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Research Article

Cloning, Expression Analysis, and Detection of the Vitellogenin in the Chinese Black Sleeper *Bostrychus sinensis*

Yangyang Zhang,[1] Kun Meng,[1] Tingting Zhou,[1] Lei Chen,[2] Tiansheng Chen,[2] Min Long,[1] and Kefu Zhou[1]

[1]*Key Laboratory of Ministry of Education for Coastal and Wetland Ecosystems, College of the Environment and Ecology, Xiamen University, Xiamen 361102, Fujian, China*
[2]*Fisheries College of Jimei University, Jimei University, 43 Yindou Road, Jimei District, Xiamen City, Fujian Province, China*

Correspondence should be addressed to Kefu Zhou; zhkefu@xmu.edu.cn

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Endocrine disruptors in marine environments represented by estrogens lead to reverse health phenomena. To obtain a more effective way to reflect and detect environmental estrogens pollution, a method was developed to obtain the full-length cDNA coding *vitellogenin* gene in *B. sinensis*, induced by 17β-estradiol (E2) solution. We have downloaded 16 fish gene sequences from the NCBI database and designed PCR primers accordingly. Based on the quantitative real-time PCR method (qRT-PCR), we analyze the differences in gene expression under the conditions of different E2 exposure times in the low, middle, and high-dose groups. The full-length cDNA consists of 4738 nucleotides with a reading frame encoding 1540 amino acid residues. In vitro recombinant plasmids were constructed and transferred to *E. coli* BL21 for vitellogenin expression. Efficient fusion expression was obtained by IPTG at 16°C, and the expressed target protein (680 amino acids, 75 kDa) existed in a soluble state, accounting for more than 25% of the total soluble protein. We prepared monoclonal antibodies using established immunohistochemistry to detect vitellogenin expression sites in sexually mature female fish. Our study shows that the expression sites of Vg in sexually mature female fish are mainly distributed in the fishtail, hepatopancreas, intestine, muscle, ovary, and pronephric kidney. In conclusion, the vitellogenin from *B. sinensis* could be used as a biomarker of environmental estrogens to achieve rapid detection in the marine environment and the subsequent experiments of development in colloidal gold strips after this research would be established to provide a highly efficient and convenient detection method for environment pollution.

1. Introduction

In recent years, environmental estrogens (EEs) as an endocrine disrupting chemical (EDC) have attracted extensive concern [1] and were divided into two categories including natural and synthetic estrogens [2]. Studies have shown that fish exposed to estrogenic chemicals may lead to population-related effects [3], including reduced production of viable offspring and male sexual reversal [4]. EEs can be transmitted to organisms through inhalation, food intake, and direct contact [5]. Due to its high lipophilic nature, it accumulates in adipose tissue and has a long half-life [6], making the pollution phenomenon to deteriorate [7].

Vitellogenin (Vg), a precursor protein, has become a common biomarker used to determine the level of estrogen or estrogen analogue contamination in oviparous animals [8] and is produced by the liver of oviparous vertebrates in response to estrogen [9]. The Vg gene is silenced in male and juvenile fish [10], while it is activated in the stimulation of exogenous estrogen compounds [11]. The Vg synthesized in male fish is delivered into the blood and has kept at a high level because of no ovaries [12]. Therefore, the abnormal elevation of Vg in male fish can indirectly indicate the pollution status of estrogens in the aquatic environment [13]. In previous studies, the natural estrogen (E2, Estradiol) was the preferred hormone for inducing fish feminization,

which has been demonstrated in previous research [14]. E2 is highly potent with high environmental persistence and a tendency to bioconcentrate in organisms [15] and can disrupt reproductive processes in fish at low concentrations that occur in the aquatic environment [16].

At present, most of the studies about the cloning and expression of the Vg gene in fish focus on freshwater [17, 18]. However, freshwater is different from marine environments due to diverse physiological structure of fish living to some extent. Therefore, it is more accurate to monitor the changes of the marine environment based on the marine fish.

The Chinese Black Sleeper, *Bostrichthys sinensis,* belonging to Periforms, Eleotridae, *Bostrichthys* [19], is a warm-water euryhaline fish distributed in the southeast coast of China and Taiwan Strait of China [20], which is sensitive to estrogen pollutants. Because of its strong settlement [21], it can reflect the pollution situation along the estuarine in a certain region for a long time. According to the long-term detection of the research found that *B. sinensis* is sensitive to environmental changes, especially estrogen [22]. Low concentrations of estrogen can cause changes in the gonads or related physiological and biochemical indicators [23].

Therefore, to obtain a more effective method for detecting EE contamination, the changes in relative expression under different E2 concentrations were studied to enhance the healthy development of the mariculture and marine environment.

2. Materials and Methods

2.1. Fish Husbandry. B. sinensis is kept in the laboratory. E2 was added to the first, second, and third groups of male fish with estradiol concentrations of the low-dose group (10 μg/L), medium-dose group (50 μg/L), and high-dose group (100 μg/L), respectively. The fourth and fifth groups were males and females without estradiol as controls, and all groups had 12 fish.

Samples were divided into five treatment groups with each group reared in a 30 cm × 20 cm×15 cm glass container with 3 L filtrated seawater. The values of temperature and salinity were 25°C and 5‰ to 10‰, respectively. Adjust the biological rhythm to day: night = 10 : 14.

2.2. Acquisition of the Full-Length cDNA of BsVg. Total RNA was extracted from the liver tissue of the male *B. sinensis* induced by E2 solution and was reversely transcribed into cDNA based on the protocol 5× All-In-One MasterMix (with AccuRT Genomic DNA Removal Kit).

We have downloaded 16 fish gene sequences from the NCBI database and designed PCR primers accordingly. The primers are Vg-CDS-F: ATGAGAGYNGTTGTRCTWGC// Vg-CDS-R: CAGCCTTTCCACAAGWCCAC. The Vg cDNA was amplified, and PCR products were conducted through agarose gel electrophoresis. The cDNA fragment was cloned into T4 vector and transformed into *E. coli* [24]. According to the results of the blue-white spot screening, a few colonies were selected for amplification. Then, the plasmid was extracted and sequenced by Xiamen Bosheng Biotechnology Company.

The full-length cDNA sequence of vitellogenin expression was further determined by designing RACE PCR primers at both ends. The designed primer sequence is Vg51 (1232–1253 bp): GGTAGGCTCCAACAGGTGAAGA, Vg52 (1047–1067 bp): TTGATGAGAGTCGGCAGCACG, Vg31 (3778–3799 bp): ACTGGAAGCTCTGCGTTGATGG, and Vg32 (4239–4261 bp): TTGACGACGGGCACATTA CCAC.

During the step, Vg31 + XP1378 was extended for 2 min. Vg51 + XP1379 was extended for 1.5 min. At a temperature of 60°C, each cycle was expanded for 25 cycles. PCR was repeated with 1 μL of each product of the abovementioned process, in which the extension time of Vg32 + XP1378 and Vg52 + XP1379 was 1.5 min, and the cycle time was 20 times at 60°C. The final product was subjected to agarose gel electrophoresis and sequencing.

The abovementioned two sequencing results were spliced to obtain the preliminary sequence (Annex 1), and the sequencing results were compared (Annex 2).

2.3. Cloning, Construction, and Protein Induction of Recombinant Plasmid. For expression of Vg, two primers were designed for cloning in Table 1 and the reaction procedure of the PCR experiment is shown in Table 2. The Vg PCR fragment of 825 bp was amplified and cloned into the T4 vector, and positive clone was confirmed by sequencing. The 825 bp fragment was released with SalI/ BamHI from T4 vector and inserted into pMAL-c5x plasmid that was digested with SalI/BamHI.

The recombinant pMAL-Vg-c5x plasmid was transformed into an *E. coli* BL21 (DE3) bacterial strain [24]. The strain was subjected to thermal shock at 42°C for 45 s and was placed on ice for 2 min and then coated with a plate (100 μg/mL ampicillin). After incubation at 37°C overnight, suitable colonies were selected. Four induction conditions of 16°C, 20°C, 28°C, and 37°C were applied, and the optimal effect was compared. Finally, 16°C was determined as the best condition for the following large amount of induction and conduct the mass induction.

Samples collected by centrifugation were placed in the Bioruptor ultrasonic crushing apparatus. The crushing conditions were as follows: continuous 15 s at 4°C and suspension of 15 s for a total length of 2 min for 20 consecutive cycles. The supernatant and precipitation were separated by centrifugation for 20 min at a speed of 1006.2 x g at 4°C. The protein concentration was measured by using the Qubit™ Protein Assay Kit, and the proteins were further identified by SDS-PAGE electrophoresis.

2.4. Immunohistochemistry and Histological Examination Procedure. The purified protein (Vg) was transferred to Xiamen Bo sheng Biological Company for monoclonal antibody preparation. Following the SABC kit instructions (SA1020) to detect the expression site of Vg in sexually

Table 1: The primers designed for the cloning of an 825 bp fragment.

Primer name	Primer sequence (5′-3′)
Vg3.1-SalI-F1	GGCGGCCGCGATATCGTCGACgttaagatcag tgctgccgcg
Vg3.1-BamHI-R	ACCTGCAGGGAATTCGGATCCtcataggagca tcttcatgcac

Table 2: Reaction procedure of PCR.

Cycles	Reaction temperature	Reaction
	95°C	3 min
35 ↑	95°C	15 s
	75°C	15 s
	72°C	300 s
	72°C	5 min
	4°C	∞

mature female fish, the prepared monoclonal antibody above was used for immunohistochemistry.

We fix the intestine, muscle, fishtail, hepatopancreas, ovary, and pronephric kidney in 4% paraformaldehyde. The main procedures included tissue dehydration, embedding, sectioning, and HE staining, and the tissue dehydration is shown in Table 3.

After embedding, paraffin sections were made. The section thickness was set to 5 μm~10 μm for sectioning, and the thickness was adjusted according to the sectioning effect. The slices were rinsed in 30% ethanol solution for a while and spread in warm water at 24°C. After the slices were removed, they were baked in a constant temperature oven at 60°C for 2 h and then dewaxed and rehydrated, as shown in Table 4.

The dewaxed slices were rinsed with running water for HE staining. Paraffin sections dyed completely by HE must be thoroughly dehydrated and transparent before sealing with neutral resin. The HE dyeing procedure is shown in Table 5.

IHC experiments were performed using the pressure cooker method and the antigen repair method, referring to the SABC kit instructions (SA1020).

In the IHC experiment, the powder form of antigen repair solution was dissolved in distilled water and heated to boiling in an autoclaved pot. The sections were immersed in the antigen repair solution and soaked in an autoclaved pot at 121°C for 5 min. The sections were treated with 1‰ TritonX-100 for 10 min, and the sections were washed with 0.01 mol/L PBS three times for 3 min each on a shaker. The sections were incubated with 3% hydrogen peroxide for 10 min at room temperature and washed with 0.01 mol/L PBS three times for 3 min each on a shaker, and the blank control group and experimental group of each section were marked clearly with a histochemical pen.

The samples were incubated in a 37°C constant temperature incubator for 30 min with a drop of 5% BSA blocking solution and then dried. The samples were

Table 3: Organization dehydration program.

Reagents	Time
50% ethanol	20 min
70% ethanol	30 min
80% ethanol	30 min
95% ethanol	1.0 h
100% ethanol I	15 min
100% ethanol II	10 min
100% ethanol + 1/2 xylene	5 min
Xylene I	10 min
Xylene II	10 min
1/2 xylene + 1/2 paraffin	1.0 h
Paraffin I	1.5 h
Paraffin II	1.5 h
Paraffin III	2.0 h

Table 4: Water dewaxing program.

Reagents	Time (min)
Dimethylbenzene III	5
Dimethylbenzene II	5
Dimethylbenzene I	2
1/2 dimethylbenzene + 1/2 ethanol	2
100% ethanol III	2
100% ethanol II	2
100% ethanol I	2
95% ethanol	2
85% ethanol	1
70% ethanol	1
50% ethanol	1
50% ethanol	1

Table 5: HE staining program.

Reagents	Effects	Time
Hematite dyeing solution	Dyeing	5 min
0.5% chemicalbookcheck	Color separation	2 s
1% aqueous ammonia	Return to blue	1 min
1% eosin	Dyeing	15 s
80% ethanol	Rinse	15 s
95% ethanol	Rinse	15 s

incubated in a 37°C constant temperature incubator for 1 h with a drop of primary antibody (1 : 500 PBS). The sections were washed three times with cryophilized PBS (0.01 mol/L) on a shaker for 3 min each.

Biotin-labeled goat antimouse IgG (1 : 100) was dropped and incubated at 37°C for 30 min, and then it was washed three times with PBS for 3 min each. SABC (1 : 100) was added drop, incubated at 37°C for 30 min, and washed with PBS three times for 3 min each. During color development, DAB reagent components A and B in the DAB horseradish peroxidase kit were added to each section with a drop of 100 μL at 1 : 1, and the reaction was performed in a 37°C incubator for 5 min (yellow color was considered as positive reaction). After completing the above operation, the DAB liquid on the glass slide was washed off with running water and 100 μL hematoxylin dyeing solution was added for 30 s. After color development, the residual liquid on the glass slide was washed off with running water. Drop the sealer (glycerin: distilled water/PBS = 9 : 1) on one side of the glass slide and gently place the cover slip to avoid bubbles.

2.5. Exposure Experiment. During the feeding process, the water was changed every 2 days. On the 3rd, 8th, 11th, and 14th days, three fish were randomly captured in one group to obtain the liver and RNA was extracted and transcribed into cDNA for qRT-PCR. The relationship between E2 different concentrations at different times and Vg expression of *B. sinensis* was studied. The qRT-PCR assays were referred to the BlasTaq™ 2X qPCR MasterMix kit, and the procedures are shown in Table 6.

3. Results

3.1. The Full-Length cDNA of BsVg. We download 16 fish gene sequences with close genetic relationships from the NCBI database and design PCR primers by DNAMAN for comparison. The cDNA PCR product from the female *B. sinensis* liver was conducted agarose gel electrophoresis using the primers mentioned above. Then, the cDNA sequence of Vg was further prepared by RACE PCR (Figure 1(b)). According to the size of the Vg, the molecular weight was determined to be about 4600–5000 bp (Figure 1(a)), so the strip was recycled and sent for sequencing. The full-length cDNA of *BsVg* and its encoding amino acid sequence are shown in Annex 1, and agarose gel electrophoresis is shown in Figure 1. The homology of the amino acid sequences of *BsVg* is shown in Annex 2.

We obtain the full-length cDNA of *BsVg*, which fills the blank of the Vg gene bank in *B. sinensis*. The BLAST comparison with other similar fish shows that the similarity was between 47.28% and 65.28%, and the specificity was maintained at more than 30%.

3.2. Expression and Purification Recombinant Plasmid. The soluble protein was obtained by prokaryotic expression of the fusion protein based on the cloned fragment (825 bp) from the full-length cDNA of *BsVg* (4738 bp).

Table 6: List of qRT-PCR program.

Stage	Temperature (°C)	Time
Predegeneration	95	3 min
40 times cycle	95	10 s
	60	10 s
	72	10 s
The third stage	95	30 s
	60	5 s
	95	5 s

The Vg PCR fragment of 825 bp was amplified and cloned into T4 vector and was released with SalI/BamHI from T4 vector and inserted into pMAL-c5x plasmid, which was digested with SalI/BamHI (Figure 2).

The recombinant plasmid was transferred to *E. coli* BL21 for Vg expression. Efficient fusion expression was obtained by IPTG (0.05 mM) at an induced temperature of 16°C, and the expressed target protein (680 amino acids, 75 kDa) existed in a soluble state, accounting for more than 25% of the total soluble protein. SDS-PAGE analysis of the induced expression protein at different temperatures is shown in Figure 3. The maltose binding protein (MBP) fusion expression system and the selected 16°C induction conditions determined that the fusion protein was highly expressed and easy to purify by using the affinity chromatography column.

The highly expressed protein obtained at 16°C could be detected in the supernatant with fewer inclusion bodies. It is in line with our expectations and provides a good foundation for future experiments.

The recombinant Vg fusion protein of *B. sinensis* was further purified by a maltose-binding protein-tag purification column (Dextrin Beads 6FF). The purified product was identified by SDS-PAGE (Figure 4).

Enzymatic digestion of fusion protein monomers with Factor Xa was performed at 20°C, and the time was 28, 32, and 36 h. Fusion protein and Factor Xa are mixed at 20 : 1 (concentration ratio). The results of SDS-PAGE analysis showed that the fusion protein without digestion almost had no target protein Vg at a molecular weight of 35 kDa, while the target protein appeared at 35 kDa after digestion and increased with the extension of digestion time (Figure 5). The obtained protein concentration (1.25 mg/mL) could be accurately measured by using the Qubit™ Protein Assay Kit.

3.3. Immunohistochemical (IHC) Tissue Section Detection. Monoclonal antibodies and purified Vg were identified by Western blot in Figure 6. Following the SABC kit instructions to detection, the expression site of Vg in sexually mature female fish using the monoclonal antibody was prepared by Xiamen Bo sheng Biological Company. We detected Vg expression in the fishtail, hepatopancreas, intestine, muscle, ovary, and pronephric kidney. The results of hematoxylin-eosin staining are shown in Figure 7, and the morphology of the entire cell tissue is clearly visible.

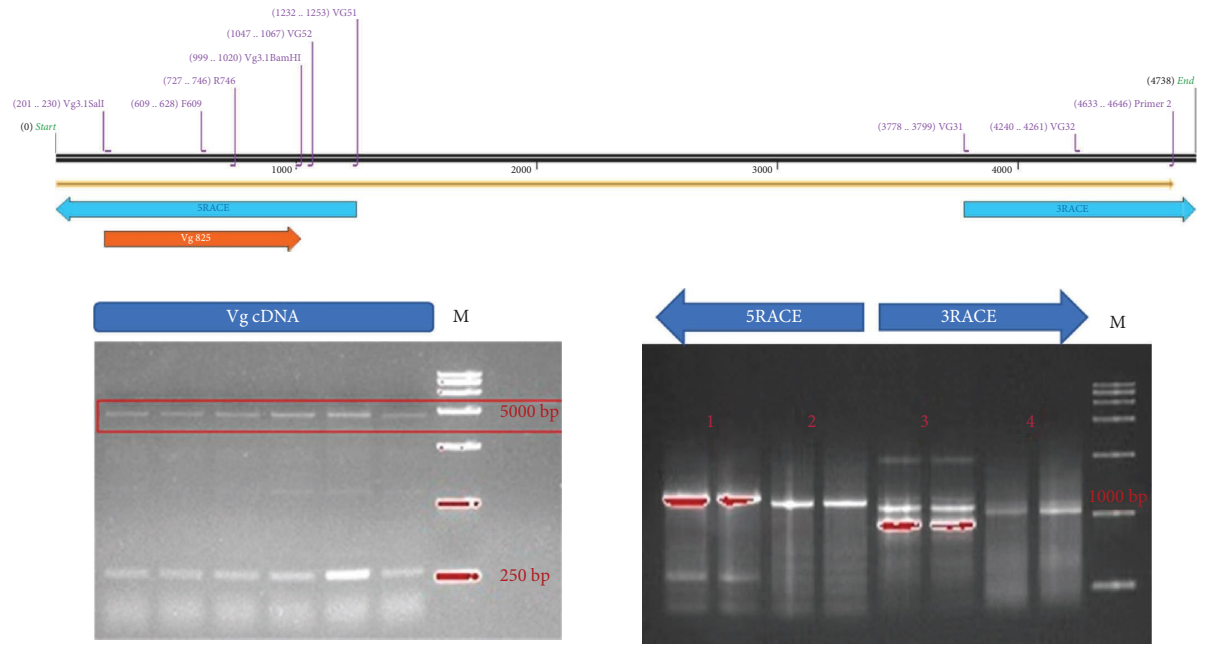

FIGURE 1: Electrophoresis of Vg cDNA (a) and RACE PCR fragment (b) from liver tissue. (a) the full-length cDNA electrophoresis of vitellogenin from *B. sinensis*. (b) RACE PCR gel electrophoresis. 1–4: different colonies after connection with T4 vector and transformed into *E. coli*.

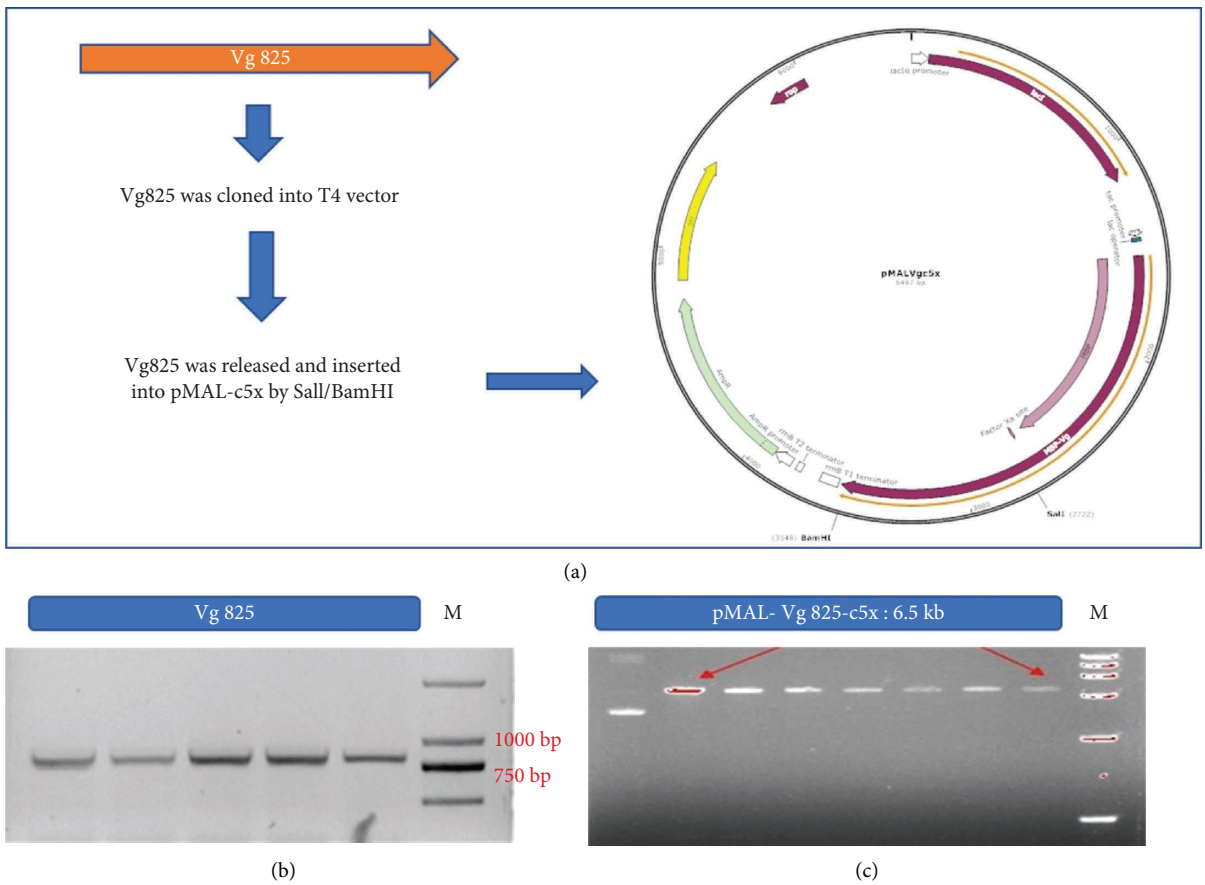

FIGURE 2: Illustration of recombinant plasmid. (a) construction of recombinant plasmid; (b) electrophoresis of Vg825 fragment by PCR; (c) identification of pMAL-Vg825-c5x vector.

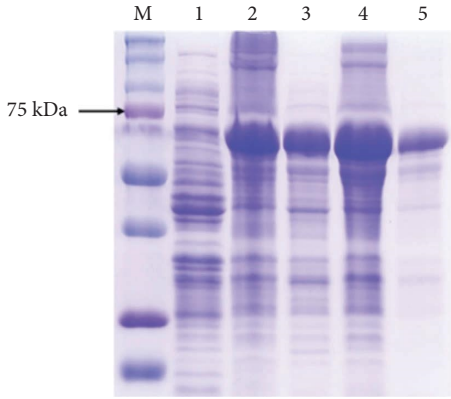

Figure 3: SDS-PAGE analysis of induced expression under different temperature conditions M: molecular marker; lane 1: not induced at 16°C; lane 2: induction at 16°C; lane 3: induction at 20°C; lane 5: induction at 28°C; lane 5: induction at 37°C.

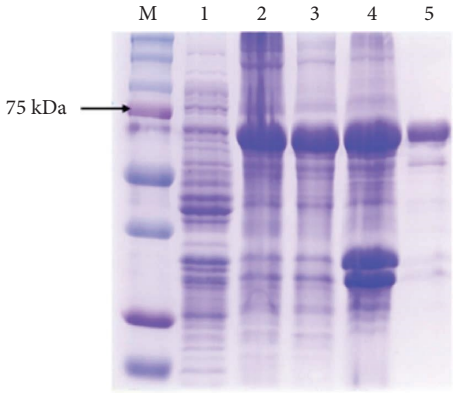

Figure 4: Purified fusion protein by maltose binding protein-tag purification column and SDS-PAGE isolation. M: molecular marker; lane 1: not induced induction at 16°C; lane 2: induction at 16°C; lane 3: supernatant after centrifugation; lane 4: precipitation after centrifugation; lane 5: purified fusion protein Vg.

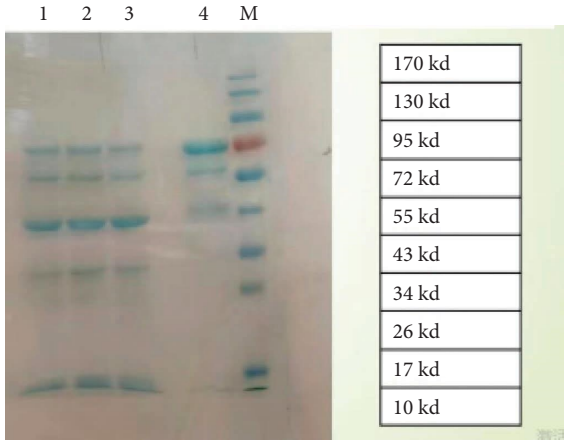

Figure 5: Results of SDS-PAGE analysis of purified fusion protein after MBP was removed by maltose enzyme digestion. M: molecular marker; lane 1–3: enzymatic digestion at 20°C for 28 h, 32 h, and 36 h; lane 4: fusion protein without enzyme digestion was placed at 20°C for 28 h.

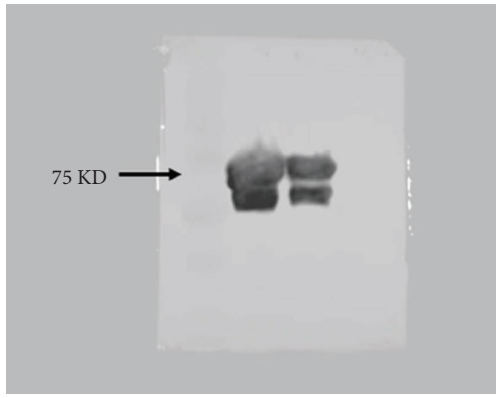

Figure 6: Western blot of monoclonal antibodies and purified Vg.

Immunohistochemical tissue sections show that the monoclonal antibody prepared has certain specificity and can be well applied to tissue detection in Figure 8.

According to the staining results, the expression sites of Vg in sexually mature female fish are mainly distributed in the fishtail, hepatopancreas, intestine, muscle, ovary, and pronephric kidney. The tail has an obvious dyeing effect, and the main guess is that the tail has abundant blood vessels.

3.4. qRT-PCR Analysis. Based on the full-length cDNA of *BsVg*, qRT-PCR assays were used to analyze the relative expression of Vg with E2 induced.

The low-dose group (10 μg/L) was found to increase on day 3, peak on day 8, and then decrease gradually. The medium-dose group (50 μg/L) rose to the highest level on day 8 and then gradually declined, but its level was the highest on day 14 in all groups. The high-dose group (100 μg/L) had a low level on day 3, a high level on day 8 and day 11, and a significant decrease on day 14 (Figure 9).

Analysis of Vg expression in different concentrations of E2 and different exposure time by qRT-PCR indicated that Vg was not or low expressed in normal male fish, but it was expressed in female fish. However, the expression level of male fish began to increase with time, there was an increase in E2 concentration, and the expression level was higher than that of Vg in female fish. It was proved that the Vg and its expression in male *B. sinensis* could accurately reflect prolonged pollution status and the degree of environmental estrogens.

4. Discussion

At present, in the process of life science research and the production of biological products [8], the preparation of recombinant proteins using expression vectors is one of the most important technologies [25]. The construction of an effective expression vector is the basic requirement for the expression of target genes [24], and it is also an important factor affecting the gene expression level and protein activity [14].

In addition, *B. sinensis* is a commercially important fish in southeastern China and Taiwan Strait of China [26] for its

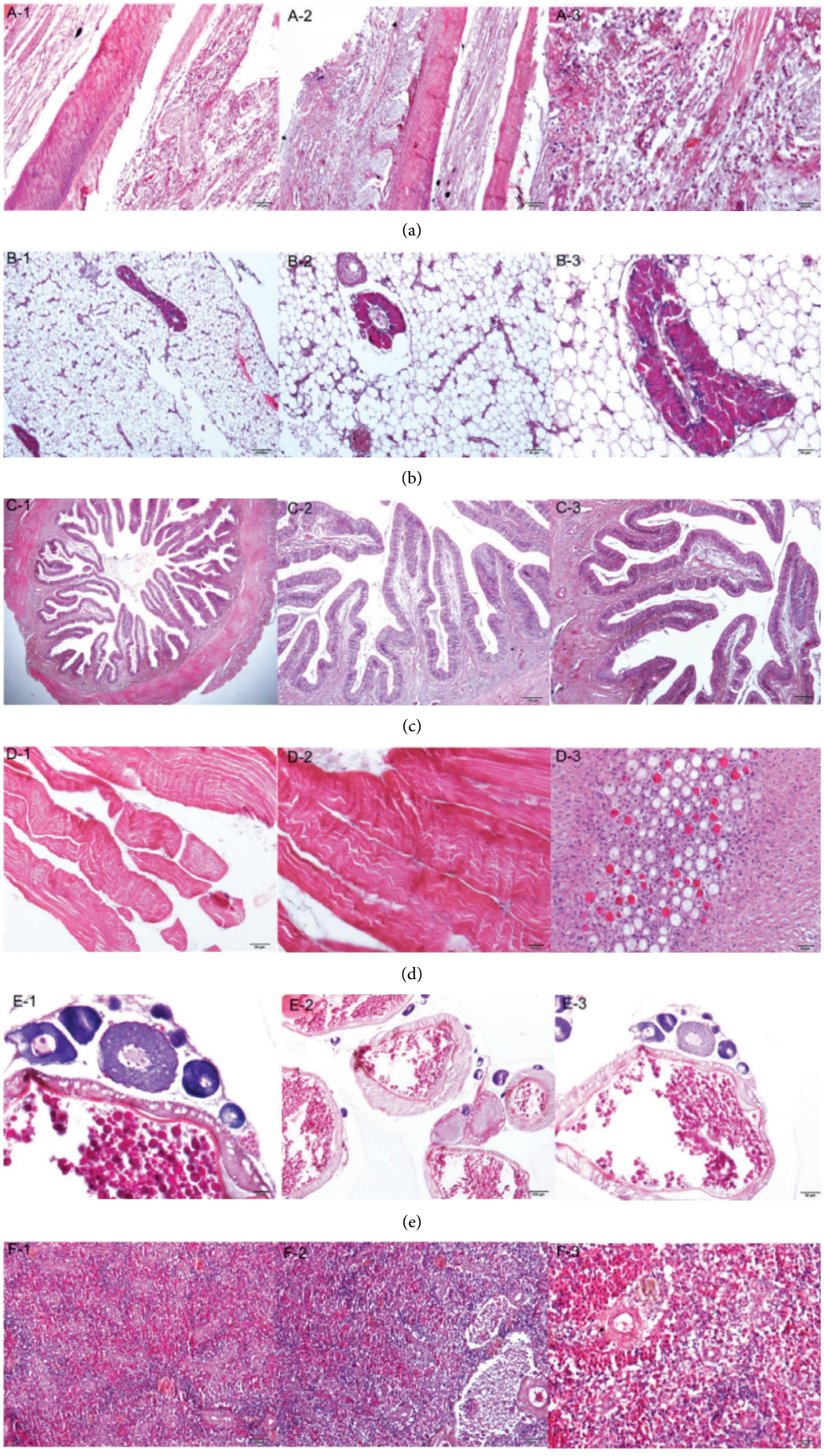

FIGURE 7: HE staining of different tissues in female *B. sinensis* (a) fishtail (b) hepatopancreas (c) intestine (d) muscle (e) ovary (f) pronephric kidney.

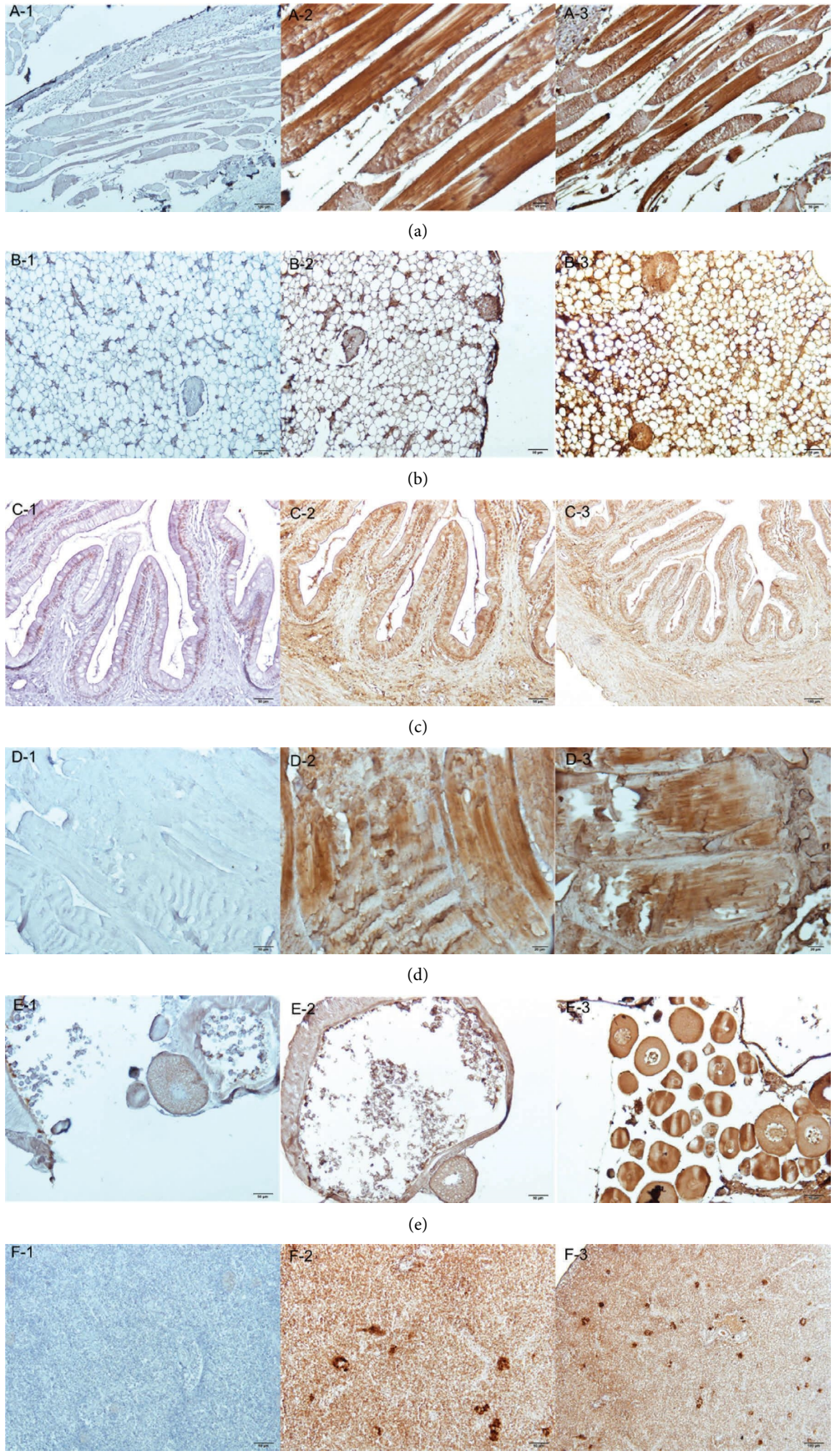

FIGURE 8: IHC of different tissues in female *B. sinensis* (a) fishtail (b) hepatopancreas (c) intestine (d) muscle (e) ovary (f) pronephric kidney 1: negative control 2: positive control 3: positive control.

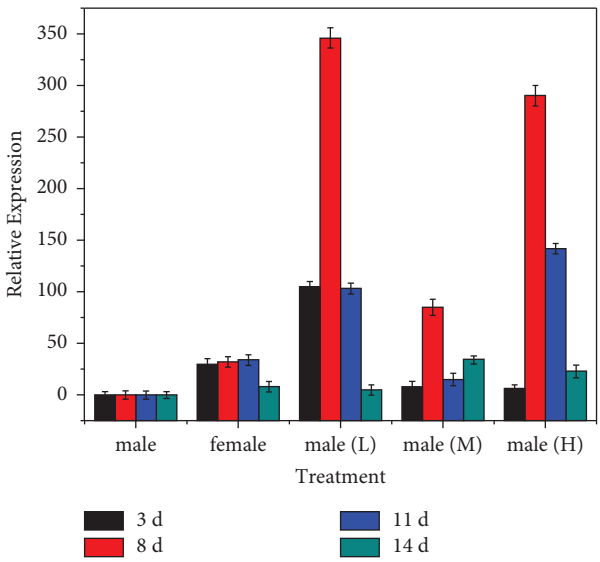

Figure 9: qRT-PCR analysis of relative expression of *BsVg* L: low-dose group, 10 μg/L; (M) medium-dose group, 50 μg/L; (H) high-dose group, 100 μg/L male: normal male fish; female: normal female fish; ordinate: relative expression. 0–400; 3 d: treatment for 3 days; 8 d: treatment for 8 days; 11 d: treatment for 11 days; 14 d: treatment for 14 days.

delicious taste, rich nutrition, and a believed traditional medical function of promoting wound healing. Nowadays, this species is gradually becoming an important mariculture fish in the coastal areas of Zhejiang, Fujian, and Guangdong provinces, China [22]. However, such as other farming fish species in China [27], *B. sinensis* has suffered from many kinds of estrogen contamination. Therefore, better understanding the mechanisms underlying the Vg response of *B. sinensis* to EEs will help us develop strategies for the management of the environment and enhance the healthy development of the mariculture of this commercial fish species.

In our research, total RNA was extracted from the liver tissue of male *B. sinensis*, and the full-length cDNA of *BsVg* was obtained by reverse transcription amplification. The Vg fusion protein with MBP-tag was formed by the construction of a recombinant plasmid, and the optimal induced expression condition (16°C, 0.05 mM IPTG) was found. The highly expressed protein obtained at 16°C could be detected in the supernatant and precipitation after centrifugation, mainly in the supernatant. It provides a good foundation for our future experiments.

In this experiment, the obtained Vg was transferred to the Xiamen Bosheng Biological Company for preparation of monoclonal antibodies. The expression of Vg in sexually mature female fish was mapped by the immunohistochemistry assay. The results show that Vg mainly distributed in the fishtail, hepatopancreas, intestine, muscle, ovary, pronephric kidney, and the tail is most obvious.

Based on the full-length cDNA of *BsVg*, the relative expression of Vg induced by E2 was analyzed by the qRT-PCR method. Three groups of different concentrations of E2 exposure were designed in 3, 8, 11, and 14 days from the *B. sinensis* liver. Results show that the Vg expression and exposure concentration have an obvious dose effect relationship, and the most obvious expression is on the eighth day of the exposure cycle.

Therefore, the regulation and expression of Vg could better reflect the pollution status of environmental estrogen, and it is more accurate and practical for environmental monitoring and evaluation as a biomarker of environmental estrogen [10].

Our study shows that Vg could be used as a biological monitoring index in marine environments, and the full-length cDNA of *BsVg* was obtained. Meanwhile, localization and quantitative detection methods of Vg expression were also achieved. The subsequent experiments of development in colloidal gold strips after this research would be established to provide a highly efficient and convenient environmental pollution detection method [28], and the colloidal gold strip depends on the monoclonal antibody prepared in this paper. At present, the detection range and type of the strip are still under further testing, and we will continue to carry out relevant research in this respect.

5. Conclusion

The full-length cDNA cloning of the *BsVg* gene and the expression and purification of the corresponding fusion protein of this gene through recombinant plasmid construction were realized in this study. The prepared antiVg monoclonal antibody of *B. sinensis* enables the localization detection of Vg in its tissues. The expression level of the *Vg* gene can effectively reflect the estrogenic effects of environmental estrogens on *B. sinensis*. The experimental results of this study provide a solid scientific basis for the application of *B. sinensis* and their Vg in the monitoring of aquatic environmental estrogen pollution, and the prepared monoclonal antibody can be used for the research of the colloidal gold detection method subsequently.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval

These animal experiments were approved by the Institutional Animal Care and Use Committee and were in strict accordance with good animal practice as defined by the Xiamen University Laboratory Animal Center (XMULAC20200022).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Supplementary Materials

Annex 1: the full-length sequence cDNA of *BsVg*. Annex 2: comparison of amino acid homology between *B. sinensis* and other fishes in GeneBank. (*Supplementary Materials*)

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