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The bacteriolytic mechanism of an invertebrate-type lysozyme from mollusk *Octopus ocellatus*

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ABSTRACT

As an important economic mollusk in coastal areas, *Octopus ocellatus* dependents on innate immune system to resist the invasion of microorganisms. Lysozyme is a crucial effector owing to its significant lytic activity against bacterial pathogens during the immune responses. In this study, characteristic and immune function of an I-type lysozyme from *O. ocellatus* (OoLyz) was investigated. OoLyz shared a close relationship with the lysozymes from other bivalve mollusks. The mRNA of OoLyz exhibited a broad transcript in different tissues/organs, and with the greatest expression in hepatopancreas. The expression of OoLyz was significantly raised when *O. ocellatus* was infected by *Vibrio anguillarum* or *Micrococcus luteus*, suggesting OoLyz participated in innate immune response of host. Prokaryotic recombinant OoLyz (rOoLyz) exhibited obvious bacteriolysis ability towards both gram-negative bacteria *V. anguillarum* and *Escherichia coli*, and gram-positive bacteria *M. luteus* and *Staphylococcus aureus*. The bacteriolysis activities of rOoLyz towards gram-negative but not gram-positive bacteria was heat stable, indicating that OoLyz might clear gram-positive bacterium by enzyme-dependent mechanisms, but eliminate gram-negative microbe via enzymatic activity independent way. Scanning electron microscopy analysis showed that rOoLyz destroyed microbes by damaging cell wall. More importantly, the fact that rOoLyz could directly degrade the peptidoglycan, further revealed its bactericidal mechanism as a muramidase. Our results revealed the essential role of I-type lysozyme in the innate immunity of *O. ocellatus*, and shed new light to understand the mechanism of immune defense of mollusks.

1. Introduction

Innate immunity is an essential part of defense system against infection in invertebrates. As an immune related molecule, lysozyme plays crucial roles in the front line of the host defense due to its lytic activity [1,2]. Up to now, three subclasses of lysozymes have been identified in animal [3], including the Chicken (C-) type lysozyme in vertebrates and insects [4], the Invertebrate (I-) type lysozyme in invertebrates and the Goose (G-) type from vertebrates like bird, fish and mammal [5]. There is very limited homology in their primary structure, but their three-dimensional structures are surprisingly similar, all with a representatives α/β structure [6].

Lysozyme performs a broad range of functions in immune system due to its ability of hydrolyzing the β-1,4 linkage between two amino sugars of the bacterial peptidoglycan, *N*-acetylmuramic acid and *N*-acetylglucosamine to lyse invading microbes [6]. An increasing number of studies have illustrated the resistant activities of lysozyme to microbial pathogens [7–10], especially gram-positive bacteria [11,12]. The broad antibacterial activity of lysozyme was determined by two distinct mechanisms. One is catalytic activity dependent [6], while the other is enzymatic activity independent, which was also believed due to structural factors [13,14]. And some lysozymes remain bactericidal after heat-denatured [15,16]. A destabilase-lysozyme from medicinal leech was the first I-type lysozyme with combined enzymatic and non-enzymatic antibacterial action [17], thereafter, the *Stichopus japonicus* lysozyme was confirmed the enhanced inhibitive activities to *Micrococcus lysodeikticus* and *Vibrio parahaemolyticus* after heat [18]. Except for the direct antibacterial ability, lysozyme could also induce or cooperate with some other immune factors, for example complement, interferon, C-reactive protein to eliminate the invading pathogens

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[8,9,19].

Considering the significant role lysozyme played in innate immunity, cumulative studies have been focused on this molecule in invertebrates. Recently, invertebrate lysozymes from *Procambarus clarkii* [20], *Marsupenaeus japonicus* [21], *Scylla paramamosain* [22] were conformed to involve in the antibacterial immunity, while the I-type lysozyme in some mollusks exhibiting lytic and isopeptidase activity [23–25]. To summarize, these results indicate that lysozyme is potentially involved in immune responses to protect the invertebrates from invaders. Although obvious progress has been achieved in the research of lysozyme from invertebrates, the characteristic and antibacterial mechanism of cephalopodous lysozyme are still poorly understood. In present study, an I-type lysozyme (OoLyz) was identified from mollusk *Octopus ocellatus*, an economic octopus specie in China [26], with the aim to analyze the molecular and structural characteristic of OoLyz, reveal its expression profiles both in tissue/organ and after bacterial stimulation, and figure out its immune function during the defense against bacterial infection.

2. Materials and methods

2.1. *Octopus ocellatus and bacterial strains*

Healthy *O. ocellatus* (15–20 cm in length and 34–38 g in weight), were purchased from a seafood market in Yantai, Shandong Province, China. They were kept in aerated seawater at 18–20 °C for 7 days before the experiments. The seawater was renewed twice a day. *Micrococcus luteus* and *Staphylococcus aureus* were cultured in LB medium, while *Escherichia coli* and *Vibrio anguillarum* were culture in LB and 2216E medium, respectively.

2.2. *Acquisition of full-length OoLyz cDNA*

Blast analysis revealed that a complete cDNA sequence of 678 bp in the *O. ocellatus* cDNA library we constructed before [30] was with high similarity to I-type lysozyme. The corresponding colony was picked up and re-sequenced using primer M13-F and M13-R (Table 1). The full-length cDNA was designed as OoLyz and deposited in GenBank under accession number MK801671.

2.3. *Sequence analysis of OoLyz*

The educed amino acid sequence of OoLyz was performed online with the Expert Protein Analysis System and BLAST. Multiple sequence alignment was performed using the Clustal X1.83 and the Sequence Manipulation Suite (SMS), while phylogenetic tree was constructed with OoLyz and lysozymes from other species by Neighbor-joining using MEGA4.1, calculated 1000 times for each branch bootstrap values. The tertiary structure of OoLyz was predicted in SWISS-MODEL and displayed in PyMOL version.

Table 1
Primer sequences used in this study.

Primer name	Primer sequences (5′-3′)
M13-F	GTAAAACGACGGCCAG
M13-R	CAGGAAACAGCTATGACC
OoLyz-RT-F	ACTCACTGTGTTGACTGCCCTCGT
OoLyz-RT-F	CTGCCACATTCTATCCATTCTTCG
Actin-RT-F	GATGATGAAGTCGCTGCTCTCG
Actin-RT-R	TCCCAGTTGGTAACAATACCGTGT
rOoLyz-F	GGATCCTCTGACGGTCCAATTCCACAGG
rOoLyz-R	CTCGAGTCACTGGCTCTTGAGACATTTCGAA
T7 Promoter-F	TAATACGACTCACTATAGGG

2.4. *Analysis of OoLyz mRNA transcript in tissues/organs by quantitative real time PCR (qPCR)*

Hemolymph was collected from six female adult *O. ocellatus* respectively with a syringe containing precooled anticoagulant (0.015 M sodium citrate, 0.45 M NaCl, 0.1 M glucose, and 0.01 M EDTA, pH 7.0), and centrifuged at 4000 rpm, 4 °C for 5 min to settle hemocytes [27]. Other tissues/organs including gonad, mantle, muscle, stomach, gill, systemic heart, kidney, branchial heart, and hepatopancreas were pulverized under liquid nitrogen. Total RNA was extracted from tissues/organs with TRIzol reagent (Invitrogen, USA). The RNA samples with well integrity, examined by agarose electrophoresis, and OD$_{260/280}$ value 1.9–2.0 were used for cDNA synthesis. The cDNA synthesis was carried out with M-MLV reverse transcriptase (Promega, USA), and the products were stored at -80 °C before the following qPCR.

A pair of OoLyz gene-specific primers, OoLyz-RT-F and OoLyz-RT-R (Table 1) were designed for qPCR. The qPCR was processed with SYBR® Premix Ex Taq™ (Takara, Dalian, China) on the Mastercycler ep realplex (Eppendorf AG, Hamburg, Germany) according to the Manual. The amplification was performed at a procedure of 95 °C for 45 s, then 40 repeats of 95 °C for 5s, 55 °C for 30 s and 72 °C for 60 s. Using β-actin as internal control, the $2^{-\triangle\triangle CT}$ method was employed to analyze the mRNA transcript of OoLyz [28]. Significance analysis was performed by *t*-test.

2.5. *Analysis of OoLyz transcript after bacterial challenge*

After temporary culture for 7 days, 120 individuals of *O. ocellatus* were divided into 3 groups randomly in 20 L aerated tanks. 50% female and 50% male in each group was used to avoid the possible effects of gender differences. One group was for blank control, and the other two for bacterial challenge experiment. The bacterial dose used for immune challenge is based on our previous study, which could activate the innate immune response of *O. ocellatus* but cause very few deaths [30,31]. The overnight bacterial cultures with OD$_{600}$ of 0.5–0.7 were diluted to coat the agar plates, and the colonies of bacteria were counted to calculate the bacteria CFU. The *O. ocellatus* were exposed to the fresh seawater suffused with 1×10^7 CFU/mL live *V. anguillarum* and *M. luteus*, respectively Six *O. ocellatus* from each group were sampled randomly. Hemocyte was extracted at 6, 12, 24 and 48 h post challenge, followed by total RNA extraction, cDNA synthesis and qPCR as described above. Statistical significance was defined as $P < 0.05$ and extremely significant as $P < 0.01$.

2.6. *Prokaryotic recombinant expression and protein purification*

The mature peptide of OoLyz exclude signal peptide were amplified by a pair of gene-specific primers rOoLyz-F and rOoLyz-R (Table 1). The corresponding fragment of the PCR product was then gel-purified and connected to His-tagged pEASY-E1 Expression Kit modified from pET (Transgen Biotech, Beijing, China). After sequencing validation, the constructed recombinant plasmid was transformed into Trans-T1 competent cell (Transgen Biotech, Beijing, China). Positive clones were further sequenced with T7 Promoter-F and rOoLyz-R (Table 1). Then recombinant plasmid was extracted and re-transformed into *E. coli* BL21 (DE3) (Transgen Biotech, Beijing, China). The recombinant OoLyz (designed as rOoLyz) was induced by IPTG (1 mmol/L) in LB medium with 100 μg/ml ampicillin for 4 h. The rOoLyz was purified via a Ni^{2+} chelating Sepharose column and refolded in the gradient urea-TBS glycerol buffer at 4 °C for 12 h (containing 6, 5, 4, 3, 2, 1, and 0 M urea in turn). The purity and concentration of purified rOoLyz were examined by 12% SDS-PAGE and BCA method, respectively [32].

2.7. *Bacteriolytic activity and heat stability of rOoLyz*

Four bacteria strains of that are usually used in the functional

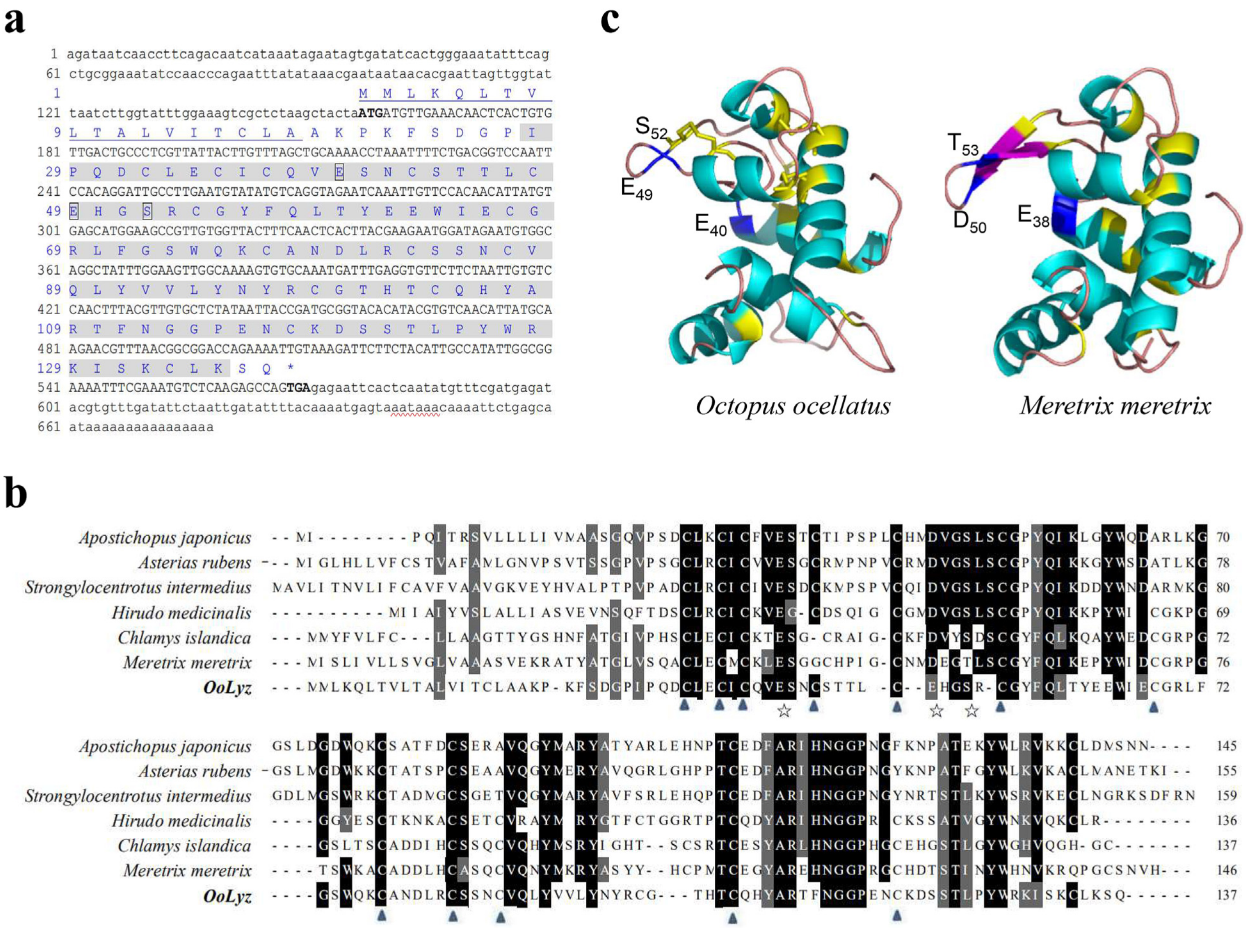

Fig. 1. Molecular sequence characteristics and evolutionary analysis of OoLyz. a: Nucleotide and deduced amino acid sequence of OoLyz cDNA. Numbers to the left of each row referred to nucleotide or amino acid position. The putative signal peptide is underlined. The shaded area is the OoLyz domain predicted by SMART. The wavy line is drawn under the tailing signal. Bold font indicates the start codon and stop codon. Enzyme active site is boxed up. b: Multiple sequence alignment of OoLyz with other I-type Lysozymes from *Apostichopus japonicas* (ABK34500.2), *Asterias rubens* (AAR29291.1), *Strongylocentrotus intermedius* (AEW10548.1), *Hirudo medicinalis* (AGJ83864.1), *Chlamys islandica* (CAB63451.1), *Meretrix meretrix* (ADL27913.1). Identical amino acids are in the black shadow, and 70% similar amino acids are shaded in gray. Twelve cysteine residues are labeled with triangles (▲). Three enzyme active sites are labled by asterisk (*). c: Predicted spatial structure of lysozyme from *O. ocellatus* and *Meretrix meretrix* in SWISS-MODEL. Green, helixes; purplish red, sheets; orange, loops; blue, enzyme active sites; yellow, cysteine residues and disulfide bonds. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

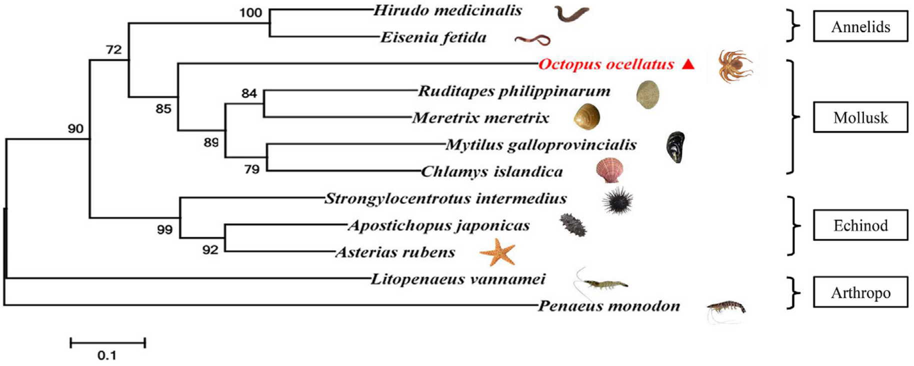

Fig. 2. A phylogenetic tree of OoLyz with lysozymes from other species including *Hirudo medicinalis* (AAA96144.1), *Eisenia feti da* (AGJ83864.1), *Ruditapes philippinarum* (ACU83237.1), *Meretrix meretrix* (ADL27913.1), *Mytilus galloprovincialis* (BAF63423.1), *Chlamys islandica* (CAB63451.1), *Strongylocentrotus intermedius* (AEW10548.1), *Apostichopus japonicas* (ABK34500.2), *Asterias rubens* (AAR29291.1), *Litopenaeus vannamei* (ABD65298.1), *Penaeus monodon* (ACZ63472.1).

studies of invertebrate biological molecules [33,34], including *S. aureus, M. luteus, V. anguillarum and E. coli*, were cultured to examine bacteriolytic activity of rOoLyz in accordance with the method described previously with modification [35]. Briefly, microbes were resuspended in PBS (pH 7.4) with an absorbance of 0.5 at 600 nm (OD_{600}). Then 15 µl of rOoLyz on concentration of 0.084 mg/ml was mixed with 200 µl of bacterial suspension and OD_{600} were measured once every 2 min.

To test whether the bacteriolytic activity of OoLyz is enzymatic activity dependent, rOoLyz was inactivated in boiled water for 30 min, and the bacteriolytic activity analysis was then performed as above.

2.8. Scanning electron microscopy (SEM) analysis

The *V. anguillarum* was collected and cleansed with PBS. 200 µl of bacterial suspension was added to 400 µl of rOoLyz (about 400 µg) and

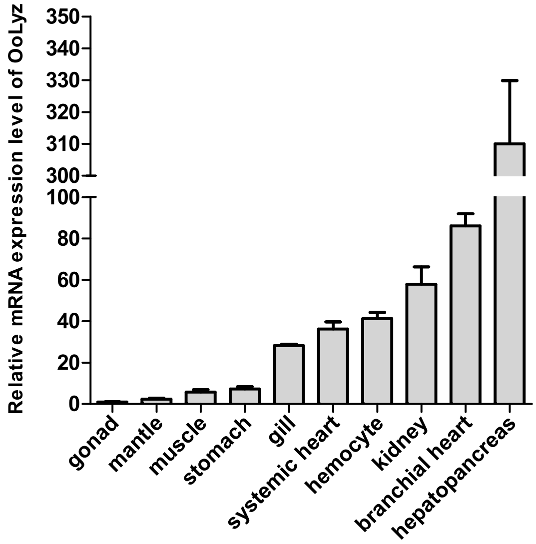

Fig. 3. The mRNA expression level of OoLyz in different tissues/organs of *O. ocellatus* detected by qPCR. Transcript levels in mantle, muscle, stomach, gill, systemic heart, hemocyte, kidney, branchial heart, and hepatopancreas are normalized to that of gonad. Vertical bars represent the mean ± standard error (N = 6).

PBS, respectively. The samples were incubated in 37 °C for 2 h and then fixed with 2.5% glutaraldehyde. After desalination the bacterial particles were dehydrated with gradient ethanol at the concentration of 30, 50, 70, 90, 100% (v/v), respectively for 15 min. They were collected and resuspended in 50 μl of t-butanol. After frozen and lyophilization, the samples were analyzed by scanning electron microscopy.

2.9. Muramidase activity of rOoLyz

To test whether the bacteriolytic activity of OoLyz is associated with muramidase activity, 100 μl of peptidoglycan (2 mg/m1 in 50 mM PBS, pH7.4) was mixed with equal volume of rOoLyz, and incubated in 37 °C for 30 min. PBS instead of rOoLyz was used as negative control. The absorbance at 540 nm was recorded as a manifestation of turbidity.

3. Results

3.1. Characteristics of OoLyz

The full-length cDNA of OoLyz is a 678 bp fragment covering a 156 bp 5′ untranslated regions (UTR) and a 111bp 3′ UTR with a poly (A) tail. The 411 bp open reading frame (ORF) region encoded 137 amino acids, containing a signal peptide (M_1-A_{18}), and a destabilase domain from X_{19} to X_{130} (Fig. 1 a).

BLAST analysis proved that Oolyz sequence shared obvious similarity with other I-type lysozymes, for instance 44% and 43% with the lysozyme from *Ruditapes philippinarum* (ACU83237.1) from *Chlamys islandica* (CAB63451.1), respectively. Multiple sequence alignments showed that OoLyz shared high homology with I-type lysozymes in other species, in particular the twelve conserved cysteine residues. Distinguished from other species, the second amino acid in enzyme active center in *O. ocellatus* was glutamate (E) instead of asparagine (D) (Fig. 1 b).

3.2. Structure and phylogenetic characteristic of OoLyz

The predicted tertiary structure of OoLyz was in comparison with the I-type lysozyme from *Meretrix meretrix* (Fig. 1c). They shared a similar structure with seven α-helix and nonregular coil but not two β-sheets. The fixed location of enzyme active centers in two molecules were well conserved, though residues were not exactly the same. The first active center E_{40} of OoLyz was located at the beginning of α-helix and exposed to the surface of the space structure. While the last two E_{49} and S_{52} were in nonregular coil. Four disulfide bonds (C_{37}–C_{43}; C_{48}–C_{54}; C_{67}– C_{87}; C_{77}–C_{83}) were well conserved to maintain the stability.

In the phylogenetic tree, OoLyz was first clustered with I-type lysozymes from lamellibranchia, and then clustered with annelids. Furthermore, it was grouped with echinoderm, and sub-clustered with arthropod lysozymes. The phylogenetic tree demonstrated that the evolutionary relationship of lysozyme was in accordance with the concept of traditional taxonomy (Fig. 2).

3.3. The mRNA transcript profile of OoLyz in tissues/organs

The OoLyz expression pattern in tissues/organs was measured by qPCR. OoLyz expression was found in all tested tissues/organs with different level, including muscle, hepatopancreas, stomach, kidney, gill, mantle, branchial heart, systemic heart, hemocyte and gonad (Fig. 3). OoLyz exhibited relative lower expression level in gonad, mantle, muscle and stomach, with the lowest level in gonad (Fig. 3). While it expressed relative higher in gill, systemic heart, hemocyte, kidney, branchial heart and hepatopancreas, with the highest level in hepatopancreas, which was 310-fold as compared to gonad (Fig. 3).

3.4. Temporal transcript profiles of OoLyz after bacterial challenge

To reveal the potential involvement of OoLyz in immune response, temporal transcript profile of OoLyz in hemocyte was examined after bacterial challenge. Based on our previous studies, a bacterial dose that

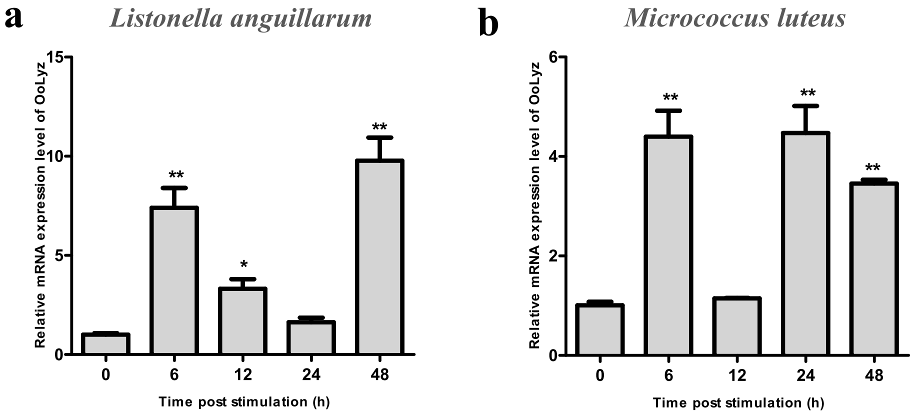

Fig. 4. Temporal expression of OoLyz mRNA in *O. ocellatus* hemocytes at 6, 12, 24 and 48 h after bacteria stimulation. a: *V. anguillarum* stimulation; b: *M. luteus* stimulation. The values are shown as mean ± S.E. (N = 6) (*: $P < 0.05$, **: $P < 0.01$).

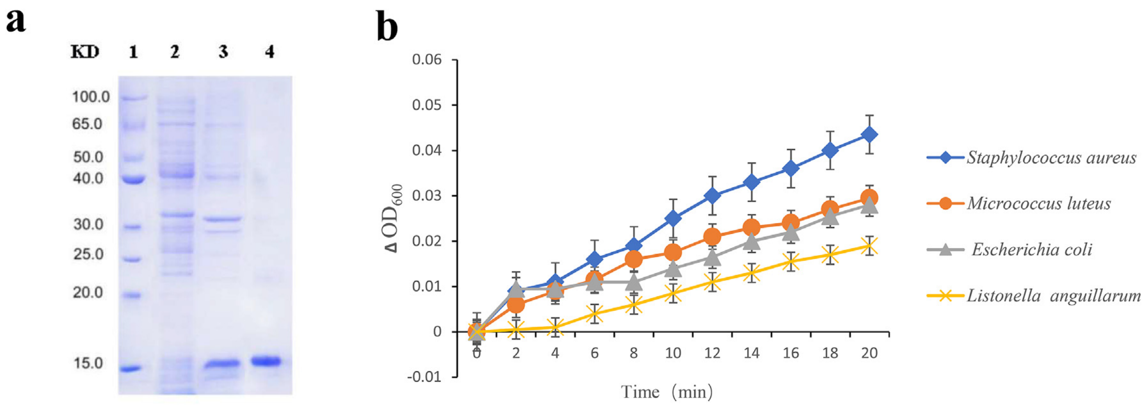

Fig. 5. SDS-PAGE analysis and the lytic activity of rOoLyz. a: lane 1: protein molecular marker; lane 2: prokaryotic expression of rOoLyz in *E. coli* BL21 (DE3); lane 3: induced rOoLyz; lane 4: purified rOoLyz. b: Four kinds of bacterial suspensions were incubated with rOoLyz, and OD$_{600}$ were measured once every 2 min. The changes of OD$_{600}$ represent the dissolution of bacteria. The ordinate is the decrease of OD$_{600}$ (origin OD - incubated OD). The squares, circles, triangles and the crosses are for *E. coli*, *V. anguillarum*, *M. luteus*, and *S. aureus*, respectively.

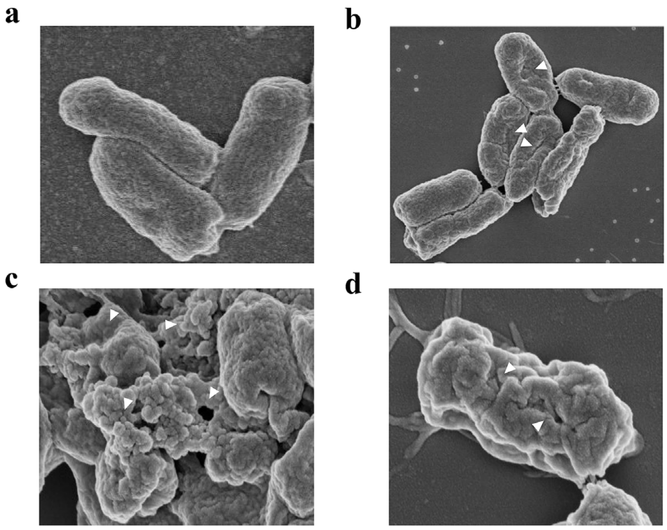

Fig. 6. Scanning electron microscopy analysis the effects of rOoLyz on *V. anguillarum*. a: *V. anguillarum* in the control group. b–d: deformed *V. anguillarum* treated with rOoLyz. The lesions of bacterium are pointed by white triangles. The *V. anguillarum* was mixed with rOoLyz and PBS, and incubated in 37 °C for 2 h for scanning electron microscopy.

will arise innate immune response but cause few deaths was used. The animal did not show any clinical signs within 48 h of infection. Stimulation of both *V. anguillarum* and *M. luteus* significantly induced the expression of OoLyz (Fig. 4). The transcript level of OoLyz was raised significantly to 7.3-fold of the control at 6 h after *V. anguillarum* challenge ($P < 0.01$) (Fig. 4 a). Then it slightly decreased at 12 h after stimulation, which was 3.3-fold of the control ($P < 0.05$). After declining to normal expression level compared to control group at 24 h after stimulation, the transcript of OoLyz up-regulated for the second time and peaked (9.7-fold, $p < 0.01$) at 48 h post infection. In accordance with the condition of *V. anguillarum* stimulation, the expression level of OoLyz up-regulated twice within 48 h post *M. luteus* challenge (Fig. 4 b). OoLyz expression level climbed up to 4.3-fold of the control at 6 h ($P < 0.01$) and declined to the normal level rapidly at 12 h. The second up-regulation occurred at 24 h that was about 4.4-fold ($P < 0.01$), and the high expression continued to 48 h ($P < 0.01$) (Fig. 4 b).

3.5. Recombination and purification of rOoLyz

The rOoLyz with a His-tag was prepared to investigate its potential function in immune defense. After induction by IPTG, OoLyz was expressed in *E. coli* BL21 (DE3) with high-efficiency by showing an obvious rOoLyz band at about 15.5 kDa (Fig. 5 a). The rOoLyz was purified with a Ni^{2+} chelating sepharose column. As measured with BCA protein assay kit (Beyotime, China), the concentration of purified rOoLyz was 1.2 mg/ml.

3.6. Bacteriolytic activity of rOoLyz

The OD$_{600}$ of the tested four kinds of bacteria all declined gradually in the 20 min after incubated with rOoLyz (Fig. 5 b). The OD value of *S. aureus* and *M. luteus* decrease 0.0435 and 0.0295 compared to the original value, while the OD value of *V. anguillarum* and *E. coli* decreased by 0.028 and 0.019, respectively. As control, OD value of bacteria suspension that without recombinant protein did not change (data not shown). These results indicated the bacteriolytic activity of rOoLyz to both gram-positive and gram-negative bacteria (Fig. 5 b).

3.7. SEM observation of rOoLyz mediated bacteriolysis

To further confirm whether the decline of OD value was associated with bacteriolytic activity, rOoLyz-treated *V. anguillarum* was observed by SEM analysis. In contrast to the glabrous and holonomic bacterium in control group (Fig. 6 a), *V. anguillarum* changed significantly after the rOoLyz-treatment (Fig. 6 b-d). The bacterium became shriveled, sunken and the surface turned to be rough (Fig. 6 b & d). Some stacked *V. anguillarum* even had so severe a distortion with the content flowed out (Fig. 6 c).

3.8. Heat stability of rOoLyz

The heat-inactivated rOoLyz was also used for bacteriolytic activity to reveal whether this activity was enzymatic activity dependent. After 30 min' inactivation in boiled water, rOoLyz could still lyse *V. anguillarum* and *E. coli* (Fig. 7 a & b), but not *S. aureus* and *M. luteus* (Fig. 7 c & d). These observations suggested that the bacteriolytic activity of rOoLyz to gram-positive bacteria rather than gram-negative bacteria was enzyme-dependent.

3.9. Muramidase activity of rOoLyz

The hydrolysis ability toward peptidoglycan of rOoLyz that demonstrated the muramidase activity was examined. After an hour incubation, the OD$_{540}$ of peptidoglycan treated with rOoLyz decreased significantly, while the absorbance of the control group did not change within testing phage (Fig. 8), suggesting the obvious muramidase

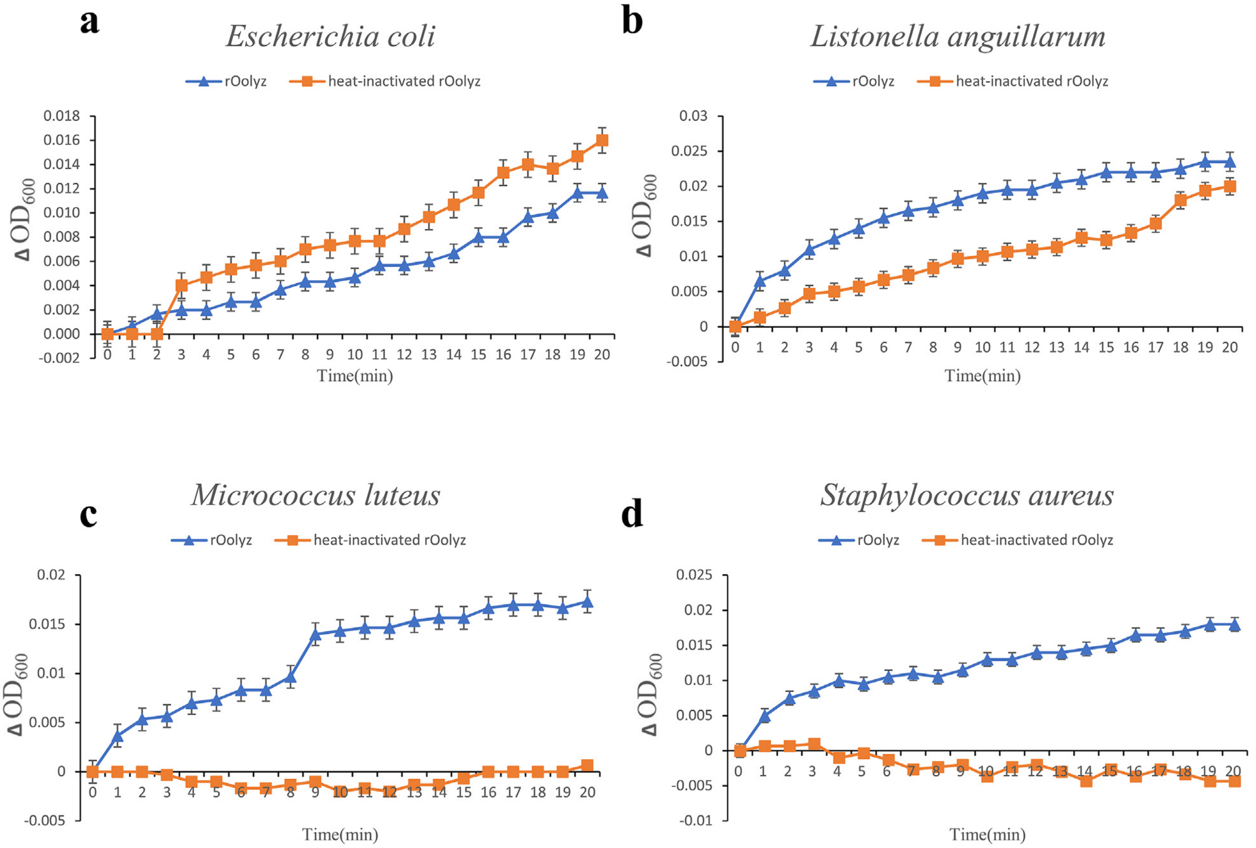

Fig. 7. Lytic activity of heat-inactivated rOoLyz. Four kinds of bacterial suspensions were mixed with rOoLyz and heat-inactivated rOoLyz, respectively. The changes of OD_{600} represent the dissolution of bacteria. The ordinate is the decrease of OD_{600} (origin OD - incubated OD). The triangles and squares stand for rOoLyz and heat-inactivated rOoLyz, respectively. a: *E. coli* substrate. b: *V. anguillarum* substrate. c: *M. luteus* substrate. d: *S. aureus* substrate.

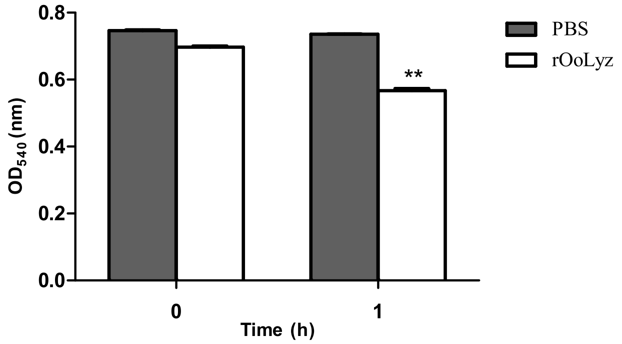

Fig. 8. Muramidase activity assay of rOoLyz. The peptidoglycan was mixed with rOoLyz and PBS, and OD_{540} was measured after 37 °C for 30 min. The asterisks indicate the muramidase activity assay of rOoLyz is significant different from the control. (**: $P < 0.01$).

activity of rOoLyz.

4. Discussion

The mollusk *O. ocellatus* is a typical invertebrate lacking adaptive immunity, it therefore relies mainly on innate immunity to defense and eliminate the invading pathogens. As an important lysosomal enzyme, lysozyme participates in humoral immunity and plays an important role in invertebrates' defense. Thus far, three kinds of lysozymes are established [36]. C-type and G-type lysozymes are both in vertebrates and invertebrates [3,4], while I-type lysozyme is unique to invertebrates [5]. I-type lysozyme has been widely detected in bivalve mollusks [24,37–39], echinoderms [40] and arthropods [41,42]. In this study, the cDNA encoding an I-type lysozyme was identified from *O. ocellatus* and recombinantly expressed. Cladogram analysis indicated that OoLyz displayed highest homology with bivalve mollusks. Like other I-type lysozymes, OoLyz coded a signal peptide at the N-terminus [40] and a conserved medicinal leech destabilase domain [43]. Multiple alignments showed twelve highly conserved cysteine residues and three relatively conserved enzyme active sites in OoLyz. This observation revelated cysteine residues may keep the stability of this protein in high osmolarity seawater and digestive organ [44]. The second active site is not aspartic like other I-type lysozyme [45], but glutamate in OoLyz, which made little difference to the bactericidal activity. In addition, the tertiary structure dipicted the major enzyme centers and sites that contribute to the strong bactericidal. As part of it, four disulfide bonds formed between pairs of cysteines help to hold the stability of the OoLyz spatial structure. Three active sites were exposed to the surface so that OoLyz could optimize its hydrolytic activity to β-1,4 linkage in peptidoglycan.

The expression of lysozyme in hepatopancreas has been predominantly detected in some marine bivalve, such as *Sinonovacula constricta* [46], *Venerupis philippinarum* [47] and *Meretrix meretrix* [24]. And lysozyme of *Crassostrea gigas* has also been found to higher express in digestive organs [48]. According to the mRNA transcripts pattern of OoLyz, it was widely expressed in tissues/organs, especially highly expressed in hepatopancreas, the gland that is functionally equivalent to the liver and pancreas in vertebrates. To avoid the scare and spraying ink, immersion with bacterial suspension instead of injection was used to infect the *O. ocellatus*. As the lysozymes in other marine mollusks [24,25,47], the mRNA expression of OoLyz was obviously induced by the stimulation of both gram-positive and gram-negative bacteria, indicating its potential participation in anti-bacterial immune response. Interestingly, the mRNA expression of OoLyz after the bacteria infection ascended twice by time as data shown. We thus speculated that OoLyz performed different function during the response to exogenous microbial stimulation. The first increase might associate with the stress response against pathogen, while the second up-regulation probably because OoLyz participated in the bacterial clearance process.

Lysozyme in some insect resists gram-positive bacteria merely [49], however, the majority of lysozyme have effects on both gram-positive and gram-negative bacteria [13,23]. In our study, the rOoLyz displayed the bacteriolytic activity to both gram-positive bacteria and gram-negative bacteria. The rOoLyz still hold the activity against gram-negative bacteria after high temperature, whereas this activity against gram-positive bacteria was impaired, demonstrating that antimicrobial effect of rOoLyz against gram-negative bacteria not only due to enzymatic activity. This feature was consistent to the previous studies in *Stichopus japonicus* [50]. The denatured lysozyme probably has an intrinsic structural motif lethal to the bacteria by targeting its membrane [15]. Whilst, some reports speculated that cationic and hydrophobic properties may also contribute to the non-enzymatic antibacterial activity [51–53]. It has been demonstrated that a helix-loop-helix peptide repertoire acting separately or in concert provided this potency of C-type lysozymes [52]. However, I-type lysozymes that lack the helix-loop-helix peptide also showed the non-enzymatic antibacterial activity, whose ability was not contributed by the single α 1-helix peptide but by peptide fragments acting in concert [17]. As shown in SWISS-MODEL, we speculate that non-enzymatic antibacterial activity of rOoLyz relied on the peptide fragments acting in concert, when the conformation of a domain in the spiral peptide region changed after heated.

The bacteriolysis mechanism of marine lysozyme is still an enigma that needs a further exploration. Several studies have revealed the bacteriolytic activity of lysozymes to gram-positive bacteria using electron microscope [54,55]. Here, we are more interested in the bacteriolytic activity of OoLyz to gram-negative bacteria *V. anguillarum*, which is a bacterial pathogen seriously endangers aquatic animals. As a helpful proof, the SEM showed the microcosmic apparent condition of gram-negative bacteria that treated with rOoLyz. It detailed the antibacterial activity of rOoLyz manifested as the cracking deformation of the bacterium. Gram-negative bacteria has complex cell walls containing lipids, polysaccharides, proteins and few peptidoglycan in the outer layer [56]. The muramidase activity resulted in the degradation of the murein layer and the mechanical strength decrease of the bacterial cell wall and ultimately causing the damage of bacteria by lysis [57]. And gram-negative bacteria was susceptible to the lytic action of lysozyme after the outer membrane has been destroyed [58–60]. Accordingly, we hypothesized the bacteriolytic effect of OoLyz against gram-negative bacteria reside in both muramidase activity and non-enzymatic factors.

We observed the bacteriolytic activity of rOoLyz to gram-positive bacteria was a bit stronger than gram-negative bacteria in the test of restraining bacterial growth. To make clear the causes, the peptidoglycan degradative capacity has been detected. Peptidoglycan, as we know, was the main component of gram-positive bacterial cell wall [61]. The gram-negative bacteria cell wall was much more complex, and contained less peptidoglycan than gram-positive bacteria. The degradation test indicated that rOoLyz has obvious peptidoglycan hydrolytic capacity, which interpreted the disparity antimicrobial activity between gram-positive and gram-negative bacteria. The sophisticated cell wall of gram-negative bacteria may hinder part of direct degradative effect of lysozyme toward peptidoglycan [62].

In our present study, the molecular characteristic, temporal expression profiles and enzymatic properties of OoLyz had been investigated. The results illustrated the essential role of I-type lysozyme in innate immunity of mollusk *O. ocellatus*. We further proposed two different mechanisms of bacteriolytic activity, according to the non-enzymatic activity and peptidoglycan degradative capacity. We presumed that OoLyz killed the gram-positive bacteria through muramidase activity, while cleared gram-negative bacteria through both muramidase activity and non-enzymatic activity. Our study provided additional evidence to the mechanisms of restraining bacterial growth by marine lysozyme, and was helpful to further understand the innate immunity of mollusks.

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