Purification of Vitellogenin and Egg Yolk Protein, and Changes of Vitellogenin Concentration during the Ovulation Period in Elkhorn Sculpin, *Alcichthys alcicornis*

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This study was conducted to determine the serum vitellogenin (VTG) concentration changes during the ovulation period in elkhorn sculpin, *Alcichthys alcicornis*. The results of sepacryl S-300 showed that the molecular weight of VTG could be 380,000. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis may indicate that the purified VTG consists of three subunits with molecular weights of 180,000, 118,000 and 85,000, respectively. Yolk protein purified from the egg extracts was eluted on an equilibrated sephacryl S-300 column, and its molecular weight was estimated 250,000. The precipitation lines of the female serum against the antiserum of the egg extracts were fused completely by immunoelectrophoresis and immunodiffusion analysis. VTG was detected in the serum, and hepatocytes from males injected with 17 β-estradiol (E_2). Furthermore, VTG was immunochemically similar to yolk proteins. The concentration of VTG was high before ovulation ($9.80 \pm 0.81 \sim 11.02 \pm 0.09$ mg/ml), and then decreased rapidly after ovulation (less than 6.19 ± 0.59 mg/ml). This study suggested that VTG was synthesized in the liver by the action of E_2 and released to blood, and then incorporated into oocytes.

Key words : elkhorn sculpin, vitellogenin, ABC, 17 β-estradiol, immunoelectrophoresis, ovulation

Introduction

It is well known that the female-specific serum protein appears in the blood of sexually maturing female in a number of teleosts as well as other nonmammalian vertebrates. The female-specific serum protein, generally vitellogenin (VTG), is synthesized in liver by the action of estrogen released into the blood stream and transported to the developing ovary where it serves as the precursor of egg yolk protein. VTG can be induced in males or immature females by treating with estrogen, which is a calcium- and ion-binding glycolipophosphoprotein (Wallace and Selman, 1981; Mommsen and Walsh, 1988). VTG has been identified and partially characterized by immunochemical procedures (Aida et al., 1973; de Vlaming et al., 1980). Many investigators described characteristics of vitellogenesis by comparative studies between VTG and related egg yolk protein using various immunochemical techniques (Hara, 1976; Campbell and Ider, 1980; Hara et al., 1980; Takemura et al., 1991; Choi, 1995). It was reported that VTG synthesis at the begining of spawning period in the ovary was increasing, and reaching to its maximum concentration during or right before the spawning period, then decreasing after the spawning period in other species (Teranishi et al., 1981; Quinitio et al., 1989; Takemura et al., 1991). However, there have been no reports on the characteristics of VTG and yolk proteins in elkhorn sculpin, and on the determination of its change during the ovulation period. Therefore, this study was conducted to determine the immunochemical properties of the purified egg yolk protein, VTG in the blood of estrogen-treated male, and the related egg yolk protein

purified from elkhorn sculpin. Changes of serum VTG concentrations, and a physiological indicator for measurements of vitellogenic activities during the ovulation were also investigated.

Materials and Methods

Experimental fish

Elkhorn sculpin, *A. alcicornis*, were sampled once a week after fish were captured by bottom-trawl along the coast of Usujiri, Hokkaido, Japan. They were kept in an outdoor 1 ton tank filled with running sea water at Usujiri Fisheries Laboratory, Hokkaido University. After anesthetizing in 0.01% ethyl p-aminobenzoate solution, blood samples were obtained from the dorsal aorta with a 2.5 ml syringe. All blood samples were clotted at room temperature for 1 hour and centrifuged at 1,500 g for 15 minutes at 4℃ to prepare serum, and then stored at −80℃ until analysis.

To obtain egg extract, one part of the ovary containing vitellogenic oocytes was homogenized in 0.01 M phosphate-buffered saline (PBS), centrifuged at 8,000 g for 20 minutes at 4℃, and stored at −80℃ until analysis.

Preparation of specific antiserum

A specific antiserum against egg extract (a-E) was prepared according to the method of Takemura et al. (1991). A mixture of 0.5 ml of egg extract and an equal volume of emulsifier (Freund's complete adjuvant) was injected to the back of a rabbit intradermally once a week for 4 weeks. Four parts of a-E were absorbed with one part of pooled male serum (absorbed a-E, ab. a-E) to remove serum components.

Hormone treatment to males

One milligram of 17 β-estradiol (E_2) was dissolved in 0.5 ml of absolute ethanol and then diluted in an equal volume of saline. One microgram of E_2 solution per gram body weight was injected to mature males intramuscularly. For control, physiological saline was injected to mature males.

Immunohistochemical staining

A part of liver of male injected with E_2 was fixed in periodate-lysine-para-formaldehyde (PLP) solution for 2 hours, rinsed in PBS at 4℃ for 2 days and then embedded in paraffin (M.P. 56~58℃). Serial sections at 4 μm were stained immunohistochemically, with the avidin-biotin-peroxidase complex (ABC) according to the method of Hsu et al. (1981).

Electrophoresis and immunodiffusion

Immunoelectrophoresis, 6% of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and double immunodiffusion were performed on 1.2% agarose (Litex HSA, Denmark) according to the method of Grabar and Williams (1953), Laemmli (1970), Ouchterlony (1953), respectively.

Chromatography

The pooled female serum was applied to a hydroxylapatite (Pharmacia-LKB, Sweden) column (2.0×20 cm), the column was equilibrated with 0.4 M potassium phosphate buffer, pH 6.8 containing 0.05% phenylmethyl sulfonyflouride, and then protein was eluted with 1.2 M potassium phosphate buffer, pH 6.8. Chromatography with sephacryl S-300 (Pharmacia-LKB, Sweden) column (2.6×100 cm) was performed with 20 mM Tris-HCl buffer, pH 8.0, containing 2% NaCl and 0.1% NaN_3. The flow rate was adjusted at 16 ml/h. Protein in each fractionation was measured at 280 nm by using spectrophotometer (Hitachi, U-3000). The determination of molecular weight was done using a sephacryl S-300 column (2.6×100 cm) with thyroglobulin (mol. wt 669,000), apoterrine (mol. wt 443,000), catalase (bovine liver) (mol. wt 240,000), aldolase (mol. wt 160,000) and BSA (mol. wt 67,000) as marker proteins.

Measurement of VTG concentrations

To measure the serum levels of VTG, single radial immunodiffusion was performed according to the Mancini et al. (1965). Serum of each mature female were collected once a week. To obtain the standard curve for VTG, eight serial dilutions of the purified VTG (16 mg/ml) were placed in different wells. VTG concentrations of each fish were determined by the single radial immunodiffusion analysis.

Statistical analysis

All results are expressed as mean ± SEM. The changes of VTG concentrations were assessed by Student *t*-test.

Results

Purification of vitellogenin

Two peaks, the first peak (Fraction No. 10) and the second peak (Fraction No. 27) by using hydroxylapatite column chromatography, were collected and concentrated. Further purification of VTG was accomplished by using sephacryl S-300 column chromatography. The elution patterns were shown in Figs. 1 and 2, and these are showing the peak (Fraction No. 60) contained female-specific serum protein. From the eluted position, it can be estimated that VTG was a molecular weight of about 380,000. The analysis of SDS-PAGE of purified female-specific serum protein are shown in Figs 3 and 4, and these are showing three main bands which migrated at positions corresponding to molecular weights of 180,000, 118,000 and 85,000, respectively.

Purification of egg yolk proteins

Extract of egg yolk protein was applied on a sephacryl S-300 column chromatography. Two peaks, one small peak at the void volume and the other large peak followed by the small peak, were obtained (Fraction No. 51. and 65). Fraction No. 65 reacted against ab. a-E. The second large peak (Fraction No. 65)

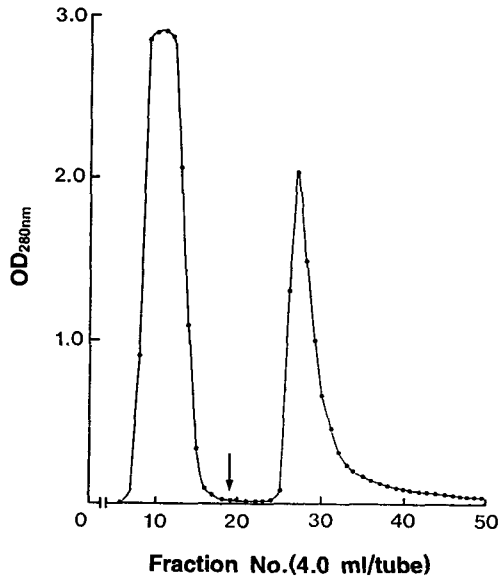

Fig. 1. Gel filtration profile on a Hydroxylapatite column of female serum. The proteins were eluted with 0.4 M potassium phosphate buffer (PPB), pH 6.8 in the first step, and in the second step (arrow) protein was elutesd with 1.2 M PPB, pH 6.8

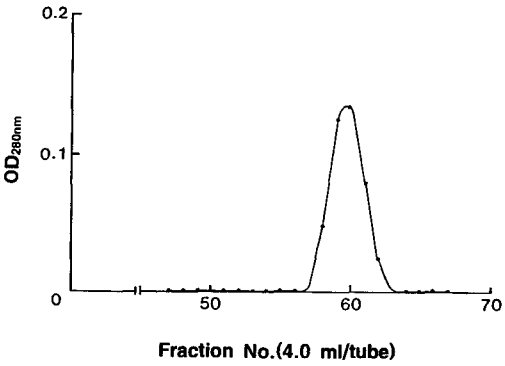

Fig. 2. Gel filtration profile on a Hydroxylapatite column was applied to sephacryl S-300 column equilibrated with 0.02 M Tris-HCl buffer, pH 8.0 containing 2% NaCl and 0. 1% NaN_3.

was collected and concentrated, and the protein could be egg yolk proteins (Fig. 5). From the eluted position, it was estimated that the molecular weight of this protein was 250,000.

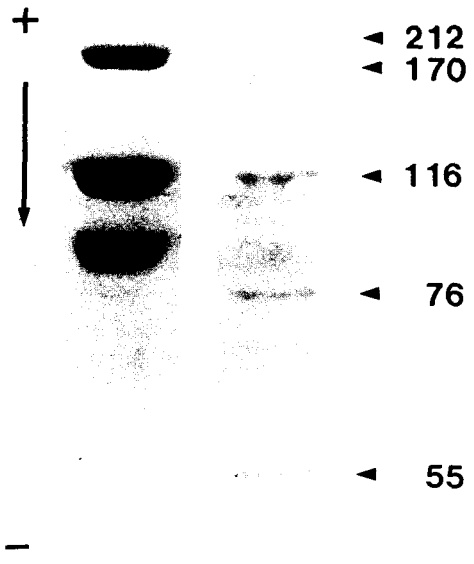

Fig. 3. SDS-polyacrylamide gel electrophoresis of purified female-specific serum protein (A) and, the molecular weights ($\times 10^3$) were mobilities from base relative to the standard proteins used (B). Arrow correspond to the standard proteins used; Hemocyanin (trimer) M.W. 212,000, Myogloblin; M.W. 170,000, β-Galactosidase, E. Coli.; M.W. 116,000, Human transferrin; M.W. 76,000, Glutamate dehyrogenase subunit; M.W. 55,440.

Induction of VTG in male serum by E_2 treatment

Immunoelectrophoretic patterns of serum from male fish treated with E_2 have shown that there was one precipitation line against the ab. a-E. The hepatic cells of elkhorn sculpin treated E_2 were stained by the immunohistochemical method (Fig. 6).

Immunological relationship of VTG and egg yolk protein

Immunoelectrophoretic and immunodiffusion patterns of the purified VTG and egg yolk protein against the ab. a-E are shown in Figs 7 and 8. The purified VTG and egg yolk proteins were reacted with the ab. a-E.

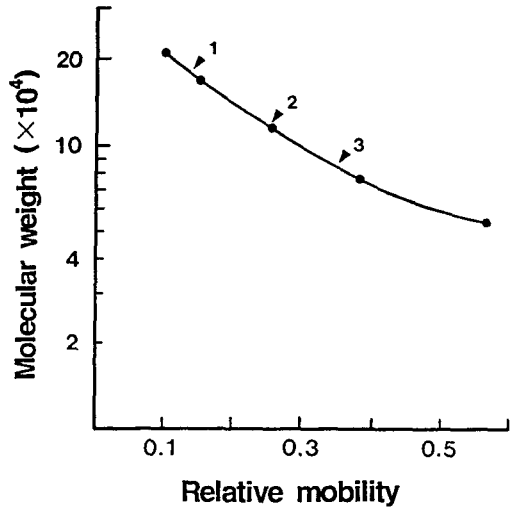

Fig. 4. Semilogarithmic plot of the molecular weights of marker proteins. The black circles correspond to the standard proteins used; subunit 1 (180,000), subunit 2 (118,000), subunit 3 (85,000). Arrow correspond to the standard proteins used; Hemocyanin (trimer) M.W. 212,000, Myogloblin; M.W. 170,000, β-Galactosidase, E. Coli.; M.W. 116,000, Human transferrin; M.W. 76,000, Glutamate dehyrogenase subunit; M.W. 55,440.

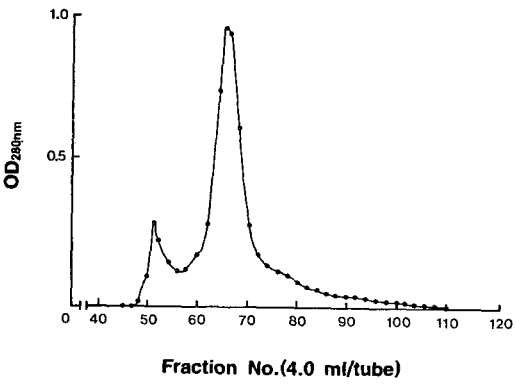

Fig. 5. Gel filtration profile on a hydroxylapatite column was applied to sephacryl S-300 column of egg extract with 0.02 M Tris-HCl buffer, pH 8.0 containing 2% NaCl and 0.1% NaN_3.

Changes of VTG concentrations during the ovulation period

Changes of VTG in serum during the ovulation period were shown in Fig. 9.

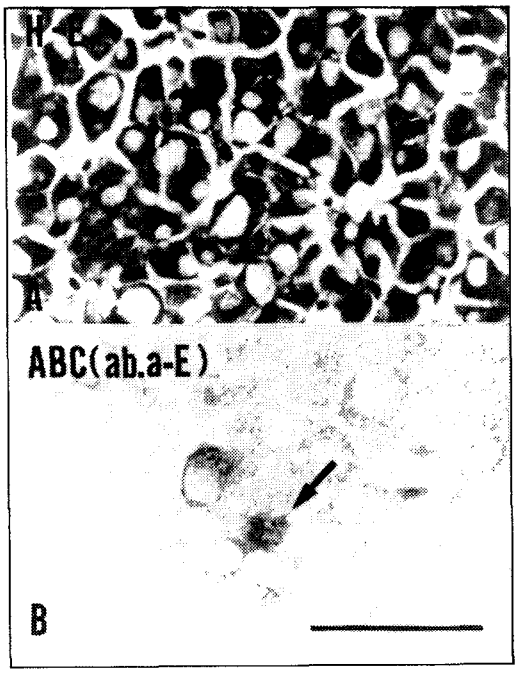

Fig. 6. Immunohistochemical staining of the liver in male with ab. a-E.
A; Male hepatic cells were injected by the physiological saline solution and stained with H · E method. B; Hepatic cells of the E_2 treated were stained with ABC method. Scale bar is 50 μm.
ab. a-E; Specific antiserum. Arrow is an indication of specific reaction by the immunohistochemical staining method in cytoplasm of male hepatic cells.

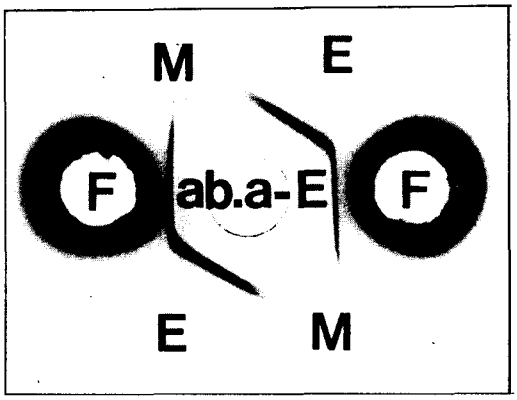

Fig. 7. Precipitin reaction of serum and egg yolk proteins against ab. a-E. MS; Male serum, FS; Female serum, E; Egg yolk proteins, ab. a-E; Specific antiserum.

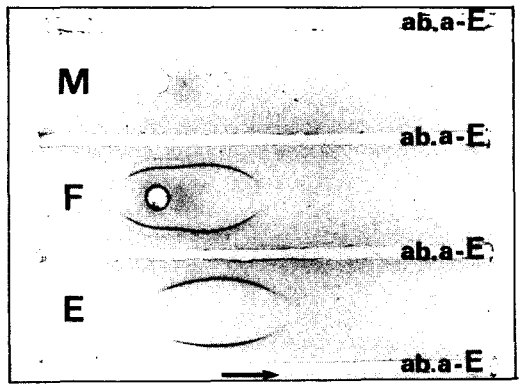

Fig. 8. Immunoelectrophoretic patterns of serum and egg yolk protein against ab. a-E. MS; Male serum, FS; Female serum, E; Egg yolk proteins, ab. a-E; Specific antiserum.

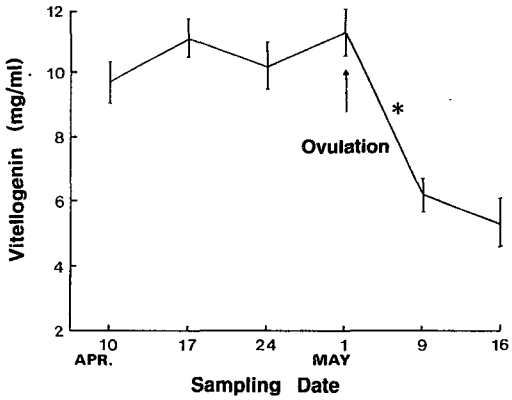

Fig. 9. Changes of vitellogenin from female elkhorn sculpin at the stage of ovulation. Vertical bars indicate the means ± SEM for ten fishes. * Significantly different at $p<0.05$, compared to the value before the ovulation (1st May).

VTG concentrations were maintained, but there was no statistical difference at high levels (9.8 ± 0.81~11.22 ± 0.09 mg/ml) before ovulation (from 10th April to 1st May). However, there was a significant decrease in VTG concentrations (less than 6.19 ± 0.59 mg/ml, $p<0.05$) after ovulation.

Discussion

In the present study, VTG from this fish was purified by using hydroxylapatite gel chromatography during the ovulation period. Hydroxylapatite gel chromatography was used to purify VTG from chum salmon (Hara, 1976), japanese eel (Hara et al., 1980), white-spotted charr (Hara et al., 1984).

Lipovitellin (Lv) type (Markert and Vanstone, 1971; Hara and Hirai, 1978; Campbell and Idler, 1980) and phosvitin (Pv) type (Mano and Lipmann, 1966; Markert and Vanstone, 1971; Campbell and Idler, 1980) of the egg yolk proteins have been purified from the ovary of fish. In salmonidae fish, the existence of egg yolk protein called as β-component including Lv and Pv, was recognized (Jared and Wallace, 1968; Market and Vanstone, 1971). It was reported that VTG was a precusor of egg yolk protein by using immunological techniques (Hara and Hirai, 1978; Hara et al., 1980, 1984).

In the present study, the antigenicity of egg yolk protein is immunologically similar to that of VTG. A reverse experiment using an antiserum against the egg yolk protein also indicated that a high similarity existed between VTG and egg yolk protein.

Jared and Wallace (1968) reported that the component of Pv type protein did not exist in some marine fish. However, it is well known that phosphorus is an essential element for embryogenesis in mammalian. The alkali-labile phosphorus from the egg extract in elkhorn sculpin was not detectable (Unpublished data). And it is possible that Pv of elkhorn sculpin may have little phosphorus concentration. It is considered that the selection of isotope method to improve the sensitivity was necessary with the selection of proper filtration equipment, for detection of Pv type component. However, Choi (1995) reported that the Pv type protein was detected in marine fish, fusilier, by the method of Martin and Doty (1949).

The concentration of VTG was maintained high for one week after the last ovulation and then decreased rapidly, indicating that there would be some VTG synthesis in the hepatocytes for a certain period after ovulation.

Generally, concentration of E_2 showed the similiar pattern to that of VTG, and concentration of E_2 was reached to its peak about 4 weeks prior to ovulation, and then decreased during the ovulation period in rainbow trout (Kobayashi et al., 1987) and arctic charr (Mayer et al., 1992). However, Guiguen et al. (1993) and Choi (1995) reported that concentration of VTG and E_2 were increased significantly during the spawning period in sea bass and fusilier. Whereas, this study indicated that the high concentration of VTG during ovulation could induce egg development which would activate VTG synthesis subsequently.

From the detected VTG by SDS-PAGE and immunoelectrophoresis against the ab. a-E, the existence of female-specific serum protein components in the serum of female elkhorn sculpin may has been confirmed by this study.

Recently, few female-specific serum proteins which were different from VTG normally, were investigated by Hara et al. (1980) and Takemura et al. (1991). However, Takemura et al. (1991) mentioned that VTG had no relationship with egg yolk protein and egg membrane in the blood of viviparous fish.

In this study, we can not examine further more about the female-specific serum protein in this fish. Therefore, the more detailed studies on hormonal and/or histological changes are required in elkhorn sculpin.

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