tyler et al.,(1996).

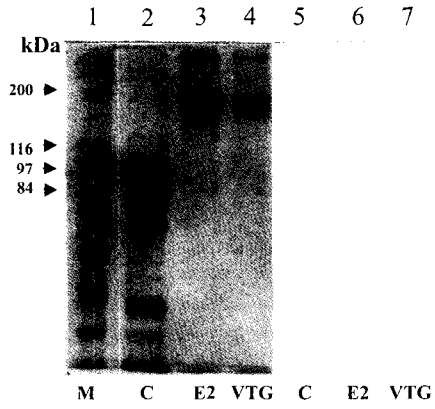

Fig. 4. Western blot analysis using purified VTG of rockfish (*Sebastes schlegeli*). Lane 1, 2, 3 and 4 are results of SDS-PAGE. Lane 5, 6 and 7 are results western blotting. Lane 1, molecular weight marker; Lane 2 and 5, E_2 untreated male serum; Lane 3 and 6, E_2 treated male serum; Lane 4 and 7, purified VTG.

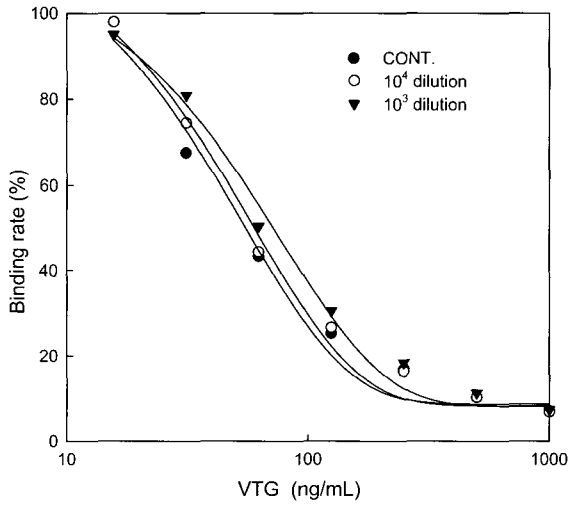

Fig. 5. Determination of optimal antigen concentration. Standard curves affected by dilution male sera added into serial dilution.

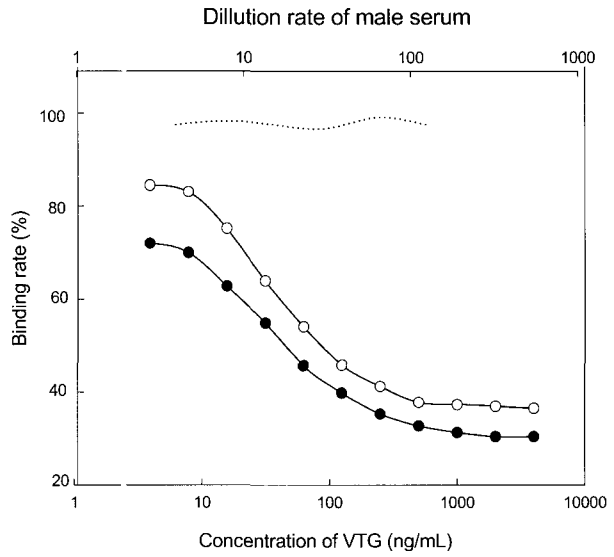

Fig. 6. Change in binding rate of VTG and serial dilutions of mature male serum (○ standard curve, ● mature female serum, ---- mature male serum).

values were 7.7, 7.5, 8.1 and 9.8% (N=11) (Table 3), and the inter-assay CV value were 7.5, 7.4, 8.2 and 11.2% (N=5) (Table 4). The recovery was between 80 and 106% (Fig. 7).

Table 3. The intra-assay coefficients of variation for measuring of serum VTG in the rockfish (*Sebastes schlegeli*)

Sample	A	B	C	D
Value of measurement (mg/mL×10^{-2})	25	132	360	752
	23	139	372	653
	26	132	382	721
	26	155	400	782
	27	163	425	817
	28	142	340	721
	30	154	353	693
	28	145	346	731
	28	159	423	882
	29	160	346	871
	30	143	384	885
Mean	27.2	147.7	375.6	768.5
SD	2.1	11.1	30.3	75.1
CV (%)	7.7	7.5	8.1	9.8

SD, standard deviation CV, coefficient of variation

Discussion

We purified and characterized the rfVTG and developed the sandwich ELISA system for quantifying estrogenic responses. Pre-treatment of male rockfish with estradiol-17β induced the appearance of a high-weight protein in plasma. This protein was present in plasma of estradiol treated male but not in untreated males. Therefore, we purified the protein and partially characterized it. Its characteristics were compared with those of teleost VTG. Confirmed VTG was used for specific antibody preparation. The rfVTG was showed monomer form about 188 kDa by gel-filtration chromatography and SDS-PAGE. Similarity was found in other species such as 180 kDa for Sea bass (Mananos et al., 1994), 175 kDa for Rainbow trout (Babin, 1992). In male tilapia, injection of estradiol-17β induced plasma protein of 200 kDa and 130 kDa mass by SDS-PAGE and had two monomer forms of VTG, 130 kDa and 180 kDa (Ding et al., 1989). A female specific serum protein was reported in white-edged rockfish (*S. taczanowskii*) and two female specific proteins reacted with antiserum against egg proteins (Takemura et al., 1991). However, there was no further information on its purification, molecular properties, and specific characterization in this species.

The purified rfVTG showed some similarities in amino acid composition with other oviparous teleosts. Its content of non-polar amino acid (alanine, isoleucine, leucine, proline) contained (52.1%) was lower to that reported for VTG in other teleost (-58%). However, considerably high level of serine (8.45%, versus 5.5-7.6%) and cysteine (2.94% versus 0-1.69%) were observed. But the abundance of the these non-polar amino acid residues may be due to VTG's ability to transport endogenous lipids in its highly hydrophobic core. However, our analysis of the VTG amino-acid composition showed characterization that cysteine and lysine was present at relatively high level and differ with striped bass, Fathead minnow and carp (0-1.0%) while similar to other marine fishes (ocean poat and cod) (1.32-1.69%). Leucine was present at relatively lower than other species (5.97%, versus 9.5-12.3%). This suggests the point of dissimilarities in the amino-acid requirement of different marine fish larvae and perhaps this information should be taken into account when formulating a starter diet for young marine fish (Zuxu et al., 1996). Sequence of the N-terminal amino acid was evaluated further to confirm identification of rockfish VTG. The identification of amino acid of the VTG showed greater than 58% to 84% similarity with other oviparous teleost and we found a highly conservative N-terminal amino acid sequence compared to other vertebrate.

Table 4. The inter-assay coefficient of variation for measuring of serum VTG in rockfish (*Sebastes schlegeli*)

Sample	Value of VTG concentration each assay (mg/mL×10^{-2})					Mean	SD	CV (%)
	1	2	3	4	5			
A	25	28	29	27	24	26.4	2.0	7.5
B	143	146	151	171	157	153.1	11.3	7.4
C	373	394	431	405	459	412.4	33.7	8.2
D	782	752	967	917	940	871.5	97.6	11.2

SD, standard deviation CV, coefficient of variation

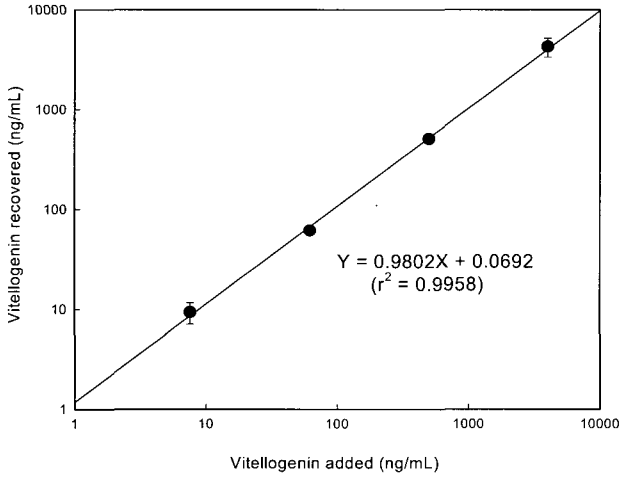

Fig. 7. Recovery test for the VTG added to pooled male serum of rockfish (*Sebastes schlegeli*).

This seems to support the hypothesis that VTG genes of non-mammalian vertebrates are highly conservative (Lee et al., 1992). The degree of conservation about amino acid of VTG in oviparous teleost fish was similar to those reported in *Xenopus*. These conserved resign of teleost is recognition signal resign, play on important role as specific uptake and cleavage of VTG into the egg proteins, lipovitellin and phosvitin (Folmar et al., 1995). Therefore, we think that this conserved sequence information could be useful not only for design and/or analysis of rockfish but also for development of a highly sensitive antibody in marine fish.

In general, the VTG gene is present but not expressed in normal males and it is thought that circulating levels of estradiol are too low to trigger its expression. Recently, electrophoresis has been used to detect putative VTG in plasma of male tilapia, (Ding et al., 1989) and northern blot analysis has been used to detect mRNA of VTG in the liver by estrogen in eel (Okumura et al., 2002). Exposure of male fish with estrogenic compounds, however, triggers VTG production in considerable amounts (Sumpter and Jobling, 1996). In our studies, the sandwich ELISA system was developed using monoclonal and polyclonal antibodies against rfVTG. The rfVTG ELISA system was sensitive with a working range between 3.2 ng/mL and 1,000 ng/mL. The sensitivity was comparable to values reported of other species, including *S. alpinus* (1 ng/mL) (Nishi et al., 2002), *P. promelas* (3 ng/mL) (Parks et al., 1999), *Danio rerio* (3 ng/mL) (Fenske et al., 2001) and *O. mykiss* (20 ng/mL) (Bon et al., 1997). But the sensitivity of the ELISA system was higher than *Pleuronectes vetulus* (10 ng/mL) (Lomax et al., 1998), *Solea vulgaris* (12 ng/mL) and *Z. viviparous* (5 ng/mL) (Korsgaard et al., 1998). Several RIAs developed for teleost VTG showed similar sensitivities (Bon et al., 1997; So et al., 1985; Tyler and Sumpter et al., 1990). The Rockfish sandwich ELISA system achieved 96.8% recovery. The intra-assay coefficients of variations were 7.5 to 9.7%. and inter-assay coefficients of variations were 7.4 to 11.2%. These variations were similar to values of other VTG ELISA systems (Bon et al., 1997; Korsgaard et al., 1998; Lomax et al., 1998). The ELISA for perch VTG showed high inter-assay coefficient (24%) because the use of polyclonal antibody might overestimate in contrast to use of monoclonal antibodies, which will give lower value (Mark et al., 2003). Therefore, our data indicate that the system is more sensitive and stable sandwich ELISA system. In conclusion, the specific rfVTG sandwich ELISA system will be used not only to monitor of the presence of estrogenic compounds in wild life but also in studying the physiological mechanisms involved in VTG uptake in viviparous fish.

References

Andrew, E.G., M.G. John, T.B. James and H.E. Barbara.

1992. Monoclonal antibody based immunoassay of vitellogenin in the blood of male channel catfish (*Ictalurus punctatus*). Comp. Biochem. Physiol., 101B, 441-446.

Babin, P.J. 1992. Binding of thyroxine and 3,5,3'-triodothyroxine to trout plasma lipoproteins. Am. J. Physio ., 262E, 712-720.

Boehlert, Y. 1991. Rockfishes of the Genus Sebastes: Their reproduction and early life history. Klower Academic Publishers. Developments in environmental biology of fishes II. Boston, London, pp. 49~56.

Bon, E., L. Barbe and J.L Rodriguez. 1997. Plasma vitellogenin levels during the annual reproductive cycle of the female rainbow trout (*Oncorhynchus mykiss*): establishment and validation of an ELISA. Comp. Biochem. Physiol., 117B, 75-84.

Danie, P.L. T.R. William, D.M. James and L.J. Lyndal. 1998. An enzyme-linked immunosorbent assay (ELISA) for measuring vitellogenin in English sole (*Pleuronectes vetulus*): Development, validation and cross-reactivity with other pleuronectids. Comp. Biochem. Physiol., 116B, 425-436.

De Vlaming, L., H.S. Wiley, G. Delahunty and R.A. Wallace. 1980. Goldfish (*Carassius auratus*) VTG: induction, isolation, properties and relationship to yolk proteins. Comp. Biochem. Physiol., 67B, 613-623.

De Vlaming, L., D. Baltz, S. Aderson, R. Fitzgerald, G. Delahunty and M. Barkley. 1983. Aspects of embryo nutrition and excretion among viviparous embiotocid teleost: Potential endocrine involvements. Comp. Biochem. Physiol. 76A, 189-198.

Ding, J.L., P.L. Hee and T.J. Lam. 1989. Tow forms of vitellogenin in the plasma and gonads of male *Oreochromis aureus*. Comp. Biochem. Physiol., 93B, 363-370.

Fenske, M., R. van Aerle, S. Brack, C.R. Tyler and H. Segner. 2001. Development and validation of a homologous zebrafish (*Danio rerio* Hamilton-Buchanan) vitellogenin enzyme-linked immunosorbent assay (ELISA) and its application for studies on estrogenic chemicals. Comp. Biochem. Physiol., 129, 217-232.

Folmar, L.C., N.D. Denslow, R.A. Wallace, G. LaFlueur, T.S. Gross, S. Bonomelli and C.V. Sullivan. 1995. A highly conserved N-terminal sequence for teleost VTG with potential value to the biochemistry, molecular biology, and pathology of vitellogenesis. J. Fish Biol., 46, 255- 263.

Finn, R.N., L. Ronnestad and H.J. Fyhn. 1995. Respiration, nitrogen and energy metabolism of developing yolk-sac larvae of Atlantic halibut (*Hippglossus hippoglossus* L.). Comp. Biochem. Physiol., 111A, 647-671.

Fyhn, H.J. and B. Serigstad. 1987. Free amino acids as energy substrate in developing eggs and larvae of the cod *Gadus morhua*. Mar. Biol. 96, 335- 341.

Hara, A., K. Yamauchi and H. Hirai. 1980. Studies on female-specific serum protein (VTG) and egg yolk protein in Japanese eel (*Anguilla japonica*). Comp. Biochem. Physiol. 65B, 315-320.

Harlow, E. and D. Lane, 1988. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Mark, H., M. Wiesmann, B. Allner and H. Sauerwein. 2003. Vitellogenin in carp (*Cyprinus carpio*) and perch (*Perca fluviatilis*): Purification, characterization and development of an ELISA for the detection of estrogenic effects. Sci. Total Environ., 20, 93-103.

Johnsen, H.K., H. Tveiten, N.P. Villassen and A.M. Arnesen. 1999. Arctic charr (*Salvelinus alinus*) VTG: Development and validation of an enzyme-linked immunosorbent assay. Comp. Biochem. Physiol., 124B, 355-362.

Kanungo, J., T.R. Petrino and P.A. Wallace. 1990. Oogenesis in *Fundulus heteroclitus*. VI. Establishment and verification of condition for vitellogenin incorporation by oocytes in vitro. J. Exp. Zool., 25, 313-321.

Kishida, M. and J.L. Specker. 1993. VTG in tilapia (*Oreochromis mossambicus*): Induction of two forms by estradiol, quantification in plasma and characterization in oocyte extact. Fish Physiol. Biochem., 12, 171-82.

Korsgaard, B. and I.M. Petersen. 1979. Vitellogenin, lipid and carbohydrate metabolism during vitellogenesis and pregnancy in the blenny (*Zoarces viviparous* L.). Comp. Biochem. Physiol., 63B, 245-251.

Korsgaard, B. and K.L. Petersen. 1998. Vitellogenin in *Zoarces viviparous*: Purification quantification by ELISA and induction by estradiol 17β and 4-nonylphenol. Comp. Biochem. Physiol., 120C, 159-166.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-685.

Lee, K.B.H., E.H. Lim, T.J. Lam and J.L. Ding. 1992. Vitellogenin diversity in the perciformes. J. Exp. Zool., 264, 100-106.

Lomax, D.P., W.T. Roubal, J.D. Morre and L.L. Johnson. 1998. An enzyme-linked immunosorbent assay (ELISA) for measuring vitellogenin in English sole (*Pleruonectes vetulus*): Development, validation and cross-reactivity with other pleuronectids. Comp. Biochem. Physiol., 121, 425-436.

Mananose, E., S. Zanuy, F. Le Menn, M. Carrillo and J. Nunez. 1994. Sea bass (*Dicentrarchus labrax* L.) vitellogenin. I. Induction, purification and partial characterization. Comp. Biochem. Physiol., 107B, 205-216.

Matsubara, T., N. Ohkubo, T. Andoh, C.V. Sullivan and A. Hara. 1999. Two forms of vitellogenin, yielding two distinct lipovitellins, play different roles during oocyte maturation and early development of barfin flounder, *Verasper moseri*, a marine teleost that spawns pelagic eggs. Dev. Biol., 213, 18-32.

Moscoconi, G.O., R.M. Carnevali, A.M. Nabissi and M. Polzonetti. 1998. Gilthead seabream (*Sparus aurata*) vitellogenin: Purification partial characterization and

validation of on enzyme-linked immunosorbent assay (ELISA). Gen. Comp. Endocrinol., 110, 252-261.

Nish, K., M. Chikae, Y. Hatano, Y. Mizukami, H.M. Yamashita and R. Sakakibara. 2002. Development and application of monoclonal antibody-based sandwich for quantification of Japanese medaka (*Oryzias latipes*) vitellogenin. Comp. Biochem, Physiol., 132, 161-169.

Ng, T.B. and D.R. Idler. 1983. Yolk formation and differentiation in teleost fishes. In Fish. Physiol. Academic. Press. New Yolk, 4, 373-404.

Okumura, H., T. Todo, S. Adachi and K. Yamauahi. 2002. Changes in hepatic VTG mRNA levels during oocyte development in the Japanese eel, *Anguilla japonica*. Gen. Comp. Endocrinol., 125, 9-16.

Parks, L.G., A.O. Cheek, N.D. Denslow, S.A. Heppell, J.A. McLachlan, G.A LeBlanc and C.V. Sullivan. 1999. Fathead minnow (*Pimephales promelas*) VTG: Purification, characterization and quantitative immunoassay for the detection of estrogenic compounds. Comp. Biochem. Physiol. 123C, 113- 125.

Rodriguez, J.L., M. Kah, O. Geffard and F. Le Menn. 1989. Enzyme-linked immuno-sorbent assay (ELISA) for sole (*Solea vulgaris*) Vitellogenin. Comp. Biochem. Physiol., 92, 741-746.

Rønnestad, I. and H.J. Fyhn. 1993. Metabolic aspect of free amino acids in developing marine fish eggs and larvae. Rev. Fish. Sci., II, 239-259.

Rnnestad, I., E.P. Groot and H.J. Fyhn. 1993. Compartmental disruption of free amino acids and protein in developing yolk-sac larvae of Atlantic halibut (*Hippglossus hippoglossus*). Mar. Biol., 116, 349-354.

Selman, K. and R.A. Wallace. 1983. Oogenesis in *Fundulus heteroclitus*, III. Vitellogenesis. J. Exp. Zool. 226, 441-457.

Selman, K. and R.A.Wallace. 1989. Cellular aspects of oocyte growth in teleosts. Zool. Sci. 6, 211-31.

Sherry, J., M. Fielden, P. Hodson, B. Burnison and K. Solomon. 1999. An ELISA for brown trout (*Salmo trutta*) VTG and its use in bioassays for environmental estrogens. Sci. Total. Environ., 225, 13-31.

So, Y.P., D.R. Idler and S.J. Hwang. 1985. Plasma vitellogenin the landlocked Atlantic salmon, *Salmo salar*: Isolation, homologous radioimmunoassay and immunological crossreactivity with vitellogenin from other teleosts. Comp. Biochem. Physiol., 81B, 63-71.

Sumpter, J.P. and S. Jobling. 1996. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ. Health Perspect., 103, 173-178.

Takemura, A., A. Hara and K. Takano. 1991. Immunochemical identification and partial characterization of female-specific serum proteins in white-edged rockfish, *Sebastes taczanowskii*. Environ. Biol. Fish., 30, 49-56.

Tao, Y., A. Hara, R. Hodson, L.C. Woods and C.V. Sullivan. 1993. Purification, characterization and immunoassay of striped bass (*Morone saxatilis*) VTG. Fish Physiol. Biochem., 12, 31-46.

Tyler, C.R. and Sumpter. 1990. The purification and partial characterization of carp *Cyprinus carpio*, VTG. Fish Physiol. Biochem., 8, 111-120.

Utarabhand, P. and P. Bunlipatanon. 1996. Plasma VTG of grouper (*Epinephelus malabaricus*): Isolation end properties. Comp. Biochem. Physiol., 115C, 101- 10.

Wallace, R.A. 1985. Vitellogenesis and oocyte growth in non-mammalian vertebrates. In: Developmental Biology, Pergamon Press, Elmsford, New York, pp. 1127-1177.

Wallace, R.A. and K. Selman. 1985, Major protein changes during vitellogenesis and maturation of Fundulus. Dev. Biol. 110, 492-498.

Wiegand, M.D. 1982. Vitellogenesis in fishes. In Reproductive Physiology of Fish Pudoc Wageningen, The Nerherlands, pp. 136-146.

Zuxu, Y. and W.C. Laurence. 1996. A biochemical characterization of vitellogenins isolated from the marine fish ocean pout (*Macrozoarces americanus* L.), lumpfish (*Cyclopterus lumpus*) and Atlantic cod (*Gadus morhua*). Comp. Biochem. Physiol., 113B, 247-253.

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