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Two duplicated chicken-type lysozyme genes in disc abalone *Haliotis discus discus*: Molecular aspects in relevance to structure, genomic organization, mRNA expression and bacteriolytic function

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ABSTRACT

Lysozymes are crucial antibacterial proteins that are associated with catalytic cleavage of peptidoglycan and subsequent bacteriolysis. The present study describes the identification of two lysozyme genes from disc abalone *Haliotis discus discus* and their characterization at sequence-, genomic-, transcriptional- and functional-levels. Two cDNAs and BAC clones bearing lysozyme genes were isolated from abalone transcriptome and BAC genomic libraries, respectively and sequences were determined. Corresponding deduced amino acid sequences harbored a chicken-type lysozyme (LysC) family profile and exhibited conserved characteristics of LysC family members including active residues (Glu and Asp) and GS(S/T) DYGIFQINS motif suggested that they are LysC counterparts in disc abalone and designated as abLysC1 and abLysC2. While *abLysC1* represented the homolog recently reported in Ezo abalone [1], abLysC2 shared significant identity with LysC homologs. Unlike other vertebrate *LysC*s, coding sequence of *abLysC*s were distributed within five exons interrupted by four introns. Both *abLysC*s revealed a broader mRNA distribution with highest levels in mantle (*abLysC1*) and hepatopancreas (*abLysC2*) suggesting their likely main role in defense and digestion, respectively. Investigation of temporal transcriptional profiles post-LPS and -pathogen challenges revealed induced-responses of *abLysC*s in gills and hemocytes. The *in vitro* muramidase activity of purified recombinant (r) abLysCs proteins was evaluated, and findings indicated that they are active in acidic pH range (3.5–6.5) and over a broad temperature range (20–60 °C) and influenced by ionic strength. When the antibacterial spectra of (r)abLysCs were examined, they displayed differential activities against both Gram positive and Gram negative strains providing evidence for their involvement in bacteriolytic function in abalone physiology.

1. Introduction

Abalones are one of the commercially important, highly prized marine gastropods. In recent decades, commercial production of these molluscs has been severely threatened by pathogenic diseases caused by bacteria. As a consequence, mass mortalities and greater economic losses have been documented [2,3]. Furthermore, emergence of drug-resistant bacteria due to the exploitation of antibiotic usage in farm production has accelerated the devastating consequences of infectious diseases. Accordingly, revealing the characteristics of key effector molecules responsible for antimicrobial mechanisms in terms of genetics might give novel insights into molluscan immunity. Practical applicability of tools developed based on this knowledge could potentially help to improve disease control strategies in molluscan aquaculture. Unfortunately, information regarding the molecular aspects and functional mechanisms related to molluscan immunity are much limited when compared to arthropods and vertebrates.

Unlike vertebrates that possess adaptive immunity, invertebrates including molluscs solely rely on innate immunity. Molluscan innate immune responses are the first line of host-defense that includes cellular responses mediated by hemocytes and humoral responses encaging antimicrobial proteins as the

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primary effector molecules against pathogen invasion [4,5]. Lysozyme (EC3.2.1. 17; muramidase), a well-known antimicrobial hydrolase enzyme, is distributed in a wider range of phylogenetically diverse biota from bacteriophage to humans. Lysozymes exert their biological function by enzymatically cleaving the β-(1,4)-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine moieties of peptidoglycan, a polymer compound exclusively found in bacterial cell wall and lyse the bacteria [6]. Hence, the principal role that lysozyme plays in most of the living systems is biodefense to protect the hosts from invading bacteria. Moreover, solubilized peptidoglycan fragments resulting from lysozyme-mediated degradation may significantly modulate the downstream immune pathways [7]. Besides, bactericidal property of lysozymes could be independent of its muramidase activity [8,9]. As a secondary role, lysozymes are considered to be involved in digestion of bacteria as feed, which also utilizes its ability to collapse bacterial cell wall. The marine environment is enriched with microorganisms, and molluscs must have to coexist with potential pathogens. Therefore, it was proposed that aquatic lysozyme may participate in dual-roles of defense and digestion in marine animals including cultured molluscs [10].

Lysozymes are traditionally classified into six sub-types based on their molecular properties, functional- and evolutionary-aspects: chicken-type (LysC), goose-type (LysG), invertebrate-type (LysI), phage-type, bacterial-type and plant-type lysozymes. LysC, LysG and LysI are commonly identified in animal kingdom, while LysC being the archetype model. To date, nearly 16 *LysI*s and 6 *LysG*s have been described from different molluscan species [11–16]. It was believed that *LysC* distribution in invertebrates is limited to arthropods and cephalochordates until Ding et al. reported the first molluscan *LysC* homolog from Ezo abalone recently [1].

We discovered two distinct variants of chicken-type lysozymes from disc abalone, *Haliotis discus discus* (*abLysC1* and *abLysC2*) using genomic sequencing and transcriptome analyses. This study is devoted toward the following main objectives: (1) to characterize *abLysC*s at molecular level and to elucidate its classification, (2) to determine the genomic organization of *abLysC*s, (3) to investigate basal-transcription and temporal mRNA expression after LPS- and pathogen-challenges and (4) to clone and express the recombinant abLysCs in order to demonstrate their muramidase activity and antibacterial spectra.

2. Materials and methods

2.1. Chemicals, reagents and bacteria

Reagents used in genomic experiments including *Taq* polymerase, SYBR Ex *Taq* and restriction enzymes were purchased from TaKaRa Bio, Japan. Molecular markers were provided by Enzynomics, Korea. Primers used in this study were synthesized by Integrated DNA technologies, Inc, USA. In cloning and expression of recombinant protein, pMAL-c2X expression vector and pMAL protein fusion and purification system were employed, respectively (NEW ENGLAND BioLabs® Inc.) [18]. All the kits used during cloning for PCR- and gel-purification and plasmid extraction were obtained from Bioneer, Korea or QIAGEN, Germany. All the molecular biology grade chemicals were provided by Sigma, USA. The bacterial strains used in the bacterial challenge experiments and lytic assays were obtained from the Korean Collection for Type Cultures (KCTC).

2.2. cDNA and genomic DNA isolation of abalone chicken-type lysozymes

We previously constructed and sequenced a normalized transcriptome library of disk abalone. Total RNA was isolated from whole healthy abalone tissues using Tri Reagent™ (Sigma) and mRNA was purified from it using FastTrack® mRNA isolation kit (Invitrogen). First strand cDNA was synthesized using Creator™ SMART™ cDNA library construction kit, Clontech and normalized using Trimmer-Direct cDNA normalization kit, Evrogen. The shotgun transcriptome database was established based on data obtained from sequencing the library using Roche 454 platform and a GS-FLX™ technology (DNA Link, Inc.). BLAST search of candidate contigs on NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) led us to identify two lysozyme homologs which were homologous to earlier defined chicken-type lysozymes, and we designated them as *abLysC1* and *abLysC2*, respectively.

We have also custom built a BAC library for disc abalone (Lucigen®, USA). Briefly, the genomic DNA extracted from abalone gills was randomly sheared and inserts with an average size of 120 kb were ligated into pSMART® BAC vectors. We recovered around 92160 independent clones, arrayed in microtiter plates and grown individually. Aliquots of grown cultures were systematically pooled with other clones located in same plate, row and column. These pools were further combined to form 20 super pools.

In order to screen the BAC library and determine the location of a clone of interest, a 2-step PCR approach was adopted as per manufacturer's suggestion, employing gene specific primers designed based on cDNA sequence (Supplementary Table 1; LysC2-F3/R4). Then, genomic DNA isolated from positive clone was confirmed to possess the specific gene using colony PCR. Finally, 15 different positive clones of different abalone genes were pooled and subjected to GS-FLX™ sequencing (Macrogen, Korea). Complete genomic sequences and putative promoter region of *abLysC2* were obtained from the sequencing data.

2.3. Molecular and genomic characterization of abLysCs

The CDSs and corresponding amino acid sequences of *abLysC*s were obtained from cDNAs using DNAssist 2.0. Homologous *LysC* sequences were retrieved from GenBank by subjecting abLysC sequences to BLASTp search. The exon-intron structure of *abLysC*s was determined by aligning mRNAs to genomic sequences using the Spidey program (http://www.ncbi.nlm.nih.gov/spidey/), and then compared with other reported *LysC* gene structures. The web-tools such as AliBaba2.1, TFSEARCH 1.3 and/or TESS were used to identify potential transcription factor binding sites in the 5′-flanking regions of approximately 2 kb. Deduced abLysC amino acid sequences were characterized by different tools available in ExPASy server (http://www.expasy.org/). Pairwise and multiple alignments were performed using the Needle tool and ClustalW2 at the EBI (http://www.ebi.ac.uk/Tools/), respectively. The tertiary structures of abLysCs were generated based on the ab-initio homology modeling strategy, using the online server I-TASSER and visualized using RasMol 2.7.5.2 [17,18]. A phylogenetic tree composed of different lysozyme sequences from various lineages was constructed using the neighbor joining (NJ) method embedded in MEGA 5.0 platform, and bootstrapped for 5000 replications.

2.4. Abalone, pathogens and lysozyme substrates

Healthy disc abalones with average weight of 50 ± 2 g were obtained from 'Youngsoo' farm (Jeju, Korea), and acclimated in 250 L flat bottom tanks filled with aerated sea water at a salinity of 33‰ at 20 ± 1 °C in Marine and Environmental Research Institute of Jeju National University. Animals were fed with fresh sea weed, *Undaria pinnatifida*, and after one week of acclimation, they were used in experiments.

To determine the immune response of *abLysC*s, different challenge experiments were devised. For bacterial challenges, a Gram