# Immunochemical Properties of Vitellogenins and Egg Yolk Proteins in Female Fusilier, *Caesio diagramma*

Cheol Young Choi, Young Jin Chang, Akihiro Takemura* and Kazunori Takano*

*Department of Aquaculture, National Fisheries University of Pusan, Pusan, 608-737, Korea*
**Tropical Biosphere Research Center, University of the Ryukyus, Sesoko, Okinawa 905-02, Japan*

# 농어목 어류, *Caesio diagramma*의 vitellogenin과 난황단백의 면역화학적 특성

최철영 · 장영진 · 竹村明洋* · 高野和則*

부산수산대학교 양식학과

*琉球大學 熱帶生物圈硏究センタ-

**This study was conducted to compare the immunochemical properties of female-specific serum proteins (vitellogenin, VTG) and egg yolk proteins in female fusilier, *Caesio diagramma*.**

**VTG of fusilier was identified and characterized by using immunochemical analysis. Two types of VTG (VTG1 and VTG2) reacted clearly with antiserum against egg proteins, were confirmed in the serum of mature female. The results of sephacryl S-300 showed that the molecular weights of VTG1 and VTG2 were 560,000 and 410,000, respectively.**

**Yolk proteins, E2 and E3, were isolated from egg extracts, and molecular weights of them were estimated 410,000 and 170,000, respectively.**

**The treatment of 17β-estradiol ($E_2$) to males has induced the synthesis of VTG of which immunological characteristics seems to be similar to the yolk proteins. The results suggest that VTG can be synthesized in the liver by the action of $E_2$ stimulation, and incorporated into the oocytes through the blood circulation. The level of serum $E_2$ was moderately high throughout the spawning period of June. The level of serum VTG was also sustained at high in May and June. The concentration changes of serum $E_2$ and VTG were corelated to the ovarian development in female fusilier.**

**The results indicated that $E_2$ may have some important roles for the vitellogenesis in female fusilier. Also, the VTG can be a precursor protein of yolk not only because it could be synthesized in the liver then incorporated into the oocytes but also because an egg yolk protein had the similiar molecular weights and antigenecity with VTG.**

**Key words ; Female fusilier, *Caesio diagramma*, EIA, Vitellogenin, Immunoelectrophoresis**

## Introduction

It is well known that a female-specific serum protein appears in the blood of sexually matured female during the normal breeding period in a number of teleosts (Wallace and Selman, 1981). The female specific serum proteins collectively named

VTG, and they have been identified in the blood of a number of teleosts by immunological, electrophoretic, chromatographic, or ultracentrifugal methods (Wallace, 1978 ; Wiegand, 1982). Proteins detected in female serum during the vitellogenesis had different physical and chemical forms from serum proteins found in male (Hara, 1978). VTG is synthesized in the liver by the action of estrogen, released into blood and then accumulated in the gonad as vitelline to stimulate the gonadal development (Wallace, 1978 ; Nath and Sundararaj, 1981). Thus, the examination of quantitative changes of VTG during the reproductive cycle of fish has a meaning in which it can give the important clue for observing the changes of vitellogenesis in fish. Many investigators described characteristics of vitellogenesis by comparative studies between VTG and related egg yolk proteins using various immunochemical techniques (Hara, 1978 ; Campbell and Ider, 1980 ; Hara et al., 1984 ; Takemura et al., 1991 ; Choi et al. 1995). The increase of VTG synthesis was reported at the begining of spawning period in the ovary, reaching to its maximum concentration during or right before the spawning period, and decreasing after the spawning period (Teranishi et al., 1981 ; Quinitio et al., 1989 ; Takemura et al., 1991).

The level of $E_2$ can be increased significantly during the spawning period (Moore, 1979). However, in case of goldfish and arctic charr, it was reported that consistency of $E_2$ was arrived to its peak 1 month prior to the spawning period in the sexually mature fishes (Kobayashi et al., 1986 ; Mayer et al., 1992).

Therefore, the changes of serum steroid hormones and VTG were investigated as the physiological indicators of vitellogenic activities.

## Materials and methods

*Experimental fish*

Female fusilier, *C. diagramma* used in this study were captured by fishing once a month from April to December, 1991, along the coast of Sesoko Island, Okinawa, Japan. They were kept in outdoor 45 ton tank at Tropical Biosphere Research Center, University of the Ryukyus. After anesthetizing in 0.01% ethyl p-aminobenzoate solution, blood samples were obtained from the caudal vasculature. All blood samples were clotted at room temperature for 30 minutes, and centrifuged at 3,000 rpm at 4℃ for 15 minutes to prepare serum, and then stored at -80℃ until analyzed.

To obtain egg extract, one part of the ovary containing vitellogenic oocytes was homogenized in 0.01 M phosphate-buffered saline (PBS), centrifuged at 10,000 rpm at 4℃ for 20 minutes and stored at -80℃ until analyzed.

*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) were 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 (ab. a-E) to remove serum components.

*Hormone treatment to male*

One mg of $E_2$ was dissolved in 0.5 ml of absolute ethanol and diluted in an equal volume of saline. One μg of $E_2$ solution per gram of body weight was injected to mature males intramuscularly. For control, physiological saline was injected into mature males.

*Electrophoresis and immunodiffusion*

Immuno-electrophoresis, 7.5% of polyacrylamide gel electrophoresis (native PAGE), rocket immunoelectrophoresis and double immunodiffusion were performed on 1.2% agarose (Litex HSA, Denmark) according to the method of Ornstein and Davis (1964),

Weeke (1973), Grabar and Williams (1953) and Ouchterlony (1953), respectively.

*Gel filtration*

Gel filtration was performed at 4℃ by using sephacryl S-300 (Pharmacia-LKB, Sweden) column (2.6×100 cm). The column was equilibrated 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).

*Estimation of alkali-labile phosphorous*

The eluates of the gel filtration were determined to estimate the concentration of alkali-labile phosphorus according to the method of Martin and Doty (1949). These fractions were measured by using spectrophotometer at 720 nm.

*Measurement of 17β-estradiol ($E_2$) in the serum*

Serum levels of $E_2$ were measured by the enzyme immunoassy (EIA) according to the method of Hibi et al. (1984). The standard of antibody solution was prepared from 0.05 to 12.8 ng/ml with BSA-PBS buffer. The maximum-binding wells were used to determine the enzyme activity bound to antiserum in the absence of free-$E_2$, of which well was served as the 'zero' standard (Bo) in determining the percent of bound (B/Bo), which is the average absorbance of the sample or standard divided by the average absorbance of the 'zero' standards on the same plate. A dose-response curve was generated by plotting the percent bound (B/Bo×100) versus log concentration.

*Ovarian maturation*

Ovarian maturation of fusilier fish were described in previous study (Choi et al. 1996).

## Results

*Identification of vitellogenin*

Immuno-electrophoretic patterns of serum derived from mature female and egg extract against ab. a-E are shown in Fig. 1. Two precipitation lines were observed in both female serum and the egg extract. The elution pattern of the mature female serum on sephacryl S-300 column is shown in Fig. 2. Five peaks containing a small peak at void volume and four large distinct peaks (F1, F2, F3 and F4) were obtained.

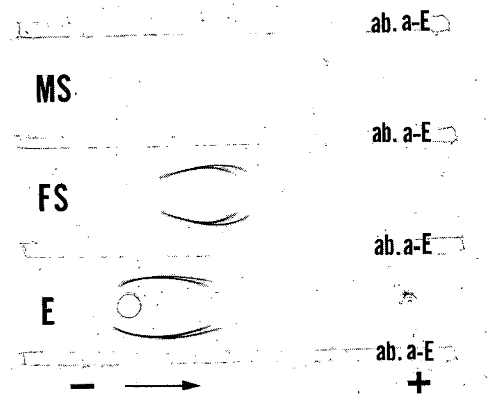



**Fig. 1. Immuno-electrophoretic patterns of serum and egg yolk proteins against ab. a-E. MS ; Male serum, FS ; Female serum, E ; Egg yolk proteins, ab. a-E ; Specific antiserum.**

The molecular weight (M.W.) of these four peaks were 560,000, 410,000, 170,000, and 130,000, respectively. Rocket immunoelectrophoresis with ab. a-E was carried out for each fractions to identify female specific serum proteins (Fig. 3). Two peaks of gel filtration were obtained and corresponded to F1 and F2.

*Identification of egg yolk protein*

An extract of egg applied on sephacryl S-300 is shown in Fig. 4. Four distinct peaks (E1, E2, E3 and E4) were obtained. Their M.W. were about 410,000 for E2 and 170,000 for E3, respectively. Among these peaks, E2 and E3 reacted against antibody to a-E (Fig. 5). When the amount of phosphorus was measured in each fractions, two phos-

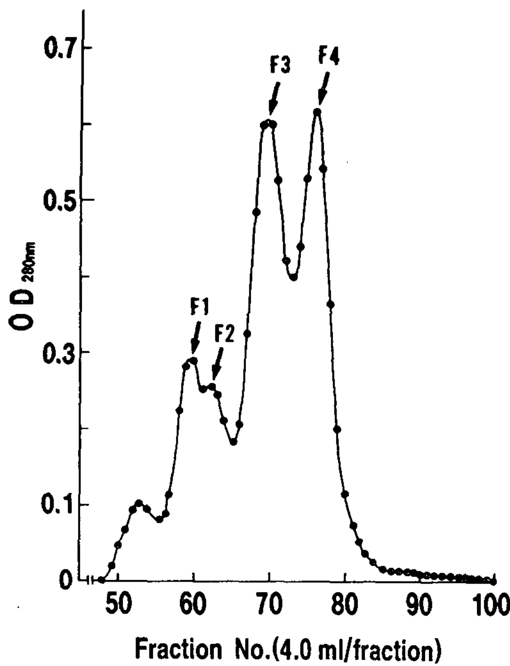



**Fig. 2. Gel filtration profile of serum from female using sephacryl S-300 column.**

phorus peaks (fraction No. 63 and 74) were obtained (Fig. 6). The first peak was corresponded to E2.

*Induction of vitellogenin in male serum by treating with $E_2$*

7.5% polyacrylamide gel electrophoresis (native PAGE) was performed for the sera of female, male, $E_2$-treated male, and egg extract (Fig. 7). Many protein bands were observed in all samples when compared the pattern of the male serum to that of the $E_2$-treated male. Two new bands were appeared in the serum from $E_2$-treated male. Female serum had the corresponding bands to them. Serum from $E_2$-treated male and nontreated male were applied to the sephacryl S-300 column. Two new peaks were not observed in serum from nontreated male, but appeared in serum from $E_2$-treated male (Fig. 8). Among these peaks, M2 and M3 reacted against ab. a-E. The fraction numbers of the peaks were corresponded to female-specific serum proteins identified in female serum (see Fig. 3).

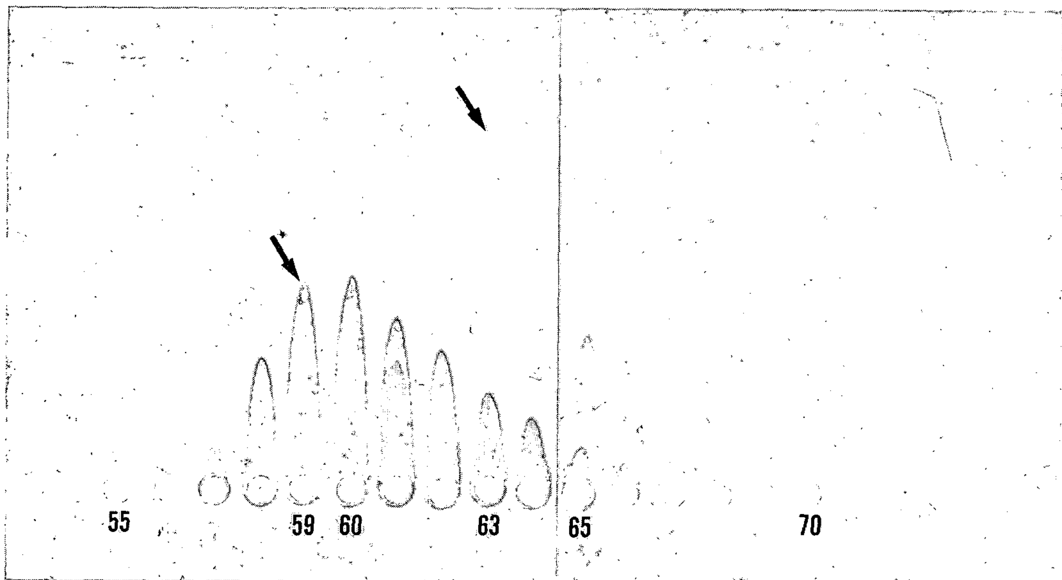



**Fig. 3. Rocket immuno-electrophoresis of each fractions with ab. a-E for identify female-specific serum pretein (VTG). Arrows indicate peaks of the VTG.**

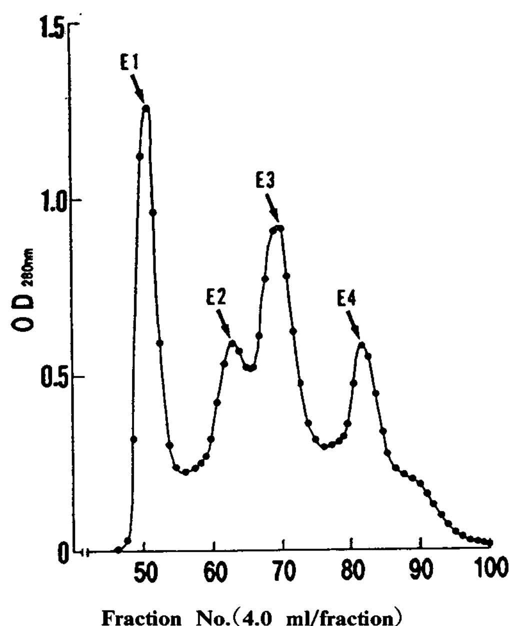



Fig. 4. Gel filtration profile of egg extract from fusilier, *Caesio diagramma* using sephacryl S-300 column.

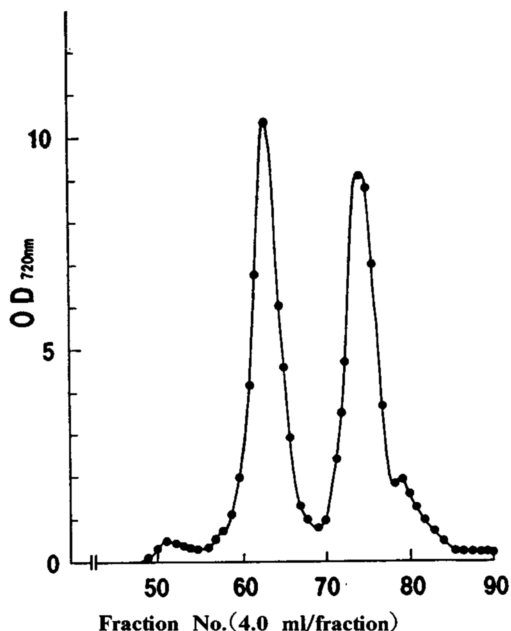



Fig. 6. Gel filtration profile of alkali-labile phosphorus content in egg extract fractions separated by sephacryl S-300 column.

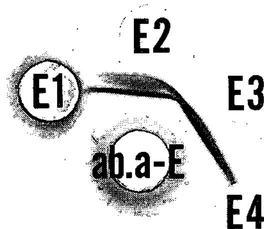



Fig. 5. Precipitiation reaction of fractions obtained from peaks (E1, E2, E3, E) on sephacryl S-300 column against specific antiserum (ab. a-E).

*Monthly changes of vitellogenin*

Monthly changes of VTG are shown in Fig. 9. VTG concentrations of each fish were expressed as a relative value against 100% of one fish undergoing active vitellogenesis in June. The concentrations of VTG were maintained high from April (11.17 ± 6.18%) to June (28.35 ± 9.04%). There was a rapid decrease in its concentration in July (3.38 ± 0.38%). From October to December, the levels remained very low (less than 1.90 %).

*Monthly changes of $E_2$ concentration*

$E_2$ concentrations were relatively high in May (2.4 ng/ml) and June (2.1 ng/ml). There was a decrease in its concentration in October (1.3 ng/ml)(Table 1). $E_2$ concentration was undetectable in November. $E_2$ concentration was decreased after vitellogenesis.

## Discussion

In this study, VTG from the female fish was identified by using immunochemical attempts in *C. diagramma.* On immuno-electrophoresis with ab. a-E, two precipitation lines were formed in serum of female *C. diagramma* undergoing an active vitellogenesis (May and June).

$E_2$ concentration was increased significantly during the spawning period of May and June. However, there was a little diffe-

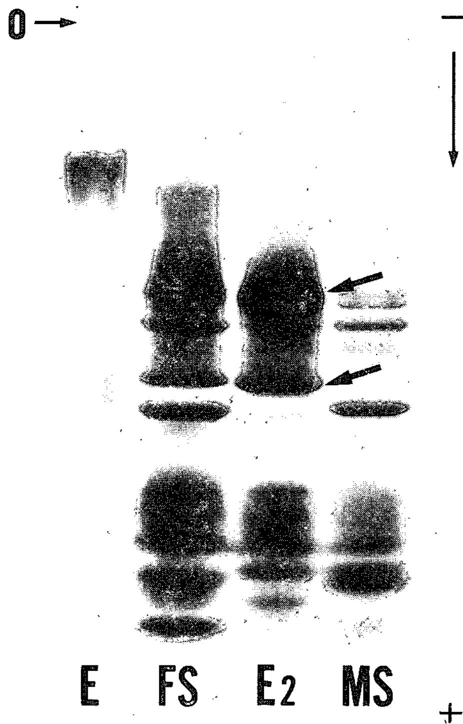



Fig. 7. Electrophoretic patterns of serum proteins derived from *Caesio diagramma* on native PAGE. MS ; Male serum, FS ; Female serum, E ; Egg yolk proteins, $E_2$ ; $E_2$-treated male.

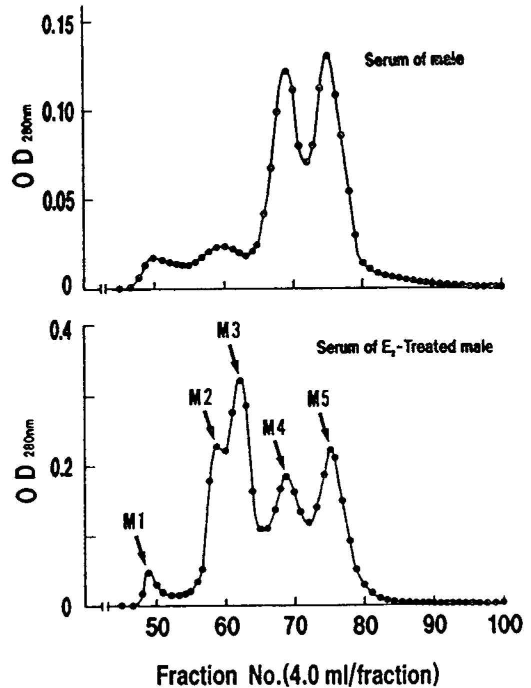



Fig. 8. Gel filtration profile of sera of male and $E_2$-treated male using sephacryl S-300 column.

rence from the tendency of increase and decrease in $E_2$ concentrations which was widely known in Cyprinidae fishes and Salmonid fishes. In rainbow trout (Kobayashi et al., 1987), the consistency of $E_2$ was reached to its peak about 4 weeks prior to ovulation, and then decreased during the ovulation period. In case of arctic charr, Salvelinus alpinus, it was reported that there was not big change of $E_2$ consistency in immature fish, whereas $E_2$ consistency was reached to its peak 1 month prior to spawning in mature fish, also observed in marine fish (Mayer et al., 1992).

The result was similar to that from sea bass, *Lates calcarifer* (Guiguen et al., 1993). Moore (1979) reported that sea bass, *L. calcarifer* was subtropical and tropical fishes

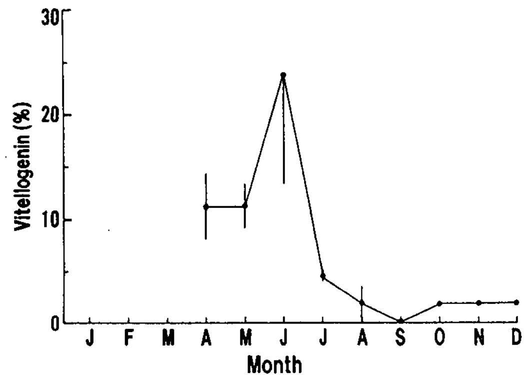



Fig. 9. Monthly changes of vitellogenin in female fusilier, *Caesio diagramma*. The mark with vertical bars indicate the mean±SEM.

to live in the Indian ocean, and that it was protandorous hermaphoroditic species. Based on these results from the study on Perciformes fish inhabiting in the tropic or subt-

**Table 1. Monthly changes of 17β-estradiol ($E_2$) concentrations determined by enzyme immunoassay (EIA) in the serum of *Caesio diagramma*.**

| Variation | Month | Mean $E_2$ Concentration (ng/ml) | Number of Determination | C. V* (%) |
|---|---|---|---|---|
| Within assay | Apr. | 1.4 | 3 | 7.8 |
| | May | 2.4 | 3 | 1.2 |
| | Jun. | 2.1 | 3 | 12.9 |
| | Jul. | 1.7 | 3 | 8.6 |
| | Aug. | -** | 3 | - |
| | Sep. | 1.4 | 3 | 9.6 |
| | Oct. | 1.3 | 3 | 4.4 |
| | Nov. | - | 3 | - |

* Coefficient of variation (standard deviation/mean×100)
** No data

ropical zones, $E_2$ might be secreted in the oocyte during the spawning period which was different from the other fish species. In the studies on sea bass, the concentration of serum $E_2$ sustained significantly high from October to February during the spawning period. The monthly changes of $E_2$ was shown the tendency to the change of VTG. VTG can be synthesized in the liver, it can be supposed that the developing process of matured oocyte is very short. It is considered that this phenomenon might be one of the characteristics of fish inhabiting in the tropic or subtropical zones.

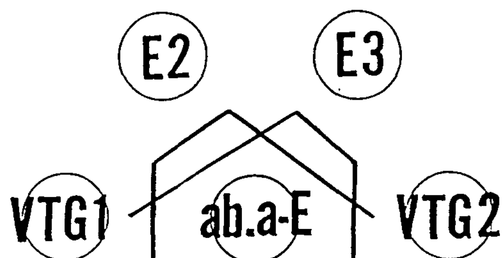
Four peaks (E1, E2, E3 and E4) were shown from the present chromatographic analysis in the egg during an active vitellogenesis. Since E2 and E3 reacted with ab. a-E, which were determined as yolk proteins of *C. diagramma.* On the contrary, E1 and E4 did not react with the antiserum and seemed not to be yolk proteins. E1 eluted near to void volume. Thus, this protein might be a macromolecules with high lipid content. Detailed natures about E4 remained unclear.

*C. diagramma* has two types of VTG and egg yolk proteins. It is well established that the eggs of teleosts, containing yolk proteins which are termed lipovitellin (Lv) type and phosvitin (Pv) type have been isolated in the ovary of fish (Wallace, 1978 ; de Vlaming, 1983). In general, Lv contains low content of phosphorus, whereas Pv contains high content of phosphorus and serine. It has been found that Lv has considerably higher M.W. than Pv. The M.W. of Lv was estimated to be 300,000 for *Oncorhynchus mykiss* (Hara and Hirai, 1978 ; Campbell and Idler, 1980), and 500,000 for *Psudopleuronectes americanus* (Ng and Idler, 1979). On the other hand, the M.W. of Pv was determined to of 43,000 for *O. mykiss* (Campbell and Idler, 1980). In this study, the M.W. of E2 and E3 were estimated to be 410,000 and 170,000, respectively. Judged from this results, it may be suggested that E2 and E3 are both Lv-like yolk proteins. Moreover, since E2 fused with E3 immunologically, it can be suggested that E2 has a dimeric structure of E3. It is likely that a Pv-like protein can be detected in the egg of *C. diagramma.* E2 had alkali-labile phosphorus in this fish. Instead of Pv, therefore, E2 might have a role as a reservoir of phosphorus in *C. diagramma.* But, Jared and Wallace (1968) has reported that the component of Pv type protein does not exist in marine fish. Choi et al.(1995) has also reported that Pv type protein was undetected in elkhorn sculpin, *Alcichthys alcicornis.*

It became obvious from this study that both VTG and two yolk proteins(E2 and E3) had an intimate relationship, because

the precipitation lines fused each other completely (Fig. 10). As mentioned above, there were two types of VTG, E2 and E3. Each VTG had corresponding E2 and E3. The following formula were based on the results of M.W. and immunodiffusion for the two types of VTG and egg yolk proteins in *C. diagramma* ;

VTG1(560,000) = E2′ (410,000)
 + E3′ (170,000) = 3E
VTG2(410,000) = E2″ (410,000)
 = 2E3″ 2(170,000)




| | M. W. |
|---|---|
| VTG1 | 560,000 |
| VTG2 | 410,000 |
| E2 | 410,000 |
| E3 | 170,000 |

VTG1 = E2 + E3
VTG2 = E3′ + E3′ = E2′

**Fig. 10. Schematic formula for the molecular weights between VTG and egg yolk proteins.**

It is suggested that VTG can be a precursor protein of yolk not only because it could be synthesized in the liver then incorporated into the oocytes but also because an egg yolk protein had the similiar molecular weights and antigenecity with VTG.

In this study, since two types of VTG were not able to isolate, the characteristics of each VTG remained unclear. In order to obtain more informations, detail analysis of amino acid compositions of VTG and yolk proteins are required.

## 요 약

농어목 어류, *C. diagramma* 암컷의 vitellogenin과 난황단백의 면역화학적 특성을 서로 비교하였다. 면역화학적 방법에 의해 *C. diagramma* 암컷의 혈청에서 vitellogenin 1 (VTG1)과 vitellogenin 2 (VTG2)를 동정하였으며, sephacryl S-300 gel 여과법에 의하여 측정된 분자량은 각각 560,000과 410,000이었다. 암컷의 난소 추출액에서는 두 가지 형태의 난황단백인 E2와 E3가 분리되었으며, 이들의 분자량은 각각 410,000과 170,000이었다.

17β-estradiol ($E_2$)을 주사한 수컷의 혈청과 간조직을 abidine-biotin complex (ABC) 방법으로 분석한 결과, vitellogenin과 유사한 물질이 검출되었다. 이상의 결과로 부터, 외인성 $E_2$에 의해 간에서 합성된 vitellogenin은 혈액 속으로 방출된 후 난소로 이동되는 것으로 추정되었다. 암컷의 $E_2$ 혈중농도는 산란기인 6월에 최고치를 나타내었고, vitellogenin 상대농도도 5~6월에 최고치를 나타내었다.

$E_2$와 vitellogenin의 변화는 본 종의 난소성숙과 밀접한 관계가 있으며, 난황형성에 있어서도 중요한 역할을 하고 있음을 알 수 있었다. 암컷 혈청 VTG2와 난소추출액 E2의 분자량이 서로 같고, vitellogenin이 간에서 합성되어 난소내로 이동하는 점으로 보아, 난소내 난황단백 성분의 전구물질은 혈청내의 vitellogenin인 것으로 추정되었다.

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