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A bifunctional invertebrate-type lysozyme from the disk abalone, *Haliotis discus discus*: Genome organization, transcriptional profiling and biological activities of recombinant protein

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ABSTRACT

Lysozyme is an important enzyme in the innate immune system that plays a vital role in fighting microbial infections. In the current study, we identified, cloned, and characterized a gene that encodes an invertebrate-type lysozyme from the disk abalone, *Haliotis discus discus* (*abLysI*). The full-length cDNA of *abLysI* consisted of 545 bp with an open reading frame of 393 bp that encodes 131 amino acids. The theoretical molecular mass of mature abLysI was 12.3 kDa with an isoelectric point of 8.03. Conserved features in other homologs, such as catalytic sites for lytic activity (Glu^{30} and Asp^{41}), isopeptidase activity (His^{107}), and ten cysteine residues were identified in abLysI. Genomic sequence analysis with respect to its cDNA showed that *abLysI* was organized into four exons interrupted by three introns. Several immune-related transcription factor binding sites were discovered in the putative promoter region. Homology and phylogeny analysis of abLysI depicted high identity and closer proximity, respectively, with an annelid i-type lysozyme from *Hirudo medicinalis*, and indicated that abLysI is a novel molluscan i-type lysozyme. Tissue-specific expressional studies revealed that *abLysI* is mainly transcribed in hepatopancreas followed by mantle. In addition, *abLysI* mRNA expression was induced following bacterial (*Vibrio parahaemolyticus* and *Listeria monocytogenes*) and viral (viral hemorrhagic septicemia virus) challenges. Recombinantly expressed abLysI [(r)abLysI] demonstrated strong lytic activity against *Micrococcus lysodeikticus*, isopeptidase activity, and antibacterial activity against several Gram-positive and Gram-negative bacteria. Moreover, (r)abLysI showed optimum lytic activity at pH 4.0 and 60 °C, while exhibiting optimum isopeptidase activity at pH 7.0. Taken together, these results indicate that abLysI is potentially involved in immune responses of the disk abalone to protect it from invaders.

1. Introduction

Lysozyme (EC 3.2.1.17), a well-known bacteriolytic enzyme, is extensively distributed in biota. Their key role is to lyse bacteria by hydrolyzing the 1,4-β-linkages in a bacterial cell wall compound called peptidoglycan (Qasba and Kumar, 1997). Various names, including muramidase and N-acetylmuramide glycanhydrolase, have been attributed to the function of lysozyme. Thus, the major function of lysozyme is host defense, as it acts as an antimicrobial and immune-modulating agent (Jolles and Jolles, 1984). In addition, lysozyme displays digestive activity (Dobson et al., 1984) or non-enzymatic activity (Cong et al., 2009; Zavalova et al., 2006) in some species.

Three distinct types of lysozyme, namely, goose type (LysG), chicken type (LysC), and invertebrate type (LysI) have been identified in the animal kingdom based on differences in their structural and biological functions. Interestingly, LysI has been characterized only in invertebrates even though other lysozyme types have been identified in both vertebrates and invertebrates. The existence of LysI was first reported by Jolles and Jolles from starfish (*Asterias rubens*), which is a marine invertebrate, based on a comparison of its N-terminal amino acid sequence with other known lysozymes (Jolles and Jolles, 1975). The first complete amino acid sequence of a LysI was described in 1999 by Ito for a marine bivalve *Tapes japonica* (Ito et al., 1999), although several LysIs have been previously purified and partially characterized (Myrnes and

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Johansen, 1994; Perin and Jolles, 1972). Subsequently, a number of LysI variants have been reported for several invertebrate phyla, including Annelida (Joskova et al., 2009), Nematoda (Mallo et al., 2002), Arthropoda (Paskewitz et al., 2008), Echinodermata (Cong et al., 2009), and Mollusca (Matsumoto et al., 2006).

In addition to its lytic activity, some LysIs exhibit isopeptidase activity. It has been previously demonstrated that LysIs harboring a "destabilase domain" can exhibit isopeptidase activity (Callewaert and Michiels, 2010). In this reaction, lysozyme can hydrolyze the bonds between glutamine γ-carboxamide and the lysine ε-amino group in fibrin, which is involved in blood coagulation (Zavalova et al., 1996). Medicinal leech (*Hirudo medicinalis*) secretes isopeptidase (also called destabilase) during blood ingestion in order to prevent blood clotting (Zavalova et al., 1996). Meanwhile, isopeptide bonds have been identified in the peptidoglycan of bacterial cell wall. These isopeptide bonds are located between D-glutamic acid and lysine residues in peptides, which connect each straight chain of 1,4-β-linked N-acetylmuramic acid and N-acetylglucosamine in an annular fashion and are suspected of being targets of isopeptidases (Takeshita et al., 2003).

Abalone, a marine gastropod mollusc, is an economically valuable species in Eastern Asia, including in the Republic of Korea. During the past few years, a great loss of abalones has been reported due to several factors such as polluted environments, global climate change, and the occurrence of diseases that are caused by microbial pathogens (Cai et al., 2008; Chang et al., 2005; Goggin and Lester, 1995; Liu et al., 2001). As a filter-feeding organism, abalone can easily be exposed to various potential pathogens in aquatic environments. Bacterial infection is one of the major threats to the health status of abalones. Vibriosis (Elston and Wood, 2006) and blister or white spot necrosis (Li et al., 1997) are among the types of diseases that have been identified in abalone. A better understanding of the immune defense responses of abalone might be helpful to improve its health condition by reducing its vulnerability to the pathogenic infections.

In the present study, we isolated and characterized both the cDNA and genomic sequences of an invertebrate type lysozyme from abalone, *Haliotis discus discus*, which shows high similarity with other LysI of various invertebrate species. We cloned, recombinantly expressed, and purified the abalone LysI by using a pMAL fusion protein system in order to study its bactericidal activity against different types of Gram-positive and Gram-negative bacterial species, as well as to study its isopeptidase activity. Tissue-specific mRNA expression was performed for several tissues in healthy abalone, and temporal transcriptional expressions were analyzed kinetically after challenging the animals with bacterial, viral, and immune stimulatory agents.

2. Materials and methods

2.1. Identification of full-length cDNA

The full-length cDNA sequence of the disk abalone lysozyme gene was extracted from a database that was established in our laboratory by employing a Roche 454 Genome Sequencer FLX System (GS-FLX™) (Droege and Hill, 2008). In brief, total RNA was extracted from healthy abalone with Tri Reagent™ (Sigma–Aldrich, Missouri, USA) and processed with a FastTrack® 2.0 mRNA isolation kit (Invitrogen, USA). To synthesize and normalize the first-strand cDNA, a Creator™ SMART™ cDNA library construction kit (Clontech, USA) and a Trimmer cDNA normalization kit (Evrogen, Russia) were used, respectively. The BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST) was applied to confirm the gene, which was designated as *abLysI*.

2.2. Identification of the genomic sequence of abLysI

The genomic sequence of *abLysI* was determined from our BAC library, which was constructed on our behalf by Lucigen® Co. (USA). As previously reported (Premachandra et al., 2012), a PCR-based screening method was adopted to isolate the correct clone with gene-specific primers (Table 1) according to the manufacturer's protocol and sequenced by means of the GS-FLX® approach (Macrogen, Korea).

2.3. Bioinformatics analysis of abLysI sequence

The *abLysI* cDNA sequence was analyzed using the BLAST algorithm at NCBI (http://www.ncbi.nlm.nih.gov/blast) and searched to identify orthologs. Multiple sequence analysis was performed using the ClustalW2 program (http://www.Ebi.ac.uk/Tools/clustalw2) (Thompson et al., 1994). The Expert Protein Analysis System (http://www.expasy.org/) was used to analyze the deduced amino acid sequence, whereas SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) was employed to predict the signal peptide of the abLysI protein. To predict the disulfide bonds in abLysI, http://clavius.bc.edu/~clotelab/DiANNA/ was used (Ferre and Clote, 2005). For phylogenetic analysis, sequence alignment was performed with the abLysI deduced amino acid sequence and corresponding sequences from various animals by using the ClustalW program. Subsequently, a neighbor-joining tree was constructed by using MEGA version 5.05 (Tamura et al., 2011) based on the above alignment. Bootstrap trials were replicated 5000 times to derive the confidence value for the phylogeny analysis. The exon–intron regions of the genomic sequence were analyzed with the Spidey program (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/). TFSEARCH ver1.3 (http://www.cbrc.jp/research/db/TFSEARCH.html) was utilized to predict the different transcriptional factor-binding sites in the 5′-flanking region of the *abLysI* gene.

2.4. Structural modeling of abLysI

A tertiary (3D) structural model of abLysI was generated by submitting the amino acid sequence to the automated mode of Swiss-Model (Arnold et al., 2006). A 1.78 Å resolution crystal structure of NAG-bound lysozyme from *Meretrix lusoria* (Chain A; PDB No. 3ab6) was selected from the PDB database and used as a template.

Table 1
Description of primers used in this study.

Primer name	Application	Sequence (5′-3′)
abLysI-F	ORF amplification	$(GA)_3$gaattcATGTTGAAGCTCGCAATCTTTGCTGTT
abLysI-R	ORF amplification	$(GA)_3$aagcttTCAACCGATGTGTGTCTGGACCTTG
abLysI-RT-F	qPCR amplification & BAC screening	AGTTGCGTCAGGGCCTACTTGAAT
abLysI-RT-R	qPCR amplification & BAC screening	AGTACCCGAGTGTGTTGCTGTGTA
abRibI-F	qPCR Internal reference	TCACCAACAAGGACATCATTTGTC
abRibI-R	qPCR Internal reference	CAGGAGGAGTCCAGTGCAGTATG

Restriction enzyme sites of the cloning primers are in lowercase and underlined.