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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A R T I C L E  I N F O

Article history:
Received 13 February 2014
Accepted 25 August 2014
Available online 18 September 2014

Keywords:
Affinity purification
Lysozyme peptide
Molecular modeling
Recombinant protein

A B S T R A C T

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.

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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-acetylg glucosamine 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 microbialic 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 fetida 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,
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 using the template of the primers were designed to amplify the SjLys-C gene from nucleotide nitrogen and the contents were homogenized. The total RNA was isolated following standard procedures[37]. The recombinant plasmid, pMD18-T-SjLys was 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.

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 in an ice bath. The sonicated supernatant containing 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-PAGE. 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, Pseudomonas nigricans 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 × 10⁸ 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) pLYS5 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 strain were tested (data not shown).

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 contains 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 EcoI site) and HS-C-2 (containing EcoRI 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) pLYS5. 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 Nif" ⁷ 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) pLYS5 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 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 SjLys-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) pLYS5. 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 tested 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. nigricans 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% and 20.0% against M. lysodeikticus, V. parahaemolyticus and V. splendidus as compared to the antimicrobial spectrum of the native SjLys-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
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 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. In this study, the GROMACS structure of SjLys-C at 100°C was the average structure of 10 ns molecular dynamics simulation time. 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. 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 and low-production yield in yeast host. 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, which all of these rare codons are frequently used in the sequence of SjLys-C.
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 Ni²⁺ 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 product. 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. nigriéaciens and A. hydrophila, were used because all of these are the common pathogenic bacteria in aquaculture, especially V. splendidus and P. nigriéaciens 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.

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 destabilise-lysozyme non-enzymatic area. The results showed that the destabilise-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 (H₁), Asn38–Gly50, loop (Lp), Gly51–Asp57, and α-helix 2 (H₂), Pro58–Cys70. 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.

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.

### Conflict of interest

The authors declare no conflict of interest.

### Financial support

This research was supported by the Natural Science Foundation of China (31072224), the Special Fund for Marine Scientific Research in the Public Interest (201405003-3) and the Major Science and
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