Gene 674 (2018) 134–142

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

A Lysin motif (LysM)-containing protein from Hong Kong oyster, *Crassostrea hongkongensis* functions as a pattern recognition protein and an antibacterial agent

Jun Li[a,b], Yang Zhang[a,b], Yuehuan Zhang[a,b], Fan Mao[a,b,c], Shu Xiao[a,b], Zhiming Xiang[a,b], Haitao Ma[a,b], Ziniu Yu[a,b,*]

[a] Key Laboratory of Tropical Marine Bio-Resources and Ecology, Guangdong Provincial Key Laboratory of Applied Marine Biology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou 510301, China
[b] South China Sea Bio-Resource Exploitation and Utilization Collaborative Innovation Center, China
[c] University of Chinese Academy of Sciences, 19A Yuquan Road, Beijing 100049, China

ARTICLE INFO

Keywords:
Lysin motif
Crassostrea hongkongensis
Peptidoglycan binding
Antibacterial activity

ABSTRACT

Lysin motif (LysM)-containing proteins are a family of carbohydrate-binding modules and are generally regarded as chitin- and peptidoglycan-binding proteins. In the present study, a novel LysM-containing protein, designated as *ChLysM*, was cloned and identified in a marine mollusk, *Crassostrea hongkongensis*. The full-length cDNA of *ChLysM* consists of 1129 bp, with an open reading frame of 861 bp encoding a 286 amino acid polypeptide. The deduced protein had a calculated molecular mass of 32.66 kDa and a pI of 8.16. SMART analysis indicated that ChLysM has one Lysin motif and a transmembrane region in the C-terminal residues. Tissue distribution analysis of *ChLysM* revealed high expression in gills and hemocytes. The upregulated transcripts of *ChLysM* in response to bacterial challenge suggest that ChLysM is involved in innate immunity against pathogen infection. The recombinant protein of ChLysM was found to bind to various kinds of peptidoglycans from *Staphylococcus aureus*, *Bacillus subtilis* and *Saccharomyces cerevisiae*, as well as binding strongly to both Gram-positive and Gram-negative bacteria. Moreover, ChLysM displayed broad-spectrum antibacterial activity against both Gram-positive bacteria (*S. aureus* and *S. haemolyticus*) and Gram-negative bacteria (*Escherichia coli* and *Vibrio alginolyticus*). Collectively, these results indicate that ChLysM is a pattern recognition molecule with bacterial growth-inhibiting activity in immune defense of *C. hongkongensis*.

1. Introduction

Mollusks, like all invertebrates, lack an adaptive immune system, and depend on the innate immune system to defend against invading pathogens (Beutler, 2004). During the innate immune response, recognition of non-self is the first and most crucial step in mounting immune responses (Medzhitov and Janeway, 2002). It is mediated by pattern recognition receptors (PRRs), which can recognize conserved pathogen-associated molecular patterns (PAMPs) on the surface of foreign intruders such as bacteria, fungi and viruses (Gordon, 2002).

The lysin motif (LysM) domain, a small protein domain of 40 amino acids, is an ancient and ubiquitous protein module that functions in peptidoglycan recognition and binding (Gust et al., 2012). It was initially identified in bacterial cell wall degrading enzymes of Bacillus phage Θ29 (Garvey et al., 1986). Similar motifs were subsequently observed in the peptidoglycan hydrolase of *Enterococcus faecalis* (Beliveau et al., 1991). Carbohydrate recognition is important for many essential biological processes, such as bacterial cell wall degradation, response to bacterial and viral infections, and pathogen defense. As a result, LysM modules containing one or more Lysin motifs can be found almost in all living organisms except archaea (Buist et al., 2008). In prokaryotes, LysM domains are widely distributed in many bacterial enzymes that are involved in cell wall degradation. These enzymes play an essential role in remodeling of cell wall peptidoglycans (PGN) during bacterial cell division (Steen et al., 2003). Several LysM domains are also involved in bacterial pathogenesis and symbiosis. The extracellular

Abbreviations: cDNA, DNA complementary to RNA; RNA, Ribonucleic acid; DNA, deoxyribonucleic acid; kDa, Kilodalton; nt, nucleotide; s, second; h, hour; NCBI, National Center for Biotechnology Information; SMART, Simple Modular Architecture Research Tool; EF1α, elongation factor 1 alpha; PCR, Polymerase chain reaction; bp, base pair; IPTG, Isopropyl β-D-1-thiogalactopyranoside

* Corresponding author at: South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou 510301, China.
E-mail address: carlzyu@scsio.ac.cn (Z. Yu).

https://doi.org/10.1016/j.gene.2018.06.091
Received 4 March 2018; Received in revised form 11 June 2018; Accepted 26 June 2018
Available online 28 June 2018
0378-1119/

protein p60 of *Listeria monocytogenes*, a LysM protein, is a major virulence factor of the intracellular bacterium (Dussurget et al., 2004). Intimin, an outer membrane protein in enteropathogenic *Escherichia coli*, also contains a LysM domain and is involved in attachment to mammalian cells (Bateman and Bycroft, 2000). In plants, LysM domains have been shown to bind to both peptidoglycan and chitin. In *Arabidopsis*, a LysM-containing receptor-like kinase was shown to be involved in defense signaling in response to fungal pathogens by binding to chitin, a β1-4 linked homopolymer of *N*-acetylglucosamine (Miya et al., 2007; Liu et al., 2012a). In rice, CEBiP, a LysM-containing receptor-like protein, played a key role in the perception and transduction of chitin oligosaccharide elicitor (Kaku et al., 2006). LysM-containing proteins are also common in animals. In zebrafish, two distinct subfamilies called LysMD and OXR were identified and shown to be highly conserved across vertebrates (Laroche et al., 2013a). Shi et al. showed that in red swamp crayfish, PcLysM could recognize different microorganisms and played a role in the innate immune responses against bacterial infection (Shi et al., 2013). In kuruma shrimp, a new Lysin motif and putative peptidoglycan-binding domain-containing protein was found to regulate the expression of some antimicrobial peptide genes and facilitate *V. anguillarum* clearance in vivo (Shi et al., 2016).

Despite a wealth of knowledge on the role of LysM proteins in vertebrates, scarce information is available concerning the biological activities and physiological roles of these proteins in invertebrates, especially mollusks, the second most diverse group of animals. Thus, in the present study, the first mollusk LysM domain-containing gene was identified and characterized from the Hong Kong oyster *Crassostrea hongkongensis*, a commercially important marine shellfish in China. Our results will shed light on the role of ChLysM and may help to elucidate the molecular mechanisms of immune defense in oysters.

2. Materials and methods

2.1. Oysters challenge and tissues collection

Two-year-old Hong Kong oysters with an average shell height of 10.0 cm were obtained from a local farm in Zhanjiang, Guangdong Province, China. Oysters were acclimated to aerated sand-filtered seawater at 24–25 °C for one week before processing.

The bacterial challenge experiment was performed according to our previous work (Li et al., 2016). Briefly, oysters received an injection of 100 μL (approximately 1×10^8 bacterial cells) of *Vibrio alginolyticus* (Gram-negative bacteria), *Staphylococcus haemolyticus* (Gram-positive bacteria) or phosphate buffered saline (PBS) as a control. After injection, the oysters were returned to the seawater tanks and 5 individuals from each group were randomly sampled at 3, 6, 12, 24, 48 and 72 h post-injection. The hemocytes from both treatment and control groups were collected for RNA extraction.

To analyze tissue distribution, total RNA was extracted from random chosen adult oysters, including adductor muscles, hearts, mantles, digestive glands, gills, gonads and hemocytes.

2.2. cDNA cloning and sequence analysis of ChLysM

A partial cDNA sequence of *ChLysM* was retrieved from our Hong Kong oyster transcriptional database. To obtain the full-length cDNA of *ChLysM*, the SMART™ RACE cDNA amplification kit (Clontech, Japan) was used, following our previous description (He et al., 2011). The primers used in this study are listed in Table 1. RACE products were cloned to pGEM-T Easy Vector (Promega, USA) and sequenced using T7 and SP6 primers on an ABI PRISM 3730 Genetic Analyzer (Perkin Elmer, USA). The full-length cDNA sequences were obtained by combining the 3′- and 5′-end sequences.

The ChLysM sequence was analyzed using the BLAST algorithm at NCBI (http://www.ncbi.nlm.nih.gov/blast) and the Expert Protein Analysis System (http://www.expasy.org/). Protein motifs were predicted with SMART (http://smart.embl-heidelberg.de). A neighbor-joining (NJ) phylogenetic tree was constructed based on the full-length amino acid sequences of known LysM proteins using the MEGA version 5.0 with 1000 bootstrap replicates.

Table 1
Sequences of primers used in this study.

Primer	Sequence (5′-3′)	Comment
ChLysMR1	AATTCCACAACTTCATCTTCTTGAAC	5′ RACE of ChLysM
ChLysMR2	CCTAGTTGCTGGTAGCTGTAAGGTTT	
ChLysMF1	GTAAAGGGGGACCAAAACCTACACAG	3′ RACE of ChLysM
ChLysMF2	GATCCAGGACCAAGATTTTTATGCTT	
UPM-long	CTAATACGACTCACTATAGGGCAAGCAGTGGT ATCAACGCAGAGT	First PCR for 5′ and 3′ RACE
UPM-short	CTAATACGACTCACTATAGGGC	
NUP	AAGCAGTGGTATCAACGCAGAGT	Nested PCR for 5′ and 3′ RACE
ChLysMF3	ACGTGGATCCATGAGTTCAAGAAAGTCCAG	
ChLysMR3	TCGAGTCGACTCATCCATTATTCTGACCTGC	Vector for ChLysM-pET28a
ChLysMF4	GTGTATGTGTTTGGTAATGCT	Real-time-PCR of ChLysM
ChLysMR4	CGGACATCCATACTGTAATGAA	
EF1α-F	GCTCCACCCAACATCACCACTG	Real-time-PCR of EF1α
EF1α-R	ACGGATTTCCTTTACGGACACG	

"F" indicates forward primer and "R" indicates reverse primer.

2.3. Transcriptional analysis of ChLysM

Total RNA was extracted from different oyster tissues with TRIzol Reagent (Invitrogen, USA) as described in the manufacturer's protocol. To synthesize cDNA, 1 μg total RNA was reverse-transcribed using PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Japan) and oligo(dT)-adaptor as primer.

The expression patterns of *ChLysM* were determined by quantitative real-time PCR (qRT-PCR) in a Light-Cycler 480 II System (Roche, USA). *EF1α* was selected as the internal control. The primers used in this assay are listed in Table 1. The qRT-PCR reaction was carried out in a total volume of 20 μL, containing 10 μL of 2× SYBR Green Master Mix (Applied Biosystems), 1 μL of the diluted cDNA, 1 μL of each primer (10 μmol L^{-1}) and 7 μL of DEPC-water. Each sample was run in triplicate along with the internal control gene. Dissociation curves of amplification products were generated to assess the specificity of the PCR amplification. The PCR efficiencies for the target and internal control gene (*EF1α*) were approximately equal and expression levels of *ChLysM* were calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). And the data f was done using student's *t*-test.

2.4. Recombinant expression and purification of ChLysM

The Open Reading Frame (ORF) of *ChLysM* was generated by PCR using primer pairs containing *Bam*HI and *Sal*I at the 5′-terminal and 3′-terminal ends, respectively (Table 1). After digestion, the target product was subcloned into a pET-28a vector. The positive clones were screened by PCR and verified by further DNA sequencing. The recombinant plasmid (pET-28a-ChLysM) was transformed into *E. coli* BL21 (DE3) (Novagen). Positive transformants were incubated in LB medium with 100 mg mL^{-1} kanamycin at 37 °C with shaking at 200 rpm. When the culture media reached an OD600 of 0.5–0.6, the cells were incubated for an additional 4 h after induction with IPTG at a final concentration of 1 mM. The recombinant ChLysM protein containing a 6 × His-tag was purified by affinity chromatography using nickel-nitrilotriacetic acid agarose (Ni-NTA) resins (Qiagen, Germany) following the manufacturer's protocol. The purified recombinant protein was analyzed by