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# Antimicrobial peptide, hdMolluscidin, purified from the gill of the abalone, *Haliotis discus*

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ABSTRACT

A 4.7 kDa antimicrobial peptide was purified from the acidified gill extract of the Abalone, *Haliotis discus*, by cation-exchange and $C_{18}$ reversed-phase high performance liquid chromatography (HPLC). Comparison of the amino acid sequences and molecular weight of this peptide with those of other known antimicrobial peptides revealed that this antimicrobial peptide have high sequence homology with that of cgMolluscidin and was designated hdMolluscidin. hdMolluscidin *is composed of 46 amino acid residues* containing several dibasic residue repeats like K–K or K-R. hdMolluscidin showed potent antimicrobial activity against both Gram-positive bacteria including *Bacillus subtilis* and *Staphylococcus aureus* (minimal effective concentrations [MECs]; 0.8–19.0 μg/mL) and Gram-negative bacteria including *Aeromonas hydrophila*, *Escherichia coli, Pseudomonas aeruginosa, Salmonella enterica, Shigella flexneri,* and *Vibrio parahemolyticus* ([MECs]; 1.0–4.0 μg/mL) without hemolytic activity. However, hdMolluscidin did not show any significant activity against *Candida albicans*. The secondary structural prediction suggested that hdMolluscidin might not form an ordered or an amphipathic structure. hdMolluscidin did not show membrane permeabilization or leakage ability. The full-length hdMolluscidin cDNA contained 566-bp, including a 5'-untranslated region (UTR) of 63-bp, a 3′-UTR of 359-bp, and an open reading frame of 144-bp encoding 47 amino acids (containing Met). cDNA study of hdMolluscidin suggests that it is expressed as a mature peptide. Our results indicate that hdMolluscidin could relate to the innate immune defenses in abalone and it may not act directly on bacterial membrane.



## 1. Introduction

Marine invertebrates, especially benthic species such as mussels, oysters, and abalones, live in a microbe-rich water environment and are constantly exposed to high concentrations of pathogenic microbes. To adapt dangerous environments or to combat potential invaders, marine invertebrates should need and develop effective defense systems. Marine invertebrates rely solely on innate immune factors in humoral and cellular immunity but lack an acquired immunity based on T-lymphocyte subsets and clonally derived immunoglobulins [1]. The cellular immunity is mediated by immune cells via phagocytosis, encapsulation, and respiratory burst (e.g., hemocytes) and humoral immunity is mediated by lectins, agglutinins, antimicrobial proteins/peptides and lysosomal enzymes in plasma [1,2]. Among these innate defenses, there is increasing evidence that antimicrobial polypeptides (AMPs), constituting peptides and small proteins, play a crucial role in innate immunity [1].

The Abalone, *Haliotis discus*, is one of the highest valued commercial shellfish species in Korea and also supports a significant aquaculture industry [3]. However, a number of pathogens containing bacteria, parasites and viruses have devastated abalone populations in East Asia containing China, Korea, and Japan. Up to date, several researches have been performed to find the relationships between the presence of innate immune factors such as antimicrobial peptides and pathogenic infections. However, still, relatively little information is known about the immune defense mechanisms in the abalone and how the levels of pathogenic microbes may be controlled by the host.

Antimicrobial polypeptides (AMPs), one of the most common

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innate defense factors, are composed of peptides or small proteins that are encoded by different genes and typically possessed broad-spectrum antimicrobial activity. Up to date, three major types of antimicrobial peptides including defensins, histone-related peptides (abhisin), and hemocyanin-derived AMP (haliotisin), have been identified from the abalone species, and have been confirmed their antimicrobial role in innate immunity [4–6]. However, the studies on the antimicrobial peptides including these peptides in abalone have almost been done in the limited gene level but there is not enough research on the protein level.

Here, we report the purification and characterization of a novel antimicrobial peptide, hdMolluscidin, from the gill of the Abalone, *Haliotis discus,* in the protein level, and investigation of cDNA cloning, secondary structure prediction, and its action mechanism.

## 2. Materials and methods

### 2.1. Tissue extraction

Four adult farmed abalones, 9.0–10.0 cm in shell length, were obtained from Jeju Island in October 2009, Korea. As soon as they arrived in the laboratory, each abalone was processed individually. The shell was gently washed with tap water and used for tissue extraction. Gill was then harvested and placed into a 15 mL sterile polypropylene tube on wet ice until 4 mL of gill tissue from a total of 4 abalones was harvested. The gill tissue was then added to a 50 mL beaker having 16 mL of pre-heated 1% acetic acid (HAc). The tissue was immediately boiled for 5 min, cooled on wet ice and homogenized on wet ice for 2 min (speed #4, Polytron PT1200 C homogenizer, Kinematica AG, Switzerland). After homogenization, samples were centrifuged at 15,000 g for 45 min at 4 °C. The supernatants were pooled (total of 13 mL) and stored at −70 °C until use.

### 2.2. Proteolytic digestion of crude extract to determine if antimicrobial compounds were proteinaceous

Susceptibilty of the antibacterial activity of crude gill extract to proteolytic digestion was determined by incubation of gill extract with 250 μg/mL crystalline trypsin (Fisher Scientific, Fairlawn, NJ) for 60 min at 37 °C. Antibacterial testing of the extract before and after protease treatment was done with the ultrasensitive radial diffusion assay (URDA) against *Bacillius subtilis* KCTC1021 and *Escherichia coli* D31.

### 2.3. Ultrasensitive radial diffusion assay (URDA)

The antibacterial activity of the purified peptide was assessed as described previously [7]. We tested the purified peptide against Gram-positive bacteria including *Bacillus subtilis* strains (*Bacillus subtilis* KCTC1021 and *B. subtilis* RM125) and *Staphylococcus aureus* RM4220, and Gram-negative bacteria including *Aeromonas hydrophila* KCTC2358, two *Escherichia coli* strains (*Escherichia coli* D31 and *E. coli* KCTC1116), *Pseudomonas aeruginosa* KCTC2004, *Salmonella enterica* KCTC2514, *Shigella flexneri* KCTC2517, and *Vibrio parahemolyticus* KCCM41664 and one yeast, *Candida albicans* KCTC7965. All were grown overnight for 18 h in trypticase soy broth (TSB) or in sabouraud dextrose broth (SDB) at the appropriate temperature (25 °C for *Aeromonas hydrophila* and *Vibrio parahemolyticus,* and 37 °C for the others). After overnight incubation, the bacterial and *Candida albicans* suspensions were diluted to a McFarland turbidity standard of 0.5 (Vitek Colorimeter #52-1210, Hach, Loveland, Colorado) corresponding to ~$10^8$ CFU/mL for bacteria and ~$10^6$ CFU/mL for *C. albicans*. One-half milliliter of diluted bacterial or *C. albicans* suspension was added to 9.5 mL of underlay gel containing $5 \times 10^6$ CFU/mL or $5 \times 10^4$ CFU/mL in 10 mM phosphate buffer (pH 6.6) with 0.03% TSB or 0.03% SDB and 1% Type I (low EEO) agarose. The purified peptide was serially diluted 2-fold in 5 μL of acidified water (0.01% HAc) and each dilution was added to 2.5 mm diameter wells made in the 1 mm thick underlay gels. After incubation for 3 h at either 25 °C (for *A. hydrophila* and *V. parahemolyticus*) or 37 °C (for the others), the bacterial or the yeast suspension was overlaid with 10 mL of double-strength overlay gel containing 6% TSB or 6% SDB with 10 mM phosphate buffer (pH 6.6) in 1% agarose. Plates were incubated for an additional 18–24 h and then the clearing zone diameters were measured. After subtracting the diameter of the well, the clearing zone diameter was expressed in units (0.1 mm = 1 U). The minimal effective concentration (MEC, μg/mL) of the purified peptide was calculated as the X-intercept of a plot of units against the $\log_{10}$ of the peptide concentration [8]. Piscidin 1, an α-helical AMP isolated from hybrid striped bass (*Morone saxatilis* × *Morone chrysops*) was used as a positive control [9].

### 2.4. Antimicrobial peptide purification

Acidified gill extracts were pooled and loaded onto a TSK-Gel SP-5PW HPLC column (7.5 × 75 mm; Tosoh, Tokyo, Japan) equilibrated with 10 mM PB (pH 6.0). Separation was achieved using a linear gradient of buffer A (10 mM PB, pH 6.0) and buffer B (1.0 M NaCl in 10 mM PB, pH 6.0) from 0 to 1.0 M NaCl for 100 min and eluted with buffer B for 20 min at a flow rate of 1 mL/min, and monitored at 220 nm. Fractions were hand collected and loaded onto a CapCell-Pak $C_{18}$ reversed-phase column (5 μm, 300 Å, 4.6 × 250 mm). The sample was eluted with a linear gradient of 5–55% acetonitrile in 0.1% TFA for 50 min at a flow rate of 1 mL/min, and the eluate was monitored at 220 nm. The separated peaks were hand collected, dried under vacuum, dissolved in 0.01% HAc, and tested for antibacterial activity against *B. subtilis* KCTC1021 by the ultrasensitive radial diffusion assay. The activity peak was finally purified to homogeneity using a CapCell-Pak $C_{18}$ reversed-phase column (5 μm, 300 Å, 4.6 × 250 mm). The sample was finally eluted with a linear gradient of 5–30% acetonitrile in 0.1% TFA for 25 min at a flow rate of 1 mL/min, and the eluate was monitored at 220 nm. The purified peptide was hand-collected, dried under a vacuum, dissolved in 0.01% HAc, tested for antibacterial activity against *B. subtilis* KCTC 1021 using the URDA and used for further characterization.

### 2.5. Hemolytic activity assay

The hemolytic activity of the synthetic peptides was determined using human red blood cells (RBCs, blood type O) [10]. The RBCs were collected from citric acid-treated blood by centrifugation at 3000 g for 5 min and washed three times with 10 mM phosphate buffer (PB) (pH 7.4) containing 150 mM NaCl to remove the plasma and the buffy coat. A suspension of 3% hematocrit in buffer with or without peptides was incubated for 60 min at 37 °C. Hemolysis was expressed as the hemoglobin content obtained from the absorbance of the supernatant at 542 nm after centrifugation at 3000 g for 5 min. A 100% hemolysis was determined by the hemoglobin release after the addition of 0.1% Triton X-100. The hemolysis percentage of the peptides was calculated by the following formula:

$$\%\text{Hemolysis} = [(\text{Abs}_{542\text{nm}} \text{ in the peptide solution} - \text{Abs}_{542\text{nm}} \text{ in buffer})/ \times (\text{Abs}5_{42\text{nm}} \textit{ in } 0.1\% \text{ Triton X} - 100 - \text{Abs}_{542\text{nm}} \text{ in buffer})] \times 100$$

Hemolytic assay was also performed in triplicates and the