Fish and Shellfish Immunology 86 (2019) 516–524

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short communication

Comparative study of β-thymosin in two scallop species *Argopecten irradians* and *Chlamys farreri*

Mengqiang Wang[a,c], Baojie Wang[a], Mei Liu[a], Keyong Jiang[a], Lei Wang[a,b,d,*]

[a] CAS Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, 266071, China
[b] Laboratory for Marine Biology and Biotechnology, National Laboratory for Marine Science and Technology, Qingdao, 266237, China
[c] Research Platform for Marine Molecular Biotechnology, National Laboratory for Marine Science and Technology, Qingdao, 266237, China
[d] CAS Center for Ocean Mega-Science, Chinese Academy of Sciences, Qingdao, 266400, China

ARTICLE INFO

Keywords:
β-thymosin
Innate immunity
Immunomodulation

ABSTRACT

The β-thymosin (Tβ) proteins participate in numerous biological processes, such as cell proliferation and differentiation, anti-inflammatory and antimicrobial mechanism. To date, Tβ proteins have been well studied in vertebrates, especially mammals. While limited Tβ or Tβ-like proteins have been reported in invertebrates. Moreover, rare information of Tβ or Tβ-like proteins is available in scallop species yet. In the present study, two Tβ homologues, *Ai*Tβ and *Cf*Tβ, were identified and characterized from two scallop species bay scallop *Argopecten irradians* and Zhikong scallop *Chlamys farreri*. They were both 41 amino acid peptide and contained one THY domain, a highly conserved actin-binding motif and two conserved helix forming regions. Tissue distribution and expression profiles of their mRNA transcripts were roughly similar yet different in detail, while their recombinant proteins exhibited different immunomodulation activity on the downstream immune parameters. These results collectively indicated that the function of Tβ family in scallop were functionally differentiated.

1. Introduction

Thymosin was first isolated from calf thymus in 1965, initially named as thymic hormone and then termed as thymosin according to its immunomodulation function on the immune system as a potential thymic lymphocytopoietic factor [1,2]. Thymosin represents a family of highly conserved small acidic polypeptides with molecular weights ranging from approximately 1 kDa–15 kDa [3]. This protein family could be divided into three main groups based on their isoelectric points (pI) as well as their capacity to stimulate lymphocytopoiesis, including α-thymosins with pI less than 5.0, β-thymosins with pI range from 5.0 to 7.0 and γ-thymosins with pI higher than 7.0 [3]. Among these groups, β-thymosin (Tβ) plays significant roles in various physiological processes [2], such as attachment and spreading of endothelial cells [4], cerebellum development [5], macrophage inhibition [6], stimulation of hormone releasing factor secretion [7], T-lymphocyte differentiation [8] and so on.

Tβ is a family of highly conserved polar polypeptides consisting of 40–44 amino acid residues with about 5 kDa molecular mass, and over 15 types of Tβs have been reported, among which, Tβ4 has been characterized from evolutionarily diverse organisms, ranging from echinoderms to mammals, and plays important role in body development and immune responses [2–6]. To date, Tβ genes have been well studied in mammals, however, limited Tβ or Tβ-like genes have been reported in invertebrates. Moreover, in marine invertebrates, the studies on Tβ or Tβ-like genes were limited to only several species. For examples, in red swamp crayfish *Procambarus clarkia*, *Pc*Thys were reported to protect hosts from white spot syndrome virus (WSSV) infection and play important roles in antiviral immune response [9]. A thymosin repeated protein1 (*Cq*TRP1) could reduce the replication of WSSV in red claw crayfish *Cherax quadricarinatus* [10]. Three thymosin-repeat proteins from kuruma shrimp *Marsupenaeus japonicas* have been proved to have a close relationship with WSSV infection [11]. Two new Tβ members, *Es*TRP1 and *Es*TRP2, have been identified in Chinese mitten crab *Eriocheir sinensis*, could response to pathogen or injury stimulation and enhance human cell proliferation [12]. A Tβ homologue (ab-TMSB) from gastropod abalone *Haliotis diversicolor supertexta* could be significantly induced by lipopolysaccharides (LPS) challenge and involved in the immune responses [13], while the stimulation of LPS, polyinosinic:polycytidylic acid (poly IC) and *Vibrio parahaemolyticus* could induce the expression of Tβ homologue (*Hd*Tβ) in gill and hemocytes in disk abalone *Haliotis discus discus* [14]. The

* Corresponding author. CAS Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, 266071, China.
E-mail address: wanglei@qdio.ac.cn (L. Wang).

https://doi.org/10.1016/j.fsi.2018.11.050
Received 26 September 2018; Received in revised form 15 November 2018; Accepted 19 November 2018
Available online 20 November 2018
1050-4648/

recombinant Tβ (rcgTβ) from Pacific oyster *Crassostrea gigas* exhibited significant antimicrobial activities [15], and a Tβ4 (*Ch*Tβ4) is involved in production of hemocytes and immune defense of Hong Kong oyster *Crassostrea hongkongensis* [16]. Additionally, the Tβ proteins exhibited an extracellular function in the central nervous system of California sea hare *Aplysia californica* [17]. To our best knowledge, rare information of Tβ or Tβ-like genes is available in scallop species till now [18].

The scallop family consists of over three hundred species identified and characterized in worldwide oceans and lakes and is one important fauna of bivalve not only at commercial and ecological levels, but also in terms of basic biology and biological evolution research [19–22]. Among all the scallop species, the Zhikong scallop *Chlamys farreri* is a native and important cultivated scallop species in North China and has accounted for more than 60% of the total scallop production in China, while the bay scallop *Argopecten irradians* was introduced from North America to China in 1982 and became an important marine cultured scallop species in China [23]. In the past two decades, Zhikong scallops suffered from severe summer mortalities [24,25], then the production of bay scallops considerably increased, due to its rapid growth rate and short grow out time [26]. Many efforts have been made to understand the mechanisms of the immune responses in scallop against pathogenic bacteria and identify genes involved in innate immunity [27–31]. The comparative study of Tβ and its effect on innate immune system between the two scallop species would not only provide a potential development of biological infectious disease control strategies in scallops farming, but also enhance the understanding of the potential functions of Tβ in invertebrate innate immunity. In the present study, two Tβ genes (abbreviated as *Ai*Tβ and *Cf*Tβ, respectively) have been identified and investigated in both *A. irradians* and *C. farreri*, with the main objectives (1) to compare their molecular structural features and mRNA expression profiles, (2) to validate their recombinant protein activities, and (3) to investigate their possible immunomodulation functions in scallop innate immunity.

2. Materials and methods

2.1. Scallops, microbe stimulation and samples collection

Adult scallops of both *A. irradians* and *C. farreri* were purchased from a local farm in Qingdao, China, and maintained in aerated seawater at about 20 °C. For each scallop species, approximately two hundred individuals were used for microbe stimulation assay. Being acclimated for about two weeks, fifty individuals defined as Gram-negative bacteria stimulation group were maintained in tanks containing live bacteria *Vibrio splendidus* strain JZ6. Another fifty individuals treated as Gram-positive bacteria stimulation group were kept in tanks containing live bacteria *Staphylococcus aureus* strain 33025 (SanYao, China). The third fifty individuals were immersed in live fungi-containing tanks with yeast *Pichia pastoris* strain GS115 (C18100, ThermoFisher, USA), and employed as fungi stimulation group. The final concentration of microbes used in this assay was all 1.0×10^8 colony forming units (CFUs) per 1 mL, and the concentration of microbes was tested as previously described [32]. The rest untreated individuals cultured in aerated seawater were used as the control group. Five individuals from every group were randomly sampled at 0, 3, 6, 12, 24 and 48 h post stimulation (hps). The hemolymphs were collected from the adductor muscle using sterile syringes and centrifuged at 800 *g*, 4 °C for 10 min to harvest the hemocytes. Hemocytes, muscle, mantle, gill, hepatopancreas and gonad from five untreated individuals were collected to determine the mRNA transcripts distribution of *Ai*Tβ and *Cf*Tβ.

2.2. RNA isolation, cDNA synthesis and full-length cDNA cloning

Raw RNA was isolated from all the samples of *A. irradians* and *C. farreri* with TRIzol Reagent (15596018, ThermoFisher, USA). The first-strand cDNA was synthesized using SuperScript IV Reverse Transcriptase (18090010, ThermoFisher, USA) with RNase-free DNase I (AM2222, ThermoFisher, USA) treated raw RNA as template and adaptor primer-oligo (dT) as primer (Table S1). The reaction was performed at 55 °C for 1 h, terminated by heated at 80 °C for 5 min, then a homopolymeric tail was added using terminal deoxynucleotidyl transferase (TdT, EP0161, ThermoFisher, USA) and dCTP (10217016, ThermoFisher, USA) and subsequently stored at −80 °C before used. By screening the bay scallop spat whole body ZAP Express Library data in National Centre for Biotechnology Information (NCBI), an expression sequence tag (EST) sequence (CN783117) homologous to previously identified Tβ genes was revealed and selected for further cloning the full-length cDNA sequence of *Ai*Tβ. While in our previous study, a transcript homologues to previous identified Tβ genes was revealed in *C. farreri* via assembling and screening publicly available EST sequences and transcriptomic reads [33], and this transcript was selected for further cloning the full-length cDNA of *Cf*Tβ. Gene-specific primers, *Ai*Tβ-RACE-R1/2, *Ai*Tβ-RACE-F1/2, *Cf*Tβ-RACE-R1/2 and *Cf*Tβ-RACE-F1/2 (Table S1), were designed using Primer Premier 5.00 according to these two sequences to clone the 5′ and 3′ cDNA ends of both *Ai*Tβ and *Cf*Tβ by rapid-amplification of cDNA ends (RACE) technique, respectively. All PCR amplifications were performed in a Veriti 96-Well Fast Thermal Cycler (4375305, ThermoFisher, USA). The PCR products were gel-purified with Monarch DNA Gel Extraction Kit (T1020S, NEB, USA) and then ligated into the pMD 19-T Vector (6013, Takara, Japan). Being transformed into competent cells *Escherichia coli* strain DH5α (9057, Takara, Japan), positive recombinants were picked by anti-Ampicillin selection and verified via PCR screening using vector primers RV-M and M13-47 (Table S1). Five positive clones were sequenced with a 3730XL automated sequencer (ThermoFisher, USA) by SangonBiotech (Shanghai) Co., Ltd (China).

2.3. Bioinformatical analysis of cDNA and protein sequences

The protein sequences of both *Ai*Tβ and *Cf*Tβ were deduced and analyzed by Lasergene 7.1.0.44 using the EditSeq module. BLAST+ 2.8.0 (blast.ncbi.nlm.nih.gov/Blast.cgi) was employed to conduct the similarity search for protein sequences. The presence and location of both signal peptides and function domains were predicted with SignalP 4.1 Server (www.cbs.dtu.dk/services/SignalP) and Simple Modular Architecture Research Tool (SMART) 8.0 (smart.embl-heidelberg.de), respectively. Clustal Omega 1.2.4 (www.clustal.org/omega) and Sequence Manipulation Suite 2.0 (www.bioinformatics.org/sms2) were used to perform and visualize multiple sequence alignments. A phylogenic tree was generated with MEGA-X 10.0.1 (www.megasoftware.net) using Neighbor-Joining (NJ) method with the parameters as following: Poison model, Uniform rate and Completed deletion. Bootstrap trials were replicated 1000 times to derive confidence value for the phylogeny analysis.

2.4. Analysis of mRNA expression patterns via quantitative real-time PCR

The mRNA transcripts of *Ai*Tβ and *Cf*Tβ in sampled tissues and their temporal expression pattern in hemocytes of two scallop species stimulated with various microbes were investigated by quantitative real-time PCR (qPCR) technique. All qPCR reactions were carried out with SYBR premix ExTaq (RR420, Takara, Japan) using 100 ng cDNA as template in a QuantStudio 5 Real-Time PCR System (ThermoFisher, USA). All the primers for qPCR listed in Table S1 were designed using PerlPrimer 1.1.21, and the efficiency of these primers were analyzed using serial two-fold dilutions of cDNA with the threshold cycle (C_T) slope method to confirm that all pairs of primers exhibit similar efficiency [34,35]. By now, β-actin and EF-1α have been used as internal control for Zhikong scallop, while β-actin was almost the only available internal control for bay scallop [22]. So, in the present study, we employed β-actin as internal control for these two scallop species, and the

mRNA expression of target gene was normalized to that of β-actin for each sample. The relative mRNA expression levels of target gene were calculated via comparative C_T method ($2^{-\Delta\Delta Ct}$ method) [36], presented as mean ± SD (n = 5) and subjected to one-way analysis of variance (ANOVA) followed by a multiple comparison using IBM SPSS Statistics 23.0.0.0, and p values less than 0.05 were considered as statistical significant.

2.5. Purification and antimicrobial activity of prokaryotic recombinant AiTβ and CfTβ

The cDNA fragment encoding the mature peptide of *Ai*Tβ or *Cf*Tβ was amplified using gene specific primers *Ai*Tβ-CDS-F/R or *Cf*Tβ-RACE-F/R (Table S1) and ligated to p*EASY*-Blunt E2 Expression Vector (CE211, TransgenBiotech, China). Being transformed into phage resistant chemically competent cells *E. coli* strain *Trans*1-T1 (CD501, TransgenBiotech, China), positive recombinants were picked by anti-Ampicillin selection and verified via PCR screening using vector primers T7 and T7ter (Table S1). The recombinant plasmids, p*EASY*-Blunt E2/*Ai*Tβ or p*EASY*-Blunt E2/*Cf*Tβ, were isolated with Monarch Plasmid Miniprep Kit (T1010S, NEB, USA) and transformed into chemically competent cells *E. coli* strain BL21(DE3) (CD601, TransgenBiotech, China). The positive transformants, *E. coli* BL21(DE3)/p*EASY*-Blunt E2/*Ai*Tβ or *E. coli* BL21(DE3)/p*EASY*-Blunt E2/*Cf*Tβ, were incubated in Overnight Express Instant TB Medium (71491, MerckMillipore, USA) containing 100 mg L^{-1} ampicillin (GG101, TransgenBiotech, China) and shook at 220 rpm, 28 °C for 24 h according to previous reports [37]. The recombinant proteins (abbreviated as r*Ai*Tβ and r*Cf*Tβ, respectively) were purified using *ProteinIso* Ni-NTA Resin (DP101, TransgenBiotech, China) under natural condition. The resultant proteins were separated using NuPAGE 12% Bis-Tris Protein Gels (NP0346BOX, ThermoFisher, USA) with NuPAGE MES SDS Running Buffer (NP0002, ThermoFisher, USA), stained by Protein Stains H Kit (C510041, SangonBiotech, China) and visualized via Gel Doc EZ imager (Bio-Rad, USA). After removing the endotoxin with ToxiEraser Endotoxin Removal Kit (L00338, Genscript, USA), the concentration of purified protein was quantified by Enhanced BCA Protein Assay Kit (P0010, Beyotime, China) and the obtained protein was stored at −80 °C.

The antimicrobial activity of purified r*Ai*Tβ or r*Cf*Tβ was evaluated against Gram-negative bacteria (*E. coli* strain DH5α, *Vibrio splendidus* strain JZ6 and *Vibrio paraheamolyticus* strain E1) and Gram-positive bacteria (*Micrococcus luteus* strain 28001 and *Staphylococcus aureus* strain 33025) by Kirby-Bauer disk diffusion susceptibility assay according to previous reports with slight modification [15,38]. Briefly, *E. coli* and *S. aureus* were cultured in LB Broth (HB0128, HopeBio, China) at 37 °C. *V, splendidus*, *V. paraheamolyticus* and *M. luteus* were cultured in 2216E Liquid Medium (HB0132, HopeBio, China) at 28 °C. For parallel experiments, gel plates were prepared with these bacteria at final concentration of 5.0×10^6 CFU mL^{-1}. Drops (20 μL) of Phosphate-Buffered Saline (PBS, 0.14 mol L^{-1} NaCl, 3 mmol L^{-1} KCl, 8 mmol L^{-1} Na_2HPO_4, 1.5 mmol L^{-1} KH_2PO_4, pH = 7.4, 10010023, ThermoFisher, USA), r*Ai*Tβ (100 μg mL^{-1} in PBS), r*Cf*Tβ (100 μg mL^{-1} in PBS) or Zeocin Selection Antibiotic (100 μg mL^{-1}, Zeocin is highly effective in a variety of organisms, including mammalian and insect cell lines, as well as in yeast, bacteria, and plants) were applied to thick filter paper (diameter: about 6 mm) on individual plates, and then incubated at 28 °C. Being cultured for 24 h, the diameters of the cleared zones were measured and presented as mean ± SD (n = 3) and subjected to one-way analysis of variance (ANOVA) followed by a multiple comparison using IBM SPSS Statistics 23.0.0.0, and p values less than 0.05 were considered as statistical significant.

2.6. Recombinant protein stimulation and quantification of immune parameters

For each scallop species, approximately two hundred individuals were used for recombinant protein stimulation assay. After acclimated for two weeks, these individuals were equally and randomly divided into four groups. The individuals in three groups received an injection of 100 μL PBS, Bovine Serum Albumin Fraction V (BSA, ST023, Beyotime, China, 100 μg mL^{-1} in PBS), r*Ai*Tβ (100 μg mL^{-1} in PBS) or r*Cf*Tβ (100 μg mL^{-1} in PBS), respectively. The untreated individuals were employed as the control group. The injected individuals were returned to seawater immediately and five individuals were randomly sampled from each group at 0, 3, 6, 12, 24 and 48 h post injection (hpi). The hemolymphs were collected and four kinds of immune parameters were measured, including total hemocytes count (THC), bacterial counts in hemolymph, superoxide dismutase (SOD) activity and catalase (CAT) activity. THC were performed to determine the total number of hemocytes per milliliter of scallop hemolymph. One hundred microliters of hemolymph was counted using a hemocytometer (HausseScientific, USA) and examined with a light microscopy (CX41, Olympus, Japan) according to previous reports [16]. For bacterial counts in hemolymph, one hundred microliters of hemolymph from experimental and control individuals was collected and immediately plated onto a 2216E agar plate. The plates were incubated at 28 °C for 36 h, and the colony counts were recorded for every plate. The hemolymphs were centrifuged at 800 *g*, 4 °C for 10 min to harvest scallop serum, and activities of SOD and CAT in scallop serum were examined with Total Superoxide Dismutase Assay Kit (S0101, Beyotime, China) and Catalase Assay Kit according to the manufacturer's directions, respectively. Protein concentration of scallop serum was measured using Enhanced BCA Protein Assay Kit. The activities of SOD and CAT were represented as specific activity (U mg^{-1}). All data of immune parameters were presented as mean ± SD (n = 5) and subjected to one-way analysis of variance (ANOVA) followed by a multiple comparison using IBM SPSS Statistics 23.0.0.0, and p values less than 0.05 were considered as statistical significant.

3. Results

3.1. Sequence features, homology and phylogeny relationship of AiTβ and CfTβ

The full-length cDNA sequences of *Ai*Tβ and *Cf*Tβ was obtained and submitted to GenBank under the accession numbers KU301763 and KY356883, respectively. The complete cDNA sequence of *Ai*Tβ was composed of 533 bp with an open reading frame (ORF) of 126 bp encoding a 41 amino acid peptide with a calculated molecular weight of 4.596 kDa and a pI of 6.571 (Fig. 1A, Table S2). While the full-length cDNA sequence of *Cf*Tβ was 530 bp with a 126 bp ORF encoding a 41 amino acid peptide with a predicted molecular mass of 4.692 kDa and a pI of 5.170 (Fig. 1B, Table S2). The polyadenylation signal (AATAAA) was located at upstream of the polyA tail of both *Ai*Tβ and *Cf*Tβ sequences (Fig. 1A and B). The deduced protein sequences of both *Ai*Tβ and *Cf*Tβ contained one THY domain (located at V^7/I^7 to A^{41}) with a highly conserved actin binding motif (located at L^{18} to T^{23}) as well as two conserved helix forming regions before (located at V^7/I^7 to V^{10}) and after (located at K^{32} to K^{39}) the actin binding motif (Fig. 1A, B, Fig. 1C, Table S2). BLAST + search revealed that *Ai*Tβ shared 88% identity with Tβ from *Sycon raphanus*, while *Cf*Tβ had 89% identity with its homologue from *Exaiptasia pallida* (Table S2), and the identity between *Ai*Tβ and *Cf*Tβ was as high as 85%. Additionally, phylogenetic analysis showed that *Ai*Tβ and *Cf*Tβ clustered with each other and then formed a sister branch to their homologues from the Genus *Haliotis* (Fig. 1D).

3.2. Tissue distribution and temporal expression of AiTβ and CfTβ mRNA transcripts after the stimulation of different microbes

The qPCR technique was employed to detect the tissue distribution of *Ai*Tβ and *Cf*Tβ mRNA transcripts using β-actin gene as internal

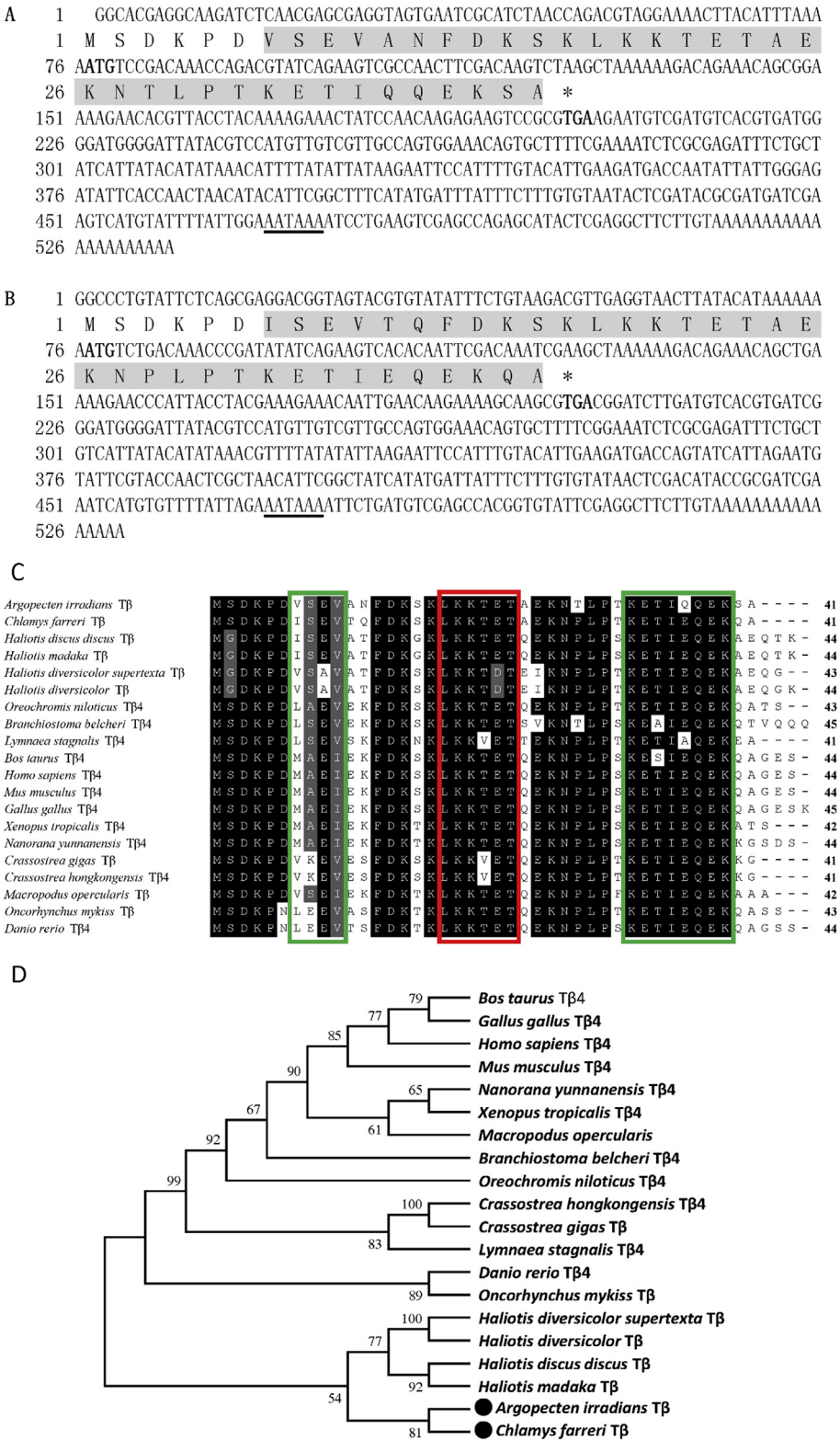

Fig. 1. Sequence features of *Ai*Tβ and *Cf*Tβ. **A**: Nucleotide sequences and deduced amino acid sequences of *Ai*Tβ from *Argopecten irradians*. The nucleotides and amino acid residues were numbered along the left margin. The THY domain was in shade. The polyadenylation signal (AATAAA) was underlined. The asterisks indicated the stop codon. **B**: Nucleotide sequences and deduced amino acid sequences of *Cf*Tβ from *Chlamys farreri*. The nucleotides and amino acid residues were numbered along the left margin. The THY domain was in shade. The polyadenylation signal (AATAAA) was underlined. The asterisks indicated the stop codon. **C**: Multiple alignments of *Ai*Tβ and *Cf*Tβ with previous known Tβs deposited in GenBank, the sequences and their accession numbers are as follows: *Argopecten irradians* Tβ, ANG56308; *Bos taurus* Tβ4, NP_001106702; *Branchiostoma belcheri* Tβ4, AAK72482; *Chlamys farreri* Tβ, ATO74506; *Crassostrea gigas* Tβ, AKC01246; *Crassostrea hongkongensis* Tβ4, AKS39780; *Danio rerio* Tβ4, NP_001124169; *Gallus gallus* Tβ4, NP_001001315; *Haliotis discus discus* Tβ, AFO64974; *Haliotis diversicolor supertexta* Tβ, ABW04622; *Haliotis diversicolor* Tβ, ABU53029; *Haliotis madaka* Tβ, ALU63762; *Homo sapiens* Tβ4, NP_066932; *Lymnaea stagnalis* Tβ4, ABB85285; *Macropodus opercularis* Tβ, AAL47854; *Mus musculus* Tβ4, NP_067253; *Nanorana yunnanensis* Tβ4, ABQ12775; *Oncorhynchus mykiss* Tβ, NP_001117822; *Oreochromis niloticus* Tβ4, XP_005463144 and *Xenopus tropicalis* Tβ4, NP_001037883. The black shadow region indicated positions where all sequences share the same amino acid residue. Similar amino acids are shaded in grey. Gaps are indicated by dashes to improve the alignments. Conserved actin-binding motif and two conserved helix forming regions were boxed in red and green, respectively. **D**: Consensus neighbor-joining phylogenetic based on the amino acid sequences of Tβs from different organisms. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. All positions containing gaps and missing data were eliminated. The numbers at the forks indicated the bootstrap value (in %). The sequences and their accession numbers are as follows: *Argopecten irradians* Tβ, ANG56308; *Bos taurus* Tβ4, NP_001106702; *Branchiostoma belcheri* Tβ4, AAK72482; *Chlamys farreri* Tβ, ATO74506; *Crassostrea gigas* Tβ, AKC01246; *Crassostrea hongkongensis* Tβ4, AKS39780; *Danio rerio* Tβ4, NP_001124169; *Gallus gallus* Tβ4, NP_001001315; *Haliotis discus discus* Tβ, AFO64974; *Haliotis diversicolor supertexta* Tβ, ABW04622; *Haliotis diversicolor* Tβ, ABU53029; *Haliotis madaka* Tβ, ALU63762; *Homo sapiens* Tβ4, NP_066932; *Lymnaea stagnalis* Tβ4, ABB85285; *Macropodus opercularis* Tβ, AAL47854; *Mus musculus* Tβ4, NP_067253; *Nanorana yunnanensis* Tβ4, ABQ12775; *Oncorhynchus mykiss* Tβ, NP_001117822; *Oreochromis niloticus* Tβ4, XP_005463144 and *Xenopus tropicalis* Tβ4, NP_001037883.

control. In the dissociation curve analysis for each analyzed gene, there was only one peak at the corresponding melting temperature, indicating that the PCR products were specifically amplified (data not shown). The mRNA transcripts of both *Ai*Tβ and *Cf*Tβ were detectable in all the tested tissues, and the highest mRNA expression level of *Ai*Tβ was found in hemocytes followed by gill, which was 9.19-fold ($p < 0.05$) and 6.09-fold ($p < 0.05$) of that in gonad, respectively (Fig. 2A). Similarly, *Cf*Tβ was also most abundant in hemocytes followed by hepatopancreas, which was 8.68-fold ($p < 0.05$) and 5.37-fold ($p < 0.05$) of that in muscle, respectively (Fig. 2B).

The temporal mRNA expression profiles of both *Ai*Tβ and *Cf*Tβ in hemocytes post microbe stimulations were examined via qPCR. The

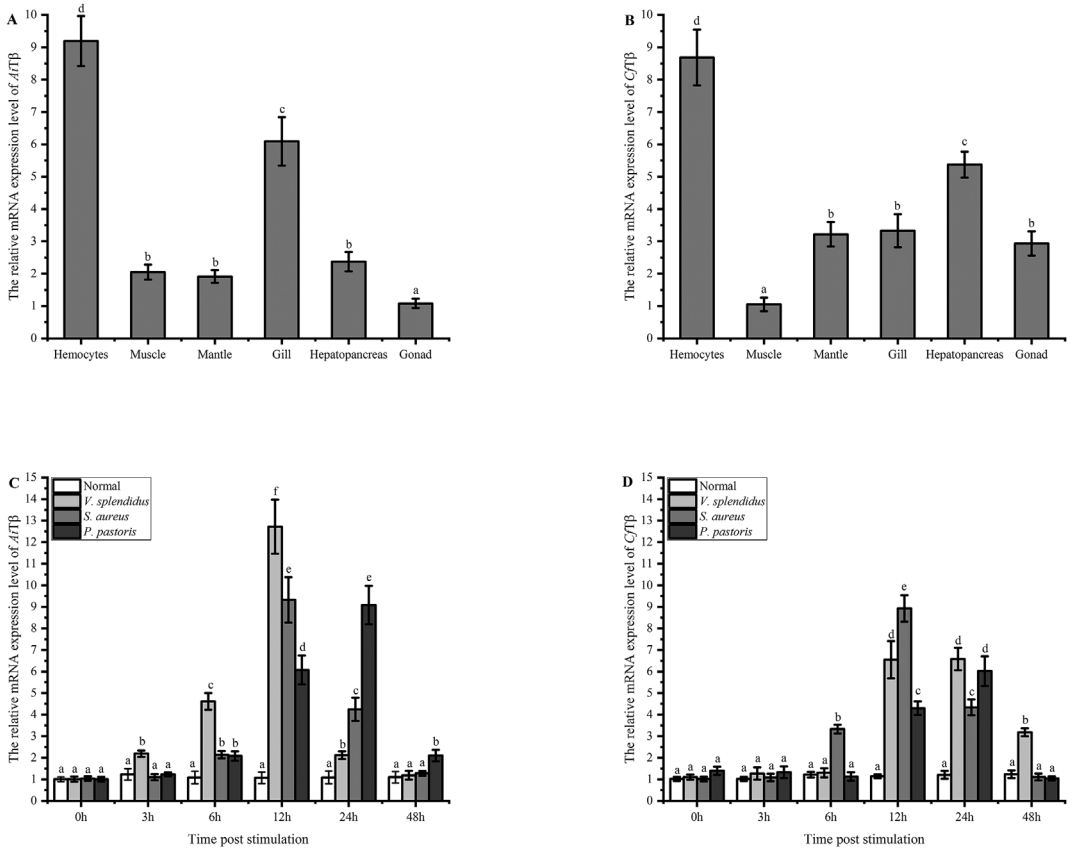

Fig. 2. Tissue distribution and temporal expression profiles of *Ai*Tβ and *Cf*Tβ mRNA transcripts post live microbes stimulation. **A**: Tissue distribution of *Ai*Tβ mRNA transcripts. The transcript level in gill, hepatopancreas, hemocytes, mantle and muscle of five adult scallops is normalized to that of gonad. **B**: Tissue distribution of *Cf*Tβ mRNA transcripts. The transcript level in gill, gonad, hepatopancreas, hemocytes and mantle of five adult scallops is normalized to that of muscle. **C**: Temporal expression profiles of *Ai*Tβ mRNA transcripts in bay scallop hemocytes post live microbes stimulation. **D**: Temporal expression profiles of *Cf*Tβ mRNA transcripts in Zhikong scallop hemocytes post live microbes stimulation. The β-actin gene was used as an internal control to calibrate the cDNA template for each sample. Vertical bars represent mean ± SD (n = 5) and bars with different characters were significantly different ($p < 0.05$).

mRNA transcripts of both *Ai*Tβ and *Cf*Tβ in hemocytes increased after microbe stimulation (Fig. 2C and D). In general, the responses of *Ai*Tβ were more intensely and rapidly than those of *Cf*Tβ. For instants, the mRNA transcripts of *Ai*Tβ significantly increased at 3 hps (2.19-fold, $p < 0.05$), reached the peak at 12 hps (12.71-fold, $p < 0.05$) and finally dropped to the origin level at 48 hps (Fig. 2C). The mRNA transcripts of *Cf*Tβ began to increase at 12 hps (6.55-fold, $p < 0.05$), kept in similar level at 24 hps (6.58-fold, $p < 0.05$) and decreased to 3.18-fold ($p < 0.05$) at 48 hps (Fig. 2D). In the *S. aureus* stimulation group, the mRNA transcripts of *Ai*Tβ and *Cf*Tβ were both significantly induced at 6 hps (2.15-fold and 3.33-fold, respectively, $p < 0.05$), reached to the highest levels at 12 hps (9.32-fold and 8.93-fold, respectively, $p < 0.05$), and finally decreased to the origin level at 48 hps (Fig. 2C and D). Being stimulated by *P. pastoris*, the mRNA transcripts of *Ai*Tβ were significantly induced at 6 hps (2.08-fold, $p < 0.05$) and reached the peak at 24 hps (9.09-fold, $p < 0.05$) and down-regulated but still higher than the origin level at 48 hps (2.10-fold, $p < 0.05$) (Fig. 2C). While the mRNA expression level of *Cf*Tβ was up-regulated at 12 hps (4.30-fold, $p < 0.05$), reached to the highest level at 24 hps (6.02-fold, $p < 0.05$) and finally down-regulated to the origin level at 48 hps (Fig. 2D).

3.3. Antimicrobial activities of rAiTβ and rCfTβ

To investigate the potential activities of *Ai*Tβ and *Cf*Tβ, the recombinant plasmid p*EASY*-Blunt E2/*Ai*Tβ or p*EASY*-Blunt E2/*Cf*Tβ were transformed into *E. coli* strain BL21(DE3). Being auto-induced, the whole cell lysates were separated by SDS-PAGE, and distinct bands of r*Ai*Tβ and r*Cf*Tβ were revealed (Fig. 3A, Fig. 3B). The antimicrobial activities of r*Ai*Tβ and r*Cf*Tβ were preliminarily estimated against five kinds of bacteria by a simplified Kirby-Bauer disk diffusion susceptibility assay. Antimicrobial activities against *V. splendidus* (diameter: 16.33 ± 0.76 mm, $p < 0.05$) and *M. luteus* (diameter: 16.73 ± 0.46 mm, $p < 0.05$) were observed with r*Ai*Tβ, while r*Cf*Tβ exhibited antimicrobial activities against *M. luteus* (diameter: 18.83 ± 0.29 mm, $p < 0.05$) (Fig. 3C).

3.4. Immunomodulation activities of rAiTβ and rCfTβ

To investigate the potential immunomodulation activities of *Ai*Tβ and *Cf*Tβ, r*Ai*Tβ and r*Cf*Tβ were administered to the adductor muscles of corresponding scallop, respectively. The THC and bacterial counts in hemolymph were counted as previously described. The CAT and SOD activities were measured using corresponding commercial reagent kit, respectively. After r*Ai*Tβ injection, the hemocytes concentration of bay scallop significantly increased at 6 hpi (5.77 ± 0.39 cells mL^{-1}, $p < 0.05$), peaked at 12 hpi (6.82 ± 0.37 cells mL^{-1}, $p < 0.05$) compared to the control groups, gradually declined and recovered to the original level at 48 hpi (Fig. 4A), while r*Cf*Tβ stimulated an increase in circulating hemocytes of Zhikong scallop at 12 hpi (4.76 ± 0.33 cells mL^{-1}, $p < 0.05$) and 24 phi (5.79 ± 0.34 cells mL^{-1}, $p < 0.05$) (Fig. 4B). Being stimulated with r*Ai*Tβ, the bacterial counts in bay scallop hemolymph significantly decreased at 12 hpi (118 ± 11 CFUs per 50 μL, $p < 0.05$), touched the bottom at 24 hpi (70 ± 9 CFUs per 50 μL, $p < 0.05$), and gradually recovered but still lower than the original level at 48 hpi (130 ± 13 CFUs per 50 μL,

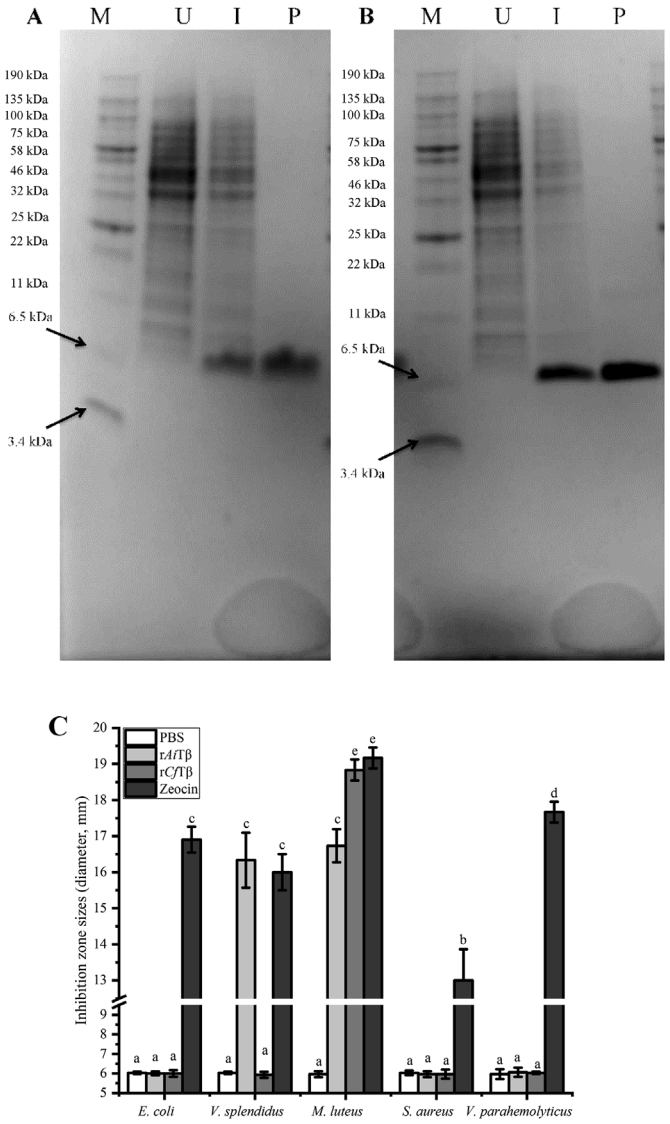

Fig. 3. Prokaryotic recombinant expression and purification of *Ai*Tβ and *Cf*Tβ. **A**: SDS-PAGE analysis of the r*Ai*Tβ in *E. coli* strain BL21(DE3). Lane M was the Unstained Protein Standard, Broad Range (P7708, NEB, USA). Lane U was the supernatant of un-induced bacteria lysate. Lane I was the supernatant of auto-induced bacteria lysate. Line P was the purified recombinant protein. **B**: SDS-PAGE analysis of the r*Cf*Tβ in *E. coli* strain BL21(DE3). Lane M was the Unstained Protein Standard, Broad Range (P7708, NEB, USA). Lane U was the supernatant of un-induced bacteria lysate. Lane I was the supernatant of auto-induced bacteria lysate. Line P was the purified recombinant protein. **C**: The antimicrobial activity of r*Ai*Tβ and r*Cf*Tβ against different microbes. The diameters of the cleared zones were measured and presented as mean ± SD (n = 3) and bars with different characters were significantly different ($p < 0.05$).

$p < 0.05$) (Fig. 4C). While injection of r*Cf*Tβ could significantly inhibit the proliferation of bacteria present in Zhikong scallop hemolymph at 24 hpi (113 ± 13 CFUs per 50 μL, $p < 0.05$) and 48 hpi (124 ± 15 CFUs per 50 μL, $p < 0.05$) (Fig. 4D). After the bay scallops were treated with r*Ai*Tβ, the CAT activity in serum significantly increased at 12 hpi (259.33 ± 18.48 U mg^{-1}, $p < 0.05$), reached the peak at 24 hpi (324.48 ± 23.19 U mg^{-1}, $p < 0.05$), and recovered to the original level at 48 hpi (Fig. 4E). After r*Cf*Tβ stimulation, the CAT activity in serum of Zhikong scallop rapidly increased at 6 hpi (273.40 ± 21.09 U mg^{-1}, $p < 0.05$), peaked at 12 hpi (364.54 ± 19.77 U mg^{-1}, $p < 0.05$), and maintained at high levels at 24 hpi (295.19 ± 14.12 U mg^{-1}, $p < 0.05$) and 48 hpi (273.04 ± 24.84 U mg^{-1}, $p < 0.05$) (Fig. 4F). The injection of r*Ai*Tβ could only induce the SOD activity in serum of bay scallop at 24 hpi (303.59 ± 14.64 U mg^{-1}, $p < 0.05$), compared with the control groups (Fig. 4G). While injection with r*Cf*Tβ, the SOD activity in serum of Zhikong scallop significantly increased at 3 hpi (299.78 ± 32.98 U mg^{-1}, $p < 0.05$), peaked at 6 hpi (403.94 ± 33.93 U mg^{-1}, $p < 0.05$), gradually recovered and finally recovered to the original level at 48 hpi (Fig. 4H).

4. Discussion

Tβ stands for a family of highly conserved polar 5 kDa peptides and is involved in multiple physiological processes [39], such as cell proliferation [40], reactive oxygen species (ROS) production [41], as well as immune defense mechanism [42]. However, the roles and functions of Tβ in scallops are still rare and fragmentary. Here, we have identified and characterized Tβ homologues from scallop species bay scallop *A. irradians* and Zhikong scallop *C. farreri*. Their sequence features, high similarities and significant phylogenetic relationship with their previously identified orthologues collectively confirmed that they were new members of invertebrate Tβ family and may have similar functions with Tβs from other invertebrates.

The tissue distribution of both *Ai*Tβ and *Cf*Tβ mRNA transcripts and their temporal expression patterns post bacterial stimulation were determined and compared via qPCR technique. Both *Ai*Tβ and *Cf*Tβ mRNA transcripts were constitutively expressed in various tissues, including hemocytes, muscle, mantle, gill, hepatopancreas and gonad, and they were both mostly detectable in hemocytes, which are thought to play pivotal roles in the recognition for nonself components, the elimination of foreign particles and the kill of pathogenic invaders [43]. Similarly, the highest mRNA expression levels of Tβs were also observed in hemocytes from other mollusk species, such as ab-TMSB from *H. diversicolor supertexta* [13], *Hd*Tβ from *H. discus discus* [14] and ChTβ4 from *C. hongkongensis* [16]. Furthermore, similar with the observation in *H. diversicolor supertexta* and *C. hongkongensis* [13,16], gill exhibited the second largest content of *Ai*Tβ in bay scallop, while the second largest content of *Cf*Tβ was detected in hepatopancreas in Zhikong scallop. Gill was considered as the first line against invading pathogens in fish or lower animals, and a recent research achievement demonstrated that tubules of gill filaments could be the potential hematopoietic position in mollusk [44], while hepatopancreas acted as the central immune regulating organ in crustaceans and mollusks [22]. So, the diversified tissue distribution of mRNA transcripts of *Ai*Tβ and *Cf*Tβ should be assumed to be related with similarities and differences of their potential functions, and the higher mRNA expression levels of *Ai*Tβ and *Cf*Tβ in hemocytes, gill and hepatopancreas implied their pivotal functions in the innate immune system in scallops.

Hemocytes are believed to be major immune cells responding to invading pathogens mainly through phagocytosis in mollusks and usually selected to investigate the expression fluctuation of innate immune related genes [45]. Coincidentally, both *Ai*Tβ and *Cf*Tβ were mostly detected in hemocytes. So, in the present study, hemocytes were also selected to confirm the temporal expression patterns of *Ai*Tβ and *Cf*Tβ mRNA transcripts post various microbes stimulation, and it has been observed that their mRNA transcripts could be significantly induced by all the three kinds of microbe stimulation, indicating that they were involved in the innate immune response against microbial pathogens of scallops. In agreement with our observation, the mRNA expression levels of Tβs in common carp *Cyprinus carpio* [46], disk abalone *H. discus discus* [14], gastropod abalone *H. diversicolor supertexta* [13], Golden pompano *Trachinotus ovatus* [47], Hong Kong oyster *C. hongkongensis* [16] and red swamp crayfish *P. clarkia* [9] were also significantly induced in responses to various immune stimulation. However, the responses of *Ai*Tβ was more rapidly and intensely than those of *Cf*Tβ against all the three kinds of microbe. The difference in their mRNA expression pattern might be related with their different

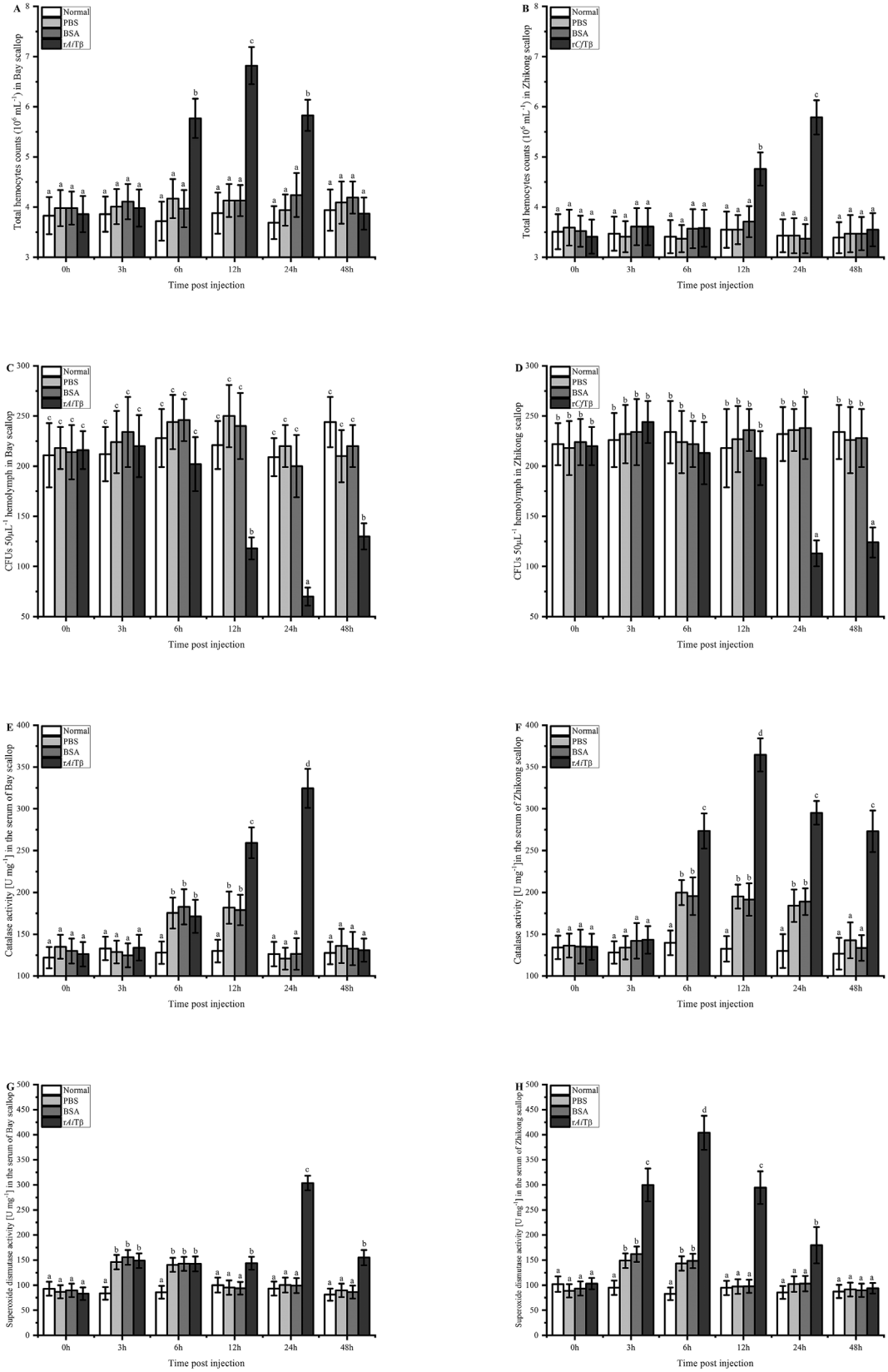

Fig. 4. The immunomodulation activities of r*Ai*Tβ and r*Cf*Tβ. **A**: Total hemocytes count of bay scallop after injection with r*Ai*Tβ. **B**: Total hemocytes count of Zhikong scallop after injection with r*Cf*Tβ. **C**: Bacterial counts in hemolymph of bay scallop after injection with r*Ai*Tβ. **D**: Bacterial counts in hemolymph of Zhikong scallop after injection with r*Cf*Tβ. **E**: Catalase activity in serum of bay scallop after injection with r*Ai*Tβ. **F**: Catalase activity in serum of Zhikong scallop after injection with r*Cf*Tβ. **G**: Superoxide dismutase activity in serum of bay scallop after injection with r*Ai*Tβ. **H**: Superoxide dismutase activity in serum of Zhikong scallop after injection with r*Cf*Tβ. Each values was shown as mean ± SD (n = 5) and bars with different characters indicated significant difference ($p < 0.05$).

roles in the innate immune system in scallops and also indicate the defensive strategies were diverse between these two scallop species.

Multiple biological activities of Tβ have been identified in vertebrates, but information about its function in invertebrates were not well documented, especially in marine invertebrates. In a previous research, recombinant cgTβ protein (rcgTβ) exhibited significant antimicrobial activity against *E. coli* [15]. Similar phenomena were observed in present study that r*Ai*Tβ performed antimicrobial activities against *V. splendidus* and *M. luteus*, while r*Cf*Tβ could inhibited the growth of *M. luteus*, indicating *Ai*Tβ and *Cf*Tβ might be involved in the antimicrobial processes in scallops. However, compared with the authentic antimicrobial peptide [48], the inhibitive concentration of *Ai*Tβ and *Cf*Tβ was rather high and the protein could not be so concentrated apparently in the normal physiological condition of scallop. It was suspected *Ai*Tβ and *Cf*Tβ were mainly involved in immune defense via their potential immunomodulation activities, while their antimicrobial activities were of adjuvant.

To further investigate the potential immunomodulation functions of *Ai*Tβ and *Cf*Tβ, r*Ai*Tβ and r*Cf*Tβ were injected to the adductor muscles of corresponding scallop, respectively. And four kinds of immune parameters have been quantified. The invertebrate hemocytes known as immune actors were believed to play pivotal roles in innate immune responses, such as engulfing bacteria via phagocytosis and producing ROS [22,49]. In previous reports, THC significantly increased after the stimulation by recombinant ChTβ4 in *C. hongkongensis* [16], while r*Es*TRP2 from Chinese mitten crab *E. sinensis* could significantly accelerate the growth of human hepatocellular carcinoma cells [12]. In the present study, the hemocytes concentration of bay scallop significantly increased after r*Ai*Tβ injection, while the enhancement of r*Cf*Tβ on THC in Zhikong scallop was weaker. Correspondingly, r*Ai*Tβ performed higher inhibitory activity on the proliferation of bacteria in the hemolymphs of bay scallop than that of r*Cf*Tβ in Zhikong scallop. Consistent with the observation in Hong Kong oyster, the enhancement on THC and the inhibitory activity on the proliferation of bacteria in the hemolymphs exhibited positive correlation to each other [16]. And the difference between *Ai*Tβ and *Cf*Tβ on these two immune parameters indicated that bay scallop might have a higher level of immune potential than Zhikong scallop, which was agreed with the results of a comparative study of immunity between these two scallop species [50].

Accumulating evidence indicates that ROS can mediate various signaling pathways and immune responses to eliminate foreign participates or kill invading pathogens, but excessive ROS will cause serious downstream reactions and even threaten the survival of organisms [51]. So, it is important for all the aerobic organisms to eliminate excessive ROS and maintain redox homeostasis. In a previous study, it was clearly confirmed that ChTβ4 could significantly increase the mRNA expression of antioxidant enzymes and meanwhile inhibit the production of ROS in *C. hongkongensis* [16]. In the current research, both r*Ai*Tβ and r*Cf*Tβ could obviously induce the activities of CAT and SOD in corresponding scallop, respectively. This observation is consistent with previous reports that Tβ was involved in cellular ant oxidation activities to inhibit the accumulation of ROS [52]. While the higher enhancement effect of r*Cf*Tβ in Zhikong scallop indicated *Cf*Tβ might be inclined to act as an immune modulator to maintain redox homeostasis.

In conclusion, two Tβ homologues, *Ai*Tβ and *Cf*Tβ, have been identified and investigated in bay scallop *A. irradians* and Zhikong scallop *C. farreri*, respectively. And they shared similar sequence features, their mRNA tissue distribution and expression profiles were roughly the same yet different in detail, while their recombinant proteins exhibited different immunomodulation activity on various downstream immune parameters. All these results indicated that both *Ai*Tβ and *Cf*Tβ were new members of invertebrate Tβ family and involved in innate immune responses against invading pathogens infection via enhancing circulating hemocytes multiplication, inhibiting microbiota proliferation and inducing the activities of antioxidant enzymes. While the diversity of their immunomodulation activities indicated the function of Tβ protein has differentiated in scallops and bay scallop might have a higher level of immune potential than Zhikong scallop.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (U1706209), Aoshan Innovation Project in Science and Technology from National Laboratory for Marine Science and Technology (2016ASKJ07), Science and Technology Service Network Plan (STS) Major Deployment Project (KFZD-SW-106) and STS Regional Centre Project (Fujian province) from Chinese Academy of Sciences. We would like to thank the expert reviewers for their constructive suggestions and enlightening comments during the revision.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.050.

References

[1] A.L. Goldstein, History of the discovery of the thymosins, Ann. NY. Acad. Sci. 1112 (2007) 1–13.
[2] E. Hannappel, β-Thymosins, Ann. NY. Acad. Sci. 1112 (2007) 21–37.
[3] T. Huff, C.S. Müller, A.M. Otto, R. Netzker, E. Hannappel, β-Thymosins, small acidic peptides with multiple functions, Int. J. Biochem. Cell Biol. 33 (2001) 205–220.
[4] T. Kobayashi, F. Okada, N. Fujii, N. Tomita, S. Ito, H. Tazawa, T. Aoyama, S.K. Choi, T. Shibata, H. Fujita, M. Hosokawa, Thymosin-β4 regulates motility and metastasis of malignant mouse fibrosarcoma cells, Am. J. Pathol. 160 (2002) 869–882.
[5] B.G. Border, S.C. Lin, W.S.T. Griffin, S. Pardue, M. Morrison-Bogorad, Alterations in actin-binding β-thymosin expression accompany neuronal differentiation and migration in rat cerebellum, J. Neurochem. 61 (1993) 2104–2114.
[6] V.T. Nachmias, Small actin-binding proteins: the β-thymosin family, Curr. Opin. Cell Biol. 5 (1993) 56–62.
[7] A.L. Goldstein, M. Badamchian, Thymosins: chemistry and biological properties in health and disease, Expet Opin. Biol. Ther. 4 (2004) 559–573.
[8] M. Paulussen, B. Landuyt, L. Schoofs, W. Luyten, L. Arckens, Thymosin beta 4 mRNA and peptide expression in phagocytic cells of different mouse tissues, Peptides 30 (2009) 1822–1832.
[9] X.Z. Shi, L.J. Shi, Y.R. Zhao, X.F. Zhao, J.X. Wang, β-Thymosins participate in antiviral immunity of red swamp crayfish (*Procambarus clarkii*), Dev. Comp. Immunol. 51 (2015) 213–225.
[10] D.L. Li, X.J. Chang, X.L. Xie, S.C. Zheng, Q.X. Zhang, S.A. Jia, K.J. Wang, H.P. Liu, A thymosin repeated protein1 reduces white spot syndrome virus replication in red claw crayfish *Cherax quadricarinatus*, Dev. Comp. Immunol. 84 (2018) 109–116.
[11] J.Y. Ma, L.W. Ruan, X. Xu, Z.M. Gao, Molecular characteristics of three thymosin-repeat proteins from *Marsupenaeus japonicus* and their responses to WSSV infection, Acta Oceanol. Sin. 35 (2016) 44–50.
[12] Y.C. Gai, J.M. Zhao, L.S. Song, L.L. Wang, L.M. Qiu, X.X. Ning, X.D. Zheng, Y. Zhang, C.K. Mu, Y. Zhang, L. Li, Two thymosin-repeated molecules with structural and functional diversity coexist in Chinese mitten crab *Eriocheir sinensis*, Dev. Comp. Immunol. 33 (2009) 867–876.
[13] L.J. Wu, X.Z. Wu, Molecular cloning and expression analysis of a β-thymosin homologue from a gastropod abalone, *Haliotis diversicolor supertexta*, Fish Shellfish Immunol. 27 (2009) 379–382.
[14] S.R. Kasthuri, H.K.A. Premachandra, N. Umasuthan, I. Whang, J. Lee, Structural characterization and expression analysis of a beta-thymosin homologue (Tβ) in disk abalone, *Haliotis discus discus*, Gene 527 (2013) 376–383.
[15] B.H. Nam, J.K. Seo, M.J. Lee, Y.O. Kim, D.G. Kim, C.M. An, N.G. Park, Functional analysis of Pacific oyster (*Crassostrea gigas*) β-thymosin: focus on antimicrobial activity, Fish Shellfish Immunol. 45 (2015) 167–174.
[16] J. Li, Y.H. Zhang, Y. Liu, Y. Zhang, Z.M. Xiang, F.F. Qu, Z.N. Yu, A thymosin beta-4 is involved in production of hemocytes and immune defense of Hong Kong oyster, *Crassostrea hongkongensis*, Dev. Comp. Immunol. 57 (2016) 1–9.
[17] E.V. Romanova, M.J. Roth, S.S. Rubakhin, J.A. Jakubowski, W.P. Kelley, M.D. Kirk, N.L. Kelleher, J.V. Sweedler, Identification and characterization of homologues of vertebrate β-thymosin in the marine mollusk *Aplysia californica*, J. Mass Spectrom. 41 (2006) 1030–1040.
[18] D. Safer, P.K. Chowrashi, β-thymosins from marine invertebrates: primary structure and interaction with actin, Cell Motil. Cytoskelet. 38 (1997) 163–171.
[19] S. Wang, J.B. Zhang, W.Q. Jiao, J. Li, X.G. Xun, Y. Sun, X.M. Guo, P. Huan, B. Dong, L.L. Zhang, X.L. Hu, X.Q. Sun, J. Wang, C.T. Zhao, Y.F. Wang, D.W. Wang, X.T. Huang, R.J. Wang, J. Lv, Y.L. Li, Z.F. Zhang, B.Z. Liu, W. Lu, Y.Y. Hui, J. Liang, Z.C. Zhou, R. Hou, X. Li, Y.C. Liu, H.D. Li, X.H. Ning, Y. Lin, L. Zhao, Q. Xing, J.Z. Dou, Y.P. Li, J.X. Mao, H.B. Guo, H.Q. Dou, T.Q. Li, C. Mu, W.K. Jiang, Q. Fu, X.T. Fu, Y. Miao, J. Liu, Q. Yu, R.J. Li, H. Liao, X. Li, Y.F. Kong, Z. Jiang, D. Chourrout, R.Q. Li, Z.M. Bao, Scallop genome provides insights into evolution of bilaterian karyotype and development, Nat. Ecol. Evol. 1 (2017) 120.

[20] Y.L. Li, X.Q. Sun, X.L. Hu, X.G. Xun, J.B. Zhang, X.M. Guo, W.Q. Jiao, L.L. Zhang, W.Z. Liu, J. Wang, J. Li, Y. Sun, Y. Miao, X.K. Zhang, T.R. Cheng, G.L. Xu, X.T. Fu, Y.F. Wang, X.R. Yu, X.T. Huang, W. Lu, J. Lv, C. Mu, D.W. Wang, X. Li, Y. Xia, Y.J. Li, Z.H. Yang, F.L. Wang, L. Zhang, Q. Xing, H.Q. Dou, X.H. Ning, J.Z. Dou, Y.R. Li, D.X. Kong, Y.R. Liu, Z. Jiang, R.Q. Li, S. Wang, Z.M. Bao, Scallop genome reveals molecular adaptations to semi-sessile life and neurotoxins, Nat. Commun. 8 (2017) 1721.
[21] C. Li, X. Liu, B. Liu, B. Ma, F.Q. Liu, G.L. Liu, Q. Shi, C.D. Wang, Draft genome of the Peruvian scallop *Argopecten purpuratus*, GigaScience 7 (2018) giy031.
[22] L.S. Song, L.L. Wang, H. Zhang, M.Q. Wang, The immune system and its modulation mechanism in scallop, Fish Shellfish Immunol. 46 (2015) 65–78.
[23] X.T. Huang, K. Bi, W. Lu, S. Wang, L.L. Zhang, Z.M. Bao, Genomic in situ hybridization identifies parental chromosomes in hybrid scallop (Bivalvia, Pectinoida, Pectinidae) between female *Chlamys farreri* and male *Argopecten irradians irradians*, Comp. Cytogenet. 9 (2015) 189–200.
[24] S.L. Liu, X.L. Jiang, X.K. Hu, J. Gong, H. Hwang, K.S. Mai, Effects of temperature on non-specific immune parameters in two scallop species: *Argopecten irradians* (Lamarck 1819) and *Chlamys farreri* (Jones & Preston 1904), Aquacult. Res. 35 (2004) 678–682.
[25] X.L. Zhang, M.Y. Zhu, R.X. Li, Z.L. Wang, B. Xia, L.H. Zhang, Density-dependent mortality of the scallop *Chlamys farreri* (Jones & Preston) in grow-out culture, Aquacult. Res. 37 (2006) 842–844.
[26] J. Greenawalt-Boswell, T.K. Frazer, C.A. Jacoby, W.S. Arnold, Mortality and exploitation rate estimates for the recreational bay scallop fishery off the gulf coast of Florida, USA, N. Am. J. Fish. Manag. 27 (2007) 1230–1242.
[27] X.L. Meng, X. Tian, M. Liu, G.X. Nie, K.Y. Jiang, B.J. Wang, L. Wang, The transcriptomic response to copper exposure by the gill tissue of Japanese scallops (*Mizuhopecten yessoensis*) using deep-sequencing technology, Fish Shellfish Immunol. 38 (2014) 287–293.
[28] R.J. Li, R. Zhang, L. Zhang, J.J. Zou, Q. Xing, H.Q. Dou, X.L. Hu, L.L. Zhang, R.J. Wang, Z.M. Bao, Characterizations and expression analyses of NF-κB and Rel genes in the Yesso scallop (*Patinopecten yessoensis*) suggest specific response patterns against Gram-negative infection in bivalves, Fish Shellfish Immunol. 44 (2015) 611–621.
[29] X.L. Meng, X. Tian, G.X. Nie, J.L. Wang, M. Liu, K.Y. Jiang, B.J. Wang, Q.Q. Guo, J.R. Huang, L. Wang, The transcriptomic response to copper exposure in the digestive gland of Japanese scallops (*Mizuhopecten yessoensis*), Fish Shellfish Immunol. 46 (2015) 161–167.
[30] J. Wang, R.J. Wang, S.Y. Wang, M.R. Zhang, X.L. Ma, P.P. Liu, M.W. Zhang, X.L. Hu, L.L. Zhang, S. Wang, Z.M. Bao, Genome-wide identification and characterization of TRAF genes in the Yesso scallop (*Patinopecten yessoensis*) and their distinct expression patterns in response to bacterial challenge, Fish Shellfish Immunol. 47 (2015) 545–555.
[31] H.B. Guo, Y.P. Li, M.W. Zhang, R.J. Li, W.R. Li, J.R. Lou, Z.M. Bao, Y.F. Wang, Expression of Cathepsin F in response to bacterial challenges in Yesso scallop *Patinopecten yessoensis*, Fish Shellfish Immunol. 80 (2018) 141–147.
[32] K. Peñuelas-Urquides, L. Villarreal-Treviño, B. Silva-Ramírez, L. Rivadeneyra-Espinoza, S. Said-Fernández, M.B.D. León, Measuring of Mycobacterium tuberculosis growth: a correlation of the optical measurements with colony forming units, Braz. J. Microbiol. 44 (2013) 287–290.
[33] M.Q. Wang, L.L. Wang, Z.H. Jia, Q.L. Yi, L.S. Song, The various components implied the diversified Toll-like receptor (TLR) signaling pathway in mollusk *Chlamys farreri*, Fish Shellfish Immunol. 74 (2018) 205–212.
[34] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29 (2001) e45.
[35] M.Q. Wang, B.J. Wang, M. Liu, K.Y. Jiang, L. Wang, Molecular characterization of a defender against apoptotic cell death 1 gene (*Cf*DAD1) from the mollusk *Chlamys farreri*, ISJ - Invert. Surviv. J. 15 (2018) 294–301.
[36] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C_T method, Nat. Protoc. 3 (2008) 1101–1108.
[37] M.Q. Wang, B.J. Wang, M. Liu, K.Y. Jiang, L. Wang, Sequence features, expression profiles and biochemical characteristics of a sigma class glutathione S-transferase gene (AiGSTσ) from bay scallop *Argopecten irradians*, ISJ - Invert. Surviv. J. 15 (2018) 19–30.
[38] P.D. Midolo, J. Turnidge, J.R. Lambert, J.M. Bell, Validation of a modified Kirby-Bauer disk diffusion method for metronidazole susceptibility testing of *Helicobacter pylori*, Diagn. Microbiol. Infect. Dis. 21 (1995) 135–140.
[39] D. Safer, V.T. Nachmias, Beta thymosins as actin binding peptides, Bioessays 16 (1994) 473–479.
[40] P. Pradelles, Y. Frobert, C. Créminon, H. Ivonine, E. Frindel, Distribution of a negative regulator of haematopoietic stem cell proliferation (AcSDKP) and thymosin, β4 in mouse tissues, FEBS Lett. 289 (1991) 171–175.
[41] J.D. Young, A.J. Lawrence, A.G. MacLean, B.P. Leung, I.B. McInnes, B. Canas, D.J.C. Pappin, R.D. Stevenson, Thymosin β 4 sulfoxide is an anti-inflammatory agent generated by monocytes in the presence of glucocorticoids, Nat. Med. 5 (1999) 1424–1427.
[42] A.L. Goldstein, Y. Asanuma, J.R. Battisto, M.A. Hardy, J. Quint, A. White, Influence of thymosin on cell-mediated and humoral immune responses in normal and in immunologically deficient mice, J. Immunol. 104 (1970) 359–366.
[43] C.J. Bayne, Phagocytosis and non-self recognition in invertebrates, Bioscience 40 (1990) 723–731.
[44] Y.Q. Li, X.R. Song, W.L. Wang, L.L. Wang, Q.L. Yi, S. Jiang, Z.H. Jia, X.Y. Du, L.M. Qiu, L.S. Song, The hematopoiesis in gill and its role in the immune response of Pacific oyster *Crassostrea gigas* against secondary challenge with *Vibrio splendidus*, Dev. Comp. Immunol. 71 (2017) 59–69.
[45] L. Canesi, G. Gallo, M. Gavioli, C. Pruzzo, Bacteria-hemocyte interactions and phagocytosis in marine bivalves, Microsc. Res. Tech. 57 (2002) 469–476.
[46] Z.G. Xiao, J. Shen, H. Feng, H. Liu, Y.P. Wang, R. Huang, Q.L. Guo, Characterization of two thymosins as immune-related genes in common carp (*Cyprinus carpio* L.), Dev. Comp. Immunol. 50 (2015) 29–37.
[47] Y. Sun, X.J. Chen, Y. Xu, Q.H. Liu, X. Jiang, S.F. Wang, W.L. Guo, Y.C. Zhou, Thymosin β4 is involved in the antimicrobial immune response of Golden pompano, *Trachinotus ovatus*, Fish Shellfish Immunol. 69 (2017) 90–98.
[48] M.Q. Wang, B.J. Wang, K.Y. Jiang, M. Liu, L. Wang, An i-type lysozyme (*Cf*LyzI) involved in innate immunity is essential for the survival of *Chlamys farreri* during *Vibrio* stimulation, ISJ - Invert. Surviv. J. 15 (2018) 285–293.
[49] M.Q. Wang, B.J. Wang, K.Y. Jiang, M. Liu, X.W. Shi, L. Wang, A mitochondrial manganese superoxide dismutase involved in innate immunity is essential for the survival of *Chlamys farreri*, Fish Shellfish Immunol. 72 (2018) 282–290.
[50] L.L. Wang, Q. Gao, F.F. Shi, C.Y. Yang, L.M. Qiu, H. Zhang, Z. Zhou, L.S. Song, The comparative study of immunity between two scallop species *Chlamys farreri* and *Argopecten irradians*, ISJ - Invert. Surviv. J. 10 (2013) 141–150.
[51] M. Schieber, N.S. Chandel, ROS function in redox signaling and oxidative stress, Curr. Biol. 24 (2014) R453–R462.
[52] C.F. Spurney, H.J. Cha, A. Sali, G.S. Pandey, E. Pistilli, A.D. Guerron, H. Gordish-Dressman, E.P. Hoffman, K. Nagaraju, Evaluation of skeletal and cardiac muscle function after chronic administration of thymosin β-4 in the dystrophin deficient mouse, PLoS One 5 (2010) e8976.