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Short communication

Molecular characterization and expression analysis of cathepsin C in Chinese giant salamander (*Andrias davidianus*) after *Aeromonas hydrophila* infection

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ABSTRACT

Background: Cathepsin C (CTSC) (dipeptidyl peptidase I, DPPI), is a member of the papain superfamily of cysteine proteases and involves in a variety of host reactions. However, the information of CTST in Chinese giant salamander (*Andrias davidianus*), an amphibian species with important evolutionary position and economic values, remained unclear.
Results: The full-length salamander CTSC cDNA contained a 96 bp of 5′-UTR, a 1392 bp of ORF encoding 463 amino acids, and a 95 bp of 3′-UTR. The salamander CTSC possessed several sequence features similar to other reported CTSCs such as a signal peptide, a propeptide and a mature peptide. The active site triad of Cys, His and Asn were also found existing in salamander CTSC. Salamander CTSC mRNA was constitutively expressed in all the examined tissues with significantly variant expression level. The highest expression of CTSC was in intestine, followed with stomach, spleen, lung and brain. Following *Aeromonas hydrophila* infection for 12 h, salamander CTSC was significantly up-regulated in several tissues including lung, spleen, brain, kidney, heart, stomach and skin.
Conclusion: CTSC plays roles in the immune response to bacterial infection, which provided valuable information for further studying the functions of CTSC in salamander.

1. Introduction

Cathepsin C (CTSC), also known as dipeptidyl peptidase I (DPPI), is a member of the papain superfamily of cysteine proteases [1], which is synthesized as an inactive precursor (zymogen), and is activated by a nonautocatalytic excision of an internal activation peptide within the N-terminal pro-peptide. The activated CTSC is consisted by "heavy" chain (231–394 amino acides) and "light chain" (395–463 amino acids) [2]. The activated CTSC may involve in a variety of host reactions, including intracellular protein degradation, cell growth, neuraminidase activation, and platelet factor XIII activation [3]. In addition, CTSC was found functioning as serine proteases in immune effector cells (mast cells, neutrophils, and lymphocytes) [4], and was essential for the interleukin (IL)-1-dependent sterile inflammatory response [5], indicating it may play roles in immune process.

Current studies on CTSC were mainly carried out in mammals, but were scarce in other animals. *Penaeus monodon* CTSC was up-regulated by lipopolysaccharide (LPS) [6], and *Fenneropenaeus chinensis* CTSC was up-regulated by *Vibrio anguillarum* and the white spot syndrome viruses (WSSVs) [7], implying that CTSC involves in immune defense against pathogens.

Chinese giant salamander is the largest extant urodela amphibian species. With the success of breeding of salamander, it has become an important economical aquatic species in central and western China. However, reports on the disease caused by pathogens in salamander, especially bacterial diseases, increased greatly in recent years. The bacterial diseases have become a hindrance to the healthy development of salamander industry [8]. Using immune strategies, e.g. developing vaccine for specific pathogen to treat or prevent the animal diseases has drawn great attention of researcher [9]. However, clearly understanding the animal immune system is a key condition for these strategies. Thus, in the present study, the salamander CTSC gene was cloned, and its expression in tissues of normal salamanders and *Aeromonas hydrophila* infected salamanders was analyzed,

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providing useful information for fully understanding the immune function of salamander CTSC in response to bacteria.

2. Materials and methods

2.1. Animal and bacterial infection

Normal Chinese giant salamanders (average body weight of 200 g) were purchased from a farm in Hubei province, China, and acclimated in aerated water tanks for one week before experiments. *A. hydrophila* (strain 4LNC209) was kindly provided by Professor Aihua Li (Institute of Hydrobiology, Chinese Academy of Sciences). Salamanders were randomly divided into two groups. One group was bacterial infected group, in which salamanders were injected intraperitoneally with *A. hydrophila* at a dose of 1.5×10^6 cfu/100 g body weight, and another group was control group, in which animals were injected with the same amount of PBS solution [8]. Ten tissues including liver, spleen, intestine, muscle, brain, stomach, kidney, lung, heart and skin from three animals in each group were sampled at 12 h post injection.

2.2. RNA extraction and reverse transcription

Total RNA of liver was isolated using Trizol reagent (Invitrogen, USA) according to manufacturer's instruction. Then, total RNA was reverse-transcribed using First Strand cDNA Synthesis Kit (Thermo Scientific, USA) based on manufacture's instruction.

2.3. Gene cloning of salamander CTSC

To obtain the salamander CTSC cDNA sequence, the liver transcriptome of salamander was searched by tBLASTn software using human CTSC (GenBank accession No. AAQ08887) as query bait. A sequence of 3215 bp in length was got, and this sequence was further analyzed using Translate at the ExPasy website (http://www.expasy.org/tools) to gain the potential open reading frame (ORF) and untranslated regions (UTRs). Then, specific primers were designed based on potential 5′-UTR and 3′-UTR, and polymerase chain reaction (PCR) amplification was done using liver cDNA as template. The PCR products were sequenced to confirm the correctness of sequences. Primers used in this study were listed in Table 1.

2.4. Sequence analysis

The amino acid (aa) of the nucleotide sequence was deduced using Translate at the ExPasy website (http://www.expasy.org/tools). The multiple alignments of aa sequences were done using Clustal O software (http://www.ebi.ac.uk/tools/msa/clustalo) and decorated with BoxShade software (http://www.ch.embnet.org/software/BOX_form.html). Protein sequence identity was calculated by MatGAT 2.02 software [10]. Isoelectric points and molecular weights were predicted with the ProtParam program (http://web.expasy.org/protparam). The signal peptide was predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP). The protein motif was identified with PROSITE database (http://prosite.expasy.org/scanprosite/). Phylogenetic tree was constructed using Neighbor-Joining (N-J) method by Mega 7.0 software and the bootstrap was set as 10,000 to test the confidence of branch topology [11]. 3-D structure of CTSC was predicted by SWISS-MODEL workspace (https://swissmodel.expasy.org/) and the quality of structure was evaluated with PROCHECK program (http://www.ebi.ac.uk/thornton-srv/software/PROCHECK/).

Table 1
Primers for gene clone and expression analysis.

Primer	Sequence (5′-3′)	Application
adCTSC-F1	CATGTGACCTTCTGGAATCCAT	Gene clone
adCTSC-R1	GCATTGAAAATGTCAGAGTACATGG	Gene clone
adCTSC-F2	GTGGCCGCAACTCCCATATT	Gene expression
adCTSC-R2	GCCACAGGATGCTTGGTTTCG	Gene expression
adActin-F	CCACTGCTGCCTCCTCTT	Gene expression
adActin-R	GCAATGCCTGGGTACATG	Gene expression

2.5. Expression of CTSC in tissues of normal and A. hydrophila infected salamander

Expressions of CTSC in tissues of normal and *A. hydrophila* infected salamanders were detected using real-time quantitative PCR. Total RNA of each tissue mentioned above was extracted using Trizol reagent (Invitrogen, USA) according to manufacturer's instruction and then was reverse-transcribed using PrimeScript® reagent kit with gDNA eraser (TaKaRa, Japan) according to manufacturer's protocol. Real-time qPCR was performed using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA) with SYBR Premix Ex Taq™ (TaKaRa, Japan) according to our previous studies [12,13]. Briefly, the cDNA fragments of CTSC and *β-actin* were amplified by RT-PCR. The amplicons in same equal molar amounts were serially ten-fold diluted and run along with the cDNA test samples on the same 96-well PCR plate as quantitative reference. The expression of CTSC in each tissue of normal salamander was normalized to the expression level of β-actin and expressed ad arbitrary units [12,13]. The expression change of CTSC in tissues of *A. hydrophila* infected salamanders was expressed as fold change according to our previous studies [12,13].

3. Results

3.1. Sequence analysis of salamander CTSC

The salamander CTSC cDNA was 1583 bp in length, with a 96 bp of 5′-UTR, a 1392 bp of ORF encoding 463 amino acids, and a 95 bp of 3′-UTR (Fig. 1). The predicted molecular mass and theoretical isoelectric point of salamander CTSC was 52.55 kDa and 5.8, respectively. The deduced amino acid of salamander CTSC shared high sequence identity with that of human (69.8%), mouse (69.0%), turtle (74.0%) and zebrafish (66.2%). Sequence alignment revealed that there existed a 23 aa of signal peptide, a 207 aa of long propeptide region (position of 24–230 aa), and a 233 aa of mature peptide region (position of 231–463 aa) in salamander CTSC (Fig. 2). The mature protein was consisted of a 164 aa of heave chain (position 231–394 aa) and a 69 aa of light chain (position of 395–463 aa), among which contained three conserved catalytic active sites (Cys^{258}, His^{405} and Asn^{427}) (Fig. 2). In addition, three potential N-glycosylation sites were found in salamander CTSC, with two in the proregion (at position 28 and 55) and one in the mature peptide (at position 276).

Further, phylogenetic tree analysis showed that vertebrates' CTSC were clustered into one clade, which was separated clearly from clade of CTSK, CTSS, CTSL, CTSH, CTSA, CTSD and CTSB. In the CTSC clade, salamander CTSC showed close relationship with turtle CTSC, which was in line with the results of sequence identity analysis. In each clade of the phylogenetic tree, the position of each sequence was in agreement with that of traditional taxonomy (Fig. 3).

3.2. Gene synteny analysis of vertebrates' CTSC loci

The gene synteny of vertebrates' CTSC loci was analyzed using Genomicus (v85.01) software. Results showed that the CTSC loci were highly conserved in vertebrates. The conserved genes near CTSC were almost found in all vertebrates, such as RAB138, GRM5, and TRY. In addition, the transcriptional directions of genes in this loci were compatible (Fig. 4). However, the gene linked with CTSC was different in lamprey (Fig. 4).

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catgtgaccttctggaatccatctgtataccgggaaagcgtttctaatcttcgtttgcccgtcccagctttgacc
ggattgcaacttctgctggccATGGCTCTGCGCCTTTCCCTCTTTCTGCTGTCGGCGCTGCTGATGTGCGGGGTG
                      M  A  L  R  L  S  L  F  L  L  S  A  L  L  M  C  G  V
CGCCTCGCCCGGGCGGACACTCCGGCCAACTGCAGCTTCCAAGACCTGGAGGGCACCTGGCTCTTTAGCGTTTGG
R  L  A  R  A  D  T  P  A  N  C  S  F  Q  D  L  E  G  T  W  L  F  S  V  W
AGGGGGCCCGGTGACGCCCAGAACCGGGACATCAACTGCTCCAGCCTGGGTCCTGTGGAAAACAAATTCACTGTC
R  G  P  G  D  A  Q  N  R  D  I  N  C  S  S  L  G  P  V  E  N  K  F  T  V
CATCTTCAGAAGCTTCACTTGGCTCAAGATGACATGGGGAATTCTGGTTTCTTCACTTTAATTTACAACCAAGGT
H  L  Q  K  L  H  L  A  Q  D  D  M  G  N  S  G  F  F  T  L  I  Y  N  Q  G
TTTGAAGTTGTAATTAATGACTACAAGTGGTTTGCATTTTTTAAGTATGAAGTACATGGCCAAAATGTGACCAGT
F  E  V  V  I  N  D  Y  K  W  F  A  F  F  K  Y  E  V  H  G  Q  N  V  T  S
TACTGCCATGAGACTCTTCCTGGGTGGGTCCATGATGTGTTGGGCCAGAACTGGGCCTGCTTTGTTGGGAAAAAA
Y  C  H  E  T  L  P  G  W  V  H  D  V  L  G  Q  N  W  A  C  F  V  G  K  K
GTATCATCTGCACCCTCCATTGTGAACACAGTGCGGTCTTATGAACATTGGGACCGATTTTCCAAGAGACGCTAC
V  S  S  A  P  S  I  V  N  T  V  R  S  Y  E  H  W  D  R  F  S  K  R  R  Y
ATGTACAACCCTGACTTTGTTGATGCTATTAACTCGGTTCAGAAATCTTGGAAAGCAACTACCTATGAAGAATAT
M  Y  N  P  D  F  V  D  A  I  N  S  V  Q  K  S  W  K  A  T  T  Y  E  E  Y
GAAATGCTCACCCTCGGAGAACTGCTTCAAAGGGCTGGTGGCCGCAACTCCCATATTCCCAGACAGCCAAAGCCT
E  M  L  T  L  G  E  L  L  Q  R  A  G  G  R  N  S  H  I  P  R  Q  P  K  P
GCTCCATTGGCAGAAGGGTCTATTGGAGTCTCAGGTCTTCCAGAGTCCTGGGATTGGAGAAATGTTAACGGAGTC
A  P  L  A  E  G  S  I  G  V  S  G  L  P  E  S  W  D  W  R  N  V  N  G  V
AACTATGTCAGTCCTGTTCGAAACCAAGCATCCTGTGGCAGTTGTTATTCATTTGCCACGATGGGCATGTTGGAA
N  Y  V  S  P  V  R  N  Q  A  S  C  G  S  C  Y  S  F  A  T  M  G  M  L  E
GCTAGAATTCGGATCCGGACGAACAATTCCCAGACGCCTATTCTCAGCCCTCAACAGGTTGTATCCTGTAGTGAG
A  R  I  R  I  R  T  N  N  S  Q  T  P  I  L  S  P  Q  Q  V  V  S  C  S  E
TACGCTCAAGGGTGTGATGGAGGATTTCCATACCTTATCGCAGGAAAATACATTCAAGATTTTGGAATCGTTGAG
Y  A  Q  G  C  D  G  G  F  P  Y  L  I  A  G  K  Y  I  Q  D  F  G  I  V  E
GAGGAATGTTTCCCTTACATTGGCACAGATTCTCCATGCACACTTAAAGAAGACTGTTACCGGTACTATACCTCA
E  E  C  F  P  Y  I  G  T  D  S  P  C  T  L  K  E  D  C  Y  R  Y  Y  T  S
GAGTACCATTATGTTGGAGGGTTTTATGGGGGTTGCAATGAAGCACTGATGAAATATGAACTTGTCAAACATGGG
E  Y  H  Y  V  G  G  F  Y  G  G  C  N  E  A  L  M  K  Y  E  L  V  K  H  G
CCTTTGGCTGTTGCTTTTGAAGTTTATGATGACTTCTTACATTATAGAGAAGGAATCTATCACCATACTGGACTG
P  L  A  V  A  F  E  V  Y  D  D  F  L  H  Y  R  E  G  I  Y  H  H  T  G  L
CAGGATCGTTTTAACCCATTTGAGCTGACAAATCATGCAGTTCTGCTTGTAGGATATGACCGTGATCACACAACA
Q  D  R  F  N  P  F  E  L  T  N  H  A  V  L  L  V  G  Y  D  R  D  H  T  T
GGGGAAAATTATTGGGTGATAAAAAATAGCTGGGGCTCTTCATGGGGTGAATATGGTTATTTCAGAATCCGCAGA
G  E  N  Y  W  V  I  K  N  S  W  G  S  S  W  G  E  Y  G  Y  F  R  I  R  R
GGTACTGATGAGTGTGCAGTTGAAAGCATAGCTGTGGCTGCAACACCTATACCAAAACTATAAatctgggcatgc
G  T  D  E  C  A  V  E  S  I  A  V  A  A  T  P  I  P  K  L  *
agagctctggaggcctcattttcagaagcagtcaataacattgtcactattaaaattaccatgatctcagactaa
aacaatgc
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Fig. 1. Nucleotide and deduced amino acid sequences of salamander CTSC. The start code and stop code was boxed. The stop code in the 3′-UCT was double underlined.

3.3. 3-D structure of salamander CTSC

The 3-D structure of salamander CTSC was constructed by comparative protein modeling method by SWISS-MODEL software using human CTSC structure (PDB No. 3PDF) as template. Similar to human CTSC structure (Fig. 5C), salamander CTSC possessed classical structure of papain superfamily, which was consisted of exclusion domain at N-terminal (D^{24}-G^{230}) (Fig. 5A) and papain-like

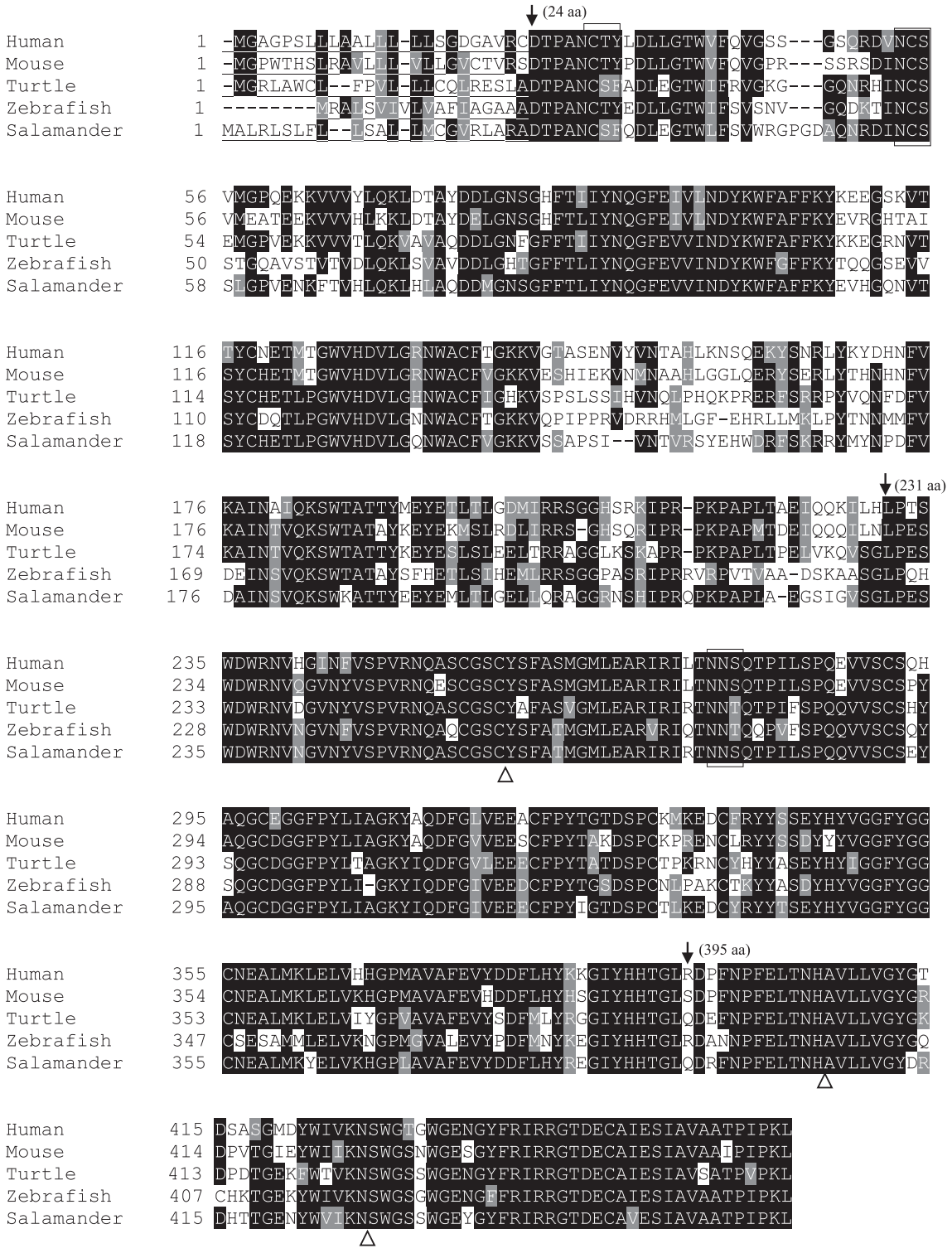

Fig. 2. Multiple alignments of CTSC. The multiple alignment was produced using ClustalO, and conserved amino acids shaded using BoxShade software. The signal peptide was underlined. Three catalytic active sites (Cys ^{258}, His^{405} and Asn^{427}) was marked by white triangles under the sequences. The arrows indicate the cleavage sites of signal peptide, propeptide, the heavy chain and light chain of the mature peptide.

structure at C-terminal (L^{231}-L^{463}) (Fig. 5B) [14]. Three catalytic active sites (Cys^{258}, His^{405} and Asn^{427}) at papain-like structure distributed on the surface of CTSC and closed to each other in the space (Fig. 5).

3.4. Tissue distribution of salamander CTSC transcripts

The tissue expression of CTSC in normal salamanders was analyzed using real-time qPCR. Results showed that salamander CTSC mRNA

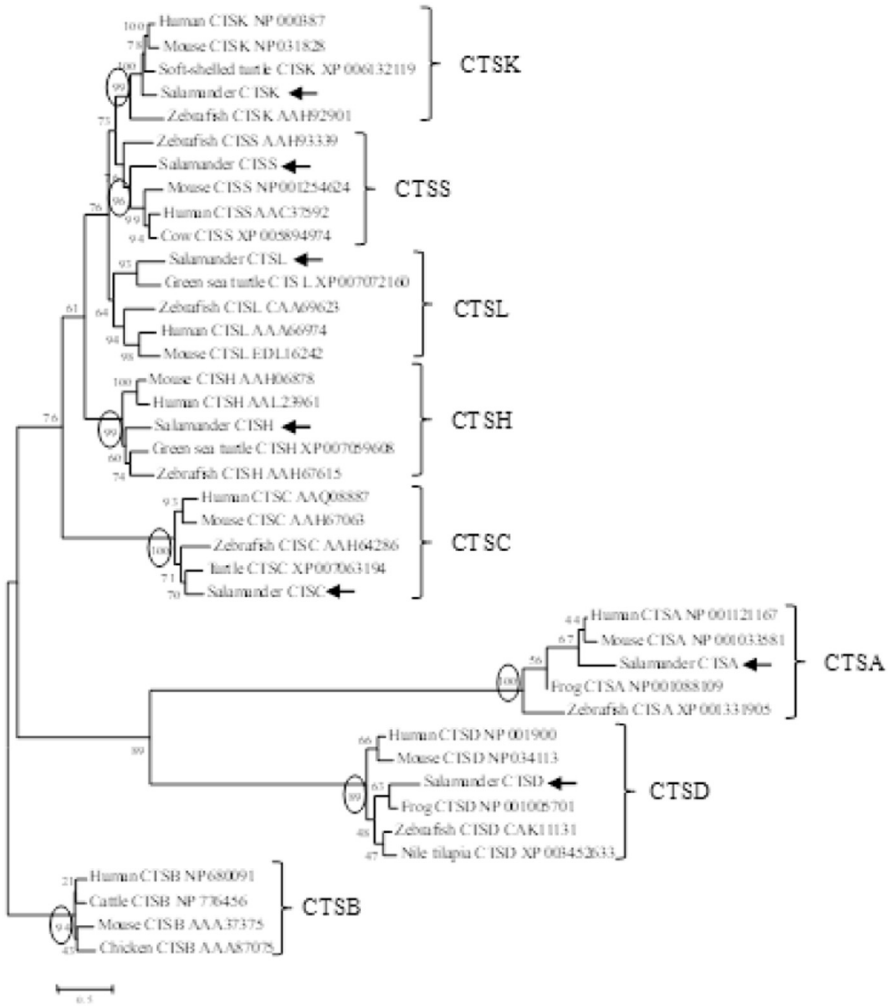

Fig. 3. Phylogenetic tree analysis of vertebrates' cathepsin genes. The tree was constructed using amino acid multiple alignments and the neighbor-joining method within the MEGA7.0 program. Node values represented percent bootstrap confidence derived from 10,000 replicates. The evolutionary distances were computed using the JTT matrix-based method. The accession number for each sequence was given after the molecular type and species name.

was constitutively expressed in all the ten examined tissues with significantly variant expression level. The highest expression of CTSC was in intestine, followed with stomach, spleen, lung and brain. There was a low-level expression of CTSC in liver, heart, muscle, kidney and skin (Fig. 6).

3.5. Expression changes of salamander CTSC after A. hydrophila infection

Following *A.hydrophila* infection for 12 h, the ten tissues mentioned above were selected and real-time qPCR was employed to check the expression changes of CTSC. As shown in Fig. 7, salamander CTSC was significantly up-regulated in lung, spleen, brain, kidney, heart, stomach and skin ($P < 0.05$), but remained no change in liver, intestine, muscle ($P > 0.05$).

4. Discussion

In this study, the cathepsin C gene was cloned from Chinese giant salamander (*Andrias davidianus*), an amphibian species with important evolutionary position and economic values [8]. Sequence analysis revealed that salamander CTSC possessed several conserved sequence features, similar to other reported CTSCs [2, 15]. Salamander CTSC contained three functional domains: a signal peptide, a propeptide and a mature peptide. The mature peptide was consisted of heavy chain and light chain. Three conserved catalytic active sites (Cys^{258}, His^{405} and Asn^{427}) were found in salamander CTSC and other vertebrates' CTSCs [16]. Further, in the 3-D structure of salamander CTSC, these three active residues were distributed on the surface of monomer, which made it easy to bind to and hydrolyze the substrate [17]. In addition, three potential N-glycosylation sites were found in salamander CTSC, with conserved positions and sequences as in other vertebrates' CTSCs, revealing that these sites might be glycosylated serving as recognition signals for targeting the enzyme to lysosomes [15]. Moreover, gene synteny analysis revealed that the genes linked CTSC were highly consistent, revealing that CTSC was highly conserved during evolution.

It was speculated that cysteine cathepsins were evolved from a common ancestor gene by gene duplication events [18]. Our phylogenetic tree analysis showed that salamander CTSC and other CTSCs were clustered together into one clade, which was separated from clades of other cysteine cathepsins, revealing that the relationship between CTSC and other cysteine cathepsins was far.

As that in mammalian species, salamander CTSC was found to be constitutively expressed in all the ten examined tissues, revealing that CTSC might function in a broad of tissues. However, the expression level of salamander CTSC in each tissue was different. Salamander

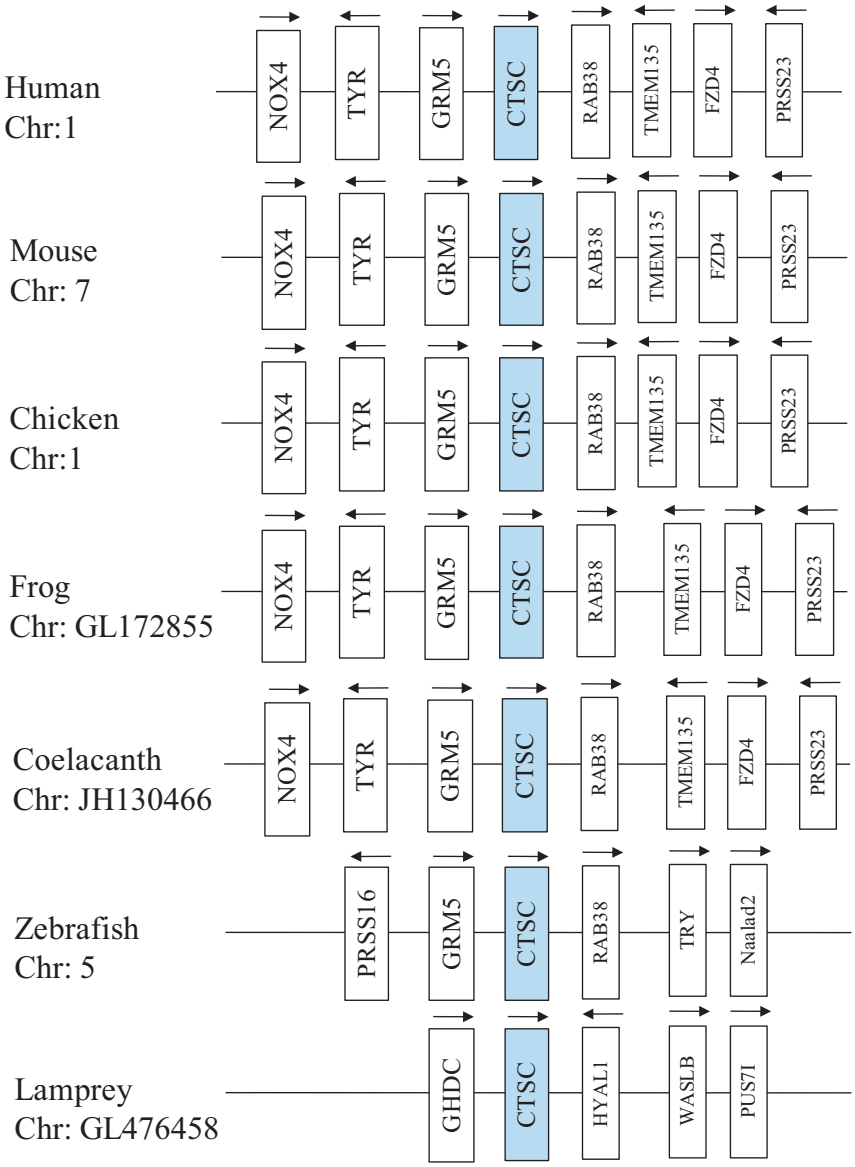

Fig. 4. Gene synteny analysis of vertebrates' CTSC loci. The transcriptional direction of each gene was marked by arrow up the box.

CTSC was highly expressed in intestine, stomach, spleen and lung. Similar case was also found for that of mammalian CTSC. Human CTSC was expressed mainly in lung, kidney [19,20], while mouse CTSC was abundant in the liver [15]. These results implied that the expression pattern of CTSC might be species-specific. Intestine was important mucosal immune related organ of amphibian, with many immune genes expressed in it [21,22]. Highly expression of salamander CTSC in intestine suggested that CTSC might play role in the mucosal immune response.

To gain the immune function of salamander CTSC, we further examined the expression changes of CTSC following *A. hydrophila* infection for 12 h. Salamander CTSC was significantly up-regulated in lung, spleen, brain, kidney, heart, stomach and skin ($P < 0.05$), indicating that CTSC was exactly involved in the immune response of bacterial infection, potentially serving as inducible acute-phase protein. Similar results were also observed in other animals. Black tiger shrimp CTSC was up-regulated by LPS stimulation and reached the maximum level at 4 h post-stimulation [6]. Rozor clam (*Sinonovacula constricta*) CTSC was significantly up-regulated in digestive gland, mantle, and gill tissues [19]. Mammalian CTSC was found existing in mast cells, neutrophils, cytotoxic T lymphocytes and natural killer (NK) cells also confirmed its role in immune progress [4,23].

In conclusion, the gene of cathepsin C was cloned and its sequence features were characterized in Chinese giant salamander. Salamander CTSC was constitutively expressed in all examined tissues with significantly variant expression level. Following *A. hydrophila* infection for 12 h, salamander CTSC was up-regulated in some tissues, indicating CTSC might play immune functions in response to bacterial infection. These results provided valuable basis for studying the functions of CTSC in salamander.

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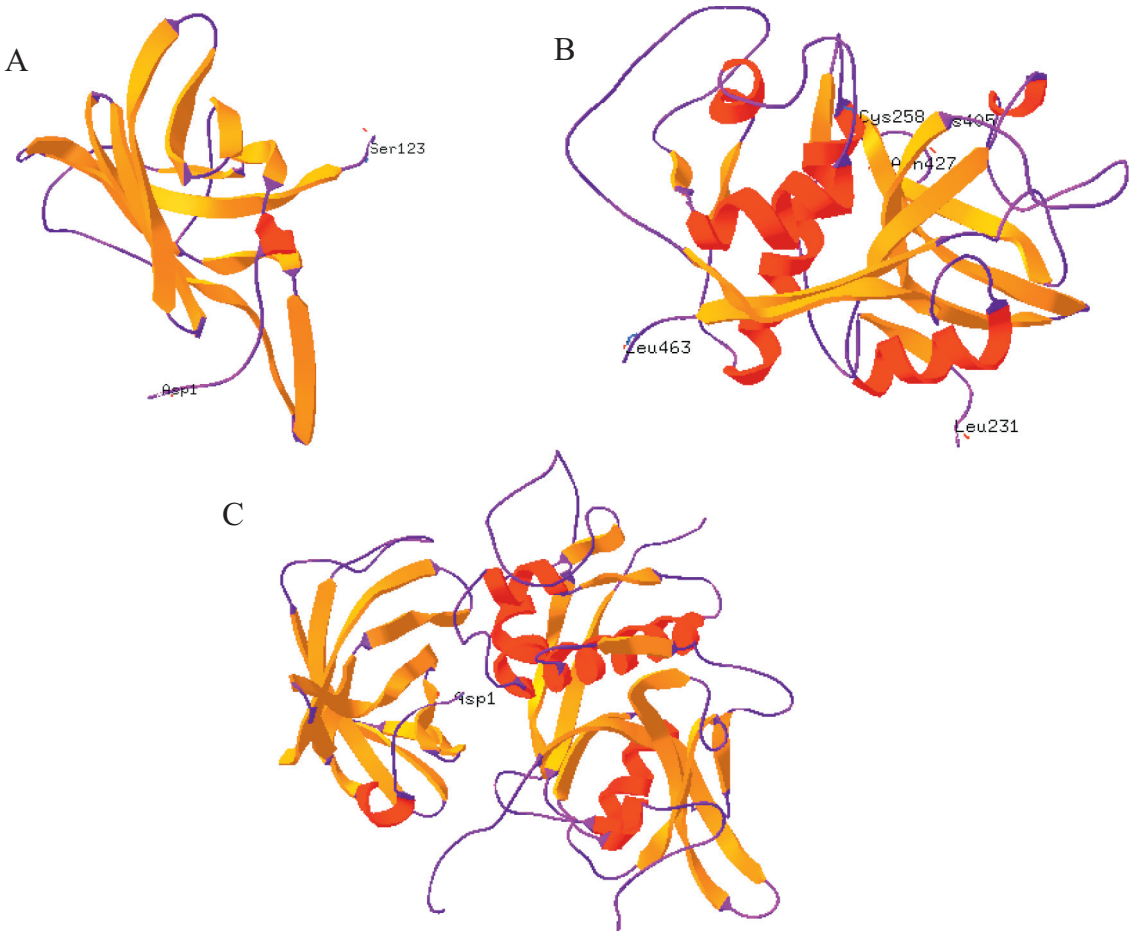

Fig. 5. Comparative analysis of 3-D structure of salamander CTSC (A and B) and human CTSC. The structure of salamander CTSC was constructed by comparative protein modeling method by SWISS-MODEL software using human CTSC structure (PDB No. 3PDF) as template. (A) Exclusion domain at N-terminal (D^{24}-G^{230}) of salamander CTSC; (B) papain-like structure (L^{231}-L^{463}) of salamander CTSC; (B) human CTSC structure.

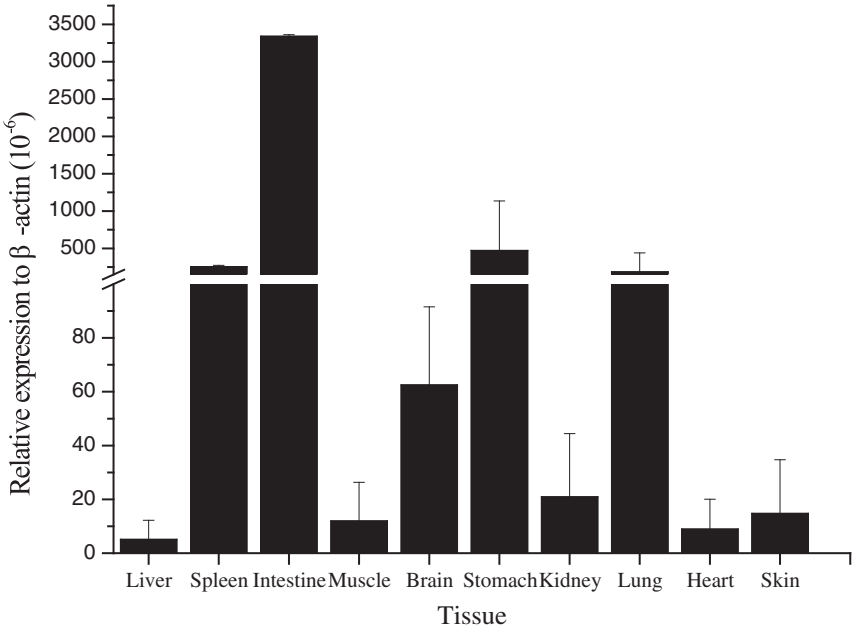

Fig. 6. Tissue expression of CTSC in normal salamander. Expression of salamander CTSC was respectively measured by real-time qPCR and normalized to that of β-actin in each tissue.

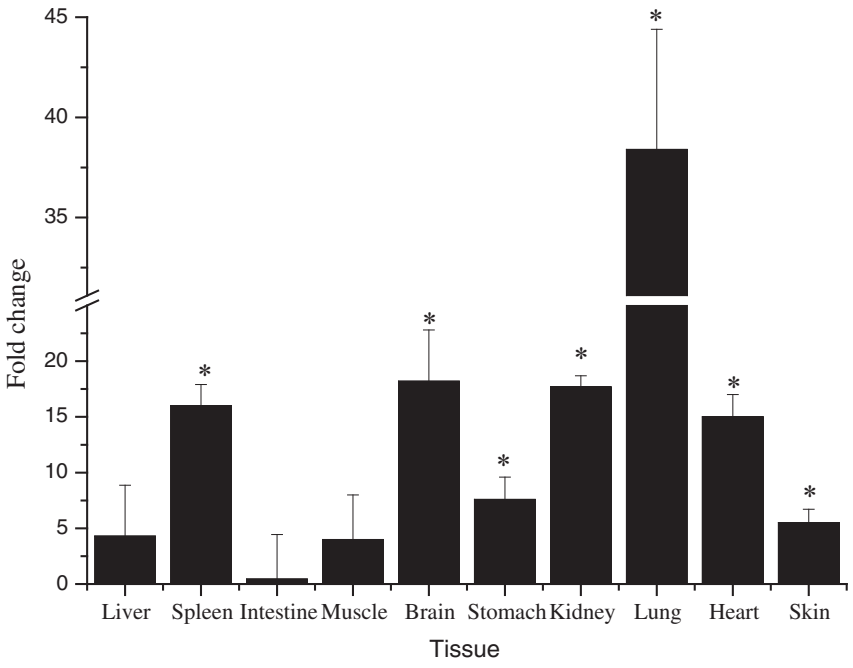

Fig. 7. Tissues expression of salamander CTSC at 12 h post *A. hydrophila* infection. Salamanders in the bacterial infected group were injected intraperitoneally with *A. hydrophila* at a dose of 1.5×10^6 cfu/100 g body weight. Control animals were injected with same amount of PBS solution. Ten tissues from three animals in each group were sampled at 12 h post injection. The expression change of salamander CTSC in each tissue was expressed as fold changes according to previous study [8]. $^*P < 0.05$.

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Conflict of interest

The authors declare no conflict of interest.

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