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Molecular characterization and expressing analysis of the c-type and g-type lysozymes in Qihe crucian carp *Carassius auratus*

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ABSTRACT

Lysozyme as an important nonspecific immune factor, can kill bacteria by hydrolyzing β-1,4-glycosidic linkages of peptidoglycan layer, and plays an important role in innate immune response against pathogen infection. In the present study, we report molecular cloning, tissue distribution and functional characterization of the c-type and g-type lysozymes in Qihe crucian carp *Carassius auratus* (designated as *Ca-clys* and *Ca-glys*, respectively). The full-length of *Ca-clys* and *Ca-glys* cDNA were cloned using RT-PCR and RACE methods. Catalytic and other conserved residues, required for functionality, were identified by multiple sequence alignment and structure predicted. The findings indicating the Ca-clys with signal peptide sequence, while the Ca-glys without, imply that the two isozymes function in different sites of cell. Phylogenetic analysis revealed that *Ca-clys* and *Ca-glys* genes evolve at different rates. Moreover, spatial expression analysis showed that *Ca-clys* transcript was most abundant in kidney and least in gill. However, the expression level of *Ca-glys* was significantly lower compared with *Ca-clys* in various tissues, which was the most abundant in spleen and least in brain. After intraperitoneal injection with *A. hydrophila* and lipopolysaccharide (LPS), the mRNA levels of *Ca-clys* and *Ca-glys* were generally up-regulated in liver and gill, but indicated the different expression changes in spleen, kidney and head kidney. With regard to the lysozyme activity, it was showed that the total enzyme activities generally increased in liver, gill, spleen, and head kidney after stimulation. These results confirmed that both *Ca-clys* and *Ca-glys* play an important role in non-specific immunity after *A. hydrophila* invasion. In this study, it was speculated that expressions of Ca-clys and Ca-glys were regulated in different patterns against pathogens.

1. Introduction

Lysozyme, as an important immune factor in the innate immune system, can catalyze the hydrolysis of β-(1,4)-glycosidic between the N-acetyl glucosamine and N-acetyl muramic acid in the peptidoglycan layer of bacterial cell wall, causing bacterial cell lysis and protecting organisms against the invasion of bacterial pathogens [1]. Based on their structural, catalytic, and immunological difference, lysozymes can be categorized into six types: chicken-type (c-type), goose-type (g-type), invertebrate-type (i-type), plant-type, bacterial-type and T4 phage-type [2–4]. The c-type and g-type of lysozymes exist in the fish and other vertebrate [2]. It has been demonstrated that lysozyme functions mainly in innate immunity against the invasion of bacterial pathogen [5,6]. Apart from their antimicrobial activity, lysozymes have also been reported to perform many other functions, such as digestion [7], complement together [8], antiviral [9], antitumor [10] and anti-inflammatory [11]. Therefore, lysozyme plays an important role in innate immunity and physiological activities.

The c-type lysozyme in fish was first reported in *rainbow trout* [12], and then founded in *Scophthalmus rhombus* [13], *Solea senegalensis* [14], *Epinephelus coioides* [15] and so on. Generally, the c-type lysozyme contains two conserved catalytic sites (Glu and Asp), conserved cysteine residues, forming disulfide bonds, and a N-terminal signal peptide, indicating to be a secret protein. In natural, most organisms only have one c-type lysozyme type. However, during the evolution, various c-type lysozymes had differentiated

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after gene replication and chromosome doubling in some organisms. There were three kinds of c-type lysozymes in *Oreochromis aureus*, and their three-dimensional structures were similar, but the expression and function were different [16]. In rainbow trout, two kinds of c-type lysozymes (I and II type) were reported, with only one amino acid difference between them, but indicated the significant difference in the biological functions [12]. The two c-type lysozymes exist in the abalone (abLysC1 and abLysC2) with the homology of 81.5% [17].

The g-type lysozyme was discovered in various species of fish, such as *Oplegnathus fasciatus* [18], *Scophthalmus maximus* [19], *Epinephelus coioides* [20], and *Dicentrarchus labrax* [21], the molecule of which, without the signal peptide, was usually bigger than that of c-type [2]. It possessed three conserved catalytic sites (Glu, Asp, and Asp), lacked the disulfide bond and N-terminal signal peptide, which was supposed to be an intracellular protein. In *Gadus morhua*, there were two kinds of g-type lysozymes (codg1 and codg2), indicating the different transcription initiation sites [22]. In Ictalurus punctatus, three kinds of g-type lysozymes (Lyg, LygI1 and LygI2) were found, with the homology of 71%–83% among them, and indicated the different expression levels in various tissues. Therefore, it was predicted that the cooperation among them could enhance the immune ability [23]. Currently, in most fish, such as *Scophthalmus rhombus* [13], *Ictalurus punctatus* [23], and *Ctenopharyngodon idellus* [24], the lysozyme was found to exist in two forms: c-type and g-type.

Qihe crucian carp *Carassius auratus*, as the important commercial fish, are widely cultured in the northern region of Henan province. In recent years, the intensive aquaculture with the high density always results in the decrease of immune level and evenly incurs the occurrence of fish disease. Up to now, the immune defense is thought as one of the most efficient ways in preventing from the disease. The lysozyme, as an important innate immunity factor, was generally founded in skin mucus of fish. However, molecular structure and expressing analysis of the c-type and g-type lysozymes have been scarcely studied in freshwater fish. In this study, the c-type and g-type lysozymes in *C. auratus* (designated as *Ca-clys* and *Ca-glys*, respectively) were cloned by reverse transcript PCR (RT-PCR) and the rapid amplification of cDNA ends (RACE) methods, the expression changes was determined using the quantitative real time - PCR (qRT-PCR) method, and the lysozyme activity was measured using the turbidimetey method. In this study, the aim is to find the differences by the comparison of gene sequence and molecular structure between *Ca-clys* and *Ca-glys*, as well as the expression profile after stimulation with *A. hydrophila* and LPS respectively. Based on the expression responses of *Ca-clys* and *Ca-glys to pathogen*, the new insights were shed on the prevention from pathogen infection.

2. Material and methods

2.1. Fish and bacteria

Qihe crucian carp *Carassius auratus*, with the body weight of 50 ± 3 g, were obtained from the breeding farm in Hebi city Henan province, which were acclimated in aerated water at 23 ± 1.5 °C for two weeks before experiments.

2.2. RNA extraction and reverse transcription

Total RNA was extracted from the liver of *C. auratus* using RNAiso Plus (Takara, Dalian, China) according to the instruction of manufacture. The quality of RNA was evaluated by 1.5% agarose gel electrophoresis. The first-strand cDNA was synthesized using the liver total RNA with the HiFi-MMLV cDNA Kit (Takara, Dalian, China). The reaction system contains 2 μL total RNA, 4 μL dNTP Mix, 2 μL Primer Mix, 4 μL 5 × RT Buffer, 2 μL DTT, 1 μL HiFi-MMLV, and 5 μL RNase-Free Water in a final volume of 20 μL. The reaction condition was at 42 °C for 45 min and 85 °C for 5 min.

2.3. Full-length cDNA cloning and sequencing of Ca-clys and Ca-glys

The primers to amplify the intermediate fragment were designed according to the conserved sequences of c-type lysozymes from *Cyprinus carp* (Gene Bank ID: AB027305), *Ctenopharyngodon idellus* (Gene Bank ID: EU835654), *Danio rerio* (Gene Bank ID: BC114260), and *C. auratus* (Gene Bank ID: KJ703111), and g-type lysozyme from *C. carp* (Gene Bank ID: AB084624), *C. idellus* (Gene Bank ID: EU835653), and *D. rerio* (Gene Bank ID: NM_001002706). The forward and reverse primers for c-type lysozyme were named as the primer CF and CR respectively, and for the g-type lysozyme were the primer GF and GR (Table 1). The amplification was performed by the 2 × Taq Master Mix with an initial denaturation at 94 °C for 3 min, followed by 30 cycles of 30 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C, a final extension for 3 min at 72 °C. The products were analyzed on 1.5% agarose electrophoresis and purified by DNA gel extraction kit (Takara, Dalian, China). The purified DNA was cloned into the pMD-19T vector (Takara, Dalian, China) and then transformed into *Escherichia coli* DH5a for sequencing.

The 3′ and 5′ RACE primers were designed based on the intermediate sequence. The 3′ RACE primers, combining with the forward gene-specific primer C3′ F, G3′ F and the 3′ RACE kit reverse primer 3′ Oligo (T) (Table 1), were used to clone the 3′ end of c-type lysozyme (*Ca-clys*) and g-type lysozyme (*Ca-glys*), respectively. The PCR reaction was performed at 94 °C for 3 min, followed by 30 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 3 min. The PCR product was used as the template for the nested PCR. The *Ca-clys* and *Ca-glys* nested PCR were performed respectively with the gene-specific primers C3′ NF, G3′ NF and 3′ Olig (supplied by the kit). The PCR reacting conditions were same as the first round PCR. The expected DNA fragment was purified and cloned into pMD-19T vector, which was transferred into the bacteria DH5α, being cultured for sequencing. The 5′ RACE for *Ca-clys* and *Ca-glys* were performed using the designed 5′ RACE primers, and the gene-specific primer C5′ R, G5′ R and the 5′ RACE

Table 1
The primers used in this study.

Primer	Sequence (5′–3′)
CF	CGCTKTGATGTTGTYCGTAT
CR	CCAGGTRTCCCATGMTTTCA
GF	AAACTRGCTGAGCATGATCTGG
GR	TGGGCTGKGGCMACAACATC
C3′ F	GGGACTTGATGGCTTTGAGGGATTC
C3′ NF	CTCATTGTGAAAACCGAAGGACTG
G3′ F	GAAATGTCCTGAATAATGGATGGTC
G3′ NF	CAGGACCACTGGAAAAGACTACTC
3′ Oligo(T)	CTGATCTAGAGGTACCGGATCCTTTTTTTTTTTTTTTT
3′ Olig	CTGATCTAGAGGTACCGGATCC
C5′ R	GATTTCAGTCCTTCGGTTTTCACAA
C5′ NR	CCTGTGGGTCTTATACTTACTCTCC
G5′ R	CTGGCGACAACACCATTGGAGTAGT
G5′ NR	CTCTGGTCTCTCTGGATATGATGGC
5′ Oligo(T)-Adaptor	GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT
5′ -Adaptor	GACTCGAGTCGACATCG
qPCR-CF	GAGGGATTCTCACTTGGCAACTAT
qPCR-CR	TTCAGTCCTTCGGTTTTCACAATG
qPCR-GF	TGTGGTCCCAAGAGCAATG
qPCR-GR	TGGCAACAACATCATTGGAGTAGTC
β-actin F	TCACACCTTCTACAACGAGCTGCG
β-actin R	GAAGCTGTAGCTCTCTCGGTCAG

kit primer 5′ Oligo(T)-Adaptor, and the nested PCR was performed using the primer C5′ NR, G5′ NR and 5′-Adaptor (Table 1). The amplifying procedure for 5′ RACE was similar with the 3′ RACE. The full-length *Ca-clys* and *Ca-glys* genes were assembled respectively with the DNAMAN 5.2 software.

2.4. Bioinformatic analysis

The cDNA and the deduced amino acid sequences of Ca-clys and Ca-glys were analyzed, respectively, with the BioEdit software. The opening reading frame was predicted with the NCBI ORF Finder (www.ncbi.nlm.nih.gov/gorf/gorf.html). The isoelectric point and molecular weight were predicted by the EXPASY (http://www.expasy.org/). The signal peptide was predicted by the SignalP (http://www.cbs.dtu.dk/services/SignalP/). The protein structure and function region were analyzed by the software Phyre 2 and SPDBV 4.01. The multiple sequence alignment was performed using the software DNAMAN. Phylogenetic tree was constructed using the neighbor-joining method in the software MEGA 5.05, with 1000 bootstrap replications.

A

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   1  gatctgaactcctcttcagatattgagagtgattttgtgagtagcactgcagagATGAGG
   1                                                        M  R
  61  GTTGCTGTTGTTGTCTTGTGTCTGATGTGGCTGTGCGTGTGTGAGAGCCGCAGGCTGGGT
   3   V  A  V  V  V  L  C  L  M  W  L  C  V  C  E  S  R  R  L  G
 121  CGCTGTGATGTCGCCCGTATCTTCAAGCGAGAGGGACTTGATGGCTTTGAGGGATTCTCA
  23   R  C  D  V  A  R  I  F  K  R  E  G  L  D  G  F  E  G  F  S
 181  CTTGGCAACTATGTGTGCACGGCCTACTGGGAGAGTAAGTATAAGACCCACAGGGTGCGT
  43   L  G  N  Y  V  C  T  A  Y  W  E  S  K  Y  K  T  H  R  V  R
 241  TCAGCTGATGTTGGGAAAGACTATGGAATCTTCCAGATAAACAGTTTTAAATGGTGCGAT
  63   S  A  D  V  G  K  D  Y  G  I  F  Q  I  N  S  F  K  W  C  D
 301  GACGGCACTCCAGGTGGAAAGAACCAGTGCAAAATACCCTGTGCAGATTTGCTAAAGGAT
  83   D  G  T  P  G  G  K  N  Q  C  K  I  P  C  A  D  L  L  K  D
 361  GACCTGAAAGCTTCAGTTGAATGTGCAAAGCTCATTGTGAAAACCGAAGGACTGAAATCA
 103   D  L  K  A  S  V  E  C  A  K  L  I  V  K  T  E  G  L  K  S
 421  TGGGACACCTGGAGTAGTTACTGTAAGGGGCGTAAGATGACACGCTGGGTGAAAGGATGT
 123   W  D  T  W  S  S  Y  C  K  G  R  K  M  T  R  W  V  K  G  C
 481  GAGGAGCACTAAtaaggccttgtctggcaacaggagctttaattagctatcatgctttca
 143   E  E  H  *
 541  gactatgtgctactgcatttaaactcttggtttatatgacttttaaaaactaaaagacat
 601  tgtcattattaacatttgtaaccttgatcttgagatgtcaattaaaagttcttcccaatt
 661  gttaaaaaaaaaaaaaaaa
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B

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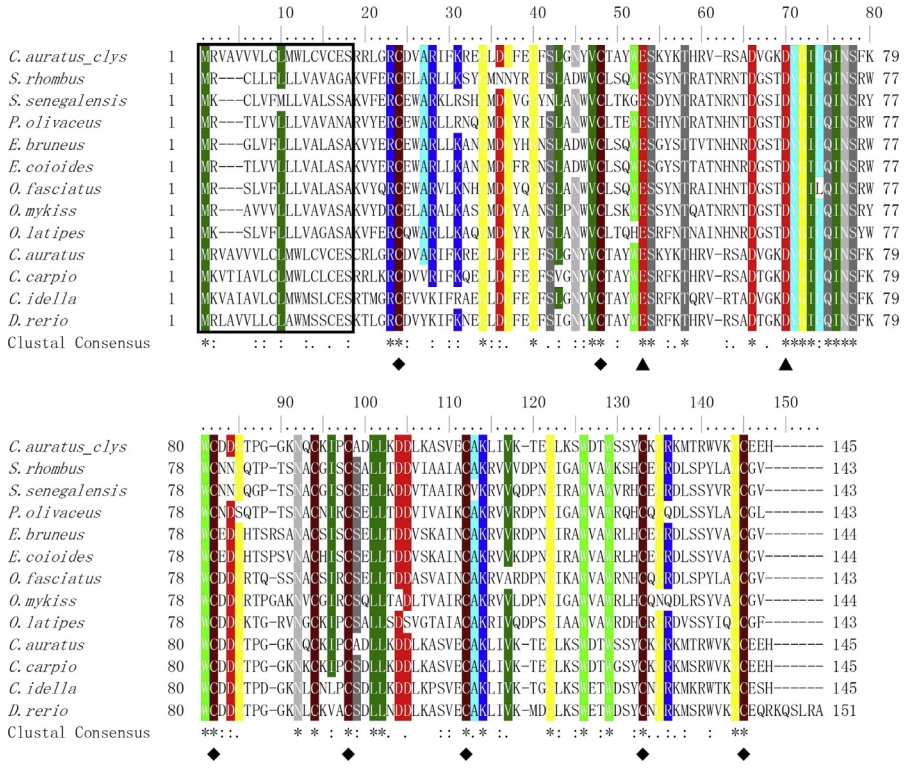
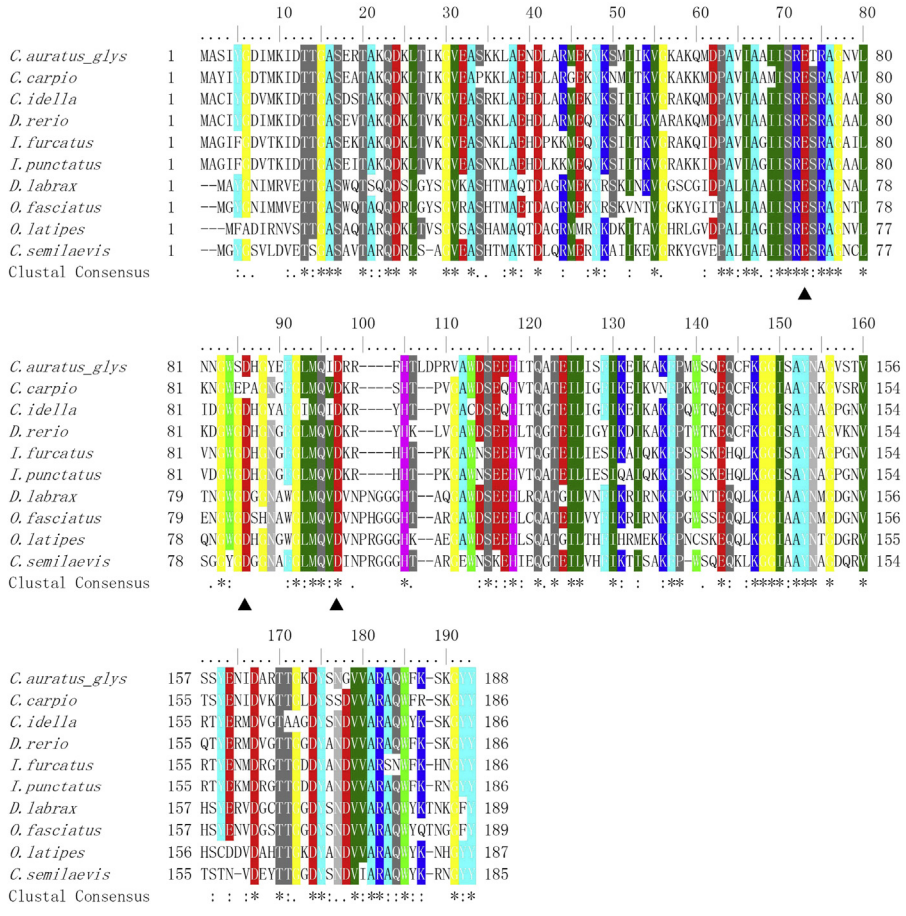
   1  gaaaagtaacaaggctcatcaaactctgtgatctgctctttcacagtagttctgcatcaac
  62  atcATGGCAAGCATCTATGGAGACATCATGAAAATAGACACCACCGGTGCATCAGAGAGG
   1      M  A  S  I  Y  G  D  I  M  K  I  D  T  T  G  A  S  E  R
 122  ACAGCAAAACAAGACAAATTAACCATAAAGGGAGTTGAAGCCTCTAAAAAACTGGCTGAG
  20   T  A  K  Q  D  K  L  T  I  K  G  V  E  A  S  K  K  L  A  E
 182  AATGATCTGGCCCGGATGGAGAAATACAAGAGTATGATCATCAAAGTTGGCAAAGCAAAG
  40   N  D  L  A  R  M  E  K  Y  K  S  M  I  I  K  V  G  K  A  K
 242  CAGATGGACCCGGCTGTGATTGCTGCCATCATATCCAGAGAGACCAGAGCCGGAAATGTC
  60   Q  M  D  P  A  V  I  A  A  I  I  S  R  E  T  R  A  G  N  V
 302  CTGAATAATGGATGGTCTGATCATGGCTATGAATTTGGCCTCATGCAGATTGACAGGCGC
  80   L  N  N  G  W  S  D  H  G  Y  E  F  G  L  M  Q  I  D  R  R
 362  TTCCACACTCTAGATCCACGCGTTGCATGGGACAGTGAGGAGCATATCACACAAGCTACA
 100   F  H  T  L  D  P  R  V  A  W  D  S  E  E  H  I  T  Q  A  T
 422  GAGATACTCATTAGCTTCATAAAAGAAATTAAGGCAAAATTTCCCATGTGGTCCCAAGAG
 120   E  I  L  I  S  F  I  K  E  I  K  A  K  F  P  M  W  S  Q  E
 482  CAATGCTTTAAAGGGGGAATATCAGCCTACAATGCAGGTGTGAGTACCGTCAGTTCATAT
 140   Q  C  F  K  G  G  I  S  A  Y  N  A  G  V  S  T  V  S  S  Y
 542  GAGAATATCGATGCCAGGACCACTGGAAAAGACTACTCCAATGGTGTTGTCGCCAGAGCC
 160   E  N  I  D  A  R  T  T  G  K  D  Y  S  N  G  V  V  A  R  A
 602  CAGTGGTTCAAAAGCAAGGGTTACTGAagaagaaaagccttaaattagtataaggagtag
 180   Q  W  F  K  S  K  G  Y  *
 662  gcctacaaccactcaagattataagtctttatttttaataaccattttaagagtttgtat
 722  tttcaaaatatgacaatattaatgcagtgaatttcctgtaatgatggtaataaaaatcat
 782  ctatttactcaaaaaaaaaaaaaaa
```

Fig. 1. The c-type (A) and g-type (B) lysozyme cDNA sequences and deduced amino acid sequences in *C. auratus*. The letters in box indicated the start codon (ATG) and "*" indicated the stop codon.

A


C. auratus_clys
S. rhombus
S. senegalensis
P. olivaceus
E. bruneus
E. coioides
O. fasciatus
O. mykiss
O. latipes
C. auratus
C. carpio
C. idella
D. rerio
Clustal Consensus

B


C. auratus_glys
C. carpio
C. idella
D. rerio
I. furcatus
I. punctatus
D. labrax
O. fasciatus
O. latipes
C. semilaevis
Clustal Consensus

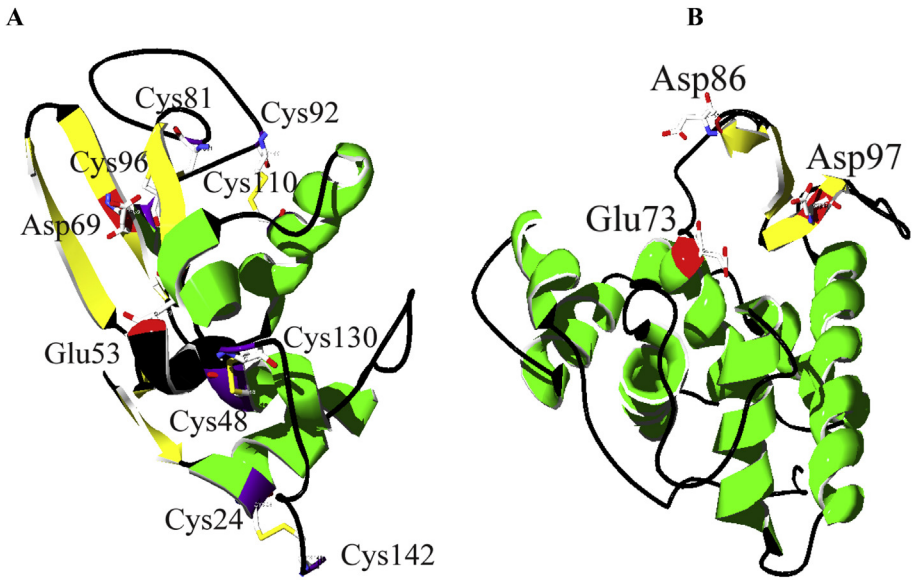


Fig. 3. Three-dimensional structures of c-type and g-type lysozymes *in C. auratus*. (A) c-type lysozyme, two enzymatic active sites are shown in red (Glu53, Asp69), eight cysteine residues are shown in purple, forming four pairs of disulfide bond. (B) g-type lysozyme, three enzymatic active sites are shown in red (Glu73, ASP86, Asp97). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.5. The expressing of the gene Ca-clys and Ca-glys in various tissues

The mRNA expression levels of *Ca-clys* and *Ca-glys* gene were determined in the various tissues (kidney, head kidney, intestine, heart, spleen, liver, muscle, brain, skin, and gill) of the health *C. auratus*, using the quantitative real-time PCR (qRT-PCR). The total RNA was extracted respectively according to the protocol described as above. Based on the obtained sequences in this study, the designed primer qPCR-CF and qPCR-CR were used to determine the expressing of *Ca-clys*, and qPCR-GF and qPCR-GR were used to determine the expressing of *Ca-glys*. The expression of reference gene *β-actin* was measured by the primer β-actin F and β-actin R (Table 1). The qRT-PCR reaction was performed in the ABI 7500 system with the Ultra SYBR Mixture (Takara, Dalian, China). A 20 μL reaction volume contained 10 μL of 2 × Ultra SYBR Mixture, 1 μL of each primer, 1 μL of diluted cDNA from the respective tissue and 7 μL RNase free ddH_2O. The amplifying program was at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. The relative gene expression level of the target gene to internal reference gene in the same tissue was analyzed using the $2^{-\Delta\Delta ct}$ method [25]. The quantitative real-time PCR analysis was performed in triplicates, and the values were given as mean ± SD (n = 3) in terms of relative mRNA expression, and all statistical analysis were implemented in the software SPSS.

2.6. Lysozyme activity assays in C. auratus

The skin mucous was scraped and collected by a clean microslide, and then homogenated with 0.75% sodium chloride, and centrifuged at 4 °C, at 1000 rpm, for 20 min, the supernatant was preserved at −20 °C. The blood was collected from caudal artery by a sterile syringe, and stratified for several hours, and the upper layer serum was collected for analysis. The health fish was scissored to sample the different tissues, which were homogenated with the 0.75% sodium chloride according to 1:9 (1 mg tissue: 9 mL buffer), and then centrifuged at 4 °C, at 1000 rpm for 20 min. The supernatant was used to determine the lysozyme activity. The bacterial suspension was configured as the following: the *micrococcus lysodeikticus* were dissolved with the 0.067 M phosphate saline buffer to the final concentration 0.2 mg/mL.

Lysozyme activity was determined with the turbidimetry method described by Xun et al. [26]. In brief, 0.1 mL serum or interstitial fluid was mixed respectively with 3 mL of bacterial suspension, and the initial OD_{570} designated as A1, was measured by spectrophotometer. The mixture was incubated in a water bath at 28 °C for 30 min, then transferred into the ice bath to stop the reaction, measured the OD_{570}, designated as A2. The content of protein, designated as P, was detected by the method of comassie blue staining [27]. The lysozyme activity was defined as one activity unit that the value of OD_{570} drop 0.001 per minute per mg tissue protein, at 28 °C, at pH 6.4. The lysozyme activity was calculated according to the formula of (A1-A2)/0.001/30/P.

2.7. The responses of lysozyme in fish challenged with A. hydrophilia and LPS

The bacterial pathogen *A. hydrophilia* were cultured in LB medium at 28 °C, then washed with 0.75% saline and resuspended in 0.75% saline to 5.35 × 10^7 CFU/mL. The experimental fish were

Fig. 2. Multiple alignment of the deduced amino acid sequences of the c-type (A) and g-type (B) lysozymes from Qihe crucial carp *C. auratus* and other species. (A) Alignment of the c-type lysozymes including c-type lysozyme amino acid sequences from other species: *C. auratus* (AID68653), *C. idella* (ACF41166), *C. carpio* (BAA95698), *D. rerio* (AAI14161), *E. bruneus* (AEB31354), *O. mykiss* (NP_001118188), *O. fasciatus* (ADZ44620), *O. latipes* (NP_001156629) and *P. olivaceus* (Q9DD65). (B) Alignment of the g-type lysozymes including g-type lysozyme amino acid sequences from other species: *C. idella* (ACF41165), *C. carpio* (BAB91437), *D. rerio* (XP_002664417), *D. labrax* (CBJ56263), *I. furcatus* (ADO28271), *I. punctatus* (NP_001187748) and *O. fasciatus* (Q8JFR1). Identical nucleotides are shaded in black background. Residues in red and green background indicate higher levels of amino acid similarity. The signal peptide is shown in black box, catalytic residues are shown in symbol of "▲", cysteines are shown in symbol of "◆". (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

grouped randomly, with 15 fish each group, and cultured in aerated tanks in triplication. With regard to the bacterial stimulation, each fish was injected intraperitoneally (i.p.) with 200 μL of *A. hydrophilia* at a dose of 5.35×10^7 CFU/mL. For the lipopolysaccharide (LPS) stimulation, each fish was injected with 200 μL of LPS (1 mg/mL) in 0.75% saline. In the control, each fish was injected with 200 μL of 0.75% physiological saline. Fish were randomly sampled from each group at 0, 3, 6, 12, 24 and 48 h after injection, and the tissue samples (liver, gill, spleen, kidney and head kidney) were collected immediately. Total RNA extraction, cDNA synthesis, qRT-PCR and lysozyme activity assay were performed as described above. The expression levels of *Ca-clys* and *Ca-glys*, in the differently challenged groups, were converted to fold changes relative to the control at the same time points. The data were expressed as means ± SD (n = 3). Statistical differences were analyzed using one-way ANOVA and Tukey multiple comparisons, implemented in software SPSS 20.0.

3. Result

3.1. Sequence analysis of Ca-clys and Ca-glys genes

Complete cDNA sequence of *Ca-clys* and *Ca-glys* genes were obtained by RT-PCR, 3′-RACE and 5′-RACE (Fig. 1). The full-length of *Ca-clys* cDNA contains a 54 bp 5′ -untranslated region (UTR), a 187 bp 3′ −UTR, and an open reading frame (ORF) of 438 bp encoding 145 amino acids with a predicted molecular mass of 1.66 kDa and a theoretical isoelectric point (*pI*) of 8.86. The deduced amino acid sequence of *Ca-clys* contains a putative signal peptide of 18 residues at the N-terminus. A multiple sequence alignment with other fish reveals that it contains two essential catalytic residues (Glu 53 and Asp 69), and the identified several important sequence signatures in *Ca-clys* (Fig. 2A). The predicted three-dimensional structure reveals eight conserved residues, which can form four disulfied bonds (Fig. 3A). Prediction by SignalP3.0 reveals that the deduced protein contains a signal peptide with 18 amino acids at N-terminus.

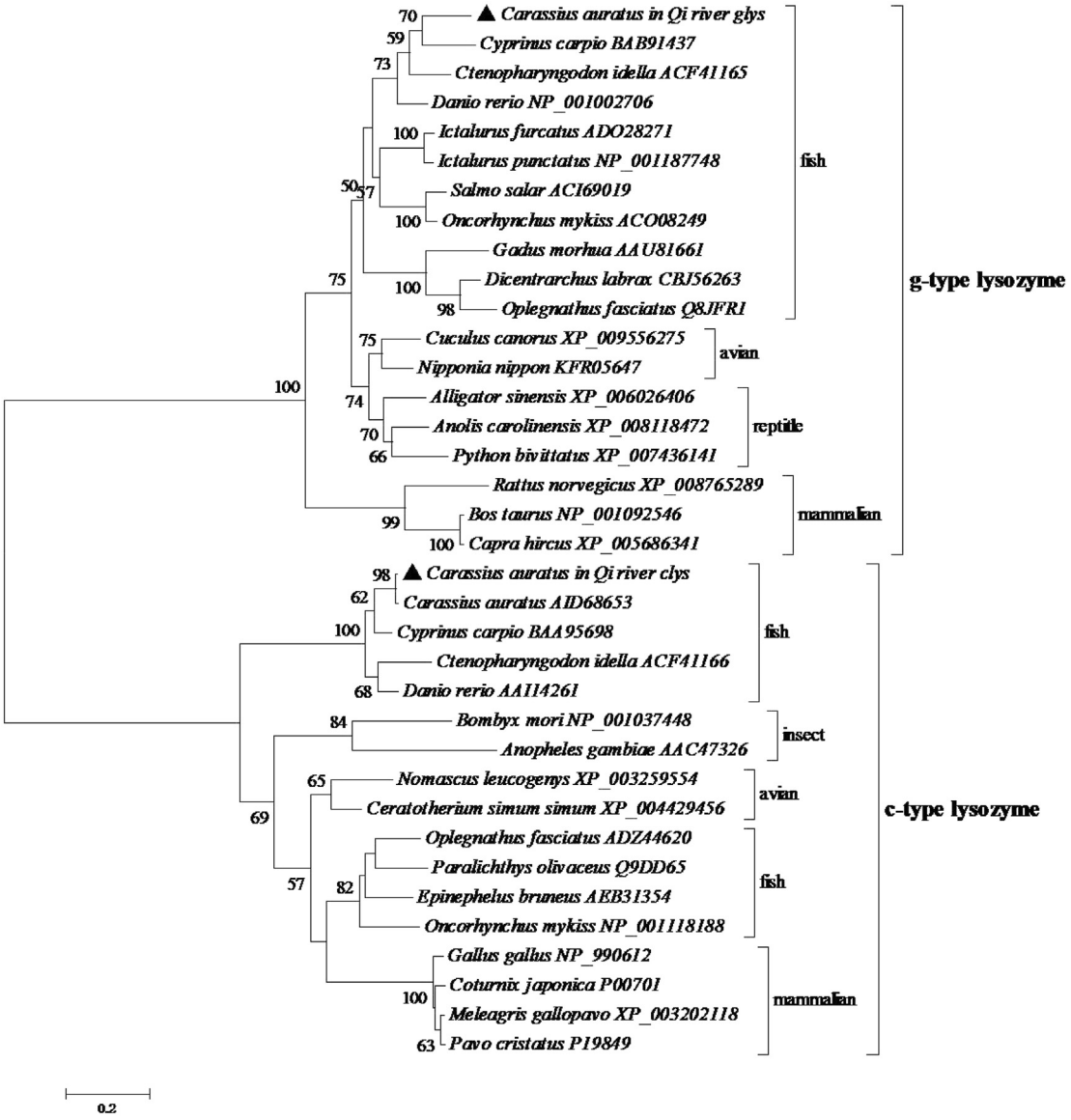


Fig. 4. The constructed NJ tree based on c-type and g-type lysozymes amino acid sequences deposited in GenBank in *C. auratus* and other organisms. Only bootstrap values of higher than 50% are indicated at the nodes. "▲" shows the c-type and g-type sequences in *C. auratus*.

The full length of *Ca-glys* cDNA is 806 bp, containing a 64 bp of 5′ -UTR, a 564 bp of ORF encoding 187 amino acids, and a 178 bp of 3′ -UTR. The full-length *Ca-glys* cDNA sequence is deposited in GenBank in Accession No. KM100713.1. The predicted molecular mass and *pI* were 2.09 kDa and 9.04, respectively. The multiple sequence alignment shows that it contains three conserved catalytic residues (Glu 73, Asp 86, and Asp 97) and a cysteine residue, but no disulfied bonds, and lack of the N-terminal signal peptide (Figs. 2B and 3B).

Based on Ca-clys and Ca-glys amino acid sequences in *C. auratus* and other organisms, the constructed phylogenetic tree (Fig. 4) revealed that Ca-clys and Ca-glys were respectively clustered into one branch. The c-type lysozymes in fish were divided into two clusters: one involved in the fish in Cypriniformes, and the another cluster contained other fishes. With regard to the Ca-glys, four main branches (fish, avian, reptitle and mammalian) have been clustered respectively, and *C. auratus* was the closest to *Cyprinus carpio*.

3.2. Expression levels of Ca-clys and Ca-glys and lysozyme activity in the tissues of C. auratus

Expression levels of *Ca-clys* and *Ca-glys* were detected in tissues (or organs) of *C. auratus*. The results showed that the highest expression of *Ca-clys* was found in kidney (316.7 fold higher than in the liver), followed by head kidney and intestine (188.6 and 11.9 fold higher than in the liver, respectively), the lowest expression levels were detected in the gill (4 fold lower than in the liver) (Fig. 5A).

In contrast, *Ca-glys* was expressed ubiquitously with no pronounced differences in transcript levels among tissues. Only in the spleen and intestine, mRNAs were 2.2 and 2.1 fold higher than in the liver, respectively. Lower expression levels were tested in muscle, kidney and brain (Fig. 5B). Generally, *Ca-glys* showed lower expression levels than *Ca-clys* in same tissue.

The lysozyme activity was detected in different tissues in *C. auratus* (Fig. 5C), which was the highest in the skin mucous, being significantly higher than in the other tissues ($P < 0.05$) and was the lowest in brain.

3.3. mRNA expressions after A. hydrophilia and LPS challenge

The changes of expression levels of gene *Ca-clys* and *Ca-glys* were analyzed in five tissues of fish challenged with *A. hydrophilia* and LPS using qRT-PCR method. The expression patterns of gene *Ca-clys* and *Ca-glys* were different in various tissues (Fig. 6). Expression level of *Ca-clys* was significantly up-regulated in liver after 6 h for the injections of LPS ($P < 0.01$), and after 3 h for the injection of *A. hydrophilia* ($P < 0.01$), compared to the control (Fig. 6, A). Expression levels increased significantly in gill of fish treated by *A. hydrophilia* after 3 h ($P < 0.01$), and by LPS only at 3 h, 6 h, and 12 h ($P < 0.01$) (Fig. 6, B). With regard to the *Ca-clys* in spleen, expression levels increased significantly at 3 h, 6 h, 12 h, and 24 h after challenged with LPS and *A. hydrophilia* ($P < 0.01$ or $P < 0.05$) (Fig. 6, C). However, the expression levels of gene *Ca-clys* were down-regulated or no change in kidney and head kidney after LPS and *A. hydrophilia* challenge (Fig. 6, D and E).

With regard to the expression level of *Ca-glys* in liver and gill, it showed the similar changes with *Ca-clys*, *Ca-glys* mRNA levels increased significantly in liver after 3 h after LPS or *A. hydrophilia* challenge ($P < 0.01$ or $P < 0.05$) (Fig. 6, F). In gill, *Ca-glys* levels significantly increased at 3 h–24 h after LPS challenge, and at 6 h–48 h after *A. hydrophilia* challenge ($P < 0.01$) (Fig. 6, G). While in spleen, *Ca-glys* transcripts showed decrease or no change after LPS and *A. hydrophilia* infection (Fig. 6, H). With regard to the *Ca-glys* in kidney, express levels were up-regulated significantly after 6 h with *A. hydrophilia* challenge, and only at 3 h and 6 h with LPS challenge ($P < 0.01$) (Fig. 6, I). For the *Ca-glys* in head kidney, significant up-regulations were observed after 3 h in response to *A. hydrophilia* and after 6 h in response to LPS (Fig. 6, J), which showed a difference with *Ca-clys*. These data indicate that two kinds of lysozyme might play the different roles against bacteria challenge.

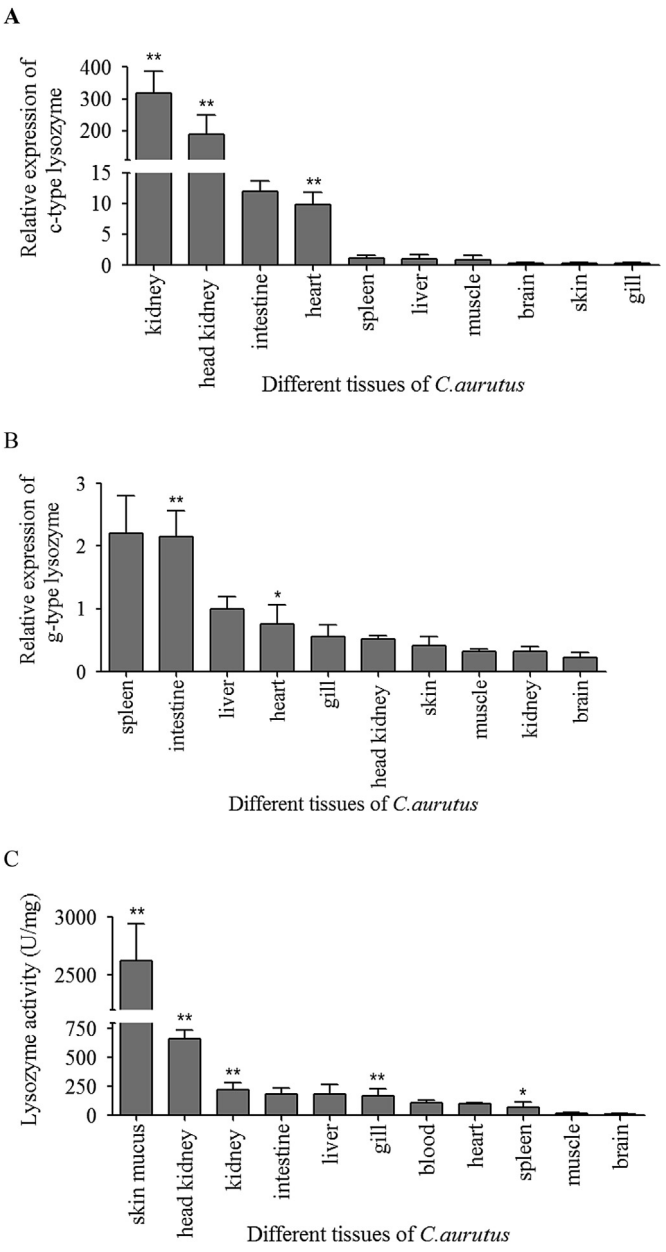


Fig. 5. Expressing level and activity of lysozyme in the different tissues of *C. aurutus*. The quantitative real-time PCR analysis was performed in triplicates, and the data were presented by mean + standard deviation (M + SD) (n = 3). The significant analysis was implemented in SPSS20.0 software, compared to the values in liver. (A) c-type lysozyme. (B) g-type lysozyme. (C) Lysozyme activity in eleven tissues. The significant difference was represented as "*" ($P < 0.05$) or "**" ($P < 0.01$).

3.4. Responses of lysozyme activity to A. hydrophilia and LPS challenge

The lysozyme activities were investigated in five tissues of *C. auratus* challenged by LPS and *A. hydrophilia*, in this study, which significantly increased after 3 h in liver, and after 6 h in gill, spleen

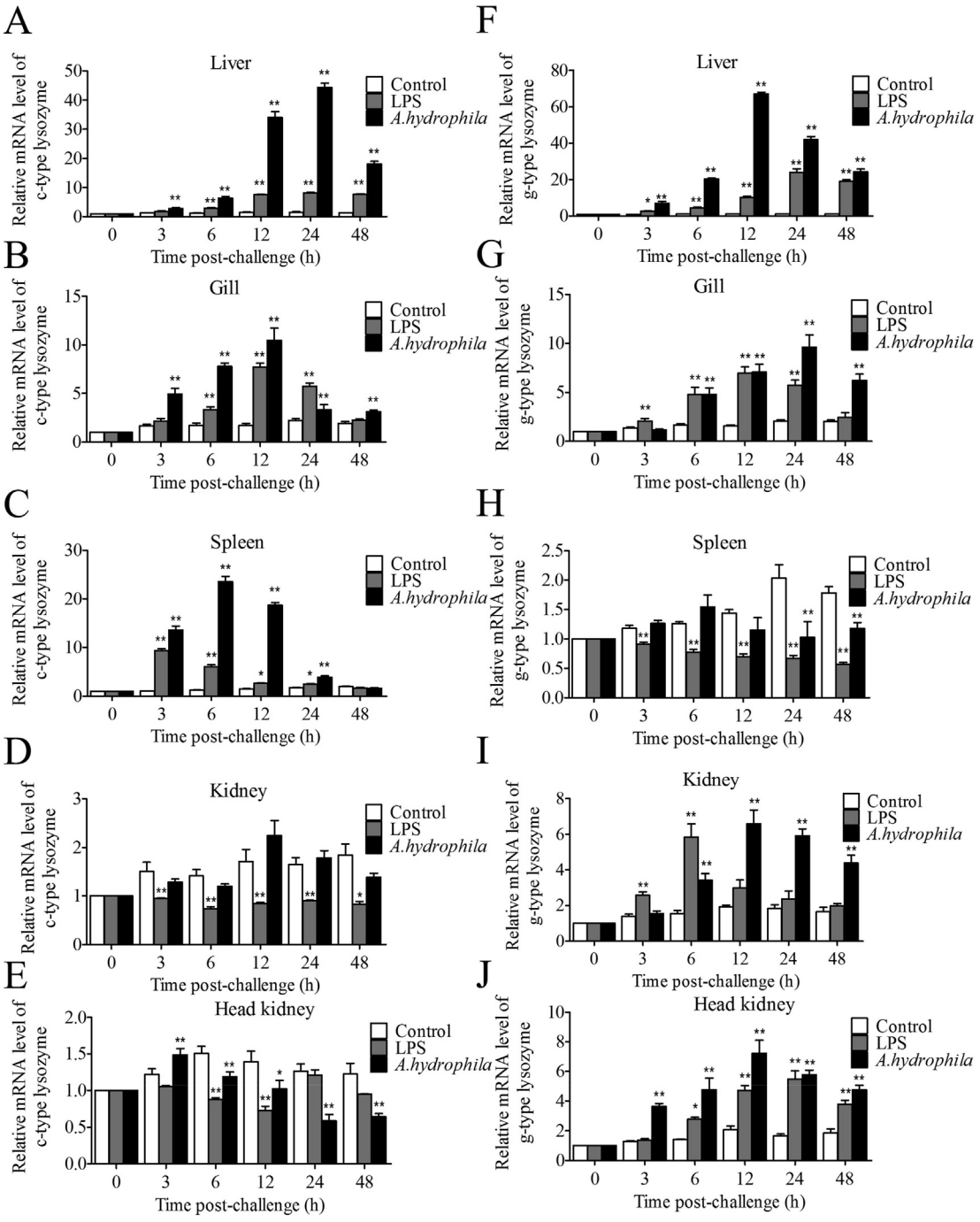


Fig. 6. Expression levels of lysozymes in five tissues of *C. aurutus* at different time points after challenged by LPS and *A. hydrophila*. The quantitative real-time PCR analysis was performed in triplicates, and the values are presented by mean ± SD (n = 3). Significant difference between the treatment and the control at each sampling point was indicated with "*" ($P < 0.05$) or "**" ($P < 0.01$). C-type lysozyme expression levels of liver (A), gill (B), spleen (C), kidney (D), and head kidney (E) at 0–48 h post-challenge were presented on the left; g-type lysozyme expression levels of liver (F), gill (G), spleen (F), kidney (J), and head kidney (J) at 0–48 h post-challenge, were presented on the right.

and head kidney ($P < 0.01$ or $P < 0.05$) (Fig. 7, A, B, C, E). In liver and gill, the lysozyme activities showed the similar changes with the mRNA expression levels in *Ca-clys* or *Ca-glys*, with a significant up-regulation at 6 h–48 h. In kidney, lysozyme activity changes were complex, indicating an increase only at 3 h with LPS challenge, and only at 24 h with *A. hydrophilia* challenge (Fig. 7, D).

4. Discussion

The innate immune system represents the important defense against pathogen infection, in which the c-type and g-type lysozymes are very important components. In this study, the full-length cDNA of *Ca-clys* and *Ca-glys* were cloned in *C. auratus*. The amino acid sequences of Ca-clys and Ca-glys in *C. auratus* were similar with the reported corresponding sequences in other fishes. For instance, the cDNA of *c-type* lysozyme in *Ctenopharyngodon idellus*

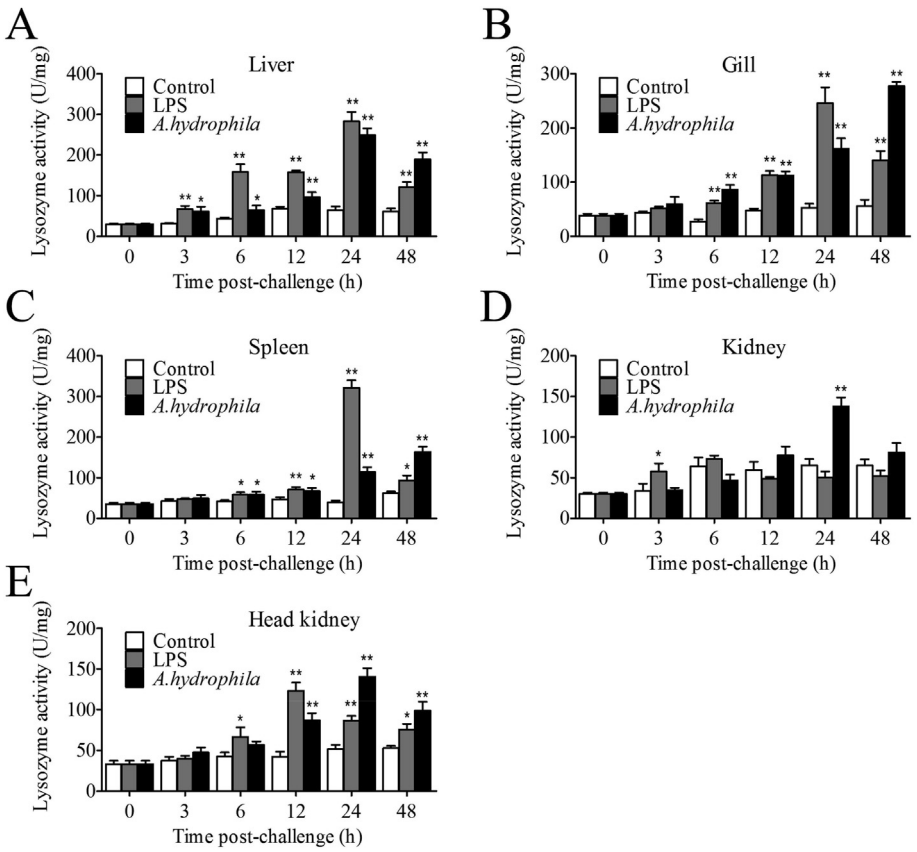


Fig. 7. Lysozymes activities in five tissues of *C. aurutus* at different time points after challenged by LPS and *A. hydrophila*. Lysozyme activities are shown respectively in liver (A), gill (B), spleen (C), kidney (D) and head kidney (E) at 0–48 h post-challenge.

was 685 bp, encoding 145 amino acids [24] and the cDNA of *g-type* lysozyme in *Cyprinus carpio* was 853 bp, encoding 185 amino acids [28]. The Ca-clys contains two conserved catalytic site Glu and Asp, located in 53 and 69, respectively, which plays an important role in the structure stability and catalytic activity [29]. The c-type lysozyme contains eight conserved cysteine residues, which can form four pairs of disulfide bond, being crucial for maintaining the stability of the protein structure [30]. In addition, it contains 18 amino acid residues of N-terminal signal peptide. Based on the existence of disulfide bond and signal peptide, it was suggested that the Ca-clys is a secreted protein. The Ca-glys protein lacks the N-terminal signal peptide and conserved cysteine residues, which contains three conserved catalytic sites (Glu, Asp and Asp), located in 73, 86 and 97, respectively. Similar result has been found in *Siniperca chuatsi* [31]. These conserved catalytic sites have the conservative function in the role of enzyme. The number of Cys of g-type lysozyme in fish is changeable, for instance, no Cys in *Paralichthys olivaceus* [32] and *Epinephelus coioides* [33], one Cys in *Cyprinus carpio* [28] and *Salmo salar* [34]; two cys in *Danio rerio* [35] and *Solea senegalensis* [36]. However, the Ca-glys in *C. auratus* lacks the N-terminal signal peptide. Therefore it is suggested that the enzyme Ca-glys is an intracellular enzyme.

In the phylogenetic tree, the c-type lysozymes in fishes were clustered into two clades. The fish in Cypriniformes form one branch, and the other fish clustered another branch. With regard to the difference between the lysozyme Ca-clys and Ca-glys, it was indicated the lower homology in the same fish, only with 13.37% in *C. auratus*, 8.6% in grass carp, and 12.43% in common carp. Therefore, it was demonstrated that there exists the significant difference in sequence or structure between *Ca-clys* and *Ca-glys*. Based on phylogenetic analysis, the Ca-clys and Ca-glys were respectively clustered into the different branches (Fig. 4). Therefore, it was speculated that the Ca-clys and Ca-glys perform the different functions in fish.

In most fish species, c-type and g-type lysozymes have been found to be expressed in all examined tissues [1]. The gene *Ca-clys* was expressed much higher in kidney and head kidney of *C. auratus*, so it was suggested that *Ca-clys* plays an important role in the immune defense (Fig. 5A). The c-type lysozyme was also extensively expressed in fish, such as *Paralichthys olivaceus*, being higher expressed in head kidney, kidney and spleen [37], while in *Scophthalmus rhombus*, it was higher expressed in stomach and liver [13]. The *Ca-glys* was ubiquitously expressed in different tissues in *C. auratus* (Fig. 5, B). The expressing patterns of g-type were different between fish and higher vertebrate (e.g. bird and mammal), which was restrictedly distributed in birds and mammals. In chicken, the g-type lysozyme was expressed in bone marrow and lung, but not in the oviduct, and it was provided an explainable evidence for the absence of g-type lysozyme in chicken egg white [38]. Two g-type lysozymes present in mouse, and the g1 lysozyme was expressed only in the tongue, whereas g2 was expressed in the skin [35]. The extensive expression of g-type lysozyme presents in fish. In *Cynoglossus semilaevis*, the g-type lysozyme was expressed in the intestine, skin, brain, gill, stomach, muscle, liver, kidney, ovary, blood, spleen, and heart [39]. In *Scophthalmus maximus*, it was expressed in spleen, heart, brain, gill, kidney, muscle, blood, and liver [19]. It has been proved that the lysozymes in fish are widely distributed, but expression levels are different among the various tissues of fish or the different species. It might result in the difference in non-specific immunity against the

pathogen invasion.

The surface mucous, as the first line of immune defense, performs the important function to kill bacteria [40]. In this study, lysozyme activity is highest in the surface mucous, indicating the strong ability to kill bacteria. Lysozyme activity is the secondly highest in kidney and head kidney. Therefore, it is indicated that the lysozyme plays an important role in immune defense in fish. Gill, as a respiring organ directly contacting with the environmental water, was the first invasion site for pathogen bacteria [41,42]. In the gill and intestine, where the pathogens easily infect, the lysozyme activities are much higher. It is demonstrated that the lysozyme plays an important role in preventing invasion from bacteria in these organs and tissues. In addition, lysozyme activity is also affected by the species, environmental factor and feeding [43,44].

The non-specific immune response will be promoted in fish, while being stimulated by the outside pathogen. In this study, the expression levels of lysozyme present the difference among the different tissues of *C. auratus*, indicating the difference in performing the non-specific immune defense for the different tissues. The different responses were shown to bacteria or bacterial component in *C. auratus* after injected with the *A. hydrophilia* and LPS. In the previous studies, the expression of g-type lysozyme increased in head kidney of *Dicentrarchus labrax* injected with the LPS and *Photobacterium damselae* L [45]. The c, g1 and g2-type lysozyme genes were drastically increased in liver, spleen and head kidney of *Ictalurus punctatus* injected with the *Edwardsiella tarda*, while the g-type lysozyme increased in head kidney [23]. With regard to *Paralichthys olivaceus* injected with *Edwardsiella tarda*, the g-type lysozyme mRNA levels increased in intestine, heart, and blood, while the c-type lysozyme increased in head kidney, spleen and ovary [32,37]. Based on the previous studies on the different expression responses of lysozyme, it has been indicated that the c-type and g-type lysozyme exist synergistic effect on bacterial invasion in various tissues [25]. In this study, the lysozyme activity increased in tissues or organs of *C. auratus* after challenged with *A. hydrophilia* and LPS. The lysozyme activities increased in early stage and then declined in liver, spleen, and kidney of the *Cyprinus carpio* exposed to 3.192 mg/L of paraquat [46]. This result is consisted with the changes of mRNA expression levels of lysozyme. Therefore, it is speculated that the expression of the lysozyme gene will be promoted in fish after stimulated by pathogen or the other chemical factors, and then increase lysozyme synthesis and activity. The mRNA expression levels and enzyme activities of c-type and g-type lysozymes increase respectively in various tissues after pathogen invasion. It was demonstrated that the c-type and g-type lysozyme play an important role in immune defenses against the bacteria invasion.

In conclusion, in Qihe Crucian carp *C. auratus*, between the c-type and g-type lysozymes, it is indicated the obvious difference in sequence and structure, implying that the different functions are performed by two lysozymes, which are expressed extensively in various tissues. The mRNA expression levels of lysozymes and enzyme activities exhibit the different expression profiles in tissues of *C. auratus* after challenged with *A. hydrophila* and LPS. It is suggested that the differences between *Ca-clys* and *Ca-glys* exist in the defense against the invading pathogens. With regard to the molecular mechanism to perform function in c-type and g-type lysozymes needs to be further investigated in the future.

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