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Upregulation in response to infection and antibacterial activity of oyster histone H4

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ABSTRACT

Several histones and histone-derived peptides have been shown to have antimicrobial activity and a potential role in innate immune defenses. A histone H4 sequence was identified in a subtractive suppression library containing genes upregulated in American cupped oysters, *Crassostrea virginica,* in response to challenge with the protozoan parasite *Perkinsus marinus*. Oyster histone H4 protein levels significantly increased in hemocyte lysates and cell free hemolymph of oysters experimentally challenged with *P. marinus*. The complete histone H4 coding sequence of *C. virginica* was cloned into a *Saccharomyces cerevisiae* yeast expression system and recombinant expression was confirmed using SDS-PAGE analysis and western blot. Delivery of yeast cells expressing recombinant oyster histone H4 into the gut of brine shrimp, *Artemia salinas,* challenged with a streptomycin resistant strain of *Vibrio anguillarum* resulted in a significant and dose-dependent decrease in the load of *V. anguillarum*. Purified recombinant histone H4 showed antimicrobial activity against *V. anguillarum* and *Escherichia coli* at micromolar concentrations, but did not affect the viability of *P. marinus* in culture. These results support the role of histone H4 in the defense of oysters against bacterial infection and validate the use of a novel oyster antimicrobial H4 in a yeast feed-based delivery system for the treatment of bacterial infections in aquaculture applications.

1. Introduction

Oysters, an economically and ecologically important species worldwide, are affected by a variety of infectious diseases. Among the major threats to the American cupped oyster, *Crassostrea virginica,* are the protozoan parasites *Perkinsus marinus* and *Haplosporidium nelsoni,* causative agents of Dermo and MSX diseases respectively, as well as mortality of larvae and juveniles caused by bacterial pathogens such as vibrios and the alpha-proteobacterium *Roseovarius crassostreae* [1–4]. One approach to managing diseases has been the development of disease-resistant oysters using selective breeding, which has proven especially successful for the prevention of MSX [1,2].

Knowledge of the mechanisms involved in the immune defenses of oysters against parasitic and bacterial infection would facilitate the development of disease-resistant oyster lines and other disease management strategies. Invertebrates do not possess an acquired immunity system and instead rely on innate defense mechanisms. In bivalves, which have an open circulation system, the circulating hemocytes are the primary host defense cells. Hemocytes are capable of non-self recognition and phagocytosis including the generation of effector molecules such as oxygen radical intermediates [5], nitric oxide [6], and antimicrobial peptides [7,8], among others. In oysters, several studies have been carried out in order to identify genes that are upregulated upon infection and could have antimicrobial activity. In *C. virginica*, oyster antimicrobial peptide defensin (AOD) was identified with a high sequence homology to defensins found in the mussels *Mytilus edulis* and *Mytilus galloprovincialis* [9]. A similar defensin has also been discovered in the Pacific oyster *C. gigas* using an expressed sequence tag approach [10]. In both cases the defensins were constitutively expressed without experimental challenge. More recently a proline-rich peptide was identified in the hemocytes of *C. gigas* that showed strong synergistic antimicrobial activity with oyster defensin [11]. Other antimicrobial molecules

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discovered in oysters include *C. virginica* plasma lysozyme [12], *C. gigas* bactericidal/permeability-increasing protein [13], and peptidoglycan recognition proteins, which are speculated to have antimicrobial properties [14]. Serine protease inhibitors cvSI-1 and cvSI-2 may also play a role in *C. virginica* host defense against *P. marinus* proliferation [15,16].

The identification in our laboratory of a fragment coding for histone H4 from a subtractive library containing genes upregulated in hemocytes of oysters experimentally infected with the protozoan parasite *P. marinus* (unpublished) led us to speculate a potential role for histone H4 in oyster immunity. Although the primary role of histones is in the structure of chromatin, there is substantial evidence that histones have an important role in antimicrobial defenses in a range of species. Intact Histone H1 was described as an important antimicrobial protein in the liver, intestine and stomach of Atlantic salmon (*Salmo salar*) [17]. In the rainbow trout (*Oncorhynchus mykiss*) antimicrobial activity in skin secretions is due to intact trout histone H2A [18] and oncorhyncin II, an antimicrobial peptide derived from histone H1 [19]. High levels of core histone proteins H2A, H2B, H3 and H4 were also found in the hemocytes of the Pacific white shrimp and demonstrated antimicrobial activity against a wide range of bacteria [20]. In marine mollusks, antimicrobial histone H2A has been indentified in both the scallop *Chlamys farreri* [21], and in the disk abalone *Haliotis discus discus* where it was induced following bacterial infection [22]. The role of intact histone H1 [23], H2A and H2B [24,25], and H3 and H4 [26] in immune defenses against bacterial infection has also been shown in mammals. A novel antimicrobial process involving histones are neutrophil extracellular traps (NETs), chromatin based threads coated in histones which trap and kill bacteria in both humans [26,27] and possibly fish [28].

We present here the identification of a gene coding for histone H4 in American cupped oysters and the characterization of the patterns of protein production in response to challenge with the protozoan parasite *P. marinus*. In addition, we explore the potential role in innate immune defenses using the brine shrimp *Artemia salinas* as an experimental model.

2. Materials and methods

2.1. Cloning and sequencing of RACE products

A fragment sequence of histone H4 from a subtractive library (Clontech, USA) containing genes upregulated in tissues of oysters experimentally infected with *P. marinus* was used to design primers for the amplification of the 5′ and 3′ ends of oyster histone H4 using the SMART™ RACE cDNA amplification kit (Clontech, USA). cDNA was prepared from total RNA from the hemocytes of oysters using the first strand cDNA synthesis protocol and H4tq reverse primer, 5′-GGCGTGCTCTGTGTAGGTCACAG CATCG-3′ (Integrated DNA Technologies, USA). RACE PCR master mix was prepared following manufacturers instructions. The PCR reaction was run on an MJ Research PTC-100 thermocycler (USA) and visualized on a 1.5% agarose gel under UV. RACE reaction product was cloned using the TOPO TA Cloning® Kit (Invitrogen, USA), following manufacturer's instructions. Several colonies were selected and cultured overnight in LB broth containing 100 μg/ml of ampicillin at 37 °C with constant agitation. Plasmid DNA was purified using the Wizard® *Plus* SV DNA Purification Kit (Promega, USA). Plasmid DNA concentration was measured using an Eppendorf Biophotometer. Fifty fmol of plasmid DNA was sequenced with 5 μM of M13 Forward or Reverse primer. Sequencing was carried out at the University of Rhode Island's Genomics and Sequencing Center using a Beckman Coulter CEQ 8000 Genetic Analysis System (Beckman, USA). Based on the 5′ DNA sequence of the first RACE PCR, a second regular PCR amplification was carried out using the H4FullF primer [5′-AGAGTAAATATGTCTGGACGTGGTAA-3′], located upstream of the start codon, and the H4FullR reverse primer [5′-TCAGGGTGTTTAACCACCG-3′], located downstream of the stop codon, with the following parameters: an initial denaturation of 2 min at 94 °C; 25 cycles of 94 °C for 1 min, 53 °C for 1 min and 72 °C for 1 min; finishing with a final extension of 72 °C for 7 min. The resulting product was visualized on an agarose gel, cloned into the TOPO vector, and sequenced. The DNA and amino acid sequences were identified and compared with that of the protein entries deposited in GenBank using the BLASTN and BLASTX programs. Alignments of the protein sequences were carried out using CLUSTALW on SDSC Workbench (workbench.sdsc.edu).

2.2. Antimicrobial assays

The antimicrobial activity of recombinant *Xenopus laevis* histones H2B and H4 (Millipore, USA) was tested against the Gram-positive bacteria *Micrococcus luteus*, *Staphylococcus aureus* (kindly provided by J. Sperry, University of Rhode Island, USA), and the Gram-negatives *Escherichia coli* ZK4 and *Vibrio anguillarum* M93Sm (streptomycin resistant, Sm^R, kindly provided by D. Nelson, URI). All bacterial stocks were kept at −80 °C in 15% (v/v) glycerol. Each bacterial strain was streaked onto an LB agar plate (LB with 20% NaCl for *V. anguillarum*) and incubated overnight at 28 °C (37 °C for *E. coli*). After incubation, a single colony of each strain was inoculated into the appropriate liquid medium and incubated again overnight at the optimum temperature with shaking. The bacteria were diluted 1:100 in appropriate media and grown until the optical density (OD) was approximately 0.1 (exponential growth) at an absorbance of 595 nm on a SpectraMax 340 Spectrophotometer (Molecular Devices, USA). Bacteria were then further diluted with poor broth [1% (w/v) bactotryptone/0.5% (w/v) sodium chloride (1% for *V. anguillarum*), pH 7.2] until the OD_{595} was approximately 0.01. Ninety μl of bacteria were added to replicate wells of a 96 well plate along with 15 μl of the appropriate histone H4 or H2B (5 and 10 μM). A negative control of sterile distilled water was also included. The plate was incubated at 30 °C (37 °C for *E. coli*) with shaking, and the OD_{570} read at 0, 7, and 24 h.

The parasite *P. marinus* (strain HCTR from Ninigret Pond, RI, USA) was kindly donated by D. Bushek (Rutgers University, NJ, USA), and the antimicrobial test was assayed using a variation of the plate microdilution assay [29]. *P. marinus* was cultured in DME/HAM media with 65 mM HEPES, 17.9 mM sodium bicarbonate, 2% fetal bovine serum and 100 units of Penicillin-Streptomycin (Sigma, USA). Results are expressed as percentage of growth inhibition compared to controls.

2.3. Experimental infection of oysters with P. marinus

American cupped (*C. virginica*) and Pacific (*C. gigas*) oysters from an area free of *P. marinus* were obtained from a commercial source (Taylor Shellfish Company, WA, USA). Oysters were placed into separate 50 L tanks with 28 psu artificial seawater (ASW) (Instant Ocean®, USA) at 15 °C and acclimated for two weeks at 20 °C. Oysters were fed daily with a mixture of *Isochrysis, Pavlova, Tetraselmis,* and *Thalassiosira* spp. (Reed Mariculture Inc., USA). Absence of *P. marinus* in the oysters was confirmed by tissue incubation in Ray's fluid thioglycollate medium (RFTM) [30]. *P. marinus* was cultured as described above. Oyster shells were notched at the edge 24 h prior to parasite inoculation to allow a small hole [31]. Oysters were injected through the notch into the adductor muscle with 5×10^5 parasites per gram oyster wet weight diluted in 100 μl of filtered artificial sterile sea water (ASW) or with just 100 μl of ASW using a syringe fitted with a 27-gauge needle. After injection,