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High-level soluble expression of the functional peptide derived from the C-terminal domain of the sea cucumber lysozyme and analysis of its antimicrobial activity

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ABSTRACT

Background: The sea cucumber lysozyme belongs to the family of invertebrate lysozymes and is thought to be a key defense factor in protecting aquaculture animals against bacterial infection. Recently, evidence was found that the sea cucumber lysozyme exerts broad spectrum antimicrobial action *in vitro* against Gram-negative and Gram-positive bacteria, and it also has more potent 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 amino acid residues 70–146 of the sea cucumber lysozyme in *Stichopus japonicus* (SjLys-C) was heterologously expressed in *Escherichia coli* Rosetta(DE3)pLysS.
Results: The fusion protein system led to over-expression of the soluble and highly stable product, an approximate 26 kDa recombinant SjLys-C protein (rSjLys-C). The present study showed that rSjLys-C displayed strong antimicrobial activity against the tested Gram-positive and Gram-negative bacteria. In particular, the heat-treated rSjLys-C exhibited more inhibitive activity than the native rSjLys-C. The structural analysis of SjLys-C showed that it is 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 is a highly soluble product and has a strong antimicrobial activity. Therefore, gaining a large quantity of biologically active rSjLys-C will be used for further biochemical and structural studies and provide a potential use in aquaculture and medicine.

1. Introduction

Among the antimicrobial peptides, lysozyme, having strong bactericidal capability, is considered as the major component of the innate immune system of many organisms and plays an 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 in the cell wall of Gram-positive bacteria and eventually results in killing of bacteria by lysis [1,3]. In addition, soluble fragments released by lysozyme degradation of peptidoglycan may play a role in immunomodulation in both vertebrates and invertebrates [4,5,6]. Moreover, lysozyme can also kill Gram-negative bacteria and inactivate viruses through a mechanism independent of its muramidase activity [7,8,9,10,11,12]. Furthermore, it has been proved that some bactericidal peptides derived from hen egg white, 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 have been 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 in the starfish *Asterias rubens* [24]. Current knowledge has confirmed that the i-type lysozymes occur in the phyla of molluscus (*e.g.* several bivalve species, *Tapes japonica*, *Mytilus edulis*, *Crassostrea gigas*, *Ostrea edulis*, *Crassostrea virginica*) [21,25,26,27,28], annelids (*e.g.* earthworm *Eisenia foetida* and *Eisenia andrei*, medicinal leech *Hirudo medicinalis*) [25,29,30], echinoderms (*e.g.* starfish *A. rubens*, sea cucumber *Stichopus japonicus*) [7,31], nematodes (*e.g. Caenorhabditis* species, *Caenorhabditis elegans*,

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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 in view of its enzymatic and non-enzymatic activities against both Gram-positive and Gram-negative bacteria. The best 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 evaluate the isopeptidase and lysozyme activities *in vitro* and determined the crystal structure [34,35,36]. However, the antibacterial activity of lysozymes is not completely dependent on the muramidase and isopeptidase enzymatic activity, making the understanding of the immune role of i-type lysozymes more challenging.

In an attempt to elucidate the functional significance of the sea cucumber lysozyme as an effective antimicrobial peptide used in aquaculture farming and food preservation, we had over-expressed the mature peptide of the sea cucumber *S. japonicus* (SjLys) in *Escherichia coli*. However, the over-expression of SjLys led to the recombinant protein in insoluble form. This could prevent the subsequent protein analysis and application due 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 wide 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 total RNA, One Step RNA PCR Kit (AMV) used in RT-PCR and all enzymes used for the genetic experiments were purchased from TaKaRa Biotechnology (Dalian, China). The expression strain *E. coli* Rosetta(DE3) pLysS and 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 sea 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 molecular biology techniques were performed following standard procedures [37].

2.2. Isolation and synthesis of SjLys-C gene

The intestines of the sea cucumber *S. japonicus* were frozen with liquid nitrogen and the contents were homogenized. The total 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 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 single step reaction. Total volume reaction of 50 μL was done in triplicates and contained 5 μL of 10× One Step RNA PCR Buffer, 10 μL of $MgCl_2$ (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 dH_2O. The thermocycle conditions were used as follows: reverse transcription at 50°C for 30 min, then initial denaturation at 94°C for 2 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 give pMD18-T-SjLys-C and the sequence of the DNA insert was confirmed by DNA sequencing.

2.3. Recombinant plasmid construction

The expression vector pET-32a(+) and the recombinant plasmid pMD18-T-SjLys were digested with *Nco* I and *EcoR* I, and ligated at 16°C overnight. The ligation products were used to transform *E. coli* DH5α by the heat 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 confirm the presence of in-frame insertion. The construct pET-32a(+)-SjLys-C was used 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. In the meantime, the strain pET-32a(+)/Rosetta(DE3)pLysS without the target DNA was used as a control 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 constant 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 done in 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 point, induction was done with 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 5 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 carried out using 1 mL HisTrap Hp column, a Ni^{2+}-NTA affinity chromatography column. The HisTrap Hp column was washed by 10 volumes of double distilled water and equilibrated with 10 volumes of the binding buffer (20 mM Na_3PO_4, 500 mM NaCl, 40 mM imidazole, pH 7.4). The sample of above filtered supernatant containing the recombinant protein was passed through the column at a flow rate 1.0 mL/min. The column was washed with the binding buffer to remove contaminating proteins, and then the rSjLys-C was eluted by the elution buffer (20 mM Na_3PO_4, 500 mM NaCl, 150 mM imidazole, pH 7.4). The eluted fractions were collected and finally dialyzed by 7 kDa cut-off dialysis bag against PBS (pH 7.4) to wipe off imidazole. The product in dialysis bag was lyophilized for use as 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% skim 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 same 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 as 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 3.0×10^9 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 the antimicrobial activities were evaluated by measuring the diameter of inhibition zone. The results were mean values with standard deviation. The data 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 rSjLys-C and heat-treated rSjLys-C for the same test bacteria was made on the basis of the *P*-values ($\alpha = 0.05$).

2.7. Hydrophobicity and hydrophilicity analyses

Gene sequence of SjLys-C was translated into amino acid (aa) sequence by DNAstar7.1 Lasergen Editseq. Hydrophobicity and hydrophilicity of SjLys-C were analyzed by the online tool (http://web.expasy.org/protscale/). The Hphob./Kyte & Doolittle scale is applied for delineating 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 SWISS-MODEL server (http://swissmodel.expasy.org/). The molecular modeling software GROMACS4.6 was used to perform the average tertiary structure of SjLys-C at different temperatures. The file conversion between the PDB file (SjLys-C.pdb) and the GROMACS files (processed.gro, topol.top, posre.itp) was performed *via* the standard GROMACS pdb2gmx method [39]. The temperature was adjusted by Berensen's coupling algorithm. And the atomic distance of α-carbon atoms between the two 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 sea 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 different 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 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.

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

The recombinant plasmid pET-32a(+)-SjLys-C was transformed into *E. coli* Rosetta(DE3)pLysS. Upon induction with IPTG, the rSjLys-C protein was over-expressed (Fig. 2, lane 2). The molecular weight of the rSjLys-C was shown to be approximately 26 kDa as expected, 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 was found that the rSjLys-C was mostly in the supernatant as a soluble form rather than in sonicated precipitate (Fig. 2, lane 4). The rSjLys-C was purified by one-step Ni^{2+} affinity chromatography as a single 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 in supernatant after sonication, which demonstrated that the rSjLys-C produced a soluble product as the major expression profile.

The expressed protein was further confirmed by Western blot analysis (Fig. 3). The results showed that the rSjLys-C had a specific immune response with Penta-His monoclonal antibody at the position of about 26 kDa, whereas no cross-reaction occurred in 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 is the target peptide.

Analysis of hydrophobicity and hydrophilicity of SjLys-C containing 77 aa residues was done to speculate the reason of its soluble expression. According to the Kyte-Doolittle calculation [38], it was found that the hydrophilic residues of SjLys-C accounted for 87% of all amino 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 is a highly hydrophilic peptide and more likely gains a water soluble product.

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 as the test microorganisms. A negative control was used 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 was found that both native rSjLys-C and heat-treated rSjLys-C could inhibit the growth of all the test bacteria. Further analysis showed that the native rSjLys-C displayed a remarkable inhibitory effect on the growth of *M. lysodeikticus*, *V. parahaemolyticus* and *V. splendidus*, and to a lesser extent on the growth 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 growth of the most test bacterial strains. In particular, 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. In addition, the experiment confirmed that the negative control did not entail any growth inhibition against any tested bacteria (data not shown).

3.4. Molecular modeling of SjLys-C with temperature variation

To explore the molecular mechanism of the more potent antimicrobial activity of SjLys-C with an increase of temperature, the

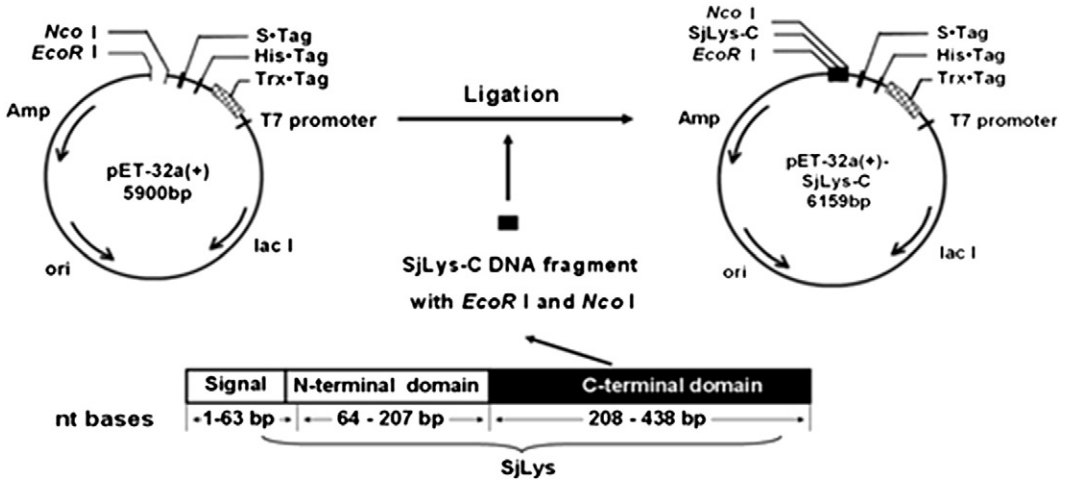

Fig. 1. Schematic diagram of construction of the expression plasmid pET-32a (+)-SjLys-C. The SjLys protein consists of a signal sequence, N-terminal region and C-terminal region as shown in the box and their nucleotide bases were numbered. The inserted DNA fragment for construction of the recombinant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

average 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 according to the GROMACS algorithm (Fig. 5a) when the initial PDB structure of SjLys-C was performed by energy minimization as an input file. The required time from the initial PDB structure to the energy-minimized altered structure in GROMACS files was about several seconds [40]. In this study, the GROMACS structure of SjLys-C at 100°C was the average structure of 10 ns molecular dynamics simulation time (Fig. 5b). The results also showed that the distance 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, leading to its more stability.

4. Discussion

For invertebrate marine animals that constantly contact microorganisms in the environment, lysozymes and the antibacterial peptides are particularly important in 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 view 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 have been encountered in the expression of antimicrobial i-type lysozyme because of producing insoluble inclusion bodies in *E. coli* host [36,41,42] and low-production yield in yeast host [43]. Therefore, using a functional peptide derived from the partial region of i-type lysozyme would provide an effective way to produce a large quantity of active protein with a cost-effective 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 the soluble rSjLys-C was achieved in a large amount. Four aspects were considered to gain the achievement. Firstly, *E. coli* Rosetta(DE3) pLysS was chosen as transforming host strain for the rSjLys-C expression. This is because Rosetta(DE3)™ host strain was designed to enhance the expression of proteins that contain codons rarely used in *E. coli*, such as AGA, AUA, CUA, and GGA [44], which all of these rare

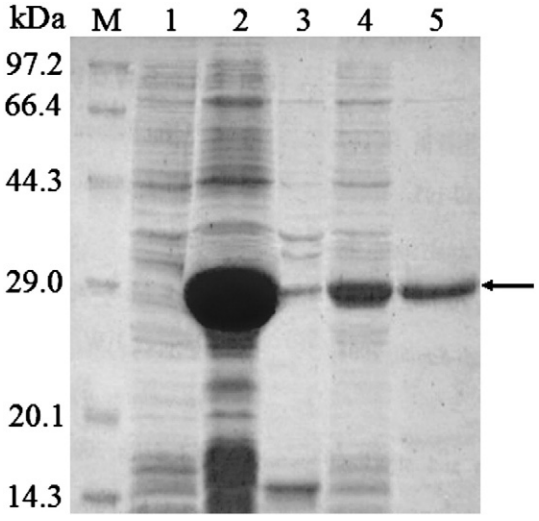

Fig. 2. Analysis of the rSjLys-C expression by SDS-PAGE. Lane M: Protein molecular weight marker; lane 1: Non-induction control; lane 2: Total cellular proteins induced by IPTG for 10 h; lane 3: Total cellular proteins in precipitate after sonication; lane 4: Total cellular proteins in supernatant after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

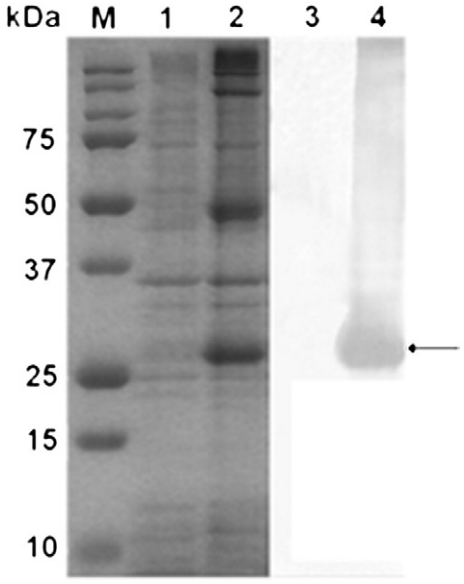

Fig. 3. Analysis of the rSjLys-C expression by Western blotting. Lane M: Precision plus protein marker; lane 1: Non-induction control; lane 2: Total cellular proteins induced by IPTG for 10 h; lane 3: Same sample as lane 1 detected by Western blotting; lane 4: Same sample as lane 2 detected by Western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

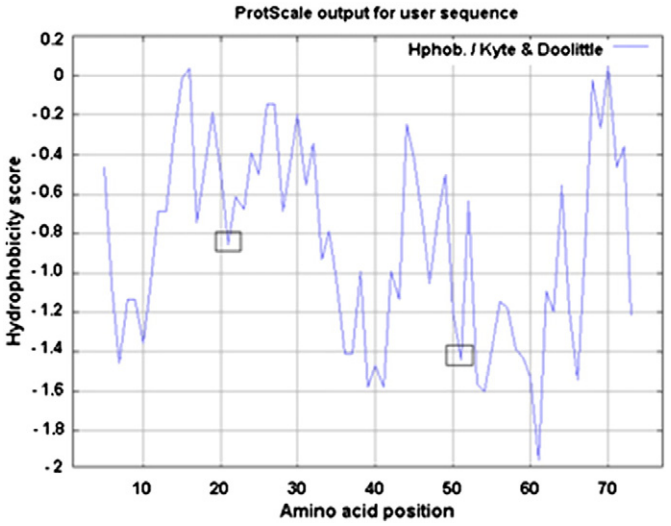

Fig. 4. Hydrophilicity and hydrophobicity profiles of SjLys-C. The Hphob./Kyte & Doolittle scale is the higher value of the hydrophobic aa (>0 is indicated hydrophobicity, while <0 is indicated hydrophilicity). The zones of aa 16–22 and 46–50 were shown in boxes.

codons are present in the SjLys-C gene. Secondly, to avoid the toxicity of the rSjLys-C to the host strain and obtain the soluble expressing recombinant protein, an expression vector pET-32a(+) was used in this study. Prokaryotic expression vector pET-32a(+) has a affinity His-tag with 6 histidines and a solubility-enhancing Trx-tag which translates into thioredoxin [45]. The recombinant protein can be purified by Ni^{2+} affinity chromatography, and this one-step purification method makes it a simple and high efficient way to collect pure recombinant product. Thirdly, the modified medium composition for cultivation of the genetic engineering strain to express the rSjLys-C was done with the addition of 1.0% glucose in LB liquid medium. The aim of adding glucose is to maintain the stability of the recombinant plasmid and improve the expressed protein solubility and folding efficiency [46]. Lastly, it was confirmed that the SjLys-C is a highly hydrophilic peptide based on hydrophobicity and hydrophilicity analysis. Therefore, it is expected that solubility of the target protein will be improved with the increase of hydrophilicity of amino acid residues.

In the study, three Gram-positive bacteria were used for the test microorganisms because *M. lysodeikticus* is a substrate for lysozyme reaction [47], and *S. aureus* and *B. cereus* are the food poisoning pathogens [48,49]. Five Gram-negative bacteria, *V. parahaemolyticus*, *V. splendidus*, *P. aeruginosa*, *P. nigrifaciens* and *A. hydrophila*, were used because all of these are the common pathogenic bacteria in aquaculture, especially *V. splendidus* and *P. nigrifaciens* which are causative pathogens for skin ulcerative syndrome in sea cucumber [50,51]. One of the current results showed that the rSjLys-C had effectively inhibitory action against the food poisoning pathogens *S. aureus* and *B. cereus*. This may indicate that the lysozyme C-terminal peptide of the sea cucumber could be used as a candidate of food preservatives because it is specific for bacterial cell walls and harmless to humans. On the other 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 high temperature. Taken together, these results indicate that the recombinant SjLys-C possessed a wide range of antimicrobial activity spectra against both Gram-positive and Gram-negative bacteria. And this is the first report that the lysozyme C-terminal peptide of the sea cucumber has the potent inhibitory effects against the devastating pathogens in sea cucumber aquaculture farming.

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.

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

In conclusion, we have been able to obtain soluble and active recombinant SjLys-C in sufficient amounts for further biochemical and structural studies. This work also provided an effort to assess its application in large-scale production. It is predicated that the peptide product of SjLys-C will be a potent antimicrobial agent and have a potential use in aquaculture and food industry.

Structural analysis of SjLys showed that the C-terminal region of SjLys did not contain the domain coding for muramidase (glycosidase) activity [7]. Therefore, the results of the antimicrobial activity of the rSjLys-C in this study indicated that SjLys-C may 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 because of 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 a single helix peptide but not multiple helix peptide. On the basis of analysis by PyMOL software, we found that the three-dimensional structure of SjLys-C contains a HLH motif, *i.e.* α-helix 1 (H_1), Asn^{38}–Gly^{50}; loop (Lp), Gly^{51}–Asn^{57}; and α-helix 2 (H_2), Pro^{58}–Cys^{70}. Therefore, it is speculated that the HLH motif played an important role in mechanism of non-enzymatic antimicrobial action of SjLys-C. The more potent antimicrobial activity of the heat-treated SjLys-C indicated that the structure standing somewhere else in SjLys-C had conformational changes which are favorable for enhancing antimicrobial activity. To confirm 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 heat 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 other hand, the SjLys-C protein has more compact structure at 100°C because of the shortened atomic distance between the active residues of Ser18 and His48. Meanwhile, it has been demonstrated in the study that the active residues Ser18 and His48 was buried in the hydrophilic region. Therefore, it is concluded that the reduced distance between the two active sites of SjLys-C would enhance the hydrophilic interaction which might strengthen its antibacterial activity after being heated in boiled water.

Conflict of interest

The authors declare no conflict of interest.

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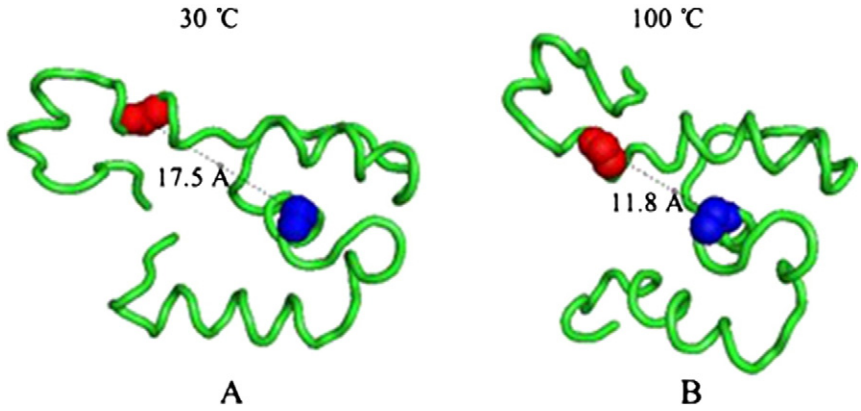

Fig. 5. The modeling structures of SjLys-C at different temperatures by molecular dynamics simulation.

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Author contributions

Proposed the theoretical frame: LC; Conceived and designed the experiments: LC, WL, YC; Software development: LC; Contributed reagents/materials/analysis tools: CL, WS; Wrote the paper: LC, WL; Performed the experiments: LC, WL, YW, CL, YC, LD, WS, JM; Analyzed the data: YW, CL, LD.

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