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Antibacterial Action of New Antibacterial Peptides, Nod1 and Nod2, Isolated from *Nordotis discus discus*

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ABSTRACT: Abalone is a valuable seafood in the aquaculture industry worldwide as it is rich in protein. However, to date, research on the functional proteins of abalone is lacking. Herein, we report two peptides with antibacterial activity from *Nordotis discus discus*. The purification of peptides was performed by solvent extraction, ultrafiltration, and reverse-phase high performance liquid chromatography. The N-terminal amino acid sequences of the isolated antibacterial peptides, named as Nod1 and Nod2, were identified by Edman degradation and did not show any similarity to other proteins and peptides in databases based on results of BLAST homology analysis. Molecular masses of Nod1 and Nod2 were 6145.06 and 6360.07 Da, respectively, as determined by mass spectrometric analysis. The two peptides displayed pH-dependent antibacterial activity against various bacteria that was more potent at pH 5.4 than pH 7.4, but they did not inhibit fungal growth at either pH levels. Their antibacterial activity was due to membranolytic action, which was assayed by SYTOX-green uptake. In addition, both peptides were virtually noncytolytic for human erythrocytes and mammalian cells.

KEYWORDS: *abalone, antibacterial peptide, pH-dependent, membranolytic action, marine invertebrate*

INTRODUCTION

The development of new antibiotics against pathogenic bacteria has become a significant problem in modern medicine.[1,2] Because of the quick adaptation of pathogenic microbes to conventional antibiotics, much effort has been made to identify new antibiotics with novel mechanisms of action to overcome this resistance. Among them, antimicrobial peptides (AMPs) are important candidates because their mechanism of action is comparable to that of conventional antibiotics.[3,4] AMPs in nature act as defensive materials against the invasion of bacterial and fungal pathogens.[5] They have been isolated from diverse sources, ranging from unicellular to multicellular microorganisms, and more than 1500 sequences have been reported. AMPs exert their killing action via membrane-damage/disruption of microbial cells in combination with intracellular activity.[6−8] In addition to their direct microbicidal action, AMPs promote the accumulation of immune cells such as macrophages, neutrophils, and lymphocytes.[9]

Invertebrate animals possess efficient innate immune systems to defend themselves against invading foreign materials due to their lack of antibodies and an acquired immune response.[10,11] Especially, marine invertebrates are good sources of novel AMPs as they are surrounded by specific environments characterized by low temperature, elevated pressure, high density of pathogenic microbes, and high salinity, in comparison to terrestrial invertebrates.[11] Thus far, about 40 AMPs have been identified from marine invertebrates, and their sequences are very variable except for the defensin family.[11−13] Specifically, AMPs have been characterized from hemocytes and coelomocytes in several taxa of marine invertebrates, including Annelida,[14,15] Arthropoda,[16,17] Cnidaria,[18] Echinodermate,[19] Mollusca,[20,21] and Tunicata.[22,23] A single species may produce several peptides having different sequences or isoforms. The diverse sequences of peptides may be due to adaptation to various environments.

In the present study, we isolated two antibacterial peptide isoforms from *Nordotis discus discus*, commonly known as abalone, belonging to the taxon Mollusca. Abalone has long been a valuable food source for humans and is an important mariculture item due to its palatable taste and applicability as a medicine.[24,25] Therefore, it possesses high economic and pharmacological value. In this study, two isolated peptides named Nod1 and Nod2 exhibited pH-dependent antibacterial activity via membranolytic activity.

MATERIALS AND METHODS

Materials. Abalone was obtained at Wando-gun, South Korea. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Sigma-Aldrich (St Louis, MO, USA). Acetonitrile and water (HPLC grade) was obtained from Burdick & Jackson Inc. (Muskegon, MI). Sytox-green was acquired from Molecular Probes (Eugene, OR). All other reagents were of analytical grade.

Isolation of Antibacterial Peptides. After washing with distilled water, whole body of abalone (250 g) was ground in liquid nitrogen. Solvent-soluble proteins (102.80 g) were extracted with organic solvent (50% methanol, v/v) at 4 °C for 3 h. To remove solvent-insoluble materials, the extracts were centrifuged (12000 rpm) at 4 °C for 20 min, after which the supernatants were freeze-dried. The supernatants with antimicrobial activity were successfully purified by ultrafiltration through a 10 kDa cutoff membrane, followed by solid phase extraction (SPE) and reverse-phase high performance liquid chromatography (RP-HPLC) on C_{18} resin. Briefly, the freeze-dried

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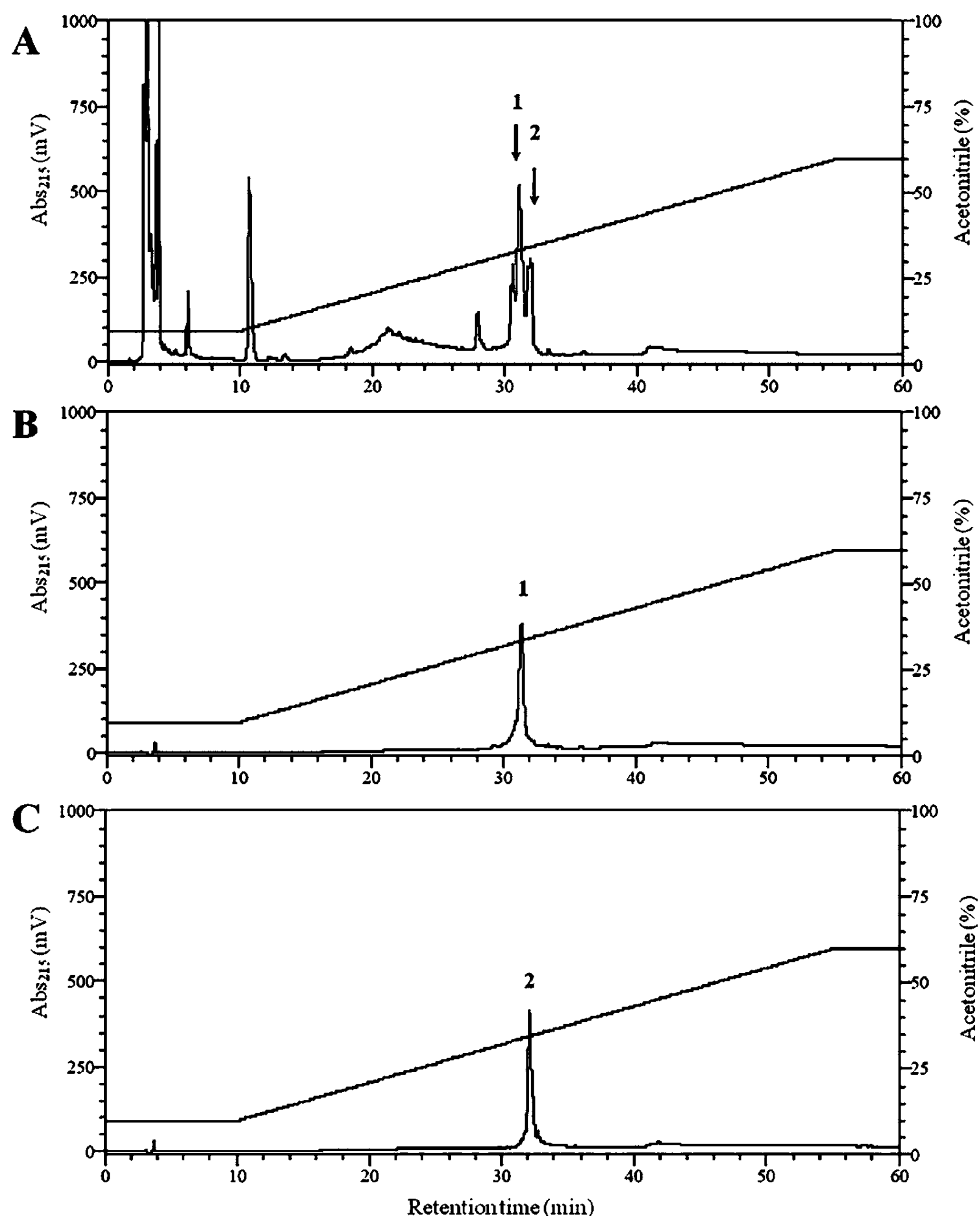

Figure 1. RP-HPLC purification of peptides. The 40% fraction from SPE was further purified through a C18 column (4.6 mm × 250 mm; Vydac, Hesperia, CA) previously equilibrated with 10% acetonitrile (ACN) containing 0.1% (v/v) TFA. Elution was achieved using a linear gradient of acetonitrile in 0.1% TFA. Absorbance was monitored at 214 nm. (A) Peaks of Nod1 (1) and Nod2 (2) are indicated by arrows. (B,C) RP-HPLC rechromatography of peak 1 and 2 fractions.

powders dissolved in distilled water were passed through a 10 kDa cutoff membrane in order to obtain peptides with a low molecular mass. The ultrafiltered materials were then subjected to SPE (Alltech Maxi-Clean Cartridges, C_{18}), after which fractions were eluted stepwise with 20%, 40%, and 100% (v/v) acetonitrile in water. The 40% fraction with higher antibacterial activity was further purified through a C_{18} column (4.6 mm × 250 mm; Vydac, Hesperia, CA) previously equilibrated with 10% acetonitrile (ACN) containing 0.1% (v/v) TFA. Peptides were separated using a gradient of 10−60% acetonitrile for 45 min at a flow rate of 1 mL/min at 40°C. Elutes were monitored by measuring the absorbance at 214 nm. Each of the peak fractions was collected and assayed for antibacterial activity. The peak fractions with strong antimicrobial activity were identified and pooled. The purity of the peptide was confirmed by the presence of a single band on 16.5% Tricine SDS-PAGE.

Tricine Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE). Electrophoresis of peptides with low molecular mass was performed according to the method of Schagger and Jagow[26] on 16.5% polyacrylamide separating gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue G-250. The molecular weight marker in Tricine SDS-PAGE consisted of triosephosphate isomerase obtained from rabbit muscle (26.6 kDa), myoglobin from horse heart (17 kDa), α-lactalbumin from bovine milk (14.2 kDa), aprotinin from bovine lung (6.5 kDa), insulin Chain B oxidized from bovine (3.5 kDa), and bradykinin (1 kDa).

Protein Determination. The molecular masses of the peptides were determined using a matrix-assisted laser desorption ionization mass spectrometer (MALDI II, Kratos Analytical Ins.). The concentration of proteins was measured using a BCA kit (Pierce, Rockford, IL).

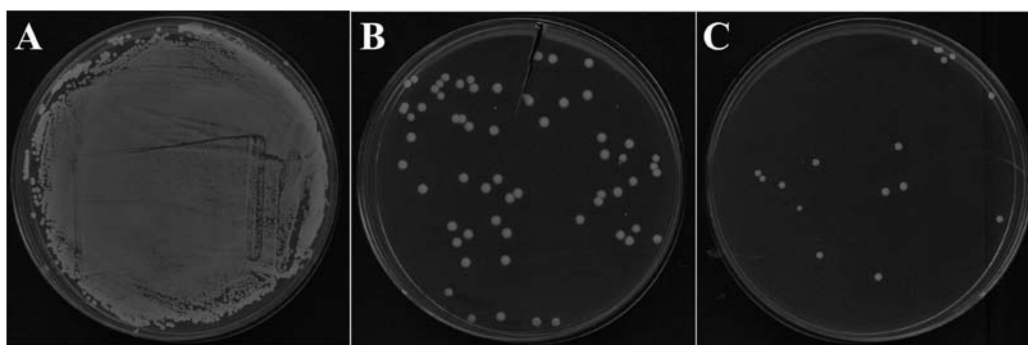

Figure 2. Antibacterial activities of Nod1 and Nod2. Exactly 250 μg/mL of isolated peptides (peaks 1 and 2 in Figure 1) were incubated with *E. coli* cells. (A) control, (B) Nod1, (C) Nod2.

Table 1. Summary of Isolation of Nod1 and Nod2 from Abalone Body

Step	Amount of crude proteins (mg/250 g powder)		Yield	
	Nod1	Nod2	Nod1	Nod2
1. Extraction with 50% (v/v) MetOH	102.80		100	
2. Ultrafiltration (under 10 kDa)	56.51		54.97	
3. SPE separation	21.32		20.74	
4. RP-HPLC	3.83	2.94	3.72	2.86

Peptide Identification. N-Terminal amino acid sequencing of peptides blotted on a polyvinylidene difluoride (PVDF) membrane was analyzed at the Korea Basic Science Institute (Seoul, Korea) via automated Edman degradation[27] using a pulse liquid automatic sequencer (Applied Biosystems Inc., Procise 491).

Assay for Antifungal Activity. To determine the antifungal activity of the purified peptides against *Aspergillus flavus* (Korean Collection for Type Cultures; KCTC 6905) and *A. parasiticus* (KCTC 6598), fungal spores from 10-day-old cultures grown on a PDA plate at 28 °C were collected using 0.08% Triton X-100.[28] Spores were diluted to a concentration of 4×10^4 spores/mL in half-strength RPMI 1640-MOPS buffer (pH 5.4 and 7.4). Then 50 μL of each spore suspension were then placed in flat-bottomed polystyrene 96-well plates (Nunc, Roskilde, Denmark), after which 50 μL of peptides serially diluted 2-fold in the same buffer was added to yield a final spore concentration of 2×10^4 spores/mL. After 24–36 h of incubation at 37 °C, hyphal growth of spores was monitored microscopically by inverted light microscopy. Absorbance was also measured at 600 nm using a VersaMax microtiter reader (Molecular Devices, CA, USA).[28]

Assay of Antibacterial Activity. *Escherichia coli* (American Type Culture Collection; ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *E. coli* O157 (ATCC 43895), *Pseudomonas aeruginosa* (ATCC 15692), and *Bacillus subtilis* (KCTC 1998) were cultured in trypticase soy broth (TSB) under agitation at 37 °C. The antimicrobial activities of the peptides were determined by microdilution assay. Briefly, bacteria were collected in midlog phase and suspended in 10 mM sodium phosphate (pH 5.4 and 7.4). Two-fold serial dilutions of the peptides were then distributed into sterile 96-well plates. An aliquot of the cell suspension (5×10^5 CFU/mL) was then added to each well, after which the plates were incubated at 37 °C for 4 h. At the end of incubation, 50 μL of the 20-fold diluted sample was plated onto TSB agar plates and incubated overnight, after which the results were confirmed.[29]

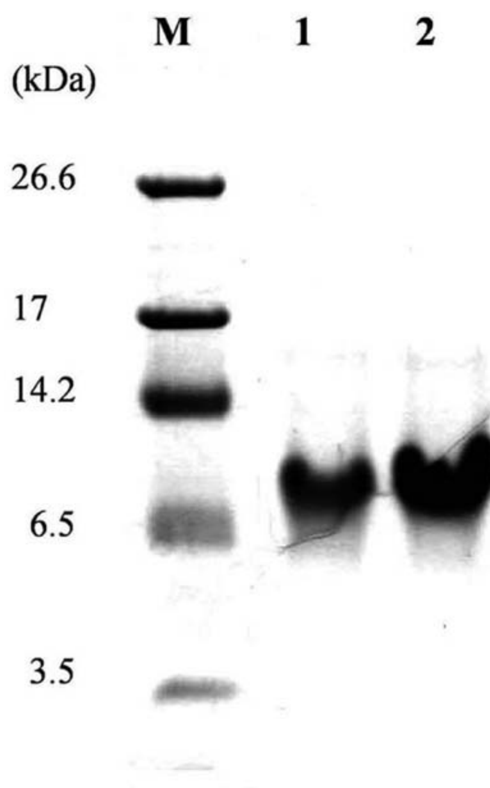

Figure 3. Tricine SDS-PAGE of Nod1 and Nod2 peptides. Peptides were analyzed by Tricine-SDS-PAGE on a 16.5% separating gel with Coomassie brilliant blue G staining. Lane M: Molecular mass marker proteins from Sigma. Lane 1: Nod1 (10 μg). Lane 2: Nod2 (10 μg).

Hemolysis of hRBCs (Human Red Blood Cells). Hemolytic activities of the peptides were measured on heparin using hRBCs collected from healthy donors. Fresh hRBCs were rinsed three times in PBS (1.5 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 135 mM NaCl, pH 7.4). Proteins dissolved in PBS were then added to 100 μL of stock hRBCs suspended in PBS (final RBC concentration, 8% v/v). The samples were next incubated with agitation for 60 min at 37 °C, followed by centrifugation for 10 min at 800*g*. The absorbance of the supernatants was assessed at 414 nm. Next, hRBCs in PBS (A_{blank}) and 0.1% Triton X-100 (A_{triton}) were used as the negative and positive controls, respectively. Percent hemolysis was calculated according to the equation:

$$\%\text{hemolysis} = [(A_{sample} - A_{blank})/(A_{triton} - A_{blank})] \times 100$$

Each measurement was conducted in triplicate.[30]

Cell Culture and Cytotoxicity. To examine the cytotoxic effects of the peptides, NIH/3T3 (mouse fibroblast) cells were cultured in

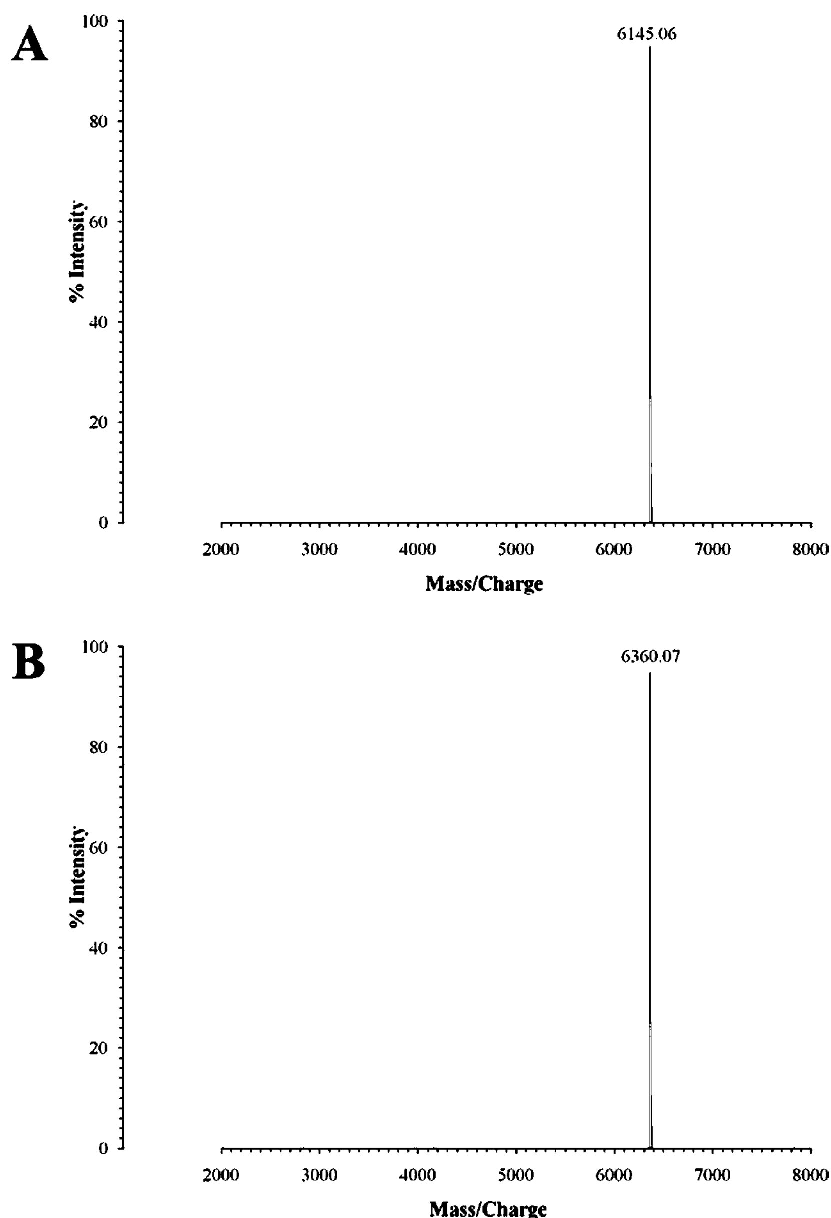

Figure 4. Mass analysis of peptides. Molecular masses of Nod1 and Nod2 peptides were determined by using MALDI-TOF MS spectrometry. (A) Nod1, (B) Nod2.

Dulbecco's Modified Eagle Medium (DMEM) supplemented with antibiotics (100 U/mL of penicillin, 100 μg/mL of streptomycin) and 10% fetal calf serum at 37 °C in a humidified chamber in an atmosphere containing 5% CO_2. Growth inhibition was evaluated by MTT assay for the measurement of viable cells. A total of 4×10^3 cells/well was seeded onto a 96-well plate, followed by incubation for 24 h. Peptides serially diluted 2-fold with DMEM were then added to the plate, followed by incubation for 24 h at 37 °C. then 10 μL of 5 mg/mL MTT was added to each well, followed by incubation for an additional 4 h. The supernatants were then aspirated, after which 50 μL of DMSO was added to the wells in order to dissolve any remaining precipitate. Absorbance was then measured at a wavelength of 570 nm using a microtiter reader.[30]

$$\%\text{survival} = [(A_S - A_B)/(A_C - A_B)] \times 100$$

where A_B is the absorbance of the blank (without cultured cells), A_C is the absorbance of the control (without peptide), and A_S is the absorbance of the mixture containing cultured cells and peptide.

SYTOX Green Uptake Assay. *E. coli* cells were grown to mid-logarithmic phase at 37 °C, washed, and suspended (2×10^7 cells/mL) in 10 mM sodium phosphate buffer (pH 5.4). The cells were then incubated with 0.5 μM SYTOX green for 15 min in the dark.[30] After the addition of peptides at appropriate concentrations, the increases in fluorescence, based on binding between the cationic dye and intracellular DNA, were monitored in a time-dependent manner (excitation wavelength of 485 nm and emission wavelength of 520 nm).

RESULTS AND DISCUSSION

Purification of the Antibacterial Peptides. Proteins of high molecular weight were denatured during purification using C_{18} RP-HPLC, after which their functions either decreased or completely disappeared due to their solvent-insoluble properties. In this study, we attempted to isolate peptides of low molecular weight. For this, solvent extracts were prepared by methanol extraction from whole body of abalone, *Nordotis discus discus*. The extracts were separated into above and below 10 kDa fractions via ultrafiltration, after which the below

10 kDa fractions with antibacterial activity (not shown data) were subjected to SPE (C_{18}). Bound materials were eluted stepwise with 20%, 40%, and 100% acetonitrile, and in the next step, the 40% fraction with antibacterial activity was subjected to RP-HPLC separation. Two major peaks (1 and 2, arrowed in Figure 1A) were fractioned in the first round of RP-HPLC purification, and pure peptides were obtained in the second round of purification (Figure 1B,C). The purified peptides were shown to possess antibacterial activity against *E. coli* (Figure 2) at high concentration (250 μg/mL). Peak 1 and 2 peptides were termed Nod1 and Nod2, respectively. As summarized in Table 1, the yields of Nod1 and Nod2 were 3.83 (3.72% recovery) and 2.94 (2.86% recovery) mg per 250 g of abalone crude protein powder, respectively. As abalone body is rich in proteins, more proteins could be extracted if carried out in aqueous buffer. However, as the extraction solution in this study was methanol, the total amount of extracted proteins was low.

Identification of Nod1 and Nod2. Homogeneity of the purified peptides was confirmed by tricine SDS-PAGE, which resulted in a single band (1 and 2 in Figure 3) ranging from 6.5 to 14.2 kDa. However, the exact molecular masses of the peptides could not be determined by SDS-PAGE. Therefore, we determined the molecular masses of Nod1 and Nod2 by MALDI-TOF MS analysis to be 6145.06 and 6360.07, respectively (Figure 4). To determine their amino acid sequences, we analyzed 35 amino acids at the N-terminus of each peptide by the Edman degradation method. The 35 residues of the N-terminal sequences of the peptides were determined as follows: Nod1, NH_2-AMSTTDCFTYLEGPFFISDGSVVVDNCVRRRMLLD and Nod2, NH_2-AMSTFACFTYLEGPFLISPFSVVVDNNVRRRMLLL. As shown in Figure 5, the

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     * * * * · · * * * * * * * * * · * * · · * * * * * * · * * * * * * *
nod1 A M S T T D C F T Y L E G P F F I S D G S V V V D N C V R R R M L L D 35
nod2 A M S T F A C F T Y L E G P F L I P F G S V V V D N N V R R R M L L L 35
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Figure 5. Sequences of the isolated peptides Nod1 and Nod2 from *Nordotis discus*. The N-terminal 35 amino acids were sequenced by the Edman degradation method.

N-terminal amino acids of Nod1 and Nod2 were similar, indicating that they are isoform peptides. Their amino acid sequences were not identical to those of any protein in the BLAST homology analysis (http://www.ncbi.nlm.nih.gov/blast), indicating that they are novel antibacterial peptides.

pH-Dependent Antibacterial Activities of Nod1 and Nod2. To investigate the antimicrobial spectrum of Nod1 and Nod2, microdilution assay was performed against five bacterial and two filamentous fungal strains under pH 5.4 and 7.4 buffer conditions. As shown in Table 2, the two peptides were inactive against the fungal strains *Aspergillus flavus* and *A. parasiticus* at pH 5.4 and 7.4. Interestingly, both peptides displayed pH-dependent antibacterial activity, which was higher at pH 5.4 compared to pH 7.4. The MICs of Nod1 and Nod2 toward all tested bacteria ranged from 10 to 20 μM at pH 5.4, but they increased to over 80 μM at pH 7.4. The higher activities of Nod1 and Nod2 under acidic conditions suggest that they may possess cysteine residues in their amino acid sequences. Antibacterial activity in an acidic environment may be therapeutically useful in numerous areas, namely cosmetics or food safety. Furthermore, antibacterial peptides active under acidic conditions have been reported to have protective effects against

Table 2. Minimum Inhibitory Concentrations of the Two Peptides against Bacterial and Fungal Strains[a]

Microorganism	MIC (μM)			
	Nod1		Nod2	
	pH 5.4	pH 7.4	pH 5.4	pH 7.4
Bacteria				
E. coli	20	>160	20	160
E. coli O157	20	160	20	160
P. aeruginosa	20	>160	10	80
S. aureus	10	>160	10	160
B. subtilis	20	160	20	160
Fungi				
A. flavus	>320	>320	>320	>320
A. parasiticus	>320	>320	>320	>320

[a]Antimicrobial activities of the peptides were measured by microdilution assay. Bacteria were collected in mid-log phase and suspended in 10 mM sodium phosphate (pH 5.4 and 7.4). To measure antifungal activities of the purified peptides, fungal spores from 10-day-old cultures grown on a PDA plate at 28 °C were collected using 0.08% Triton X-100. Spores were diluted to a concentration of 4×10^4 spores/mL in half-strength RPMI 1640-MOPS buffer (pH 5.4 and 7.4).

infection by pathogenic bacteria, as some human tissues, namely the skin, urinary tract, oral cavity, and vagina, have physiologically acidic pHs.[31] This finding might be useful when these peptides are applied as a skin therapy, as the pH of skin is approximately 5.4.[32]

Noncytotoxic Effects of Nod1 and Nod2. For a peptide to be developed as an antibiotic agent, its cytotoxicity must be low. Hemolysis and cytotoxicity of the purified peptides were assessed against hRBC and NIH3T3 cells. The hemolytic activities of Nod1 and Nod2 were determined to be 1.61% and 4.57% at 400 μM, respectively, whereas melittin as a negative control displayed 83.6% hemolysis at 3.13 μM (Figure 6A).

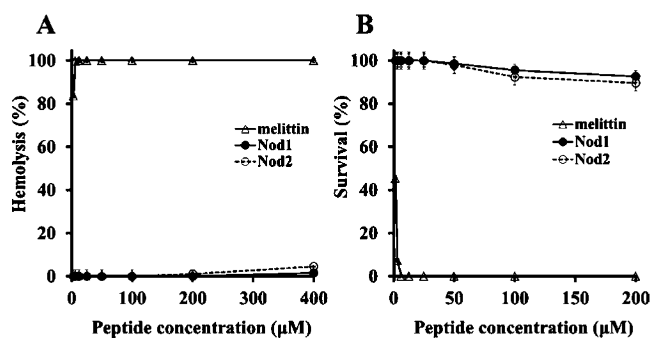

Figure 6. Hemolytic and cytotoxic effects of Nod1 and Nod2 peptides. Dose-dependent hemolytic and cytotoxic activities of Nod1 and Nod2 against human red blood cells (A) and NIH3T3 cells (B) were evaluated using melittin as a negative control.

In addition, the survival rates of NIH3T3 cells were 92.64% (Nod1) and 89.61% (Nod2) at 200 μM (Figure 6B). Therefore, Nod1 and Nod2 were shown to be noncytotoxic peptides. Further, it appears that Nod1 and Nod2 possess remarkable antimicrobial effects against a variety of microbial cells without hemolytic activity or cytotoxicity.

Membrane-Permeable Action of Nod1 and Nod2. To investigate the antibacterial actions of two peptides, we carried out membrane-permeable assay based on the entrance of SYTOX green dye into bacterial cells, which indicates disruption of the cytoplasmic membrane.[30] Emitted fluorescence of this cell-impermeable dye increases upon binding to nucleic acids. Each peptide was incubated with this dye at 20 μM and 0.5 μM, respectively, with *E. coli* cells and the results were observed under a fluorescence microscope. As shown in Figure 7, control cells in the absence of peptide did not display green fluorescence (Figure 7A), whereas *E. coli* cells in the presence of peptide expressed remarkable fluorescence (Figure 7B,C). This result suggests that the two peptides exerted growth inhibition against bacterial cells via membranolytic action.

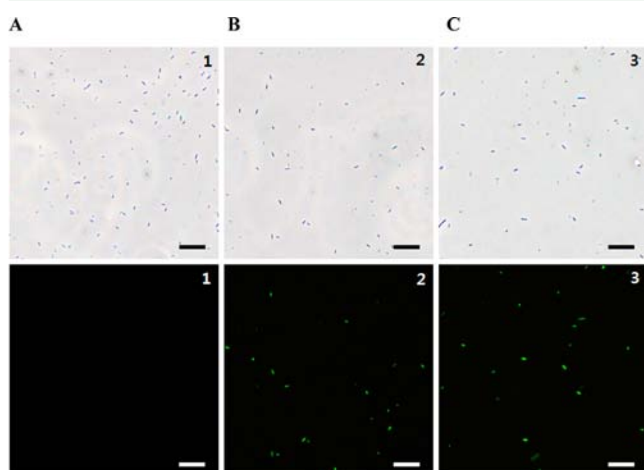

Figure 7. Membrane-permeable activities of Nod1 and Nod2 peptides. Peptides (20 μM) dissolved in 10 mM sodium phosphate (pH 5.4) buffer were incubated with *E. coli* cells for 15 min and then visualized by fluorescence inverted microscopy. (A) control, (B) Nod1, (C) Nod2. 1: Bright field microscopy. 2: Fluorescent field microscopy.

In summary, the two peptides termed Nod1 and Nod2 were successfully isolated from *Nordotis discus discus*. We showed that these peptides possess potent antibacterial activity against pathogenic bacteria under acidic pH levels within 10−20 μM of the MIC. Additionally, their cytotoxic effects were shown to be very low. Lastly, the molecular masses of Nod1 and Nod2 were determined to be 6145.06 and 6360.07 Da, respectively, by MALDI-TOF/MS analysis. They further inhibit bacterial growth by membrane-permeable action.

Several nontoxic peptides, mostly of bacterial or biomaterial origin (e.g., from seaweeds, barley seeds, cabbage leaves, or *Streptomyces*), have been purified and are now commercially available for use against human pathogens.[33] Specifically, Nod1 and Nod2 peptides have been shown to efficiently inhibit human pathogens. Here, we found that Nod1 and Nod2 had antimicrobial activities against *A. flavus*, which can negatively affect the storage of grains and also act as a human pathogen.[34] Further, Nod1 and Nod2 have been shown to inhibit *A. parasiticus*, a mold known to produce aflatoxin. Aflatoxins are a group of naturally occurring mycotoxins, and a high level of exposure causes acute hepatic necrosis, eventually resulting in cirrhosis and/or carcinoma of the liver.[35] Lastly, *E. coli* O157, an enterohemorrhagic strain and foodborne pathogen, was shown to be inhibited by Nod1 and Nod2.[36] Thus, Nod1 and Nod2 have a variety of beneficial and therapeutic uses. We expect that their acid-active nature may encourage their application in the field of antibiotic skin therapy.

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Author Contributions

⊥These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

AMP, antimicrobial peptide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SPE, solid phase extraction; RP-HPLC, reverse-phase high performance liquid chromatography; PVDF, polyvinylidene difluoride; KCTC, Korean Collection for Type Cultures; ATCC, American Type Culture Collection; TSB, trypticase soy broth; hRBCs, human red blood cells

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