Fish and Shellfish Immunology 87 (2019) 371–378

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Thymosin participates in antimicrobial immunity in zebrafish

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ARTICLE INFO

Keywords:
Thymosin
Innate immunity
Antimicrobial function
Zebrafish

ABSTRACT

Thymosin hormones, which were shown to be involved in immune system development and differentiation in previous studies, have antimicrobial functions in different animals. Zebrafish are a useful model for immunology research. Although thymosin has been reported to be involved in the embryonic development of zebrafish, it is necessary to uncover the antimicrobial function of thymosin in zebrafish. In this study, we expressed thymosin β (Tβ) in zebrafish *in vitro* and studied its antimicrobial function. The Tβ protein consists of 45 amino acids and is conserved among its family members, especially the actin-binding motif (LKKTET). *Tβ* was expressed in all tested tissues and was highly expressed in the brain, liver and hindgut. After *Aeromonas hydrophila* challenge, the *Tβ* transcript level increased in the skin, liver, kidney, spleen, thymus, foregut, gills and midgut. Purified recombinant thymosin β (rTβ) protein was used to study the antimicrobial mechanism. rTβ could inhibit the growth of *Staphylococcus aureus*, *Aeromonas hydrophila*, *Vibrio anguillarum*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. rTβ also binds to and agglutinates certain bacteria. Further study showed that rTβ could combine with the polysaccharides from gram-negative and gram-positive bacterial walls. All results suggested that the Tβ of zebrafish plays a significant role in innate antibacterial immune responses.

1. Introduction

Thymosins were originally discovered and isolated from calf thymus [1]. Based on their different isoelectric points, they are divided into three groups: α-thymosin (pH < 5.0), β-thymosin (5.0 < pH < 7.0) and γ-thymosin (pH > 7.0) [2]. Thymosins are a group of highly conserved small peptides containing 10 to 50 amino acids and are found in many organisms from echinoderms to mammals.

The functions of thymosins involve different physiological activities, such as cell differentiation, cytokine secretion, neural development, cancer progression and antiviral response. Cell differentiation is essential for individual development. Thymosin β4 (Tβ4) can promote the differentiation of progenitor cell lines to cardiomyocytes [3]. However, Prothymosin α (ProTα) localizes on the inner acrosomal membrane of mature spermatozoa [4]. This suggests that ProTα plays an important role in gametogenesis. In human natural killer cells, the overexpression of Tβ4 stimulates interferon-γ expression and secretion [5]. However, native or recombinant ProTα variants potently induce type I interferon expression and then restrict human immunodeficiency virus-1 replication in macrophages [6]. Tβ4 overexpression regulates the nervous system in the developing chicken embryonic optic tectum [7]. Tα1 can inhibit breast cancer, melanoma and lung cancer [8,9]. Interferon-Tα1 recombinant protein could suppress hepatitis B virus (HBV) replication inducks [10].

Tβ, as an actin-binding protein, is also involved in immune responses. In invertebrate animals, Tβ participates in anti-pathogen immune responses. Thymosin3 expression increased in *Marsupenaeus japonicus* after *Vibrio anguillarum* or *Staphylococcus aureus* challenge [11]. A recombinant fragment of the Tβ of sea urchin could restrain the growth of *P. aeruginosa* by inhibiting biofilm formation [12,13]. The Tβ of pacific oyster inhibited gram-negative *Escherichia coli* [14]. In the Hong Kong oyster, the *Tβ* transcript level was upregulated after *Vibrio alginolyticus* challenge. Additionally, the knockdown of Tβ expression resulted in a significant decrease in haemocytes [15]. In Chinese mitten crabs, after *Listonella anguillarum* challenge, the Tβ-like protein expression profile increased in haemocytes [16]. In *Helicoverpa armigera*, Tβ protein expression was upregulated at the metamorphic stage in the integument or fat body after bacterial challenge [17]. Interestingly, *Tβ* expression is also involved in the immune responses induced by viral invasion. Tβ protected crayfish from white spot syndrome virus infection and played an important role in the antiviral immune response [18].

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https://doi.org/10.1016/j.fsi.2019.01.040
Received 29 October 2018; Received in revised form 16 January 2019; Accepted 25 January 2019
Available online 28 January 2019
1050-4648/ Published by Elsevier Ltd.

In mammals, Tβ could improve *P. aeruginosa*-induced keratitis by addressing both the infectious pathogen and deleterious host response [19]. In the liver injury mice induced by chronic ethanol and lipopolysaccharide (LPS), Tβ prevented inflammation and oxidative stress [20]. The antimicrobial activity of chemically synthesized human Tβ was tested *in vitro* against plankton and biofilms of *Staphylococcus* spp. and *P. aeruginosa* [21]. The structure and function of platelets is similar to granulocytes, which participate in antimicrobial host defence. The Tβ from platelets exhibited antimicrobial activities against *S. aureus* [22]. Tβ10 could be upregulated by *Mycobacterium bovis* and resulted in bovine macrophage apoptosis. Additionally, Tβ4 greatly improved recovery from HBV-induced hepatitis [23].

In teleosts, Tβ4 was identified in golden pompano, and the purified recombinant Tβ4 could inhibit the growth of *Vibrio harveyi* [24]. To date, two thymosins have been characterized as immune-related genes in common carp [25]. In zebrafish, Tβ was cloned, and its functions were researched in previous studies. Tβ4 regulated cardiac valve formation via endothelial-mesenchymal transformation in zebrafish embryos [26]. During zebrafish development, Tβ expression was tightly correlated with neuronal growth and differentiation [27]. Additionally, Tβ4-sulfoxide attenuated inflammatory cell infiltration in the wounded zebrafish [28]. However, the antimicrobial function of the zebrafish Tβ should be elucidated.

In this study, we aimed to uncover the antimicrobial function of Tβ in zebrafish. Tβ mRNA was detected in all tested tissues, especially in the brain, liver and hindgut. After *A. hydrophila* challenge, Tβ expression was upregulated. Recombinant Tβ could inhibit bacterial growth and agglutinate several bacteria. Additionally, recombinant Tβ could bind to bacteria and the polysaccharides from different bacteria. These results indicated that Tβ plays a vital role in zebrafish antibacterial immunity.

2. Materials and methods

2.1. Fish cultivation

Wild zebrafish (6 months) were raised in a recirculating freshwater system at 28 °C. Fish were fed three times per day with Artemia during experiments with 14 h/10 h light dark cycles. In the experiment, zebrafish were removed from the recirculating system and cultivated in individual tanks.

2.2. Zebrafish challenge and tissue collection

The experimental zebrafish were challenged by a bacterial solution containing 10^{10} CFU/l (CFU, colony-forming unit) *A. hydrophila* as in a previous study [29]. Before dissection, the fish were anaesthetized in 100 ng/ml MS222 (Sigma, USA) for 5 min. Different tissues were collected, including skin, gills, kidney, spleen, thymus, liver, foregut and midgut at 0 h, 3 h, 6 h, 12 h, 24 h, 36 h, 48 h and 72 h after challenge. Each sample contained 5 fish. The tissues were separated under an anatomical lens (Lica S6D, Germany).

2.3. Total RNA extraction and cDNA synthesis

All samples were homogenised in homogenizers, and the total RNA was extracted using Trizol Reagent (TIANGEN, China) [30]. The first-strand cDNAs were reverse-transcribed using the FastQuant RT Kit (TIANGEN, China) as previously described [31,32]. The synthesized sample was diluted 10-fold for expression pattern analysis.

2.4. Quantitative real-time PCR (qRT-PCR) analysis

The transcript levels of *Tβ* at different time points after *A. hydrophila* challenge were evaluated with qRT-PCR by using RT-TM-F/RT-TM-R primers (Table 1). qRT-PCR was performed on a LightCycler 72 Real-time PCR system (QiaGen, Germany) with FastStart DNA Master SYBR Green I (TransGen, China) [33]. The PCR programme was as follows: 95 °C for 10 min; 40 cycles of 94 °C for 15 s and 60 °C for 60 s; and a melting curve analysis from 72 °C to 95 °C [34]. The expression profiles of *Tβ* were produced using the $2^{-\triangle\triangle CT}$ method [35,36]. The *β-actin* transcript level was used as a common control. Each test was repeated in three independent experiments.

Table 1
Sequences of primers used in this study.

Primers	Sequence (5′-3′)
RT- TM-F	CATTCACTGCTTTACGCT
RT- TM-R	GGTTCTTTTCTTGGGTCT
β-actin-F	CAGATCATGTTTGAGACC
β-actin-R	ATTGCCAATGGTGATGAC
BD-TM-EcoRI-F	GGAATTCATGGCCGACAAACCCAAC
BD-TM-XhoI-R	CCGCTCGAGTCACGGTGTGGACTCTCC

2.5. Bioinformatics analysis

The amino acid sequence alignments were built using the GeneDoc software. The potential structure of the Tβ protein was built using the SWISS-MODEL server (http://swissmodel.expasy.org/). The protein structure of Tβ9 (Protein Data Bank [PDB] accession no. 1hj0.1.A) was used as a template. Furthermore, the phylogenetic tree of the β-thymosins (Tsβ) was constructed by the neighbour-joining (NJ) method in MEGA7.0 [37].

2.6. Recombinant β-thymosin (rTβ) expression and purification

The reverse transcribed cDNA was used as a template to amplify the *Tβ* ORF using the designed primers BD-TM-EcoRI-F and BD-TM-XhoI-R (Table 1). PCR was performed using the following settings: 95 °C for 3 min; 32 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and a final extension step of 72 °C for 10 min. All the products were analysed by 1% agarose electrophoresis, and the fragments were purified with a PCR product purification kit (TIANGEN, China). The *Tβ* DNA fragment was inserted into the pET-32a (+) plasmid [38]. The recombinant plasmid was transformed into *Escherichia coli* Rosetta competent cells, which were incubated at 37 °C in lysogeny broth (LB) culture media containing 100 μg/ml ampicillin. Recombinant protein expression was induced with 0.5 mM isopropyl-β-d-thiogalactoside (IPTG). The rTβ protein was purified by nickel column affinity chromatography (GE, USA) as described previously [39]. The recombinant protein was quantified by 12.5% SDS polyacrylamide gel electrophoresis (SDS-PAGE).

2.7. Liquid bacteriostatic assay

Eight kinds of bacteria were used in the liquid bacteriostatic test, including the gram-positive bacteria *S. aureus*, *Bacillus thuringiensis*, *Bacillus megaterium* and *Bacillus subtilis* and the gram-negative bacteria *A. hydrophila*, *V. anguillarum*, *K. pneumoniae* and *P. aeruginosa* (as a gift from JX Wang's lab). The bacteria were cultivated overnight at 37 °C and then washed twice with phosphate buffer saline (PBS, 0.1 mol/l Na_2HPO_4, 0.1 mol/l $Na_2H_2PO_4$, pH = 7.5). Bacteria were diluted with PB medium (1% Tryptone, 0.5% NaCl), and the final concentration was 10^5 CFU/ml. The bacterial suspension (180 μl) was added to a 96-well plate. The rTβ recombinant protein solution (20 μl, 60 μg/ml) was incubated with bacteria in 96-well plates. His-tag protein was used as a control. The 96-well plate was shaken gently for 48 h. Each test was repeated three times. The absorbance at 620 nm was read by a microplate reader (Molecular Devices, FilterMax F3, USA).

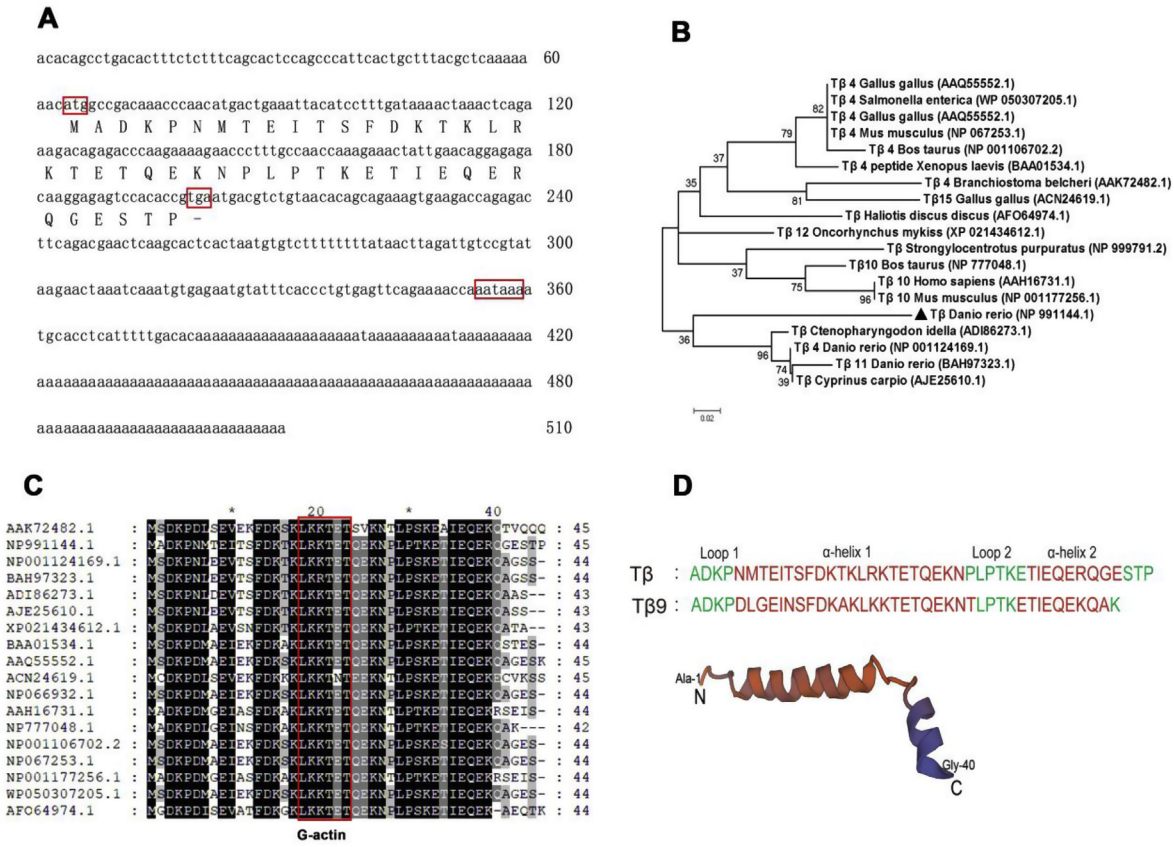

Fig. 1. The sequence information and structure model of the Tβ from zebrafish and phylogenetic tree of the Tsβ. (A) Nucleotide and amino acid sequences of the zebrafish Tβ. The translation initiator codon (atg), stop codon (tga) codons and polyadenylation signal are indicated by red boxes. (B) Phylogenetic tree of the Tsβ from different species. The neighbour-joining (NJ) distance tree was established. Branch confidence levels are built on 1000 bootstrap replicates. (C) The Tsβ from different species were aligned using GeneDoc. Conserved amino acids are indicated in dark (=100%) and light grey (≧75%) area. The G-actin binding domain is indicated by the red box. (D) Alignment of the Tβ amino acid and the structure model. The code colours represent: red, a-helices 1 and 2; green, loops 1 and 2. Protein quaternary structure of the Tβ9 was used as a template. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.8. Bacterial agglutination assay

Eight bacteria, including the gram-positive and gram-negative bacteria described above, were used for the bacterial agglutination assay. Bacteria were shaken overnight at 37 °C and then inoculated in LB medium (1:100) for 2 h until reaching the logarithmic phase. The bacteria were centrifuged at 5000 × *g* for 5 min and then resuspended in TBS (10 mM Tris-HCl, pH 7.5, and 150 mM NaCl) to OD_{600} = 0.4 [40]. The bacterial suspension (20 μl) was added to a 96-well plate. The rTβ protein solution (20 μl, 100 μg/ml) was incubated with the bacteria for 30 min at 37 °C in 96-well plates. His-tag protein was used as a control. All assays were performed in triplicate. Bacterial agglutination was observed by inverted microscopy (Lecia, MDi1, Germany).

2.9. Bacterial binding assay and Western blot

The eight bacteria described above were used in the protein binding assay. Recombinant rTβ protein (1 mg/ml, 20 μg) was added into 1 ml of bacterial suspension (in TBS, OD_{A600} = 1) and incubated for 30 min at room temperature with gentle rotation. The incubated bacteria were washed with TBS 4 times and then washed with a 7% SDS solution. Both the eluted solution and sedimentary bacteria were quantified by Western blot using the His-tag monoclonal antibody (Proteintech, USA) as described in a previous method [41]. The proteins in the supernatant were separated using 12.5% SDS-PAGE and then transferred onto PVDF membranes. The membranes were blocked for 1 h with 5% nonfat milk (in TBS) and incubated with the His-tag antibody (1:5000 in 5% nonfat milk, Proteintech, USA) overnight. After washing 3 times with TBST (100 mM NaCl at pH 7.5, 10 mM Tris-HCl, and 0.01% Tween 20), goat anti-mouse IgG conjugated with HRP (1:10,000 in TBS, Proteintech, USA) was added and incubated for 2 h. After washing 3 times with TBST, the ECL chemiluminescent reagent (Beyotime, China) was used to detect the binding ability with a gel imager (AI 600, GE, USA) [42].

2.10. Polysaccharide-binding assay

The binding activities of the recombinant rTβ protein to LPS from *E. coli 055:B5*, peptidoglycan (PGN) from *S. aureus* and lipoteichoic acid (LTA) from *S. aureus* were analysed by enzyme linked immunosorbent assay (ELISA). The LPS, PGN and LTA were coated on the bottom of 96-well plates. The polysaccharides were dissolved in water and added into 96-well plates (50 μl, 80 μg/ml). The plates were incubated for 12 h at 37 °C and then at 60 °C for 30 min. The coated plates were blocked for 2 h with 1 mg/ml BSA in TBS and washed 4 times with TBS. The rTβ protein solution (0, 0.005, 0.01, 0.1, 1, or 2 μg/ml) in 0.1 mg/ml BSA was added to the coated plate wells for 2 h at 25 °C. A His-tag protein solution was used as the control group. Each well was washed 4 times using TBS and incubated with His-tag antibody (1:2000 in 0.1 mg/ml BSA, Proteintech, USA) for 2 h. After washing 4 times, HRP-conjugated anti-mouse IgG (1:10,000, Proteintech, USA) was added and incubated for 1 h [43]. After washing 4 times, the ECL chemiluminescent reagent (Beyotime, China) was added to each well. The chemiluminescent intensity of each well was read by a microplate reader (FilterMax F3,

Molecular Devices, USA).

2.11. Statistical analysis of data

Data represent the results of at least three independent experiments. Student's *t*-test was used to determine other statistically significant results. Differences were considered significant at $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (**).

3. Results

3.1. Characterization of Tβ cDNA and phylogenetic tree

The Tβ cDNA of zebrafish contains 510 base pairs (bp), including a 5′-untranslated region (UTR) of 63 bp and a 309 bp sequence with a polyadenylation signal at the 3′-UTR. The ORF of Tβ is 138 bp and encodes 45 amino acids (Fig. 1A). According to the BLAST analysis, the cloned Tβ was consistent with NP991144. To analyse the molecular evolutionary relationship of Tsβ, we constructed a phylogenetic neighbour-joining (NJ) tree. The Tβ of zebrafish was assigned to a branch cluster with fish (Fig. 1B). This suggested that the Tsβ from teleosts had high similarity. To uncover other Tβ paralogous genes, the Tsβ in zebrafish genome was shown in Table 2.

3.2. Alignment and structure model of Tβ protein

To identify Tβ, the aligned amino acid sequences were compared to multiple alignments of the Tsβ from different species (Fig. 1C). The high similarity of Tsβ (75%–100%) suggested that Tsβ is highly conserved from vertebrates to invertebrates, specifically at the G-actin binding site (in red box).

To obtain a model structure, a 3-dimensional structure was predicted (Fig. 1D). The Tβ modelling structure had two α-helical domains, ^{5}NMTEITSFDKTKLRKTETQEKN^{26} and ^{33}TIEQERQGE^{41}, and two loop domains, ^{1}ADKP^{4} and ^{27}PLPTKE^{32}.

3.3. Tissue distribution and expression profiles of Tβ

To examine the tissue distribution of Tβ in zebrafish, its mRNA level was quantified by qRT-PCR. The results showed that Tβ was distributed in all the selected tissues (Fig. 2A). Tβ was highly expressed in the brain, liver, hindgut, muscle, thymus, midgut, skin and kidney. However, less Tβ was detected in the spleen, gills and foregut.

To determine whether Tβ is involved in antibacterial immune responses, qRT-PCR was performed to evaluate the expression profiles of different tissues after *A. hydrophila* challenge. The results showed that the *Tβ* mRNA level quickly increased in the gills, liver and midgut 3 h after *A. hydrophila* challenge (Fig. 2C, G and J). In the foregut, the *Tβ* mRNA level increased after 6 h (Fig. 2H). However, in the skin, kidney and thymus, the *Tβ* mRNA level increased after 12 h (Fig. 2B, D and F). The *Tβ* mRNA level increased after 24 h in the spleen (Fig. 2E). These results suggested that *Tβ* could participate in antibacterial innate immune responses. Interestingly, the *Tβ* mRNA level increased earlier in the gut than in the kidney, thymus and spleen. However, the expression profiles had various shapes in the different tissues after *A. hydrophila* challenge.

3.4. Liquid bacteriostatic ability of rTβ

To test the antibacterial activity of Tβ, recombinant Tβ protein (rTβ) was expressed (in *E. coli* Rosetta) and purified. A band corresponding to rTβ was observed at approximately 25 kDa (Fig. 3A). The His-tag protein was also expressed and purified as the control protein. A liquid bacteriostatic assay was performed *in vitro*. The results showed that rTβ could inhibit the growth of the gram-positive bacterium *S. aureus* as well as the gram-negative bacteria *A. hydrophila, V. anguillarum*, *K. pneumoniae* and *P. aeruginosa* (Fig. 3B). The inhibitory effect was more significant for *K. pneumoniae* and *P. aeruginosa*. However, an inhibitory effect was not observed for *B. thuringiensis*, *B. megaterium* and *B. subtilis*. These results indicated that rTβ could inhibit the growth of gram-positive and gram-negative bacteria. Interestingly, the inhibitory effect was more significant for the gram-negative bacteria.

3.5. Bacteria agglutinating ability of rTβ

To examine the impact of rTβ on bacteria, a bacterial agglutination assay was performed. The results showed that the bacteria *S. aureus*, *A. hydrophila*, *V. anguillarum*, *K. pneumoniae* and *P. aeruginosa* were gathered (Fig. 4). These results suggested that the rTβ protein could agglutinate different bacteria. However, the agglutinating phenomenon was more significant for the gram-negative bacteria.

3.6. Bacteria and polysaccharide-binding ability of rTβ

To investigate the binding ability of rTβ, a bacterial binding assay was first performed. The results showed that rTβ could strongly bind to *S. aureus*, *A. hydrophila*, *V. anguillarum*, *K. pneumoniae* and *P. aeruginosa* (Fig. 5A). However, the binding effect for *S. aureus* and *P. aeruginosa* was more significant than that of the other bacteria. The polysaccharide-binding ability was also determined. Three polysaccharides, LPS, LTA and PGN, were isolated from different bacterial walls. The results showed that rTβ could significantly bind to the polysaccharides (Fig. 5B and C). Under low protein concentrations, rTβ could better bind with LPS. Interestingly, under higher protein concentrations, the ability of rTβ to bind to LTA and PGN increased.

4. Discussion

In this study, we investigated the antimicrobial function of Tβ in zebrafish. This study showed that Tβ can participate in the innate immune responses induced by *A. hydrophila*.

Tsβ has high similarity among mammals, invertebrates, and teleosts. Tβ is a ubiquitous protein family that is present in most tissues and cell lines, such as blood platelets, neutrophils, macrophages, and other lymphoid tissues in mammals [44,45]. In shrimp, *Tβ* is highly expressed in haemocytes and heart [11,18]. In the pacific oyster, *Tβ* was expressed at the highest level in the mantle [14]. A previous study showed that *Tβ*4 was highly expressed in the gills, spleen and head kidneys of *Trachinotus ovatus* (golden pompano) [24]. In this study, we found that *Tβ* is highly expressed in the liver as well as in the brain and hindgut of zebrafish. These results indicate that *Tsβ* is ubiquitously expressed in different tissues. However, different species have specific tissue distribution.

Table 2
The Tβ paralogous genes in zebrafish.

Gene names	Chromosome location	Amino acid numbers	Reference sequences
Tβ	21	45	NM_205581 (GenBank)
Tβ1	14	46	NM_001165918 (GenBank)
Tβ2	14	45	B8A6B8 (UniProt)
Tβ4 X-linked	9	44	NM_001130697 (GenBank)

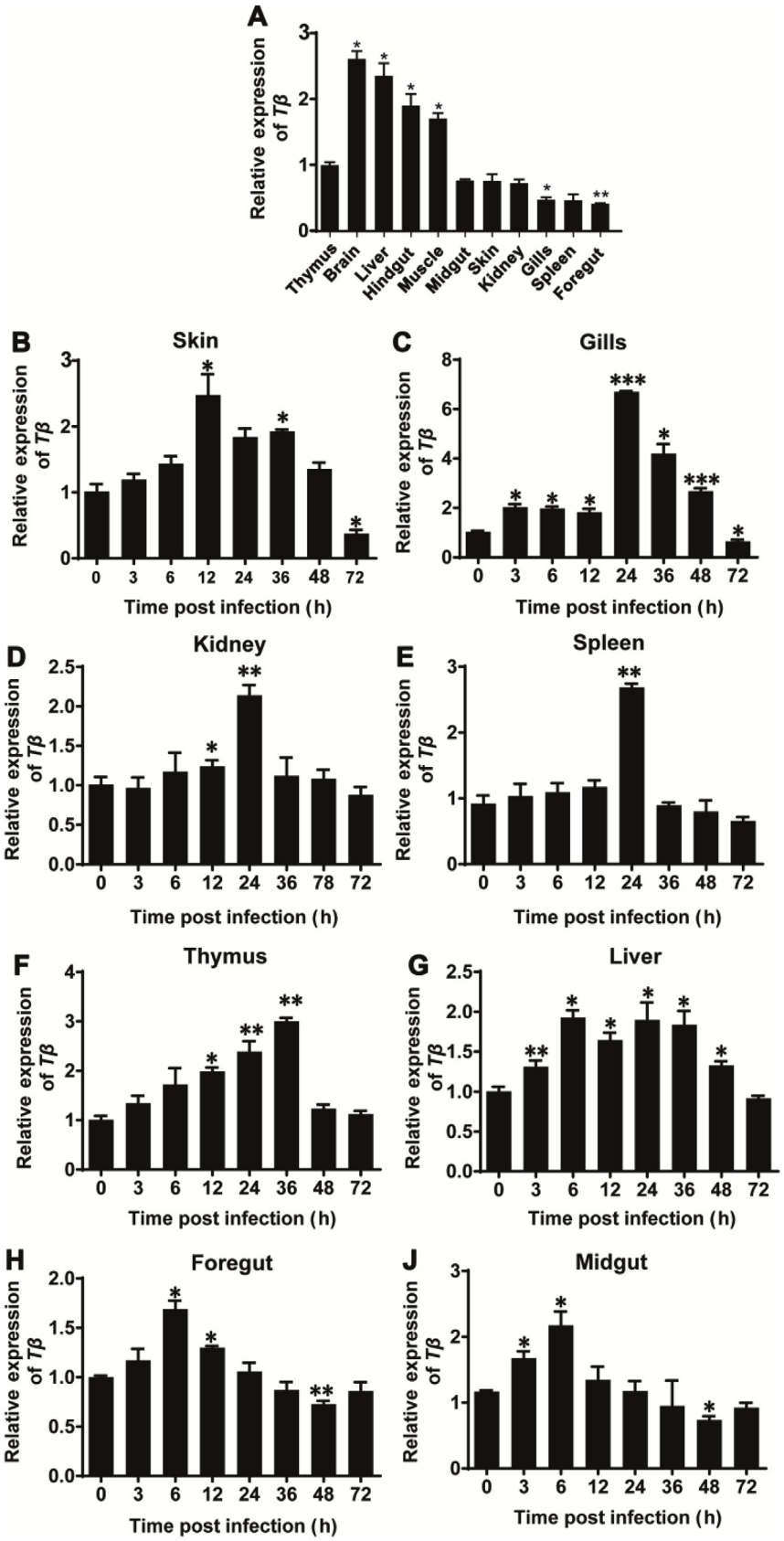

Fig. 2. The tissue distribution and expression profiles of Tβ. (A) QRT-PCR analysis of the transcription levels in different tissues of unchallenged zebrafish. The Tβ transcription level in the thymus was used as the control. (B–J) Transcriptional patterns in the skin, gills, kidney, spleen, thymus, liver, foregut and midgut after *A. hydrophila* challenge. *β-actin* was used as the internal control. The presented data are from three independent assays. ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

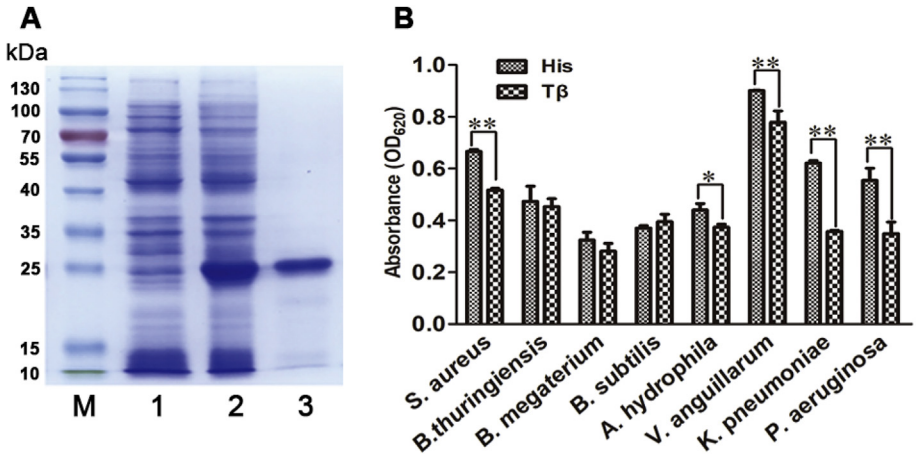

Fig. 3. Expression, purification and the liquid bacteriostatic ability of rTβ. (A) rTβ was expressed and purified from Rosetta. Horizontal characters represent the following: M, Marker; 1, non-induced sample; 2, induced sample; and 3, purified rTβ. (B) The ability of rTβ to inhibit bacteria. In this test, eight bacteria were used: *S. aureus*, *B. thuringiensis*, *B. megaterium*, *B. subtilis*, *A. hydrophila*, *B. anguillarum*, *K. pneumoniae*, and *P. aeruginosa*. His-tag protein was used as a control. Three independent assays were performed in this test. $^{**}p < 0.01$ and $^{*}p < 0.05$.

Tsβ functions are involved in immune responses. In invertebrates, Tsβ participates in antimicrobial and antiviral activities. In pacific oysters, mRNA expression peaked 48 h after *Vibrio* spp. infection. In silkworm, *Tβ* was significantly decreased starting 24 h after BmNPV challenge, which indicated that Tβ could exert a direct antiviral effect [46]. In carp, Tβ could upregulate the expression of T lymphocyte-related genes after infection with spring viremia of carp virus [25]. This indicated that Tβ enhanced the immune response and modulated the development of T lymphocytes in teleosts. In zebrafish, Tβ4 regulates embryonic cardiac valve formation [26]. In this study, we found that *Tβ* expression was upregulated in several tissues in zebrafish after *A. hydrophila* challenge (Fig. 2). This indicates that Tβ is involved in antimicrobial responses. Additionally, according to the mRNA transcript profiles, the *Tβ* expression increased in intestine was earlier than kidney, thymus and spleen. This probably indicates that the high expression of the *Tβ* arose earlier in the mucosal immunity tissue than in systemic immunity tissues.

In invertebrates, Tβ could inhibit *S. aureus* and *V. anguillarum* growth and increase the survival rate of infected shrimp [11]. The Tβ from the sea urchin has antistaphylococcal biofilm activity [47]. In humans, the Tβ4 fragment EIEKFDKSKLK showed antibacterial activity against *P. aeruginosa* [21]. According to the results of the liquid bacteriostatic assay in this study, Tβ in zebrafish could also inhibit the growth of *S. aureus*, *V. anguillarum* and *P. aeruginosa*. Additionally, Tβ could also inhibit the growth of *A. hydrophila* and *K. pneumoniae*. Interestingly, among the gram positive bacteria used in this study, only the growth of *S. aureus* was inhibited. This showed that the different bacteria are differentially sensitive to the effect of Tβ. These results indicate that Tβ in zebrafish is an antimicrobial peptide (AMP) molecule involved in innate immune responses.

To play a role in antimicrobial function, AMPs should interact with microbial cell membranes. Then, the interaction will lead to dynamic change in the structures and topologies of the microbes [48,49]. In this process, AMPs could inhibit essential cellular processes, including nucleic acid synthesis, cell wall synthesis, protein synthesis and enzymatic activities [50,51]. A previous study showed that the polysaccharides of bacterial cell walls contributed to the stiffness of bacteria and biofilm formation [52,53]. In this study, a bacterial agglutination assay showed that Tβ in zebrafish could agglutinate several bacteria, including *S. aureus*, *V. anguillarum*, *A. hydrophila*, *K. pneumoniae* and *P. aeruginosa* (Fig. 4). Additional assays showed that the Tβ in zebrafish could bind to these bacteria, especially *S. aureus* and *P. aeruginosa*. Additionally, we found that the Tβ in zebrafish could bind to the polysaccharides from different bacterial cell walls (Fig. 5). These results suggest that the Tβ in zebrafish is a kind of AMP involved in antimicrobial immune responses.

In conclusion, *Tβ* was expressed ubiquitously in zebrafish, and its transcript level was upregulated after *A. hydrophila* challenge in several tissues. Tβ could inhibit and agglutinate several bacteria, especially *S. aureus,* and the tested gram-negative bacteria. In addition, Tβ could bind to bacteria directly. Further study indicated that Tβ could also

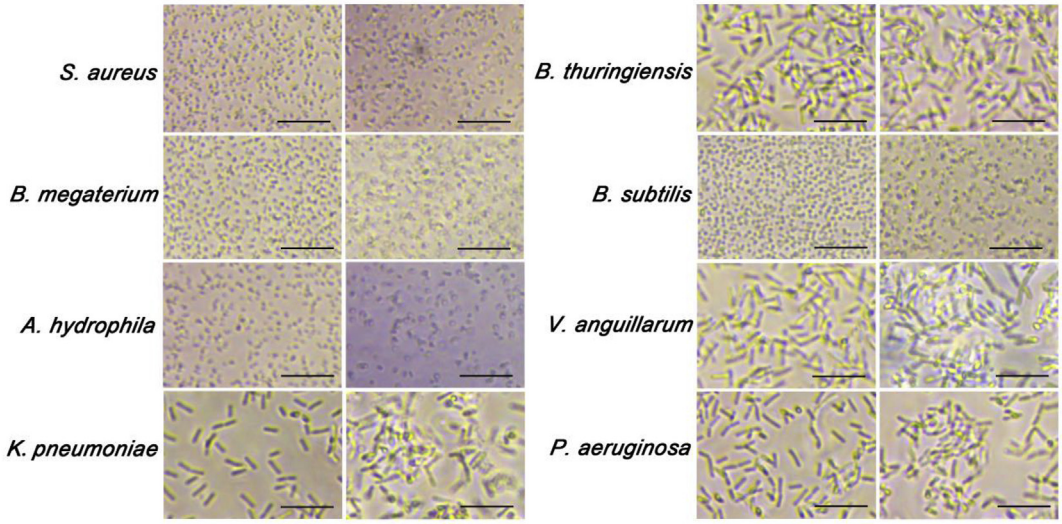

Fig. 4. The ability of rTβ to agglutinate bacteria. Bacterial agglutination test of rTβ. His-tag protein was used as the control. The images were obtained from a microscope (Lecia, MDi1, Germany). The eight bacteria described above were used in the tests. Scale bar = 20 μm.

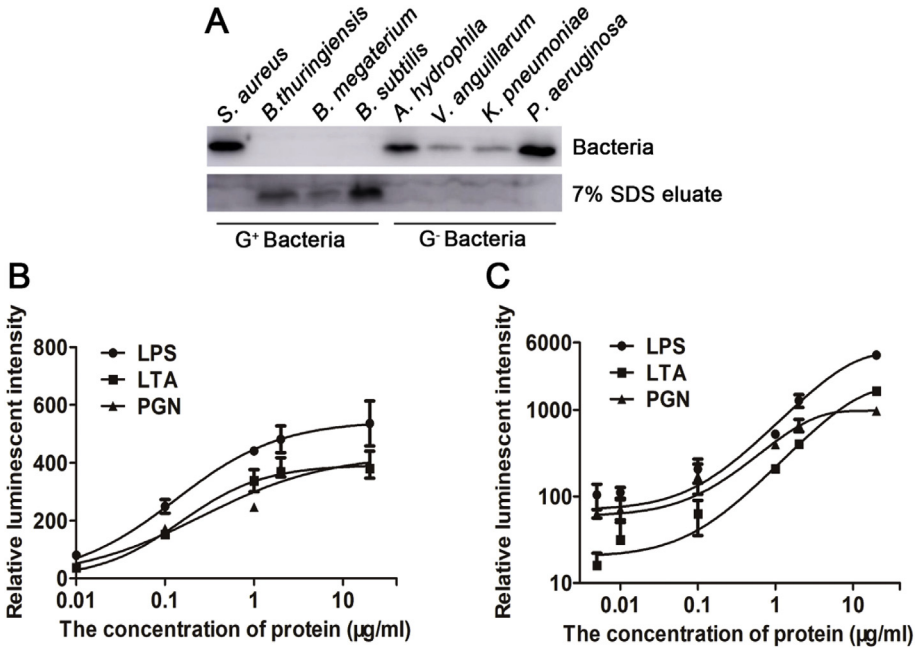

Fig. 5. Bacteria and polysaccharide binding ability of rTβ. (A) Western blot detected the bacterial binding. The samples of the precipitated bacteria and 7% SDS eluate are shown. (B) The His-tag was used as the control. (C) RTβ bound to polysaccharides. Three polysaccharides (LPS, LTA and PGN) were used for the ELISA analysis. Three independent assays were performed in the test.

bind to the polysaccharides from different bacteria. Therefore, Tβ, as a kind of AMP, could participate in innate immune responses in zebrafish.

Acknowledgements

This work was supported by grant from Shandong University Scientific Research Project (No. J18KB081).

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