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Background: Lysozyme is ubiquitous in many organisms and functions primarily in bacterial lysis.

Results: The absence of lysozyme in shrimp resulted in the increase of bacteria in the hemolymph leading to mortality.

Conclusion: Lysozyme is essential to shrimp survival particularly in regulating bacterial communities in the hemolymph.

Significance: A clear understanding of the immune effectors in shrimp, such as AMPs, is vital in ensuring resistance against bacterial pathogens.

Lysozyme is an enzyme that cleaves the $\beta$-1,4-glycosidic linkages between $N$-acetylmuramic acid and $N$-acetylglucosamine in peptidoglycan, leading to bacterial lysis. Recently, lysozyme has been found to have anti-HIV and anti-cancer properties in mammals. However, most functional analyses were done in vitro using purified or recombinant lysozyme protein. Here, we used RNA interference to silence c-type lysozyme expression in penaeid shrimp, Marsupenaeus japonicus, to analyze the function of lysozyme in vivo. Silencing of lysozyme expression by dsRNA lysozyme (dsLYZ) led to 100% mortality without any artificial bacterial infection in 5 days. Lysozyme deficiency caused the number of hemocytes in hemolymph to decrease from $1.3 \times 10^7$ to $2.3 \times 10^5$ cells/ml and caused the number of bacteria to increase from 78 to 764 colony-forming units/ml. Suppression of bacterial growth using oxytetracycline and kanamycin showed improvement in mortality, suggesting that shrimp mortality post-dsLYZ injection can be attributed to bacterial growth in the shrimp hemolymph. The majority of the bacteria, identified by 16 S rRNA analysis, were Gram-negative species such as Vibrio and Pseudomonas. Furthermore, PKH26 staining showed that the dsLYZ-injected shrimp were unable to eliminate non pathogenic Escherichia coli or Staphylococcus aureus in 24 h. These data suggest that c-type lysozyme in shrimp serves to regulate the growth of bacterial communities, particularly Gram-negative bacteria, in the hemolymph.

Aquatic organisms are in constant contact with a plethora of bacteria, many of which are pathogenic. Shrimp are protected from aquatic pathogens by their innate immune system (1). The innate immune system in shrimp has both cellular and humoral responses. Although both responses start with the recognition of microbial components, such as pathogen-associated molecular patterns, the cellular response is mediated mainly by hemocytes that directly attack pathogens, whereas the humoral response is mediated by the release of immune components into the hemolymph. The cellular response includes phagocytosis, nodule formation, and encapsulation, whereas the humoral response includes the prophenoloxidase (proPO)$^2$ cascade, blood coagulation, and the release of antimicrobial peptides (AMPs).

Recently, there has been increasing interest in the roles of AMPs in the invertebrate immune response. AMPs are small cationic molecules that form the first line of host defense against pathogenic infections; they possess potent antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, parasites, and some viruses (2–4). They are classified according to their size, conformational, and amino acid composition: 1) cysteine-rich, amphiphilic $\beta$-sheet peptides, 2) cysteine-disulfide ring peptides with and without amphiphilic tails, 3) amphiphilic $\alpha$-helical peptides without cysteine, and 4) linear peptides with one or two predominant amino acids (5). AMPs are present in a wide variety of organisms, from single-celled microbes to humans (5, 6). Penaeid shrimp have several types of AMPs, including penaeidin (7–9), crustin (10–12), anti-lipopolysaccharide factor (13–15), histone (16), hemocyanin (17, 18), and lysozyme (19–24).

Lysozyme is a bacteriolytic enzyme that is found in both prokaryotes and eukaryotes. It mainly hydrolyzes the $\beta$-1, 4-glycosidic linkages between $N$-acetylmuramic acid and $N$-acetylglucosamine in peptidoglycan leading to bacterial lysis (25). Based on their structural, catalytic, and immunological properties, lysozymes are classified into six types: chicken-type lysozyme (c-type), goose-type lysozyme (g-type), plant lysozyme, T4-phage lysozyme (phage-type), and invertebrate type lysozyme (i-type). Lysozyme super family proteins have additional roles in digestion (26), tumor inhibitory activity (27), and anti-HIV activity (28).

In shrimp, lysozyme was found to display antimicrobial activity against both Gram-negative and -positive bacteria including Vibrio species that are pathogenic to shrimp (19, 21, 23, 24, 25). Meanwhile, injection of recombinant lysozyme protein protected blue shrimp from white spot syndrome virus, a virulent pathogen in shrimp, infection (29).
In this study we used RNA interference (RNAi) to suppress the expression of a c-type lysozyme in shrimp in order to analyze further its function in vivo. Our results on the localization and anti-bacterial function of lysozyme confirm that it has an important role in crustacean immunity.

**EXPERIMENTAL PROCEDURES**

Shrimp—Kuruma shrimp (Marsupenaeus japonicus) used in this study were purchased from a commercial shrimp farm in Miyazaki, Japan. All shrimp were screened for signs of infectious diseases and kept for at least 2–3 days in artificial seawater maintained at 25 °C and 30 parts per thousand before all experimental procedures. Shrimp were fed daily with a commercial shrimp feed.

Preparation of Double-stranded RNA—Specific double-stranded RNA (dsRNA) was generated in vitro using a T7 Riboprep MAX Express System (Promega) following the manufacturer’s instructions. Briefly, gene-specific primers with or without the T7 promoter sequence were designed to produce sense and antisense strands separately. The primer sequences are shown in supplemental Table S1. PCR products were purified with GenElute™ PCR Clean-Up kit (Sigma). The purified products were transcribed to yield single-stranded RNAs. Equal amounts of the single-stranded RNAs were annealed to produce double-stranded RNAs that were further purified and quantified for the in vivo experiment. Thirty μg of dsRNA was injected into a 10-g shrimp. Controls samples were injected with either PBS or GFP-dsRNA.

Expression Analysis—Shrimp tissues (eye, gills, hemocytes, intestine, lymphoid organ, muscle, and stomach) were collected at specified time points. Total RNAs were isolated using RNAiso (Takara Bio Inc., Japan), and cDNAs were synthesized using Moloney murine leukemia virus reverse transcriptase (Invitrogen) following the manufacturer instructions. RT-PCR analysis was performed using Moloney murine leukemia virus reverse transcriptase (Invitrogen) following the manufacturer instructions. RT-PCR analysis was conducted to check the gene expression patterns and confirm gene silencing. PCR conditions consisted of an initial denaturation at 95 °C for 5 min followed by 28 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. Transcripts were visualized and analyzed in 1% agarose gel electrophoresis.

For the Western blot analysis, shrimp tissues (gills, hemocytes, intestine, lymphoid organ, muscle, and stomach) were homogenized in lysis buffer (20 mm Tris-HCl, pH 8.0, 150 mm NaCl, 1 mm EDTA, 1% Tween 20, 1 mm PMSF), mixed with 2 × SDS sample buffer, boiled for 5 min, and briefly centrifuged. Twenty μl of each sample was separated by 18% SDS-PAGE. After transfer onto nitrocellulose membrane, Western blotting was performed using mouse anti-shrimp lysozyme serum (1: 5000 in blocking buffer) and HRP-conjugate anti-mouse IgG (Rockland) as a secondary antibody (1:10000 in blocking buffer). Finally, membranes were reacted with a chemiluminescent substrate. The recombinant lysozyme was prepared as described earlier (19).

Lysozyme Activity Assay—Lysozyme activity of hemocytes was assayed by the lysoplate method (30). A gel plate was prepared containing 1% agarose in 50 mm phosphate buffer (pH 6.2) and 1 mg/ml Micrococcus luteus (Sigma). Hemocytes were homogenized in PBS. Ten μl of homogenate was applied to a well on a plate, and the plate was incubated at 25 °C for 24 h. Lytic activity was indicated by cleared zones around the well.

Antibiotics Treatment—To suppress bacterial growth, antibiotics (oxytetracycline and kanamycin) were added to 60-liter tanks to a final concentration of 50 mg/liter. Control shrimp were reared in normal water. For each treatment, mortality was recorded daily.

Ability of Shrimp Hemolymph to Eliminate Bacteria—Escherichia coli and Staphylococcus aureus were cultured in LB medium until A[600] = 1, fixed with 0.5% formalin solution for 24 h, washed twice with phosphate buffered saline, stained with PKH26 fluorescent dye following the manufacturer’s instructions, and injected into shrimp at 1 day post-dsLYZ injection. The number of PKH-stained E. coli or S. aureus in the hemolymph was determined at 3, 6, and 24 h post-injection.

Furthermore, to analyze bacterial growth in shrimp, GFP-expressing E. coli was injected to shrimp at 1 day post-dsLYZ injection. The number of GFP-expressing E. coli in the hemolymph was determined at 1, 2, 3, and 4 days post-injection.

**RESULTS**

Expression Analysis and Lytic Activity—RT-PCR analysis showed that lysozyme gene is expressed in shrimp immune-related organs such as gills, hemocytes, and lymphoid organ (Fig. 1A). All three of these tissues also showed lytic activity against M. luteus (Fig. 1C). Western blot analysis detected lysozyme in the hemocytes but not in the serum (Fig. 1B), which explains why no lytic activity was observed in the serum (Fig. 1C). dsGFP injection did not affect lysozyme gene expression (supplemental Fig. S1).

RT-PCR showed that injection of dsLYZ suppressed lysozyme mRNA from day 1 (Fig. 1D). Western blot analysis also showed that lysozyme protein was suppressed in the gills, hemocytes, and lymphoid organ at day 3 post-dsLYZ injection (Fig. 1E). As expected, suppression of lysozyme gene and protein expression caused the loss of lytic activity against M. luteus (Fig. 1F).

Shrimp Mortality, Hemocyte Counts, and Bacterial Counts—Silencing of lysozyme by dsLYZ resulted in 100% mortality within a few days, whereas the dsGFP- and PBS-injected groups suffered little mortality (Fig. 2A). Statistical analysis showed that mortality rates between dsLYZ-injected and control shrimp is highly significant (p = 3.39 × 10^-14).

In the 3 days after injection of dsLYZ, the number of circulating hemocytes gradually decreased from 1.3 × 10^7 to 2.3 × 10^6 cells/ml (Fig. 2B), whereas the number of bacteria in the hemolymph gradually increased from 78 to 764 colony-forming units/ml (Fig. 2C).

To analyze the bacterial community present in lysozyme-silenced shrimp, bacterial colonies were sequenced using 16 S rDNA. Although it is not suited for quantitative study, it effectively showed the profile of bacteria in the shrimp hemolymph. From the 918 16 S rRNA fragments that were collected, 774 (84.6%) gene fragments were from Gram-negative bacteria, whereas 60 sequence (6.7%) were from Gram-positive bacteria (supplemental Fig. S2). In Gram-negative bacteria, 162 sequences (17.7%) were found to be from Vibrio sp., and 80 (8.8%) were from Escherichia sp. A small number of Gram-
positive bacteria and cyanobacteria were detected such as *Finegoldia* sp., *Staphylococcus* sp., *Nocardia* sp., and *Lyngbya* sp.

**Antibiotic Treatment**—It was also not clear whether shrimp mortality was caused by bacterial growth alone or simply the lack of the lysozyme gene that could be essential to shrimp survival. To address this concern, antibiotics oxytetracycline and kanamycin were added in water. Oxytetracycline is a commonly used antibiotic in shrimp aquaculture. However, drug-resistant bacteria have been reported in shrimp ponds. Therefore, in addition to oxytetracycline, kanamycin, a drug not used in shrimp aquaculture, was used to suppress bacterial growth. DsLYZ injected shrimp kept in water treated with oxytetracycline and kanamycin showed 24–70% improved survival (Fig. 3) and is statistically significantly different from control ($p < 0.0001299$).

**Bacterial Clearance**—To elucidate the role of shrimp lysozyme in vivo, nonpathogenic *E. coli* (Gram-negative) and (Gram-positive) *S. aureus* were injected into lysozyme-silenced shrimp. dsLYZ-injected shrimp showed higher *E. coli* counts and weaker bacterial clearing ability ($1.8 \times 10^5$ cells/ml at 3 h and $1.8 \times 10^5$ cells/ml at 24 h post-injection) than those injected with either PBS or GFP ($6.5 \times 10^5$ cells/ml at 3 h and $6.0 \times 10^5$ cells/ml at 24 h; $3.1 \times 10^5$ cells/ml at 3 h and $2.0 \times 10^4$ cells/ml at 24 h post-injection, respectively) (Fig. 4A). In the case of *S. aureus*, the dsLYZ-injected group also showed a much weaker bacterial clearing ability ($1.2 \times 10^5$ cells/ml at 3 h and $2.0 \times 10^4$ cells/ml at 24 h post-injection) than both PBS- and GFP-injected groups ($5.3 \times 10^5$ cells/ml at 3 h and $2.0 \times 10^4$ cells/ml at 24 h; $6.0 \times 10^4$ cells/ml at 3 h and $3.3 \times 10^3$ cells/ml at 24 h, respectively) (Fig. 4B). Although lysozyme-deficient shrimp were able to clear formalin-killed *E. coli* and *S. aureus*, higher numbers of these bacteria were observed in lysozyme-deficient shrimp at each time point. Furthermore, when control and lysozyme-silenced shrimp were injected with GFP-expressing *E. coli*, the number of *E. coli* in the hemolymph decreased in the former and gradually increased in the latter (Fig. 4C).

**DISCUSSION**

RNAi, the introduction of dsRNA into cells to interfere with gene function, was first reported in *Caenorhabditis elegans* (31) and has since been a staple in the analysis of gene function in many organisms. Introduction of dsRNA causes sequence-specific cleavage of target genes or inhibits transcription (32–35). In this study we used dsRNA specific to shrimp lysozyme to suppress lysozyme gene expression and understand the function of this gene in vivo.

Shrimp hemocytes play a crucial role in the immune system and are involved in the mediation of both humoral and cellular responses. An earlier *in situ* hybridization analysis shows that shrimp lysozyme is expressed mainly in granular and semi-granular hemocytes (36). Here we showed that shrimp c-type lysozyme was expressed not just in the hemocytes but also in the gills and lymphoid organ. In the shrimp hemolymph, lysozyme expression is confined to the hemocytes. The lymphoid organ is a primary site of bacterial accumulation and bacteriostasis and accommodates hemocytes that in turn facilitates bacterial phagocytosis (37, 38). Shrimp gills meanwhile play a multifunctional role and were reported to be a site of the formation of hemocyte nodules during foreign particle injection.
Thus, the detection of lysozyme in the gills and lymphoid organ might have been because of the hemocytes present in these tissues.

Injection of a dsRNA specific to lysozyme effectively suppressed mRNA and protein expressions of this gene. Along with the silencing of lysozyme expression, dsLYZ-injected shrimp showed decreased lytic activity against *M. luteus*, suggesting that shrimp lysozyme is involved in bacterial elimination.

Silencing of lysozyme also resulted in a decreased number of hemocytes and increased number of bacteria, particularly Gram-negative bacteria, leading to shrimp mortality. Silencing of proPO also caused shrimp mortality without bacterial infection (40), whereas suppression of clottable protein and transglutaminase rendered shrimp more susceptible to infection (41). These results suggest that the components of shrimp immunity are closely correlated. The presence of bacteria in healthy shrimp is not surprising as bacteria have been found in the hemolymphs of other crustaceans, including horseshoe crab (42), blue crab (43–45), and penaeid shrimp (46). The interaction between crustaceans and the microorganisms present in their bodies, however, is not yet fully understood.

When lysozyme-silenced shrimp were reared in oxytetracycline- and kanamycin-treated water to suppress bacterial growth, shrimp mortality improved. This indicates that the increase of bacteria endemic in shrimp triggered by the absence of lysozyme led to shrimp mortality.

We also conducted microarray analysis to analyze the changes in the expression of other shrimp genes after dsLYZ injection. Our results showed that the absence of lysozyme caused the increase in expression of some putative immune-related genes such as histone, double-stranded binding protein, proteases, protease inhibitors, and hemocyanin (supplemental Table S3). On the other hand, not much change was observed in proPO, transglutaminase, and clottable protein (supplemental Table S3). These components are key molecules in melanization and blood coagulation, and their expressions were observed phenotypically because hemolymph of lysozyme-silenced shrimp exhibited melanization and coagulated immediately when withdrawn (supplemental Fig. S3). Suppression of proPO was previously shown to down-regulate the expressions of shrimp antimicrobial peptides such as penaeidin, crustin, and lysozyme (40). The fact that suppression of proPO suppresses lysozyme, but lysozyme does not suppress proPO suggests that proPO is a regulator of AMP gene expression.

Earlier we mentioned that lysozyme recombinant protein has been shown to exhibit lytic activity against both Gram-positive bacteria and Gram-negative bacteria. Interestingly, whereas shrimp did not lose bacterial clearance ability, lysozyme-silenced shrimp showed weaker elimination ability to Gram-negative and Gram-positive bacteria. Incomplete loss of bacterial

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**FIGURE 2. Effect of lysozyme silencing in shrimp.** A. Survival rate in shrimp injected with dsLYZ, dsGFP, and PBS is shown. The dsRNA was administered at a dose of 3 μg/g shrimp body weight, and an equal volume of PBS was injected into control samples. Thirty shrimp were used per treatment. The survival curve was determined using the Kaplan-Meier method, and the survival times of each experimental group were statistically analyzed by Log rank test. Total hemocyte counts (B) and total bacterial counts (C) in shrimp injected with dsLYZ, dsGFP, and PBS is shown. Total hemocyte counts and total hemocyte counts were determined from three separate shrimp per group. Shrimp were kept in 25 °C. Vertical bars represent the means ± S.E. Asterisks indicate significant difference compared with control (p < 0.05).

**FIGURE 3. Effect of antibiotics on the survival of dsLYZ-injected shrimp.** The dsLYZ was administrated at a dose of 3 μg/g shrimp body weight. Antibiotic treatment consisted of 50 mg/liter each of kanamycin and oxytetracycline. Thirty shrimp were used per treatment. The survival curve was determined using the Kaplan-Meier method, and the survival times of each experimental group were statistically analyzed by log rank test.
lysozymes (accession numbers GQ478703 and GQ478704) and the dsRNA sequence we used to silence the expression of the c-type lysozyme. The ClustalW result shows that the dsRNA c-type lysozyme sequence is 46 and 45% identical to shrimp i-type lysozyme 1 and 2, respectively. This suggests that silencing of the c-type lysozyme was gene-specific.

In summary, the absence of lysozyme led to a decrease in hemocyte counts, increased bacterial counts in the hemolymph, and loss of lytic activity. It also caused the up-regulation of certain immune-related genes. The bacterial profile in lysozyme-deficient shrimp showed a proliferation of Gram-negative bacteria but was also found to have weaker elimination ability against both Gram-negative and Gram-positive bacteria. Taken together, our results show that lysozyme is an essential component of shrimp immunity, particularly in regulating bacterial community in the hemolymph, and suggest that it functions collectively with other immune-related genes in conferring immunity.

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