Identification and characterization of a goose-type lysozyme from sewage snail *Physa acuta*

Yunhai Guoa, b, Hongxuan He c, *

a National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, Shanghai 200025, China
b Key Laboratory of Parasite and Vector Biology of the Chinese Ministry of Health, WHO Collaborating Centre for Malaria, Schistosomiasis and Filariasis, Shanghai 200025, China
c Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China

A R T I C L E   I N F O

Article history:
Received 22 December 2010
Received in revised form 16 May 2014
Accepted 19 May 2014
Available online 29 May 2014

Keywords:
Freshwater snail
*Physa acuta*
Goose-type lysozyme
Real-time PCR
Antimicrobial activities

A B S T R A C T

Freshwater snail *Physa acuta* has been considered as an important invasive species and medical mollusc. Field investigation has shown that this snail could survive better than other snails in polluted water bodies. To understand the immune mechanisms of *P. acuta*, suppression subtractive hybridization hepatopancreas cDNA library has been constructed with bacterial challenge. In this study, a full-length cDNA of a novel goose-type lysozyme (PALysG) has been identified from *P. acuta* by EST and RACE technique. The conservative structure domains share high homology with other molluscan g-type lysozymes including the SLT domain, the substrate binding sites, the catalytic residues, three alpha-helices structures and six molluscan specific cysteines. Meanwhile, PALysG is the first record of goose-type lysozyme in Gastropoda. Real-time PCR indicated that PALysG mRNA had been expressed significantly at high levels in hepatopancreas for 8–48 h. PALysG recombinant protein displayed the lytic activity of g-type lysozyme with other organisms against *Micrococcus lysodeikticus*.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

The freshwater snail *Physa acuta* (Mollusca, Gastropoda) which is known for having been introduced to more than 70 countries in the world [1,2], has evolved the unique ability to survive in aquatic habitats with extreme microbial stress such as drains, sewage pools, and seriously polluted water bodies [3,4]. It also can be an excellent model organism for aquatic toxicity testing [5]. This species maybe play a suitable model organism for investigations of the immune mechanisms against microbial stress of invasive alien species.

Innate immune system is of great importance to protect invertebrates against a wide range of microbial pathogens. Lysozyme is an important antibacterial protein based on hydrolytic activity to cleave the β-1, 4-glycosidic bond between N-acetyl-d-glucosamine and N-acetylmuramic acid in the peptidoglycan layer. Six different types of lysozymes are found including chicken-type lysozyme (c-type), goose-type lysozyme (g-type), and invertebrate-type lysozyme (i-type), plant-lysozyme, bacterial-lysozyme and phage-lysozyme [6]. Two different types of lysozymes are found in mollusc including g-type [7,8] and i-type [9].

In this study, we screen cDNA library of *P. acuta* that are induced upon immune challenge. Suppression subtractive hybridization (SSH) library has been constructed to selectively amplify and identify cDNAs that were differentially expressed in response to injected crude bacterial endotoxin (LPS). An expression sequence tag (EST) similar to g-type lysozymes has been identified from *P. acuta* by EST and RACE technique. The conservative structure domains share high homology with other molluscan g-type lysozymes including the SLT domain, the substrate binding sites, the catalytic residues, three alpha-helices structures and six molluscan specific cysteines. Meanwhile, PALysG is the first record of goose-type lysozyme in Gastropoda. Real-time PCR indicated that PALysG mRNA had been expressed significantly at high levels in hepatopancreas for 8–48 h. PALysG recombinant protein displayed the lytic activity of g-type lysozyme with other organisms against *Micrococcus lysodeikticus*.

2. Materials and methods

2.1. Animal collection and maintenance

Adult *P. acuta* were collected from Xiaoyue River, Chaoyang district, Beijing (40.0221° N, 116.2138° E) and immediately transported to the key laboratory of animal ecology and conservation

---

* Corresponding author. Tel./fax: +86 10 64807118.
E-mail address: hehx@ioz.ac.cn (H. He).

http://dx.doi.org/10.1016/j.fsi.2014.05.029
1050-4648/© 2014 Elsevier Ltd. All rights reserved.
biology, Institute of Zoology, Chinese Academy of Sciences. Snails were placed in holding tanks with recirculating freshwater maintained at room temperature until spawning. The offspring were transferred to other holding tanks until mature. Snails were fed approximately 1 g mixed fish food per 10 adult snails every second day. Food was withheld 24 h prior to experimentation.

2.2. Immune challenge of snail and collection of hepatopancreas

Fifty adult offspring snails, weighing between 140 and 190 mg, were used for immune challenge. 5 μl PBS sample volume, corresponding to 10 μg ml^-1 LPS (purified *Escherichia coli* endotoxin, Sigma, USA) per adult snail were injected into the foot using 1 ml disposable syringes. Meanwhile, another group snails injected with 5 μl PBS were used as control and maintained without bacterium challenge. Hepatopancreas from immune challenged, saline challenged snail was taken at 5 time periods, starting at 2, 4, 6, 8 and 24 h after injection. Snail was frozen in liquid nitrogen and immediately taken hepatopancreas to store at -80 °C.

2.3. Construction of cDNA libraries by SSH

Total RNA of *P. acuta* challenged with LPS and controls were extracted, respectively, by use of TRizol Reagent (Invitrogen) following the manufacturer’s protocol. The pooled RNA used for SSH was prepared by mixing equal quantity of all RNA samples together. From the total RNA, 2 mg mRNA was purified using poly (A) Tract mRNA Isolation Systems (Promega), and utilized as template, the first strand cDNA was generated using the SMART PCR cDNA Synthesis Kit (Clontech) according to the manufacturer’s protocol. The double strand cDNA was obtained by long distanced PCR with related primers. The cDNA libraries were constructed by SSH using the PCR-Select cDNA Subtraction Kit (Clontech) as described by the manufacturer. The challenged group cDNA was used for tester and the control group was used for driver.

2.4. cDNA cloning of lysozyme in *P. acuta*

By Blastx in the DNA database of NCBI, sequence comparison revealed that an EST from the SSH library of *P. acuta* had a high homology with lysozyme from the Zhikong scallop, *Chlamys farreri* (AY437875). To obtain complete sequence from the EST, rapid amplification cDNA ends (RACE) technique was utilized to amplify 5’ and 3’ ends sequences using SMART™ RACE cDNA Amplification Kit (Clontech) and gene specific primers (Table 1).

2.5. Sequence analysis

The deduced amino acid sequence from the identified cDNA sequence was called PALysG. The sequence analysis and protein domain features were performed by the Expert Protein Analysis System (http://www.ncbi.nlm.nih.gov/blast) and Simple Modular Architecture Research Tool [10], separately. Sequence alignments were computed using the ClustalX software [11], Phylogenetic tree was reconstructed using neighbor-joining method [12] by the software MEGA [13]. The relative supports of branches were tested by bootstrap analysis with 1000 replicates. Trees were showed with the software TREEVIEW. Sequences of g-type lysozymes included *Homo sapiens* (NP_783862.2), *Azumapecten farreri* (AGA95494.1), *Haliotis discus discus* (AGQ50335.1, AGQ50337.1), *A. irradians* (AAAX99791.1), *Mizuhopecten yessoensis* (AEY77130.1), *C. farreri* (AB853641.1), *Branchiostoma floridae* (XP_002614121.1), *Trichoplax adhaerens* (XP_002107677.1), *Danio rerio* (NP_001002706.1), *Gallus gallus* (NP_001001470.1), *Xenopus* (Silurana) *tropicalis* (NP_001015739.1), *Ciona intestinalis* (XP_002126087.1), *Oikopleura dioica* (CAD92344.1, CAD923242.1), and *Crassostrea gigas* (BAH66799.1).

2.6. Temporal expression patterns of PALysG mRNA in hepatopancreas

DNase-treated total RNAs were isolated from each treated individual which infected or controlled at 0, 2, 4, 8, 12, 24, 48 h by using RNA pure™ total RNA extraction kit (Biotek) according to the manufacturer's instrument. For Real-time PCR analysis, cDNAs were transcribed from RNAs treated with DNase by using an Omniscript reverse transcriptase kit (Qiagen). RT-PCR specific primers and β-actin gene primers (Table 1) were designed using the software Oligo 6, respectively. Triplicate experiments were performed for all reactions.

2.7. Recombinant expression and purification of PALysG

The *P. acuta* lysozyme gene was amplified with specific expression primers and cloned into Bac-to-Bac® Baculovirus expression system (Invitrogen) expression vector. For construction expression vector, EcoRI site and Xho I site were added to the PALysG gene recombinant expression primers, separately (Table 1). The purified PCR product were cloned into pFastBacHTa vector and sequenced to ensure in-frame insertion. pFastBacHTa/PALysG was transformed to *E. coli* DH10Bac. The cells were then spread on LB plates containing antibiotics (50 μg/ml kanamycin, 7 μg/ml gentamicin, and 10 μg/ml tetracycline), 100 μg/ml X-gal and the inducer, 40 μg/ml IPTG. The recombinant bacmid baculoviruses was extracted from the amplified *E. coli* DH10Bac, and then transfected into logarithmic growth phase Sf9 cell (preserved in our laboratory). Grace’s insect cell culture medium (Invitrogen) was utilized at 27 °C in laboratory. Recombinant protein contained 6× His tag at N-terminal according to pFast-BacHTa vector design. Ni-NTA spin columns were utilized to purify the protein. The results were identified on 12% slab SDS-PAGE.

2.8. Antimicrobial activities of PALysG

Based on Minagawa et al. [14], PALysG antimicrobial activity assay were performed using hen egg white lysozyme (HEWL) as reference by the radial diffusion method. Three bacterial strains *Micrococcus lysodeikticus*, *Staphylococcus aureus* MRSA and *E. coli* DH5α were used as substrates. The 200 μl bacteria were spread into the freshly poured 50 mm plates after the concentration was adjusted to 0.1 absorbence unit at 600 nm. Fifty μg purified protein was dropped into individual 5 mm diameter well. Radial diffusion assays were performed after 48 h incubation at 25 °C according to the radius of the clearing zone.
3. Results

3.1. Characterization of PALysG gene

Seven hundred and sixty-nine significant ESTs were sequenced from SSH library of P. acuta. A 233 bp EST was highly similar to other identified g-type lysozymes in invertebrate and vertebrate species by BLASTx analysis. Based on PA100245 sequence, 5’- and 3’-end nucleotide sequences were determined separately using RACE technique. The full-length cDNA sequence of PALysG (GenBank accession no. HQ435321) was obtained by sequences splicing. As shown in Fig. 1, PALysG cDNA 5’-end contained a 31 bp untranslated region, and 3’-end had a canonical polyadenylation signal sequence TATAAA and a poly A tail. A 597 bp open reading frame (ORF) encoded a 198 amino acids polypeptide. The predicted molecular weight and theoretical isoelectric point was 21.2 kDa and 6.38, respectively. The presumed amino acids sequence included a 15 amino acids signal peptide, a transglycosylase SLT domain (Bacterial lytic transglycosylases Domain) which contained special hydrolytic activity sites.

BLASTx results indicated that PALysG shared high similarity with other molluscan g-type lysozyme proteins, such as A. irradians (AAAX09979, 60% identity, E = 1e-69), C. farreri (ABB53641, 58% identity, E = 8e-60), C. gigas (BAH6799, 48% identity, E = 1e-42). Additionally, PALysG was clustered with other six molluscan g-type lysozymes in invertebrate and vertebrate species by multiple alignment [8,16]. Especially, PALysG shared the same six cysteine residues (Δ: Gli 79, Asp 94, Asp 105) [7,8,15], and three predicted alpha-helices structures of g-type lysozymes by multiple sequence alignment [8,16]. Additionally, PALysG shares the same SLT domain, the cysteine residues (Δ: Cys 104, Cys 113, Cys 121) with other molluscs [7,17]. Based on above descriptions, it apparently showed that PALysG should be a new g-type lysozyme.

Using 16 sequences of multiple alignments to construct phylogenetic tree, the result (Fig. 3) indicated that PALysG (ADV36303.1) was first clustered with other six molluscan g-type lysozymes. Other eight chordate animals g-type lysozyme constructed another major branch.

3.2. RT-PCR analysis

To understand the transcript levels of the PALysG lysozyme mRNA, hepatopancreas were used in different time after LPS challenge. The quantitative real-time PCR analysis results (Fig. 4) indicated PALysG mRNA was expressed significantly in 8 h, and arrived the highest level in 12 h after challenge.

3.3. Recombinant expression and antimicrobial activities of PALysG

The target protein molecular mass was 21 kDa which was consistent with the expected molecular mass by software. The antimicrobial activity of purified product was evaluated the antimicrobial activities by radial diffusion assay. As shown in Fig. 5, recombinant expression protein was inhibited significantly M. lysodichis multiplication according to the radius of the antimicrobial zone. Meanwhile, the antimicrobial capability of this protein was relatively stronger on E. coli DH5α than hen egg white lysozyme, but shared same effect on S. aureus MRSA.

4. Discussion

As an invasive species, P. acuta has been studied on distribution migration, and parasite host [1,4,18–21]. However, our study focus on the another phenomenon that P. acuta could survive better in polluted water environment than other snails. A better understanding the immune mechanisms of P. acuta might be lead to important advances in the innate immune system of invertebrate. In this study, we have constructed a suppression subtractive hybridization (SSH) library of P. acuta to selectively amplify and identify hepatopancreas cDNAs under immune challenge. A g-type lysozyme EST has identified by BLASTx and the full-length cDNA has amplified out by RACE techniques. The putative amino acid sequence (designated PALysG) shares the same SLT domain, the substrate binding sites, the catalytic residues and the three alpha-helices structures with other invertebrate and vertebrate g-type lysozyme [7,8,15]. Specially, same six cysteines with other molluscan g-type lysozyme also appear in PALysG which possibly constitute disulphide bridge to result in a compact structure [7,17]. It should further support that the six cysteines are molluscs-specific [7].

Many of g-type lysozymes have been identified from birds and fishes [15,22–28]. The g-type lysozyme has been found in invertebrate during the recent years. Until now, several g-type lysozymes have been discovered from molluscan Pelecypoda, such as...
C. gigas, C. farreri and A. irradians [7,8,17]. In this study, we have first identified a g-type lysozyme PALysG from Gastropoda. It demonstrates that this lysozyme is possibly existed extensively in molluscs. This molecular evolutionary relationship revealed the agreement with the concept of traditional taxonomy. In this molecular phylogenetic analysis, the mollusca g-type lysozyme of C. gigas was excluded. According to Itoh and Takahashi [8], the g-type lysozyme gene of C. gigas also contains peptidoglycan recognition protein (PGRP) sequence, and maybe possess simultaneously the function of bacterio-lytic and peptidoglycan recognition. The unique structure increase the likelihood of molecular evolution changing, also affect the molecular phylogenetic analysis.

Fig. 2. Multiple amino acid sequence alignment of PALysG with other g-type lysozymes from various animals. Catalytic residues and the substrate binding sites of g-type lysozymes are marked with black pentagon (●) and black triangle (▲), respectively [7]. White triangles (△) show the six cysteine residues [7,8,15]. Three boxes indicate three predicted alpha-helices structures [8,16]. Sequences used for the analysis are g-type lysozymes of Homo sapiens (NP_783862), Xenopus (Silurana) tropicalis (NP_001015739), Gallus gallus (NP_001001470), Danio rerio (NP_00102706), Branchiostoma floridae (XP_002611412), Ciona intestinalis (XP_002126087), Crassostrea gigas (BAH66798), Chlamys farreri (ABB53641), Argospecten irradians (AAK09979) and Trichoplax adhaerens (XP_002107877).

Fig. 3. Phylogenetic analysis of PALysG with other g-type lysozymes from various organism. Homo sapiens (NP_783862.2), Azumapecten farreri (AGA95494.1), Haliotis discus discus (AGQ03315), A. irradians (AAK09979), Mesoposthona yessoensis (AY777313.1), C. farreri (ABB53641), Branchiostoma floridae (XP_002611412), Trichoplax adhaerens (XP_002107767), Danio rerio (NP_001002706.1), Gallus gallus (NP_001001470.1), Xenopus (Silurana) tropicalis (NP_001015739.1), Ciona intestinalis (XP_002126087.1), Oikopleura dioica (CAD92344.1, CAD92342.1, Oikopleura dioica 100 AGQ50335.1: Halota discus discus 100 AGQ50337.1: Halota discus discus 100 AAX00979.1: Argospecten irradians 100 AGA95494.1: Azumapecten farreri 100 AAB853641:1 Chlamys farreri XP_002107877 Trichoplax adhaerens

Fig. 4. RT-PCR analysis of mRNA expression levels of PalysG in hepatopancreas at different times after LPS challenge. Vertical bars were shown as means ± S.E., n = 3.
The g-type lysozyme has been found in different molluscan tissues, such as gills, hepatopancreas, gonad, hemocytes, mantle, heart, intestine, etc. [7,8,17]. Previous studies have also suggested that g-type lysozyme had predominantly expressed in hepatopancreas of C. farreri [7]. In this study, we have succeeded to get g-type lysozyme PALysG from P. acuta hepatopancreas, and the expression pattern has been investigated in hepatopancreas with different water environment, the recombinant expression of PALysG has participated in internal defenses of microbial infection.

To understand the immune mechanism of P. acuta in polluted water environment, the recombinant expression of PALysG has been performed in baculovirus expression system to perform activity assay. The recombinant protein PALysG possessed the same lytic activity of g-type lysozyme with other organisms against M. lysodikicus. PALysG was similar to display without inhibiting capacity against S. aureus with C. farreri g-type lysozyme CFLysG [7]. Meanwhile, our radial diffusion assay indicated that PALysG had exhibited a weak defensive activity toward E. coli, which was consistent with grass carp Ctenopharyngodon idella g-type lysozyme [29]. Although g-type lysozyme has been identified from sewage snail P. acuta, the detailed knowledge need further researches, whether PALysG antimicrobial activity is stronger than other g-type lysozyme.

Acknowledgments

This work was funded by grants from CAS Innovation Program (KSCX2-YW-N-063), National Key Basic Research and Development Program of China (9732007BC0190103), USDA (0760621234), and the National Natural Science Funds (31040072).

References