A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Development of an immunological probe to measure reproductive effort of the Suminoe oyster, *Crassostrea ariakensis*

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GRADUATE SCHOOL

CHEJU NATIONAL UNIVERSITY

2009. 2.

Development of an immunological probe to measure reproductive effort of the Suminoe oyster, *Crassostrea ariakensis*

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A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science

2009. 02.

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2009. 02.

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CONTENTS

국 문 요 약

이 연구는 강굴 (*Crasostre ariakensis*)의 번식생태학적 특성을 규명하기 위해 실시되었으며, 강굴 난 특이 단백질에 대한 다클론 항체를 개발하여 강굴의 번식량을 정량적으로 측정하였다. 난 단백질에 특이적으로 발현하는 항체 개발을 위해 성숙한 강굴 암컷에서 알을 분리한 후, 알 추출물을 4회에 걸쳐 토끼에 주사하였다. 항 혈청의 교차반응 확인을 위해 double immunodiffusion test, ELISA, 그리고 western blotting을 실시하였다. 본 연구에서 개발된 항체는 초기에 강굴의 외투막, 아가미, 폐각근 및 체조직에 약한 교차반응 보였다. 이러한 교차반응은 활성화된 glutaric dialdehyde 굴 체조직 단백질을 부착시켜 면역흡착제를 준비한 뒤, 이를 항혈청과 반응시켜 제거하였다. 효소항체면역법 (enzyme-linked immunosorbent assay, ELISA) 수행 결과, 이 연구에서 개발된 rabbit anti-oyster egg IgG는 0.25-10 μg/ml의 egg 단백질을 측정할 수 있었으며, 실제 강굴에 포함된 난을 정량적으로 분석한 결과, 체내에 포함된 1% 가량의 알 까지 정량적으로 측정이 가능하였다.

강굴 번식량을 측정하기 위하여 2007년 1월부터 7월까지 섬진강 하구에서 매월 40 개체씩 채집하였다. 조직학적 관찰 결과, 1월에 처음 초기발달단계 개체가 관찰되었고, 6월과 7월에 대부분의 굴은 성성숙 단계였다 (85-87%). 또한 7월에 13%의 산란개체를 관찰할 수 있었다. 생식소중량지수 (Gonado-somatic Index, GSI)는 대부분의 암컷 개체가 초기와 후기 발달단계를 보인 4월에 0.7-14%였고, 성성숙 시기인 7월에는 17.5-67%를 나타냈다.

포란수는 ELISA를 통하여 추정된 알 전체 무게를 알 한 개 무게인 14 ng으로 나누어 추정하였다. ELISA방법에 의하여 측정된 성숙한 강굴의 알 무게

를 알 한 개의 무게로 나누어 추정한 결과, 5-7월 성숙기의 강굴은 개체 당 1.6 – 9.1 억개의 알을 포함하고 있었으며, 굴의 크기 (건중량)와 포란수 사이에는 양의 상관관계가 있었다.

이 연구에서 개발된 강굴 알 특이단백질 다클론 항체와 효소면역학적 방법 (ELISA)은 강굴의 번식량을 추정하는데 있어 매우 신속하고 민감한 방법으로 간주되며, 향후 다른 해산 유용 이매패류의 번식량 연구에도 효과적으로 사용할 수 있을 것으로 사료되었다.

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1. Introduction

The Suminoe oyster, *C. ariakensis* (Fujita 1913) is distributed in estuarine area along the coasts of northern (Yellow River) and southern China (Zhejiang, Yangjiang and Zhuhai), Japan (western Kyushu) and Korea (west and south coast) from low intertidal to depth of 15 m (Okutani, 2000; Zhou and Allen, 2003; Lam and Morton, 2004; Yoo et al., 2004; Zhang et al., 2005; Yoon et al., 2008). As an estuarine species, the Sumino oyster occurs in wide range of temperature and the salinity (Zhou and Allen, 2003; Joo, 2006; Park, 2007; An et al., 2006). Sumino oyster is also known to grow faster than any other oysters. According to Harding and Mann (2006), 1 to 5 year old *C. ariakensis* grow 27-55 mm (1 year) to 170-204 mm in shell length (5 years). Due to its fast growth and unique taste, Suminoe oyster has been considered as an aquaculture candidate in Korea (Yoo et al., 2004; An et al., 2006). Since the Sumino oyster also is proven to be more resistant to *Haplosporidium nelsoni* and *Perkinsus marinus*, the two major pathogenic parasites in Chesapeake Bay (Calvo et al., 2001; Paynter et al., 2008), the Sumino oyster is now considered to be introduced from Asian water to the Bay to replace the American oyster (Allen, 2000; Calvo et al., 2001; Grabowski et al., 2003). Accordingly numerous studies have been carried out to understand genetics, ecology and reproduction of the Sumino oysters in China and in the USA (Perdue and Erickso, 1984; Langdon and Robinson, 1996; Zhang et al., 2005; Wang and Guo, 2008; Wang et al, 2008). In contrast, only a few studies have investigated reproduction and ecology of the Sumino oyster in Korean water (Yoo et al., 2004; Joo, 2006; Park, 2007; Yoon et al., 2008).

Understanding reproductive pattern of marine bivalves such as an annual reproductive cycle and the quantity of gametes produced during spawning is crucial in the management of natural population as well as to develop an aquaculture industry. In particular,

measurement of reproductive effort, the proportion of energy or materials that oyster allocates to reproduction is crucial in the management of the brood stock either in natural population or in the hatchery (see Gosling, 2003). In oysters, reproductive effort is often measured the weight or number of the gamete using various methods (Deslous-Paoli and Heral, 1988; Pouvreau et al., 2000; Choi et al., 1993; Kang et al, 2003; Royer et al., 2008). Despite the importance, few studies have reported reproductive effort of marine bivalves due to the difficulties involved in the measurement; gonad of marine bivalve is often an integral part of the visceral mass and technically difficult to separate and measure in most cases (see Lucas, 1982). Alternatively, immunological method has been applied in the quantification of the reproductive effort. For the measurement, polyclonal antibodies are raised from the egg proteins and the reproductive effort is estimated using an enzyme-linked immunosorbent assay (ELISA) using the polyclonal antibodies (Choi et al., 1993; Kang et al., 2003; Park and Choi, 2004; Park et al., 2005; Royer et al., 2008). According to Park and Choi (2004) ELISA is sensitive enough to detect minute quantity of gametes and furthermore, it is fast and cheap.

The objective of this study is to develop an immunological probe to measure reproductive effort of the Sumino oysters in an annual reproductive cycle. The study also estimate fecundity of the Sumino oyster during spawning season based on the reproductive effort estimated using the immuno-probe.

2. Materials and methods

2.1. Study area and sampling effort

Suminoe oyster was collected monthly from an estuarine area at Seomjin River on the southern coast of Korea from January to July 2007 (Fig. 1). Upon arrival at the laboratory,

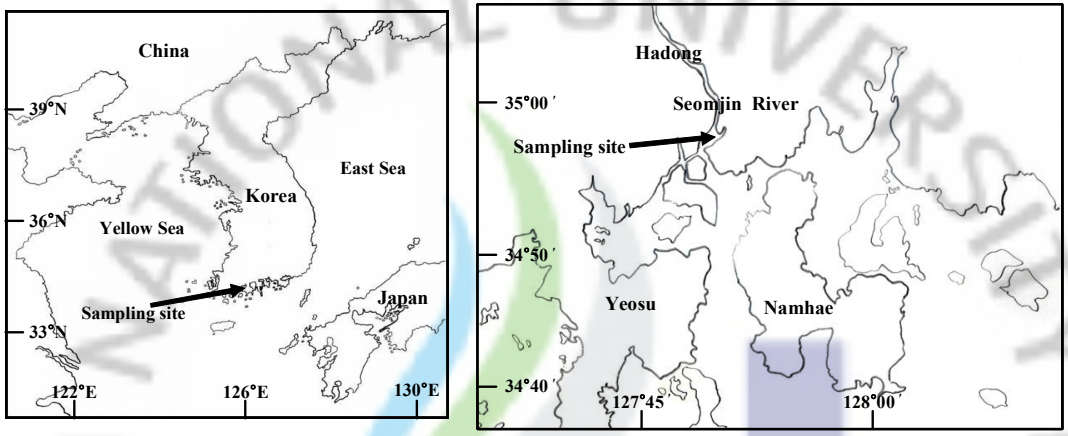

Fig. 1. Map showing the sampling location.

biometric parameters of individual oyster were recorded including shell length (SL, longest axis), shell height (SH), shell width (SW), total weight (TWT) and tissue wet weight (TWWT). The internal shell cavity volume was determined by the water displacement method according to Galtsoff (1964) to determine the condition index (CI). To compare the CI with other studies, CI-I (Yoo et al., 2004; Joo, 2006; Kang et al., 2007) and CI-II (Lawrence and Scott, 1982; Kang and Choi, 1999; Massapina et al., 1999) were calculated according to the following formula; CI-I = TWWT x 1,000/(SL x SH x SW) and CI-II = TDWT (tissue dry weight) x 1,000/internal shell cavity volume.

A longitudinal section was made in the middle of each oyster through the digestive gland, gill and mantle. The section was fixed in Davidson's fixative for 48h. The remaining tissues were lyophilized at each sampling date and kept at -70 ℃ until used.

2.2. Histology

For histological study, the previously cross-sectioned soft tissue of each oyster was fixed in Davidson's solution for 48 h. After fixation, the tissue was dehydrated and embedded in paraffin. The paraffin block was sliced to 6 μm and stained with Harris' hematoxylin and Eosin Y.

Gonad development was also analyzed quantitatively using the planimetric technique. The histological slides were scanned and the percentage gonad area (PGA, %) of each slide was measured from the digitized images using an image analyzing software. PGA was then calculated by the gonadal area divided by the total cross-sectional area except the gill (Fig. 2).

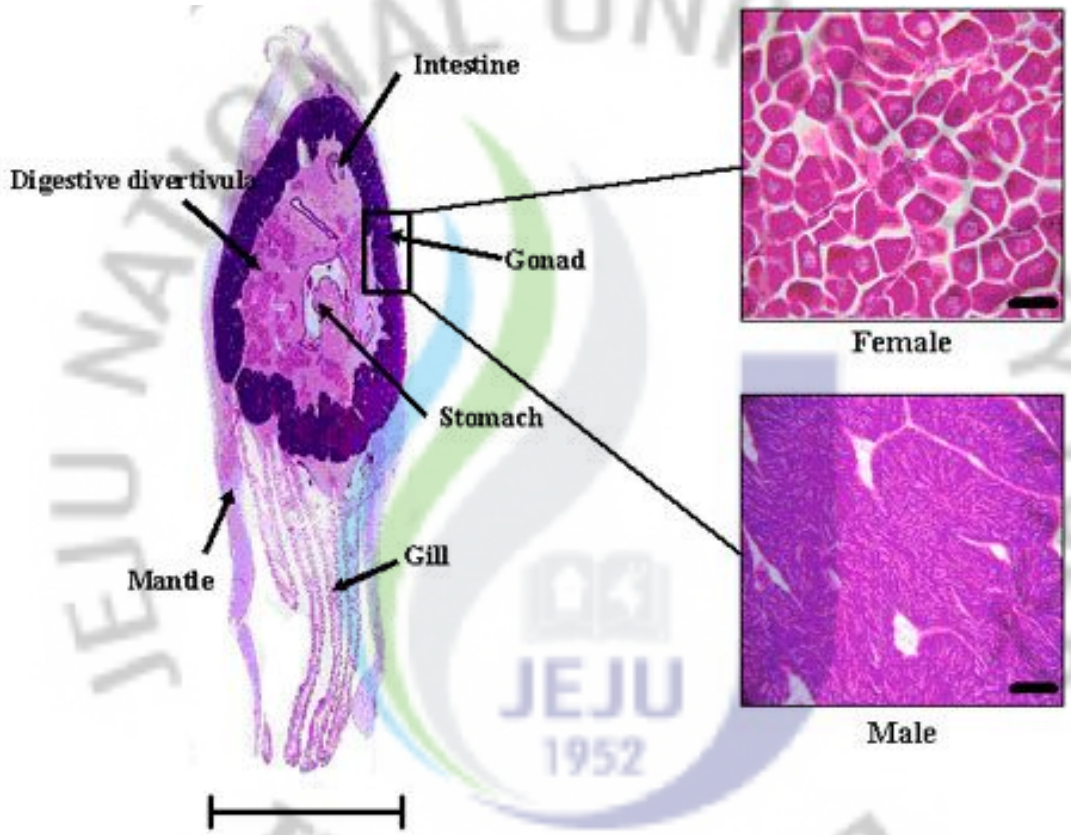

Fig. 2. Cross-section of *C. ariakensis*. Scale bar: 1cm. The female and male oysters could observe under light microscope. Scale bar: 100 μm.

2.3. Purification of oyster eggs

Fully matured market-sized (14-23 cm in shell height) female oysters were used as the source of the ripe eggs. The mature eggs were extracted from the gonad and transferred in phosphate buffered saline (PBS, pH 7.4). Crude oyster egg extracts were then filtered through a 100 μm mesh screen to remove tissue debris. Filtered egg suspensions were transferred into a 50 ml conical tube at 4 °C for 1 h, and the supernatant was decanted. This cleaning procedure was repeated five times. The purified oyster eggs were stored at -70 °C until used. To evaluate the weight of a single egg, subsample of purified eggs in a known volume of suspension were taken and counted using a haemocytometer. A same volume of eggs was then lyophilized and weighed. Total weight of eggs was then divided by the total number of eggs counted in order to determine weight of an individual egg.

2.4. Biochemical composition of the purified eggs and oyster tissue

The protein concentration in the purified eggs and in the oyster tissue was determined using Lowry et al. (1951)'s method after alkaline hydrolysis with 0.1M NaOH at 37 °C for 2 h using bovine serum albumin as the standard. Total carbohydrates were quantified photometrically by the phenol-sulfuric acid extraction method of Taylor (1995) with dextrose anhydrose as a standard. Total lipids were extracted using a mixture of chloroform and methanol (Bligh and Dyer, 1959) and measured gravimetrically. Ash content was obtained by igniting a subsample (100 mg) of homogenized tissue in a muffle furnace at 450 °C for 24 h. The estimation of the total protein, lipid, carbohydrate and ash content was based on the dry tissue weight of each individual and expressed as mg/TDWT.

2.5. Development of oyster egg-specific antibody

The development of the rabbit anti-oyster egg IgG was raised following the protocol outlined by Kang et al. (2003) (Fig. 3). After immunization, the rabbit anti serum was

assessed to check the specificity between the serum and the egg protein using a double-immunodiffusion test according to Ouchterlony and Nilsson (1978), ELISA and western blotting.

The double immuno-diffusion test indicated that the antiserum exhibited a weak cross-reactivity to the non-gonadal tissue proteins. In order to remove the non-specific antibodies in the rabbit antiserum, an immunoadsorbent was prepared by polymerizing sexually undifferentiated oyster tissue using glutaric dialdehyde according to Fuchs and Sela (1978). Twenty milliliters of the rabbit antiserum was mixed with an equal volume of the immunoabsorbent and incubated for 3 h at room temperature. Non-specific antibody in the antiserum bound to the surface of the immunoadsorbent particles during incubation while the egg-specific antibody remained free. The oyster egg-specific antibody was then isolated from the unbound fraction of the antiserum by centrifugation. The rabbit anti-oyster egg IgG in the supernatant was precipitated using 50% saturated ammonium-sulfate and the precipitate was dissolved and dialyzed in PBS (Fig. 4). The specificity of the rabbit anti-oyster egg IgG was tested again using immunofluorescence, ELISA and Western blotting, and no further cross-reaction with somatic proteins was demonstrated (Fig. 6, 7 and 9).

2.6. Quantification of oyster eggs using ELISA

An indirect enzyme-linked immunosorbent assay (ELISA) was performed to determine the amount of oyster egg protein in the lyophilized oysters following the protocol developed by Kang et al. (2003). As positive controls, the Sumino oyster egg protein was prepared in 100 ng/ml-20 µl in PBS and added to a polystyrene 96-well microplate. Sexually undifferentiated oyster tissue homogenate was also included in the plate as the negative controls. The microplate containing those controls and oyster samples was incubated at 4 °C overnight or at 37 °C for 3 h. After incubation, the wells were then washed four times with washing solution containing Triton-100 in PBS (PBST), and 150 µl of 1% bovine serum albumin was added to each well as a blocking agent. The plate was incubated for another 1 h

and washed four times with PBST-100. After incubation the oyster egg specific antibody developed in this study was applied as the primary antibody (6.8 μg/ml) and allowed one hour to react. After incubation, the plate was washed 4 times with PBST-100 to remove the unbound antibody. Goat anti-rabbit IgG alkaline phosphatase conjugate (1:1,000, Sigma) as a secondary antibody diluted in blocking buffer was added and incubated again for 1 h. Finally, p-nitrophenylphosphate (p-NPP) substrate dissolved in 0.1 M glycine buffer was added as a coloring agent and the optical density of each well in the plate was measured at 405 nm using a micro-plate reader. The quantity of egg protein in an oyster sample was then estimated from a titration curve constructed using a regression between optical densities of the oyster egg protein added in the plate as the positive control and the concentrations of the egg preparations. Based on the egg protein estimation using ELISA, the quantity of egg was then estimated by multiplying the quantity of the egg protein measured by ELISA by 1.96, the ratio of the egg protein to total egg weight estimated in this study. Finally, a weight-based gonad-somatic index (GSI) as a ratio of the egg mass to the total tissue weight, was established.

2.7. Immunofluorescence assay

An immunofluorescence assay was carried out to visualize and localize the rabbit anti-oyster egg IgG and oyster egg protein interaction. Series of histological blocks containing ripe eggs were sliced to 6 μm, deparaffinized and rehydrated. The sections were incubated with 5% bovine serum albumin (BSA) in PBST-100 as a blocking agent. After blocking, the tissue sections were incubated with the rabbit anti-oyster egg IgG (1/100 diluted) at room temperature for 1 h. The tissues were then washed five times in PBST-100, incubated with fluorescein isothiocyanate-conjugated (FITC) goat anti-rabbit IgG (1:400 dilution, Sigma) as a secondary antibody for 1 h and again washed in PBST-100. Presence of the antibody-antigen reaction in the slides was observed under a fluorescence microscope.

2.8. Characterization of Oyster egg-specific protein using western blotting

The oyster egg proteins and other oyster samples were separated according to the different sizes of protein by 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to polyvinylidene difluoride (PVDF) membrane and blocked with 5% skim milk in TBS-T (Tris-buffered saline with Twen 20) for 3 h. The membrane was then incubated with diluted (1:1,000) anti-oyster egg IgG as a primary antibody in blocking buffer overnight at 4 °C. After washing with TBS-T, goat anti-rabbit IgG horseradish peroxidase conjugated (HRP, 1/3,000 diluted with TBS-T) was added onto the membrane as the secondary antibody and incubated for 1 h at room temperature. The final antibody-antigen complexes in the blotted membrane were visualized with a luminescent reagents (Thermo Scientific).

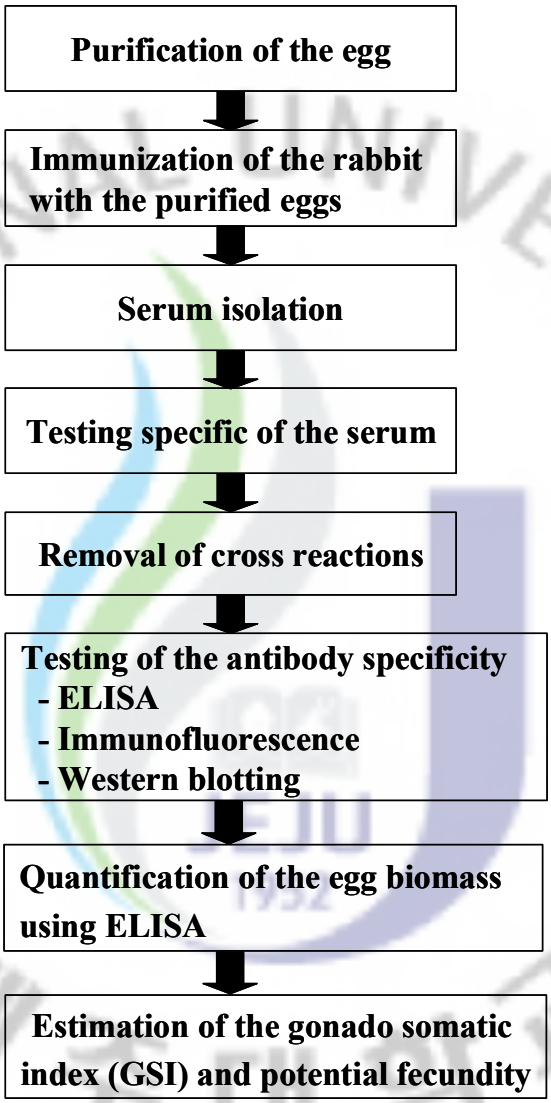

Fig. 3. Protocol used in the development of Suminoe oyster egg-specific antibody

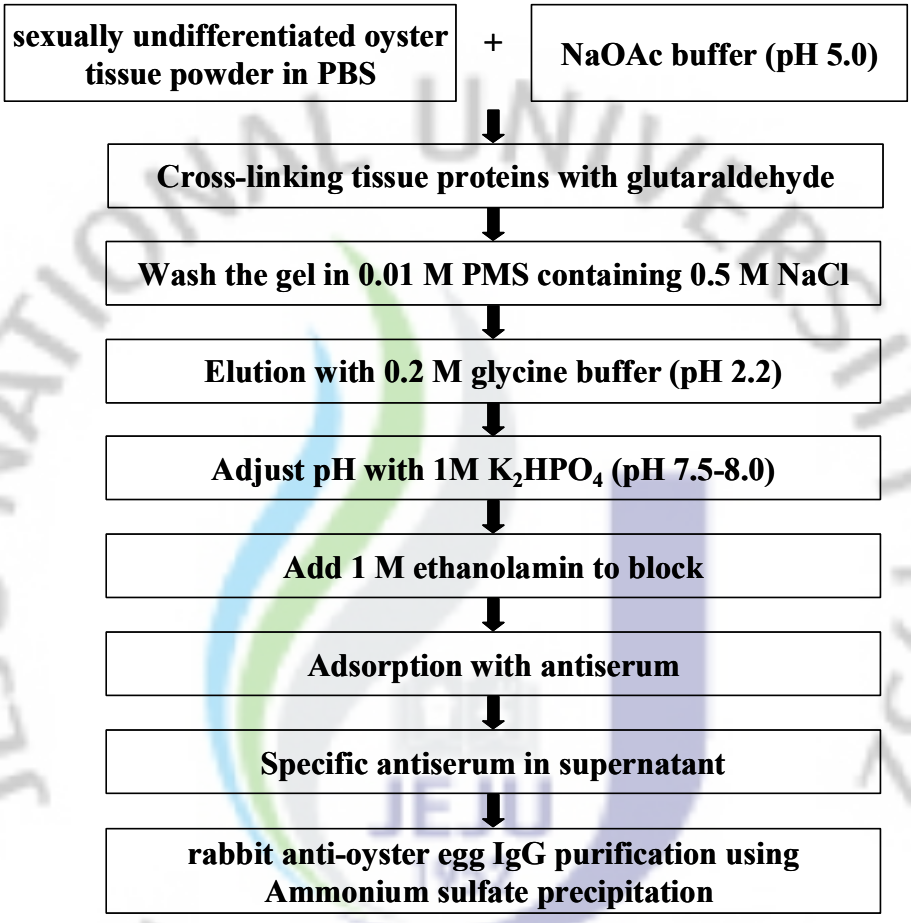

Fig. 4. Protocol used in the removing the cross-reactivity using an immunoadsorbent.

3. Results

3.1 Specificity of the antiserum

The rabbit antiserum raised from purified oyster eggs initially demonstrated a weak but recognizable cross-reactivity to the non-gonadal tissue proteins in the double immunodiffusion test (Fig. 5), ELISA and the Western blotting. After eliminating the cross reacting antibody using the immunoadsorbent, a strong precipitin line was observed between the egg protein and the antibody. In ELISA and the Western blotting, no distinguishable reaction was observed between the purified antibody and the somatic tissue protein (Fig. 7 and 9).

Indirect immunofluorescence assay performed on the mature female gonadal tissue also revealed highly specific interaction between the antibody and the egg proteins. The oocytes were stained with the fluorescent antibody but no positive staining was observed in the connective tissue, nucleus, mantle or gills (Fig. 6). It was suggested that the rabbit anti-oyster egg IgG reacts with vitellins, egg-specific proteins in animal eggs. The titration curve indicated that he anti-oyster egg IgG in ELISA detect 0.2 to 8 μg/ml of the oyster egg presented in the standard solution (Fig. 7).

3.2. Biochemical composition of the purified eggs and oyster tissue

The ripe eggs were successfully purified from the ovaries of *C. ariakensis* using different sizes of mesh screening and centrifugation. The biochemical composition of the oyster eggs is listed in Table 1. The dry weight of the individual egg was estimated to be 14 ng, and it is composed of 51.4% protein, 5.3% carbohydrate, 24.1% lipid and 9.4% ash, respectively.

Total proteins, carbohydrates, lipids and ash content in the oyster tissues used in this study are plotted on Fig. 8. The monthly mean total proteins ranged from 21-56% of the dry tissue weight. The total protein was found to be lower in winter period (January and February 24-

27%) and relatively higher when the oyster became sexually mature from March to June (36-38%). The carbohydrate level in the tissue increased from March to May then decreased in Jun. The carbohydrate values were stable during January to March and the level increased in April whereas, the carbohydrate level decreased dramatically from May (46%) to July (32%). Lipid percentage showed clear monthly changes during the study period. The values gradually increased from January to May having the highest value (7%) then the levels decreased from June to July as a similar pattern observed in the carbohydrate. Ash levels showed no remarkable difference during study period with the values ranging from 6% to 7%.

3.3. Characterization of the oyster egg-specific protein using the Western blotting

Fig. 9 shows the immunoreactive peptides in purified eggs, eggs in ripe, late developing and early developing and sexually indifferent oysters. The oyster egg proteins are complex of proteins comprising different size of peptides of approximately 150, 120, 95, 90, 82 and 55 kDa. The late developing and rape stage of oyster proteins showed the major band with molecular size of 150 and 55 kDa and very weak band of 120, 95, 90 and 82 kDa respectively. In contrast, the early developing stage and indifferent stage of oyster proteins exhibited no immunologically active bands.

3.4. Gonad development

The gametogenic pattern of the oysters was categorized into six reproductive stages (Table 2). Figure 10 shows microscopic features and frequency distribution of the gametogenic stages during the study period. In January, most of the oysters (59%) were in early developing stage, exhibiting early vitellogenic oocytes and spermatogonia. Late developing stage was observed from May to July and ripe oysters could be observed as early as in May (25%). Spawning commenced in July in both sexes when 6. 7% oysters were engaged in reproduction. In June (84.6%) and July (86.7%), the oysters were fully matured and were ready for spawning. Only one oyster was found to be an accidental functional hermaphrodite.

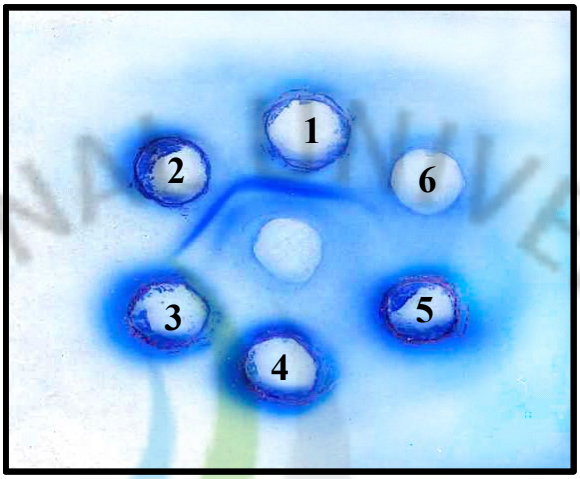

Fig. 5. Double-immunodiffusion test showing the antibody-antigen reaction. Antiserum is placed in the central well and antigens are placed in the wells around the antiserum: 1 to 2, egg protein; 3 to 4, mantle; 5, gill; 6, adductor muscle.

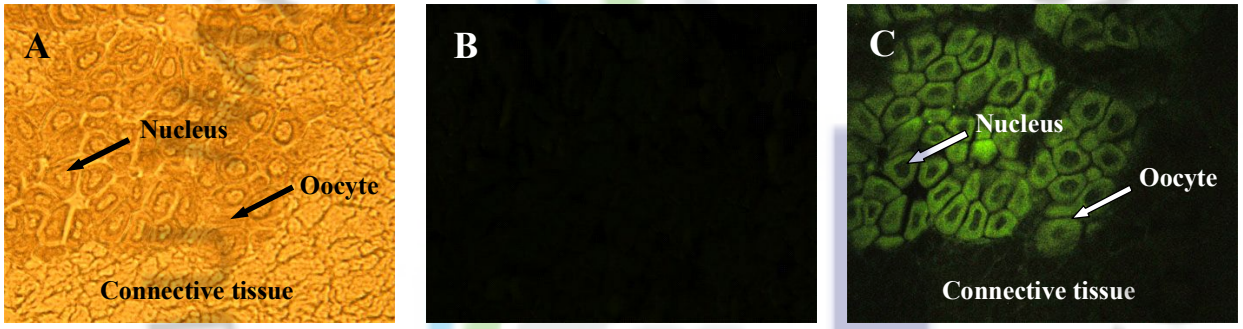

Fig. 6. Indirect immunofluorescence assay performed on sexually mature female gonad. The oocytes were detected with the anti-oyster egg IgG, followed by the addition of FITC-labeled anti-rabbit secondary antibodies. (A) Back ground, (B) Control, (C) Oyster egg – FITC

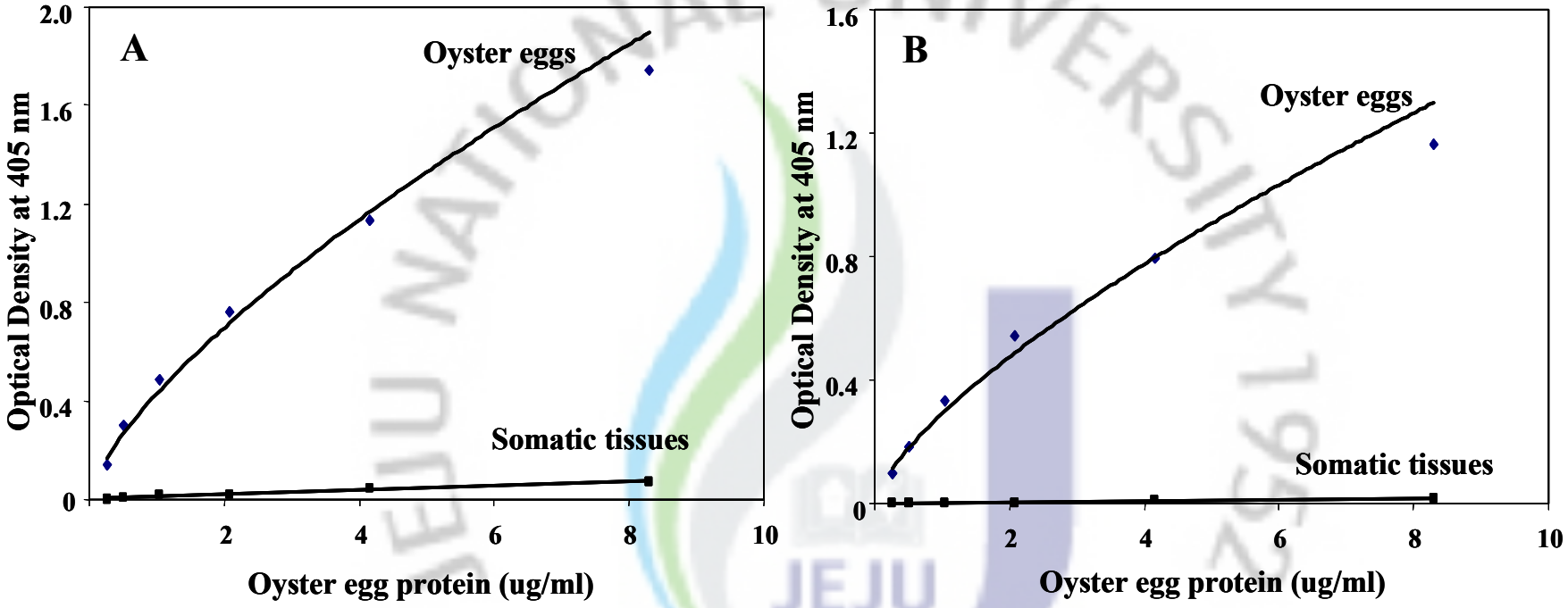

Fig. 7. A typical ELISA standard curve for quantification of the oyster egg proteins. (A) Before removal of cross-reaction with somatic tissues. (B) After removal of cross-reaction.

Table 1. Biochemical composition of the Suminoe oyster egg.

	Percent composition
Protein	51.1
Carbohydrate	5.3
Lipid	24.1
Ash	9.4
Total	89.9

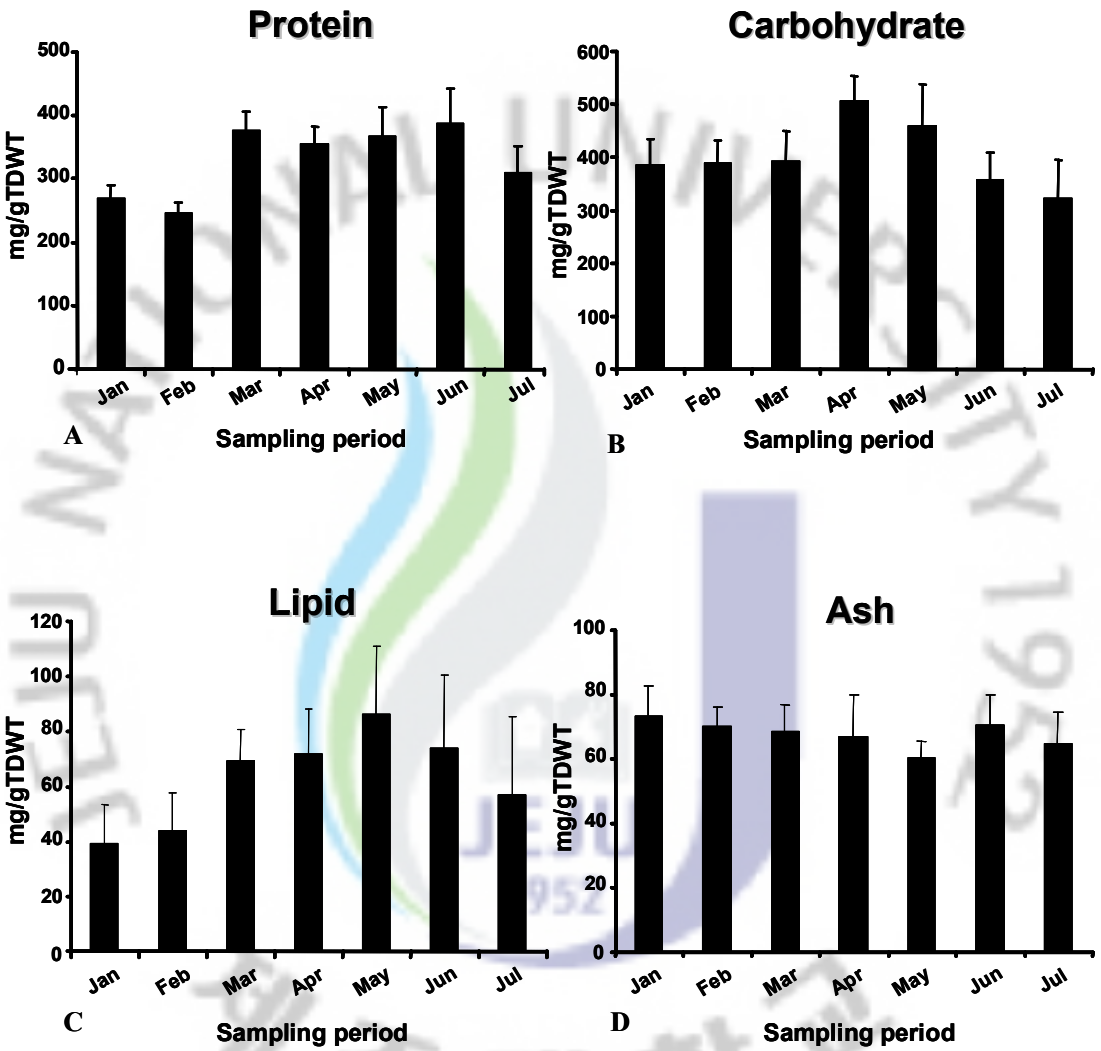

Fig. 8. Biochemical composition of protein (A), carbohydrate (B), lipid (C) and ash (D) in individual oyster during the study.

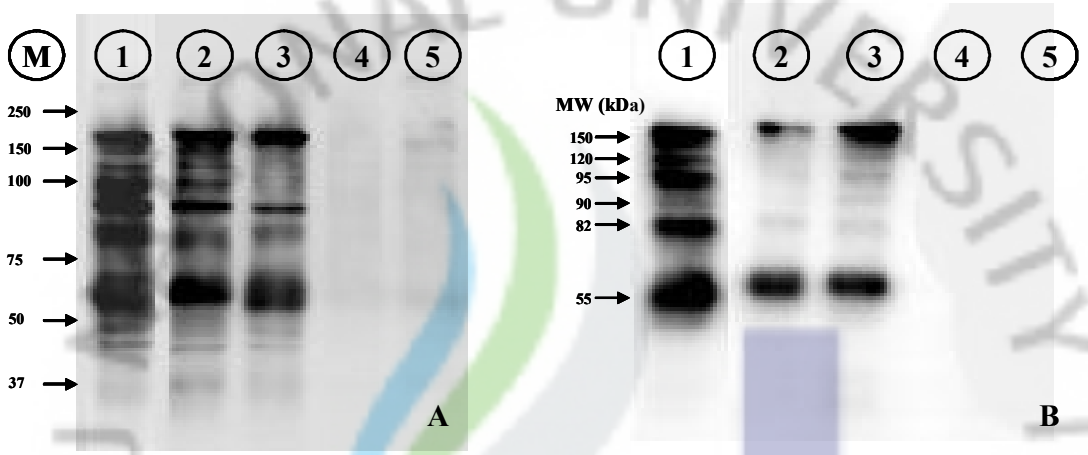

Fig. 9. Western immunoblot of the oyster egg and different development stage of the oysters. The oyster egg proteins are complex of proteins comprising different size of peptides of approximately 150, 120, 95, 90, 82 and 55 kDa. A: Before removal cross reaction, B: After removal cross reaction. M, marker; 1, egg protein; 2, ripe stage of female oyster; 3, late developing stage of female oyster; 4, early developing stage of female oyster; 5, somatic tissue.

Table 2. Description of the reproductive stages observed from an annual reproductive cycle of *Crassostrea ariakensis*

Reproductive stage	Description
Indifferent	The sex cannot be distinguishable since no germ cells are present along the folicle walls.
Early developing	The follicles are expanded and small oogonia, early vitellogenic oocyte, spermatogonia and spermatocyte can be observed.
Late developing	The oocytes increase their size and free oocytes in the lumen but most oocytes attached to the follicular walls. Every stage of gamete cells including spermatogonia, spermatocyte, spermatid and spermatozoa can be found.
Ripe	The follicles are fully packed with mature oocyte and the spermatozoa are arranged in a radial manner.
Spawning	The free ripe oocytes in the lumen are decreased and the radial arrangement of the spermatozoa become lost.

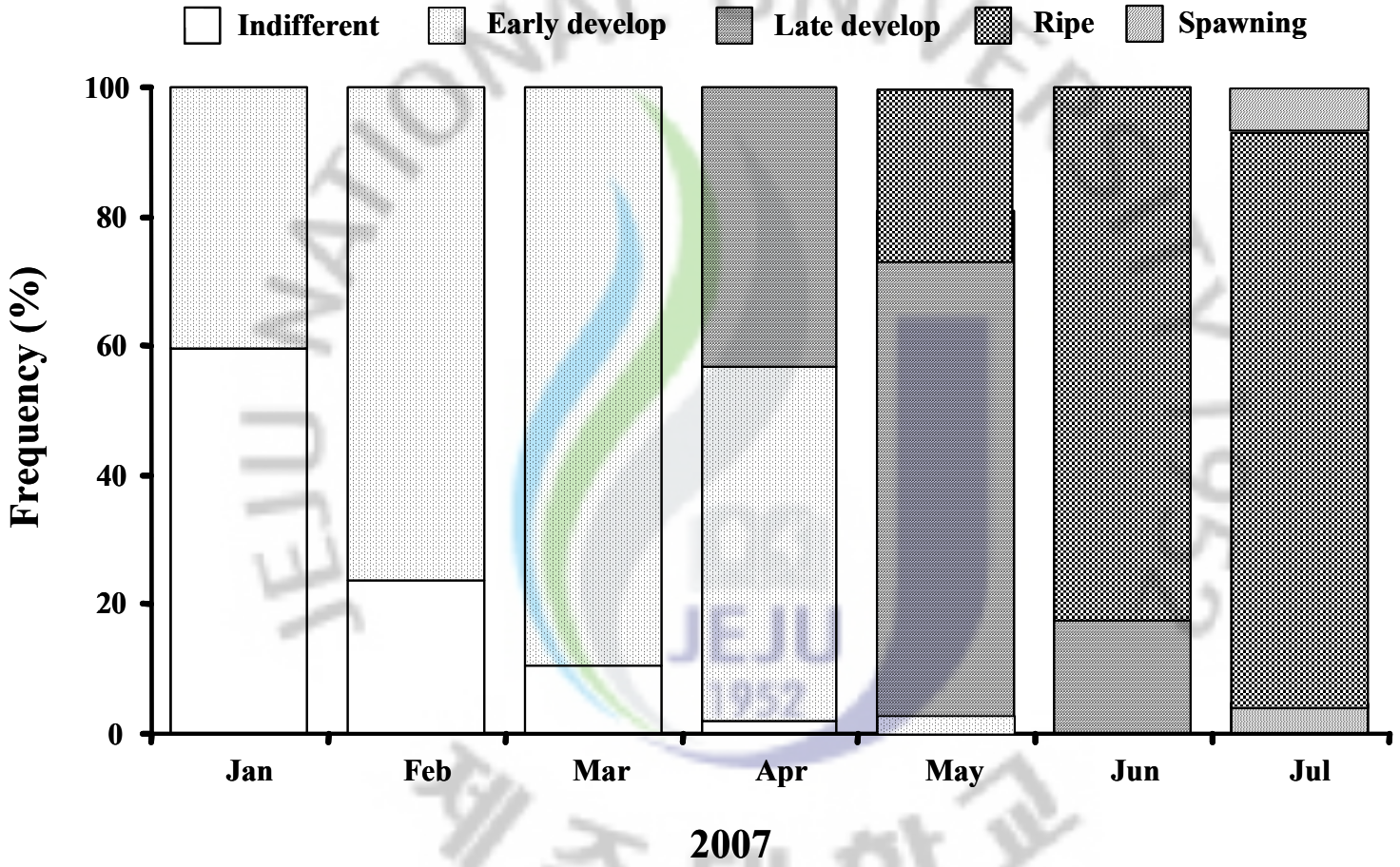

Fig. 10. Frequency of the reproductive stage of the oyster during study.

The condition index (CI) of the oysters ranged from 0.03 – 0.10 (CI-I) and 0.04 – 0.22 (CI-II). The both CI changed slightly over the study period exhibiting the peak during June when most of the oysters were fully matured in the histological preparations (Fig. 11 and Table 3). The PGA could be estimated from April when the most of oysters were in early and late developing stages. The mean value of PGA dramatically increased from April (5.1%) to June (51%) when the ovaries were fully developed (Fig. 12).

3.5. Quantitative measurement of GSI using ELISA

Figure 13 shows the monthly mean GSI measured using ELISA from January to July 2007. The reproductive effort of the oysters remained undetectable from January to March when the oysters were in indifferent and early developing stage. Of 25 female oysters collected in April, the GSI could be estimated only from 13 oysters having an average GSI of 3.62±4.42%. Other female oysters were in early and late developing stages and possibly contained eggs less than 1% of their body weight. The GSI dramatically increased from April to July as the gonad become fully mature. The highest mean GSI (66.93 %) was observed in July when most of the individuals were ready for spawning (Table 4).

Figure 14 shows the GSI measured from different reproductive stages of the Sumino oysters. The GSI was detectable in only 5 individuals in 61 of early developing oysters with a mean 1.4%. GSI noticeably increased from early developing to late developing stage (16.2%). In ripe stage, GSI could be estimated from all the oysters (38 oysters) using ELISA, ranging from 20 to 67%.

3.6. Potential fecundity of *C. ariakensis* estimated by ELISA

The potential fecundity was estimated from oysters collected during May and July when the oysters were fully ripe and ready to spawn. The potential fecundity ranged from 162 to 910 million eggs having an average of 452 million eggs among 38 ripe oysters. A positive correlation between the total dry tissue and the potential fecundity was observed (Fig. 15).

Table 3. Monthly mean shell length in mm (SL), tissue wet weight in g (TWWT), tissue dry weight in g (TDWT) and condition index (CI) of *Crassostrea ariakensis*.

Month	**N**	**SL (cm)**	**TWWT (g)**	**TDWT (g)**	**CI-1**	**CI-2**
January	40	189.2±14.6	44.62±7.75	10.24±2.12	0.076±0.012	0.1±0.02
February	40	194.6±13.9	51.04±6.90	13.17±1.94	0.068±0.013	0.12±0.03
March	40	192.9±14.7	49.97±12.10	12.81±3.43	0.055±0.012	0.11±0.04
April	40	172.8±16.7	42.65±8.82	10.50±2.23	0.078±0.012	0.12±0.02
May	40	180.1±16.3	51.48±12.72	13.63±3.36	0.07±0.014	0.13±0.03
June	40	149.3±12.4	40.87±8.07	10.35±2.19	0.083±0.014	0.15±0.03
July	30	181.1±21.3	55.38±14.67	12.71±3.49	0.075±0.016	0.11±0.02

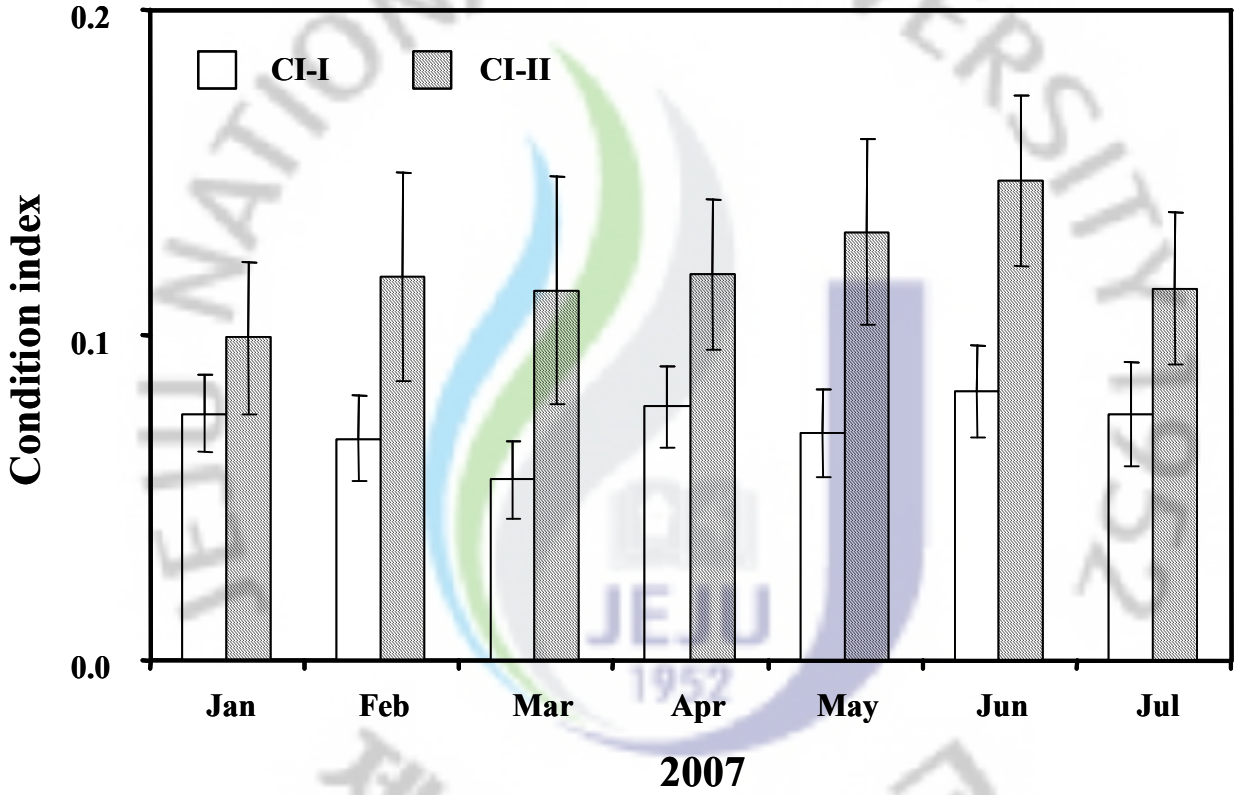

Fig. 11. Monthly changes in the condition index (CI) of *C. ariakensis*.

CI-I = TWWT x 1,000/(SL x SH x SW) and CI-II = TDWT X 1,000/internal shell cavity volume (SCV).

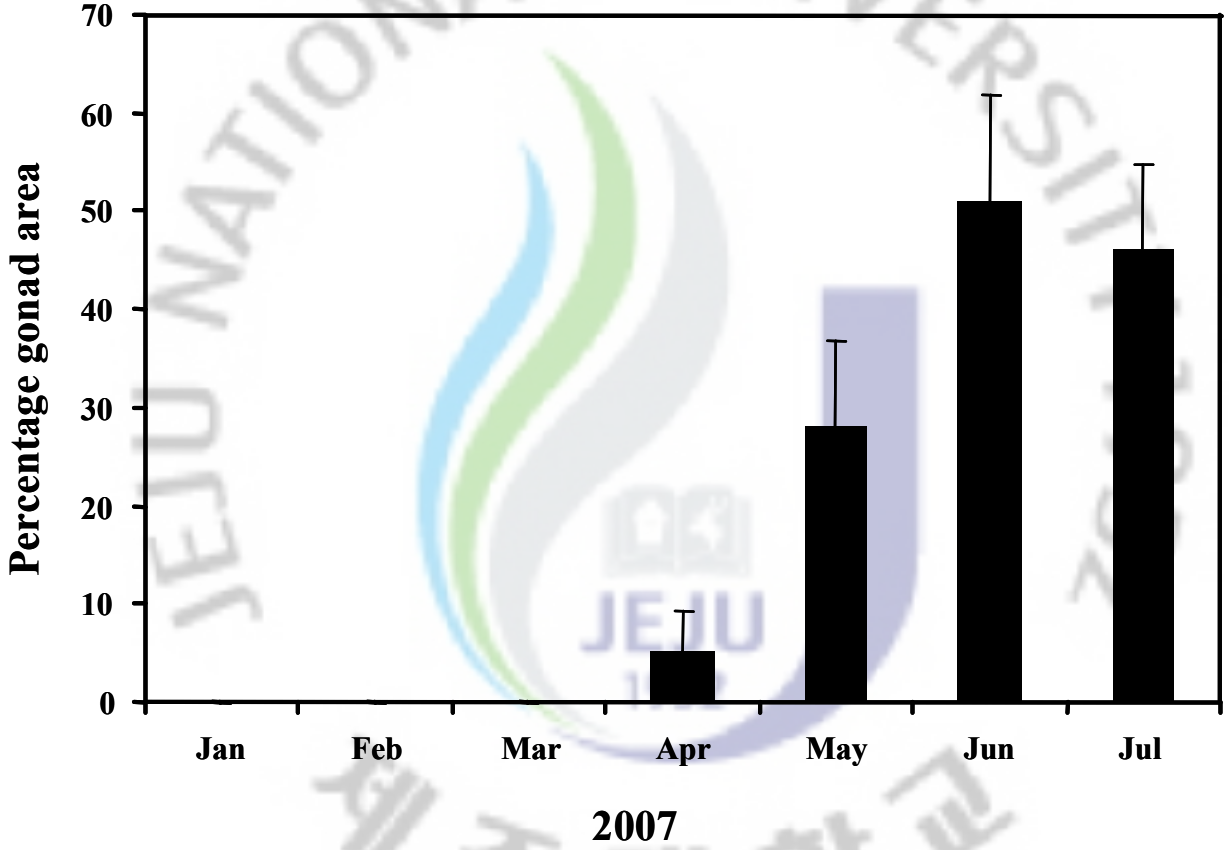

Fig. 12. Monthly mean percentage of the gonad area (PGA).

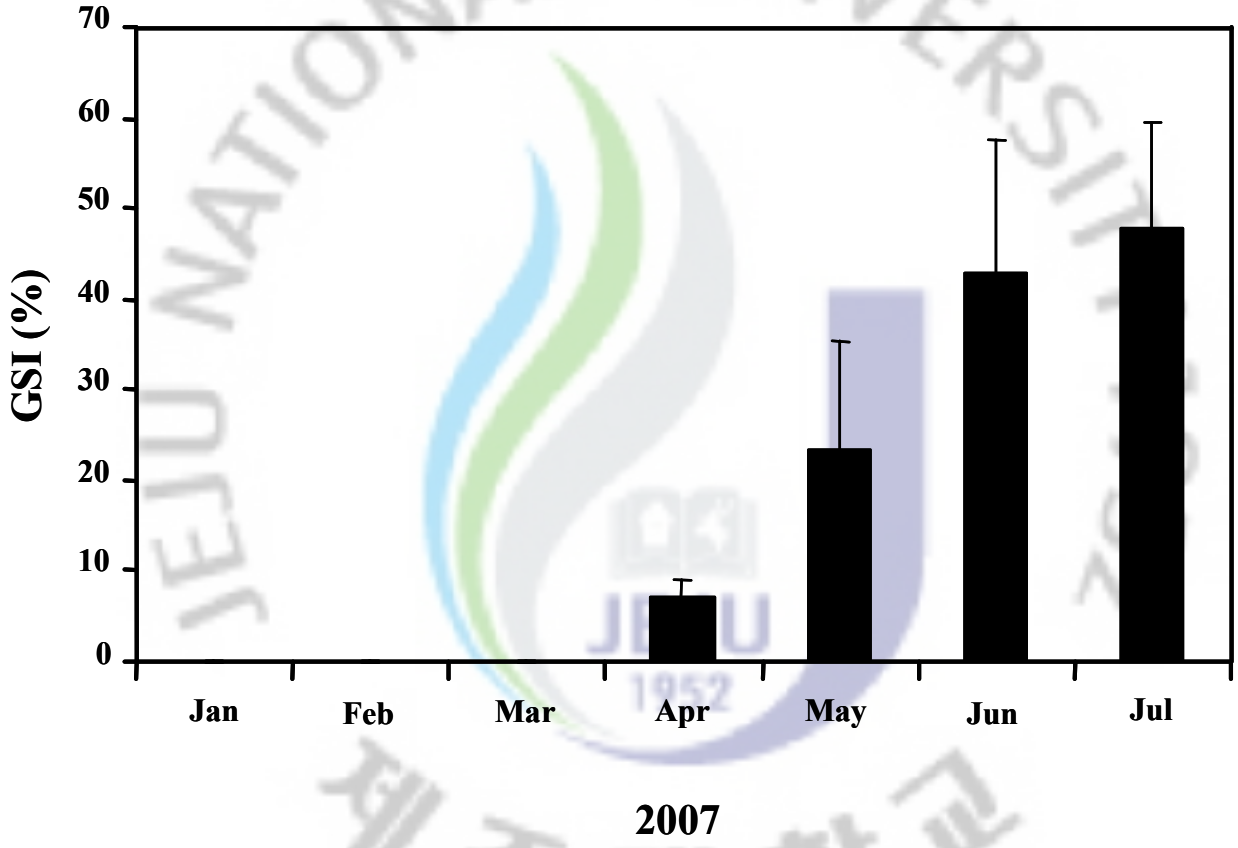

Fig. 13. Monthly changes in gonadosomatic index (GSI) of the oysters collected from Seomjin River mouth estuary.

Table 4. Gonadosomatic index (GSI) of the *C. ariakensis* estimated using ELISA.

Month	N female	Mean GSI (%)	Highest GSI (%)	Lowest GSI (%)
January	**12**	**0**	**0**	**0**
February	**16**	**0**	**0**	**0**
March	**17**	**0**	**0**	**0**
April	**25**	**3.62±4.42**	**14.03**	**0.62**
May	**21**	**23.38±12.05**	**46.22**	**1.1**
June	**18**	**42.95±14.61**	**62.97**	**14.19**
July	**20**	**47.69±11.80**	**66.93**	**17.52**

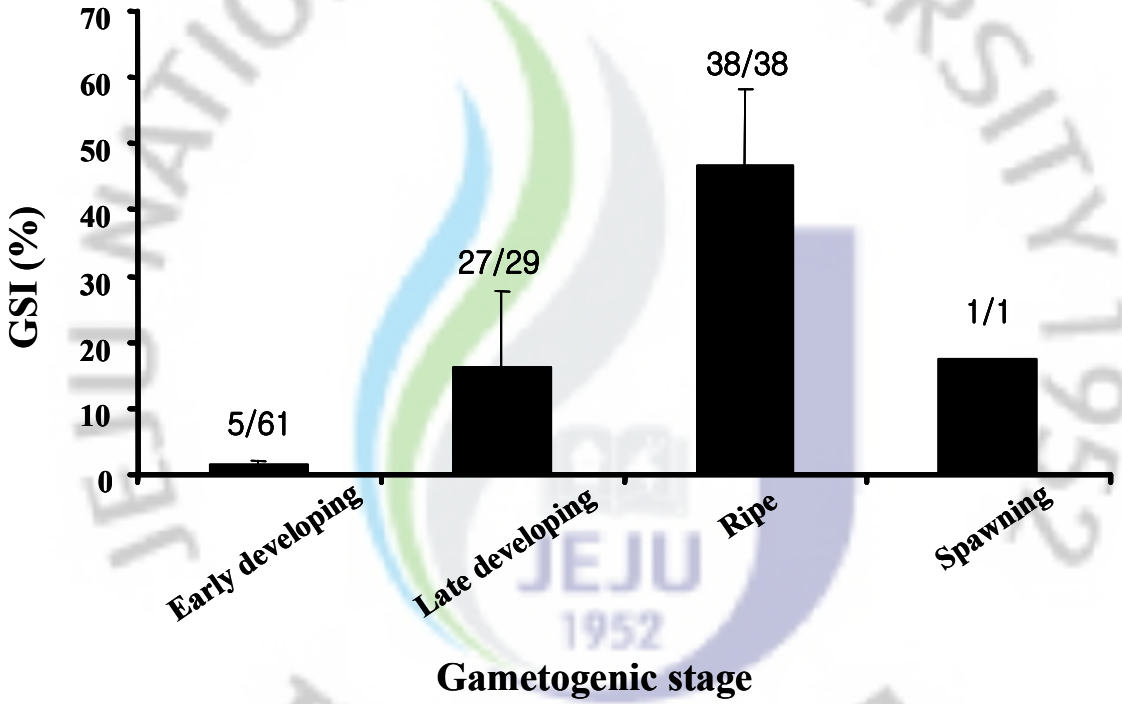

Fig. 14. Gonadosomatic index (GSI) of the Sumino oyster at different gametogenic phase.

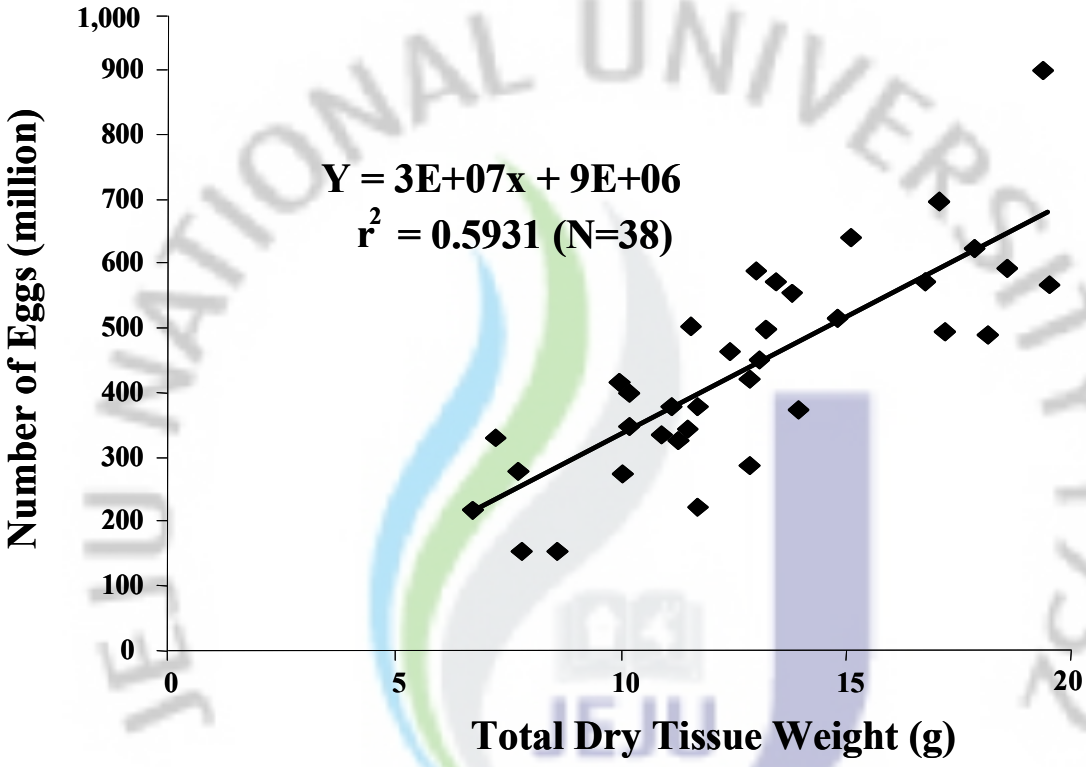

Fig. 15. Correlation between the oyster size as dry tissue weight and the potential fecundity estimated from ELISA. The potential fecundity was estimated by dividing the total amount of egg estimated using ELISA by the weight of individual egg estimated as 14 ng.

4. Discussion

4.1. Specificity of the rabbit anti-*C. ariakensis* egg IgG

Relatively few studies have investigated the proximate composition and the individual weight of marine bivalves eggs (Table 5). The proximate composition of the individual egg of *C. ariakensis* was estimated to be 51.4% protein, 5.3% carbohydrate, 24.1% lipid and ash, 9.4% respectively. Similarly major component of other marine bivalves eggs were protein followed by lipid and carbohydrate. The single dry egg weight of *C. ariakensis* estimated in this study was similar to *C. virginica* (Lee and Heffernan, 1991; Choi et al., 1993) and *C. gigas* (Massapina et al., 1999; Kang et al., 2003) eggs ranged from 12 to 13 ng. In clams such as *Mercenaria mercenaria* (Lee and Heffernan, 1991), *Saxidomus purpurdatus* (Park et al., 2005) and *Ruditapes philippinarum* (Park and Choi, 2004) eggs were higher then oyster eggs having the egg weight of 51, 95 and 22 ng respectively. The clam eggs (*R. philippinarum, M. mercenaria and S. purpurdatus*) which is heavier than oyster egg (*C. ariakensis, C. gigas and C. virginica*) might sink easily when they released in water column during spawning (Park et al. 2005).

The western blotting performed on the oyster egg proteins revealed that the egg proteins are complex of proteins comprising different size of peptides of approximately 150, 120, 95, 90, 82 and 55 kDa (Fig. 8). Several studies reported the egg-specific peptides with molecular weights of 105, 85, 66, 64, 60, 45 and 41 kDa in Pacific oysters (Suzuki et al., 1992), 76, 56, 50, 48, 18 and 17 kDa in *C. virginica* (Lee and Heffernan, 1991), 98, 87, 68, 60, 56, 36 and 19 kDa in *M. mercenaria* (Lee and Heffernan, 1991) and 330, 96, 64, 50 and 31 kDa in *R. philippinarum* (Park and Choi, 2004). These egg-specific peptides are so-called vitellines, a major part of invertebrate eggs, and are used as an energy source and nutrients during gonadal development and spawning (Lee and Heffernan, 1991; Suzuki et al., 1992).

As shown in Fig 8, the late developing and ripe stage oyster proteins showed the same pattern of bands as the egg protein while the early developing and indifferent stage

Table 5. Biochemical composition and the weight of various marine bivalve eggs.

Species	Location	Egg dry weight (ng)	Protein (%)	Carbohydrate (%)	Lipid (%)	Author
Mercenaria mercenaria	Georgia, USA	51	40	8	14	Lee and Heffernan (1991)
Sxidomus purpuratus	Geoje, Korea	95	37.44	10.83	11.4	Park et al. (2005)
Ruditapes philippinarum	Gomso Bay, Korea	22	41	-	-	Park and Choi (2004)
Crassostrea virginica	Georgia, USA	12	50	9	21	Lee and Heffernan (1991)
C. virginica	Galveston Bay, USA	13	40	-	-	Choi et al. (1993)
C. gigas	Ria Formosa, Portugal	-	44-74	7-12	16-38	Massapina et al. (1999)
C. gigas	Goseong Bay, Korea	13	41	11.7	25.5	Kang et al. (2003)
C. ariakensis	Seomjin River, Korea	14	51.1	5.3	24.1	Present study

oyster proteins exhibited no band. The early developing stage of the oysters was mostly composed of pre-vitellogenic eggs, which contained no or very low level of vitellin as outlined by Park and Choi (2004). As the Sumino oyster egg antibody was raised from the ripe eggs possibly from the yolk protein know as vitellin, the antibody did not react with vitellogenin, the precursor of vitellin, mostly present in the early developing eggs. Suzuki et al. (1992) suggested that the vitellin-like protein of *C. gigas* in the oocytes may support the occurrence of autosynthetic yolk formation inside oocytes, not in the other somatic tissues.

The immunofluorescence assay (Fig. 5), ELISA (Fig. 6) and western blotting (Fig. 8) show that the rabbit anti-oyster egg IgG was raised from vitellins in oyster eggs, as observed in other studies (Suzuki t al., 1992; Kang et al., 2003; Park and Choi, 2004; Park et al., 2005).

4.2. Reproductive effort of *C. ariakensis*

Choi et al. (1993) first utilized immunological method in the quantitative measurement of reproductive effort of the American oyster, *C. virginica*. In their study, the polyclonal antibody raised from *C. virginica* egg antibody detected 0.2-10 μg/ml egg protein solution in ELISA. Kang et al. (2003) also developed polyclonal antibody to the eggs of Pacific oyster, *C. gigas* to estimate the reproductive effort. They also found that ELISA was sensitive and fast enough to detect small quantity of the eggs present in oysters in early gametogenic stage. Immunological method was also applied in the measurement of reproductive effort of clams, *Ruditapes philippinarum* and *Saxidomus purpuratus* (Park and Choi, 2004; Park et al., 2005). Similarly, the antibodies raised from the clam eggs detected 200 ng/ml-15 μg/ml of the egg proteins in ELISA.

In this study, the same oysters were used for histology as well as for the quantification of reproductive effort. The gametogenic stage-wise GSI could successfully detect reproductive effort in late developing, ripe and spawning oyster while the most oysters in early developing stage (January to March) were not sensitive enough to detect the reproductive effort by ELISA. The egg protein of some early developing oysters was

detected in April might be due to the conversion of some vitellogenin into vitellin (Park and Choi, 2004; Uddin, 2008). As shown in Fig. 14, the GSI in ripe stage oyster was 47% and dropped to 18% in spawning stage oyster. The high value of GSI even in spawning stage leads to understand that the *C. ariakensis* was not released large amount of gametes in one time but slowly and continuously release.

Up to now, few studies on measurements of quantitative reproductive output in oysters have reported using ELISA. The American oyster, *C. virginica* in Galveston Bay indicated approximately 20% GSI during the spawning season while the highest GSI was observed to be 42% (Choi et al., 1993). The Pacific oyster, *C. gigas* in Goseong Bay showed highest GSI (67%) during spawning time (Kang et al., 2003) and *C. gigas* in France indicated 36-61% GSI of the oysters in their first, second or third years during spawning time (Royer et al. 2008). The mean GSI estimated in the present study for *C. ariakensis* during ripe stage is comparable with that of *C. gigas*.

To monitor the reproductive condition, the percentage gonad area (PGA) and GSI were measured using an image analyzing software and ELISA. Interestingly, both results showed consistent trend during study period though the PGA value was relatively higher than the GSI value. This study suggests that the PGA could be inflated compare with GSI estimated by ELISA because the PGA could contain the patches of gonad area. In other words, the more precise way of doing it is to circumscribe each patch of gonad, not simply the area where the gonad is contained. This point is emphasized by the ELISA data.

4.3. Estimation of potential fecundity using ELISA

The potential fecundity of the female oysters was evaluated only ripe oysters as proved by histology according to Uddin (2008) and calculated by dividing total egg weight measured using ELISA by the weight of an oyster egg, 14 ng. In the previous immunological studies, the fecundity was estimated in case of all females during mean peak GSI assuming that all the individuals were ripe and ready for spawning during that time (Kang et al., 2003;

Park and Choi, 2004; Park et al., 2005). Uddin (2008) suggested that the histology indicated that the individuals are often composed of different development stages during each sampling period. Consequently, the assessment of potential fecundity confirmed by histology is more accurate and higher than previous studies.

Table 6 shows the fecundity of marine bivalves reported from various studies using ELISA collected from different habitats. In this study, the potential fecundity of the oysters ranged from 162 to 910 million eggs having an average of 452 million eggs among 38 oysters. The fecundity of manila clam in Gomso Bay ranged from 0.94-11 million (Park and Choi, 2004) and the potential fecundity of the clams collected from Jeju varied from 2.42-8.97 million (Uddin, 2008). Park et al. (2005) showed that the fecundity of the *S. purpuratus* (9-31 million) from Geoje was higher than the manila clam collected from Korean waters using the same immunological technique. The fecundity estimated for *C. ariakensis* is much higher than the *C. virginica* (Choi et al,. 1993), *C. gigas* (Kang et al., 2003; Royer et al., 2008) as 3.7-65.4 million in Galveston Bay, 4-196 million in Goseong Bay and 2.6-234 million in Normandy respectively.

As shown in Table 5, the highest fecundity was observed in *C. ariakensis* among the marine bivalves estimated using ELISA and it supports that the fecundity is remarkably associated with the dry weight of samples except in *S. purpuratus* (Park et al., 2005). Fig. 15 shows that a positive correlation was observed between oyster size (total dry weight) and potential fecundity estimated from ELISA. Choi et al. (1993), Kang et al. (2003) and Royer et al. (2008) also revealed a size-dependent increase in fecundity of *C. virginica* and *C. gigas*.

4.4. Biochemical composition of the *C. ariakensis*

Seasonal changes in biochemical composition of marine bivalves are closely associated with the reproductive cycle, mostly in the natural habitat (Navarro et al., 1989; Kang et al., 2000; Marin et al., 2003; Ngo et al., 2006) and with changes in various subfractions such as protein and non-portein nitrogen; phospholioids and neutral lipids; and

Table 6. Fecundity of marine bivalves reported from various studies.

Species	Location	Shell length (mm)	Tissue dry weight (g)	Egg diameter (㎛)-histology	Individual egg dry weight (ng)	Fecundity	Author
Ruditapes philippinarum	Gomso Bay, Korea	21.11-46.50	0.2-0.8	61.19±5.66	22	0.94-11x10^6	Park and Choi (2004)
R. philippinarum	Jeju, Korea	21.6-43.3	0.3-0.5	58.93±6.87	22	2.42-8.97x10^6	Uddin (2008)
Saxidomus purpuratus	Geoje, Korea	86.94	8.4-13.2	70.81±7.52	95	9-31x10^6	Park et al. (2005)
Crassostrea virginica	Galveston Bay, USA	70-120	0.7-1.9	-	13	3.7-65.4x10^6	Choi et al. (1993)
C. gigas	Goseong Bay, Korea	74-91.6	0.9-2.9	-	13	4-196x10^6	Kang et al. (2003)
C. gigas	Normandy, France	55.2-88.6	0.7-5.9	25.8±56.7	13	2.6-234x10^6	Royer et al. (2008)
C. ariakensis	Seomjin River, Korea	125.2-230.1	10.5-13.6	-	14	162-910x10^6	Present study

glycogen and free sugars (Gabbott 1976). *C. gigas* (Kang et al., 2000) showed clear seasonal trends and patterns of each component. The proteins and carbohydrates, particularly glycogen which is considered to be the main reserves of the gamete formation are inefficient to meet the energy required for increasing gonad and tissue weight.

The total proteins of *C. ariakensis* in this study showed increasing pattern during the gonad maturation process. The highest value of protein found in June when most oysters were ripe stage accord with Ngo et al. (2006) suggestions that protein serves and energy reserve during gametogenesis. The total carbohydrate levels were sharply decreased from April (highest value) to July (lowest value) supported that carbohydrate is the main component during indifferent and early developing stage (Ngo et al., 2006). Lipids compose an important nutrient store in bivalves and it is used as an energy source during gametogenesis (Delgado et al., 2004; Fernández-Reiriz et al., 2007). The monthly variation of lipid levels was similar to the carbohydrate and the increasing levels from January to May noticeably decreased in this study. Interestingly, the lipid levels of this study were almost same patterns with *C. gigas* (Kang et al., 2000; Ngo et al., 2006). Kang et al. (2000) accepted that the glycogen is transformed into lipid for the formation of gametes with the result of highest value prior to spawning.

4.5. Gonad development

Understanding gametogenesis and subsequent annual reproductive cycle of marine bivalves is essential in the proper management of harvesting practices as well as for developing aquaculture (Menzel, 1991; Gosling, 2003).

Histological observation showed that gametogenesis of *C. ariakensis* initiated in January and most of the oysters were fully matured during June and July. Unfortunately we could not continue sampling activities from August due to the big flood and heavy rainfall at the sampling area. Spawning commenced in July (6.7%) but large scale spawning could be observed from August. According to Joo (2006), gametogenesis of *C. ariakensis* collected

from same area as present study initiated in December and spawning occurred from July (< 50%) to September which was broadly similar with the present study. The little differences in timing and duration of spawning are likely due to the exogenous factors controlling gametogenesis that may typically vary from year to year, such as water temperature, salinity and food availability (Hyun et al., 2001; Kang et al., 2000; Ngo et al., 2002; Ngo et al., 2006).

The condition factor is frequently used an index of the nutritive and health status of bivalves, based on the principle that individuals of a given length, higher weight, are in a better condition. However the studies on condition index (CI) of bivalves are limited owing to the lack of a standard formula for measuring CI (Lawrence and Scott., 1982; Kang and Choi., 1999). This study estimated CI of the *C. ariakensis* from Seomjin River in Korea using two formulas and the CI changed slightly during the study period having the peak during June when most of the oysters were fully matured (Fig. 11). The CI of small size and regular shape bivalves such as *Ruditapes philippinarum*, *Corbicular leana* and *Coecella chinensis* were estimated according CI-I formulas, whereas using the CI-II formula has measured large size bivalves especially oyster (Lawrence and Scott, 1982; Massapina et al., 1999) and Mussel (Kang and Choi, 1999). But CI-I may have some errors to estimate CI with length due to their irregular shapes and barnacles (Kang and Choi, 1999). This study suggests that the volumetric condition index (CI-II) is simple and accurate method as a standard measurement to evaluate conditions of *C. ariakensis*.

5. Conclusion

In conclusion, the anti-Sumino oyster egg antibody was developed in this study. The antibody was successfully applied in the quantification of the egg protein using ELISA. The egg protein of *C. ariakensis* was characterized with SDS PAGE and subsequent Western

blotting. Molecular mass of the polypeptide fractions of the oyster egg proteins was determined to be 150, 120, 95, 90, 82 and 55 kDa. Gametogenesis commenced in January and most of the oysters became ripe by May. Monthly mean GSI from January to April varied from 0.6 to 66.9 %. Gametogenic stage-wise mean GSI was 1.4 % in early developing oysters, 16.2 % in late developing individuals, 46.9 % in ripe oysters and 17.5 % in spawning individuals, indicating that the Sumino oysters spawn when the egg mass accumulated more than 40% of the body weight. The estimated potential fecundity of the ripe oysters ranged from 162 to 910 million eggs with an average of 452 million. The immunological method developed in this study was fast and highly sensitive to measure the eggs in the Sumino oysters in various reproductive stages.

SUMMARY

A polyclonal antibody specific to egg protein of the Suminoe oyster, *Crassostrea ariakensis* was developed in this study to assess the reproductive effort. A New Zealand white rabbit was immunized with the purified oyster egg harvested from the ripe ovaries. After two months of immunization the rabbit antiserum showed strong specificity to the egg protein in enzyme-linked immunosorbent assay (ELISA) and in a double immuno-diffusion. In ELISA, the rabbit anti-oyster egg IgG detected as little as 0.2 μg/ml of the Sumino oyster egg protein. Quantity of egg present in an oyster estimated using ELISA was finally expressed as gonad-somatic index (GSI).

GSI of Suminoe oysters were estimated monthly from January to July 2007 at Sumjin River estuary off the south coast of Korea. Histology indicated that the gametogenesis initiated in January and most oysters were mature and ready for spawning by the middle of July. GSI of oyster collected in April 0.6 to 14.0, when most female oysters were in early to late developing stage. In July, most oysters were ready for spawning GSI of the Sumino oysters at Sumjin River estuary varied from 17.5 to 67.0% with a mean of 47.7. Potential fecundity of the ripe oysters was also determined in this study, by dividing the amount of egg estimated in ELISA with a mean dry weight of single egg as 14 ng. The potential fecundity ranged from 162 to 910 million eggs and a positive correlation was found between the potential fecundity and size of the oyster.

The immunological technique applied in the quantification of reproductive effort of the Sumino oyster was successful to measure variable quantity of the eggs present in various gametogenic stages, 1-67 % in the early developing to ripe and believed to be the method of choice for the quantification.

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감사의 글

이 논문이 완성되기까지 너무나 부족한 저에게 깊은 관심과 용기를 주신 최광식 교수님께 진심으로 감사를 드립니다. 바쁘신 중에도 저의 논문을 심사해주신 이경준 교수님과 정준범 교수님께도 감사를 드립니다. 그리고 학위과정 동안 많은 가르침을 주신 이기완 교수님, 이영돈 교수님, 김기영 교수님께도 깊은 감사를 드립니다.

실험실 생활의 시작부터 지금까지도 늘 따끔한 충고와 용기를 불어넣어주시는 해양연구원 강도형 박사님과 군산대학교의 박경일 교수님께 감사의 마음을 전합니다. 석사과정 동안 많은 격려와 실험을 도와준 무척추동물양식실험실의 Dr. Ludovic Donaghy, Dr. Mausumi Adhya, Yanin Limphanont, 양현성 선배님, 이지연 누나, 김경훈 선생님, 홍현기, 정희도, 이희중 그리고 김대경 박사님, Dr. Md. Jasim Uddin, 임나래 누나, 김태호 선배, 최규성에게 고마움을 전합니다. 또한, 실험에 필요한 샘플 채집을 해주신 청아수산 구순자님께 감사 드립니다.

마지막으로 제가 멀리 제주에서 공부하고, 이 길을 걸어올 수 있도록 믿어주시고 격려해주신 부모님과 형에게 감사의 말을 전하고 싶습니다.