Heliyon 10 (2024) e24044

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Expression characteristics and in vitro antibacterial properties of C-type lysozyme in crucian carp infected with *Aeromonas salmonicida*

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ARTICLE INFO

Keywords:
Crucian carp
Lysozyme
Aeromonas salmonicida
Antibacterial activity
Immune response

ABSTRACT

Aeromonas salmonicida is an ancient fish pathogen. Lysozymes are important molecules in the innate immune system that fight bacterial infections. The expression characteristics of C-type lysozyme in crucian carp infected with *A. salmonicida* and its antibacterial effect against *A. salmonicida* had not been investigated. Thus, we used bioinformatics to analyze the gene and protein characteristics of C-type lysozymes in crucian carp. Changes in C-type lysozyme expression before and after crucian carp infection with *A. salmonicida* were detected, and the in vitro antibacterial effect of recombinant carp C-type lysozyme on *A. salmonicida* was validated. The results showed that the coding DNA sequence region of the lysozyme gene sequence was 438 bp long, encoding 145 amino acids and containing two conserved catalytic sites: Glu53 and Asp69. Phylogenetic analysis revealed that crucian carp C-type lysozymes clustered with *Cyprinus carpio* lysozyme C. After crucian carp were infected with *A. salmonicida*, the gene and protein expression of C-type lysozymes in the liver, spleen, kidney, and hindgut were significantly upregulated, with the liver showing the highest upregulation that was 15 times higher than that in the uninfected group. In addition, recombinant C-type lysozyme exhibited significant antibacterial activity against *A. salmonicida*, with an average inhibition zone radius of 0.92 cm when using 40 μg recombinant lysozyme. In conclusion, this study reveals the important role of C-type lysozymes in the innate immune response of crucian carp and provides a theoretical basis for preventing crucian carp infection with *A. salmonicida*.

1. Introduction

Crucian carp (*Carassius auratus*) is widely distributed in various water bodies in China and is highly valued for its edible and medicinal value. In recent years, high-density and intensive farming practices and the excessive use of antibiotics have led to a high incidence of disease in crucian carp. *Aeromonas salmonicida* is a pathogen that causes fish furunculosis, which causes significant economic losses to the global aquaculture industry. Over the past few decades, researchers have focused on the infection of salmonid fish by *A. salmonicida*. However, with the expanding host range and emergence of atypical strains, non-salmonid fish have become susceptible to infections [1]. In recent years, there have also been reports of *A. salmonicida* infecting crucian carp [2,3]. However, the

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https://doi.org/10.1016/j.heliyon.2024.e24044
Received 13 October 2023; Received in revised form 26 December 2023; Accepted 2 January 2024
Available online 3 January 2024

immune mechanisms in crucian carp against *A. salmonicida* remain unclear.

The fish immune system comprises innate and adaptive immunity [4]. Fish have a more primitive immune system and a less developed specific immune response than do higher vertebrates such as mammals and birds. In addition, fish are ectothermic; thus, environmental factors can influence their immunity. Therefore, the innate immune system plays a crucial role in defense against pathogen invasion in fish [5]. Notably, the knowledge of the characteristics of the innate immune response of crucian carp during pathogen infections or disease outbreaks is limited. Therefore, understanding the characteristics of the innate immune response of crucian carp to pathogens and developing effective antibiotic alternatives are vital for promoting healthy crucian carp aquaculture and formulating immunization strategies.

Lysozyme, an innate immune defense factor, is crucial in protecting aquatic animals against microbial infections [6,7]. As primitive vertebrates, osteichthyes possess two types of lysozymes: types C and G [8]. These two types exhibit significant differences in their gene structures, including the composition of introns and exons, as well as the molecular weight of the protein [9–14]. Researchers have discovered a deep cleft on the surface of lysozyme that constitutes the active site of the enzyme [15]. The antibacterial mechanism of lysozyme primarily involves the hydrolysis of the β-1,4 glycosidic linkage in cell wall peptidoglycans, resulting in cell wall lysis and eventual bacterial death [12]. However, the presence of components such as lipopolysaccharides and lipoproteins in the extracellular matrix of gram-negative bacteria [16,17] impairs the bactericidal activity of lysozymes. Lysozymes must synergistically interact with cations such as lactoferrin, defensins, and antimicrobial peptides to dissolve the lipopolysaccharides of gram-negative bacteria [12]. Furthermore, the antibacterial mechanism of lysozymes involves non-enzymatic pathways [18]. For example, it can penetrate bacterial cells and adsorb onto the bacterial cytoplasm, disrupting normal physiological metabolism and exerting antimicrobial effects [19]. It can also activate autolysins in bacterial cells, leading to bacterial lysis [20].

Research on animal lysozymes has made significant progress. However, the expression characteristics and antibacterial properties of lysozymes in crucian carp after infection with *A. salmonicida* remain unclear. Therefore, the objective of this study was to analyze the structural characteristics of C-type lysozyme in crucian carp and investigate the innate immune response of crucian carp's C-type lysozyme after being infected with *A. salmonicida*. Additionally, an in vitro antibacterial assay was conducted to evaluate the antibacterial activity of the recombinant lysozyme against *A. salmonicida*. The results of this study will provide a theoretical basis for the immunological characteristics and in vitro antibacterial activity of C-type lysozymes in the innate immune system of crucian carp.

2. Materials and methods

2.1. Experimental animals and strains

Full-sib crucian carp was purchased from a farm in Xining, Qinghai Province, China. Fish weighing approximately 180 g were transferred into a 1000-L aerated water tank and cultured at 20–22 °C for 7 d. During the experiment, two-thirds of the water was replaced every 2 d. The fish were fed pelletized feed daily at 2–3% of their body weight. A strain of atypical *A. salmonicida* NCIMB 1102 was purified from diseased crucian carp and stored in our laboratory.

2.2. Test and sample collection of crucian carp

The strain was cultivated in tryptic soy agar medium at 20 °C for 72 h; then, the bacterial suspension was collected by centrifugation at 8000×*g* for 15 min. The bacteria were washed thrice with sterile phosphate-buffered saline (PBS) and then counted on agar plates before being stored at 4 °C. Eighteen crucian carp were randomly divided into control and experimental groups, with nine fish in each group. The experimental group was intra-abdominally injected with 0.2 mL of 1.5×10^8 CFU/mL bacterial suspension; the control group was intra-abdominally injected with 0.2 mL of sterile PBS. Fish samples were collected on the 5th day after infection. Fish were anesthetized by immersion in 100 ng/mL MS-222, and their liver, kidney, spleen, and posterior intestinal tissues were dissected by opening the abdomen. The tissues were washed with sterile diethyl pyrocarbonate-treated water and stored in liquid nitrogen. Three biological replicates were used for each tissue sample, with three fish per sample. The same procedure was followed for the control group.

2.3. RNA extraction and reverse transcription

Total RNA from different samples (liver, kidney, hindgut, and spleen) was extracted using TRIzol reagent (Thermo Fisher Scientific, USA). Specifically, fresh fish tissues were ground with liquid nitrogen, and 50 mg of ground tissue was added to a 1.5-mL centrifuge tube. Next, 1 mL of TRIzol was added, and the mixture was left at room temperature (20–25 °C) for 5 min to ensure complete lysis. Next, 0.2 mL chloroform was added, and the tube was shaken for 15 s, followed by a 2-min incubation period at room temperature and centrifugation at 12000×*g* for 15 min at 4 °C. The supernatant was collected, and isopropanol (0.5 mL) was added to the tube. The liquid in the tube was gently mixed, left at room temperature for 10 min, and centrifuged at 12000×*g* for 10 min at 4 °C. The supernatants were discarded. Finally, 1 mL of 75 % ethanol was added to wash the precipitate and centrifuged at 7500×*g* for 5 min at 4 °C. The supernatant was discarded, and the pellet was air-dried before being dissolved in an appropriate amount of diethyl pyrocarbonate-treated water for concentration and purity measurements. Total RNA was then extracted and reverse-transcribed into cDNA using a first-strand cDNA synthesis kit (Roche, Basel, Switzerland).

2.4. Amplification, sequencing, and bioinformatic analysis of C-type lysozyme gene in crucian carp

Design primers were based on the coding DNA sequence of *Carassius gibelio* c-lysozyme mRNA (XM_052542666) as follows: forward primer, 5′-ATGAGGTGTGTGTTGTTG-3′; reverse primer, 5′-TAGTGCTCCTCACATCCTTTC-3′. The polymerase chain reaction (PCR) mixture with a total volume of 50 μL contained 0.5 μL of Taq polymerase (ExTaq, DRR001A, TaKaRa, Japan), 4 μL of dNTP (each 2.5 mM), 5 μL of 10 × PCR buffer, 1 μL each of forward and reverse primers (10 mM), 1 μL of cDNA template, and 37.5 μL of PCR-grade water. The reaction conditions were initial denaturation at 95 °C for 5 min; 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s, for a total of 32 cycles; and 72 °C for 10 min. Subsequently, the PCR products were gel-purified, cloned into the pMD18-T vector (D103A, TaKaRa), and sequenced by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). DNAMAN software was used to analyze DNA sequences. SignalP-5.0 software was used to predict protein signal peptides. The EditSeq module in the DNAstar7.0 green program was used to analyze the protein molecular weight and isoelectric point, SignalP 5.0 (http://www.cbs.dtu.dk/services/SignalP/) was used to predict protein signal peptides, and MEGA 6.0 was employed to perform the physiological analysis. The neighbor-joining method was used to construct a consensus tree, with the robustness of each topology checked by 1000 bootstrap replications. Phyre2 software was used to predict the protein tertiary structure [21].

2.5. Real-time quantitative PCR

The SYBR Premix Ex *Taq*II (Tli RNase H Plus) fluorescent quantitative PCR reagent kit was used for protein expression analysis. According to the manufacturer's instructions, the reaction mixture was prepared and performed using an ABI 7500 real-time fluorescent quantitative PCR instrument. The 20-μL reaction mixture consisted of 10 μL of SYBR® Premix mixture, 0.5 μL each of the forward and reverse primers (c-lysozyme F: CACTGCAGAGATGAGGGTTG; c-lysozyme R: CCATCAAGTCCCTCTCGCTT), 1 μL of diluted cDNA template, and 8 μL of ddH_2O. The reaction conditions involved an initial denaturation at 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 30 s. All detections were performed in triplicate. The *β-actin* gene was used as the reference gene, and the relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Three biological replicates were analyzed, and statistical analyses were conducted using GraphPad Prism 5 software.

2.6. Gene synthesis, expression, and polyclonal antibody preparation of crucian carp C-type lysozyme

The coding DNA sequence of the crucian carp C-type lysozyme gene was truncated to remove the signal sequence (base 1–54), and the resulting sequence was fused with the promoter synthesized by GENEWIZ Biotechnology Co., Ltd. The resulting fragment was ligated into a PET-28a vector (TaKaRa), which was then transformed into DH5a-competent cells. Plasmid extraction was performed after large-scale amplification, followed by sequencing to verify the plasmid DNA sequence. The sequenced plasmid vector was transfected into competent Rosetta cells (Solarbio, Beijing, China). Bacterial expression was induced by isopropyl-ᴅ-thiogalactopyranoside at 28 °C for 20 h. The bacterial pellet was collected by centrifugation at 12,000×*g* for 5 min. The bacteria were then resuspended in PBS, and the cell pellet was resuspended in a 4 × sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and analyzed using 12 % SDS-PAGE. Bacteria from expanded cultures were purified using a His-tagged protein purification kit (Beijing ComWin Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. The protein concentration was determined using the Bradford method.

Purified recombinant proteins were diluted with an equal volume of Freund's complete adjuvant, mixed, and emulsified. Each purified protein was subcutaneously injected into two New Zealand rabbits (male，weight about 2.2 kg) at an initial dose of 800 μg/rabbit. After 2 weeks, we performed a second immunization using 400 μg/rabbit of protein and Freund's complete adjuvant. Additional immunizations were administered weekly for 4 weeks, and the doses were increased over time. Four days after the fourth immunization, blood was collected from the heart, and serum was isolated from each sample. Antibody titers were determined using an indirect enzyme-linked immunosorbent assay. The antibody efficacy was expressed as the ratio (P/N) of the absorbance value of the sample well to the average absorbance value of the negative control wells. When the P/N was greater than two, the potency of the antibodies was indicated.

2.7. Western blot

Proteins were extracted from the liver, kidneys, hindgut, and spleen of the crucian carp before and after infection. Western blot analysis was then performed. In brief, equal amounts of protein from infected and untreated crucian carp samples were resolved using SDS-PAGE. Proteins were transferred to nitrocellulose membranes, which were blocked with 5 % non-fat dried milk in tris-buffered saline containing 0.1 % Tween 20 (TBST) for 1 h at room temperature. The membranes were incubated with the polyclonal antibody (diluted 1:8000) and anti-β-actin antibody (diluted 1:2,000, AC026, ABclonal) for 1 h at 37 °C. All antibodies were diluted using TBST. The membranes were washed thrice with TBST and labeled with goat anti-rabbit IgG (AC026, ABclonal) conjugated to horseradish peroxidase (diluted 1:5000; bs-0295G-HRP; BIOSS) for 2 h at 37 °C. The membranes were then washed thrice with PBS containing 0.1 % Tween 20, followed by visualization with Western Bright (Advantsa, San Jose, CA, USA).

2.8. In vitro bacteriostatic test

Before the experiment, the purified recombinant lysozyme was diluted with PBS to prepare five concentrations (5, 10, 20, 30, and

40 μg) of samples. Next, 0.2 mL of *A. salmonicida* suspension with a concentration of 1×10^6 CFU/mL was evenly spread on the surface of tryptic soy agar plates. Sterile Oxford cups were placed vertically on the agar surface, and different concentrations of recombinant protein (PBS as the control group) were added to each cup. The plates were then incubated at 23 °C in a biochemical incubator for 36 h, and the radii of the inhibition zones on the plates were measured. Each group was analyzed thrice.

2.9. Statistical analyses

All data were analyzed using GraphPad Prism 5 software (GraphPad Software Inc. CA, USA). All data were represented as mean ± SEM. Differences between treatments were evaluated by independent samples *t*-test.

3. Results

3.1. Sequence characteristics, phylogenetic analysis, and 3D view of crucian carp C-type lysozyme

The open reading frame of this sequence was 438 bp long and encoded 145 amino acids, including 25 basic (K and R), 18 acidic (D and E), 46 hydrophobic (A, I, L, F, W, and V), and 36 polar amino acids (Fig. 1A). The predicted molecular weight was approximately 16.58 kDa, with a theoretical isoelectric point of 8.52. The phylogenetic tree of various lysosome protein sequences revealed two independent branches, with this gene clustered together with *Cyprinus carpio* lysozyme C (LOC109090952) (Fig. 1B), which contains two conserved catalytic amino acid residues, i.e., Glu (at position 53) and Asp (at position 69), and eight conserved Cys residues that

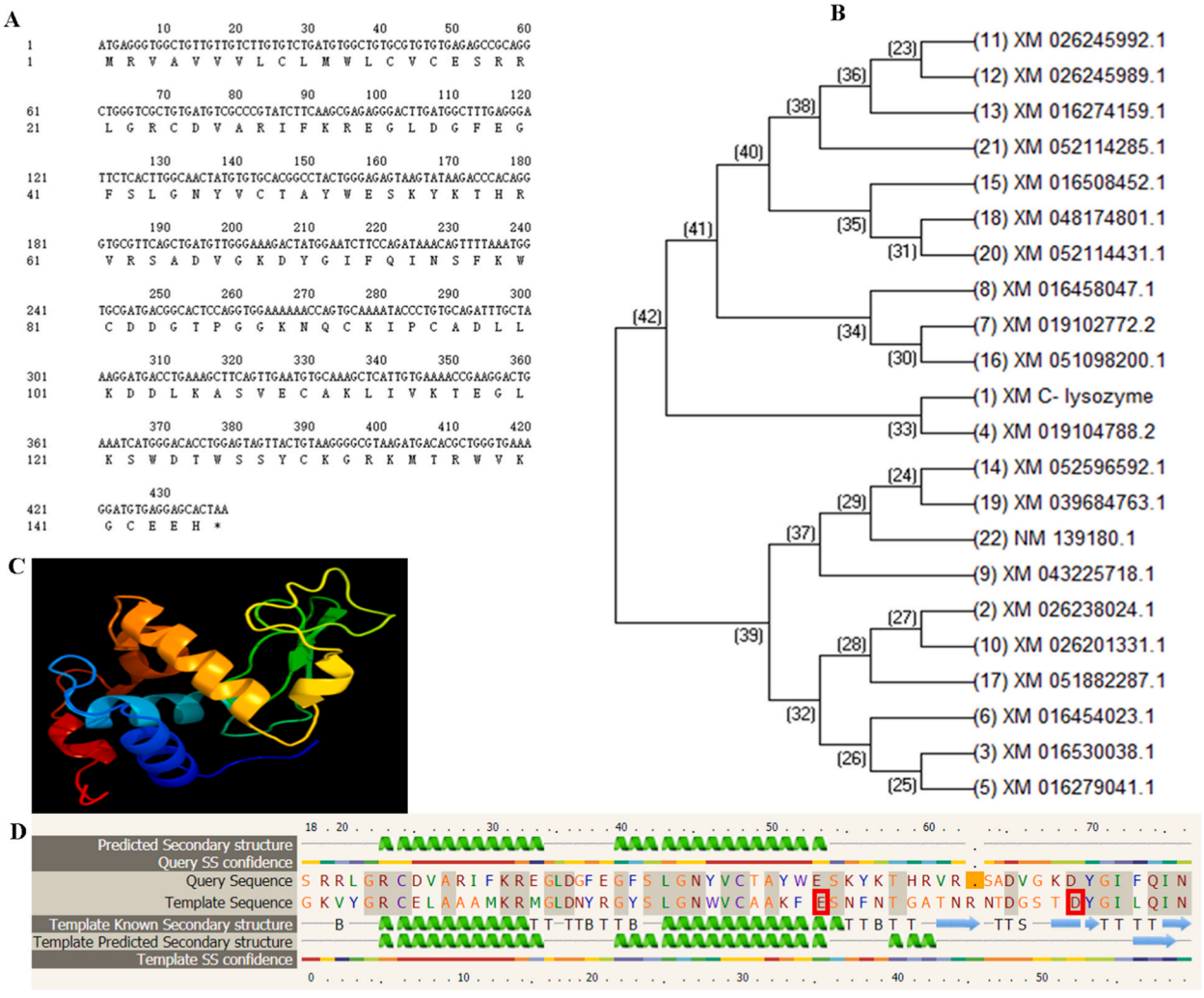

Fig. 1. (A) Nucleotide and deduced amino acid sequence of C-type lysozyme cDNA from *Carassius auratus*. (B) Phylogenetic evolutionary tree based on lysozyme DNA sequences of different fish. (C) Interactive 3D view, image colored by rainbow N→C terminus, model dimensions (Å): X: 39.124, Y: 34.815, Z: 39.487. (D) Secondary structure and conservative catalytic residues were predicted based on the template d1ghla.

can form four pairs of disulfide bonds (Fig. 1C and D). The first 18 amino acids constitute the N-terminal signal peptide, indicating that this protein is secretory.

3.2. Recombinant protein expression and polyclonal antibody

The recombinant crucian carp lysozyme C protein was analyzed using SDS-PAGE (Fig. 2A), and a molecular weight of approximately 15 kDa was determined after purification (Fig. 2B). This molecular weight is consistent with the expected size of the protein without a signal peptide. The concentration of the purified recombinant protein was 7.165 μg/mL. As an antigen, the purified recombinant protein was immunized in New Zealand White rabbits four times, successfully generating polyclonal antibodies against the lysozyme. The antibody titer was determined to be 1:16000 (Table 1).

3.3. Expression changes in C-type lysozyme genes in different tissues of crucian carp before and after infection

Under normal circumstances, the relative expression level of C-type lysozyme in the kidneys of crucian carp was the highest, i.e., 70.6 times higher than that in the liver, followed by that in the hindgut, with lower expression levels in the liver and spleen (Fig. 3). Five days after infection with *A. salmonicida*, the mRNA expression of the C-type lysozyme was significantly upregulated in four tissues of crucian carp: the liver, kidney, spleen, and hindgut (Fig. 4A, B, 4C and 4D). Among them, upregulation in the liver was the most significant, i.e., 15 times higher than that before infection ($p < 0.01$) (Fig. 4A). The kidneys showed the second highest upregulation, with a 2.5-fold increase ($p < 0.05$) (Fig. 4C).

3.4. Expression changes in C-type lysozyme protein in different tissues before and after infection

Western blot analysis showed that the expression of C-type lysozyme in the liver, spleen, kidney, and posterior intestine of crucian carp was upregulated to varying degrees after 5 d of infection with *A. salmonicida* (Fig. 5A and B) (Supplementary material 1), which is consistent with the results obtained from gene-level detection using quantitative reverse transcription-PCR. This result further validates the expression characteristics of C-type lysozymes in crucian carp after infection with *A. salmonicida* at the protein level.

3.5. In vitro antibacterial activity of recombinant crucian carp C-type lysozyme against A. salmonicida

The results of the in vitro bacteriostatic tests showed that the recombinant C-type lysozyme exhibited significant bacteriostatic activity against *A. salmonicida*. As the experimental dose was increased, the visible inhibition zone on the culture plate expanded (Fig. 6A). Using 5, 10, 20, 30, and 40 μg recombinant lysozyme, the average radius of the inhibition zone was 0.17, 0.39, 0.57, 0.71 and 0.92 cm, respectively (Fig. 6B). No visible inhibition zones were observed in the PBS-treated control group.

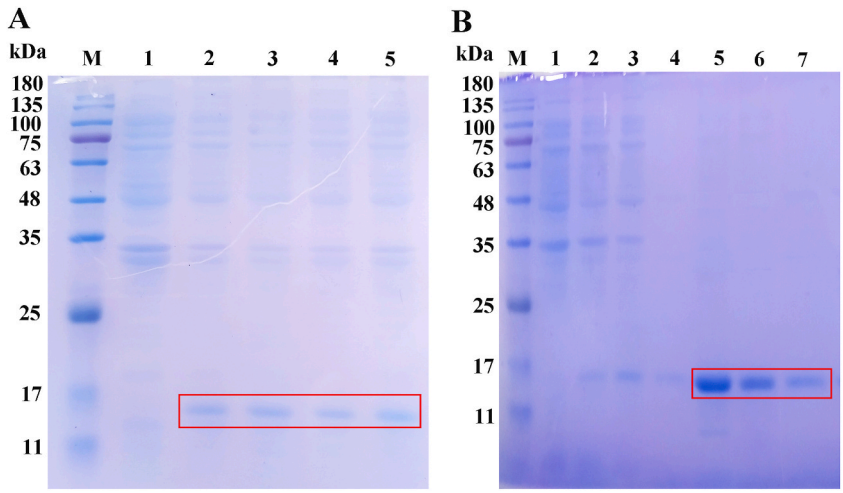

Fig. 2. SDS-PAGE analysis of the recombinant proteins of the C-type lysozyme. (A) M: Protein marker (11–180 kDa); Lane 1: Control group without IPTG; Lane 2–5: Unpurified supernatant containing recombinant lysozyme. (B) M: Protein marker (11–180 kDa); Lane 1: Control group without IPTG; Lane 2–3: Expression of unpurified recombinant lysozyme. Lane 5–7: Purified recombinant lysozyme. Each lane of the 12 % polyacrylamide gel contained 10 μL of each protein sample and was stained with Coomassie Brilliant Blue R250. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Titer of anti-serum detected by ELISA.

Dilution Ratio	OD_{450}	P/N	[a]/–
1:1000	2.41	10.04	a
1:2000	2.28	9.50	a
1:4000	1.66	6.91	a
1:8000	1.09	4.54	a
1:16000	0.92	3.83	a
1:32000	0.34	1.42	–
Negative Control	0.27	/	/
Negative Control	0.21	/	/

[a] Positive; -: negative.

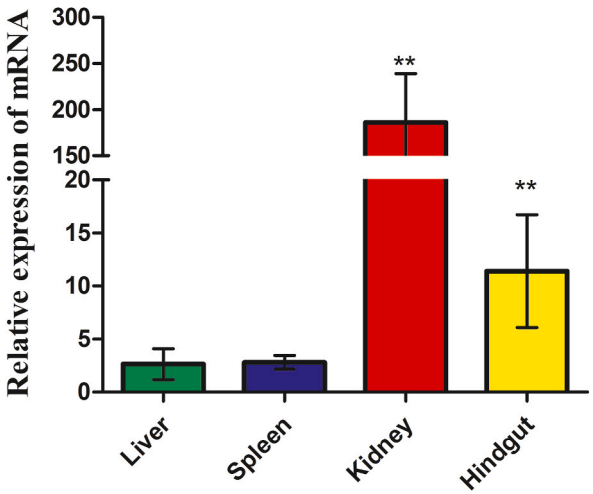

Fig. 3. Expression of C-type lysozyme mRNA in different tissues of crucian carp. Statistical analysis (Student's t-test) was conducted using GraphPad Prism software. $^{**}p < 0.01$.

4. Discussion

The innate immune system is crucial for resisting pathogenic infections, improving the overall health of aquatic animals in aquaculture, and enhancing disease resistance. Lysozyme is a key component of the natural immune system that catalyzes the hydrolysis of bacterial cell walls [22]. It is also a critical marker molecule for studying environmental pollutants [23,24], immune stimulants [25,26], and fish disease resistance [27,28]. In this study, we analyzed the gene and protein characteristics of C-type lysozymes in crucian carp. Moreover, we investigated changes in the gene and protein expression of C-type lysozymes in the liver, spleen, kidney, and hindgut of crucian carp before and after infection with *A. salmonicida*. Furthermore, we expressed and validated the antimicrobial activity of recombinant lysozyme against *A. salmonicida* in vitro. Notably, according to our literature review, this study is the first to investigate the expression characteristics and antimicrobial properties of C-type lysozymes in crucian carp after infection with *A. salmonicida*.

C-type lysozymes in fish are generally composed of four exons and three introns, with a cDNA length of approximately 600–700 bp encoding approximately 150 amino acids [29–31]. We discovered that the coding DNA sequence region of crucian carp C-type lysozyme had a total length of 438 bp, encoding 145 amino acids (Fig. 1A), with an amino acid sequence length similar to those reported in other fish species. Studies have shown that fish C-type lysozymes often contain two conserved catalytic residues, Glu and Asp, which play crucial roles in structural stability and catalytic activity. Our results showed that the crucian carp C-type lysozyme had Glu at position 53 and Asp at position 69 ((Fig. 1D), which differs from the conserved catalytic residue positions in Atlantic salmon [32], brill [13], grass carp [30], and *Scophthalmus maximus* [33]. Furthermore, the closer the phylogenetic relationships among fish species, the higher the homology of their lysozymes. Notably, crucian carp and *Cyprinus carpio* belong to the same family, Cyprinidae, and have high homology in C-type lysozymes (Fig. 1B), indicating a close evolutionary relationship between them in the phylogenetic tree. In this study, although the structure of the C-type lysozyme was predicted using Phyre2 software, the specific protein structure needs to be further elucidated using techniques such as X-ray crystallography, nuclear magnetic resonance, electron microscopy, etc.

The expression pattern of C-type lysozymes varies in different fish tissues, and C-type lysozymes mainly serve two functions: digestion and immune defense [34]. Moreover, digestive-type lysozymes are mainly non-calcium binding, with a low isoelectric point and acidic pH [29,35]. We found that the isoelectric point of the C-type lysozyme in crucian carp was relatively high at 8.52, suggesting that it does not possess the molecular characteristics of a digestive enzyme. Similar to the liver of vertebrates higher than fish, the liver

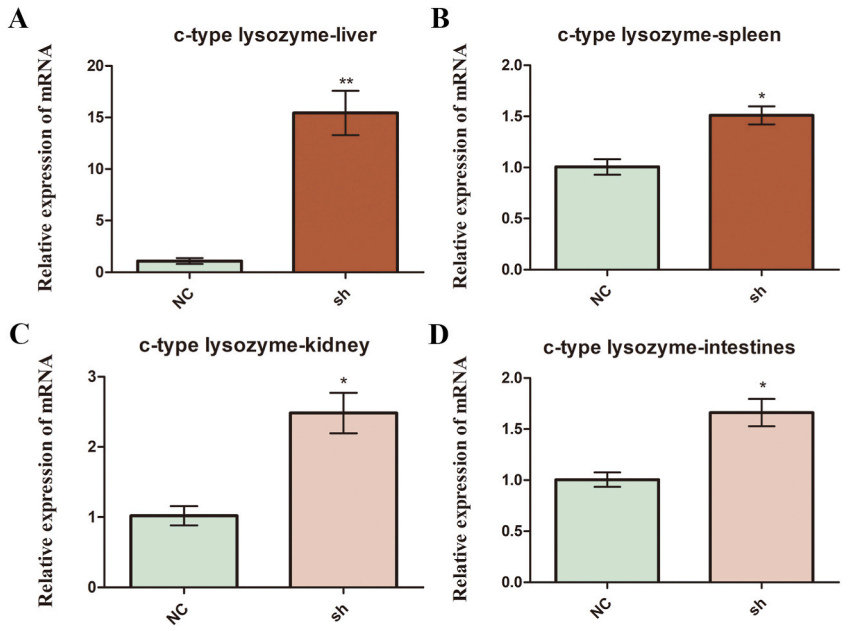

Fig. 4. Expression of C-type lysozyme mRNA in different tissues of crucian carp before and after infection by *A. salmonicida*. Expression in the (A) liver, (B) spleen, (C) kidney, and (D) intestines. Statistical analysis (Student's t-test) was conducted using GraphPad Prism software. $*p < 0.05$, $**p < 0.01$. NC: control group, sh: experimental group.

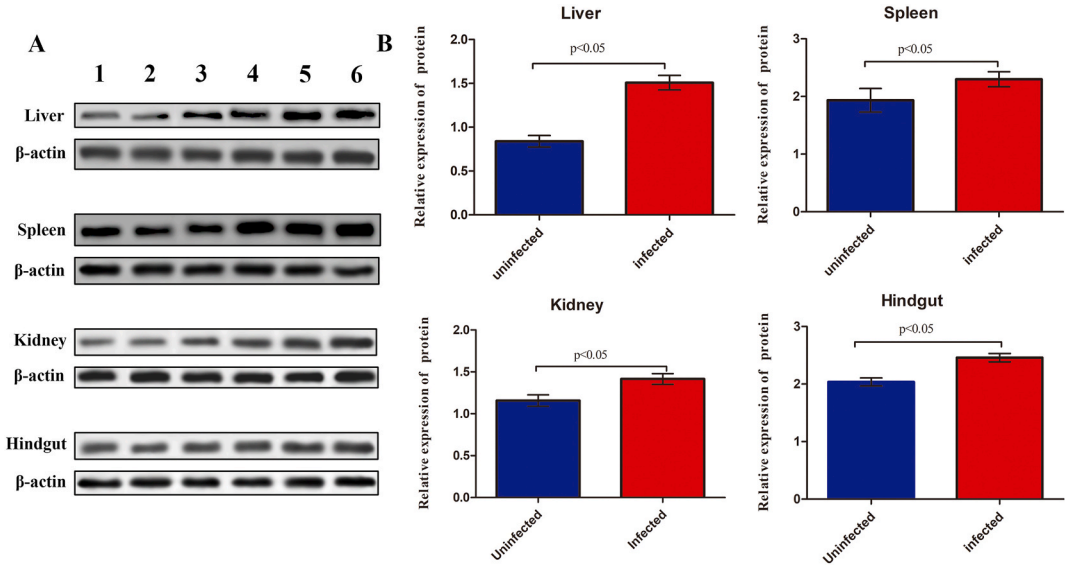

Fig. 5. The C-type lysozyme expression was up-regulated in four different tissues of crucian carp after A. salmonicida infection. (A) Representative images of the Western blot membrane showing the expression of the C-lysozyme in uninfected and infected four different tissues of crucian carp. The tissues labeled as 1, 2 and 3 represent the uninfected tissues, while the tissues labeled as 4, 5 and 6 represent the infected tissues. (B) Quantitative analysis of the expression of the C-lysozyme in uninfected and infected four different tissues after normalization to *β-actin*, three biological replications. Statistical analysis (Student's t-test) was conducted using GraphPad Prism software.

of fish is an important glandular and digestive organ that maintains physiological functions. Given the liver's considerable contact with antigens and endotoxins from intestinal microbiota and the presence of immune cells [36], the liver is considered an immune organ [37], capable of clearing microorganisms that cross the intestinal barrier and enter the liver. We found that the expression of C-type lysozyme in the liver of crucian carp infected with *A. salmonicida* was significantly upregulated by 15-fold (Fig. 4A), similar to the changes observed in grass carp after *A. hydrophila* treatment [30]. In 2013, Hooper et al. discovered that intestinal epithelial cells secrete lysozymes and other antimicrobial proteins via autophagy to resist pathogen infection [38]. We found that the expression of

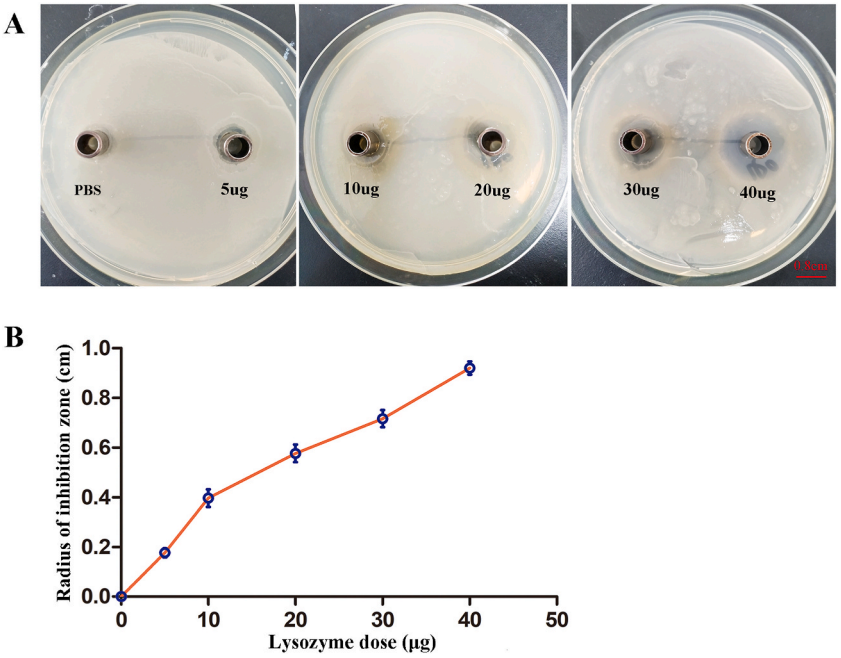

Fig. 6. Antibacterial activity of recombinant C-type lysozyme against *A. salmonicida* in vitro. (A) Different doses of recombinant lysozyme are resistant to *A. salmonicida* and form a bacteriostatic ring on a solid culture dish. (B) GraphPad Prism software was used for statistical analysis. The horizontal axis represents the dose used; the vertical axis represents the radius of the bacteriostasis circle.

C-type lysozymes was upregulated in the hindgut of infected crucian carp. This finding is related to the important immune function of the fish hindgut, and it may be related to the activation of the autophagy pathway in intestinal epithelial cells by *A. salmonicida* infection, leading to the secretion of a large amount of lysozyme to defend against pathogen invasion. In addition, the expression levels of C-type lysozymes in the spleen and kidney showed varying degrees of upregulation (Fig. 4B and C). These results demonstrate that C-type lysozymes in crucian carp play an important role in the non-specific immune response of the organism.

Due to the serious problem of bacterial resistance caused by traditional antibiotic therapies, developing new antimicrobial methods is imperative [39]. Currently, a strong emphasis is on developing rapid, effective, and environmentally friendly treatment approaches to overcome the limitations of traditional therapies [40]. One safe alternative is to rely on lysozymes for bacterial hydrolysis [41]. Similar to natural lysozymes, recombinant lysozymes show high activity and antibacterial properties [42,43]. We found that compared with that of the negative control, recombinant C-type lysozyme from crucian carp exhibits excellent in vitro antibacterial activity against *A. salmonicida*, with a diameter of inhibition zone reaching 0.92 cm when using a dose of 40 μg (Fig. 6). This demonstrated that recombinant C-type lysozyme from crucian carp can effectively inhibit the proliferation of *A. salmonicida* in vitro. However, further research is needed to determine whether this recombinant protein has antibacterial activity in vivo and how to prevent its degradation and destruction by digestive enzymes during the process of clinical application.

In conclusion, we successfully expressed recombinant C-type lysozyme in crucian carp, prepared polyclonal antibodies against lysozyme in rabbits, and found that the expression of C-type lysozyme genes and proteins in the crucian carp liver, spleen, kidney, and hindgut was upregulated during infection with *A. salmonicida*. Additionally, the recombinant lysozyme exhibited adequate antimicrobial activity against *A. salmonicida*. This research not only reveals the immune response characteristics of C-type lysozymes in crucian carp after *A. salmonicida* infection but also provides a scientific basis for future applications in medicine, food, and agriculture.

Ethics statement

This study was reviewed and approved by Science and Technology Ethics Committee of Qinghai University, with the approval number:SL-2023007. All animal experiments in this study were conducted in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and its associated guidelines, as well as the policies and legal requirements of European Communities Council Directive 2010/63/EU. Additionally, we follow the guiding principles of the Chinese Legislation on the Use and Care of Laboratory Animals.

Funding statement

This work was supported by the Youth Scientific Research Fund of Qinghai University (Number:2022-QNY-4) and National Science Foundation of China (Number:32360865).

Data availability statement

The data related to this research has been stored in Mendeley Data, Version 1 (doi: 10.17632/f5xfy7ghz7.1, https://data.mendeley.com/datasets/f5xfy7ghz7/1).

CRediT authorship contribution statement

Xiao-dong Ling: Writing – original draft, Project administration, Methodology, Funding acquisition, Conceptualization. **Jianshu Lv:** Writing – original draft, Investigation, Data curation. **Fu-ju Chen:** Visualization, Methodology, Conceptualization. **Xiao-tong Qin:** Validation, Investigation. **Mei-si Wu:** Writing – review & editing, Investigation. **Feng Bai:** Writing – review & editing, Validation. **Hui-qiong Luo:** Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank Editage (www.editage.cn) for English language editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e24044.

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