An i-type lysozyme (CfLyzI) involved in innate immunity is essential for the survival of Chlamys farreri during Vibrio stimulation

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Abstract
Lysozymes act as key components of the innate immunity in invertebrates and play a pivotal role in early defense against invading microbe infection. In this study, an i-type lysozyme homology was identified and characterized in Zhikong scallop Chlamys farreri (designated as CfLyzI). The full-length cDNA sequence of CfLyzI contained a 702 bp open reading frame (ORF), which encoded a polypeptide of 233 amino acids and contained a typical destabilase function domain. The mRNA transcripts of CfLyzI were detectable in all the investigated tissues, including hemocytes, muscle, mantle, gill, hepatopancreas and gonad with the peak level in hemocytes. Being stimulated by Vibrio splendidus, the mRNA transcripts of CfLyzI significantly increased in hemocytes. The CfLyzI-suppressed scallops turned to be more susceptible to Vibrio. All these results indicated that CfLyzI acted as an efficient effector in the innate immunity and was also essential for hosts’ survival during Vibrio stimulation in Zhikong scallop.

Key Words: Chlamys farreri; Innate immunity; Invertebrate type lysozyme

Introduction
The innate immunity, also known as non-specific immunity or in-born immunity, acts as first line for all the multicellular organisms and almost the only defense mechanism for invertebrates that protects hosts from microbial invaders (Medzhitov and Janeway Jr, 1997). Antimicrobial proteins and peptides (AMPs), also called host defense peptides (HDPs), are ancient effector molecules of innate immunity, and provide a principal first line of defense against the microbial pathogens in all living organisms (Hoffmann et al., 1999). Among all the identified AMPs, lysozyme (EC 3.2.1.17), also termed as N-acetylmuramidase glycanhydrolase or muramidase is an antimicrobial enzyme forming part of the innate immunity and also regarded as an important digestive enzyme in animals, especially in ruminant artiodactyls and filter-feeding organisms (Daffre et al., 1994; Boman, 1995).

Lysozyme makes up a large amount of proteins and peptides and ubiquitously presents in diverse organisms ranging from human to virus (Johnson, 1998). Based on catalytic characteristics, molecular features and original sources, lysozymes could be classified into several types, including bacteria type, chalaropsis type (ch-type), chicken/conventional type (c-type), goose type (g-type), invertebrate type (i-type), phage type and plant type (Jielian et al., 2017). Among all the animal origin types, c-type and g-type lysozymes are present in all the vertebrates, while invertebrates mainly produce i-type lysozymes and partially produce c-type, ch-type or g-type lysozymes (Gallewaert and Michiels, 2010). Moreover, i-type lysozymes exhibit multiple activities, such as chitinase, isopeptidase, muramidase and non-enzymatic antibacterial activities (Van Herreweghe and Michiels, 2012). Zhikong scallop Chlamys farreri (Mollusca; Bivalvia; Pteriomorphia) is a dioecious bivalve native to the coast of China, Japan and Korea, and weightily contributes to the aquaculture industry of...
northern China (Li et al., 2017b). However, scallop aquaculture industry has been experiencing mass mortality during summer period and suffering from extensive economic losses in the past decades (Mattozzo, 2016; Huang et al., 2018). The complex interactions among environment, hosts and pathogen are regarded to be the main causes for such mass mortality of cultured scallops (Wang et al., 2012). Lysozymes make a major contribution to the accomplishment of innate immune responses (Saurabh and Sahoo, 2008). In previous studies, a g-type lysozyme (designated as CfLyzG) exhibited inhibitive effect on the growth of both Gram negative and Gram positive bacteria with more potential activities against Gram positive bacteria, and its single nucleotide polymorphisms (SNPs) were associated with resistance or susceptibility to Vibrio (Listonella) anguillarum (Zhao et al., 2007b; Li et al., 2009; 2013). In the present study, an i-type lysozyme gene (designated as CfLyzI) has been cloned and investigated in Zhikong scallop, and the main objectives of the present study were (1) to characterize the molecular features of CfLyzI (2) to validate the tissue and temporal expression patterns of its mRNA transcripts, and (3) to confirm the function of CfLyzI via double strand RNA (dsRNA) mediated RNA interference (RNAi).

Materials and methods

Scallop, Vibrio stimulation, temperature stress and samples collection

Vibrio splendidus was cultured in liquid 2216E media (HB0132-1, HopeBioL, China) at 28 °C and 140 rpm overnight. The bacteria were collected by centrifugation at 4000 g for 10 min and re-suspended in filtered seawater. Approximately sixty scallops were employed for Vibrio stimulation assay. Scallop samples were purchased from a local farm in Qingdao, China, and maintained in aerated seawater at 20 °C. After acclimated for two weeks, thirty scallops were immersed with live bacteria V. splendidus at a final concentration of 1.0 × 10⁸ colony forming units (CFUs) per 1 mL, defined as Vibrio stimulation group. The rest thirty scallops were employed as the control group. Five scallops from the two groups were randomly sampled at 0, 3, 6, 12, 24 and 48 h post stimulation, respectively. For the temperature stress assay, modified from the previous report (Ding et al., 2018), the scallops were randomly divided into five groups, and treated at 10 °C, 15 °C, 20 °C (as control), 25 °C and 30 °C for 7 days, respectively. Five scallops from each group were sampled. Hemocytes, muscle, mantle, gill, hepatopancreas and gonad from five untreated scallops were collected to determine the mRNA distribution of CfLyzI.

RNA preparation and cDNA synthesis

Total RNA was isolated from the hemocytes and other tissues using RNAiso Plus (9108, Takara, Japan). The first-strand cDNA was synthesized with SuperScript IV Reverse Transcriptase (18090010, Thermo Fisher Scientific, USA) using the RO1 DNase I (M6101, Promega, USA) treated total RNA as template and adaptor primer-primer-oligo (dT) as primer (Table 1). The reactions were performed at 55 °C for 1 h, terminated by heating at 80 °C for 5 min, then a homopolymeric tail was added using dCTP (4028, Takara, Japan) and terminal deoxynucleotidyl transferase (TdT, 2230, Takara, Japan), and then stored at -80 °C till used.

Cloning the full-length cDNA of CfLyzI

In our previous studies, a transcript sequence homologues to previous identified i-type lysozymes was identified in C. farreri via assembling and screening public available expression sequence tags and transcriptomic data (Wang et al., 2018b). And this transcript sequence was selected for further cloning of CfLyzI. Four gene-specific primers, CfLyzI-RACE-R1/2 and CfLyzI-RACE-F1/2, were designed to clone the full-length cDNA of CfLyzI via 5’ and 3’ rapid amplification of cDNA ends (RACE) technique, respectively (Table 1). All the PCR reactions were performed in a MJ Mini Personal Thermal Cycler (Bio-Rad, USA), and PCR products were purified with Monarch DNA Gel Extraction Kit (T1020S, NEB, USA), ligated into the pMD18-T simple vector (D103A, Takara, Japan), and then transformed into the competent cells Escherichia coli DH5α (CB101, Tiangen, China). The positive recombinants were identified using anti-ampicillin selection and confirmed by PCR screening using the universal primers M13-47 and RV-M (Table 1). Five of the positive clones were sequenced in a PRISM 3730XL automated sequencer (Thermo Fisher Scientific, USA).

Bioinformatics analysis of CfLyzI cDNA and deduced protein sequences

Blast + 2.7.1 was employed to conduct the search for sequence similarities. The deduced amino acid sequences of CfLyzI were analyzed by Lasergene suite 7.0.0.44 using the EditSeq module. The presence and location of signal peptide sequences of CfLyzI was analyzed using SignalP 4.1 SMART 7.0 was employed to analysis the function domains. Multiple sequence alignments were generated with Clustal Omega 1.2.4 and visualized by Sequence Manipulation Suite 2.0 using the multiple alignment show module.

Quantitative real-time PCR analysis of CfLyzI mRNA expression patterns

The tissue and temporal expression patterns of CfLyzI mRNA in hemocytes were investigated by quantitative real-time PCR (qRT-PCR). All the qRT-PCR reactions were performed in a LineGene K FQD-48A Fluorescence Quantitative PCR Detection System (Bior, China) using the SYBR premix ExTaq (RR420, Takara, Japan). All the primers for qRT-PCR were listed in Table 1. For each sample, the mRNA expression of CfLyzI was normalized to that of elongation factor 1α (EF-1α). The relative abundance of CfLyzI mRNA was calculated using comparative Ct method (2ΔΔCt method) as mean ± SD (Schmittgen and Livak, 2008). The data were subjected to one-way analysis of variance (ANOVA) followed by a multiple comparison using IBM SPSS Statistics 23.0.0.0, and the p values less than 0.05 were considered statistically significant.
**Table 1 Oligonucleotide primers used in the experiments**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Brief information</th>
</tr>
</thead>
<tbody>
<tr>
<td>adaptor primer</td>
<td>GGCACACGCGTCGACTAGTAC</td>
<td>Anchor primer for 3’ RACE</td>
</tr>
<tr>
<td>adaptor primer-oligo (dG)</td>
<td>GGCACACGCGTCGACTAGTAC&lt;i&gt;g&lt;/i&gt;N</td>
<td>Anchor primer for 5’ RACE</td>
</tr>
<tr>
<td>adaptor primer-oligo (dT)</td>
<td>GGCACACGCGTCGACTAGTAC&lt;i&gt;t&lt;/i&gt;VN</td>
<td>Oligo (dT) for cDNA synthesizing</td>
</tr>
<tr>
<td>C(\varepsilon)-1a-qRT-F</td>
<td>ATCTTTCCTCCTACTCCGTTCTCT</td>
<td>Internal control for real-time PCR</td>
</tr>
<tr>
<td>C(\varepsilon)-1a-qRT-R</td>
<td>GGCAAGTTCTCAATCCCTCAATCCCA</td>
<td>Internal control for real-time PCR</td>
</tr>
<tr>
<td>C(\varepsilon)+lyz-CDS-R</td>
<td>ATGTGCAATTATATGATGCTATACTCT</td>
<td>Gene specific primer for CDS</td>
</tr>
<tr>
<td>C(\varepsilon)+lyz-CDS-R</td>
<td>CTAGCGTGTGACCAGAAACCCAT</td>
<td>Gene specific primer for CDS</td>
</tr>
<tr>
<td>C(\varepsilon)+lyz-dsRNA-Basic-F</td>
<td>GACGATAGATGGATAATACAGCAAGAT</td>
<td>Gene specific primer</td>
</tr>
<tr>
<td>C(\varepsilon)+lyz-dsRNA-Basic-R</td>
<td>CCCCACCTCATGCGTAGAGGC</td>
<td>Gene specific primer</td>
</tr>
<tr>
<td>C(\varepsilon)+lyz-dsRNA-T7-F &amp; C(\varepsilon)+lyz-qRT-F</td>
<td>GGATCCTAATCCGACTACATAGGGATCGGACATAGTAAATAATACAAGCAAGAT</td>
<td>Gene primer incorporated with T7 promoter &amp; Gene specific primer for real-time PCR</td>
</tr>
<tr>
<td>C(\varepsilon)+lyz-dsRNA-T7-R</td>
<td>GGATCCTAATCCGACTACATAGGGATCGGACATAGTAAATAATACAAGCAAGAT</td>
<td>Gene primer incorporated with T7 promoter &amp; Gene specific primer for real-time PCR</td>
</tr>
<tr>
<td>C(\varepsilon)+lyz-RACE-R1</td>
<td>ACTCTTTAGCGTGCTCATGCTGCACTCACTCA</td>
<td>Gene specific primer for RACE</td>
</tr>
<tr>
<td>C(\varepsilon)+lyz-RACE-R2</td>
<td>ATCGCGCTATTCTCTTGATGATGATGATGATGATGAT</td>
<td>Gene specific primer for RACE</td>
</tr>
<tr>
<td>EGFP-dsRNA-Basic-F</td>
<td>CGACGATTAAAGGCCCACAGT</td>
<td>GFP specific primer</td>
</tr>
<tr>
<td>EGFP-dsRNA-Basic-R</td>
<td>CTTGACAGCTGCGATGAC</td>
<td>GFP specific primer</td>
</tr>
<tr>
<td>EGFP-dsRNA-T7-F</td>
<td>GGATCCTAATCCGACTACATAGGGATCGGACATAGTAAATAATACAAGCAAGAT</td>
<td>GFP primer incorporated with T7 promoter</td>
</tr>
<tr>
<td>EGFP-dsRNA-T7-R</td>
<td>GGATCCTAATCCGACTACATAGGGATCGGACATAGTAAATAATACAAGCAAGAT</td>
<td>GFP primer incorporated with T7 promoter</td>
</tr>
<tr>
<td>M13-47</td>
<td>CAGCAGTTTCTCTCTAGTCACCAG</td>
<td>Vector primer for sequencing</td>
</tr>
<tr>
<td>RV-M</td>
<td>GACGCGATAAACAATTTCCACACAGG</td>
<td>Vector primer for sequencing</td>
</tr>
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**Knock-down of C\(\varepsilon\)+Lyz in vivo via RNAi**

T7 promoter tagged primers EGFP-dsRNA-T7-F/R and C\(\varepsilon\)+lyz-dsRNA-T7-F/R (Table 1) were used to amplify the cDNA fragments of enhanced green fluorescent protein (EGFP) and C\(\varepsilon\)+Lyz, and the resultant PCR products were purified to synthesize dsRNA. The dsRNA were produced via in vitro transcription according to the methods previously described (Wang et al., 2018a; Wang et al., 2011), and its concentration was quantified using Nanodrop Lite (Thermo Fisher Scientific, USA) and adjusted to a final concentration of 1 mg mL<sup>-1</sup>. One hundred micrograms of C\(\varepsilon\)+lyz dsRNA was injected into adductor of each scallop, and the control groups received an injection of 100 mg EGFP dsRNA or PBS, while the untreated scallops were employed as blank group. Post dsRNA injection, hemocytes from five scallops of each group were collected every 12 h and used for total RNA isolation and cDNA synthesis. The efficiency of gene silence was confirmed via qRT-PCR, and an optimum time point (36 h post dsRNA injection) was selected for the *Vibrio* stimulation and mortality comparison assay.

**Vibrio stimulation and mortality comparison**

Approximately four hundred scallops were employed for *Vibrio* stimulation and mortality comparison assay according to our previous descriptions (Wang et al., 2011; 2018a). These scallops were equally and randomly divided into four groups (one experimental group, two control groups and one blank group) and then each group was subdivided into three subgroups. At 36 h post dsRNA injection, the scallops were stimulated with live *V. splendidus* at the final concentration of 1×10<sup>8</sup> CFUs mL<sup>-1</sup>. The cumulative mortalities were recorded every 12 h. The t-test was used to verify significant differences in mortality levels between different groups, and the p values less than 0.05 were considered as statistically significant.

**Results**

The molecular features of C\(\varepsilon\)+Lyz

The complete cDNA sequence of C\(\varepsilon\)+Lyz was obtained via 5’ and 3’ RACE technique and submitted to GenBank with the accession number KU361831. It comprised 880 bp, containing a 105 bp
5’ untranslated regions (UTR), a 73 bp 3’ UTR with a poly A tail, a polyadenylation signal site (AATAAA) and an 702 bp open reading frame (ORF). This ORF encoded a polypeptide of 233 amino acid residues with a predicted molecular mass at approximately 25.19 kDa and a theoretical isoelectric point (pI) of 7.35. A signal peptide (from M1 to A20) and a typical destabilase domain (from S119 to M227) were revealed in the deduced amino acid sequence. Additionally, two specific motifs (from C123 to C127 and from W130 to K153) were also revealed (Figure 1A). Multiple sequence alignment of CfLyzI with CfLyzG showed that the homology between these two lysozymes was rather low (Figure 1B). While pairwise sequence alignment showed that CfLyzI exhibited higher homology to its invertebrate counterparts, for example, CfLyzI exhibited similarities of 60 % to i-type lysozyme of Chlamys islandica (CAB63451), 53 % to that of Crassostrea rivularis (ADY38955) and 45 % to that of Mytilus galloprovincialis (AJQ21515).

Fig. 1  Sequence features and multiple alignments of CfLyzI. A: Sequence features. The nucleotides and amino acids were numbered along the left margin. The putative signal peptide was underline. The typical function domain was in shade. The specific motifs were double underlined. The stop codon was indicated by the asterisk. The polyadenylation signal (AATAAA) site was boxed. B: Multiple alignments of CfLyzI with CfLyzG. The black shadow region stood for positions with the same amino acids. Similar sites were in grey. Gaps were indicated by dashes.
The tissue and temporal mRNA expression patterns of ClLyzI

The qRT-PCR was employed to detect the tissue and temporal mRNA expression patterns of ClLyzI. The ClLyzI mRNA could be detected in all the investigated tissues. The peak level of ClLyzI mRNA was found in hemocytes, followed by gill, which were 9.18-fold and 3.39-fold of that in gonad, respectively (p < 0.05; Figure 2A). Hemocytes were selected to test the temporal mRNA expression patterns of ClLyzI post Vibrio stimulation. The mRNA expression levels of ClLyzI were significantly up-regulated at 3 h post Vibrio stimulation (4.55-fold compared with the origin level, p < 0.05), reached to the peak level at 12 h (9.09-fold, p < 0.05), and then down-regulated to the origin level at 48 h (Figure 2B). Additionally, ClLyzI mRNA expression levels in hemocytes were temperature-dependent. Compared with the control group, the ClLyzI mRNA expression levels were stable at 15 °C and 25 °C, but significantly decreased at 10 °C and 30 °C, which was 0.53-fold and 0.22-fold compared with the origin level (p < 0.05), respectively (Figure 2C).

Cumulative mortality of ClLyzI-suppressed scallops

The effect of RNAi for ClLyzI was confirmed by qRT-PCR. Generally, 70% inhibition of mRNA expression post dsRNA injection was considered as a threshold for an effective RNAi experiment (Krueger et al., 2007). In the present study, the mRNA abundance of ClLyzI gene started to decrease at 24 h post dsRNA injection and maintained rather low (less than 0.3-fold of the origin expression level) from 36 h to 84 h (Figure 3A). So, 36 h post dsRNA injection was selected as the optimum time point for the following Vibrio stimulation and cumulative mortality assay. Without Vibrio stimulation, the final mortality rates were 9.47%, 8.92%, 8.83% and 9.29% for normal, PBS injected, EGFP-dsRNA injected and ClLyzI-suppressed scallops, respectively (Figure 3B). Being stimulated with Vibrio, the cumulative mortality of ClLyzI-suppressed scallops was significantly higher than those of control groups from 12 h post stimulation. The ClLyzI-suppressed scallops died out at 72 h, while the cumulative mortalities were 51.35%, 58.15% and 57.69% for normal, PBS injected and EGFP-dsRNA injected scallops at the same time, respectively. The semi-lethal time for ClLyzI-suppressed scallops was less than 48 h, while those of other groups were about 72 h (Figure 3C).

Discussion

I-type lysozymes play pivotal roles in invertebrate innate immunity and are considered to be the first barrier against invading microbes (Saurabh and Sahoo, 2008). In invertebrates, especially in marine invertebrates, recent research achievements indicated that i-type lysozymes were extensively involved in innate immune responses and exhibit more extensive activities than those of terrestrial invertebrates, due to various invading microbes in the aquatic environment (Jielian et al., 2017). For examples, an i-type lysozyme from the Asiatic hard clam Meretrix meretrix expressing along with larval development showed typical lysozyme activity and strong antibacterial activity against Gram negative and Gram positive bacteria, and its SNPs were correlated with the resistance to Vibrio.
Fig. 3 RNAi of CfLyzI and its effect. A: The relative abundance of CfLyzI mRNA in scallops hemocytes. Bars with different characters stood for significant difference ($p < 0.05$). B: Mortalities without *Vibrio* stimulation. C: Mortalities post *Vibrio* stimulation.
and growth of hosts (Yue et al., 2011; 2012; 2013). The intensive expression profiles and strong antimicrobial activities against bacteria of recombinant i-type lysozyme indicated its potential antibacterial roles in the sea cucumber Apostichopus japonicus (Wang et al., 2008; 2009; Yang et al., 2010). A recombinant i-type lysozyme from the white shrimp Litopenaeus vannamei showed a broad spectrum of antimicrobial properties with high antibacterial activities against Vibrio species (Chen et al., 2016), while an i-type lysozyme was potentially involved in the ontogenesis and immune defense in Kuruma shrimp Marsupenaeus japonicus (Liu et al., 2016). Additionally, the research achievements on i-type lysozymes in the eastern oyster Crassostrea virginica indicated a possible adaptive evolutionary pathway for i-type lysozymes from host defense to digestion in bivalves (Xue et al., 2004; 2007; 2010). Although many i-type lysozyme genes have been identified from marine bivalves, to our best knowledge, no i-type lysozymes have been studied in Zhikong scallop yet (Jielian et al., 2017). In the present study, a novel i-type lysozyme, CfLyzI, was identified and characterized from C. farreri. Its molecular feathers, tissue and temporal expression patterns and potential function were also investigated.

Bioinformatics analysis revealed that CfLyzI contained a typical destabilase domain as the same as previously identified H-type lysozymes, and exhibited high similarity with its invertebrate counterparts. Two specific motifs were revealed, which was as same as the observation in razor clam Sinonovacula constricta and Manila clams Venerupis (Ruditapes) philippinarum (Zhao et al., 2010; Chen et al., 2018). Moreover, similar with its counterpart from S. constricta, the deduced protein sequence of CfLyzI contained high amount of cysteine residues (8.59%, 20 among 233 residues), contributing to its stability in the high osmolality seawater (Chen et al., 2018). Its sequence characteristics, high similarity with other previously identified i-type lysozymes collectively suggested that CfLyzI is a novel member of i-type lysozymes and may have similar functions with its homologues from other invertebrates.

The i-type lysozyme functions as the essential defender against invading microbes, and its mRNA transcripts have been reported to be ubiquitously found in various tissues in marine invertebrates (Jielian et al., 2017). In the present study, the CfLyzI mRNA transcripts were detectable in all the investigated tissues and such ubiquity indicated that it would process many important physiological functions, especially as the first barrier against invading microbes in innate immunity. The peak level of CfLyzI mRNA expression was found in hemocytes, followed by gill, and the variable tissue distribution of CfLyzI mRNA transcripts would be related with tissue function. Mollusk hemocytes have been reported to be responsible for bactericidal activity by mediating numerous toxic compounds, such as lysozyme, lysosomal enzymes, nitric oxide, phenol oxidase and superoxide (Li et al., 2008). Gill was reported to be the first line against invading microbes in fish or lower animals, and a recent report demonstrated that tubules of gill filaments might be one of the potential hematopoietic positions in mollusk (Li et al., 2017a). The abundance of CfLyzI mRNA in hemocytes and gill implied its pivotal roles in the innate immunity of scallops.

Hemocytes are the major immune cells and respond to invading microbes mainly via phagocytosis in mollusks, and this tissue is usually selected to investigate the fluctuation of immune related genes (Canses et al., 2002). In the present study, hemocytes were also selected to test the temporal expression patterns of CfLyzI post Vibrio stimulation and during temperature stress. It has been reported that the mRNA scripts of i-type lysozyme could be induced by various invading microbes (Jielian et al., 2017). After the Vibrio stimulation, the expression levels of CfLyzI mRNA in hemocytes sharply increased and reached the peak at 12 h, which was consist with previous observation on other mollusks species (Chen et al., 2018). Hence, CfLyzI could participate in innate immunity via acting as an important innate immune effector to kill or eliminate invading microbes. Additionally, the mRNA expression of CfLyzI were significantly depressed at 10 °C and 30 °C, and high temperature could inhibit the mRNA expression of CfLyzI more efficiently, compared with cold shock. Such susceptible to temperature, especially to heat shock, might provide valuable insights and potential clue to a possible treatment for large scale mortalities of cultured scallops in summer.

As a major pathogen, the Gram negative bacteria Vibrio was believed to cause mass mortality of cultured scallop (Zhao et al., 2007a). A previous study revealed that SNPs of CfLyzG was speculated to be associated with the resistance or susceptibility of C. farreri to Vibrio (Zhao et al., 2007b; Li et al., 2009; 2013). In the present study, the role of CfLyzI against Vibrio infection in scallop has been evaluated via Vibrio stimulation and RNAI technique. After Vibrio stimulation, the cumulative mortality of CfLyzI-suppressed scallops significantly increased and the semi-lethal time for CfLyzI-suppressed scallops significantly shortened. These results suggested that the CfLyzI was involved in innate immunity and essential for hosts' survival during Vibrio stimulation in C. farreri.

In conclusion, the complete cDNA sequence of a novel i-type lysozyme was identified and characterized in C. farreri. Its mRNA transcripts could be significantly induced by Vibrio stimulation. The CfLyzI-suppressed scallops turn to be more susceptible to Vibrio. All these results indicated that CfLyzI was an efficient effector involved in the innate immunity and also essential for hosts' survival during Vibrio stimulation in C. farreri.

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Reference
Chen F, Wei ZX, Zhao XL, Shao YN, Zhang WW. Molecular characteristics, expression, and antimicrobial activities of i-type lysozyme from the razor clam Sinonovacula constricta. Fish Shellfish Immunol. 79: 321-326, 2018.


