INTERNATIONAL JOURNAL OF MULTIDISCIPLINARY RESEARCH AND STUDIES

VOLUME4ISSUE01

# ''HIGH-LEVEL SOLUBLE EXPRESSION OF THE FUNCTIONAL PEPTIDE DERIVED FROM THE C-TERMINAL DOMAIN OF THE OCEAN CUCUMBER LYSOZYME AND ANALYSIS OF ITS ANTIMICROBIAL ACTIVITY''

PROF.DUHAOG, F.G; PROF.ZANG, P.J. MOONG

## ABSTRACT

### Background

The sea cucumber lysozyme belongs to the family of invertebrate lysozymes and is believed to be a key defense consider protecting aquaculture animals against bacterial infection. Recently, evidence was found that the ocean cucumber lysozyme exerts broad-spectrum antimicrobial action in vitro against Gram-negative and Gram-positive bacteria, and it also has less attackable antimicrobial activity independent of its enzymatic activity. To explore the antimicrobial role of this non-enzymatic lysozyme and model its structure to novel antimicrobial peptides, the peptide from the C-terminal aminoalkanoic acid residues 70–146 of the ocean cucumber lysozyme in Stichopus japonicus (SjLys-C) was heterologously expressed in Escherichia coli Rosetta(DE3)pLysS.

### Results

The fusion protein system led to the over-expression of the soluble and highly stable product, an approximate 26 kDa recombinant SjLys-C protein (rSjLys-C). this study showed that rSjLys-C displayed strong antimicrobial activity against the tested Gram-positive and Gram-negative bacteria. particularly, the heat-treated rSjLys-C exhibited more inhibitive activity than the native rSjLys-C. The structural analysis of SjLys-C showed that it's a typical hydrophilic peptide and contains a helix-loop-helix motif. The modeling of SjLys-C molecular structures at different temperatures revealed that the tertiary structure of SjLys-C at 100°C underwent a conformational change which is favorable for enhancing antimicrobial activity.

Conclusion

These results indicate that the expressed rSjLys-C may be a highly soluble product

and encompasses a strong antimicrobial activity. Therefore, gaining an out-sized quantity of biologically active rSjLys-C are going to be used for further biochemical and structural studies and supply a possible use in aquaculture and medicine.

**Keywords**

Affinity purificationLysozyme peptide molecular modelingRecombinant protein

## 1. Introduction

Among the antimicrobial peptides, lysozyme, having the strong bactericidal capability, is taken into account because the major component of the innate system of the many organisms and plays a very important role in protecting the host species from microbial invasion [1,2]. The enzyme has muramidase (glycohydrolase) activity that catalyzes the cleavage of the glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan within the semipermeable membrane of Gram-positive bacteria and eventually ends up in the killing of bacteria by lysis [1,3]. additionally, soluble fragments released by lysozyme degradation of peptidoglycan may play a job in immunomodulation in both vertebrates and invertebrates [4,5,6]. Moreover, lysozyme may also kill Gram-negative bacteria and inactivate viruses through a mechanism independent of its muramidase activity [7,8,9,10,11,12]. Furthermore, it's been proved that some bactericidal peptides derived from hen ovalbumin, T4 phage, and human milk lysozymes have an exaggerated and broad-spectrum microbicidal activity [13,14,15,16,17,18,19].

Based on the differences in structural, catalytic, and immunological characteristics, the currently known lysozymes are classified into six distinct types: chicken-type (c-type) lysozyme, goose-type (g-type) lysozyme, invertebrate-type (i-type) lysozyme, phage lysozyme, bacterial lysozyme, and plant lysozyme [3,20,21,22,23]. The i-type lysozyme was first identified within the starfish Asterias Rubens [24]. Current knowledge has confirmed that the i-type lysozymes occur within the phyla of molluscus (e.g. several bivalve species, Tapes japonica, mytilid, Crassostrea Gigas, Ostrea edulis, Crassostrea virgin-

ica) [21,25,26,27,28], annelids (e.g. earthworm Eisenia foetida and Eisenia andrei, hirudinean Hirudo medicinalis) [25,29,30], echinoderms (e.g. starfish A. rubens, holothurian Stichopus japonicus) [7,31], nematodes (e.g. Caenorhabditis species, Caenorhabditis elegans, Caenorhabditis briggsae and Caenorhabditis remanei) [32], and arthropods (e.g. mosquito Anopheles gambiae) [33]. In recent years, the marine i-type lysozymes have gained an increased interest seeable of its enzymatic and non-enzymatic activities against both Gram-positive and Gram-negative bacteria. the most effective example of characterizing the lysozyme function as a peptidoglycan-breaking enzyme is for the marine bivalve T. japonica. These studies used the purified protein to judge the isopeptidase and lysozyme activities in vitro and determined the crystal structure [34,35,36]. However, the antibacterial activity of lysozymes isn't completely obsessed on the muramidase and isopeptidase enzymatic activity, making the understanding of the immune role of i-type lysozymes more difficult.

In a trial to elucidate the functional significance of the ocean cucumber lysozyme as a good antimicrobial peptide employed in aquaculture farming and food preservation, we had over-expressed the mature peptide of the ocean cucumber S. japonicus (SjLys) in Escherichia coli. However, the over-expression of SjLys led to the recombinant protein in insoluble form. this might prevent the next protein analysis and application thanks to restriction of the purified protein amount and its activity through undergoing denaturation and refolding of the insoluble protein. Therefore, we here reported to undertake over-expression of the soluble fusion peptide SjLys-C and evaluate the peptide antimicrobial activity against a good range of microorganisms.

## 2. Materials and methods

### 2.1. Materials

The sea cucumber S. japonicus was provided by Dalian Zhangzidao Island Fishery Group, Dalian, China.

E. coli strain DH5α, the pMD18-T vector, RNAiso™ Plus for the extraction of the

whole RNA, One Step RNA PCR Kit (AMV) employed in RT-PCR and every one enzymes used for the genetic experiments were purchased from TaKaRa Biotechnology (Dalian, China). The expression strain E. coli Rosetta(DE3)pLysS and therefore the vector pET-32a(+) were obtained from Novagen (San Diego, CA, USA). Oligonucleotide primers were synthesized and positive clones were sequenced at Beijing Genomics Institute (Beijing, China). The affinity column HisTrap HP was purchased from GE Healthcare (Piscataway, NJ, USA). PVDF membranes were from Merck KGaA (Darmstadt, Germany). All other reagents were of biochemical research grade.

The recombinant plasmid, pMD18-T-SjLys, containing the ocean cucumber lysozyme gene, was constructed and transformed in E. coli DH5α in our lab as previously reported [7]. The strain of E. coli Rosetta(DE3)pLysS was grown in LB medium (10 g tryptone, 10 g NaCl, and 5 g yeast extract in 1 L of double distilled water). Plasmid isolation and routine biological science techniques were performed following standard procedures [37].

### 2.2. Isolation and synthesis of SjLys-C gene

The intestines of the ocean cucumber S. japonicus were frozen with cryogen and also the contents were homogenized. the full RNA was isolated following the instruction of RNAiso™ Plus (TaKaRa, China). A pair of primers were designed to amplify the SjLys-C gene from nucleotide bases 208-438 of the SjLys gene (GenBank accession no. EF036468) using the template of the S. japonicus cDNA. The forward primer HS-C-1 (5′-GAATGCCATGGTGATGGGAGGTAGTCT-3′) and therefore the reversed primer HS-C-2 (5′-GTGGAATTCTGTTCAGTTGTTGCTCATGTC-3′) introduced an Nco I site and an EcoR I site (both indicated by an underline), respectively. The SjLys-C gene was synthesized by reverse transcription and PCR amplification in a very single step reaction. Total volume reaction of fifty μL was drained triplicates and contained 5 μL of 10× One Step RNA PCR Buffer, 10 μL of MgCl2 (25 mM), 5 μL of dNTP Mixture (10 mM), 1 μL of RNase Inhibitor (40 U/μL), 1 μL of AMV RNase XL (5 U/μL), 1 μL of total RNA (1 μg/μL), 1 μL of AMV-Optimized Taq (5 U/μL), 1 μL of HS-C-1 (20 μM), 1 μL of HS-C-2 (20 μM) and 24 μL of RNase free dH2O. The thermocycle conditions were used as fol-

lows: reverse transcription at 50°C for 30 min, then initial denaturation at 94°C for two min followed by 35 cycles of amplification (94°C for 30 s, 58°C for 30 s, 72°C for 1 min), and an overextension step of 72°C for 10 min. The amplification products were analyzed by electrophoresis on 1.5% agarose gel. The expected PCR product was then cloned into pMD18-T vector to administer pMD18-T-SjLys-C and also the sequence of the DNA insert was confirmed by DNA sequencing.

### 2.3. Recombinant plasmid construction

The expression vector pET-32a(+) and also the recombinant plasmid pMD18-T-SjLys were digested with Nco I and EcoR I, and ligated at 16°C overnight. The ligation products were wont to transform E. coli DH5α by the warmth shock method. Positive clones selected on the LB agar plate containing 100 μg/mL ampicillin (Amp) and 34 μg/mL chloramphenicol (Cam) were screened by PCR. Plasmid DNA from positive clones was purified and subjected to DNA sequencing to verify the presence of in-frame insertion. The construct pET-32a(+)-SjLys-C was wont to transform the expression strain E. coli Rosetta(DE3)pLysS for recombinant protein synthesis.

### 2.4. Over-expression and purification of rSjLys-C

A positive clone strain, pET-32a(+)-SjLys-C/Rosetta(DE3)pLysS, was used for the rSjLys-C expression. within the meantime, the strain pET-32a(+)/Rosetta(DE3)pLysS without the target DNA was used as an effect sample of expression. Both strains were grown in LB broth containing 100 μg/mL Amp, 34 μg/mL Cam and 10 mg/mL glucose. After 14–16 h of overnight growth with a relentless orbital shaking of 180 rpm at 37°C, each culture of 1% was inoculated into LB/Amp/Cam medium supplemented with 5 mg/mL glucose. The culture was worn out an orbital shaker at 160 rpm and 37°C until the optical density of 0.6–0.7 at 600 nm was reached. At this time, induction was finished the addition of 0.5 mM IPTG (isopropyl-β-d-thiogalactoside). The culture was continuously incubated for 10 h at 120 rpm and 28°C.

After the cultivation, the cells were harvested by centrifuging at 10,000 × g and 4°C for

15 min and re-suspended in pre-cold PBS (pH 7.4) with the addition of 1% Triton X-100. The re-suspension of cells was sonicated at 400 W for five min (sonicating 2 s and pausing 1 s) in an ice bath. The sonicated preparation was centrifuged at 15,000 × g for 15 min. The collected supernatant was filtered with a 0.22 μm filter membrane to be prepared for purification using immobilized metal affinity chromatography.

The purification procedure was allotted using 1 mL HisTrap Hp column, a Ni2 +-NTA affinity column. The HisTrap Hp column was washed by 10 volumes of double H2O and equilibrated with 10 volumes of the binding buffer (20 mM Na3PO4, 500 mM NaCl, 40 mM imidazole, pH 7.4). The sample of above filtered supernatant containing the recombinant protein was competent the column at a rate of flow 1.0 mL/min. The column was washed with the binding buffer to get rid of contaminating proteins, then the rSjLys-C was eluted by the elution buffer (20 mM Na3PO4, 500 mM NaCl, 150 mM imidazole, pH 7.4). The eluted fractions were collected and at last dialyzed by 7 kDa cut-off dialysis bag against PBS (pH 7.4) to wipe off imidazole. the merchandise in dialysis bag was lyophilized to be used because the purified rSjLys-C, and stored at -20°C.

### 2.5. Western blot analysis

Total proteins of pET-32a(+)-SjLys-C/E. coli Rosetta(DE3)pLysS produced before and after IPTG induction were analyzed by 12.5% SDS-PGAE. For Western blot analysis, all proteins were transferred to a PVDF membrane. The membrane was blocked with TBST buffer (20 mM Tris–HCl, 150 mM NaCl, 0.05% Tween-20) containing 1.5% BSA and 5% milk, and incubated overnight at 4°C. The membrane was washed twice with TBST buffer and incubated with the diluted Penta-His antibody (1:1000) for 1 h. The membrane was washed twice with TBST buffer and incubated with the identical buffer for 15 min before incubating with the diluted HRP-labeled rabbit anti-mouse IgG antibody (1:10,000) for 1 h. The PVDF membrane was washed twice with TBST buffer. Finally, the detection of the bound antibodies was performed by incubating the membrane with TrueBlue Peroxidase substrate for 1 min.

### 2.6. Antimicrobial activity assay

The antimicrobial activity of the rSjLys-C was assayed by Oxford cup method. Eight bacterial strains were used because the test microorganisms, including Gram-positive Micrococcus lysodeikticus, Staphylococcus aureus and Bacillus cereus, and Gram-negative Vibrio parahaemolyticus, Vibrio splendidus, Pseudomonas aeruginosa, Pseudoalteromonas nigrifaciens and Aeromonas hydrophila. The test strains were grown overnight at 30°C in LB medium, respectively. The lyophilized powder of the rSjLys-C was redissolved in PBS (pH 7.4) and adjusted the protein concentration to 0.5 mg/mL. The diameter of inhibition zone was measured by the cup-plate method. Each test bacterial cells were adjusted to three.0 × 109 CFU/mL in growth medium. 50 µL of cell culture was homogeneously spread onto the LB agar plate. Three oxford cups were placed on a LB agar plate. 200 µL of the rSjLys-C and heat-treated (at 100°C for 40 min) rSjLys-C was gently loaded into individual cups. Meanwhile, the purified product of the strain pET-32a(+)/E. coli Rosetta(DE3)pLysS without the target SjLys-C gene was used as a negative control. The agar plates were incubated overnight at 30°C, and also the antimicrobial activities were evaluated by measuring the diameter of the inhibition zone. The results were mean values with variance. the info were analyzed by analysis of variance (ANOVA), and a statistically significant difference was identified at the 95% confidence level. The comparison of the diameter inhibitive zones between native enjoys-C and heat-treated rSjLys-C for the identical test bacteria was made on the premise of the P-values ($\alpha = 0.05$).

### 2.7. Hydrophobicity and hydrophilicity analyses

Gene sequence of SjLys-C was translated into organic compound (aa) sequence by DNAstar7.1 Lasergen Editseq. Hydrophobicity and hydrophilicity of SjLys-C were analyzed by the web tool (http://web.expasy.org/protscale/). The Hphob./Kyte & Doolittle scale is applied for delineating the hydrophobic and hydrophilic character of the protein [38].

### 2.8. Molecular modeling analysis

The three-dimensional model of SjLys-C (SjLys-C.pdb) from Protein Data Bank (PDB) was generated by the SWISS-MODEL server (http://swissmodel.expasy.org/). The molecular modeling software GROMACS4.6 was wont to perform the typical tertiary structure of SjLys-C at different temperatures. The file conversion between the PDB file (SjLys-C.pdb) and therefore the GROMACS files (processed.gro, Topol.top, posre.it) was performed via the quality GROMACS pdb2gmx method [39]. The temperature was adjusted by Berendsen's coupling algorithm. and also the atomic distance of α-carbon atoms between the 2 active sites of SjLys-C was measured.

## 3. Results

### 3.1. Construction of recombinant expression plasmid pET-32a(+)-SjLys-C

In our previous study, the sequence of the ocean cucumber lysozyme SjLys showed that it consists of a putative N-terminal signal sequence (aa 1–21) and a mature peptide (aa 22–146). The mature peptide of SjLys contained two domains which code the various function. The N-terminal domain of SjLys (aa 22–69) showed the catalytic (glycosidase) activity, whereas the C-terminal domain (aa 70–146) was probably involved in a very non-enzymatic antibacterial activity [7].

In the present study, the DNA fragment coding C-terminal domain of SjLys-C was amplified with primers HS-C-1 (containing Nco I site) and HS-C-2 (containing EcoR I site), and inserted into the E. coli expression vector pET-32a(+) as shown in Fig. 1. The recombinant expression plasmid pET-32a(+)-SjLys-C included a 6× His-tag as a purification utility and a Trx-tag as a solubility-enhancing partner at the N-terminus.

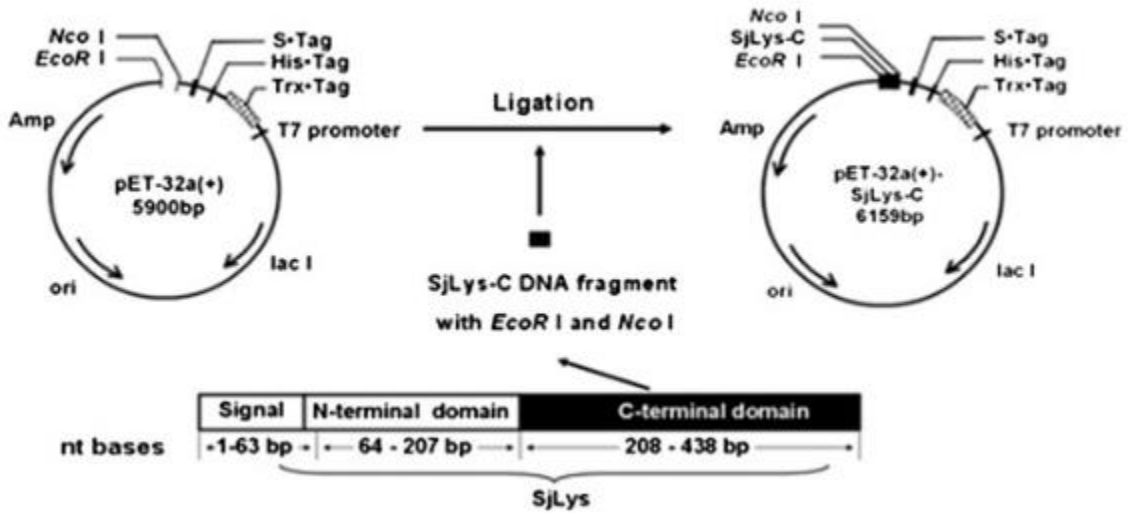


Fig. 1. Schematic diagram of construction of the expression plasmid pET-32a (+)-SjLys-C. The SjLys protein consists of a signal sequence, N-terminal region and C-terminal region as shown in the box and their nucleotide bases were numbered. The inserted DNA fragment for construction of the recombinant expression plasmid was shown in a black box.

3.2. Over-expression of soluble rSjLys-C in E. coli

The recombinant plasmid pET-32a(+)-SjLys-C was transformed into E. coli Roset-ta(DE3)pLysS. Upon induction with IPTG, the joys-C protein was over-expressed (Fig. 2, lane 2). The relative molecular mass of the joys-C was shown to be approximately 26 kDa for sure, containing 8.72 kDa of SjLys-C and 17.42 kDa of three fusion tags (His-tag, Trx-tag, and S-tag) from pET-32a(+). After sonicating the culture cells, it had been found that the joys-C was mostly within the supernatant as a soluble form instead of in sonicated precipitate (Fig. 2, lane 4). The joys-C was purified by one-step Ni2 + af-finity chromatography as one band shown on SDS-PAGE (Fig. 2, lane 5). Analysis by BandScan 5.0 showed that the rSjLys-C comprised ~ 85% of total cellular proteins, which indicated that the rSjLys-C was over-expressed in E. coli. Further analysis showed that the rSjLys-C accounted for ~ 70% of total cellular proteins within

the supernatant after sonication, which demonstrated that the rSjLys-C produced a soluble product because the major expression profile.

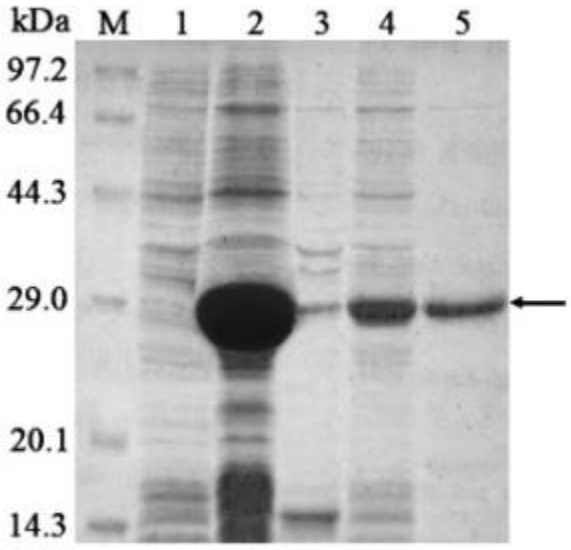


Fig. 2. Analysis of the rSjLys-C expression by SDS-PAGE. Lane M: Protein molecular weight marker; lane 1: Non-induction control; lane 2: Total cellular proteins induced by IPTG for 10 h; lane 3: Total cellular proteins in precipitate after sonication; lane 4: Total cellular proteins in supernatant after sonication; lane 5: Purified rSjLys-C by HisTrap Hp column. An arrow indicates the target rSjLys-C.

The expressed protein was further confirmed by Western blot analysis (Fig. 3). The results showed that the rSjLys-C had a selected immunologic response with Penta-His antibody at the position of about 26 kDa, whereas no cross-reaction occurred within the proteins from pET-32a(+)-SjLys-C/E. coli Rosetta(DE3)pLysS before induction. This demonstrated that the rSjLys-C expressed correctly in prokaryote E. coli, suggesting that it's the target peptide.

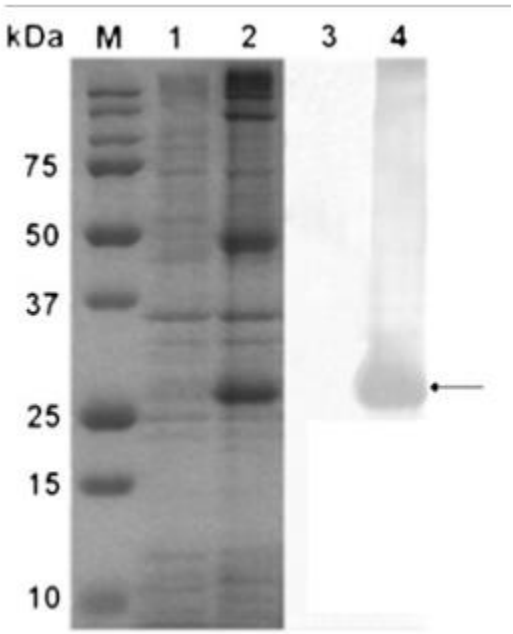


Fig. 3. Analysis of the rSjLys-C expression by Western blotting. Lane M: Precision plus protein marker; lane 1: Non-induction control; lane 2: Total cellular proteins induced by IPTG for 10 h; lane 3: Same sample as lane 1 detected by Western blotting; lane 4: Same sample as lane 2 detected by Western blotting. An arrow indicates the target rSjLys-C.

Analysis of hydrophobicity and hydrophilicity of SjLys-C containing 77 aa residues was done to invest the rationale for its soluble expression. consistent with the Kyte-Doolittle calculation [38], it absolutely was found that the hydrophilic residues of SjLys-C accounted for 87% of all aminoalkanoic acid residues. Furthermore, two active residues Ser18 and His48 in SjLys-C [7] were found to locate in two higher hydrophilicity zones (aa 16–22 and 46–50) (Fig. 4). These results indicated that the SjLys-C could be

a highly hydrophilic peptide and more likely gains a water-soluble product.

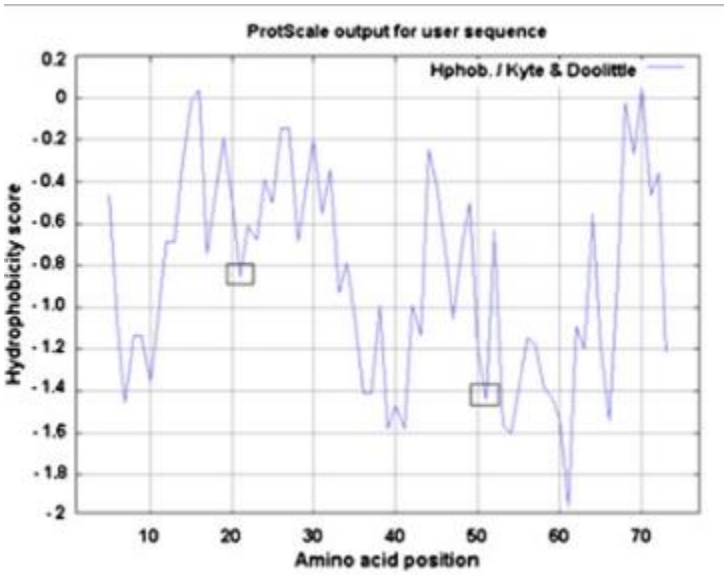


Fig. 4. Hydrophilicity and hydrophobicity profiles of SjLys-C. The Hphob./Kyte & Doolittle scale is the higher value of the hydrophobic aa (> 0 is indicated hydrophobicity, while < 0 is indicated hydrophilicity). The zones of aa 16–22 and 46–50 were shown in boxes.

### 3.3. Antimicrobial activity of rSjLys-C

The antimicrobial activity of the native rSjLys-C and heat-treated (100°C for 40 min) rSjLys-C was assayed using three Gram-positive bacteria and five Gram-negative bacteria because the test microorganisms. Negative control was employed by the purified product of the induced culture pET-32a(+) in E. coli Rosetta(DE3)pLysS. From the results of antimicrobial zone assays (Table 1), it absolutely was found that both native enjoys-C and heat-treated rSjLys-C could inhibit the expansion of all the test bacteria. Further analysis showed that the native rSjLys-C displayed a stimulating inhibitory effect on the expansion of M. lysodeikticus, V. parahaemolyticus and V. splendidus, and to a lesser extent on the expansion of S. aureus, B. cereus, P. aeruginosa, P. nigrifaciens and A. hydrophila. Meanwhile, another significant result was found that the rSjLys-C after heat treatment could more effectively inhibit the expansion of the foremost test bacterial strains. particularly, the antimicrobial activity of the heat-treated rSjLys-C was increased by 21.1% against M. lysodeikticus, 19.0% against V. parahaemolyticus and 11.4% against V. splendidus as compared to the antimicrobial spectrum of the native

rSjLys-C. additionally, the experiment confirmed that the negative control didn't entail any growth inhibition against any tested bacteria (data not shown).

Table 1. Antimicrobial activity of the rSjLys-C.

| The test bacteria | The diameter inhibitive zone (mm) | | | *p*-value |
|---|---|---|---|---|
| | rSjLys-C | Heat-treated rSjLys-C | Δ (%) | |
| *S. aureus* | 10.0 ± 0.4 | 10.5 ± 0.2 | 5.0 | 0.11882 |
| *M. lysodeikticus* | 19.0 ± 0.5 | 23.0 ± 0.6 | 21.1 | 0.00097** |
| *B. cereus* | 11.5 ± 0.2 | 12.2 ± 0.2 | 6.1 | 0.01417* |
| *V. parahaemolyticus* | 21.0 ± 0.5 | 25.0 ± 0.4 | 19.0 | 0.00029** |
| *V. splendidus* | 20.1 ± 0.2 | 22.4 ± 0.2 | 11.4 | 0.00018** |
| *P. aeruginosa* | 8.0 ± 0.5 | 8.3 ± 0.4 | 3.8 | 0.49712 |
| *P. nigrifaciens* | 9.2 ± 0.1 | 9.5 ± 0.2 | 3.3 | 0.06017 |
| *A. hydrophila* | 9.1 ± 0.1 | 9.8 ± 0.2 | 7.7 | 0.00702** |

Each value is the mean of three replicates ± standard deviation. The asterisks indicate statistically significant differences (*, $P < 0.05$; **, $P < 0.01$) from the normal samples.

$$\Delta = \frac{\text{Diameter inhibitive zone}_{\text{heat-treated rSjLys-C}} - \text{Diameter inhibitive zone}_{\text{rSjLys-C}}}{\text{Diameter inhibitive zone}_{\text{rSjLys-C}}}.$$

### 3.4. Molecular modeling of SjLys-C with temperature variation

To explore the molecular mechanism of the stronger antimicrobial activity of SjLys-C with a rise of temperature, the typical modeling of SjLys-C molecular structures at 30°C and 100°C were performed by the software GROMACS 4.6. The tertiary structure of SjLys-C at the condition of 30°C was generated in keeping with the GROMACS algorithm (Fig. 5a) when the initial PDB structure of SjLys-C was performed by energy minimization as an computer file. the specified time from the initial PDB structure to the energy-minimized altered structure in GROMACS files was about several seconds

[40]. during this study, the GROMACS structure of SjLys-C at 100°C was the common structure of 10 ns molecular dynamics simulation time (Fig. 5b). The results also showed that the gap between the active residues of Ser18 and His48 shortened from 17.5 Å to 11.8 Å when the temperature increased from 30°C to 100°C. It revealed that the SjLys-C provided more compact folding structure under the severe condition of 100°C, resulting in its more stability.

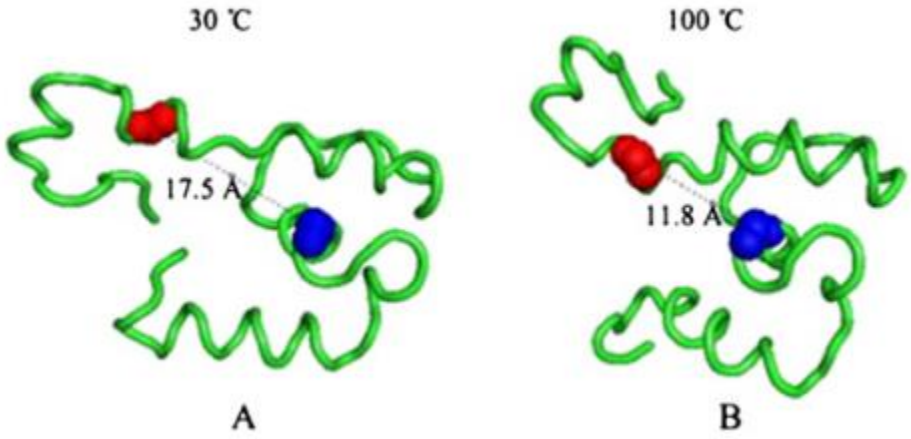


Fig. 5. The modeling structures of SjLys-C at different temperatures by molecular dynamics simulation.

## 4. Discussion

For invertebrate marine animals that constantly contact microorganisms within the environment, lysozymes and also the antibacterial peptides are particularly important within the first line of defense against the invasion of bacterial pathogens [7,11]. In recent years, the family of i-type lysozymes was well-studied in sight of its enzymatic muramidase and non-enzymatic activities against both Gram-positive and Gram-negative bacteria. The study of i-type lysozyme will increase our understanding of the regulatory process of the defense mechanisms. However, difficulties are encountered within the expression of antimicrobial i-type lysozyme due to producing insoluble inclusion bodies in E. coli host [36,41,42] and low-production

yield in yeast host [43]. Therefore, employing a functional peptide derived from the partial region of i-type lysozyme would offer an efficient thanks to produce an oversized quantity of active protein with an economical and scalable method.

In the present study, the constructed recombinant plasmid pET-32a(+)-SjLys-C was over-expressed in E. coli Rosetta(DE3)pLysS and also the soluble rSjLys-C was achieved in an exceedingly great amount. Four aspects were considered to achieve the achievement. Firstly, E. coli Rosetta(DE3)pLysS was chosen as transforming host strain for the rSjLys-C expression. this is often because Rosetta(DE3)™ host strain was designed to reinforce the expression of proteins that contain codons rarely employed in E. coli, like AGA, AUA, CUA, and GGA [44], which all of those rare codons are present within the SjLys-C gene. Secondly, to avoid the toxicity of the rSjLys-C to the host strain and acquire the soluble expressing recombinant protein, an expression vector pET-32a(+) was utilized in this study. Prokaryotic expression vector pET-32a(+) includes a affinity His-tag with 6 histidines and a solubility-enhancing Trx-tag which translates into thioredoxin [45]. The recombinant protein is purified by Ni2 + affinity chromatography, and this one-step purification method makes it a straightforward and high efficient thanks to collect pure recombinant product. Thirdly, the modified medium composition for cultivation of the gene-splicing strain to precise the rSjLys-C was through with the addition of 1.0% glucose in LB liquid medium. The aim of adding glucose is to keep up the soundness of the recombinant plasmid and improve the expressed protein solubility and folding efficiency [46]. Lastly, it absolutely was confirmed that the SjLys-C may be a highly hydrophilic peptide supported hydrophobicity and hydrophilicity analysis. Therefore, it's expected that solubility of the target protein are improved with the rise of hydrophilicity of organic compound residues.

In the study, three Gram-positive bacteria were used for the test microorganisms because M. lysodeikticus may be a substrate for lysozyme reaction [47], and S. aureus and B. cereus are the sickness pathogens [48,49]. Five Gram-negative bacteria, V. parahaemolyticus, V. splendidus, P. aeruginosa, P. nigrifaciens and A. hydrophila, were

used because all of those are the common pathogenic bacteria in aquaculture, especially V. splendidus and P. nigrifaciens which are causative pathogens for skin ulcerative syndrome in holothurian [50,51]. one among the present results showed that the rSjLys-C had effectively inhibitory action against the malady pathogens S. aureus and B. cereus. this could indicate that the lysozyme C-terminal peptide of the ocean cucumber may be used as a candidate of food preservatives because it's specific for bacterial cell walls and harmless to humans. On the opposite hand, the rSjLys-C also had remarkable antimicrobial activities against all the test pathogenic Gram-negative bacteria, especially when it showed the characteristic of more tolerant to extreme temperature. Taken together, these results indicate that the recombinant SjLys-C possessed a large range of antimicrobial activity spectra against both Gram-positive and Gram-negative bacteria. And this is often the primary report that the lysozyme C-terminal peptide of the ocean cucumber has the potent inhibitory effects against the devastating pathogens in holothurian aquaculture farming.

Structural analysis of SjLys showed that the C-terminal region of SjLys failed to contain the domain coding for muramidase (glycosidase) activity [7]. Therefore, the results of the antimicrobial activity of the rSjLys-C during this study indicated that SjLys-C could also be a peptide with non-enzymatic antimicrobial action. Ibrahim et al. [10] demonstrated that the denatured non-enzymatic lysozyme in chicken exerted antimicrobial action against Gram-positive and Gram-negative bacteria thanks to its helix-loop-helix (HLH) structure. Zavalova et al. [52] studied on antimicrobial activity of destabilase-lysozyme non-enzymatic area. The results showed that the destabilase-lysozyme was different from the c-type lysozyme, because the antimicrobial activity was worked by one helix peptide but not multiple helix peptide. On the idea of research by PyMOL software, we found that the three-dimensional structure of SjLys-C contains a HLH motif, i.e. α-helix 1 (H1), Asn38–Gly50; loop (Lp), Gly51–Asn57; and α-helix 2 (H2), Pro58–Cys70. Therefore, it's speculated that the HLH motif played a vital role in mechanism of non-enzymatic antimicrobial action of SjLys-C. The less attackable antimicrobial activity of the heat-treated SjLys-C indicated that the structure standing some place else in

SjLys-C had conformational changes which are favorable for enhancing antimicrobial activity. to verify this prediction, the results of molecular dynamics simulation showed that tertiary structure of SjLys-C kept stability under the condition of 100°C compared to 30°C. However, the comparison of structures demonstrated that several parts of the SjLys-C protein were reset after the warmth treatment. On the one hand, the expansion of the N terminal region and C terminal region resulted the exposure of two active residues (Ser18 and His48). On the opposite hand, the SjLys-C protein has more compact structure at 100°C thanks to the shortened atomic distance between the active residues of Ser18 and His48. Meanwhile, it's been demonstrated within the study that the active residues Ser18 and His48 were buried within the hydrophilic region. Therefore, it's concluded that the reduced distance between the 2 active sites of SjLys-C would enhance the hydrophilic interaction which could strengthen its antibacterial activity after being heated in boiled water.

In conclusion, we've got been able to obtain soluble and active recombinant SjLys-C in sufficient amounts for further biochemical and structural studies. This work also provided an endeavor to assess its application in large-scale production. it's predicted that the peptide product of SjLys-C are a potent antimicrobial agent and have potential use within the aquaculture and therefore the food industry.

**References**

[1]P. Jollès, J. Jollès
What's new in lysozyme research?
Mol Cell Biochem, 63 (1984), pp. 165-189, 10.1007/BF00285225
View Record in ScopusGoogle Scholar

[2]B. Masschalck, C.W. Michiels
Antimicrobial properties of lysozyme in relevancy foodborne vegetative bacteria
Crit Rev Microbiol, 29 (2003), pp. 191-214, 10.1080/713610448

CrossRefView Record in ScopusGoogle Scholar

[3]E.M. Prager, P. Jollès
Animal lysozymes c and g: an summary
P. Jollès (Ed.), Lysozymes: Model enzymes in biochemistry and biology, Birkhäuzer Verlag, Basel (1996), pp. 9-31
CrossRefView Record in ScopusGoogle Scholar

[4]J.W. Park, C.H. Kim, J.H. Kim, B.R. Je, K.B. Roh, S.J. Kim, et al.
Clustering of peptidoglycan recognition protein-SA is required for sensing lysine-type peptidoglycan in insects
Proc Natl Acad Sci U S A, 104 (2007), pp. 6602-6607, 10.1073/pnas.0610924104
CrossRefView Record in ScopusGoogle Scholar

[5]G. Sava, V. Ceschia, S. Pacor
Mechanism of the antineoplastic action of lysozyme: Evidence for host-mediated effects
Anticancer Res, 9 (1989), pp. 1175-1180
View Record in ScopusGoogle Scholar

[6]G. Sava, S. Pacor, G. Dasic, A. Bergamo
Lysozyme stimulates lymphocyte response to ConA and IL-2 and potentiates 5-fluorouracil action on advanced carcinomas
Anticancer Res, 15 (1995), pp. 1883-1888
View Record in Scopus Google Scholar

[7]L. Cong, X. Yang, X. Wang, M. Tada, M. Lu, H. Liu, et al.
Characterization of an i-type lysozyme gene from the ocean cucumber Stichopus japon-icus, and enzymatic and nonenzymatic antimicrobial activities of its recombinant protein

J Biosci Bioeng, 107 (2009), pp. 583-588, 10.1016/j.jbiosc.2009.01.016
ArticleDownload PDFView Record in Scopus Google Scholar

[8]H.R. Ibrahim, S. Higashiguchi, L.R. Juneja, M. Kim, T. Yamamoto
A structural phase of heat-denatured lysozyme with novel antimicrobial action
J Agric Food Chem, 44 (1996), pp. 1416-1423, 10.1021/jf9507147
View Record in ScopusGoogle Scholar

[9]H.R. Ibrahim, S. Higashiguchi, M. Koketsu, L.R. Juneja, M. Kim, T. Yamamoto, et al.
Partially unfolded lysozyme at neutral pH agglutinates and kills Gram-negative and Gram-positive bacteria through membrane damage mechanism
J Agric Food Chem, 44 (1996), pp. 3799-3806, 10.1021/jf960133x
View Record in ScopusGoogle Scholar

[10]H.R. Ibrahim, T. Matsuzaki, T. Aoki
Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic function
FEBS Lett, 506 (2001), pp. 27-32, 10.1016/S0014-5793(01)02872-1
ArticleDownload PDFView Record in ScopusGoogle Scholar

[11]J.A. Nash, T.N. Ballard, T.E. Weaver, H.T. Akinb
The peptidoglycan-degrading property of lysozyme isn't required for bactericidal activity in vivo
J Immunol, 177 (2006), pp. 519-526, 10.3410/f.1033209.375180
CrossRefView Record in ScopusGoogle Scholar

[12]A. Pellegrini, U. Thomas, R. Von Fellenberg, P. Wild
Bactericidal activities of lysozyme and aprotinin against Gram-negative and Gram-

positive bacteria associated with their basic character
J Appl Bacteriol, 72 (1992), pp. 180-187, 10.1111/j.1365-2672.1992.tb01821.x
CrossRefView Record in ScopusGoogle Scholar

[13]A.M. Abdou, S. Higashiguchi, A.M. Aboueleinin, M. Kim, H.R. Ibrahim
Antimicrobial peptides derived from hen egg lysozyme with inhibitory effect against Bacillus species
Food Control, 18 (2007), pp. 173-178, 10.1016/j.foodcont.2005.09.010
ArticleDownload PDFView Record in ScopusGoogle Scholar

[14]K. Düring, P. Porsch, A. Mahn, O. Brinkmann, W. Gieffers
The non-enzymatic microbicidal activity of lysozymes
FEBS Lett, 449 (1999), pp. 93-100, 10.1016/S0014-5793(99)00405-6
ArticleDownload PDFView Record in ScopusGoogle Scholar

[15]H.N. Hunter, W. Jing, D.J. Schibli, T. Trinh, I.Y. Park, S.C. Kim, et al.
The interactions of antimicrobial peptides derived from lysozyme with model membrane systems
Biochim Biophys Acta Biomembr, 1668 (2005), pp. 175-189, 10.1016/j.bbamem.2004.12.004
ArticleDownload PDFView Record in ScopusGoogle Scholar

[16]H.R. Ibrahim, K. Imazato, H. Ono
Human lysozyme possesses novel antimicrobial peptides within its N-terminal domain that focus on bacterial respiration
J Agric Food Chem, 59 (2011), pp. 10336-10345, 10.1021/jf2020396
CrossRefView Record in ScopusGoogle Scholar

[17]H.R. Ibrahim, U. Thomas, A. Pellegrini
A helix-loop-helix peptide at the upper lip of the situation cleft of lysozyme confers potent antimicrobial activity with membrane permeabilization action
J Biol Chem, 276 (2001), pp. 43767-43774, 10.1074/jbc.M106317200
ArticleDownload PDFView Record in ScopusGoogle Scholar

[18]B. Masschalck, R. Van Houdt, E.G.R. Van Haver, C.W. Michiels
Inactivation of Gram-negative bacteria by lysozyme, denatured lysozyme, and lysozyme-derived peptides under high hydrostatic pressure
Appl Environ Microbiol, 67 (2001), pp. 339-344, 10.1128/AEM.67.1.339-344.2001
View Record in ScopusGoogle Scholar

[19]A. Pellegrini, U. Thomas, N. Bramaz, S. Klauser, P. Hunziker, R. Von Fellenberg
Identification and isolation of a bactericidal domain in chicken ovalbumin lysozyme
J Appl Microbiol, 82 (1997), pp. 372-378, 10.1046/j.1365-2672.1997.00372.x
CrossRefView Record in ScopusGoogle Scholar

[20]J.J. Beintema, A.C.T. Van Scheltinga
Plant lysozymes
P. Jollès (Ed.), Lysozymes: Model enzymes in biochemistry and biology, Birkhäuser Verlag, Basel (1996), pp. 75-86
CrossRefView Record in ScopusGoogle Scholar

[21]S. Bachali, M. Jager, A. Hassanin, F. Schoentgen, P. Jollès, A. Fiala-Medioni, et al.
Phylogenetic analysis of invertebrate lysozymes and also the evolution of lysozyme function
J Mol Evol, 54 (2002), pp. 652-664, 10.1007/s00239-001-0061-6

View Record in ScopusGoogle Scholar

[22]J. Fastrez
Phage lysozymes
P. Jollès (Ed.), Lysozymes: Model enzymes in biochemistry and biology, Birkhäuser Verlag, Basel (1996), pp. 35-64
CrossRefView Record in ScopusGoogle Scholar

[23]J.V. Höltje
Bacterial lysozymes
P. Jollès (Ed.), Lysozymes: Model enzymes in biochemistry and biology, Birkhäuser Verlag, Basel (1996), pp. 65-74
Google Scholar

[24]J. Jollès, P. Jollès
The lysozyme from Asteria rubens
Eur J Biochem, 54 (1975), pp. 19-23, 10.1111/j.1432-1033.1975.tb04108.x
CrossRefView Record in ScopusGoogle Scholar

[25]Y. Ito, A. Yoshikawa, T. Hotani, S. Fukuda, K. Sugimura, T. Imoto
Amino acid sequences of lysozymes newly purified from invertebrates imply wide distribution of a completely unique class within the lysozyme family
Eur J Biochem, 259 (1999), pp. 456-461, 10.1046/j.1432-1327.1999.00064.x
View Record in ScopusGoogle Scholar

[26]N. Itoh, K.G. Takahashi
cDNA cloning and in place hybridisation of a unique lysozyme within the Pacific oyster, Crassostrea Gigas

Comp Biochem Physiol B Biochem Mol Biol, 148 (2007), pp. 160-166, 10.1016/j.cbpb.2007.05.005
ArticleDownload PDFView Record in ScopusGoogle Scholar

[27]T. Matsumoto, A.M. Nakamura, K.G. Takahashi
Cloning of cDNAs and hybridization analysis of lysozymes from two oyster species, Crassostrea gigas and Ostrea edulis
Comp Biochem Physiol B Biochem Mol Biol, 145 (2006), pp. 325-330, 10.1016/j.cbpb.2006.08.003
ArticleDownload PDFView Record in ScopusGoogle Scholar

[28]Q.G. Xue, N. Itoh, K.L. Schey, Y.L. Li, R.K. Cooper, J.F. La Peyre
A new lysozyme from the eastern oyster (Crassostrea virginica) indicates adaptive evolution of i-type lysozymes
Cell Mol Life Sci, 64 (2007), pp. 82-95, 10.1007/s00018-006-6386-y
CrossRefView Record in ScopusGoogle Scholar

[29]R. Josková, M. Šilerová, P. Procházková, M. Bilej
Identification and cloning of an invertebrate-type lysozyme from Eisenia andrei
Dev Comp Immunol, 33 (2009), pp. 932-938, 10.1016/j.dci.2009.03.002
ArticleDownload PDFView Record in ScopusGoogle Scholar

[30]L.L. Zavalova, I.P. Baskova, S.A. Lukyanov, A.V. Sass, E.V. Snezhkov, S.B. Akopov, et al.Destabilase from the hirudinean may be a representative of a unique family of lysozymes,Biochim Biophys Acta Protein Struct Mol Enzymol, 1478 (2000), pp. 69-77, 10.1016/S0167-4838(00)00006-6
ArticleDownload PDFView Record in ScopusGoogle Scholar

[31]S. Bachali, X. Bailly, J. Jollès, P. Jollès, J.S. Deutsch
The lysozyme of the starfish Asterias rubens. A paradigmatic type i lysozyme
Eur J Biochem, 271 (2004), pp. 237-242, 10.1046/j.1432-1033.2003.03915.x
View Record in ScopusGoogle Scholar

[32]H. Schulenburg, C. Boehnisch
Diversification and adaptive sequence evolution of Caenorhabditis lysozymes (Nematoda: Rhabditidae)
BMC Evol Biol, 8 (2008), p. 114, 10.1186/1471-2148-8-114
CrossRefView Record in ScopusGoogle Scholar

[33]S.M. Paskewitz, B. Li, M.K. Kajla
Cloning and molecular characterization of two invertebrate-type lysozymes from Anopheles gambiae,Insect Mol Biol, 17 (2008), pp. 217-225, 10.1111/j.1365-2583.2008.00797.x
CrossRefView Record in ScopusGoogle Scholar

[34]T. Goto, Y. Abe, Y. Kakuta, K. Takeshita, T. Imoto, T. Ueda
Crystal structure of Tapes japonica lysozyme with substrate analogue: Structural basis of the catalytic mechanism and manifestation of its chitinase activity amid quaternary structural change
J Biol Chem, 282 (2007), pp. 27459-27467, 10.1074/jbc.M704555200
ArticleDownload PDFView Record in ScopusGoogle Scholar

[35]S. Hikima, J.I. Hikima, J. Rojtinnakorn, I. Hirono, T. Aoki
Characterization and performance of kuruma shrimp lysozyme possessing lytic activity against Vibrio species
Gene, 316 (2003), pp. 187-195, 10.1016/S0378-1119(03)00761-3

ArticleDownload PDFView Record in ScopusGoogle Scholar

[36]K. Takeshita, Y. Hashimoto, Y. Thujihata, T. So, T. Ueda, T. Lomoto
Determination of the whole cDNA sequence, construction of expression systems, and elucidation of fibrinolytic activity for Tapes japonica lysozyme
Protein Expr Purif, 36 (2004), pp. 254-262, 10.1016/j.pep.2004.05.001
ArticleDownload PDFView Record in ScopusGoogle Scholar

[37]J. Sambrook, D.W. Russell
Molecular cloning: A laboratory manual
(3rd ed.), Cold Springs Harbour Laboratory Press, ny (2001)
Google Scholar

[38]J. Kyte, R.F. Doolittle
A simple method for displaying the hydropathic character of a protein
J Mol Biol, 157 (1982), pp. 105-132, 10.1016/0022-2836(82)90515-0
ArticleDownload PDFView Record in ScopusGoogle Scholar

[39]N. Sapay, D.P. Tieleman
Combination of the CHARMM27 field with united-atom lipid force fields
J Comput Chem, 32 (2011), pp. 1400-1410, 10.1002/jcc.21726
CrossRefView Record in ScopusGoogle Scholar

[40]C. Margreitter, D. Petrov, B. Zagrovic
Vienna-PTM web server: A toolkit for MD simulations of protein post-translational modifications,Nucleic Acids Res, 41 (2013), pp. 422-426, 10.1093/nar/gkt416
Google Scholar

[41]P. Supungul, V. Rimphanitchayakit, T. Aoki, I. Hirono, A. Tassanakajon
Molecular characterization and expression analysis of a c-type and two novel murami-dase-deficient i-type lysozymes from Penaeus monodon
Fish Shellfish Immununol, 28 (2010), pp. 490-498, 10.1016/j.fsi.2010.01.003
ArticleDownload PDFView Record in ScopusGoogle Scholar

[42]X. Wang, L. Cong, D. Wang, X. Yang, B. Zhu
Recombinant expression and antibacterial activity of i-type lysozyme
from holothurian Stichopus japonicus
Sheng Wu Gong Cheng Xue Bao, 25 (2009), pp. 189-194
View Record in ScopusGoogle Scholar

[43]Y. Gu, L. Cong, N. Luo
Cloning and expression of lysozyme from Stichopus japonicus in Pichia pastoris
J Dalian Polytech Univ, 29 (2010), pp. 317-320
CrossRefView Record in ScopusGoogle Scholar

[44]R. Novy, K. Yaeger, D. Held, R. Mierendorf
Coexpression of multiple target proteins in E. coli
Innovations, 15 (2002), pp. 2-6
View Record in ScopusGoogle Scholar

[45]M. Hammarström, N. Hellgren, S. Van Den Berg, H. Berglund, T. Härd
Rapid screening for improved solubility of small human proteins produced as fusion pro-teins in escherichia
Protein Sci, 11 (2002), pp. 313-321, 10.1110/ps.22102
View Record in ScopusGoogle Scholar

[46]T.H. Grossman, E.S. Kawasaki, S.R. Punreddy, M.S. Osburne
Spontaneous cAMP-dependent derepression of organic phenomenon in stationary phase plays a task in recombinant expression instability
Gene, 209 (1998), pp. 95-103, 10.1016/S0378-1119(98)00020-1
ArticleDownload PDFView Record in ScopusGoogle Scholar

[47]P. Mörsky
Turbidimetric determination of lysozyme with Micrococcus lysodeikticus cells: Reexamination of reaction conditions
Anal Biochem, 128 (1983), pp. 77-85, 10.1016/0003-2697(83)90347-0
ArticleDownload PDFView Record in ScopusGoogle Scholar

[48]Y. Le Loir, F. Baron, M. Gautier
Staphylococcus aureus and sickness
Genet Mol Res, 2 (2003), pp. 63-76
View Record in ScopusGoogle Scholar
[49]P.E. Granum, T. Lund
Bacillus cereus and its malady toxins
FEMS Microbiol Lett, 157 (1997), pp. 223-228, 10.1111/j.1574-6968.1997.tb12776.x
ArticleDownload PDFCrossRefView Record in ScopusGoogle Scholar

[50]H. Li, G. Qiao, J.Q. Gu, W. Zhou, Q. Li, S.H. Woo, et al.
Phenotypic and genetic characterization of bacteria isolated from diseased cultured holothurian Apostichopus japonicus in northeastern China
Dis Aquat Org, 91 (2010), pp. 223-235, 10.3354/dao02254
CrossRefView Record in ScopusGoogle Scholar

[51]C.Y. Zhang, Y.G. Wang, X.J. Rong
Isolation and identification of causative pathogen for skin ulcerative syndrome in Apostichopus japonicus
J Fish China, 30 (2006), pp. 118-123
View Record in ScopusGoogle Scholar

[52]L.L. Zavalova, I.P. Baskova, E.V. Barsova, E.V. Snezhkov, S.B. Akopov, S.A. Lopatin,Recombinant destabilase-lysozyme: Synthesis de novo in E. coli and mechanism of the enzyme expressed in Spodoptera frugiperda
Biochem Mosc, 69 (2004), pp. 776-781
View Record in ScopusGoogle Scholar

**AUTHOR AFFILIATION**

PROF.DUHAOG, F.G; PROF.ZANG, P.J. MOONG