Analysis and expression of Pmlyzi3 from *Penaeus monodon*

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Abstract. Lysozymes are crucial immune molecules and play an important role in innate immunity. Here, a new lysozyme named Pmlyzi3 was found from the transcriptome data of *Penaeus monodon*. The Pmlyzi3 gene was 438bp in length, encoding a 146-residue peptide and the first 19 residues constituted a signal peptide. The mature peptide contained 10 cysteines and had 7 α -helices in its N terminal. Moreover, it showed 88% identity with lysozyme-like protein from *Penaeus vannamei*. To express Pmlyzi3, pColdIV-SUMO-Pmlyzi3 plasmid was constructed by linking the Pmlyzi3 with SUMO tag, then transformed to *Escherichia coli* BL21 (DE3). By optimizing expression conditions, SUMO-Pmlyzi3 was successfully expressed in high level and purified with Ni-NTA column. Following with SUMO protease excision, pure Pmlyzi3 was obtained by removing SUMO tag, which would be helpful to study its function.

1 Introduction

Antimicrobial peptides (AMPs), as an important immune molecule, own an indispensable function in innate immunity [1]. AMPs normally exist in many kinds of organisms against a variety of bacteria, fungi, parasites, viruses and cancer cells [2], so that researchers think that it would be the best candidate for an antibiotic. Comparing with traditional antibiotics, AMPs are not easy to cause bacterial resistance [3]. Till now, over 2960 AMPs were found and 2184 of them were from animals [4].

Invertebrates depend on innate immunity to defend invading microbes because they lack adaptive immunity. It is well known that immunity was composed of cellular immunity and humoral immunity, and AMPs belonged to the humoral immunity system [5]. Several types of AMPs were found in crustaceans, including anti-lipopolysaccharide factors (ALFs), penaeidins, crustins and lysozymes [6,7]. Penaeidins, crustins and ALFs were reported in recent years [8,9].

Lysozyme, as a cationic protein, normally existed in animal, plant and microorganism. They killed microbes by destroying the β -1,4-glycoside bond which made the cell wall hydrolyze and the contents leak out. Generally, there were three types of lysozymes, including chicken-type lysozyme (c-type), goose-type lysozyme (g-type) and invertebrate-type lysozyme (i-type) [10]. The c-type was found in viruses, bacteria, plants, insects, reptiles, poultry, fish and mammals [11], g-type existed in birds, fish and scallop [13], while i-type normally was in invertebrates. There were over 20 i-type lysozymes including the first found in *Asterias rubens* [13-15].

Lysozymes were normally involved in defensive mechanisms including lysis, immune response and antimicrobial and antiviral activities [16]. Recombinant expression of c-type lysozyme from *Litopenaeus vannamei* showed notable antimicrobial activities to *Vibrio alginolyticus* and *Vibrio cholerae* [17]. The c-type and i-type lysozymes from *Penaeus japonicus* and *Penaeus monodon* also owned antimicrobial activities to gram-negative and gram-positive bacteria [10][18].

Though c-type lysozyme from *Penaeus japonicus* and *Penaeus monodon* were reported in many articles, i-type lysozyme had few reports. Hence, we obtained a new i-type lysozyme from transcriptome data of *Penaeus monodon*. Recombinant expression of this lysozyme in *Escherichia coli* and analysis of its antimicrobial activity were performed and significant results were obtained.

2 Materials and Methods

2.1 Materials

2.1.1 Vector and strain

pColdIV vector was bought from General Biology (Anhui) System Co., Ltd and competent cell of *E. coli* BL21 (DE3) was from Shanghai Local Biotechnology Co., Ltd.

2.2 Bioinformatics analysis of Pmlyzi3

The nucleotide sequence of Pmlyzi3 was obtained from transcriptome sequencing data of the hepatopancreas of

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Penaeus monodon (unpublished data). The Open Reading Frame and amino acid sequence of Pmlyzi3 were deduced by ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The signal peptide was predicted with signalP 4.0 server. DNAMAN version 6 was used for generating gene structure information. Homologous sequences of *Pmlyzi3* were obtained by Basic Local Alignment Search Tool (BLASTP) and multisequences alignment was performed with MEGA X. The physicochemical properties were predicted with the online software Protparam (<http://web.expasy.org/protparam/>).

2.3 Gene synthesis and vector construction

The mature peptide of *Pmlyzi3* was fused to the C-terminus of a SUMO protein with His-tag and overexpressed in *Escherichia coli* BL21 (DE3). The nucleotide sequence of His-SUMO-*Pmlyzi3* with *NdeI* and *SacI* cut sites in both ends was codon optimized for *E. coli* and chemically synthesized (General Biosystems, Inc., Hefei, China). The synthesized DNA was then linked to pColdIV vector by *NdeI* and *SacI* cut sites. The reconstituted plasmid was transformed to *E. coli* BL21 Rosetta (DE3), named pColdIV-SUMO-*Pmlyzi3*.

2.4 Verification of recombinant plasmid

Recombinant plasmid verified through PCR with primers of pColdIV-F and pColdIV-R. pColdIV-F was as : 5'-ACGCCATATCGCCGAAAGG-3' and pColdIV-R was as 5'-GGCAGGGATCTTAGATTCTG-3'. The PCR was performed with 94 °C, 3min; 40 cycles of 94 °C, 20s and 58 °C, 20 s; 72°C, 5min. The PCR product was checked by electrophoresis on a 1% agarose gel.

2.5 Protein expression in *E.coli*

The recombinant plasmid was transformed to *E. coli* BL21 Rosetta (DE). Adding 20 mL of overnight cultivated transgenic *E. coli* into the fresh 2 L LB broth medium containing ampicillin (50 µg/mL) and then cultivating them at 37 °C with shaking at 200 rpm until the absorbance reached 0.5 at 600 nm. IPTG was added to the culture at a final concentration of 1 mM. The induction was performed at 16°C for 6 hours. Cells were harvested by centrifugation and the cell pellets were washed and resuspended in PBS. The bacterial suspensions were then disrupted by ultrasonication for 40 min (power: 195W, open for 2 seconds, stop for 4 seconds). The supernatant (soluble fraction) was collected and analyzed by SDS-PAGE.

2.6 Optimization the expression of SUMO-*Pmlyzi3*

When induction with IPTG, culture was sampled after induced 0, 1.5, 3, 4.5, 6, 7.5 and 9h. The IPTG concentration was chosen at 0.2, 0.4, 0.6, 0.8 mM. All the samples were checked by SDS-PAGE.

2.7 Protein purification

The fusion protein was purified by Ni-NTA Sepharose Fast Flow (Ruidahenghui, Beijing, China), and the his-tagged protein was eluted with buffer contained 50 mM PBS, 300 mM NaCl and 200 mM imidazole. Elutes were further analyzed by SDS-PAGE.

The eluted protein was further dialyzed against 50mM Tris-HCl, 200mM NaCl to remove the imidazole and then quantified by Bradford reagent (Sangon, China). 100μg protein was mixed with 1U SUMO protease (General Biosystems, Inc., Hefei, China), and incubated at 4°C overnight to remove the SUMO tag. The pure Pmlyz3 was treated with 50% acetonitrile, then 100% acetonitrile to remove coomassie brilliant blue. The supernatant was discarded and the gel was treated with pancreatin (25μg/mL), then extract solution (acetonitrile and H₂O in 1:4, followed by 0.5% formic acid in total volume). The powder was obtained by ultrasonography and vacuum drying, and then dissolved in LC-MS buffer, finally identified by LC-MS.

3 Results

3.1 Identification of Pmlyzi3

Pmlyzi3 was obtained from the transcriptome after blast. The Pmplyzi3 gene was confirmed by RT-PCR and it was 438bp in length, encoding a 146-residues peptide and the first 19 residues constituted a signal peptide (Figure 1). The 127-residues mature peptide contained 10 cysteines and had 7 α -helixes in its N terminal (Figure 2). The molecular weight was 14.279 kDa and the theoretical pI was 4.62.

3.2 Alignment of *Pmlyzi3* with other lysozymes

Pmlyzi3 showed 88% identity with lysozyme-like from *Penaeus vannamei*, while 60% with lysozyme from *Eriocheir sinensis*. However, Pmlyzi3 had low identity with other lysozyme, even Penmonlys-like type from *Penaeus monodon* (Figure 3).

Fig. 1. Nucleotide and amino acid sequence of Pmlyzi3. The signal peptide was underlined; the cysteines were labeled in black triangle.

Fig. 2. Protein secondary structure of Pmlyzi3. α -helix was in green.

Fig.3. Molecular phylogenetic tree of Pmlyzi3 from *Penaeus monodon*.

The GenBank IDs of lysozymes sorted by species are as follows: *Penaeus vannamei* (Penvanlys-like: XP_027231470.1; Penvanlys-like-: XP_027212812.1; Penvanlys-like low: XP_027212212.1; Penvanlys-like type i-1: ROT76628.1; Penvanlys-like type i-1: ROT76627.1) ; *Penaeus japonicus* (Penjaplys: AMO44057.1) ; *Penaeus monodon* (Penmonlys-like type I: ACZ63471.1) ; *Eriocheir sinensis* (Erisinlys: AEU04535.1); *Mytilus galloprovincialis* (Mytgallys type I: AJQ21515.1; Mytgallys-partial: OPL33781.1; Mytgallys: AAN16210.1; Mytgallys2: BAF63423.1) ; *Procambarus clarkia* (Proclalys3 type I: AVK42824.1) ; *Limulus polyphemus* (Limpolllys-1: XP_022236369.1; Limpolllys1-l partial: XP_022236677.1; Limpolllys2-l: XP_013792023.1) ;

3.3 Vector construction, recombinant expression of Pmlyzi3

The mature peptide of Pmlyzi3 was linked with SUMO protein with His- tag, then ligated to pColdIV with *NdeI* and *SacI*. PCR results showed that SUMO-Pmlyzi3 could be amplified from pColdIV-SUMO-Pmlyzi3 with primers of pColdIV-F and pColdIV-R (Figure 4).

Fig.4. Verification of recombinant plasmid
M: DL 2000 marker; 1: negative control;
2: pColdIV-SUMO-Pmlyzi3

3.4 Expression of SUMO-Pmlyzi3

pColdIV-SUMO-Pmlyzi3 was transformed to *E.coli* BL21 (DE3) and then transformants were induced by 1.0mM IPTG for 6h, showing that SUMO-Pmlyzi3 was expressed with the weight of 25kDa and most of the recombination protein was soluble protein (Figure 5).

According to our results, the best protein expression condition was induced with 0.8mM IPTG for 4.5h.

Fig.5. Expression of SUMO-Pmlyzi3
M: protein marker; 1: without induction; 2: induction with 1.0mM IPTG for 6h; 3: supernatant after cell disruption; 4: sediment after cell disruption

3.5 Purification of Pmlyzi3

The His-SUMO-Pmlyzi3 fusion protein was expressed in *E.coli* induce with IPTG and then purified with a Ni-NTA column, finally eluted by 500mM imidazol as a pure protein (Figure 6).

Fig.6. Purification of SUMO-Pmlyzi3 by Ni-NTA column
M: protein marker; 1: sample, which is the supernatant after cell disruption; 2: flowthrough; 3: wash off protein with 100mM imidazole; 4, 5: eluted proteins with 200mM and 500mM imidazole

3.6 Obtaining Tag-free Pmlyzi3

After incubated with 0.5U SUMO protease, 50 μ g SUMO-

Pmlyzi3 was succeeded in removing SUMO tag (Figure 7). Following with LC-MS analysis, tag-free *Pmlyzi3* was confirmed that there was no other residues except that of *Pmlyzi3* (Figure 8).

Fig.7. Cleavage of SUMO tag
 M: protein marker; 1: SUMO-*Pmlyzi3*; 2: SUMO protease; 3: protein after cleavage, with SUMO and *Pmlyzi3* indicated

Protein sequence coverage: 22%

Matched peptides shown in **bold red**.

1 SSTTRGVGDD CLACMCWASS NCSMPTPVCK MNGWGEVCGP WAITEPYWID
 51 GGR**LFNNFYK** CVEDWKCNE D TVRNYLDRYV TNSDAKCQDY AR**THAGGPLG**
 101 **AWNDGTLPLYN YLVR**KDCLDYG IFTPPSV

Fig.8. LC-MS analysis of the enzyme digested product of SUMO-*Pmlyzi3*

4 Discussion

A new lysozyme named *Pmlyzi3* was found from the transcriptome data of *P. monodon* after bioinformatics analysis. Most of the lysozymes found in shrimp were c-type or i-type[19]. They owned a LYZ1 domain composed of 10 cysteines[20].

Antimicrobial peptide was hard to achieve high level expression because its molecular weight was low and they existed anti-bacterial action. The present results showed that SUMO as the molecular chaperone was helped to express antimicrobial peptide[21]. Hence, we employed SUMO as molecular chaperone to express antimicrobial peptide in *E.coli*. Considering that antimicrobial peptide was easy to form inclusion, SUMO-*Pmlyzi3* was induced in low temperature. Due to the his-tag, we can purify recombinant protein by Ni-NTA column[22]. After treated with SUMO protease, *Pmlyzi3* was freed from fusion protein so that we obtained pure *Pmlyzi3*, which would be helped to study its function.

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