Developmental and Comparative Immunology 49 (2015) 96–102

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Short communication

Identification of candidate antimicrobial peptides derived from abalone hemocyanin

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ARTICLE INFO

Article history:
Received 11 July 2014
Revised 6 November 2014
Accepted 8 November 2014
Available online 13 November 2014

Keywords:
Hemocyanin
Antimicrobial peptide
Mollusc
Haliotisin
Innate immunity
In silico

ABSTRACT

Hemocyanins present in invertebrate hemolymph are multifunctional proteins, responsible for oxygen transport and contributing to innate immunity through phenoloxidase-like activity. In arthropods, hemocyanin has been identified as a source of broad-spectrum antimicrobial peptides during infection. Conversely, no hemocyanin-derived antimicrobial peptides have been reported for molluscs. The present study describes a putative antimicrobial region, termed haliotisin, located within the linking sequence between the α-helical domain and β-sheet domain of abalone (*Haliotis tuberculata*) hemocyanin functional unit E. A series of synthetic peptides based on overlapping fragments of the haliotisin region were tested for their bactericidal potential. Incubating Gram-positive and Gram-negative bacteria in the presence of certain haliotisin peptides, notably peptides 3-4-5 (DTFDYKKFGYRYDSLELEGRS ISRIDELIQQRQEKDRTFAGFLLKGFGTSAS) led to reductions in microbial growth. Furthermore, transmission electron micrographs of haliotisin-treated bacteria revealed damages to the microbial cell wall. Data discussed here provides the first evidence to suggest that molluscan hemocyanin may act as a source of anti-infective peptides.

1. Introduction

In the absence of acquired immunity and the ability to produce clonally derived immunoglobulins, marine invertebrates rely solely on innate immunity to survive the microbiologically challenging and varied environment (reviewed by Ellis et al., 2011). Invertebrate immunity can be categorised broadly into three lines of defence: physical (exoskeleton), cellular and humoral. While cellular immunity (phagocytosis and encapsulation) is facilitated by a heterogeneous population of immune cells (hemocytes; reviewed by Smith, 2010), humoral immunity involves a battery of hemolymph-based bioactive compounds, including antimicrobial peptides (AMPs; reviewed by Zänker, 2010). AMPs are produced by certain organs (hepatopancreas, digestive tract and gills) and circulating hemocytes in response to infection, wounding or abiotic stressors such as temperature (De Zoysa et al., 2010; Tassanakajon et al., 2014). Conventional AMPs are ~15 to 60 amino acids in length, ranging from 1.5 to 8 kDa in size, positively charged (cationic), amphipathic and containing ≥30% hydrophobic residues (reviewed by Brogden, 2005; Smith et al., 2010). The majority of known AMPs come from the processing of larger inactive proteins, however, some studies suggest that biologically active proteins, such as hemocyanin (Lee et al., 2003) and hemoglobin (Ullal et al., 2008), can be altered to produce microbicidal cryptides.

Hemocyanins (Hcs) are large, multi-subunit oxygen carrier proteins found in the hemolymph of numerous arthropods and molluscs (Decker et al., 2007; Markl, 2013). Data gathered over the last decade has established Hcs as versatile macromolecules, contributing to development, homeostasis, hemostasis and immune defenses within marine invertebrates (reviewed by Coates and Nairn, 2014). Hc-derived peptides with anti-microbial properties have been recorded previously in shrimp (Destoumieux-Garzón et al., 2001; Qiu et al., 2014), crayfish (Lee et al., 2003) and a spider (Riciluca et al., 2012). To date, no Hc-derived AMPs have been observed for molluscs.

Abbreviations: AMPs, Antimicrobial peptides; FC, Functional unit; HdH1, *Haliotis diversicolor* hemocyanin 1; HtH1, *Haliotis tuberculata* hemocyanin 1; Hc, Hemocyanin; PO, Phenoloxidase.

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http://dx.doi.org/10.1016/j.dci.2014.11.008
0145-305X/

Despite the increasing number of mollusc species being brought into mariculture, particularly abalones (Hooper et al., 2007; De Zoysa et al., 2010; Zhuang et al., 2010), few studies have focused on molluscan immune defenses. In contrast, significant investment has been made in characterising shrimp and crab immunity, thereby establishing several groups of crustacean AMPs (Smith et al., 2010). In this study, we have identified a putative antimicrobial region (termed haliotisin) within the conserved loop sequence of functional unit (FU)-E of *Haliotis tuberculata* hemocyanin type 1(HtH1). Synthetic peptides based on the amino acid composition of this region inhibited the growth/replication of Gram-positive (*Bacillus subtilis*) and Gram-negative (*Erwinia carotovora*) bacteria. Furthermore, *in silico* structural modelling of the most effective haliotisin peptides (3-4-5) revealed a linear α-helical structure. Data described here advocate a role for molluscan Hc in AMP production, and in doing so, enhances our understanding of respiratory protein function in innate immunity.

2. Materials and methods

2.1. Peptide synthesis

Peptides were purchased from Genescript Inc. (Nanjing, China). Peptides were purified by HPLC, with homogeneity described as >80% from the accompanying MALDI-ToF mass spectrometry data. Peptides were dissolved in sterile phosphate buffered saline (PBS), 50 mM NaPi and 100 mM NaCl, pH 7.6. Solubility issues encountered with peptides 3 and 6 were overcome by the addition of 0.1% *n*-dodecyl β-D-maltoside (DDM).

2.2. In silico *identification of putative AMPs*

The Antimicrobial Peptide Database (Wang et al., 2009) was used initially to identify/locate potential anti-microbial regions within all eight abalone Hc FUs (a–h). HtH1 was chosen because it is more abundant than isoform HtH2 (3:1) within abalone hemolymph (Keller et al., 1999), and due to the availability of the complete cDNA sequence (GenBank: Y13219.2, Lieb et al., 2000).

2.3. Sequence alignments and structural models of FU-E

Sequences of Hc FU-E from various mollusc species were aligned using Clustal Omega (Goujon et al., 2010; Sievers et al., 2011) and edited further in ESPript 2.2 (Gouet et al., 1999, 2003): *Haliotis tuberculata* (gi:7159826, gi:27368649), *Haliotis diversicolor* (gi:255046234), *Megathura crenulata* (gi:560177105, gi:94013939), *Rapana venosa* (gi:31076723), *Nucula nucleus* (gi:57335438, gi:57335436), *Helix lucorum* (gi:346987844), *Melanoides tuberculata* (gi:549438767), *Sepia officinalis* subunit 1 (gi:88657467), *Aplysia californica* (gi:62679967), *Sepiella maindroni* (gi:543869159), *Euprymna scolopes* (gi:674268754) and *Nautilus pompilius* (gi:56710669).

Structures of abalone Hc FU-E were predicted initially using the online server I-TASSER (Zhang, 2008), and then flexibly fitted to the corresponding density map (EMDB ID: 2503) that had been segmented in UCSF Chimera using MDFF (Pattersen et al., 2004; Trabuco et al., 2008), to build the pseudoatomic model of FU-E from isomeric HdH1 (Zhu et al., 2014). The crystal structures of *Rapana thomasiana* (PDB-1LNL) and *Megathura crenulata (PDB-4BED)* were used as templates to construct the haliotisin region of HtH1 FU-E. Potential peptide cleavage sites and carbohydrate binding sites were predicted using the ExPASy Bioinformatics Resource Portal (Gasteiger et al., 2005). A 3D model of haliotisin peptides 3-4-5 (DTFDYKKFGYRYDSLELEGRSISRIDELIQQRQEKDRTFAGFLLKGFGTSAS) was assembled using the QUARK algorithm (Xu and Zhang, 2012) and edited in UCSF Chimera.

2.4. Antimicrobial assays

2.4.1. Radial diffusion assay (RDA)

The antimicrobial activity of the haliotisin peptides against Gram-positive and Gram-negative bacteria, *B. subtilis* and *E. carotovara* respectively, was performed as described previously (Banas et al., 2013). Briefly, bacteria were grown in Luria–Bertani (LB) media to mid-logarithmic phase and used for subsequent experiments at 10^5 or 10^4 colony-forming units (CFU)/mL. The bacterial suspension was applied to LB agar (2%) to produce microbial lawns. Sterile 4-mm-diameter filter discs were then placed onto the agar, with 10–20 μL (0–50 μM) of peptide added to each disc. Plates were incubated at 30 °C for 16–24 hours and monitored closely for zones of inhibition.

2.4.2. Minimal inhibitory concentration (MIC)

Bacteria were grown in LB at 30 °C and diluted to 10^6 CFU/mL in PBS, pH 7.6. Peptides were diluted in PBS also. The bacteria and diluted peptides were mixed in equal-volume, followed by incubation at 30 °C for 2–3 hours. At the end of incubation, bacteria were placed on LB agar and incubated at 30 °C overnight for measurements of minimal inhibitory concentration (MIC). The lowest concentration of peptide that resulted in the highest level of inhibition was used to define the MIC. All experiments were carried out in triplicate.

2.5. Transmission electron microscopy

To investigate the effect(s) of synthetic haliotisin-peptides on bacterial cell morphology, *B. subtilis* and *E. carotovor*a were treated with higher concentrations (2× MIC) of P3 (2 μM) and P4 (~5 μM) for 30 minutes at 30 °C. Approximately 10 μL of the bacterial/peptide suspension was loaded subsequently onto Parafilm. Next, formvar-membrane-coated copper grids were placed onto the bacteria for 5 min, covered in 2% phosphotungstic acid (PTA; negative staining) for 30 s and dried at room temperature. Samples were observed using a HITACHI H-7650 transmission electron microscope (TEM).

3. Results

The complete amino acid sequence of HtH1 (GenBank, Y13219.2) was used conveniently for *in silico* analyses and the identification of candidate AMPs. Multiple peptide sequence alignments (Clustal Ω) revealed that the putative antimicrobial region of HtH1 FU-E, termed haliotisin, is highly conserved amongst molluscs (Fig. 1A): 84% identity with HdH1, 71% with *Haliotis asinina* Hc (partial), 70–71% with Hcs 1 and 2 from *M. crenulata* and ≥58% similarity to Hcs from *N. nucleus*, *N. pompilius*, *E. scolopes*, *S. maindroni*, *A. californica*, *S. officinalis*, *H. lucorum*, *R. venosa* and *M. tuberculata*.

By employing the recently published cryo-EM structure of HdH1 (Zhu et al., 2014) and the crystal structures of *R. thomasiana* (PDB-1LNL; Perbandt et al., 2003) and *M. crenulata* (PDB-4BED; Gatsogiannis and Markl, 2009) Hcs, a homology-based model representative of HtH1 was constructed successfully (Fig. 1B). The conformational model detailed the location of FU-E (containing haliotisin) to the centre of the asymmetric unit of the Hc decamer (in agreement with Meissner et al., 2000). The haliotisin-region (C1002–L11028) is visible in a loop arrangement, between the four α-helix (core)-domain and the β-sheet (sandwich)-domain.

Eight synthetic overlapping peptides (Fig. 1C) were designed to represent a variety of net charges and % hydrophobicity (see supplementary Table S1). Each peptide (P1–P8), ranging in size from 2.2 kDa to 3.2 kDa, was tested for their ability to inhibit/prevent growth of *B. subtilis* (Gram +) and *E. carotovora* (Gram –) using radial diffusion assays (Fig. 2, supplementary Fig. S1). Peptide 3 (P3) corresponding to the internal region D1034–R1053 of Hc FU-E