



Short communication

The first molluscan TCTP in *Venerupis philippinarum*: Molecular cloning and expression analysisChenghua Li^a, Limei Qiu^b, Xuanxuan Ning^c, Aiqin Chen^a, Song Qin^a, Huifeng Wu^a, Jianmin Zhao^{a,*}^a Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, 17 Chunhui Road, Laishan District, Yantai 264003, China^b Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China^c Yantai Oceanic Environmental Monitoring Central Station of SOA, Yantai 264006, China

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ABSTRACT

Translationally controlled tumor protein (TCTP) is one of the abundant and ubiquitously expressed proteins in metazoans. In the present study, the first molluscan TCTP (denoted as VpTCTP) was identified from *Venerupis philippinarum* haemocytes by EST and RACE approaches. The full-length cDNA of VpTCTP consisted of 1148 nucleotides with an open-reading frame of 555 bp encoding 184 amino acids. The deduced amino acid sequence of VpTCTP shared high similarity with TCTPs from other species, indicating that VpTCTP should be a new member of TCTP family. Several highly conserved motifs, including 5' terminal ologopyrimidine (5'TOP) starting sequence and rich AU and AUUT elements in 3'UTR, were also identified in VpTCTP. The tissue and temporal expression of VpTCTP after *Vibrio anguillarum* challenge was recorded by quantitative real-time RT-PCR. VpTCTP transcript could be detected in all examined tissues with the highest expression level in haemocytes and the lowest in hepatopancreas. Concerning the time-course expression in haemocytes, the relative expression of VpTCTP mRNA was down-regulated sharply from 6 h to 12 h post-infection. Then, the expression level was obviously up-regulated and reached 3.4-fold to that in the control group at 48 h post challenge. As time progressed, the expression of VpTCTP recovered to the original level at 96 h. All these results indicated that VpTCTP was an acute-phase protein involved in the immune response of *V. philippinarum*.

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1. Introduction

The translationally controlled tumor protein, commonly known as TCTP, is a highly conserved protein existed in a range of eukaryotic organisms, including protozoa, yeasts, plants, nematodes and mammals. Recently, much attention has been paid to the ubiquitous protein for its multiple roles in various biologically and medically relevant processes [1]. Accumulating evidence indicates that TCTP could: 1) regulate cell growth, division, organ size and apoptosis [2–4]; 2) be involved in stress responses caused by heavy metals and heat shock [5,6]; 3) be relevant in the defense against virus or bacterial pathogen [7,8].

Solution structure analysis showed that yeast TCTP shared structural similarity to a family of guanine nucleotide-free chaperones, indicating that TCTP might be functioned as molecular chaperone [9]. Gnanasekar et al. also demonstrated that both human and parasite *Schistosoma mansoni* TCTPs could bind to a variety of denatured proteins and protect them from the harmful effects of thermal

shock [10]. Moreover, the human TCTP mRNA was found to possess a strong secondary structure and able to induce the dsRNA-dependent protein kinase R, suggesting a connection to the interferon pathway [1]. In shrimp *Penaeus japonicus* and *Penaeus monodon*, TCTP expression was both up-regulated after WSSV challenge, while the level of *P. monodon* TCTP expression was significantly decreased when shrimp showed the mortality characteristic, indicating that TCTP was a key factor involved in innate immunity [11,12].

As an important protein involved in host immunity response with chaperone activity, TCTP has not been identified from mollusk so far. In order to fill in the gap, the present study aims to: (1) clone the full-length cDNA of TCTP from *Venerupis philippinarum* (denoted as VpTCTP); (2) investigate the tissue and temporal expression profile of VpTCTP after being infected by *Vibrio anguillarum* pathogen.

2. Materials and methods

2.1. Clams and bacterial challenge

The clams *V. philippinarum* (7.5–11 g in weight) were purchased from Qingdao, Shandong Province, China. The clams were

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acclimated for a week before processing. After the acclimation period, the clams were randomly divided into six flat-bottomed rectangular tanks with 50 L capacity, each containing 50 clams. The temperature was held at 20–22 °C throughout the whole experiment. The salinity for the supplied seawater was kept at 30‰.

For the *V. anguillarum* challenge experiment, one tank served as control. The other five tanks were immersed with high density of *V. anguillarum* with final concentration of 10^7 CFU mL⁻¹. The infected clams were randomly sampled at 6 h, 12 h, 24 h, 48 h, 72 h and 96 h respectively. The haemolymphs from the control and the infected groups were collected using a syringe individually and centrifuged at 2000×g, 4 °C for 10 min to harvest the haemocytes. There were five replicates for each time point.

2.2. cDNA library construction and EST analysis

A cDNA library was constructed from the haemocytes of a clam challenged by *V. anguillarum*, using the ZAP-cDNA synthesis kit and ZAP-cDNA GigapackIII Gold cloning kit (Stratagene). Random sequencing of the library using T3 primer yielded 3226 successful sequencing reactions. BLAST analysis of all the 3226 EST sequences revealed that an EST of 448 bp was highly similar to the identified TCTP from shrimp. Therefore, this EST sequence was selected for further cloning of the full-length cDNA of TCTP from *V. philippinarum*.

2.3. RNA isolation and cDNA synthesis

Total RNA was isolated from the haemocytes of clams using the TRIzol reagent (Invitrogen). First-strand cDNA synthesis was performed according to Promega M-MLV RT Usage information with the RQ1 RNase-Free DNase (Promega)-treated total RNA (1 µg) as template and oligodT primer. The reactions were incubated at 42 °C for 1 h, terminated by heating at 95 °C for 10 min. TdT (Takara) was used to add homopolymer dCTP tails to the 5' end of the purified first-strand cDNA.

2.4. Cloning of the full-length cDNA of VpTCTP

Four specific primers, including two forward primers P1 (TGCTGACCTTATTGGTGAAAC), P2 (GATAACCAGTTGGAGGATGTAC), and two reverse primers P3 (ATGGGAATAAACTAGCCTGTAC), P4 (TTTCCACCAATAAGGTCAGCAC), were designed based on the EST to clone the full-sequence cDNA of VpTCTP. The first round PCR reaction to get 3'-end was amplified by using primers P1 and oligodT. Using 1 µl of 1:50 dilution of the first round PCR products as nested PCR template, the 3'-end nested PCR reaction was performed with primers P2 and oligodT. The PCR amplification of 5'-end of VpTCTP was carried out with reverse primers P3 and oligodG. For the second round PCR, the above PCR products were used as template, and reverse primer P6 and oligodG were used as primers.

The PCR programs were performed in a Applied Biosystem Veriti 96 well Thermal Cycler in a 25 µl reaction volume containing 2.5 µl of 10×PCR buffer, 1.5 µl of MgCl₂ (25 mmol L⁻¹), 2 µl of dNTP (2.5 mmol L⁻¹), 1 µl of each primer (10 µmol L⁻¹), 15.8 µl of PCR-grade water, 0.2 µl of Taq polymerase (5 U µl⁻¹) (Promega) and 1 µl of cDNA template. The PCR temperature profiles were 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 2 min and an additional extension at 72 °C for 10 min.

The PCR product was gel-purified and cloned into pMD18-T simple vector (Takara). After transformed into the competent cells of *Escherichia coli* JM109, the positive recombinants were identified through ampicillin selection and PCR screening with M13-47 and RV-M primers. Three of the positive clones were sequenced on an ABI3730 Automated Sequencer (Applied Biosystem).

2.5. Sequence analysis of VpTCTP

The VpTCTP gene sequence was analyzed using the BLAST algorithm at NCBI web site (<http://www.ncbi.nlm.nih.gov/blast>), and the deduced amino acid sequence was analyzed with the Expert Protein Analysis System (<http://www.expasy.org/>). The percentages of similarity and identity of VpTCTP with TCTP proteins from other organisms were calculated by the Identity and Similarity Analysis program (<http://www.biosoft.net/sms/index.html>). Protein phosphorylation sites were predicted with PROSITE scan (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_prosite.tml).

2.6. VpTCTP mRNA distribution in different tissues and temporal expression profile in haemocytes post *V. anguillarum* challenge

For the tissue distribution experiment, the clams were cultured in seawater with high density of *V. anguillarum* (10^7 CFU mL⁻¹) for 5 days. Fifty milligram of various tissues, including haemocytes, foot, gill, hepatopancreas and muscle, were dissected from three individuals, and subjected to total RNA extraction as described above. Single-strand cDNA was synthesized and diluted to 1:50 for subsequent SYBR Green assay.

The tissue distribution and temporal expression of VpTCTP transcripts in haemocytes of clams challenged with *V. anguillarum* were determined by quantitative real-time RT-PCR. Two VpTCTP gene-specific primers P5 (GAAACCCATCCAGGAGGAGCAA) and P6 (TCCTACATCAAAGCCTTCACGGAC) were used to amplify a product of 302 bp. A set of actin primers, P7 (CTCCCTTGAGAAGAGCTACGA) and P8 (GATACCAGCAGATTCCATACCC), were used to amplify a product of 121 bp served as internal control. The quantitative real-time RT-PCR was carried out in an Applied Biosystem 7500 Real-time PCR System, and performed in a total volume of 20 µl, containing 10 µl of 2×SYBR Green Master Mix (Applied Biosystems), 6 µl of the diluted cDNA mix, 0.25 µl of each primer (10 µmol L⁻¹), 3.5 µl of DEPC-treated water. The thermal profile for real-time PCR was 50 °C for 2 min and 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Dissociation curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. After the PCR program, data were analyzed with SDS 2.01 software (Applied Biosystems). To maintain consistency, the baseline was set automatically by the software. The comparative Ct method was used to analyze the relative expression levels of VpTCTP as previously described [13]. All data were given in terms of relative mRNA expression as means ± S.E. The results were subjected to *t*-test analysis and the *P* value less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. cDNA cloning and sequence analysis of VpTCTP

An 839 bp fragment containing polyA tail was cloned from the cDNA of *V. philippinarum* haemocytes by using the primers P2 and oligodT. Blastx analysis indicated the fragment was similar to the TCTP from *Fenneropenaeus chinensis* (ABB05536). The 5'-end was obtained with primer P4 and oligodG to get a 236 bp product. By overlapping the three fragments together, an 1148 bp nucleotide sequence representing the full-length cDNA of VpTCTP was assembled and deposited in GenBank under accession no. GQ384411. The complete nucleotide and deduced amino acid sequence of VpTCTP were shown in Fig. 1.

The cDNA sequence of VpTCTP contained a 555 bp ORF, flanked by a 78 bp 5' UTR with a stop codon TAA preceding the initial methionine and a 515 bp 3'UTR with a canonical polyadenylation

signal site AATAAA and a polyA tail. To be mentioned, the 5'UTR of VpTCTP started with a 5' terminal oligopyrimidine (5'TOP) (Boxed in Fig. 1), characteristic for a certain group of translationally controlled mRNAs, such as ribosomal protein mRNA. The 3'UTR harbored AT-rich regions and ATTTA elements, which was another typical feature of TCTP mRNAs [1].

The ORF encoded a polypeptide of 184 amino acid residues with a calculated molecular mass of 20.52 kD and theoretical pI of 4.57. Notably, most of the characterized TCTPs possessed average size of 170 aa. The only similar one to VpTCTP was identified from *Hydra vulgaris* with 184 aa in size (AAB18403). The difference in molecular weight was not clear to our knowledge, and was perhaps connected to the varied evolution manners and substitution rates of the amino acid residues in TCTPs [4].

3.2. Homologous analysis of VpTCTP

Blast analysis revealed that VpTCTP showed high identities with other registered TCTPs. For example, it shared 56% identity with *Drosophila virilis* (EDW59431) counterpart, 55% identity with TCTPs from *Artemia franciscana* (ABZ81535), *F. chinensis* (ABB05536) and *Cyprinus carpio* (ABC59222), 54% identity with *Danio rerio* TCTP (NP-937783). Potential phosphorylation sites were identified from VpTCTP through Prosite scan at PBIL. VpTCTP contained three potential protein kinase C phosphorylation sites [STI-x-[RK], corresponding to the amino acid positions at 38aa–40aa (SRK), 116aa–118aa (TEK) and 169aa–171aa (TIK), three casein kinase phosphorylation sites at 9aa–12aa (TGDE), 55aa–58aa (SQEE) and 71aa–74aa (SGID). Two tyrosine kinase phosphorylation sites [RK]-x(2,3)-[DE]-x(2,3)-Y were also identified from VpTCTP, located at 136aa–144aa (KYSDDVSVY) and 146aa–154aa (REGFDVGAY). Additionally, GGNPSQ (51aa–56aa) and GSLVSY (93aa–98aa) in

VpTCTP were predicted to be N-myristoylation sites, which were consensus to its typical sequence G-(EDRKHPFYW)-x(2)-[STAGCN]-{P}. Similar phosphorylation sites were also identified in TCTPs from sea perch [14] and flatfish trout [15]. These conserved motifs strongly indicated VpTCTP should be one new member of TCTP family.

3.3. Tissue-specific expression of VpTCTP

To examine the tissue distribution profile of VpTCTP, total RNA was isolated from tissues of hepatopancreas, gill, muscle, haemocytes and foot. The expression of VpTCTP transcript was predominantly detectable in the haemocytes and gills, and marginally detected in the tissues of muscle, foot and hepatopancreas (Fig. 2). Considering the abundance of VpTCTP transcript in haemocytes, the expression in other tissues may be derived from haemocyte contamination. The tissue expression of VpTCTP was contradicted to the result in shrimp, where the highest expression level was observed in hepatopancreas and lowest expression level in haemocytes [7]. The distinct difference concerning the expression profile of hepatopancreas and haemocytes might be partially explained by their entirely different response manners towards *Vibrio* challenge and species difference.

3.4. Temporal expression profile of VpTCTP transcript post *Vibrio* challenge

The temporal expression of VpTCTP transcript after *Vibrio* challenge was shown in Fig. 3. The mRNA transcript decreased sharply from 6 h to 12 h after infection (only 1/4 of the control group at 12 h). Then, the expression level was up-regulated to 3.4-fold and 3.2-fold of the control group at 48 h and 72 h respectively. At 96 h, the expression drop back to the original level. Significant differences of the expression level of VpTCTP were observed at 12 h ($P < 0.01$), 48 h ($P < 0.01$) and 72 h ($P < 0.05$) post injection compared with the control group.

TCTP had been demonstrated to be an anti-apoptotic protein and antioxidant protein [16]. In fly and mouse, TCTPs could block the normal immune responses by acting as dominant negative mutants [4]. The expression profile of VpTCTP presented here could be explained according to the suggested functions. During the early stage of bacterial challenge, host immunity response was initiated to answer up the challenge. VpTCTP, a negatively regulatory factor,

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1  GGGGGCTTTTCGGTTCGTCTTAGAAATTCGGTCAAATTGATTCAGTTGGGTAAAAATC
61  TAACATAATCCGATCAAATGATTCCTTTACAAGGATTAATGACAGGGGATGAAATGTTTC
    M I L Y K D L M T G D E M F
121 ACAGATGCATCCCCATGCATTATGAGGGTGAAGATGATTCCTGTATGTGTTGAAGGA
    T D A F P M H Y E G E D D F L V C V E G
181 AAGCTTGTATCGCGCAAGAAGGGTGAATCAATCAGTGCAGCTTATGGTGGAAACCCA
    K L V S R K K G E S I S A D L I G G N P
241 TCCACGAGGAGCAAGGAGAGGAGTGTGTGATGACACTGAGACTGTGTGACGGCATTGAT
    S Q E E Q G E E C C D D T E T V S G I D
301 GTTGTATTAGATAACCAGTTGGAGGATGTACAGGCTAGTTTATCCCATCAAAGGAAGT
    V V L D N Q L E D V Q A S L F P S K G S
361 TTAGTGAGCTACTGAAGAAGTTTGTCAAAAAGGTAGTTGATGGTCTACAGAAGAAGGT
    L V S Y L K K F V K K V V D G L T E E G
421 GATACAGAAAAATGTGATTGCTTTAAAAAGCCATGTCAGCACACATTAACAATTTGTG
    D T E K C D C F K K A M S A H I K Q F V
481 GGCATAATTCGGATGATGTTTCAGTTTATGTCGGTGAAGGCTTGTATGATGAGGATAC
    G K Y S D D V S V Y V R E G F D V G A Y
541 AAACAGGCTCCAATGATGGTGTGGTCAAGGATGGTCTACTATCAAATGTTATGCT
    K Q A P M I G V W S E D G R T I K L Y A
601 ATGAAACTGTCTAATGAAGAAAAATGCTAACCCCTGTACAGATGCCAGCAAGTCCG
    M K H C L I E E K C *
661 GAGTCTTCCAGAAGCTTAATAAAAAATGTATCATTATAAATCATGTAACAATTAATA
721 CAAGAAGAACCAGGTAGACATAACATAATTTGACTTCTGGTATTCAACAACCTCACTT
781 CAATTTTATATTACATTCACATTTAAAGACAAAATTATGTTATGTCATAATTAGGGGCT
841 TTTAAACGTACTTTTATGTTGATGTTAACTGAATTTGTTGAAATTTATGACACTTA
901 AAACGAAAAAAGAAGCTTACATGATGAAAAACCTATTAATAGGGTGTGATAAAAAAT
961 GGTGCAATAGTTTACAAATATGTTAAATTTTAAACATCTCGTGTGAAAGCCCTGTAGCG
1021 CATATATAAATCATGAAATAAAGTACCGCTTATCTGTGTTTATATCTGTGTTGTTAA
1081 AAATGATCGAGTTATCATTCAAGCCAAATAAATAATTTATTTCAAAAAAATAAAAAA
1141 AAAAAAAA

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Fig. 1. The nucleotide sequence (above) and its deduced amino acid sequence (bottom) of VpTCTP. Nucleotides are numbered from the first base at the 5' end. The 5'-terminal oligopyrimidine tract (5'-TOP) is boxed. The canonical polyadenylation signal sequence AATAAA is underlined and shadowed.

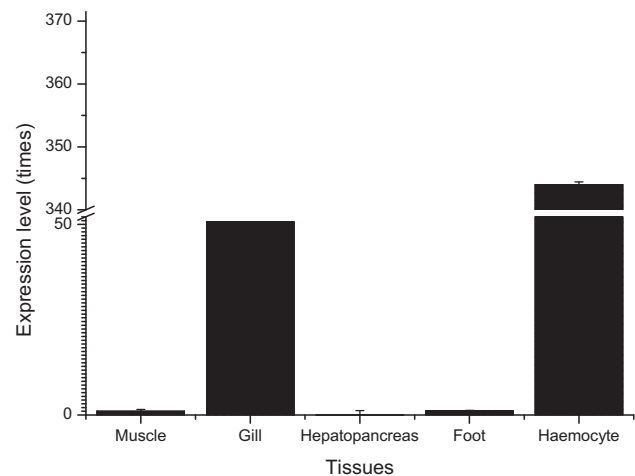


Fig. 2. Tissues expression level of VpTCTP after *Vibrio anguillarum* infection measured by quantitative real-time PCR. Each symbol and vertical bar represents the mean \pm SD ($n = 3$).

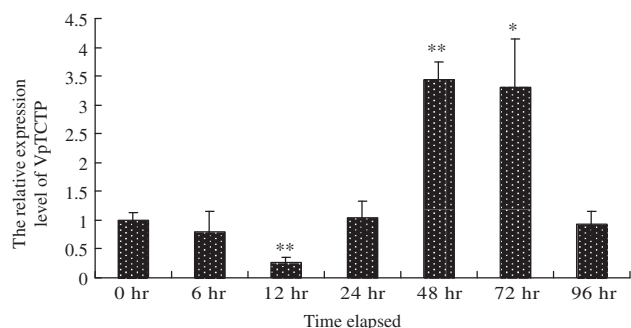


Fig. 3. Time-course expression level of VpTCTP transcript in haemocytes after *Vibrio anguillarum* infection measured by quantitative real-time PCR at 0 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h. Each symbol and vertical bar represents the mean \pm S.D ($n = 5$). Significant differences between challenged group and control group are indicated by an asterisk ($P < 0.05$) and two asterisks ($P < 0.01$), respectively.

was down-regulated to achieve the goal. During the combat between host and pathogen, haemocytes were involved in many processes to eliminate the pathogen, including wound recovery, respiratory burst, encapsulation and phagocytosis, leading to the decrease of total number of haemocytes (THC) in the end, which was observed in many other organisms [17–20]. The change of THC perhaps required the down-regulation of the expression of anti-apoptotic protein VpTCTP. As an antioxidant protein, the expression of VpTCTP must be restricted at lower level to avoid the degradation of reactive oxygen species produced at the first several hours. With time progressed, the hazard effect of cell apoptosis, immunity responses and ROS came to obvious. Therefore, the expression of VpTCTP was up-regulated to minimize the negative effects. To better understand the immune roles of VpTCTP, it would be necessary to determine the localization of VpTCTP in specific haemocytes and also the correlation between the expression level and haemocyte counts.

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References

- [1] Bommer U, Thiele B. The translationally controlled tumor protein (TCTP). *Int J Biochem Cell Biol* 2004;36:379–85.

- [2] Gachet Y, Tournier S, Lee M, Lazaris-Karatzas A, Poulton T, Bommer UA. The growth-related, translationally controlled protein P23 has properties of a tubulin binding protein and associates transiently with microtubules during the cell cycle. *J Cell Sci* 1999;112:1257–71.
- [3] Venugopal T. Evolution and expression of translationally controlled tumour protein (TCTP) of fish. *Comp Biochem Physiol B* 2005;142:8–17.
- [4] Hinojosa-Moya J, Xoconostle-Cázares B, Piedra-Ibarra E, Méndez-Tenorio A, Lucas WJ, Ruiz-Medrano R. Phylogenetic and structural analysis of translationally controlled tumor proteins. *J Mol Evol* 2008;66:472–83.
- [5] Gnanasekar M, Thirugnanam S, Zheng G, Chen A, Ramaswamy K. Gene silencing of translationally controlled tumor protein (TCTP) by siRNA inhibits cell growth and induces apoptosis of human prostate cancer cells. *Int J Oncol* 2009;34:1241–6.
- [6] Mak CH, Poon MW, Lun HM, Kwok PY, Ko RC. Heat-inducible translationally controlled tumor protein of *Trichinella pseudospiralis*: cloning and regulation of gene expression. *Parasitol Res* 2007;100:1105–11.
- [7] Wang S, Zhao X, Wang J. Molecular cloning and characterization of the translationally controlled tumor protein from *Fenneropenaeus chinensis*. *Mol Biol Rep* 2009;36:1683–93.
- [8] He N, Qin Q, Xu X. Differential profile of genes expressed in hemocytes of white spot syndrome virus-resistant shrimp (*Penaeus japonicus*) by combining suppression subtractive hybridization and differential hybridization. *Antiviral Res* 2005;66:39–45.
- [9] Thaw P, Baxter NJ, Hounslow AM, Price C, Waltho JP, Craven CJ. Structure of TCTP reveals unexpected relationship with guanine nucleotide-free chaperones. *Nat Struct Biol* 2001;8:701–4.
- [10] Gnanasekar M, Dakshinamoorthy G, Ramaswamy K. Translationally controlled tumor protein is a novel heat shock protein with chaperone-like activity. *Biochem Biophys Res Commun* 2009;386:333–7.
- [11] Bangrak P, Graidist P, Chotigeat W, Phongdara A. Molecular cloning and expression of a mammalian homologue of a translationally controlled tumor protein (TCTP) gene from *Penaeus monodon* shrimp. *J Biotechnol* 2004;108:219–26.
- [12] Rajesh S, Kiruthiga C, Rashika V, Priya R, Narayanan RB. Molecular cloning, sequence analysis and tissue expression of translationally controlled tumor protein from the WSSV-infected Indian shrimp *Penaeus indicus*. *Aquac Res* 2010;41:545–51.
- [13] Li C, Ni D, Song L, Zhao J, Zhang H, Li L. Molecular cloning and characterization of a catalase gene from Zhikong scallop *Chlamys farreri*. *Fish Shellfish Immunol* 2007;24:27–34.
- [14] Qiu L, Song L, Wu L, Xu W, Jiang S. cDNA cloning and mRNA expression of the translationally controlled tumor protein(TCTP)gene from Japanese sea perch (*Lateolabrax japonicus*). *Acta Oceanol Sin* 2005;24:113–9.
- [15] Wang J, Guo H, Zhang S, Li Yin, Guo B, Wang S. Cloning, expression and characterization of translationally controlled tumor protein (TCTP) gene from flatfish turbot (*Scophthalmus maximus*). *J Ocean Univ Chin* 2008;7:184–92.
- [16] Gnanasekar M, Ramaswamy K. Translationally controlled tumor protein of *Brugia malayi* functions as an antioxidant protein. *Parasitol Res* 2007;101:1533–40.
- [17] Cheng W, Liu CH, Chen JC. Effect of dissolved oxygen on the immune response of *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus*. *Aquaculture* 2004;232:103–15.
- [18] Chiu CH, Guu YK, Liu CH, Pan TM, Cheng W. Immune responses and gene expression in white shrimp, *Litopenaeus vannamei*, induced by *Lactobacillus plantarum*. *Fish Shellfish Immunol* 2007;23:364–77.
- [19] Li CC, Yeh ST, Chen JC. The immune response of white shrimp *Litopenaeus vannamei* following *Vibrio alginolyticus* injection. *Fish Shellfish Immunol* 2008;25:853–60.
- [20] Rodríguez-Ramos T, Espinosa G, Hernández-López J, Gollas-Galván T, Marrero J, Borrell Y, et al. Effects of *Echerichia coli* lipopolysaccharides and dissolved ammonia on immune response in southern white shrimp *Litopenaeus schmitti*. *Aquaculture* 2008;274:118–25.