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Identification and expression of the hepcidin gene from brown trout (*Salmo trutta*) and functional analysis of its synthetic peptide

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ABSTRACT

Hepcidin, a hepatic antimicrobial peptide, is a key player of the nonspecific immune system. The structure of hepcidin gene from brown trout (*Bthepc*) has been characterized at the molecular level. The 1158-bp mRNA generates a coding sequence (CDS) of 267 bp, which encodes an 88-amino acid protein. Molecular evolution analysis classified *Bthepc* to the family Salmonidae. Amino acid sequence homologies between *Bthepc* and hepcidin in other species such as *Oncorhynchus mykiss*, *Salmo salar,* and *Hucho taimen* were found to be 93.18%, 96.59%, and 92.05% respectively. The mature peptide and the signal peptide of *Bthepc* are made of 25 and 24 amino acids, respectively. Similar to the other species, eight conserved cysteines in the mature peptide of *Bthepc* are held together by four disulphide bonds. Expression profiling of *Bthepc* indicated its highest expression in the liver. Further, iron levels or inflammation did not induce the age-dependent expression of *Bthepc*. *Bthepc* mRNA expression analysis in six immune tissues (liver, gill, spleen, skin, head kidney and intestine) indicated different levels of increase when challenged with *Aeromonas salmonicida* and *Aeromonas hydrophila*. The antimicrobial activity of synthetic *Bthepc* to typical pathogens was verified *in vitro*. In addition, *Bthepc* showed moderate haemolytic activity to mammalian erythrocytes. The antimicrobial activity of *Bthepc* was attributed to the disruption of the bacterial outer membrane integrity, which was evident from our scanning electron microscopy results. In summary, hepcidin gene of brown trout was characterized, and its antimicrobial activity was verified on different levels.

1. Introduction

During the course of evolution, fishes form an indispensable branch point in the evolution of the immune system because the development of the innate and adaptive immune systems of animals began in fishes [1]. As fishes are exposed to diverse pathogens in their natural habitat, their innate immune system has adapted to the aquatic environment [2]. With the development of aquaculture in recent years, there is a rapid growth in the fisheries production on a large scale. Drug resistance of aquatic organisms continues to increase, impeding the development of aquaculture. The drug accumulation in aquatic products poses a major threat to human health. The innate immune factors such as antimicrobial peptides (AMPs), which produce numerous small peptides, form the first line of defence in the fish immune system [3,4].

Hepcidin is a regulatory hormone playing a crucial role in innate immune responses [5]. It is a cysteine-rich AMP, which exerts its role in regulating iron metabolism and antimicrobial function against pathogens [6]. The sequences and structures of hepcidin are well characterized in many vertebrates [7]. Hepcidin promoter encompasses the binding sites for NF-kB, hepatic nuclear factor, and enhancer binding protein [8]. The highly conserved hepcidin precursor in teleost contains a signal peptide, a propeptide, and a mature peptide. The signal peptide of hepcidin contains 24 amino acids and possesses antimicrobial function. The propeptide of hepcidin contains a typical R-K/R-R motif for recruitment of propeptide convertase to form the mature peptide [9] that consists of 19–31 amino acids, which harbours a typical Q-S/I-H-L/A-L motif in the N-terminus and regulates iron metabolism [6]. There are eight conserved cysteine residues forming four disulphide bonds in the C-terminus, which are implicated in the biological functions of hepcidin [10,11]. Iron storage in liver and iron level in blood circulation exert double effects on the transcription of hepcidin gene [12,13]. What's more, the expression of hepcidin is also affected by

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inflammation, which is induced by interleukin (IL) through JAK2/STAT3 signaling pathway [14]. Hepcidin disrupts the cell membrane of the pathogen [15]. Alternatively, it interacts with cell membranes to form instantaneous pores. The mature peptides enter the cells through these pores to arrest growth the pathogens by inhibiting cell wall synthesis, cellular respiration, and nucleic acid or protein synthesis [16].

Brown trout (*Salmo trutta*), which has its origin in Europe, North Africa and West Asia, has emerged as a high-quality cold water fish around the world [17]. In China, brown trout had been introduced to Tibet in the nineteenth century [18]. As a measure to protect this species, the brown trout has been enlisted as a class II protected species (Fish) in Tibet. The cultivation of brown trout has been increasing annually due to increasing demand. With the expansion of farming area and breeding density, the environmental deterioration leads to disease outbreak, especially the bacterial diseases bringing about a fatal blow to the aquaculture industries. The key molecular element for the non-specific immune response of brown trout remains to be characterized. Identification of antimicrobial activities of related immune factors in brown trout will serve as important markers for genetics and breeding and would lay a foundation for the development of novel antimicrobial agents. In the present study, the hepcidin cDNA of brown trout (*Bthepc*) was cloned and the structure of *Bthepc* mature peptide was predicted. Expression profiles of *Bthepc* challenged with *Aeromonas salmonicida* and *Aeromonas hydrophila* were analysed at various time points in different tissues to identify *Bthepc* immune response to these pathogens. The synthetic mature *Bthepc* peptide was tested for its antimicrobial activity against different pathogens *in vitro* and the mechanism of action of *Bthepc* on bacterial outer membranes is presented.

2. Materials and methods

2.1. Fish and sample collection

The brown trouts were bred and cultivated at the Bohai experimental station of the Heilongjiang River Fisheries Research Institute (Harbin, China). Experimental fishes were transferred to the 1 m^3 tanks with a recirculating water system, at 12 ± 0.1 °C, under constant photoperiod (12 Light:12 Dark). The tank was divided into three separate spaces for different fish groups. The body length and weight of experimental fishes used for the bacterial challenge studies were (25.2 ± 4.4) cm and (68.8 ± 9.5) g, respectively. The sample size for this part of the experiment was 3N = 150. The tissues were collected and immersed in RNAlater (Takara, Dalian, China) storage solution and frozen at −80 °C until further use. The liver tissues and blood were sampled from 30-month-old (30 M), 18 M, and 6 M (3N = 9) brown trouts to assess *Bthepc* expression and iron levels. All experiments were performed according to the European Communities Council Directive (86/609/EEC) norms. All fishes involved in this research were bred following the guidelines of the Animal Husbandry Department of Heilongjiang, P.R. China. All efforts were made to minimise the pain.

2.2. Molecular cloning of Bthepc

The reference nucleotide sequence of hepcidin (GenBank ID HQ711993) from *Oncorhynchus mykiss* was used to design gene-specific primers. The cDNA of brown trout liver tissue was used as the template to amplify *Bthepc*. The PCR cycling conditions were as follows: initial denaturation of 94 °C for 5 min; followed by 30 cycles of 94 °C for 30 s, annealing at 59.8 °C for 30 s, and extension at 72 °C for 1 min; with a final extension at 72 °C for 10 min. PCR products were cloned into a pMD-18T vector (Takara, Dalian, China) and the clones were confirmed by sequencing. NGSP1, GSP1, NGSP2, and GSP2 primers (Table 1) were designed based on gene sequence data. The rapid-amplification of cDNA ends (RACE) method was performed according to the SMARTer® RACE 5′/3′ kit instructions (Takara, Dalian, China). For the outer amplification of 5′/3′ ends, reagents were mixed as followed for each 50 μl PCR reaction: 10 × cDNA PCR reaction buffer 5 μl, dNTP Mix (10 mM) 1 μl, advantage 2 Polymerase Mix (50 ×) 1 μl, Diluted adaptor-ligated cDNA 5 μl, AP1 primer (10 μM) 1 μl, GSP1/GSP2 primer 1 μl, H_2O 36 μl. The first program of PCR was as followed: 94 °C for 30 s; 5 two-step cycles: 94 °C for 5 s and 72 °C for 2 min; followed by 5 two-step cycles: 94 °C for 5 s and 70 °C for 2 min; and 25 cycles: 94 °C for 5 s and 68 °C for 2 min. The second program was as follows: 94 °C for 30 s; 30 two-step cycles: 94 °C for 5 s and 72 °C for 2 min. The nested PCR were performed according to the following protocol: a. Dilute 5 μl of the outer PCR product into 245 μl of Tricine-EDTA buffer. b. using 5 μl of the diluted outer PCR product in place of the ready cDNA; 1 μl of the AP2 primer and 1 μl of NGSP1/NGSP2; fewer cycles (15 instead of 25 cycles).

Table 1
Primers used for *Bthepc* cloning and expression detection.

Primer	Sequence (5′-3′)	Purpose
ssHep-F	TGTCACTTCAATTCCAACTGACTTC	Conserved sequence cloning
ssHep-R	GTGCAGTGATAAAGACGTCAATGAG	Conserved sequence cloning
NGSP1	CTGCAACTGCTGTCACAACAAG	3′RACE
GSP1	GTTGGAAGCATTGACAGTCCAG	3′RACE
NGSP2	ATGTCTTTTTCCTGTGTCATTAGGC	5′RACE
GSP2	AGTTTGAGGTTGTGCAGTGATAAAG	5′RACE
Bthepc-F	ACCCTGTGGCTAAATGTTCAGG	Realtime-PCR
Bthepc-R	CTGTAGATTCCATTCCCAGACTCC	Realtime-PCR
IL1b-F	GAACACCTCTGAAAGTAAGGCATG	Realtime-PCR
IL1b-R	GCAGGTCCTTGTCCTTGAACTC	Realtime-PCR
IL6a-F	AGTGTACCAGCTTCTTCTTCAGCA	Realtime-PCR
IL6a-R	TGGCAGACAGGTCCTCTACTACC	Realtime-PCR
IL6b-F	ATGTTCTTCTCCAGCACATTAAGG	Realtime-PCR
IL6b-R	CTACCACCTCAGCAACCTTCATC	Realtime-PCR
IL8-F	TTCAAACTCACCACAGACAGAGAAG	Realtime-PCR
IL8-R	CTGTTGTTATCTCGCTGGTAACAAG	Realtime-PCR
βactin-F	CACAGACTACCTGATGAAGATCCTG	Realtime-PCR
βactin-R	TTCCCATCTCCTGTTCGAAGTC	Realtime-PCR

2.3. Sequence analysis of Bthepc

The amino acid sequence of *Bthepc* was predicted using the http://genes.mit.edu/GENSCAN.html tool. Multiple sequence alignment of the published sequences for hepcidin gene was performed with *Bthepc* using DNAMAN 6.0 software. The similarity of hepcidin between different species was compared using the Clustal X software [19]. Twenty-eight hepcidin amino acid sequences were used in the phylogenetic tree construction using the neighbour-joining (NJ) method in MEGA 6.0 [20] and evaluated by 1000 bootstrap replications. The signal peptide region of *Bthepc* was predicted by http://www.cbs.dtu.dk/services/SignalP/, and the transmembrane domain was analysed using the TMHMM tool. The isoelectric point (*pI*) and the molecular weight of *Bthepc* mature peptide were predicted using the Compute pI/MW tool (http://web.expasy.org/compute_pi/). The 3D protein structure prediction was performed using the Swissmodel Server (https://www.swissmodel.expasy.org/) and visualised using the Swiss-PdbViewer 4.1 software.

2.4. Iron level detection in serum and liver

Blood samples from each brown trout were collected and allowed to stand at 4 °C for 24 h. The serum samples were collected after centrifugation at 3000 × *g* for 10 min. Standard manufacturer's protocol was followed to estimate the iron concentration using the serum iron detection kit (Jiancheng, Nanjing, China). For the estimation, 1.5 ml iron chromogenic agent was added to 0.5 ml of tested serum and standard solution, respectively. The iron chromogenic agent with distilled water was considered as the blank sample. All the samples were boiled for 5 min followed by centrifugation for 10 min at 3500 r/min.

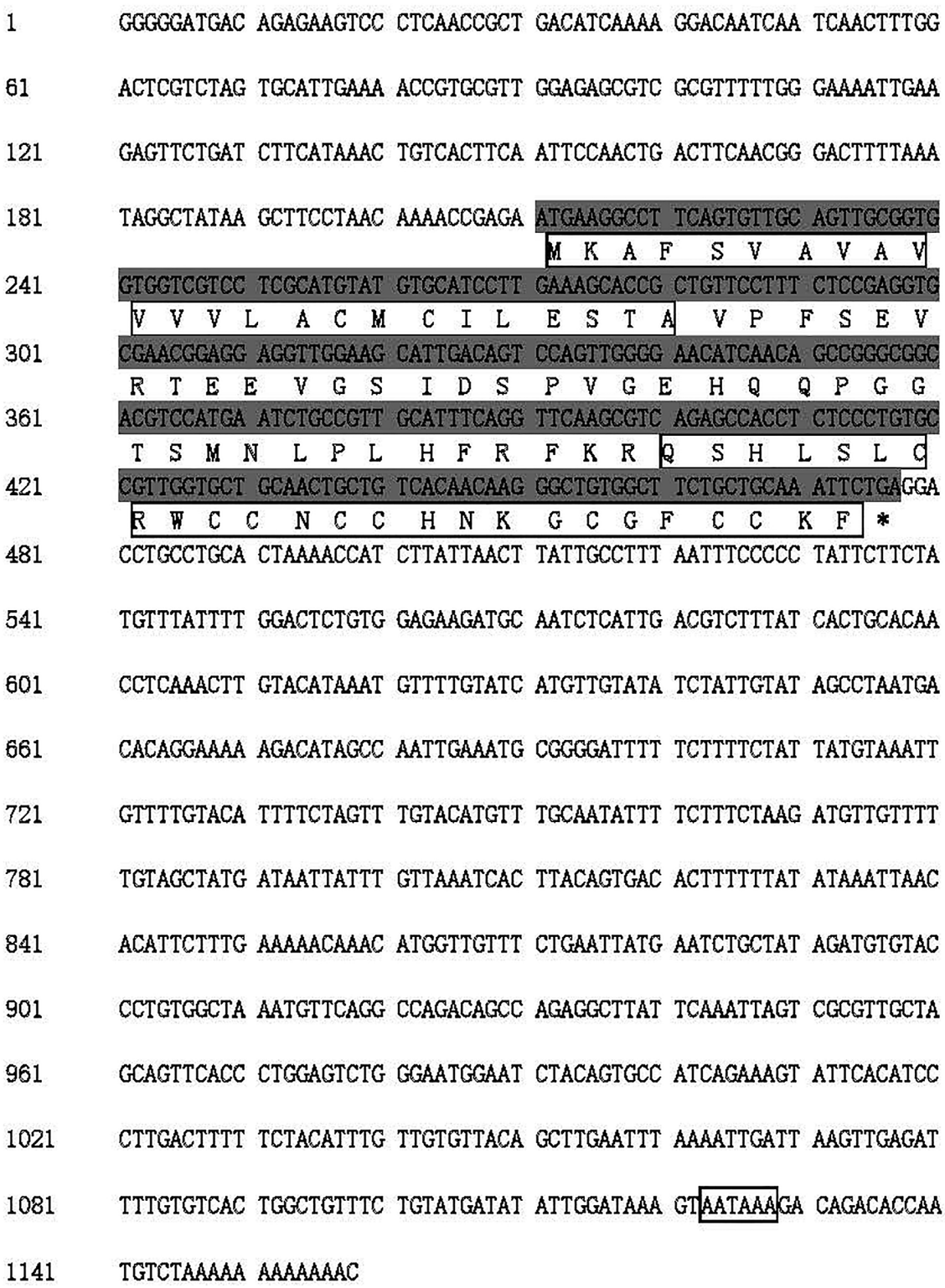

Fig. 1. Gene sequence of *Bthepc*. The coding sequence region is highlighted in grey.

The supernatants were collected to detect the optical density (OD) at 520 nm. The concentration of serum iron was calculated as follows: Serum iron (mg/L) = ($A_{serum, 520\ nm} - A_{blank, 520\ nm}$)/($A_{standard, 520\ nm} - A_{blank, 520\ nm}$) × standard solution concentration (2 mg/L). All the measurements were repeated three times. For the detection of iron level in liver, the proteins were extracted from the livers of 6M, 18M and 30M brown trouts, and the protein concentrations were measured using the nano-volume spectrophotometer (Analytik jena, Germany). The method of protein extraction form livers was as follows: liver tissue was cut into pieces, and 1 ml RIPA lysis buffer (Beyotime, China) and 10 μl PMSF (Beyotime, China) was added per 100 mg liver tissue before fully homogenized. Then homogenized samples were centrifuged at 12, 000 × *g* for 5 min, and the supernatants were transferred into new tubes, which was ready for concentration measurement. The measurement was performed as per the tissue iron detecting kit protocol (Jiancheng, Nanjing, China). The concentration of liver iron was calculated as

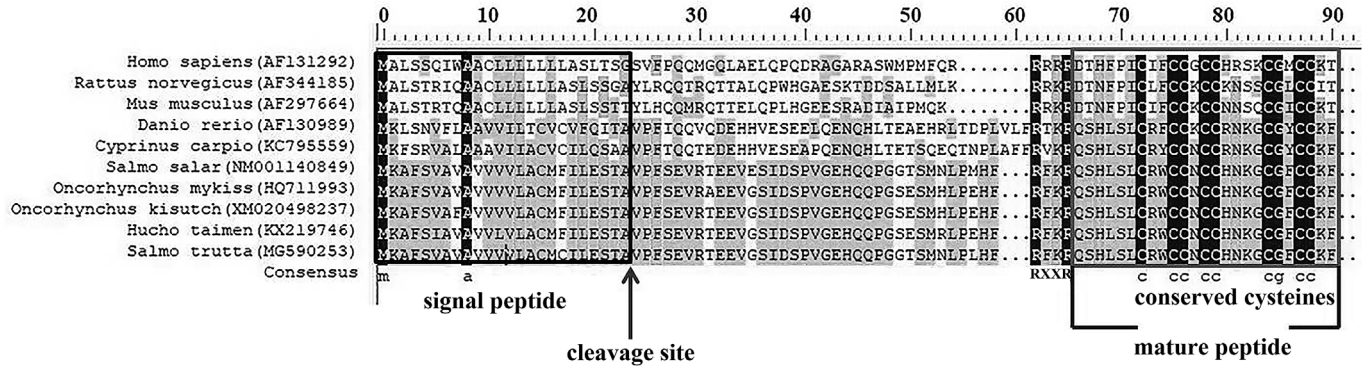

Fig. 2. Amino acid sequence alignment of hepcidin. The predicted signal peptide and mature peptide are shown in a box. The cleavage site is marked by an arrow. The eight conserved cysteines in the mature peptide are highlighted in black.

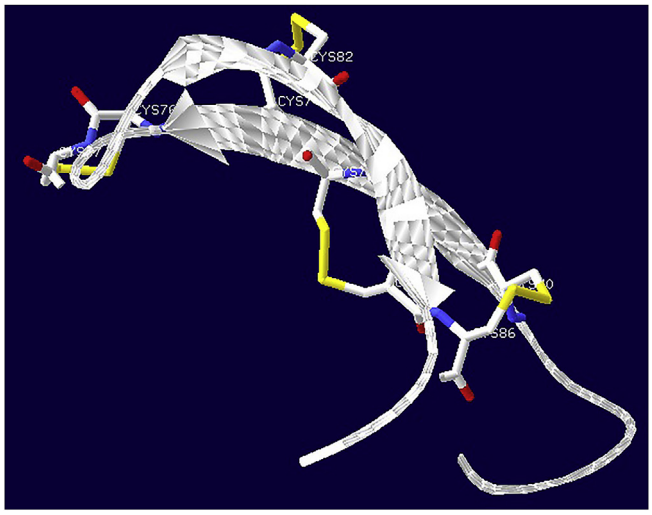

Fig. 3. Predicted 3D structure of the *Bthepc* mature peptide. Cysteine residues (CYS77-CYS76, CYS74-CYS82, CYS73-CYS85, CYS70-CYS86) are shown in blue, and side chains of cysteine residues are shown in red. The yellow ribbons represent four disulphide bonds. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

following: liver iron (mg/g) = ($A_{\text{liver, 520 nm}} - A_{\text{blank, 520 nm}}$)/($A_{\text{standard, 520 nm}} - A_{\text{blank, 520 nm}}$) × concentration $_{\text{standard solution}}$ (2 mg/L)/concentration $_{\text{liver protein}}$ (g/L). All the measurements were repeated three times.

2.5. Bacterial challenge

Brown trouts were separated into three groups. Except for the control group, the other two groups were injected intraperitoneally with *A. salmonicida* and *A. hydrophila*, respectively. Activated colony was selected to inoculate in liquid medium, which was placed in the thermostatic shaker for 12 h at 200 rpm. When the OD600 value of bacteria suspension was 1, microbial concentration was about 1×10^9 CFU. Then the microbial concentration was diluted to 1×10^6 CFU with sterile liquid medium. The bacterial suspension (1×10^6 CFU) in 100 μl PBS was injected into each fish. The control group was injected with the same volume of PBS. Six fishes were collected at each time point (0, 2, 4, 8, 12, 24, 48, and 72 h) after bacterial challenge, and six tissues with important immune roles (intestine, liver, skin, spleen, gill, and head kidney) of each fish were sampled. Sampled tissues from the control and the test groups were placed in 2 ml RNAlater (Takara, Dalian, China), and stored at −80 °C for RNA extraction.

2.6. Real-time PCR

Quantitative real-time PCR was performed using FastStart Universal SYBR® Green Master Mix (ROX). The conditions were as follows: 95 °C for 30 s; 40 two-step cycles: 95 °C for 5 s and 60 °C for 34 s; followed by 95 °C for 15 s; 60 °C for 1 min; and 95 °C for 15 s. Beta-actin was used as the reference gene. The primers were designed using Primer Premier Software 5.0. Primers used for real-time PCR are listed in Table 1. For each cDNA sample, all target and reference genes were amplified independently in triplicate on the same plate and the same experimental run. The melting curve analysis showed that there were no dimers or other non-specific PCR products in any of the reactions performed. Ct values were measured using the CFX96 C1000 touch Thermal Cycler (BIO-RAD, America).

2.7. Polypeptide synthesis

The *Bthepc* mature peptide (QSHLSLCRWCCNCCHNKGCGFCCKF) synthesized by solid phase synthesis was purchased from ChinaPeptides Company (Shanghai, China). The crude peptide was purified to 87.82%, and the peptide was identified using mass spectrometry (MS) analysis. The molecular weight of the peptide was found to be 2881.42 Da. The scrambled peptide (CLGHLCKNCCCFWQNRCCCHKSFGS) was also synthesized in the same way and used as the negative control.

2.8. Antimicrobial assay

The antimicrobial activity was tested by the disc diffusion method. The following organisms were used as target pathogens: 1) gram-negative bacteria: *Acinetobacter baumannii*, *Aeromonas salmonicida* (ATCC 33658), *Aeromonas hydrophila* (ATCC 7966), and *Pseudomonas aeruginosa*; 2) gram-positive bacteria: *Staphylococcus aureus*, *Enterococcus faecium*; and 3) the fungus *Candida glabrata*. Bacterial suspensions at the logarithmic phase were diluted to 10^6 CFU/ml and cover the 1% agar medium. The *Bthepc* peptide solution (2 mg/ml) was applied to the agar medium, and the negative drug-sensitive slips were immersed in a scrambled peptide solution. The media containing the targeted pathogens were incubated at the corresponding growth temperature for 24 h. The inhibition zone diameters were measured and all tests were repeated five times.

2.9. Haemolytic assay

The haemolytic activity of the peptide was measured as previously described [21]. Fresh anti-coagulated human blood was collected from one volunteer (Harbin, China) in a polycarbonate tube coated with heparin. The experimental protocol was reviewed and approved by the ethics committee at the Hospital of Heilongjiang Province. The erythrocytes were washed twice with PBS and then diluted to 1% in PBS.

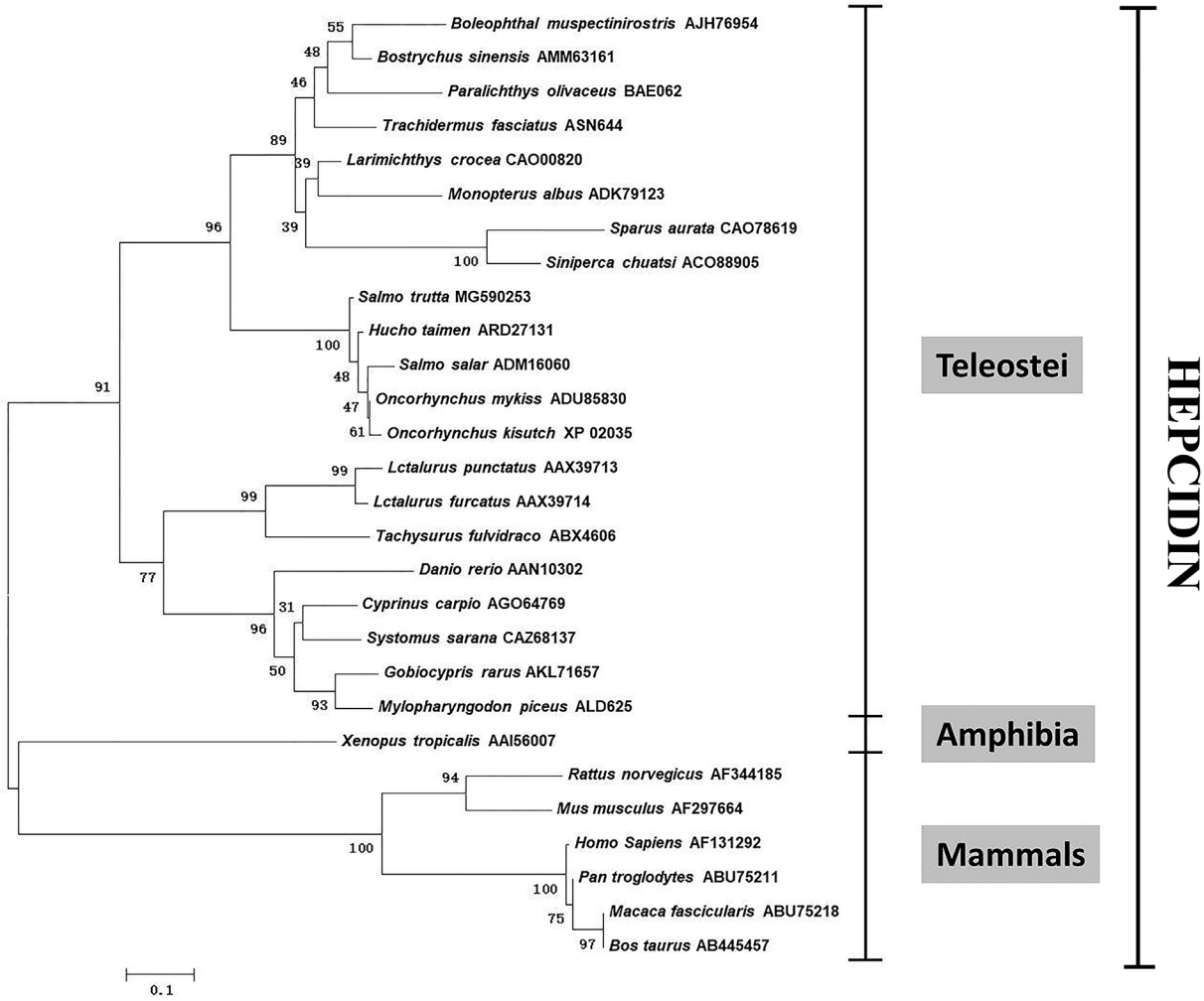

Fig. 4. Phylogenetic tree based on hepcidin amino acid sequences. Accession numbers of the sequences are shown.

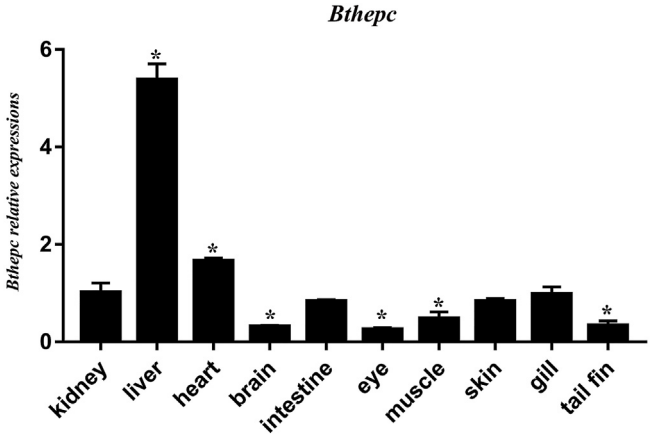

Fig. 5. Expression profile of *Bthepc* in different tissues of brown trout (*$P < 0.05$ vs. *Bthepc* expression in kidney, **$P < 0.01$ vs. *Bthepc* expression in kidney).

Erythrocytes (90 μl) were added to a 96-well plate. Thereafter, 2-fold serially diluted *Bthepc* peptide in 0.01% (v/v) acetic acid was added to each well of the sterile 96-well plate. Cells in PBS and 0.1% Triton X-100 were employed as negative and positive controls, respectively. The plate was centrifuged at 800 × *g* for 10 min after incubation at 37 °C for 2 h. The supernatants were transferred to a fresh 96-well plate, and the absorbance was measured at 405 nm. Percent of haemolysis was calculated as $[(OD_{405\ nm,\ peptide}–OD_{405\ nm,\ PBS})/(OD_{405\ nm,\ 0.1\%\ Triton\ X\text{-}100}–OD_{405\ nm,\ PBS})] \times 100$.

2.10. Scanning electron microscope

Scanning electron microscopy (SEM) experiments were performed as previously described [22]. *Aeromonas salmonicida* and *Aeromonas hydrophila* were harvested at the logarithmic phase and resuspended to an OD_{600} of 0.2 with PBS. The mixture of cells and the *Bthepc* peptide (0.5 mg/ml) was incubated at 37 °C for 60 min. The scrambled peptide was used as the negative control. The cells were washed thrice with PBS and fixed overnight with 2.5% (w/v) glutaraldehyde at 4 °C. The bacterial cells were washed twice with PBS and dehydrated through a series of ethanol concentration (50%, 70%, 90%, and 100%) for 15 min each. Upon dehydration, the dried bacterial cells were transferred to a 1:1 mixture of alcohol and tert-butanol for 30 min, followed by pure tert-butanol for 1 h. The dried bacterial specimens were coated and visualised under a field emission scanning electron microscope (HITACHI S-3400N, Japan).

2.11. Statistical analysis

The Ct value of target sequence normalized to reference sequence was calculated as a function of $2^{-\Delta\Delta Ct}$, for real-time PCR. Graphs were plotted using GraphPad Prism 7.0. Least significant difference (LSD) of one-way ANOVA was used to analyse the differences between groups.

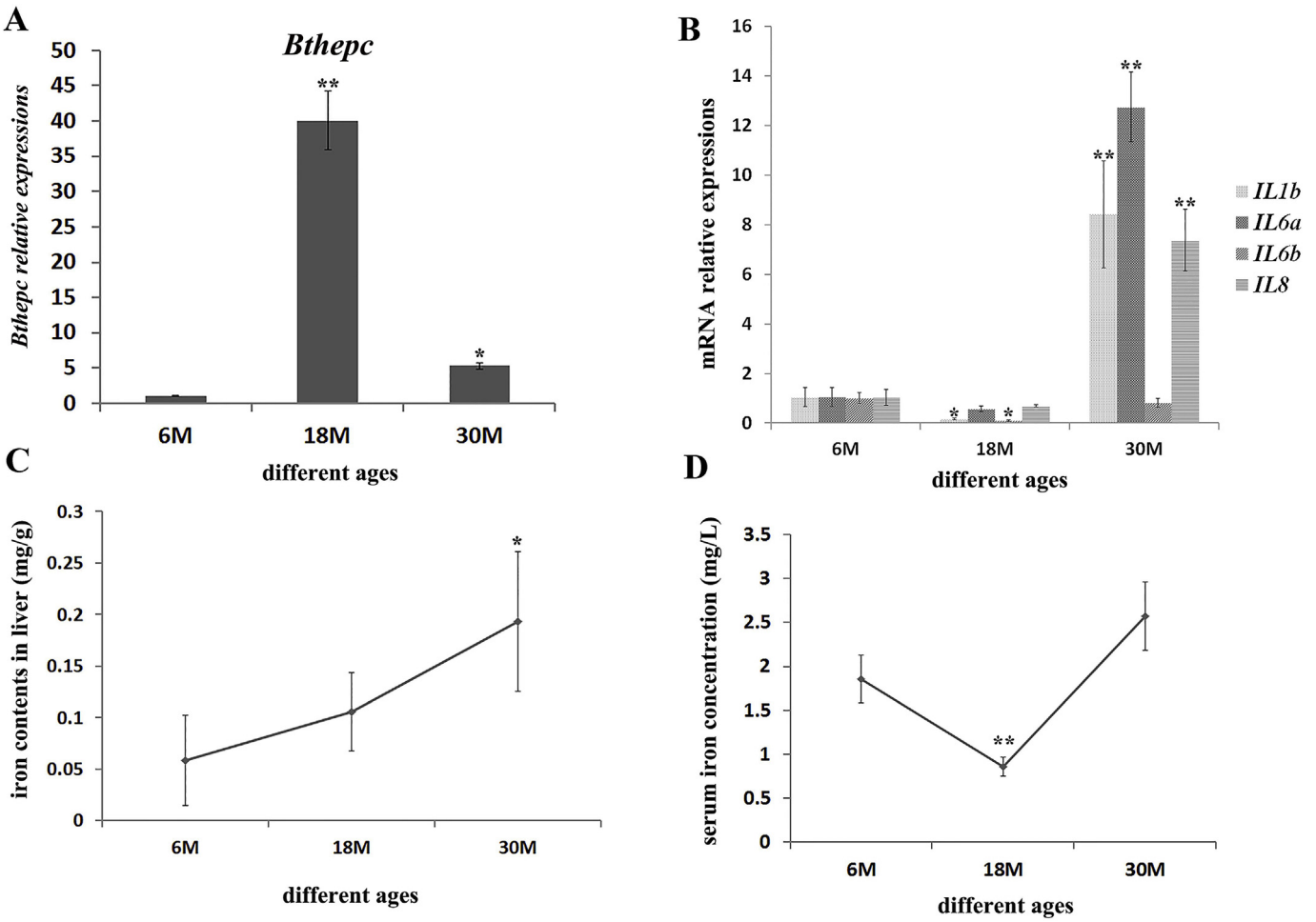

Fig. 6. Expression profile of *Bthepc* in different ages. A. The mRNA expression of *Bthepc* in the liver of 6 M (month-old), 18 M and 30 M brown trouts (*$P < 0.05$ vs. *Bthepc* expression in 6 M, **$P < 0.01$ vs. *Bthepc* expression in 6 M). B. The expression of IL1a, IL6a, IL6b, and IL8 mRNAs in the livers of 6 M, 18 M, and 30 M brown trouts (*$P < 0.05$ vs. *Bthepc* expression in 6 M, **$P < 0.01$ vs. *Bthepc* expression in 6 M). C. Hepatic iron concentration in 6 M, 18 M, and 30 M brown trouts (*$P < 0.05$ vs. iron content in 6 M). D. Serum iron concentration in 6 M, 18 M, and 30 M brown trouts (**$P < 0.01$ vs. serum iron concentration in 6 M).

Results are expressed as the mean ± S.E. Statistical analysis was performed using SPSS 13.0 for Microsoft Windows; $P < 0.05$ was considered statistical significant.

3. Results

3.1. Molecular cloning of Bthepc and phylogenetic analysis

The *Bthepc* cDNA sequence of 1158 bp length (GenBank MG590253) has been retrieved from the GenBank database (Fig. 1). The coding sequence (CDS) of *Bthepc* is 267 bp and contains 88 amino acid (aa) residues. The 5′ untranslated region (UTR) was from 1 to 210 bp, and 3′ UTR was from 478 to 1123 bp. Poly A sequence was found at the position of 1123–1128 bp. The amino acid sequence of *Bthepc* is shown in Fig. 2, in which the predicted cleavage site is located between Ala^{24} and Val^{25}, and the signal peptide is predicted from 1 to 24 aa. The mature peptide sequence is from 64 to 88 aa with a molecular weight of 2.881 kDa and a theoretical *pI* of 8.53. By comparing the amino acid sequence of *Bthepc* with the amino acid sequence of hepcidin of other species, such as *Oncorhynchus mykiss*, *Salmo salar* and *Hucho taimen,* the sequence homology was found to be 93.18%, 96.59%, and 92.05%, respectively. The predicted cleavage site of the mature peptide is between Arg^{63} and Gln^{64}. There are eight conserved cysteine residues in the *Bthepc* mature peptide forming four disulphide bonds, as evident from the 3D protein structure prediction presented in Fig. 3. The phylogeny tree was constructed based on 28 amino acid sequences of hepcidin from different species to analyse the evolutionary relationship of *Bthepc*. As illustrated in the phylogenetic tree (Fig. 4), *Bthepc* is clustered in the family Salmonidae. Mammals are located at the same branch location, whereas teleost species and amphibians are located in their respective branches. Phylogenetic analysis showed that hepcidin proteins are highly conserved during biological evolution and that their protein structures have extensive homology.

3.2. Spatiotemporal expression profile of Bthepc

The expression profile of *Bthepc* in different tissues (kidney, liver, heart, brain, intestine, eye, muscle, skin, gill, and tail fin) of brown trout of 1-year-old was examined by Real-time PCR. The experimental fish in this part were all in healthy status, which were not challenged by pathogens. The expression of *Bthepc* the liver was significantly upregulated compared to that in the other tissues. Moreover, *Bthepc* expression was relatively high in the heart, kidney, gill, intestine, and skin (Fig. 5). In order to explore its expression pattern in different stages of development of brown trout, the expression of *Bthepc* was investigated in the liver tissues of 6-month-old (6 M), 18 M, and 30 M brown trouts (Fig. 6A). Real-time PCR analysis revealed the highest expression of *Bthepc* in 18 M, with 40-fold higher expression than in 6 M, and 8-fold higher expression than in 30 M. However, considering the expression of hepcidin is affected by iron levels and inflammation status, expressions

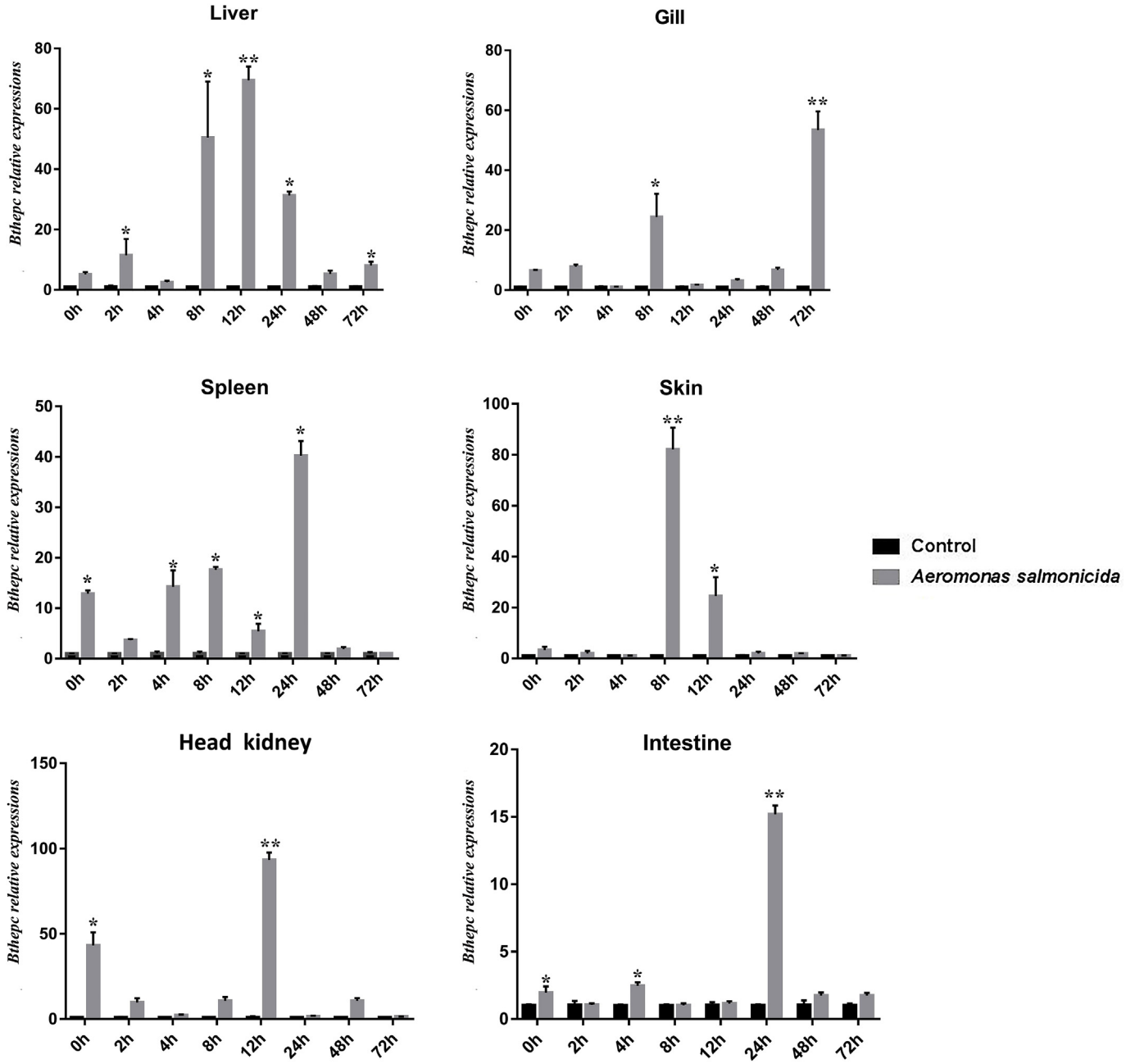

Fig. 7. Relative expression of *Bthepc* in different tissues in response to *Aeromonas salmonicida* challenge. Error bars indicate mean ± SD from three replicates measured independently (*$P < 0.05$ vs. control at the same time point, **$P < 0.01$ vs. control at the same time point).

of interleukin genes and iron levels in serum and liver at different developmental ages were analysed. It was observed that the expression of *IL1b* and *IL6b* in 18 M was significantly lower than in 6 M, whereas *IL6a* and *IL8* expression levels remained unchanged (Fig. 6B). The expression of *IL1b*, *IL6a*, and *IL8* mRNA was significantly higher in the 30 M than in 6 M brown trouts. As for the iron content in liver, it showed an increase with the age (Fig. 6C). However, the iron level in serum was significantly lower in 18 M than that in both 6 M and 30 M brown trout (Fig. 6D).

3.3. Expression of Bthepc in response to bacterial challenge

Expression of *Bthepc* in the six important immune tissues was assessed in response to challenge with *A. salmonicida* and *A. hydrophila*. *Bthepc* expression in the liver post-challenge with *A. salmonicida* reached its peak at 12 h post-infection, which was about 70-fold higher than the control group (Fig. 7). However, the expression of *Bthepc* began to decrease after 12 h but was still higher than that in the control group. Hepatic expression of *Bthepc* could not be restored to the control level even after 72 h. In the gills, the expression of *Bthepc* reached its maximum at 72 h after the challenge, which was about 55-fold higher than that in the control group. As for the spleen, the expression of *Bthepc* increased significantly at 0 h compared to control, and an increase was observed between 4 and 24 h, with a peak value at 24 h. In the skin, the expression increased from 8 h, which was 80-fold higher than that of the control. In the head kidney, the peak expression was detected at 12 h, while in the intestine, it was detected at 24 h, which were 90-fold and 15-fold higher respectively, compared to the control group. With the challenge of *A. hydrophila*, the peak *Bthepc* expression was observed at 8 h in the liver and at 12 h in the gills (Fig. 8). The

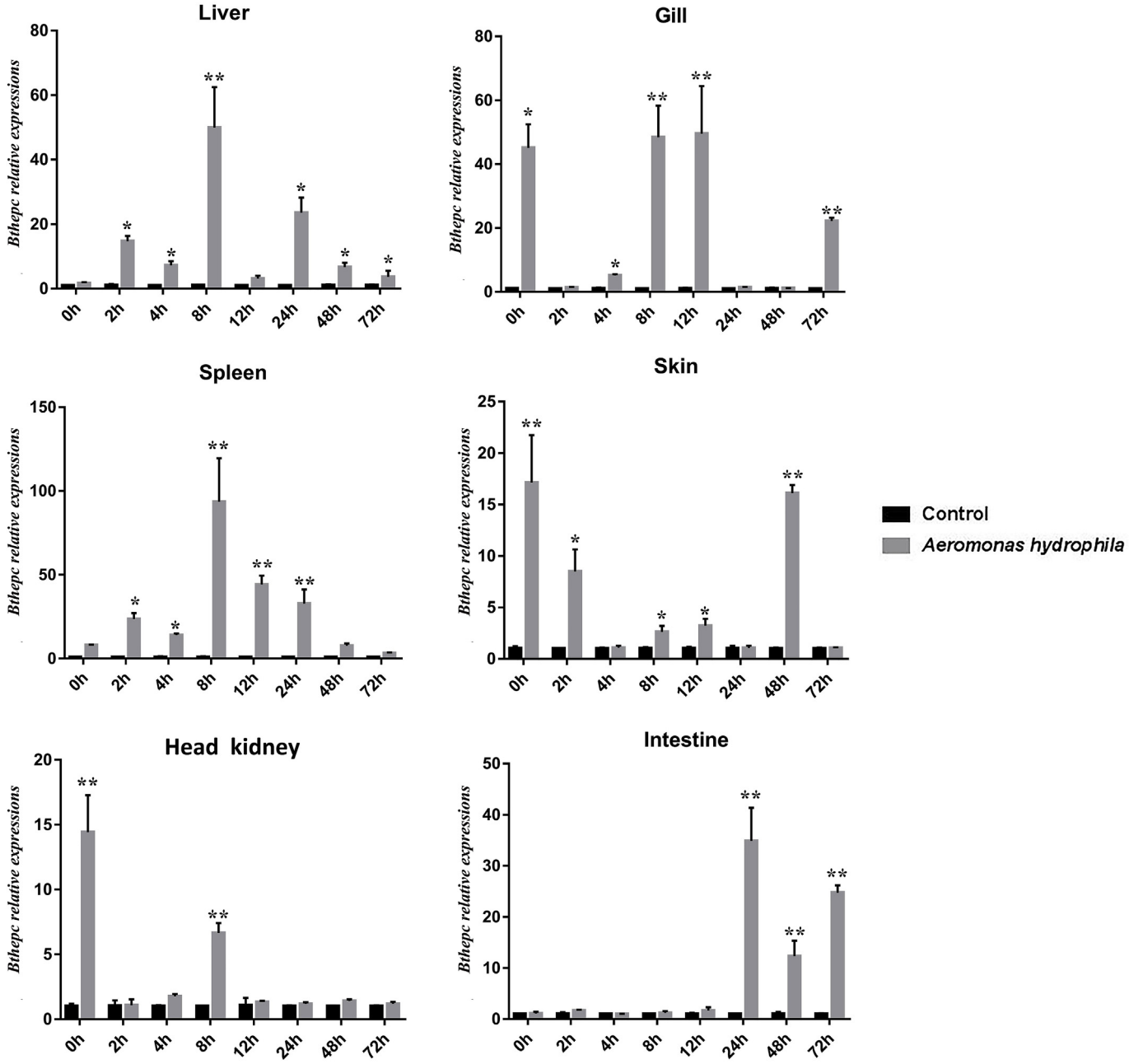

Fig. 8. Relative expression of *Bthepc* in different tissues in response to *Aeromonas hydrophila* challenge. Error bars denote mean ± SD from three replicates measured independently (*$P < 0.05$ vs. control at the same time point, **$P < 0.01$ vs. control at the same time point).

expression of *Bthepc* was increased at different time points in the spleen, the peak was observed at 8 h, which was 93-fold higher than that in the control. High expression of *Bthepc* was observed at 0 h and 48 h in the skin, at 0 h in the head kidney. In the intestine, an increase in *Bthepc* expression was observed between 24 and 72 h.

3.4. The antimicrobial and haemolytic activity of Bthepc

The mature *Bthepc* peptide was synthesized to test its antimicrobial activity using the lysoplate assay. Our results indicated that the growth of gram-negative bacteria, gram-positive bacteria, and the fungus were inhibited by the *Bthepc* peptide (Table 2). The antimicrobial activity of peptide was significantly higher than that of the scrambled peptide against all the tested pathogens in this study. The haemolytic activity of *Bthepc* was verified against the human erythrocytes (Fig. 9). Results indicated that 50% disruption of human erythrocytes occurred at a *Bthepc* peptide concentration of 34.71 μM, and a moderate haemolytic activity (1.9%–28.2%) was observed below 2.17 μM.

3.5. Antimicrobial mode of Bthepc

Hepcidin disrupts the cell membrane to rupture the pathogen. The effect of *Bthepc* on the outer membrane of major pathogens was analysed using SEM. In *A. salmonicida* cells treated with the *Bthepc* peptide, a difference in the morphology of the outer membrane was clearly noticed compared to the treated scrambled peptide (Fig. 10 A and B). The treated cell membrane became rough and presented many bumps, whereas the control cells were intact and smooth. The outer membrane of *Bthepc* peptide-treated *A. hydrophila* cells was wrinkled and disrupted, and the cells treated with scrambled peptide appeared normal (Fig. 10C and D).

Table 2
The antimicrobial activity of the *Bthepc* peptide. [1]Inhibition areas of *Bthepc* peptide were measured. [2]Inhibition areas of scrambled peptide were measured. The * indicates the difference between *Bthepc* and scrambled peptide (*P < 0.05;**P < 0.01).

Microorganism	Inhibition areas (mm^2)	
	Sthepc[1]	Scrambled peptide[2]
Gram-negative bacteria		
Acinetobacter baumannii	49.42 ± 7.55**	17.20 ± 1.93
Aeromonas salmonicida	55.50 ± 5.56*	21.36 ± 6.16
Aeromonas hydrophila	56.91 ± 7.70*	25.33 ± 5.98
Pseudomonas aeruginosa	50.32 ± 7.70**	18.69 ± 4.30
Gram-positive bacteria		
Staphylococcus aureus	49.26 ± 7.98*	22.38 ± 4.74
Enterococcus Faecium	40.52 ± 3.35*	24.27 ± 7.18
Fungi		
Candida glabrata	71.24 ± 9.84**	27.88 ± 4.56

4. Discussion

Salmo trutta is a valuable cold-water fish and understanding its inherent immune mechanism is beneficial to protect the brown trout population and for artificial breeding. Hepcidin is a key player in regulating non-specific immunity in fishes, protecting them from pathogenic attacks and injuries [23]. The broad-spectrum antimicrobial activity of hepcidin provides new insights into the marker-assisted selection of breeding and development of new antimicrobial agents by overcoming drug resistance problems. Hepcidin is involved in regulating iron metabolism [24] and exerts antimicrobial activity against major pathogens [23].

The *Bthepc* peptide with an RXXR motif is recognized by propeptide convertase for conversion into an active mature peptide, similar to other fishes [25]. Mature *Bthepc* peptide showed high similarity with hepcidin from other species. The hairpin structure formed by four disulphide bonds is important to disrupt the outer cell membrane of the pathogens [26]. In general, the attractive force between polypeptide and bacterial surface was enhanced by the electrostatic effect between cationic peptides and negatively charged cell wall of the bacterium [27,28]. Hepcidin of teleost species is located at the same phylogenetic branch as that of the amphibians and mammals. Phylogenetic analysis indicated that the *Bthepc* sequence was highly conserved.

The expression of *Bthepc* was the highest in the liver compared to other tissues [29–31]. *Bthepc* expression in the heart, kidney, gill, intestine, and skin were still relatively higher, which indicates that *Bthepc* plays an antimicrobial role in important immune tissues. Therefore, *Bthepc* was speculated to function as a non-specific immune factor to protect the brown trouts from pathogens. The expression of *Bthepc* in different developmental ages showed that the maximum level of *Bthepc* in 18 M. This excluded the effect of inflammation and hepatic iron levels. However, according to results in this study, the iron content in serum was the lowest in 18 M, it could be considered that the expression of *Bthepc* may be negatively regulated by serum iron level as in other species [32]. Interleukin constitute a very important cytokine family, which play pivotal roles in mature, activation, proliferation and immunity adjustment of organism. In our results, the expression of *IL1b*, *IL6a*, and *IL8* was significantly higher in the 30 M than in 6 M brown trout. It could be concluded that immunoregulation capability was upregulated with age in brown trout. While there was no significant different change for the expression of *IL6b*, the difference in functions of *IL6a* and *IL6b* should be thoroughly studied.

For cold-water fish cultivation and breeding, *Aeromonas* species are used as the major pathogens [33–35]. The bacterial challenge in the brown trout showed that the expression of transcript 8–24 h post-injection was significantly higher in the liver than that in the control groups, which is similar to those reported in other fishes [36]. The maximum of *Bthepc* expression under *A. salmonicida* challenge was 4 h later than *A. hydrophila*. It could be concluded that immune response to *A. hydrophila* was more rapid in liver of brown trout. Expression of *Bthepc* in the gill exhibited a peak at 72 h and 12 h when challenged

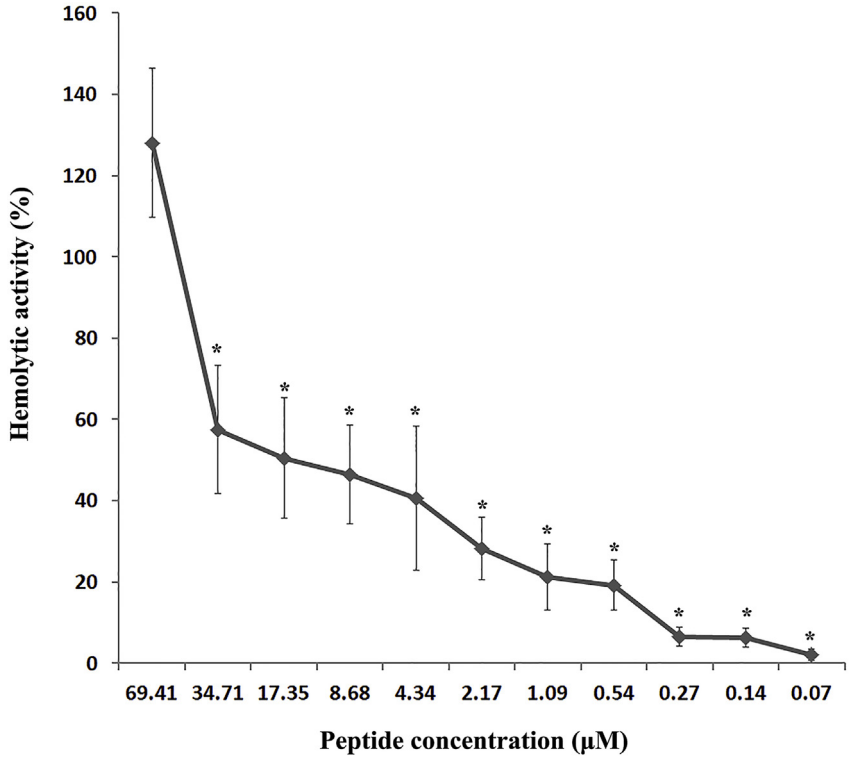

Fig. 9. Haemolytic activity of *Bthepc*. Haemolytic activity was evaluated by incubating the *Bthepc* peptide in 2-fold serial dilution with freshly isolated human erythrocytes for 2 h, followed by the estimation of the released haemoglobin at 405 nm. (*$P < 0.05$ vs. 69.41 μM).

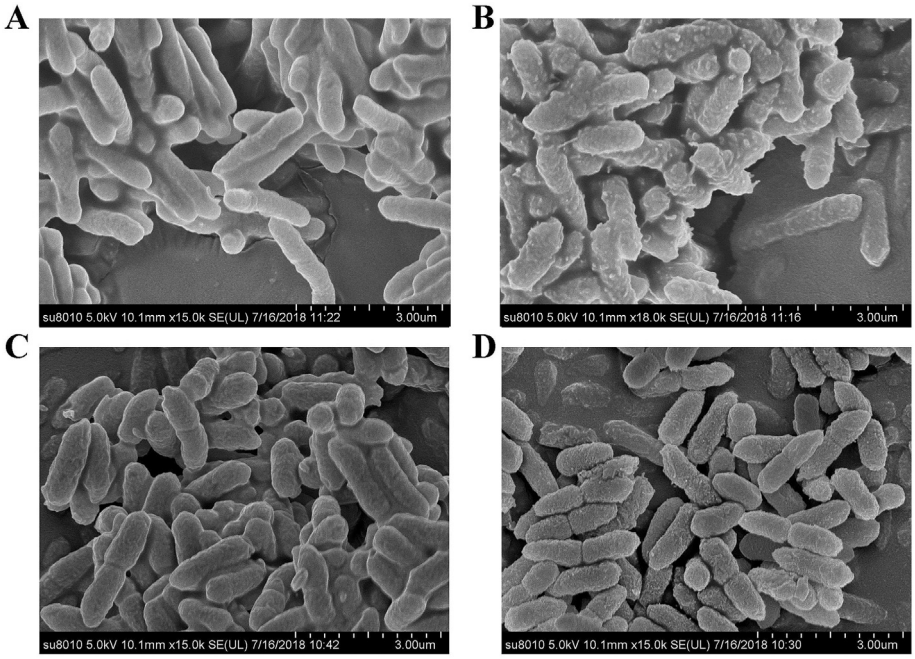

Fig. 10. Scanning electron micrographs of *A. salmonicida* and *A. hydrophila.* SEM micrographs of *A. salmonicida*: (A) Scrambled peptide; (B) *Bthepc* peptide. SEM micrographs of *A. hydrophila*: (C) Scrambled peptide; (B) *Bthepc* peptide.

with the two *Aeromonas* pathogens. The major roles of the gills include gas exchange, maintenance of electrolyte and acid base balance, and release of nitrogenous wastes [37]. The gill is one of the major immune barriers with a large mucosal area [34]. These two pathogens, *A. hydrophila* and *V. anguillarum,* invade fishes by infecting their gills [38]. The immune responses of *Bthepc* to *A. salmonicida* and *A. hydrophila* were quite different in gill brown trout. When challenged with *A. hydrophila*, expression of *Bthepc* was rapidly upregulated, while it began to increase at 8 h and reached the highest level at 72 h under the challenge with *A. salmonicida*. From this indicated that the nonspecific immunity of gill was quite different to different pathogens in brown trout. In fishes, the spleen is the major site of erythrocyte and neutrophil production, storage, and maturity. It is also a centre of melano-macrophages, which increase rapidly to protect the cells from free radical damage upon pathogen attack [39]. The presence of *Bthepc* in the spleen enables rapid immune responses at 0–2 h post-injection. During 4–24 h after challenge with the two *Aeromonas* pathogens, *Bthepc* expression maintained in a higher level in spleen. Skin is a mucosal tissue, which comes in direct contact with the external water environment. *Bthepc* expression profiles showed obvious differences of immune response after injection two *Aeromonas* pathogens in skin. The head kidney is another important lymphoid tissue of the fishes, which contains B and T lymphocytes, and granulocytes [40]. The present study indicated the rapidly increased expression of *Bthepc* in the head kidney and reached highest level at 8 h or 12 h after injection with the pathogenic bacteria. From the expression of *Bthepc*, it could be speculated that head kidney played important roles in nonspecific immune of brown trout. The intestine acts as a germinal centre for lymphocytes, constituting yet another important immune tissue in the fishes. The intestinal tissue acts as a storehouse for lymphocytes, macrophages, and granulocytes, despite lacking a complete lymphatic structure [41]. When challenged with the two *Aeromonas* pathogens, expression of *Bthepc* in the intestine increased significantly at 0–4 h and 24–72 h post-injection. The time of immune response to different pathogens in intestine of brown trout may be different. Even so, *Bthepc* in intestine plays an important role in conferring immunity against the pathogens, which provides new insights into the development of novel antimicrobial agents.

The antimicrobial activity of the *Bthepc* mature peptide was verified by the disc diffusion method. Previous studies demonstrated that the antimicrobial activity of synthetic hepcidin peptides is higher than that of the recombinant *rHtHep* [42]. Our present investigation strongly suggest that the *Bthepc* peptide efficiently inhibit the growth of the studied gram-negative bacteria, gram-positive bacteria, and fungus *in vitro*, while showing moderate haemolytic activity to mammalian erythrocytes.

One of the antimicrobial activities of AMPs is to disrupt the cell membrane [43,44], which is the main reason why it overcomes the problem of drug resistance. Through our SEM observations, the outer cell membrane of major pathogens treated with the *Bthepc* peptide exhibited wrinkled morphology, indicating the disruption of the cell membrane to release the cytoplasmic content leading to cell death.

5. Conclusions

In the present study, cDNA sequence of brown trout hepcidin gene was cloned. The protein structure and function was conserved across the species. Expression profiling of *Bthepc* indicated its highest expression in the liver. The age-dependent expression of *Bthepc* showed its highest expression at 18 M. *Bthepc* mRNA showed different levels of increase when challenged with pathogens. The antimicrobial activity of synthetic *Bthepc* to typical pathogens was verified *in vitro*. In addition, *Bthepc* showed moderate haemolytic activity in mammalian erythrocytes. This study may contribute to control of bacterial diseases of brown trout with the application of AMPs.

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