Fish & Shellfish Immunology 47 (2015) 833–846

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Mechanism study on a new antimicrobial peptide Sphistin derived from the N-terminus of crab histone H2A identified in haemolymphs of *Scylla paramamosain*

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ARTICLE INFO

Article history:
Received 27 April 2015
Received in revised form
6 October 2015
Accepted 9 October 2015
Available online 22 October 2015

Keywords:
Scylla paramamosain
Histone
Sphistin
Antimicrobial activity
Antimicrobial mechanism
Cytotoxicity

ABSTRACT

Histone H2A is known to participate in host immune defense through generating special antimicrobial peptides (AMPs), for which it has been an interesting research focus to characterize this kind of peptides in vertebrates and invertebrates. Although thousands of AMPs have been reported in variety of life species, only several AMPs are known in crabs and in particular no H2A-derived AMP has yet been reported. In the present study, a 38-amino acid peptide with antimicrobial activity was determined based on the sequence analysis of a histone H2A identified from the mud crab *Scylla paramamosain*. The histone H2A derived peptide was an AMP-like molecule and designated as Sphistin. Sphistin showed typical features of AMPs such as amphiphilic α-helical second structrue and positive charge net. The synthetic Sphistin exerted high antimicrobial activity against Gram-positive, Gram-negative bacteria and yeast, among which *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Pseudomonas stutzeri* are important aquatic pathogens. Leakage of the cell content and disruption of the cell surface were observed in bacterial cells treated with Sphistin using scanning electron microscopy. It was proved that the increasing cytoplasmic membrane permeability of *Escherichia coli* was caused by Sphistin. Further observation under confocal microscopy showed that Sphistin could combine onto the membrane of *Staphylococcus aureus*, *E. coli* MC1061 and *Pichia pastoris* but not translocate into the cytoplasm. Moreover, the affinity of Sphistin with either LPS or LTA was also testified that there was an interaction between Sphistin and cell membrane. Thus, the antimicrobial mechanism of this peptide likely exerted via adsorption and subsequently permeabilization of the bacterial cell membranes other than penetrating cell membrane. In addition, synthetic Sphistin exhibited no cytotoxicity to primary cultured crab haemolymphs and mammalian cells even at a high concentration of 100 μg/mL for 24 h. This is the first report of a histone-derived Sphistin identified from *S. paramamosain* with a specific antimicrobial activity and mechanism, which could be a new candidate for future application in aquaculture and veterinary medicine.

1. Introduction

Histones are traditionally known as the principal protein component of chromatin [1]. In the past decade, many observations suggest that histones are involved in a multitude of biological functions beyond the confines of the nuclear envelope [2]. For instance, the histone can act as endotoxin-neutralizing proteins [3,4], leucocytes stimulatory factor [5], homeostatic thymus hormone [6] as well as apoptotic signal transmitting factor [7] and also

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http://dx.doi.org/10.1016/j.fsi.2015.10.010
1050-4648/

as an antimicrobial peptide.

Since the antimicrobial activity of histones was firstly reported in 1932, histone-derived antimicrobial peptides across almost all kind of histones (histone H1, H2A, H2B, H3, and H4) have been discovered to display antimicrobial activities against bacteria, fungi, viruses and protozoa. Previous studies show that histone-derived peptides from various vertebrates, such as mammals [3,8–11], chicken [12], frog [13] and fish [14–20], all possess antimicrobial activity. Meanwhile a few studies have reported that this class of antimicrobial peptides is also identified in invertebrates, such as histone H2A fragments in scallop (*Chlamys farreri*) [21], abalone (*Haliotis discus discus*) [22], Pacific white shrimp (*Litopenaeus vannamei*) [23] and freshwater prawn (*Macrobrachium rosenbergii*) [24,25].

Some histone antimicrobial peptides are found to be a extracellular secreted antimicrobial peptide, which are purified from skin or epithelium and derived from intact histone precursors through proteolytic cleavage [26,27]. The active histone fragments are usually derived from the N-terminal part of histone. For instance, buforin I, isolated from the stomach extract of the Korean frog *Bufo bufo gargarizans*, is processed from unacetylated histone H2A by pepsin C isozymes [26]. Parasin I, isolated from the skin mucus secretions of channel catfish, is the fragmented histone processed by cathepsin D [27]. In addition to endogenous histones, synthetic and recombinant histone-derived N-terminal fragments also display potent bacterial killing activity. The reported Abhisin, a potential antimicrobial peptide derived from histone H2A of disk abalone (*H. discus discus*), is synthesized and shows antimicrobial and anticancer activity [22]. The N-terminal fragment of scallop (*C. farreri*) histone H2A peptide is expressed in *Pichia pastoris* GS115 and exerts antibacterial activity against both Gram-positive and Gram-negative bacteria [21]. Thus N-terminus of histone H2A could be an active antimicrobial peptide.

The mud crab, *Scylla paramamosain*, is an important fisheries and aquaculture species in China. As to other invertebrates, antimicrobial peptides (AMPs) play crucial roles in the natural defense system against microbial invasion in the crab. So far there are some AMPs identified in several crab species, including linear α-helical AMPs Bac-like [28] and Callinectin [29], anionic AMPs Scygonadin [30], cysteine-rich AMPs ALFs [31], Multi-domain or chimeric AMPs Crustin Hyastatin [32], Arasin [33] and GRPSp [34]. However, no histone-derived AMP has been reported and its antimicrobial mechanism is still unknown in crab so far. In the present study, based on the determined gene sequence of histone H2A in the mud crab *S. paramamosain*, a 38 aa N-terminal peptide was identified and named as Sphistin. To understand the antimicrobial activity of Sphistin, a synthetic Sphistin peptide was produced and its spectrum of activity against bacteria and yeast was measured. The bacterial binding assay and membrane integrity assay were carried out to understand its antimicrobial mechanism. The LPS and LTA binding experiment was designed in order to know the possibility of intereaction between Sphistin and negatively charged molecules located on the bacterial surface. Finally, the cytotoxicity of Sphistin on normal cells was investigated. From the study, the antimicrobial properties and mechanism of this new histone-derived antimicrobial peptide in carb will be evaluated and thus would further shed light on the innate immune system of crustaceans.

2. Materials and methods

2.1. Determination of histone H2A cDNA and genomic DNA sequences

In our previous study, a forward suppression subtractive hybridization (SSH) cDNA library was constructed from haemolymphs of *S. paramamosain* and the up-regulated genes were identified in order to isolate differentially expressed genes in response to LPS [35]. Partial sequence of mud crab histone H2A was identified during the screening of the SSH cDNA library database.

RACE PCR was performed to amplify the full-length histone H2A cDNA sequence. Specific primers were designed according to the obtained partial cDNA sequence (Table 1). RACE cDNA was prepared with an SMART RACE cDNA Amplification kit (Clontech) and was used as template for PCR. PCR conditions were as follows: 94 °C for 4 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The final extension was carried out at 72 °C for 10 min.

Total genomic DNA was prepared from the carb muscle using MiniBEST Animal Tissue Genomic DNA Extraction Kit Ver.2.0 (Takara). The genomic sequence of histone H2A was amplified by PCR reaction using primers intron F and R (Table 1), and the LA Taq DNA polymerase (Takara). The amplification conditions were: 1 min at 94 °C, 35 cycles of 10 s at 98 °C, 4 min at 68 °C, then 10 min at 72 °C for further extension.

All the expected DNA fragment was ligated to pMD18-T vector (Takara) and transformed into *Escherichia coli* DH5α. Positive clones were identified by bacterial-colony PCR and sequenced.

2.2. Sequence analysis

Nucleotide and deduced amino acid sequences of Histone H2A cDNA were analyzed using software DNAsis. The calculated molecular mass and the theoretical isoelectric point were predicted by SIB Bioinformatics Resource Portal (http://web.expasy.org/compute_pi/). Signal peptide was predicted by SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/). Protein sequences of Histone H2A from different organisms were obtained through the NCBI BLAST search program. A multiple sequence alignment was created with software Bioedit. Phylogenetic trees of the selected Histone H2A was constructed using the neighbour-joining method, and the reliability of the branching was tested using bootstrap resampling (with 1000 pseudo-replicates). The figure was drawn using the MEGA5. The gemonic sequence of Histone H2AZ from different organisms was obtained through the NCBI and a schematic representation of the exon and intron organization was drawn by DNAMAN8. The α-helical secondary structure was predicted using the DNAstar Protean program and helical wheel modeling. Moreover, the 3D structure of Sphistin was also predicted in http://swissmodel.expasy.org/interactive.

2.3. Synthesis of the Sphistin peptide sequence

A 38 aa sequence of Sphistin peptide. (MAGGKAGKDSGKAKAKAVSRSARAGLQFPVGRIHRHLK) which was derived from the N-terminus of the mud crab histone H2A was synthesized using the solid phase peptide synthesis method (TASH Co., China). The molecular masses and purity of the purified peptides were verified by mass spectroscopy and HPLC, respectively.

2.4. Microorganisms

All strains were purchased from the CGMCC with the exception of *E. coli* MC1061 and the yeast strain *P. pastoris* GS115, which were kindly provided by Dr. Chun Li and bought from Invitrogen Biotech respectively. The bacteria were cultured overnight at the appropriate temperature (28 °C or 37 °C) either on Muller-Hinton agar or marine agar 2216 (Difco). Yeast strains were grown on YPG agar (yeast extract 1%, peptone 1%, and glucose 2%) at 28 °C for 2 d for the experiments.