



Full length article

Gene identification and antimicrobial activity analysis of a novel lysozyme from razor clam *Sinonovacula constricta*Ming Guo^a, Huihui Wang^a, Yina Shao^a, Ronglian Xing^c, Xuelin Zhao^a, Weiwei Zhang^a, Chenghua Li^{a,b,*}^a School of Marine Sciences, Ningbo University, Ningbo, 315211, PR China^b Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, 266071, PR China^c College of Life Sciences, Yantai University, Yantai, 264005, PR China

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ABSTRACT

Lysozymes are important immune effectors present in phylogenetically diverse organisms. They play vital roles in bacterial elimination during early immune responses. In the present study, a second invertebrate-type (i-type) lysozyme gene from razor clam *Sinonovacula constricta* (denoted as ScLYZ-2) was cloned by RACE and nested PCR methods. The full-length cDNA sequences of ScLYZ-2 were 1558 bp, including a 5' untranslated region (UTR) of 375 bp, an open reading frame of 426 bp, and a 3'-UTR of 757 bp with polyadenylation signal sequence (AATAAA) located upstream of the poly(A) tail. SMART analysis showed that ScLYZ-2 contains a signal peptide in the first 16 amino acid (AA) sequences and a destabilase domain located from 24 to 134 AA sequences. The deduced AA sequences of ScLYZ-2 were highly similar (42%–58%) to other known lysozyme genes of bivalve species. Multiple alignments of AA sequences showed that ScLYZ-2 possesses the classical i-type lysozyme family signature of two motifs ["MDVGLSCGP(Y/F)QIK" and "CL(E/L/R/H)C(I/M)C"] and two catalytic residues (Glu³⁵ and Asp⁴⁶). Moreover, phylogenetic analysis showed that ScLYZ-2 is a new member of the i-type lysozyme family. In healthy razor clams, ScLYZ-2 was highly expressed in the hepatopancreas, followed by the gills, water pipes, and abdominal foot. Lysozyme activity and ScLYZ-2 expression levels were significantly upregulated in the hepatopancreas and gills after being infected with *V. splendidus*, *V. harveyi*, *V. parahaemolyticus* and *S. aureus* and *M. luteus*. Moreover, the recombinant ScLYZ-2 had strong antimicrobial activities against *V. splendidus*, *V. harveyi*, and *V. parahaemolyticus*. Furthermore, the minimal inhibitory concentration of the recombinant ScLYZ-2 against *V. parahaemolyticus* was 7.2 μmol/mL. Taken together, our results show that ScLYZ-2 plays an important role in the immune defense of razor clam by eliminating pathogenic microorganisms.

1. Introduction

The razor clam *Sinonovacula constricta* is commonly known as a seawater bivalve mollusk, which belongs to the Solenidae family. It is an economic species that mainly distributed in the coastal areas of Japan, China, North Korea, and Vietnam [1]. However, it has suffered from serious diseases, especially infection from *Vibrio parahaemolyticus*, causing great economic losses [2]. Like all invertebrates, the razor clam lacks adaptive immunities; thus, it relies on innate immunities to protect itself from potential pathogens in the aquatic environment [3]. Its innate immunities consist of the cellular immunities mediated by hemocytes and humoral immunities that apply antimicrobial molecules to lyse invading pathogens [4]. Antimicrobial peptides are important components of the humoral defense mechanisms of invertebrates, and

lysozymes are the most well-known antimicrobial peptides [5].

Lysozyme, also known as N-acetylmuramide glycanhydrolase or muramidase, is an alkaline glycoside hydrolase enzyme. The enzyme (EC3.2.1.17) cleaves the β-1,4 glycosidic bond between N-acetylglucosamine and N-acetylmuramic acid in the peptidoglycan of cell wall by catalysis, causing bacterial cell wall rupture and content spillage, which result in bacterial death [6]. Thus, it is a kind of spectrum antimicrobial effector molecule that exists widely in tissues, cells, and secretions of various animals, plants, and microorganisms [6,7]. Currently, according to the differences in structural, catalytic, and immunological characters, lysozymes are generally divided into six types, namely, plant lysozyme, bacterial lysozyme, T4 phage lysozyme, invertebrate-type (i-type) lysozyme, chicken-type (c-type) lysozyme, and goose-type (g-type) lysozyme [8–11]. Unlike c-type and g-type

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lysozymes that are found in various organisms, such as fish and mammals, i-type lysozyme is a novel family ascertained solely in invertebrate. Since the first i-type lysozyme gene was discovered in starfish *Asterias* [12], i-type lysozymes have attracted considerable interests, and to date, about 30 i-type lysozymes from echinoderms [13], mollusks [7], nematodes [14], and arthropods [15] have been identified.

Aside from their digesting and filtering abilities [16], i-type lysozymes have chitinase, isopeptidase, and muramidase activities and exert innate immune responses. In *Stichopus japonicus*, the recombinant lysozyme not only possesses isopeptidase and glycosidase activities but also displays strong antibacterial abilities against both Gram-positive and Gram-negative bacteria [13]. The recombinant lysozyme of *Hirudo medicinalis* possesses isopeptidase and muramidase activities against *Micrococcus lysodeikticus* conferred by independent active sites [17]. Nowadays, increasing i-type lysozyme genes have been described in bivalves such as *Sinonovacula constricta* [18], *Venerupis philippinarum* [19], *Meretrix meretrix* [20], *Crassostrea gigas* [21], and *Crassostrea virginica* [22]. Studies have confirmed that these lysozymes are expressed in almost all tissues and significantly induced by bacterial infection [23]. Moreover, several purified proteins of these lysozymes were found to have bacteriolytic activities, especially pathogens belonging to *Vibrio* spp [18–20].

Clearly, multiple isoforms of i-type lysozyme exist in bivalve species. For instance, two i-type lysozymes of *Ruditapes philippinarum* and four i-type lysozymes of *Hyriopsis cumingii* have been discovered [5,24]. In our previous study, the lysozyme gene from a razor clam (for distinction, defined as ScLYZ-1) was cloned and characterized [18]. In the present study, a second lysozyme gene (ScLYZ-2) in razor clam was discovered. The full-length cDNA, the distribution in tissues, the response profiles to *V. splendidus*, *V. harveyi*, *V. parahaemolyticus*, *S. aureus* and *M. luteus* infection, and the antibacterial activities of recombinant and folded ScLYZ-2 were investigated in this study. Results can supplement preliminary data for understanding the roles of i-type lysozymes in razor clams. Moreover, progress in the characteristic analysis of lysozymes can help us further understand the immune defense mechanism of mollusks and provide new vision for disease control in mollusk aquaculture.

2. Materials and methods

2.1. Experiment animals

One hundred healthy razor clams with body weights of 10 ± 1.4 g were purchased from a fish farm in Ningbo City of Zhejiang Province, China. The razor clams were fed in an oxygen-supplying laboratory aquaria (30 L), and the seawater conditions were controlled as follows: temperature at 15 ± 1 °C, dissolved oxygen at 6.0 ± 0.5 mg/L, salinity of 20, pH 8.0, total ammonia < 0.2 mg/L, and nitrite < 0.02 mg/L. All razor clams were acclimatized for at least three days before treatment.

2.2. Bacterial challenge and sampling

The strain of *Vibrio parahaemolyticus*, *Vibrio harveyi*, *Vibrio splendidus*, *Micrococcus luteus* and *Staphylococcus aureus* were previously stored at -80 °C in our laboratory and prepared based on previously described procedures [18]. Briefly, the melted bacterial stocks of *V. parahaemolyticus*, *V. harveyi* and *V. splendidus* were transferred into 2216E broth (1 g/L of yeast and 5 g/L of tryptone, PH 7.6) at 140 rpm and 28 °C overnight. The melted bacterial stocks of *M. luteus* and *S. aureus* were transferred into nutritional agar liquid medium (10 g/L of tryptone, beef extract 3 g/L, NaCl 5 g/L, PH 7.6) at 150 rpm and 35 °C overnight. After bacterial centrifugation at 3000 rpm for 5 min and washing three times with sterilized 0.01 M phosphate-buffered saline (PBS), the bacteria were resuspended in sterilized PBS. For the

challenge, the razor clams were averagely and randomly divided into six groups, with 50 individuals in each group. Five groups of razor clams were bathed with *V. parahaemolyticus*, *V. harveyi* and *V. splendidus*, *M. luteus* and *S. aureus* in the final concentration of 10^7 CFU/mL, and the remaining group without bath treatment was set as the control. The gills and hepatopancreas were sampled from three randomly selected individuals before infection and at 6, 12, 24, and 48 h after infection with sterilized tweezers and scissors. The challenge dose and sampling time points were selected based on previously described techniques [25,26]. For spatial expression analysis, the hepatopancreas, gills, hemocytes, abdominal foot, and water pipes were randomly sampled from three individuals in the control group. The hemolymphs were sampled at the backside of razor clams by using 1 mL sterile syringe and then centrifuged for 10 min at 4 °C and $1000 \times g$ to obtain the hemocytes. The lysate supernate of hemocytes, hepatopancreas, and gills used for detecting the lysozyme activity was prepared as follows. The hemocytes were washed and resuspended in PBS, sonicated for 2 min at 0 °C, and centrifuged at $4000 \times g$ for 10 min at 4 °C. The gills and hepatopancreas were sampled and immediately rinsed with pre-cooled PBS that contained protease inhibitors. The corresponding volume of PBS was added to shredded tissues (w/v: 1:9) and then ground thoroughly on ice in a glass homogenizer. To further lyse tissue cells, the homogenate was broken by ultrasound and centrifuged at $5000 \times g$ for 10 min. The resulting supernatant fluid was collected for determination of enzyme activity.

2.3. Cloning of the full-length *S. constricta* lysozyme-2 (ScLYZ-2) cDNA

Total RNA was extracted from the hepatopancreas of healthy razor clams with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then reverse-transcribed into first-strand cDNA using the SuperScript Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). The kit contains RNase-free DNase I, which could remove the genomic DNA. Rapid amplification of cDNA ends (RACE) was performed to clone the full-length cDNA of ScLYZ-2 with specific primers by SMARTer™ RACE cDNA Amplification Kit (TaKaRa, Otsu, Japan). Specific primers for 3' RACE, 5' RACE, and core sequences were designed according to the acquired unigenes [27] and are listed in Table 1. The PCR products were purified and ligated into a pMD19-T vector (TaKaRa, Otsu, Japan) and then transformed into competent *Escherichia coli* DH5 cells. Then, the positive clones were sequenced on an ABI 3130xl Genetic analyzer (Applied Biosystems, Waltham, USA).

2.4. Sequence and phylogenetic analysis

The sequence homology of ScLYZ-2 was analyzed using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/blast>), and the isoelectric point, net charge of the peptide, and molecular weights were calculated on the ExpASY molecular biology server (<http://www.expasy.org/tools>). The ClustalW Multiple Sequence Alignment program (<http://www.ebi.ac.uk/clustalw/>) was used to create the multiple sequence alignment and annotated using GeneDoc software (<http://www.psc.edu/biomed/genedoc>). The signal peptide of the protein was identified using the SignalP program (version 3.0) (<http://www.cbs.dtu.dk/services/SignalP>). The phylogenetic tree was constructed using the neighbor-joining (N-J) method in the MEGA 5.0 software with 10,000 bootstrap replicates. The identities between each pair of the amino acid (AA) sequences were calculated using the MatGAT package (version 2.02).

2.5. Quantification of mRNA expression

RNA extraction and cDNA synthesis from the gills, hepatopancreas, abdominal foot, water pipes, and hemocytes of healthy razor clams were performed using the method described above. The RNAs were incubated with RNase-free DNase I (Takara Bio, Otsu, Japan) and

Table 1
Primers used in this study.

Primer Name	Primer Sequence (5'-3')	Used for	amplified fragments
ScLYZ-2-3'-1	CAGTTTCCTCCCGGACCTGTTGA	3' RACE	203 bp
ScLYZ-2-3'-2	CGAATGACTTGACGTGTGC		
ScLYZ-2-5'-1	GTGTTCCCTAGCGTACGCTGGC	5' RACE	213 bp
ScLYZ-2-5'-2	CCTACTTCCATTGACAGTC		
qScLYZ-2-F	CTGCCATACTATCAAGACTGCGG	Real-time PCR	221 bp
qScLYZ-2-R	TTCAGAGCCTCCCAGTATTTGAG		
ScLYZ-2-F	CAGTTTCCTCCCGGACCTGTTGA	encoding mature peptide	378 bp
ScLYZ-2-R	TAAACAAGATGCCACATCCGG		
ScLYZ-2-BamH I-F	GGATCCAGTTTCCTCCCGGACCTGTTG	Vector construction	378 bp
ScLYZ-2-Xho I-R	CTCGAGTTAAACAAGATGCCACATCCG		
qScβ-actin-F	AAGAGCCGTGTTCCATCC	Real-time PCR	89 bp
qScβ-actin-R	AGCCTCATCTCCACATAGC		

quantified with a NanoDrop Spectrophotometer (Thermo Scientific, MA, USA). Then, the single-strand cDNA was synthesized from 1 µg of total RNA using the PrimeScript™ RT-PCR Kit (TaKaRa, Otsu, Japan). qRT-PCR was performed using the ABI 7500 PCR system. Each reaction contains 1 µL of reverse and forward primers (Table 1), 10 µL of SYBR Green Mix (TaKaRa, Otsu, Japan), and 2 µL of 1:10 diluted cDNA and RNase-free water to a final reaction volume of 20 µL. The expression of ScLYZ-2 mRNA was detected by qRT-PCR using specific primers qScLYZ-2-F and qScLYZ-2-R (Table 1), and Scβ-actin gene was used as the internal control. The reaction was performed at the following conditions: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 20 s, 72 °C for 30 s, and a final extension of 72 °C for 10 min. The amplification efficiencies of ScLYZ-2 and Scβ-actin were 97.4% and 104.4%, respectively, as determined using the standard curve drawn by the dilution series from 10² to 10⁹. Finally, the qRT-PCR single-peak products were verified by sequencing and 3% agarose gel electrophoresis. All data were given in terms of relative mRNA by the 2^{-ΔΔCT} method [28].

2.6. Expression, purification, and refolding of ScLYZ-2

Based on the gene sequences encoding ScLYZ-2, specific primers (Table 1) were designed to amplify the predicted sequences. The purified PCR products were digested with restriction endonuclease (*Bam*H I and *Xho* I) and inserted into the pET-28a expression vector to construct the recombinant plasmids pET-ScLYZ-2. Then, the recombinant plasmids pET-ScLYZ-2 were transformed into the *E. coli* BL21(DE3) competent bacteria and cultured in LB medium. After the bacteria grew to the mid-logarithmic phase, 1 mM isopropyl thiogalactoside was added and incubation at 37 °C for 8 h. Subsequently, the bacteria were harvested through centrifugation at 8000 × g and 4 °C. The obtained cell pellet was used for protein purification using HisTrap™ Ni-Agarose (GE, USA) according to the manufacturer's instructions. For refolding, the recombinant ScLYZ-2 was subjected to the stepwise dialysis method [29]. In addition, the recombinant ScLYZ-2 was dialyzed from 6, 3, 2, 1, 0.5, and 0 M urea of guanidine HCl dialysis fluid to PBS for more than 12 h at 4 °C. Moreover, the L-arginine and oxidized glutathione (GSSG) were added to obtain a high biological activity of recombinant ScLYZ-2 at the 1 and 0.5 M dialysis stage. Then, the purified and folded ScLYZ-2 (rScLYZ-2) were treated with Triton X-114 to remove the endotoxin [30]. Thereafter, the rScLYZ-2 was subjected to 12.5% SDS-PAGE for analysis, and its concentrations were determined by the Bradford method and stored at -20 °C.

2.7. Determination of enzyme activity

The activity of lysozyme in the lysate supernate of hemocytes, gills, and hepatopancreas was detected using commercial assay kits (Shanghai Tongwei Biotechnology Co., Shanghai, China) based on the manufacturer's instructions. Briefly, the lysate supernate was first

added to the microplates (48-well) precoated with lysozyme capture antibody for 1 h incubation at 37 °C. After washing, the HRP-labeled antibody was added into the microplate for 1 h at 37 °C. Color development was carried out with tetramethylbenzidine, and the absorbance was measured using an automated ELISA reader at 405 nm. The unit definition of lysozyme activity corresponded to 1 mg of phenol liberated per 100 mL of lysate supernate at 37 °C in 30 min. The lysozyme activity was expressed as U/100 mL of lysate supernate.

2.8. Antimicrobial activity assays

The disc diffusion assay was used to assess the antimicrobial activity of rScLYZ-2 based on our previous study [31]. Bacterial strains were used as substrates, including two Gram-positive bacteria (*M. luteus* and *S. aureus*) and three Gram-negative bacteria (*V. splendidus*, *V. harveyi*, and *V. parahaemolyticus*). The nutrient agar medium (15 g/L of agarose, 3 g/L of beef extract, 10 g/L of tryptone, 5 g/L of NaCl, PH 7.3 ± 0.1) containing two Gram-positive bacterial species (10⁷ CFU/mL) and the 2216E medium containing three Gram-negative bacterial species (10⁷ CFU/mL) were poured onto 90 mm plates. Then, the Oxford cup (8 mm diameter) was placed onto the solidified medium. The cups were immersed in 20, 50, and 100 µg of the rScLYZ-2 solutions with equal volumes. *M. luteus* and *S. aureus* were cultured at 28 °C for 24 h, and *V. splendidus*, *V. harveyi*, and *V. parahaemolyticus* were cultured at 35 °C for 24 h. The sterile liquid medium and PBS at equal volumes were set as the controls. Finally, the diameter of the transparent circle around each Oxford cup was measured after incubation.

The minimal inhibitory concentration (MIC) of rScLYZ-2 against *V. parahaemolyticus* was detected based on the method described previously [26]. The rScLYZ-2 proteins (28.8 µmol/L) were gradient-diluted twice in a sterile 96-well microtiter plate. Furthermore, 50 µL of *V. parahaemolyticus* suspension (0.5 × 10⁴ CFU/mL) was added in each column. The column without rScLYZ-2 was used as the control. After incubation at 28 °C overnight, the microtiter plate was read at OD₆₀₀. Each assay was performed in triplicate.

3. Results and discussion

3.1. Cloning and sequence analysis of ScLYZ-2

The full-length cDNA sequence of ScLYZ-2 (GenBank accession number: MG544120) was 1558 bp, including a 3'-untranslated region (UTR) of 757 bp with polyadenylation signal sequence (AATAAA) located upstream of the poly(A) tail, a 5'-UTR of 375 bp, and an ORF of 426 bp encoding 142 AA (Fig. 1). The isoelectric point of ScLYZ-2 is 7.90. A signal peptide of 16 AA was found in the deduced AA sequences of ScLYZ-2 by SMART program, indicating that the protein was secreted. Previous studies in various animals have demonstrated that lysozymes play key roles in the inflammatory response [32–34]. However, no study has investigated the inflammatory response of lysozyme

1 ACTTCTCGTAGTTATGTAACAGCTTACACTTATTTACGAAGAACCTCATTAAACCACT
61 TACACACTTACATCATTGGCTCTTCTTATAGTTTGACCTGATAATGAGTAATAATAGA
121 ATACAAATACTTTGCAAGATAGGTTTGTATCTGTAATAAAACGGAAGTACCTACTAGTAC
181 GGACGGAATCCGCCATTTTTGGGAATATAATGGTTTATAGTAGAACAATTTGTTATT
241 TCTAGGAATAACTGGATACATTGCATTACATATCTACAACAACACCGCCAACGTTTT
301 GAAATAAGCTACGCCATCTTTGAGACAACCGAAGACAGAAGATAGTTTACACTAGTGT
1 M L F I A V F L A V V A I A G
361 TGATTCCGCTCCAGGATGTTGTTTATTGCCGTGTTCTGGCAGTGGTGGCCATAGCTGGC
16 **G** Q F P P G P V E D D C M S C I C T M E
421 GGTCAGTTTCTCCCGACCTGTGAAGACGACTGTATGAGTTGTATCTGCACGATGGAA
36 S G C N P L D C R M D V G S L S C G Y F
481 TCCGGATGTAATCCATTAGACTGTCAATGGATGATAGGGTCCCTTCTCTGTGGATATTC
56 Q I K L P Y Y Q D C G Q P R S D L G W K
541 CAGATCAAGCTGCCATACTATCAAGACTCGGTCAACCACGCTCAGATCTGGCTGGAAG
76 G C S N D L T C A A T C V Q N Y M R R Y
601 GGTTGTTCAAGTACTTGACGTGTGACGAACTGTGTTCAAATACATGAGACGTTAT
96 V G R S G C S P T C Q T Y A R E H N G G
661 GTTGGGCGTCCAGCTGCAGTCCAACATGCCAGCTACGCTAGGGAACACAACGGTGGAA
116 P I G C R R S S T L K Y W E A L K K I P
721 CCCATTGGATGCCCGGAGCTCGACGCTGAAATACTGGGAGGCTCTGAAGAAAATCCCC
136 G C G H L V *
781 GGATGTGGCATCTGTTAAGGACATAACGTCGTCTTGGCTAGTTGTAATCAAATCCA
841 GTGCTAACTGTTTATTACTTCTTGATATGCAACTTTGTTATGATTGACAGCTCTTA
901 AATATTAAGGTGACCTGAACGTCTGTGATCTGTGTTTGTCTGTAATGTAATACGAAAC
961 AGACGTCACCTAACGTAACATAAAATCATTACGTCGCCATCCGTTAGCGGATCCATTACT
1021 AATGTAAGCAGTTTAAAGAATTTGTTAATTTTGTGTTGAATATCCGACCACCACTACA
1081 ATAACATGGCTTGAACAATTTACTTTATGCCACTCGAATGCACTTTACTGTTGGGA
1141 TACTGCCAATATGCTATAGTGTATTTATTTGTAATCTTATTATAAATAGTCTCGGCAACAT
1201 ATGTTTTTTATCGGCTTACCAAAATTAATAGATACCCTTTCTAATATCTCAAGTAGAT
1261 ATCATATGATAATTTAGCGTGGCTATTTCTTCTTTTTTCTTTGTTTGTGTAATTA
1321 CTGTCGATGCAAACTGAAAATACTATTATTACCGTCAGTCTATTGTGTTACAATCAAT
1381 CTATTTGTGAATATTAGAATTTATATACCGAAGGTGAAAGATGGACTAACAAATCTTT
1441 TACATATATCAGTGGGAATTAGAACGATCTTGTTTTATTGTGTTGTATGAAAGACG
1501 ATGCTGAATCTATAACTTAAATAAACGACTTTATTCGTCGAAACTGAAAAA

Fig. 1. Nucleotide and deduced AA sequences of ScLYZ-2 cDNA (GenBank accession number: MG544120). The start codon is marked in black bold and the stop codon is marked in asterisk. The predicted signal peptide of ScLYZ-2 is in gray shadow. The predicted lysozyme activity sequences of ScLYZ-2 were double underlined. The cysteine residues were marked in blue and two active sites were marked in red. The RNA instability motifs (ATTTA) and polyadenylation signal (AATAAA) was marked in bold italics. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in shellfish. In the present study, two copies of a cysteine-rich motif of “C-X-C-X4-C-X3-YC-X-C-X6-C-X3-C-X-X2-C” located from 72 to 113 AA were found in ScLYZ-2. The cysteine-rich motif of ScLYZ-2 is similar to a various of CXC domains motifs from plants and animals that function during inflammation [35,36]. Thus, we speculate that ScLYZ-2 may participate in the inflammatory response and should be determined in our future studies. A typical domain named pfam destabilase located from 24 to 134 AA was found in ScLYZ-2, and the gene ontology function showed that the domain has lysozyme activity. Similarly, the function of this domain was also found in other i-type lysozymes from bivalves [20,21]. The i-type lysozyme has several specific signatures, including the highly conserved region MDVGLSCGP(Y/F)QIK and the specific motif CL(E/L/R/H)C(I/M)C, high content of

cysteine residues, and approximately 11–20 kDa molecular weight [18,37–39]. In the present study, the theoretical molecular weight of the mature peptide of ScLYZ-2 was approximately 17.3 kDa. The ScLYZ-2 contains high amounts of cysteine residues (about 10%, 14 of 141 AA), which is consistent with other bivalve i-type lysozymes [16,21,40], suggesting that more cysteine residues existed in the razor clam, possibly rendering it to become more stable [41]. In addition, the highly conserved region “MDVGLSCGP(Y/F)QIK” and the specific motif “CL (E/L/R/H)C(I/M)C” were also found in ScLYZ-2, although the AA residues of “L” and “(E/L/R/H)” in the specific motif “CL (E/L/R/H)C(I/M)C” were instead “M” and “S,” respectively (Fig. 2, red line). Taken together, these signatures described above indicated that ScLYZ-2 is a new subfamily member of i-type lysozymes.

3.2. Sequence homology and phylogenetic relationships of ScLYZ-2

Blast homology analysis revealed that the AA sequence identity between ScLYZ-2 and the vertebrate lysozyme gene was fairly low. Meanwhile, ScLYZ-2 shared higher AA sequence identity (42%–58%) with the lysozyme gene of other bivalve species, with the highest identity of lysozyme gene to *R. philippinarum* and the lowest identity to *C. gigas* (Fig. 2). Moreover, the AA sequence identity between ScLYZ-2 and ScLYZ-1 was 53%, suggesting that they have similar biological functions. Previous studies have showed that i-type lysozymes could exert isopeptidase and lysozyme activities [13,16,42]. In this study, we found that region A (30–64 AA) of ScLYZ-2 was probably responsible for the lysozyme activities, and region B (77–118 AA) might be involved in isopeptidase activities. However, the isopeptidase activities of ScLYZ-2 proteins should be determined in our future studies. In addition, two families with highly conserved lysozyme catalytic residues (Glu³⁵ and Asp⁴⁶) that function in the hydrolysis of β -1,4-glycosidic bonds in the peptidoglycan of the bacterial cell wall were also found in ScLYZ-2 [43].

The N-J method was used for the phylogenetic tree construction of ScLYZ-2 with other lysozymes (Fig. 3). The tree was separated into two main clusters corresponding to invertebrates and vertebrates. Both ScLYZ-2 and ScLYZ-1 were located in the invertebrate clade. ScLYZ-1 was grouped closely with *Mytilus galloprovincialis* and *Mizuhopecten yessoensis*, whereas ScLYZ-2 was grouped closely with *C. gigas* and *C. virginica* that demonstrated the evolution, indicating that the lysozymes underwent an adaptive evolution through duplication [5,16].

3.3. Tissue distribution of ScLYZ-2 transcript in healthy razor clams

The expression of ScLYZ-2 in tissues of healthy razor clams, including hemocytes, gills, hepatopancreas, water pipes, and abdominal foot, were detected by qRT-PCR, and the expression level of ScLYZ-2 in hemocytes served as the control (Fig. 4A). ScLYZ-2 mRNA transcript was found in all of the examined tissues, with the highest expression observed in the hepatopancreas ($p < 0.05$), followed by the gills, water pipes, and abdominal foot ($p > 0.05$), indicating that ScLYZ-2 plays roles in various biological processes [19]. Hemocytes are vital immune cells that function in immune defenses [44], detoxication [45], and tissue repair [46]. However, the expression level of ScLYZ-2 in hemocytes was relatively low in the present study. Our previous study in *S. constricta* also showed the low expression of ScLYZ-1 in hemocytes [18], indicating that the hemocytes of razor clam contains low abundance of lysozyme genes. The hepatopancreas is often considered an immune organ that functions in resisting the invasion of pathogens [19]. In this study, the expression level of ScLYZ-2 in the hepatopancreas was significantly higher than that in other tissues, suggesting that it could digest various pathogens as digestive enzymes in its living marine environment filled with abundant microorganisms [39,47]. The gills are the respiratory organ of razor clam, which can filter pathogens in seawater by the protective mucus on their surface [19]. However, the ScLYZ-2 transcript in gills had the lowest expression levels. Moreover,

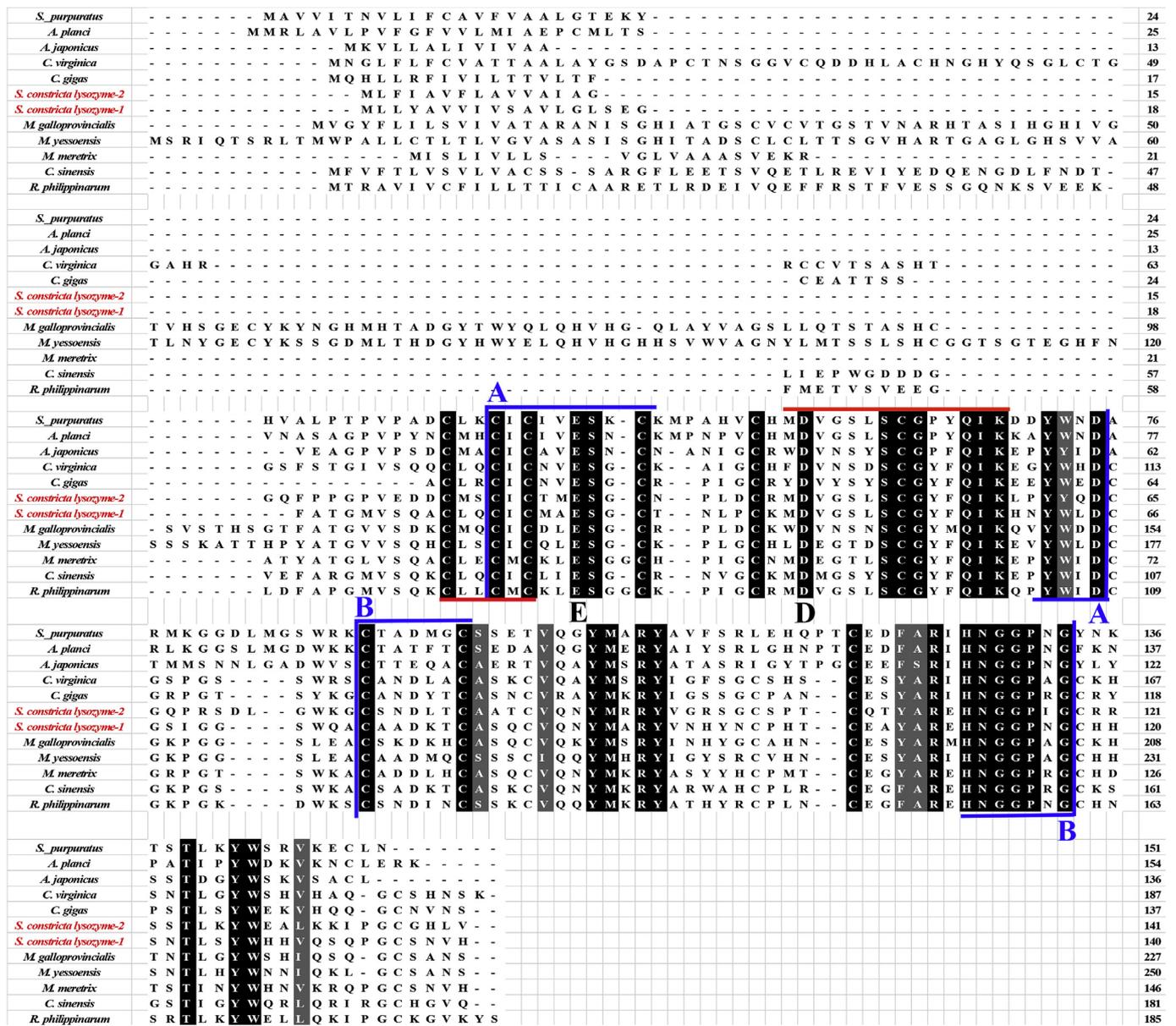


Fig. 2. Multiple alignment of the deduced AA sequences of ScLYZ-2 with other known lysozymes. The consensus residues were shaded with the threshold of more than 80% identity. Identical residues were presented in black, and similar residues were indicated in light gray. The family signature of i-type lysozymes was marked in red line. The two highly conserved lysozyme catalytic residues, namely, aspartate (D) and glutamate (E), were presented directly below the alignments. Region A was identified correlation to lysozyme activity, and Region B was identified correlation to isopeptidase activity. The AA sequences accession numbers: *Strongylocentrotus purpuratus* lysozyme (XP_788343.1), *Acanthaster planci* lysozyme (XP_022088600.1), *Homo species* lysozyme (ACO37637.1), *R. philippinarum* lysozyme (ACU83237.1), *Apostichopus japonicus* lysozyme (AHH27701.1), *Rattus norvegicus* lysozyme (AAA41551.1), *Sus scrofa* lysozyme (AAB16862.1), *C. gigas* lysozyme (BAF48044.2), *S. constricta* lysozyme (MG544119), *M. galloprovincialis* lysozyme (AJQ21515.1), *M. meretrix* lysozyme (ADL27913.1), *Cyclina sinensis* lysozyme (AET13645.1), *C. virginica* lysozyme (AB206328.1), *M. yessoensis* lysozyme (XP_021354667.1). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

ScLYZ-2 showed lower expression levels in the gills and hepatopancreas compared with ScLYZ-1, as detected in our previous study [18]. In another study, the expression of *C. virginica* lysozyme-1 gene was higher in labial palps and mantles, while the transcripts of lysozyme-2 gene were highly expressed in digestive gland tissues [48]. The differences in the expression levels of different isoforms of lysozyme gene in tissues suggested that they had different biological functions during their evolution [16,19]. Furthermore, previous studies showed that the higher the gene expression, the slower the evolution [49–52]. In the present study, the expression of ScLYZ-2 was lower than that of ScLYZ-1 detected in our previous study, suggesting that the evolution of ScLYZ-2 was faster than that of ScLYZ-1.

3.4. Transcriptional responses of ScLYZ-2 in hemocytes, gills and hepatopancreas after *V. parahaemolyticus*, *V. harveyi*, *V. splendidus*, *M. luteus* and *S. aureus* challenge

The hemocytes, gills, and hepatopancreas are the vital tissues for resisting pathogen invasion in bivalves, and the profiles of immune-related genes are usually detected in these tissues [39,53]. Therefore, qRT-PCR was used to examine the expression profiles of ScLYZ-2 in the gills, hepatopancreas, and hemocytes after pathogens challenge (Fig. 4). During *V. parahaemolyticus* challenge, the expression of ScLYZ-2 in the hepatopancreas slightly decreased at 6 h post challenge first and then gradually increased, reaching the peaks at 24 h (2.13-fold, $p < 0.05$),

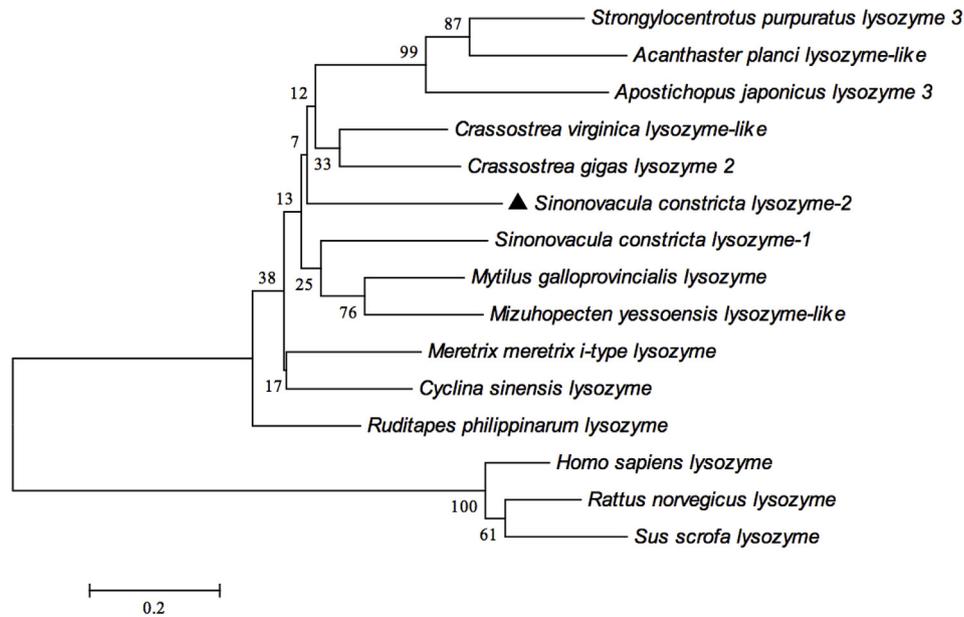


Fig. 3. Phylogenetic tree analysis of the relationship of ScLYZ-2 with other known lysozyme AA sequences from various organisms by the N-J method. Numbers at the nodes represent bootstrap proportions on 1000 replicates.

but decreased to normal levels at 48 h compared with that in the control group (Fig. 4B); The expression profiles of ScLYZ-2 in the gills and hemocytes were similar to those in the hepatopancreas, with the highest expression both at 24 h post challenge (1.53-fold in the gills, 3.53-fold in hemocytes, $p < 0.05$) compared with the control (Fig. 4C and D); Moreover, the expression of ScLYZ-2 in the gills was slowly suppressed but still maintained a high level at 48 h post challenge (1.50-fold, $p < 0.05$) compared with the control. These results

demonstrated that the mRNA level of ScLYZ-2 in the gills, hemocytes, and hepatopancreas was considerably induced after *V. parahaemolyticus* challenge and reached their peaks at 48 h. Similarly, the expression levels of ScLYZ-2 in the gills and hepatopancreas post *V. harveyi*, *V. splendidus*, *M. luteus* and *S. aureus* infection were also significantly up-regulated ($p < 0.05$) (Fig. S1). Thus, ScLYZ-2 could respond to pathogen invasion. Moreover, i-type lysozymes can shift the function from defensive to digestive through evolution [5,16]. In this study, the

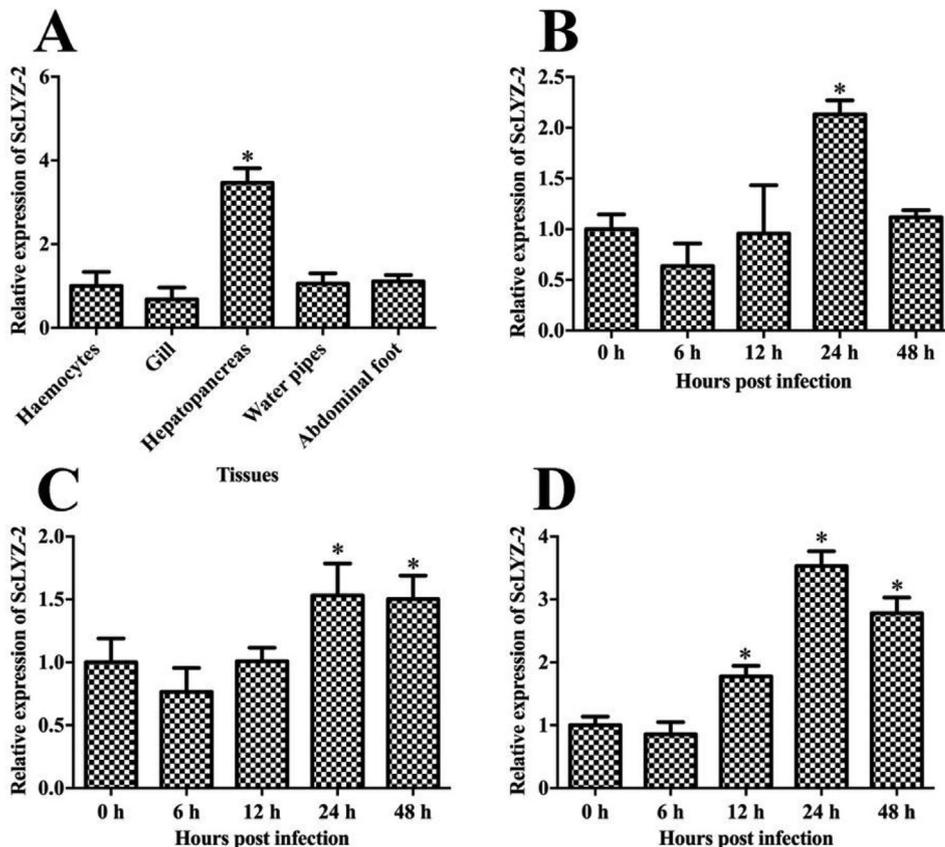


Fig. 4. Tissue distribution and time-course expression of ScLYZ-2 detection by qRT-PCR. A: Expression profiles of ScLYZ-2 mRNA in various tissues of healthy razor clams; B: Expression profiles of ScLYZ-2 in gills post *V. parahaemolyticus* infection; C: Expression profiles of ScLYZ-2 in hepatopancreas post *V. parahaemolyticus* infection. D: Expression profiles of ScLYZ-2 in hemocytes post *V. parahaemolyticus* infection. Values were presented as mean \pm SD, $n = 3$. Asterisks indicated the significant differences ($p < 0.05$).

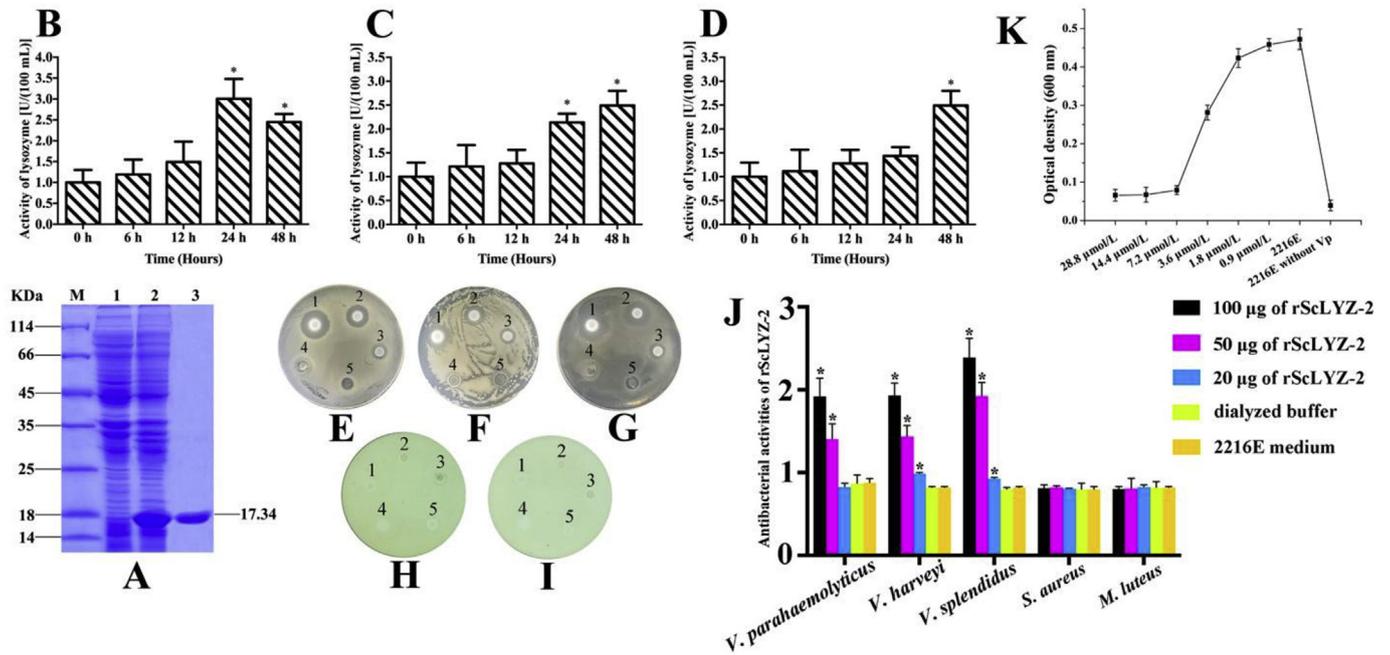


Fig. 5. Antibacterial activities of rScLYZ-2. **A:** SDS-PAGE analysis of the rScLYZ-2. Lane M: protein marker; Lane 1: recombinant clones without IPTG induction; Lane 2: recombinant clones with IPTG induction; Lane 3: purified rScLYZ-2. **B, C and D:** Activity of lysozyme in hemocytes, hepatopancreas and gill after challenge with *V. parahaemolyticus*, respectively. The results were present as the mean \pm SD, $n = 3$. Asterisks indicated the significant differences ($p < 0.05$). **E:** *V. parahaemolyticus*; **F:** *V. harveyi*; **G:** *V. splendidus*; **H:** *M. luteus*; **I:** *S. aureus*. (1): 100 μ g of rScLYZ-2; (2): 50 μ g of rScLYZ-2; (3): 20 μ g of rScLYZ-2; (4): dialyzed buffer; (5) 2216E medium; **J:** Quantitative results of antibacterial activity of rScLYZ-2 against *V. parahaemolyticus*, *V. harveyi*, *V. splendidus*, *M. luteus* and *S. aureus*. The antibacterial activity of dialyzed buffer or 2216E medium was set as the control. Results were present as the mean \pm SD, $n = 3$. Asterisks indicated the significant differences ($p < 0.05$). **K:** growth results of *V. parahaemolyticus* at different concentrations of rScLYZ-2 (OD₆₀₀). Values were presented as mean \pm SD, $n = 3$.

expression level and peak time of ScLYZ-2 in the gills and hepatopancreas post challenge were lower and later than those of ScLYZ-1, respectively. Hence, ScLYZ-2 evolved faster than ScLYZ-1, suggesting that ScLYZ-2 is mainly involved in digestive function.

3.5. Expression and purification of ScLYZ-2

After IPTG induction, gene coding ScLYZ-2 was efficiently expressed in *E. coli* BL21(DE3) with the pET-28a express vector. SDS-PAGE showed a distinct 17 kDa band (Fig. 5A), which was in agreement with the predicted molecular weight of ScLYZ-2. The histidine-tagged rScLYZ-2 with high purity was obtained after being purified with Ni²⁺ affinity chromatography under native conditions. However, the purified proteins of rScLYZ-2 were found in the form of inclusion bodies. When the step dialysis method was applied for refolding inclusion bodies [29], the soluble proteins of rScLYZ-2 were gained. The folded rScLYZ-2 was further analyzed by SDS-PAGE. The molecular weight of rScLYZ-2 was consistent with the calculated molecular weight of 17.34 kDa, and mass spectrometry also confirmed that the purified proteins were rScLYZ-2 (Fig. S2).

3.6. Antimicrobial activity of recombinant ScLYZ-2

Lysozymes are defense enzymes of the non-self recognition system in host defense reaction [54]. In this study, the ScLYZ-2 mRNA in hemocytes, gills, and hepatopancreas was significantly expressed after *V. parahaemolyticus* challenge, and the results were in line with the expression of ScLYZ-1 in our previous study [18]. Hence, both ScLYZ-1 and ScLYZ-2 mRNA can respond positively to host immune responses upon pathogen invasion. To further evaluate the antimicrobial activity of rScLYZ-2, we first examined the activity of lysozyme in the gills, hepatopancreas, and hemocytes of razor clam after *V. parahaemolyticus* challenge from protein level. The lysozyme activities in hemocytes (Fig. 5B), hepatopancreas (Fig. 5C), and gills (Fig. 5D)

significantly increased ($p < 0.05$), and all reached the maximum values at 48 h post challenge, suggesting that ScLYZ-2 protects the host from pathogens at the transcriptional and protein levels. A previous study showed that i-type lysozymes of aquatic animal exhibit activities against Gram-negative and Gram-positive bacteria [45]. Thus, we next investigated the antimicrobial activity of ScLYZ-2 against three Gram-negative bacterial species and two Gram-positive bacterial species. Results showed that rScLYZ-2 possessed strong antimicrobial activity against *V. harveyi* (1.93 ± 0.15 cm), *V. splendidus* (2.39 ± 0.23 cm), and *V. parahaemolyticus* (1.92 ± 0.22 cm) at the highest dose of 100 μ g. The rScLYZ-2 displayed moderate antimicrobial activity against *V. harveyi* (1.43 ± 0.14 cm), *V. splendidus* (1.92 ± 0.17 cm), and *V. parahaemolyticus* (1.40 ± 0.19 cm) at the dose of 50 μ g. Treatment with 20 μ g of rScLYZ-2 was less effective against *V. harveyi* (0.98 ± 0.02 cm) and *V. splendidus* (0.92 ± 0.04 cm), and no significant inhibition zone of *V. parahaemolyticus* was found (Fig. 5E–G). However, rScLYZ-2 showed no lysozyme activity for the two Gram-positive bacteria, *S. aureus* and *M. luteus* (Fig. 5H and I). The quantitative results of antibacterial activity of rScLYZ-2 against the five bacteria were also shown along with the inhibition zones on the plates (Fig. 5J). Likewise, in two i-type lysozymes from *Procambarus clarkii*, rScLYZ-1 tends to inhibit the growth of Gram-negative bacterial species but not Gram-positive bacterial species [15,18]. Conversely, two types of i-type lysozymes from *C. virginica* and lysozyme purified from *Chlamys islandica* significantly inhibited the growth of both Gram-positive and Gram-negative bacteria, such as *Bacillus cereus*, *Listeria monocytogenes*, *V. vulnificus*, and *E. coli* [16,55]. Moreover, the recombinant c-type lysozymes from *Penaeus monodon* and *Venerupis philippinarum* can also inhibit the growth of Gram-positive bacteria [26,56]. Thus, we need to determine whether rScLYZ-2 uniquely protects against Gram-negative pathogens in our future studies. Given that *V. parahaemolyticus* is an important pathogen for razor clams, we further detected the MIC of rScLYZ-2 against *V. parahaemolyticus*. The results revealed that the growth of *V. parahaemolyticus* was significantly

inhibited by the MIC of rScLYZ-2, with 7.2 $\mu\text{mol}/\text{mL}$ concentration (Fig. 5K). In conclusion, a new lysozyme gene ScLYZ-2 was cloned from the razor clam. The sequence characteristics showed that ScLYZ-2 belongs to the i-type lysozyme family. Lysozyme as a natural and safe antibacterial substance, high expression of ScLYZ-2 after *V. parahemolyticus*, *V. harveyi*, *V. splendidus*, *M. luteus* and *S. aureus* challenge and its antibacterial activity against three Gram-negative bacteria in this study, which suspected that the application of recombinant lysozyme as a green preparation to aquaculture will become a new way for the healthy breeding of aquatic animals.

Notes

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fsi.2019.03.077>.

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