Developmental and Comparative Immunology 84 (2018) 382–395

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

First characterization of an anti-lipopolysaccharide factor (ALF) from hydrothermal vent shrimp: Insights into the immune function of deep-sea crustacean ALF

Han-jie Gu [a, b, c], Qing-lei Sun [a, b], Shuai Jiang [a, b], Jian Zhang [a, b, d, *], Li Sun [a, b, **]

[a] CAS Key Laboratory of Experimental Marine Biology, Institute of Oceanology, and Center for Ocean Mega-Science, Chinese Academy of Sciences, Qingdao, China
[b] Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China
[c] University of Chinese Academy of Sciences, Beijing, China
[d] Center of Deep Sea Research, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China

ARTICLE INFO

Article history:
Received 30 January 2018
Received in revised form
18 March 2018
Accepted 19 March 2018
Available online 20 March 2018

Keywords:
Anti-lipopolysaccharide factor
Antimicrobial peptides
Rimicaris sp.
Deep-sea hydrothermal vent
Antibacterial
Antiviral

ABSTRACT

Anti-lipopolysaccharide factor (ALF) is a type of antimicrobial peptides (AMPs) with a vital role in antimicrobial defense. Although a large amount of ALFs have been identified from neritic and fresh water crustacean species, no functional investigation of ALFs from deep-sea animals have been documented. In the present study, we characterized the immune function of an ALF molecule (named RspALF1) from the shrimp *Rimicaris* sp. residing in the deep-sea hydrothermal vent in Desmos, Manus Basin. RspALF1 shares 51.5%–62.4% overall sequence identities with known shrimp ALFs and contains the conserved LPS binding domain (LBD). Both recombinant RspALF1 (rRspALF1) and the LBD-derived peptide (ALF1P1) bound to the cell wall components of Gram-negative and Gram-positive bacteria and killed a wide range of bacteria, especially those from deep-sea hydrothermal field, by damaging bacterial cellular structures. The bactericidal activities of rRspALF1 and ALF1P1 were optimal and stably maintained from 4 °C to 37 °C, which is comparable to the ambient temperature range of the habitat of *Rimicaris* sp. In addition to bacteria, rRspALF1 and ALF1P1 also exhibited anti-fungal activity. rRspALF1 and ALF1P1 exhibited high killing efficiencies, which, in terms of MIC values, were ranged between 0.25 μM and 4 μM for bacteria and 4 μM–8 μM for fungi. When introduced *in vivo*, both rRspALF1 and ALF1P1 effectively inhibited bacterial infection in shrimp and reduced the dissemination of bacterial and viral pathogens in fish. Together, these results provide the first insight into the biological property of deep-sea ALF and indicate that RspALF1 very likely plays a significant role in immune defense by functioning as a highly effective antimicrobial with a broad target range.

1. Introduction

Antimicrobial peptides (AMPs) are a diverse class of naturally existing molecules that constitute an important part of the innate immune system by functioning as a first line of defense against invading microorganisms (Sperstad et al., 2011). AMPs have broad killing effects on bacteria, fungi, viruses, parasites, and even cancerous cells (Roch, 2004). Unlike most antibiotics, which usually target specific proteins (Nguyen et al., 2011), many AMPs work on bacterial membranes or other generalized targets, and there is mounting evidence that compounds targeting fundamental structures are less likely to induce bacterial resistance than conventional antibiotics (Hancock and Patrzykat, 2002). Compared to the large number of AMPs isolated from terrestrial invertebrates (~1500), much fewer AMPs (~40) from marine organisms have been characterized (Sperstad et al., 2011).

Anti-lipopolysaccharide factor (ALF) is a type of AMP with broad-spectrum antimicrobial activities against bacteria, fungi, parasites, and viruses (Hancock and Scott, 2000). The first ALF was identified from horseshoe crab *Limulus Polyphemus* and was initially named after its inhibitory effect on the coagulation system

* Corresponding author. Institute of Oceanology, Chinese Academy of Sciences, 7 Nanhai Road, Qingdao 266071, China.
** Corresponding author.Institute of Oceanology, Chinese Academy of Sciences, 7 Nanhai Road, Qingdao 266071, China.
E-mail addresses: zhangjian@qdio.ac.cn (J. Zhang), lsun@qdio.ac.cn (L. Sun).

https://doi.org/10.1016/j.dci.2018.03.016
0145-305X/

activated by lipopolysaccharide (LPS) (Ohashi et al., 1984; Tanaka et al., 1982). Since then, a number of ALFs from different crustacean species, such as shrimp, crab, lobster, and crayfish, have been investigated (Beale et al., 2008; de la Vega et al., 2008; Liu et al., 2005, 2006). Recombinant ALFs acquired by prokaryotic as well as eukaryotic expression systems have been reported to possess antimicrobial activities (Carriel-Gomes et al., 2007; Somboonwiwat et al., 2005; Tharntada et al., 2009). ALFs contain two conserved cysteine residues that form a disulfide loop known as the LPS binding domain (LBD) (Hoess et al., 1993; Morita et al., 1985). Some synthetic LBD peptides were shown to possess antibacterial and antiviral activities (Guo et al., 2014; Nagoshi et al., 2006). It's worth noting that all documented ALFs and LBD peptides originate from neritic and fresh water crustacean species, and nothing is known about the ALFs of deep-sea crustaceans.

The deep sea is the largest habitat on earth and poorly explored in terms of potential drugs (Sperstad et al., 2011; Warrant and Locket, 2004). Shrimp of the family Alvinocarididae are found in abundance in various deep-sea hydrothermal vents and constitute the dominant faunal biomass of many hydrothermal ecosystems (Hernandez-Avila et al., 2015; Nye et al., 2013). In a previous study, we analyzed the transcriptome of the shrimp *Rimicaris* sp., a genus of Alvinocarididae family, obtained from the hydrothermal vent in Desmos, Manus Basin, and identified a large amount of genes associated with immunity (Zhang et al., 2017). In the present work, we characterized one of these immune genes, i.e., an ALF molecule named RspALF1. We investigated the *in vitro* and *in vivo* antimicrobial activities of both RspALF1 and its LBD peptide, thus providing the first insights into the biological property of ALF from deep-sea hydrothermal vent animal.

2. Materials and methods

2.1. Ethics statement

Experiments involving live animals were conducted in accordance with the "Regulations for the Administration of Affairs Concerning Experimental Animals" promulgated by the State Science and Technology Commission of Shandong Province. The study was approved by the Ethics Committee of Institute of Oceanology, Chinese Academy of Sciences.

2.2. Sequence characterization and phylogenetic analysis

The cDNA sequence of RspALF1 had been reported previously (Zhang et al., 2017). Homology searches of nucleotide and deduced amino acid sequences were performed using BLASTn and BLASTp algorithm of the NCBI (http://www.ncbi.nlm.nih.gov/blast). Signal peptide (SP) was identified using SignalP program (http://www.cbs.dtu.dk/services/SignalP). Domain and motif search was performed with the conserved domain search program of NCBI and PROSITE database (http://prosite.expasy.org/scanprosite/). The theoretical molecular mass and isoelectric point were predicted by using EditSeq in the DNASTAR (Madison, WI) software package. Physicochemical parameters of RspALF1 and ALF1P1 were predicted using ExPASy PROTPARAM bioinformatics resource portal (http://web.expasy.org/protparam). Multiple sequence alignment was created with ClustalW using BioEdit software. The spatial structures of RspALF1 was predicted by the PyMOL Molecular Graphics System (http://www.pymol.org/). Phylogenetic tree was constructed using neighbour-joining method with MEGA software version 5.1, reliability of the tree obtained was assessed by bootstrapping, using 1000 replications.

2.3. Bacterial strains and culture conditions

The Gram-negative bacteria (*Edwardsiella tarda*, *Vibrio harveyi*, *Vibrio anguillarum*, and *Pseudomonas fluorescens*), Gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus iniae*, and *Micrococcus luteus*), and fungi (*Pichia pastoris* and *Yarrowia lipolytica*) have been reported previously (Chen et al., 2013; Sun et al., 2016). The deep-sea bacteria *Pseudoalteromonas* sp., *Alteromonas* sp., and *Bacillus* sp. were isolated from the same deep-sea hydrothermal vent field as *Rimicaris* sp. (Zhang et al., 2017). *S. iniae* was cultured in TSB-YE medium (Haibo, Qingdao, China) at 28 °C; the three deep-sea strains were cultured in marine 2216E medium (Sun et al., 2015a) at 28 °C; all other bacterial strains were cultured in Luria-Bertani broth (LB) medium at 37 °C (for *M. luteus* and *S. aureus*) or 28 °C (for *P. fluorescens*, *V. anguillarum*, and *V. harveyi*); the two fungi were cultured in Yeast Extract Peptone Dextrose (YPD) medium at 30 °C.

2.4. Peptides

All peptides were synthesized by China Peptides Co., Ltd. (Shanghai, China). The cyclic peptides ALF1P1 (5′-CSFSVNPKIKRWQLYFSGNMWC-3′) and ALF1P1M1 (5′-CSFSVNPKIEEWQLYFSGNMWC-3′) had one protective flanking amino acid residue at N- and C-termini and a disulfide bond formed between the two conserved cysteine residues. The linear peptide ALF1P1M2 has the same amino acid sequence as ALF1P1 but the thiol group of the cysteine residues was methylated to disable the formation of disulfide loop. The negative control peptide P86P15 has been reported previously (Zhang et al., 2012a). All peptides were 5′-FITC-labeled and purified by high-performance liquid chromatography to 95% of purity. Lyophilized peptides were stored at −80 °C and dissolved in sterile PBS (pH 7.4) before use.

2.5. Plasmid construction and protein purification

To construct pEtRspALF1, which expresses His-tagged recombinant RspALF1 (rRspALF1), the coding sequence of RspALF1 was amplified by PCR with primers ALF1F1 (5′-GATATCATGCAGGGCCTGGGAGGCCT-3′, underlined sequence, EcoRV site) and ALF1R1 (5′-GATATCAGCATTCAACCAAGCTCTGGCT-3′, underlined sequence, EcoRV site). The PCR products were ligated with the T-A cloning vector T-Simple (TransGen Biotech, Beijing, China), and the recombinant plasmid was digested with EcoRV to retrieve the RspALF1-containing fragment, which was inserted into pET32a (Novagen, San Diego, USA) at the EcoRV site, resulting in pEt-RspALF1. *E. coli* BL21(DE3) was transformed separately with pEt-RspALF1 and pET32a (the latter for purification of rTrx tag), and the transformants were cultured in LB medium at 37 °C to mid-log phase. Isopropyl-β-D-Thiogalactopyranoside (0.4 mM) and 3% absolute ethyl alcohol were then added to the culture. After growing at 16 °C for overnight, the cells were harvested by centrifugation, and His-tagged recombinant proteins were purified under native conditions using nickel-nitrilotriacetic acid (Ni-NTA) columns (GE Healthcare, Uppsala, Sweden) as recommended by the manufacturer and as described previously (Liu et al., 2010). The protein was concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, USA) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.6. Quantitative real time reverse transcription-PCR (qRT-PCR)

For qRT-PCR analysis, gill, muscle, and hepatopancreas were taken aseptically from five individual shrimp and used for total RNA extraction with EZNA Total RNA Kit (Omega Bio-tek, Doraville, USA).