Thesis for the Degree of Master of Fisheries Science

Antimicrobial activity of Defensin-like peptide from Pacific abalone, *Haliotis discus hannai*.

by
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북방전복(*Haliotis discus hannai*)에서 유래한 디펜신 유사 펩타이드의 항균활성

Advisor: Prof. Jong-Myoung Kim
by Phil-Lip Kwon

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Antimicrobial activity of Defensin-like peptide from Pacific abalone, *Haliotis discus hannai.*

A dissertation

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Abstract

Abalone has been regarded as a high-value product in Korea's aquaculture industry.

Previous study was carried out to find differentially expressed genes associated with growth rate of Pacific abalone. For this, the abalones were divided by size and weight and using analysis of differentially expressed genes. This result identified several genes of which the expression level was significant differences between large and small-size groups. Among these genes, this study tested antimicrobial activity of Defensin-like peptide. cDNA encoding HdhDef consists of 66 amino acids (including 18 amino acids of a signal peptide and 48 amino acids of a chain) The recombinant mHdhDef with his_6-tag added to the N-terminus was induced to overexpression in *E. coli* BL21 (DE3) and purified with nickel column and then dialysis was performed. Purified

recombinant mHdhDef was tested for antimicrobial activity by URDA. The result showed that recombinant mHdhDef has antimicrobial activity against Gram-positive bacteria (*Bacillus subtilis*, *Streptococcus parauberis*) and Gram-negative bacteria (*Vibrio harveyi*, *Escherichia coli*). Several experiments were conducted to find out the mechanism of antibacterial activity. This result suggested that mHdhDef seems not to break bacterial cell wall, but interact with the bacterial cell wall.

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I . Introduction

Antibiotics have been used as an effective means to control the population of pathogenic bacteria. With increasing usages of antibiotics, there have been concerns about the treatment of bacteria with antibiotic resistance as exemplified by the appearance of so-called superbugs that have resistant to nearly all antibiotics (Neu, 1992). To overcome such a difficulty for the treatment of pathogenic bacteria by using the first-generation antibiotics including Penicillin, there has been efforts to develop the next generation antibiotics (Rotem and Mor, 2009). In addition to chemical modification of preexisting antibiotics, novel antimicrobial peptides (AMPs) from various organisms have received attention as potential next-generation antibiotics because their antimicrobial mechanisms are different from conventional antibiotics (Jenssen et al., 2006).

In this study, we identified AMP from Pacific abalone *Haliotis discus hannai*, a high-value aquaculture species in Korea. Previous study was carried out to find differentially expressed genes associated with

growth rate of Pacific abalone (Choi et al., 2022). For this, Pacific abalones grown for 200 dpf (days post fertilization) were divided by size and weight and using analysis of differentially expressed genes. The result identified several genes of which the expression level was significant differences between large and small-size groups. These include genes encoding defensin-like peptide of Pacific abalone, *Haliotis discus hannai* (HdhDef)).

Interestingly, marine invertebrates including abalones lack an adaptive immune system, thus innate immunity was essential for marine invertebrates (Loker et al., 2004). Defensins were well-known for antimicrobial peptides (AMPs) (Oppenheim et al., 2003). In general, AMPs are small and cationic peptides that play an important role in the innate immune system and they have broad-spectrum antimicrobial activity and are found in diverse organisms (Huan et al., 2020). To test the antimicrobial activity of HdhDef, cDNA encoding HdhDef consisting of 66 amino acids (including 18 amino acids of a signal peptide and 46 amino acids of a chain) were cloned into expression vector. Recombinant HdhDef overexpressed in *E. coli* was purified and tested for antimicrobial activity by URDA.

Ⅱ. Materials and Methods

1. Cloning and transformation

The full-length cDNA sequence of *Haliotis discus hannai* defensin-like peptide (HdhDef) consists of 322 bp including an untranslated region (UTR) of 121 bp and coding sequence (CDS) of 201 bp. CDS encodes 66 amino acids including signal peptide of 18 residues and, mature peptide of 48 residues, designated as mHdhDef. Gene corresponding to cDNA of mHdhDef was amplified by polymerase chain reaction (PCR). PCR primers were designed from the sequence corresponding to

the mature peptide region of HdhDef gene. PCR reaction was carried out in 20 μL of a mixture containing cDNA templates and 1 μL of forward primers and 1 μL of reverse primer in 1X HiQ-PCR Mix (Genotech, Korea). Each PCR reaction consisted of an initial denaturation at 95℃ for 3 min, together with 30 cycles of denaturation at 95℃ for 30 sec, annealing for 30 sec at 68℃, and extension at 72℃ for 30 sec. The final extension was carried out for 5 min at 72℃. The PCR products were cloned into NdeI/XhoI

restriction enzyme sites of pColdI DNA expression vector (Takara Bio Inc).

For the competent cell preparation, *E. coli* BL21 (DE3) were cultured in LB broth at 22℃ overnight with shaking (180 rpm). When the *E. coli* cells reached 0.6 of OD_{600}, the cells were chilled on ice for 10 min and centrifuged at 2,500 x*g* for 10 min at 4℃. Pellet was resuspended in transformation buffer (TB) [10 mM Hepes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, pH 6.7], followed by addition of 7% DMSO. For transformation, 100 μl of competent cells were mixed with recombinant plasmid and incubated on ice for 30 min. Upon heat-shock at 42℃ for 1 min followed by rapidly chilling on ice for 3 min, 500 μl of LB medium were added to the mixture followed by incubation at 37℃ for 1 hour. Cells were plated onto the LB agar medium containing ampicillin (100 μg/mL) and incubated at 37℃ overnight. Recombinant mHdhDef was designated as rmHdhDef.

2. Sequence analysis

The full-length amino acid sequences of HdhDef were aligned with other genes encoding defensin from other organisms searched from NCBI GenBank database. Phylogenetic trees were constructed by the

neighbor-joining method (Saitou and Nei, 1987) using the version of MEGA: 11.0.13 (Molecular Evolutionary Genetics Analysis). The reliability of each node was estimated by the bootstrap method with 1000 replications. Gaps and missing data were treated by the pairwise deletion manner.

The amino acid sequences of the phylogenetic tree were obtained from *Haliotis discus hannai* (ABF69125.1), *Haliotis madaka* (ALU63750.1), *Haliotis rufescens* (XP_046358087.1), *Haliotis discus discus* (ACZ15982.1), *Haliotis diversicolor* (QHI06028.1), *Haliotis rubra* (XP_046584092.1). Mammalian defensins from *Homo sapiens* (AAA52304) and *Mus musculus* (NP_034160.1) were used as outgroups.

The molecular mass (MW) and isoelectric point (PI) of the predicted mature peptide were calculated by ProtParam (http://www.expasy.ch/tools/protparam.html). The SWISS-MODEL prediction algorithm (https://swissmodel.expasy.org/) was applied to generate the three-dimensional (3D) structure model. Antimicrobial Peptide Calculator and Predictor at APD (http://aps.unmc.edu/AP/main.php)

3. Purification of recombinant protein

Transformed *E. coli* BL21 (DE3) cells were precultured in Luria-Bertani (LB) broth containing ampicillin (100 μg/mL) overnight at 37℃ with shaking 200 rpm. Precultured cells were diluted 1:100 with LB broth containing ampicillin (100 μg/mL) at 37 ℃ with shaking (200 rpm) until the concentration of cells reached about 0.5 of OD_{600}, and then quickly cool the culture to 15℃ in ice for 30 minutes. After cooling, expression of recombinant mHdhDef was induced with isopropy1 β-D-thiogalactoside (IPTG) (final concentration of 0.5 mM) at 18℃ for 20 hours. Cells were harvested by centrifugation at 5000 x*g* at 4℃ for 20 minutes.

Harvested cells were resuspended in LEW buffer (50 mM NaH2PO4, 300 mM NaCl, pH 8.0). After several freeze-thaw cycles, the cells were sonicated on ice at 20% amplitude (Scientz-IID, SCIENTZ Biotechnology, Ningbo, China) for 20 minutes, and then additionally add $CaCl_2$ and DTT (final concentration of 10 mM, and 2 mM respectively). The lysate was divided into soluble proteins and insoluble proteins by centrifugation at 5000 x*g* at 4℃ for 20 minutes. The pellet containing the most of the target proteins was solubilized in LEW buffer containing 8 M urea. The suspension was sonicated on ice

at 20% amplitude for 20 minutes, and then additionally add $CaCl_2$ and DTT (final concentration of 10 mM, and 2 mM respectively) and centrifuged at 5000 x*g* at 4℃ for 20 minutes. The supernatant was diluted to a concentration of 2 M urea. The supernatant was loaded onto a nickel column (Protino® Ni-TED) and washed with LEW buffer containing 2 M urea three times. Recombinant protein mHdhDef was eluted by using LEW buffer containing 2 M Urea and 250 mM Imidazole. Finally, 5 mL of purified mHdhDef was obtained.

4. Dialysis of purified proteins

To remove denaturants and refold denatured rmHdhDef protein, stepwise dialysis was performed by dialysis Membrane (Spectra/Por® 3 Dialysis Tubing, 3.5 kD MWCO) at room temperature.

5 steps of dialysis were performed with decreasing stepwise concentration: 1 M Urea in LEW buffer, 0.5 M Urea in LEW buffer, 0 M Urea in LEW buffer, LEW buffer/ 0.1 M Tris (pH 8.0) buffer, 0.1 M Tris (pH 8.0) buffer. Each dialysis step was performed for 2 hours respectively. Finally, purified mHdhDef was obtained in 0.1 M Tris (pH 8.0).

Dialyzed mHdhDef was run through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then the concentration of protein was estimated by bovine serum albumin (BSA) as standard.

5. Antimicrobial activity (URDA)

Antimicrobial activity was tested by using ultrasensitive radial diffusion assay (URDA) (Lehrer et al., 1991). Bacterial cells were cultured in tryptone soy broth (TSB) overnight at 37 ° C or 30 ° C with shaking.

Overnight cultured cells were diluted with 20 mM phosphate buffer (containing 0.03% TSB) to 84%T (≒ 1×10^8 CFU/mL). The suspension (0.4 mL) of the bacterial cells were mixed with 7.1 mL of underlay gel (containing 20 mM phosphate buffer, 0.03% TSB, and 1% agarose). Total 7.5 mL of mixed underlay gel was poured into a petri dish (90 mm of diameter, 15 mm of height) and set at room temperature for 10 minutes. After the mixture was completely solidified, 2.5 mm of wells were punched. For examining an

antibacterial activity, 5 μl of the sample was loaded to each well (2.5 mm of diameter, 1 mm of thick) and set at room temperature for 30 minutes, until all of the sample wells were completely diffused and absorbed into each sample well. Each sample was loaded once again. and absorbed for 3 hours at room temperature. Total, 7.5 mL of overlay gel (containing 20 mM phosphate buffer, 6% TSB, and 1% agarose) was poured into the underlay gel. The petri dish was incubated at 37 ° C or 30 ° C for 8 hours, antimicrobial activity was visualized as a clear zone of each sample well.

Subtracting 2.5 mm of the sample well' s diameter, a clear zone was measured.

Antimicrobial activity was compared with ampicillin as a positive control. TDW and 0.1 M tris (pH 8.0) were used as negative and positive control respectively.

6. Bacterial agglutination assay

An agglutination test of bacterial cells was performed to test whether antimicrobial peptides induce bacterial agglutination of bacterial cells (*E. coli* expressing green fluorescent protein (GFP) and *Bacillus subtilis*) with $CaCl_2$ (Zhang et al., 2018). Bovine serum

albumin (BSA) was used as a negative control.

Bacterial cells were cultured in tryptone soy broth (TSB) at 37 ° C for 4 hours and harvested by centrifugation at 12000 x*g* at 4 ° C for 10 min. Harvested bacterial cells were resuspended in tris-buffered saline (TBS) at an OD_{600} of 0.6.

Resuspended cells were mixed (1:1) with purified rmHdhDef and incubated at 28 ℃ for 1 hour in the presence of final concentrations of 10 mM $CaCl_2$. For staining, 4′,6-diamidino-2-phenylindole (DAPI) was additionally added to a mixture of *Bacillus subtilis* cells for staining. Using fluorescence microscopy, bacterial agglutination was observed.

7. Bacterial binding assay

To investigate the binding of mHdhDef with bacterial cells, bacterial binding assay was performed (Zhang et al., 2018). Bacterial cells were cultured in tryptone soy broth (TSB) at 37 ℃ for about 5 hours and harvested by centrifugation at 12000g at 4 ℃ for 10 min. Harvested bacterial cells were resuspended in tris-buffered saline (TBS) at an OD_{600} of 1.0. To make the mHdhDef sample soluble, mHdhDef was mixed with DM. After that, the mixture was added to resuspended bacterial cells and incubated at room temperature for 30 min with shaking. Finally, the mixture was divided into supernatant,

and pellet by using centrifugation. Each sample was analyzed by immunoblotting. An anti-his tag antibody was used to analyze each samples.

8. Outer membrane permeabilization assay

The outer membrane permeabilization activity of antimicrobial peptides was determined by 1-N-phenylnaphthylamine (NPN) uptake assay (Falla et al., 1996; Hancock et al., 1991). *E. coli* ML35 cells were precultured in tryptic soy broth (TSB) overnight at 37 ℃ with shaking. And then precultured cells were diluted in TSB and grown to $O.D_{600}$ of 0.4-0.6. The cells were harvested by centrifugation and washed with 5 mM HEPES buffer (5 mM KCN, 5 mM glucose, pH 7.2). And then the cells were resuspended to $O.D_{600}$ of 0.4-0.6 and 1 mL of resuspended cells was mixed with 20 μl of 0.5 mM NPN and 10 μl of antimicrobial peptides. The mixture was measured for 3 min using a Fluorescence spectrometer (Fluorolog-QM, Horiba) with excitation wavelength of 350 nm and an emission wavelength of 420 nm. Piscidin was used as positive control.

9. DNA binding assay

To investigate whether antimicrobial peptides bind to DNA molecules, a DNA binding assay (Fried, 1989; Nam et al., 2014) was performed with a slight modification. Antimicrobial compounds are mixed with a DNA marker, and DNA retardation was analyzed by using agarose gel electrophoresis. If antimicrobial peptides bind to DNA molecules, the retardation of DNA band was inhibited. For the control, 150 ng of DNA Ladder Marker (Enzynomics, Daejeon, Korea) was mixed with 1.5 μg or 3 μg of antimicrobial peptides and then leave it at room temperature for 30 minutes. After the electrophoresis in 1.5% agarose gels, DNA was visualized upon staining with 1 μg/mL ethidium bromide (EtBr).

Ⅲ. Results and Discussion

1. Cloning and transformation

Based on the results of NCBI BLAST, the full-length mRNA sequence was obtained (Figure 1). The sequence encoding HdhDef consists of 322 base pairs encoding 66 amino acids including a signal peptide (1-18 region) and a mature peptide (19-66 region). The theoretical isoelectric points (pI) and molecular weight (Mw) of HdhDef were 7.65 and 6,874 Da respectively. The mature peptide of HdhDef was composed of 48 residues and its theoretical isoelectric points (pI) and molecular weight (Mw) were 7.85 and 5,020 Da, respectively.

To express the mature peptide of HdhDef, DNA corresponding to cDNA of mHdhDef was amplified by PCR (Figure 2). Upon cloing PCR products into pColdI DNA expression vector, the recombinant pColdI DNA expression vectors were transformed into *E. coli* BL21(DE3) cells.

(A)

```
1                                                    attcctgaagtcaacatg
                                                                     M   1
19  aaactgcttctgctctctttggtaattgccattgtcggtatggccgatgcggcctccctccagaag
      K  L  L  L  L  S  L  V  I  A  I  V  G  M  A  D  A  A  S  L  Q  K  23
85  cgcgtcacctgcgacctgctgtccttccagatcggtggattctccttcggtgattctgcctgtgct
      R  V  T  C  D  L  L  S  F  Q  I  G  G  F  S  F  G  D  S  A  C  A  45
151 gctcattgcattgttcttcaccacaacggtggacactgcagcaatggcgtctgtgtctgtcggtaa
      A  H  C  I  V  L  H  H  N  G  G  H  C  S  N  G  V  C  V  C  R  *  66
217 acagttatttcagacttacactgcagatcaacatctgtgtatcattgactcttcaataaacagat

283 tataaagctttaaaaaaaaaaaaaaaaaaaaaaaaaaaa
```

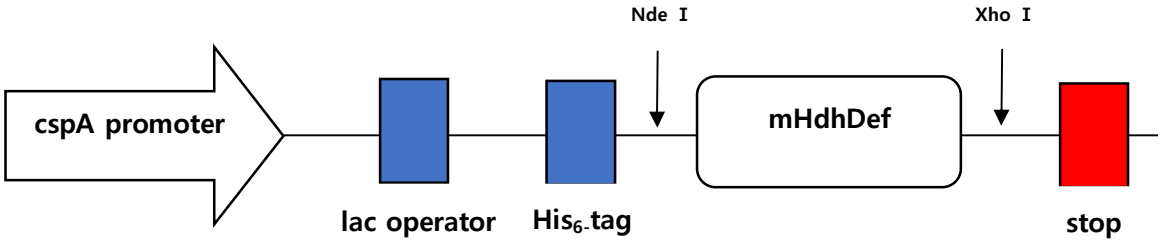
(B)

Length 66
Mass (Da) 6,874

```
        10        20        30        40        50
MKLLLLSLVI AIVGMADAAS LQKRVTCDLL SFQIGGFSFG DSACAAHCIV LHHNGGHC
```

Figure 1. (A) The full-length mRNA sequence of *Haliotis discus hannai* and its coding sequence. Amino acid sequences were shown as upper cases. (B) Amino acid sequences of HdhDef, blue underline indicates hydrophobic, red underline indicates polar amino acid residues, respectively.

(A) pCold DNA I −mHdhDef



(B)

(C)

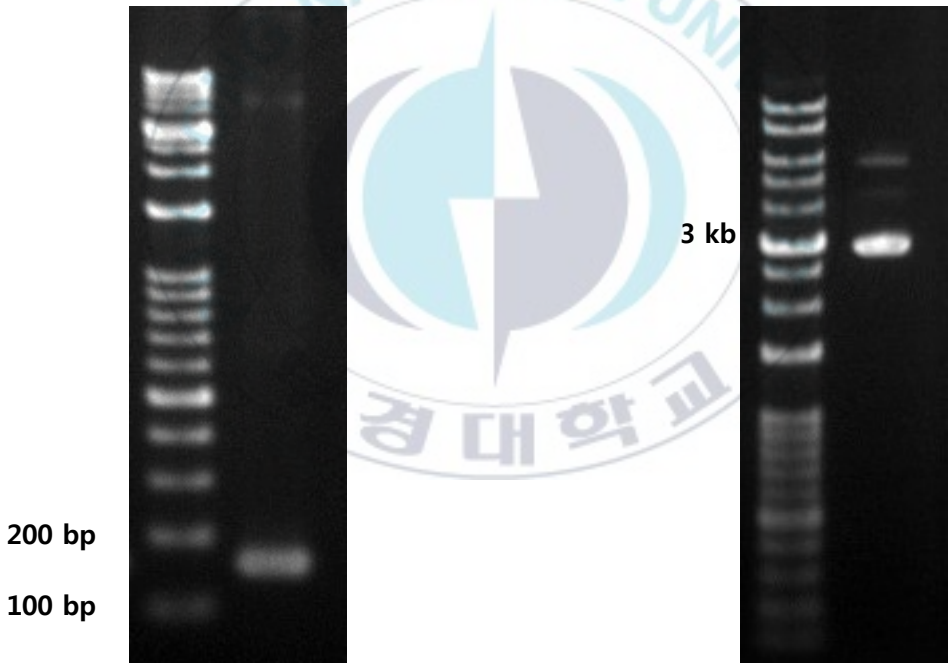


Figure 2. (A) A Schematic representation of the constructed pColdI−mHdhDef expression vector. (B) cDNA of mHdhDef was run on a 2% agarose gel. (C) The constructed pColdI−mHdhDef was run on a 1.5% agarose gel.

2. Multiple sequence alignment and phylogenetic tree analysis

Translated amino acids sequences of HdhDef sequence was obtained from NCBI database (Figure 3). HdhDef encoded 66 amino acids. Amino acid sequence from HdhDef exhibited identities of 98.48% with *Haliotis madaka* (Genbank ALU63750.1), identities of 84.85% with that of *Haliotis rufescens* (NCBI reference sequence XP_046358087.1), identities of 84.85% with *Haliotis discus discus* (Genbank ACZ15982.1), identities of 81.82% with *Haliotis diversicolor* (Genbank QHI06028.1), identities of 72.73% with *Haliotis rubra* (NCBI Reference Sequence XP_046584092.1).

The domain of invertebrate defensin contains six cysteine residues which might form three disulfide bonds in the mature peptide of invertebrate defensin. Multiple sequence alignment of HdhDef was performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Figure 3).

Phylogenetic trees were constructed with Vetigastropoda defensin and mammalian defensin (Figure 4). The phylogenetic tree also indicated a similarity between HdhDef and other Vetigastropoda defensins but a broad distinction from mammalian defensin. HdhDef was grouped with *Haliotis madaka.*

The prediction of the HdhDef 3D structure was obtained by UniPro

(https://www.uniprot.org/peptide-search) (Figure 5.)

Predicted mature peptide region of HdhDef included a one alpha helix and two beta sheets.

(A)

(B)

```
Haliotis rubra              MKLLLLCLVVVIVAMADAASIEKRITCDLLSFTIMGNSIGDSACAAKCLITKHSGGHCSG  60
Haliotis discus hannai      MKLLLLSLVIAIVGMADAASLQKRVTCDLLSFQIGGFSFGDSACAAHCIVLHHNGGHCSN  60
Haliotis madaka             MKLLLLCLVIAIVGMADAASLQKRVTCDLLSFQIGGFSFGDSACAAHCIVLHHNGGHCSN  60
Haliotis rufescens          MKFLLLCLVIAFVGMSDAASLQKRVTCDLLSLQIMGNSFGDSACAAHCIGLHHSGGHCSG  60
Haliotis discus discus      MKFLLLCLVIAFVGMSDAASLQKRVTCDLLSLQIMGNSFGDSACAAHCIGLHHSGGHCSG  60
Haliotis diversicolor       MKFLLLCLVIAFVGMSDAASIQKRVTCDLLSLQIMGNSFGDSACAAHCIGLSHSGGHCSG  60
                            **:***.**:.:*.*:****::**:******: * * *:*******:*:   *.*****.

Haliotis rubra              GVCVCR  66
Haliotis discus hannai      GVCVCR  66
Haliotis madaka             GVCVCR  66
Haliotis rufescens          GVCVCR  66
Haliotis discus discus      GVCVCR  66
Haliotis diversicolor       GVCVCR  66
                            ******
```

Figure 3. (A) Amino acid sequences of HdhDef. The red bar indicates signal peptide, yellow bar indicates mature peptide, purple bar indicates domain of invertebrate defensin. (B) Multiple alignment of amino acid sequences was performed by using Clustal Omega.

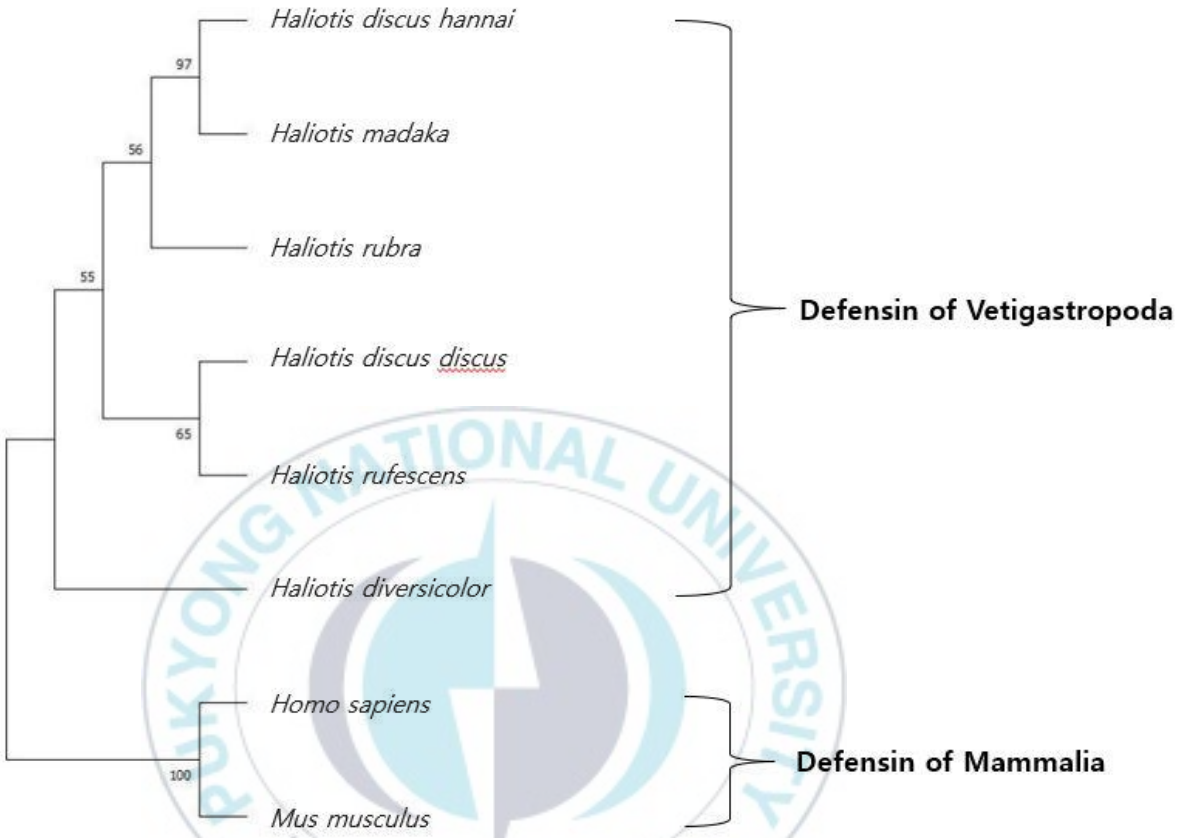


Figure 4. Construction of neighbor-joining tree based on amino acid sequence of Defensin from Vetigastropoda and mammals. Bootstrap values are indicated for each node. Taxonomic groups are indicated on the right. Defensin sequences from *Haliotis discus hannai* (ABF69125.1), *Haliotis madaka* (ALU63750.1), *Haliotis rufescens* (XP_046358087.1), *Haliotis discus discus* (ACZ15982.1), *Haliotis diversicolor* (QHI06028.1), *Haliotis rubra* (XP_046584092.1). mammalian defensins from *Homo sapiens* (AAA52304) and *Mus musculus* (NP_034160.1) were used as outgroups.

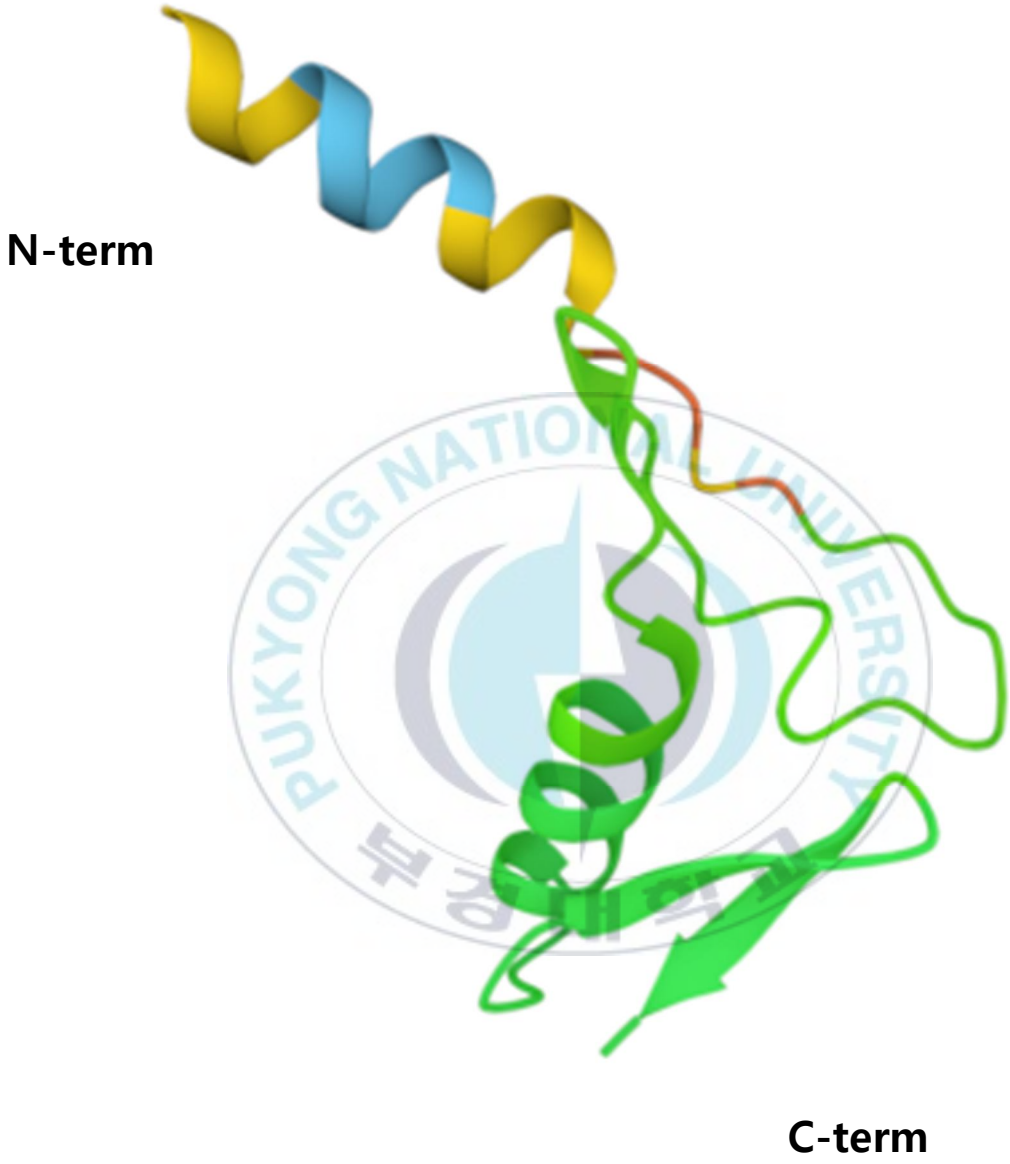


Figure 5. The predicted 3-dimensional structure of HdhDef based on AlphaFold Protein Structure Database. The colorful region of the N-terminus indicates the signal peptide (1-18 amino acids), green region indicates the mature peptide (19-66 amino acids) of HdhDef.

3. Recombinant protein purification

N-terminal His-tagged recombinant mHdhDef was overexpressed in *E. coli* BL21 (DE3) cells. Molecular weight of mHdhDef was estimated to be 8.8 kDa. Overexpression of mHdhDef was induced at 18° C, 120 rpm with 0.5 mM IPTG. The amount of mHdhDef was mostly overexpressed as an insoluble protein. For purification, insoluble protein was solubilized in 8 M urea and purified by using a nickel column (Protino® Ni-TED). Recombinant mHdhDef was eluted in LEW buffer (with 2 M Urea and 250 mM Imidazole). Stepwise dialysis was performed to increase proper refolding of proteins. Finally, purified mHdhDef was obtained in the presence of 100 mM Tris (pH 8.0)

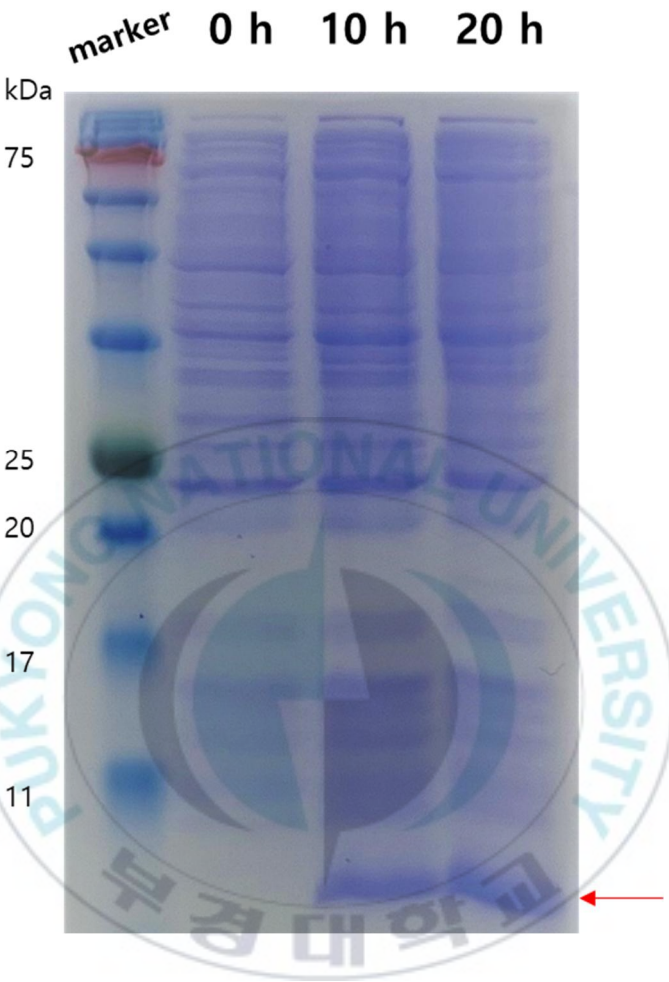


Figure 6. Expression profile of the recombinant construct pCold I –mHdhDef transformed into *Escherichia coli* BL21 (DE3). Overexpression of mHdhDef was induced by 0.5 mM IPTG at 18 ℃ for 20 hours. BL21 (DE3) cell lysate was run on SDS–PAGE gel to confirm the overexpression of mHdhDef. Each lane indicates the time after induction of IPTG: 0 hour, 10 hours, and 20 hours, respectively. The red arrow indicates the mHdhDef.

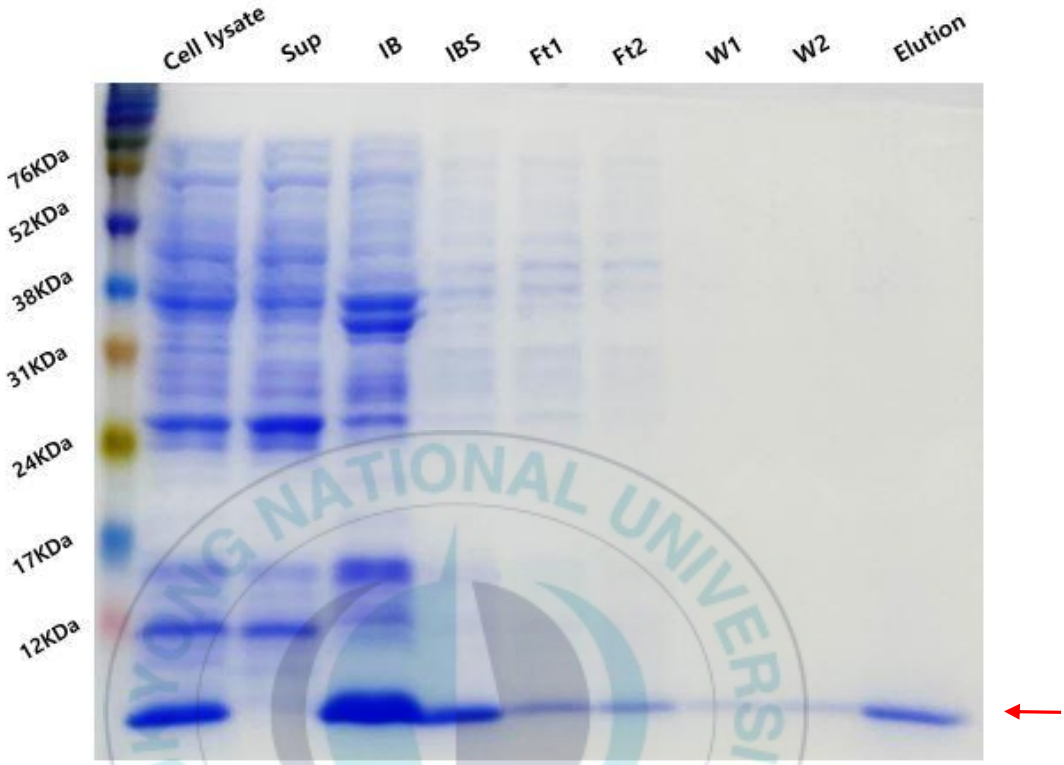


Figure 7. Purification profile of the recombinant protein run on a 15 % SDS-PAGE gel. *E. coli* BL21 cells were sonicated to purify mHdhDef and then lysate was divided into supernatant and inclusion body. The inclusion body was solubilized in urea and then divided into supernatant and inclusion body upon centrifugation. The supernatant was poured into a nickel column and obtained purified mHdhDef. Each lane indicates protein marker, together with cell lysate, Sup: supernatant, IB: inclusion body, IBS: inclusion body sup, Ft: flow through, W: wash, Elution. The red arrow indicates the mHdhDef

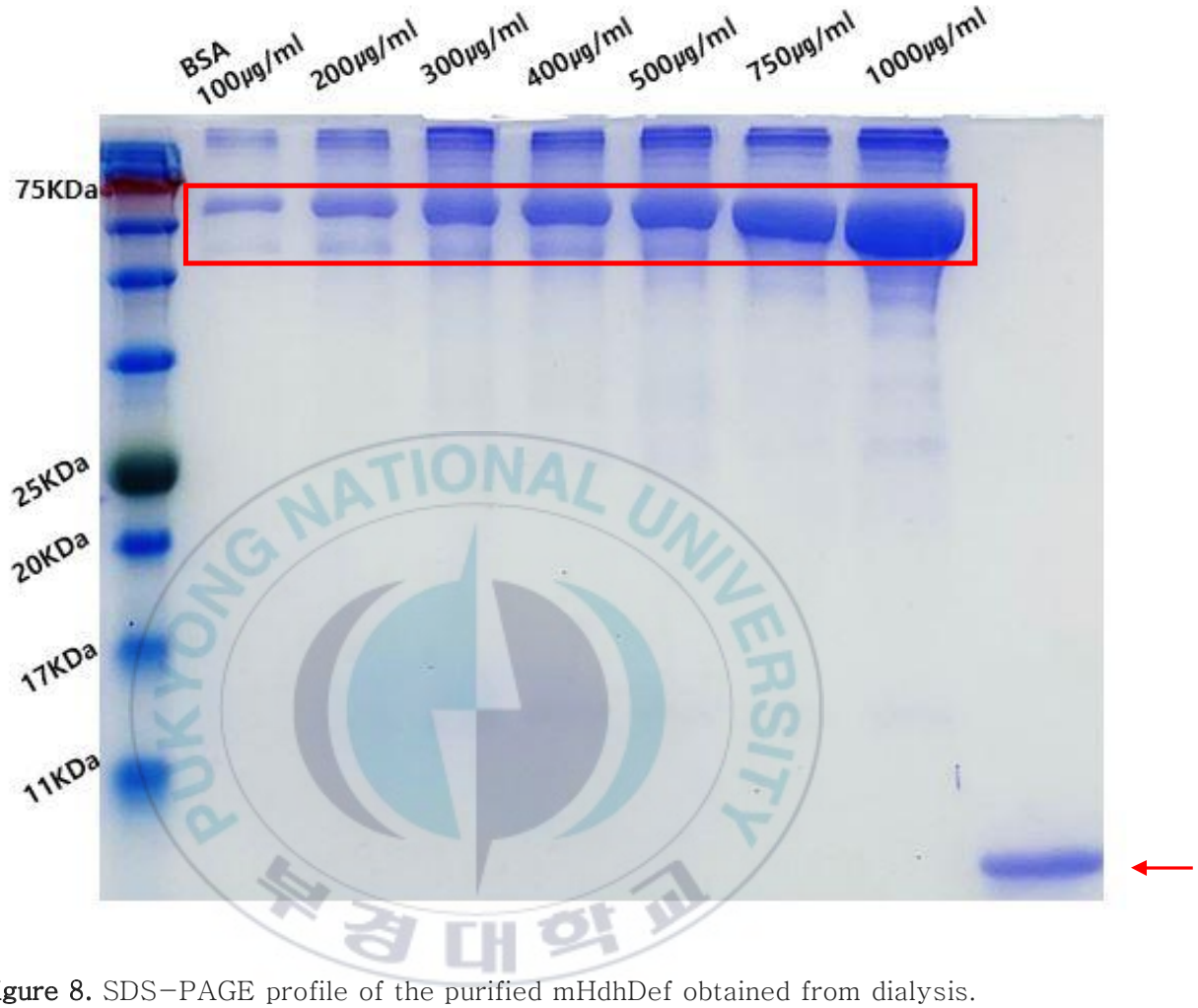


Figure 8. SDS-PAGE profile of the purified mHdhDef obtained from dialysis. After dialysis, purified mHdhDef was in 100 mM Tris and run on a SDS-PAGE gel. The concentration of purified mHdhDef sample was compared with BSA.

4. Antimicrobial activity (URDA)

The antimicrobial activity of purified mHdhDef was determined by using

ultrasensitive radial diffusion assay (URDA). The result showed that mHdhDef has antimicrobial activity against Gram-positive bacteria (*Bacillus subtilis* KCTC1021, *Streptococcus parauberis* KCTC3651) and Gram-negative bacteria (*Vibrio harveyi* ATCC 14126, *Escherichia coli* ML35).

The sample was loaded into each well with increasing concentration from minimal inhibitory concentration (MIC, μg/mL). After 8 hour incubation, the sample well having antimicrobial activity formed a clear zone and the distilled water, 100 mM Tris (pH 8.0) was used as negative control, respectively. Ampicillin was used to compare the antimicrobial activity of mHdhDef.

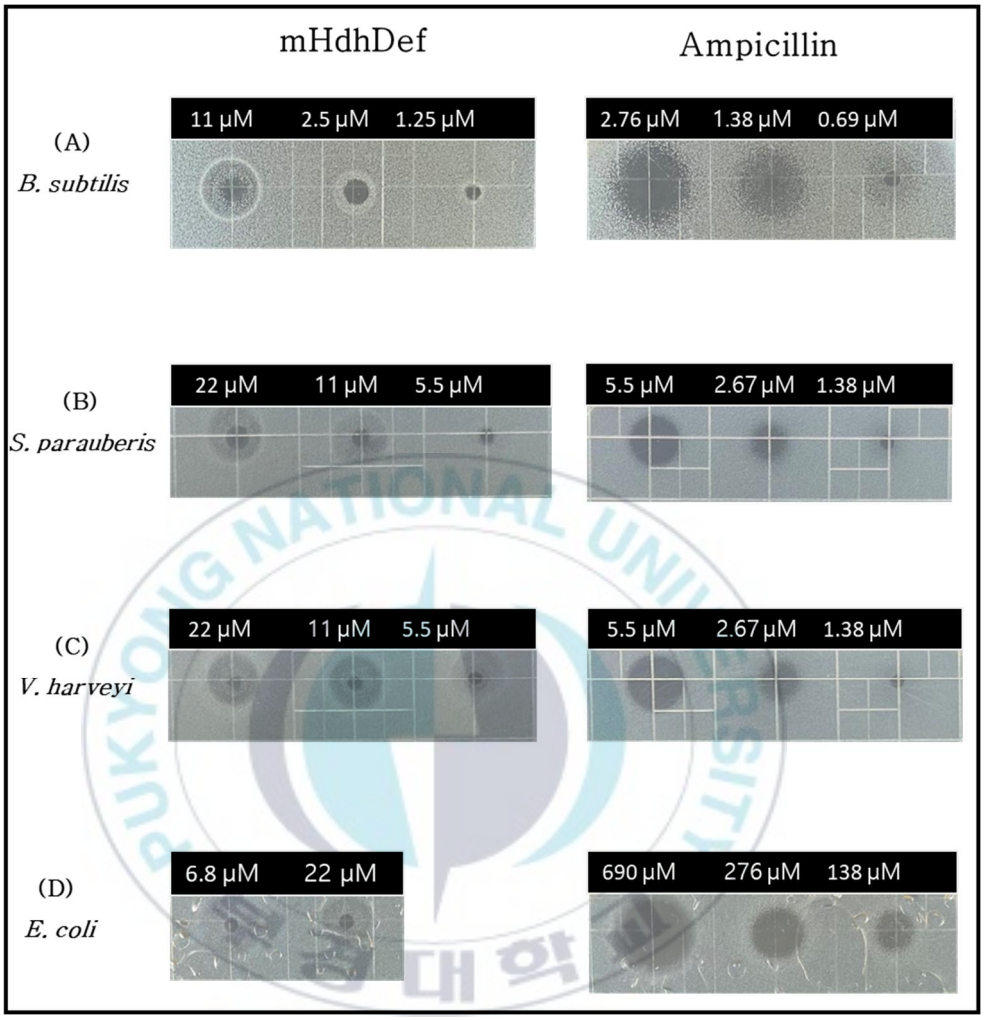


Figure 9. Antimicrobial activity of the recombinant mHdhDef. The sample was loaded into each well with increasing concentration from minimal inhibitory concentration. (A) *Bacillus subtilis.* (B) *streptococcus parauberis.* (C) *Vibrio harveyi.* (D) *Escherichia coli.*

Microbes	Gram	Minimal effectives concentration (μg/ml) [μm]	
		rmHdhDef	Ampicillin
B. subtilis	+	11 [1.25]	0.25 [0.69]
S. parauberis	+	49 [5.5]	0.5 [1.38]
E. coli	–	196 [22]	50 [138]
V. harveyi	–	49[5.5]	0.5 [1.38]
M. luteus	+	-	2.5 [6.9]
S. enterica	–	-	5 [13.8]
E. piscicida	–	-	35 [96.6]
V. alginolyticus	–	-	30 [82.8]

Table 1. Antimicrobial activity of mHdhDef and ampicillin. The recombinant mHdhDef showed antimicrobial activity against *B. subtilis, S. parauberis, E. coli, V. harveyi*, but not against *M. luteus, S. enterica, E. piscicida, V. alginolyticus* in the mHdhDef concentration of 1000 μg/mL.

5. Bacterial agglutination assays

We tested whether defensin could bind to bacterial cells using a centrifugation assay in which the supernatant containing the unbound protein was separated from the pellet containing bacteria bound to defensin. Although most of defensin was present in the fraction containing precipitated bacterial cells after centrifugation, the purified defensin tended to self-precipitate under our experimental conditions. To further confirm the specific binding of the recombinant protein to the bacterial cells using an assay based on centrifugation, we inhibited the defensin self-precipitation by using dodecyl maltoside, which is widely utilized for membrane protein solubilization. The result showed that defensin was detected in the supernatant but did not precipitate, indicating that dodecyl maltoside adequately solubilized the purified defensin in the absence of bacteria (Figure 9). In contrast, most of the defensin was detected in the pellet containing bacterial cells upon the addition of either Gram-negative *E. coli* or Gram-positive *B. subtilis*. These results show that defensin bound to bacterial cells.

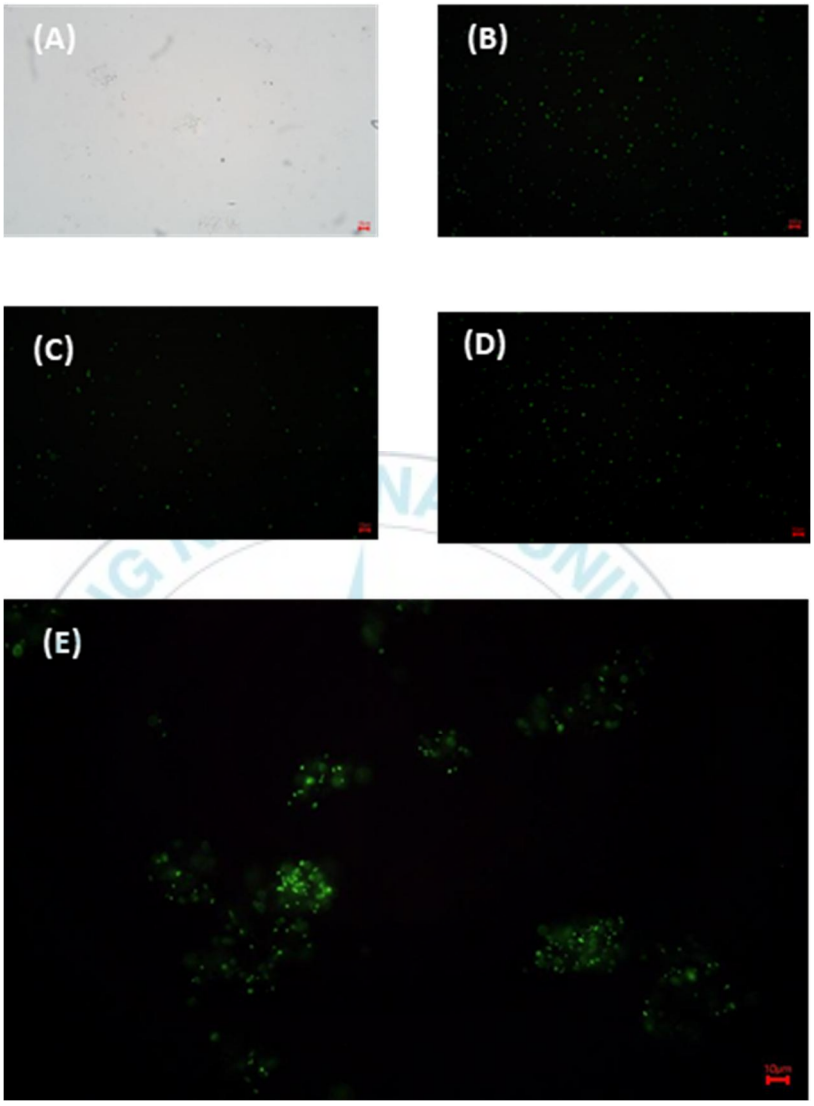


Figure 10. Microscopic analysis of the bacterial agglutination. Experiment was carried out with mHdhDef in *E. coli* BL21-GFP cells in a $CaCl_2$-dependent manner. Bacterial agglutination tests were performed on *E. coli* BL21-GFP cells mixed with antimicrobial peptides and $CaCl_2$ and then mixtures were incubated at room temperature for 1 hour. **(A)**, **(B)**, **(C)**, **(D)** were used as negative control. **(A)**: TBS buffer was mixed with mHdhDef and $CaCl_2$, **(B)**: *E. coli* BL21-GFP cells were mixed with 0.1 M Tris and $CaCl_2$, **(C)**:

E. coli BL21-GFP cells were mixed with BSA and $CaCl_2$, (D): *E. coli* BL21-GFP cells mixed with mHdhDef and triple distilled water. (red scale bar: 10 μm).

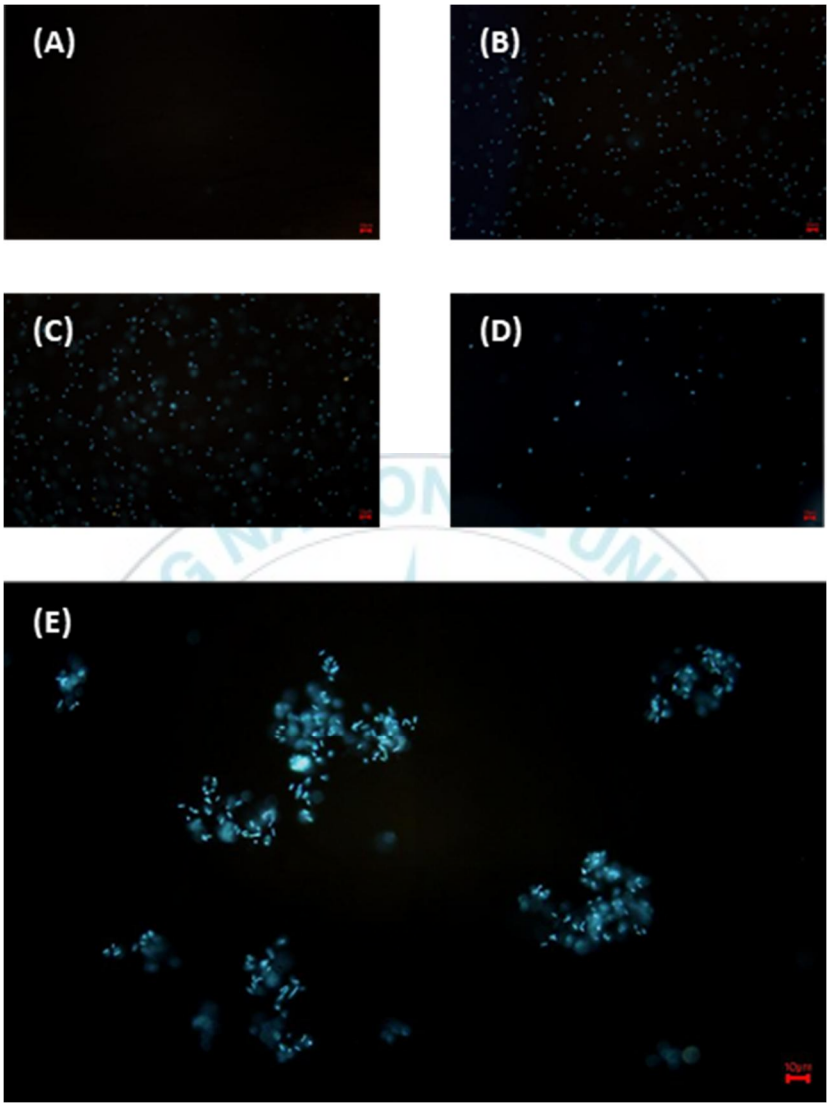


Figure 11. Bacterial agglutination assay of the recombinant defensin by microscopic and fluorescence microscopy by using. *Bacillus subtilis* was stained with DAPI to observe the cells. Bacterial agglutination tests were performed on *Bacillus subtilis* cells mixed with antimicrobial peptides and $CaCl_2$ and then mixtures were incubated at room temperature for 1 hour. **(A)**, **(B)**, **(C)**, **(D)** were used as negative control. **(A)**: TBS buffer was mixed with mHdhDef and $CaCl_2$, **(B)**: *Bacillus subtilis* cells were mixed with

0.1 M Tris and $CaCl_2$, (C): *Bacillus subtilis* cells were mixed with BSA and $CaCl_2$, (D): *Bacillus subtilis* cells mixed with mHdhDef and triple distilled water. (red scale bar: 10 μm).

6. Bacterial binding assay

We tested whether rAbCTLD could bind to bacterial cells using a centrifugation assay in which the supernatant containing the unbound protein was separated from the pellet containing bacteria bound to rAbCTLD. Although most of rAbCTLD was present in the fraction containing precipitated bacterial cells after centrifugation, the purified rAbCTLD tended to self-precipitate under our experimental conditions (data not shown). To confirm the specific binding of the recombinant protein to the bacterial cells using an assay based on centrifugation, we inhibited the rAbCTLD self-precipitation by using dodecyl maltoside, which is widely utilized for membrane protein solubilization. We found that rAbCTLD was detected in the supernatant but did not precipitate, indicating that dodecyl maltoside adequately solubilized the purified rAbCTLD in the absence of bacteria (Figure 9). In contrast, most of the rAbCTLD was detected in the pellet containing bacterial

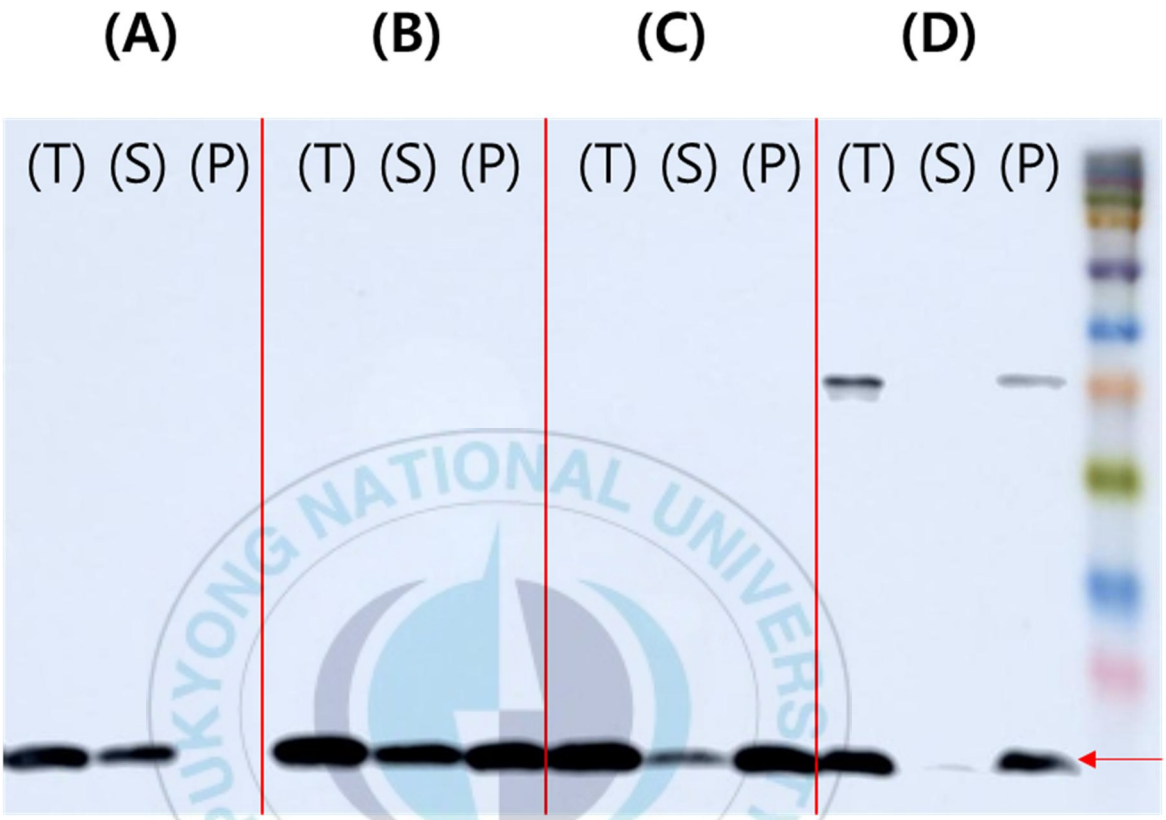


Figure 12. Bacterial binding analysis of the purified mHdhDef. Assay was carried out with recombinant protein dissolved in dodecyl maltoside (final concentration of 0.05%) and then mixed with *E. coli* BL21-GFP. **(A)** Negative control, *E. coli* cells were replaced with TBS buffer. **(B)** Purified mHdhDef was mixed with 1 X *E. coli* cells and divided by centrifugation. **(C)** Purified mHdhDef was mixed with 10 X *E. coli* cells and divided by centrifugation. **(D)** Purified mHdhDef was mixed with 100 X *E. coli* cells and divided by centrifugation. **(T)** total sample. **(S)** supernatant. **(P)** pellet.

7. Outer membrane permeabilization assay

The permeabilization of the *E. coli* ML 35 outer membrane was observed by the NPN uptake assay. This was based on the fact that If antimicrobial peptides break the outer membrane of Gram−negative bacteria, NPN emits fluorescence in hydrophobic environments.

The NPN was excited at 350 nm and fluorescence intensity was recorded at 420 nm. Upon addition of mHdhDef to the mixture of NPN and *E. coli* ML35 cells, fluorescence was measured for 3 minutes. While the fluorescence was drastically increased upon addition of piscidin, only a slight increase in was observed upon addition of mHdhDef. This result suggested that mHdhDef has little, if any, outer membrane permeabilization ability.

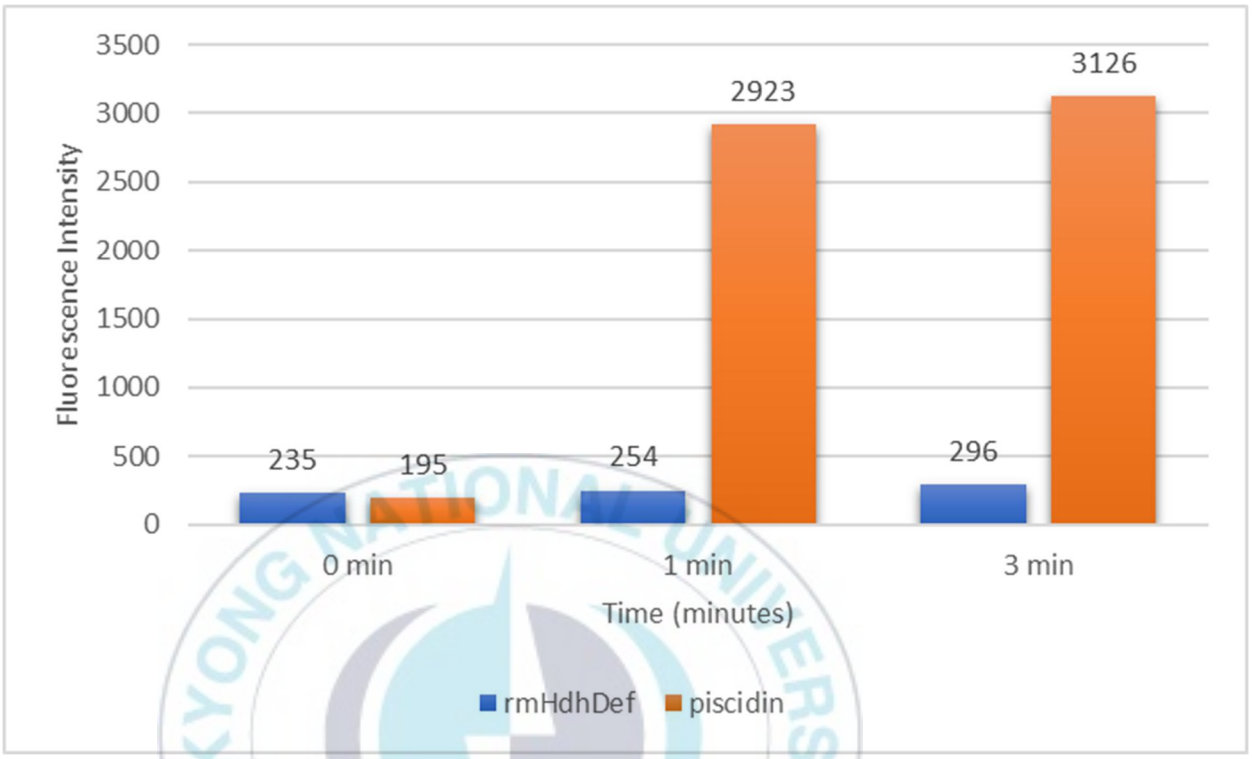


Figure 13. Bacterial outer membrane permeability of mHdhDef was monitored for 3 minutes. The blue bar graph indicates mHdhDef (150 μg/mL) and the orange bar graph indicates piscidin (150 μg/mL).

8. DNA binding assay

DNA binding ability of mHdhDef was conducted using a commercial marker DM3200 ExcelBand™ 1KB Plus (0.1−10 kb) DNA Ladder. Agarose gel electrophoresis (containing 1.5% agarose gel and EtBr) was used for analysis.

While an addition of piscidin which was known to have a DNA-binding ability, showed an inhibitory migration of DNA molecules, mHdhDef showed no binding to DNA molecules, which is evident in the migration of DNA bands along the agarose gel.

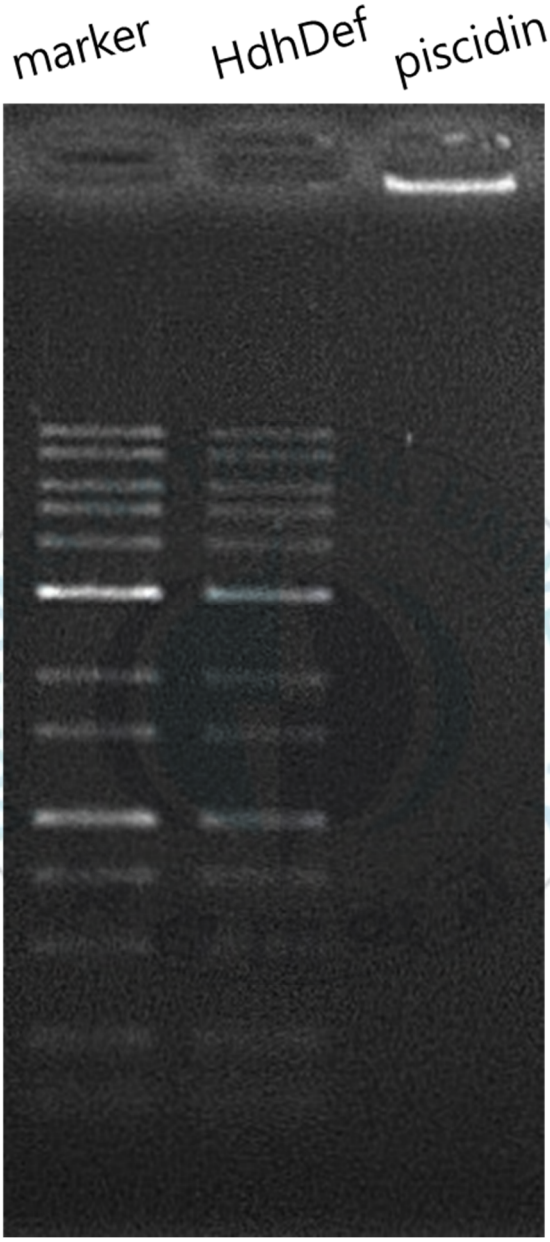


Figure 14. DNA binding ability of mHdhDef. 4 μg of the antimicrobial peptide was mixed with 350 ng of DNA marker. Piscidin exhibited potent DNA binding ability, DNA band was inhibited on the agarose gel. On the other hand, mHdhDef exhibited no potent DNA binding ability.

Ⅳ. Discussion

Defensin-like peptide of *Haliotis discus hannai* was shown to be related with growth of Pacific abalone. Moreover, it exhibits antimicrobial activity against Gram-positive bacteria (*Bacillus subtilis, Streptococcus parauberis*), and Gram-negative bacteria (*Vibrio harveyi, Escherichia coli*). HdhDef might play a role of the innate defense, so it leads to the normal growth of abalone. The mature peptide of HdhDef consists of 48 amino acid residues which were predicted to form one alpha helice and two beta sheets. The antimicrobial mechanism of mHdhDef seems not to break bacterial cell wall, but interact with the bacterial cell wall.

The antimicrobial activity of mHdhDef was not remarkable as compared with other antimicrobial peptides, thus finding the region of antimicrobial activity was needed to improve the minimal inhibitory concentration (MIC) of mHdhDef (Koo et al., 2008; Nam et al., 2014; Seo et al., 2013).

An additional experiment of the hemolytic assay was needed to determine the possibility of mHdhDef being used as antibiotic.

Ⅴ. Abstract (Korean)

북방전복(Haliotis discus hannai)에서 유래한 디펜신 유사 펩타이드의 항균 활성

권 필 립

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전복은 국내 양식산업에 있어 고부가가치 종으로 여겨진다. 선행연구에서 전복의 치패를 각각 크기별로 그룹을 나눈 후 RNAseq을 이용하여 그룹별로 발현차이를 보이는 유전자 분석을 실시한 결과 큰 그룹과 작은 그룹간의 유의미한 발현차이를 보이는 속성장 관련 유전자를 몇 가지 확인하였다. 본 실험에서는 속성장 관련 유전자중 Defensin-like peptide의 항균활성능을 확인하였다. 해당 유전자의 coding sequence는 201 bp로 66 개의 아미노산을 암호화하며 18 개의 signal peptide와 48 개의 mature peptide로 구성된다. mature peptide의 n말단에 his_6-tag이 추가된 재조합 단백질을 *E. coli* BL21 (DE3)에서 과발현을 유도하였고 nickel column으로 정제후 투석을 거쳤다. 정제된 재조합 단백질은 URDA를 통해 그람양성균과 그람음성균에 각각 항균활성 실험을 진행하였고 그람양성균인 *Bacillus subtilis, Streptococcus parauberis* 와 그람음성균 *Escherichia coli, Vibrio harveyi* 에 항균활성을 보였다. 항균활성 메커니즘을 알아보기 위하여 몇 가지 실험을 진행하였고 그 결과 세균의 세포막을 파괴하기보다는 세포막과 상호작용을 통해서 항균활성능을 가지는것으로 보여진다.

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