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Interaction of Apidaecin Ib with Phospholipid Bilayers and its *Edwardsiella* Species-specific Antimicrobial Activity

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Apidaecin Ib had strong antimicrobial activity against several tested Gram-negative bacteria including *Escherichia coli*, *Enterobacter cloacae*, and *Shigella flexneri* (MECs; 0.3-1.5 μg/mL), but showed no activity against all the tested Gram-positive bacteria including *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus* and one yeast, *Candida albicans* (MECs; >125 μg/mL). Interestingly, this peptide showed potent antibacterial activity only against *Edwardsiella* species (MECs; 0.6-3.6 μg/mL) among the tested fish pathogenic bacteria through a bacteriostatic process and showed no significant hemolytic activity. Apidaecin Ib took an unordered structure in all environments and also had very weak membrane perturbation activity even at 25 μM. Anti-*Edwardsiella* activity of apidaecin Ib is stronger than those of other antimicrobial polypeptides or antibiotics, but its activity is salt-sensitive. These results suggest that apidaecin Ib has *Edwardsiella* species-specific antibacterial activity and could be applied as new preventive or control additives for *Edwardsiella* species infection in freshwater fish aquaculture.

Key Words : Apidaecin Ib, *Edwardsiella* species, Alternatives, Fish pathogenic bacteria

Introduction

Antimicrobial polypeptides (AMPPs) have been considered as a new type of alternatives due to their different action mode on bacterial membrane from conventional antibiotics, and have been heralded for being less harmful for the environment and consumers than antibiotics and non-inducible resistant bacteria.[1-3] During the past decades, numorous researchers have discovered AMPPs from not only land organisms but also marine organisms and have studied on the application of them as alternatives to antibiotics. However, much of the researches have been focused on the development of new alternatives for foods or terrestrial animals such as humans and poultrys, and very few studies have been performed for marine animals.[4] Recently, research interests have been turned to the development of new alternatives for improving infectious diseases in fish or shellfish aquaculture.[5] Especially, several researchers are trying to develop new therapeutic agents or alternatives to control bacterial infections in fish aquaculture. Interestingly, the fish pathogens are usually Gram-negative bacteria.[6] It is required that the development of new drugs or alternatives to antibiotics for treatment of or control of bacterial fish diseases might be focused on materials which have Gram-negative bacteria specific antibacterial spectrum.

Apidaecins isolated from lymph fluid of the honeybee, *Apis mellifera*, are proline-rich antibacterial peptides containing 18 amino acid residues and exhibits a broad range antibacterial activity against Gram-negative bacteria through bacteriostatic process, but show no activity against Gram-positive bacteria.[7-9] In this regard, apidaecin-like peptides have recently been considered as a good candidate for the new alternatives against fish Gram-negative bacteria such as *Aeromonas* spp., *Edwardsiella* spp., and *Vibrio* spp. Although apidaecins have been applied recently as feed additives instead of antibiotics for common carp basal ditets for investigation of its effectiveness on growth performances and immune function, there is no report for the screening of antibacterial activity of apidaecins against specific fish pathogenic bacteria, espicially *Edwardsiella* species.[10]

E. tarda, *E. hoshinae*, and *E. ictaluri* are generally known as *Edwardsiella* species and *E. tarda* and *E. ictaluri* which is predominantly found in freshwater fish, infect fish and cause diseases.[11,12] They cause edwardsiellosis/emphysematous putrefactive disease leading to mass mortality in various fish and result in tremendous economic losses from aquaculture industry.[11,13] *Edwardsiella* species infection has recently become one of the most serious threats to fish aquaculture.[14,15] Up to date, to treat *Edwardsiella* species infections in fish aquaculture, antibiotics drugs or other chemicals are widely used both on a preventive and curative basis.[5] The development of alternatives to antibiotics which is less dangerous for aqueous animals, consumers and environment, and which do not lead to bacterial resistance, is an essential need for a sustainable aquaculture. To achieve this, peptide antibiotics meet the purpose of the development of a new

alternative.

In this study, to evaluate the possibility of use apidaecin Ib, one of apidaecin family, as an alternative for treatment of or control of *Edwardsiella* diseases, we examined the antibacterial activity of apidaecin Ib against several *Edwardsiella* species.

Materials and Methods

Chemical Reagent. For peptide synthesis, Fmoc-resin and Fmoc (9-fluorenylmethoxycarbonyl)-protected amino acids were purchased from Advanced Chem Tech (Louisville, KY, USA). Diisopropylcarbodiimide (DIPCI), 1-hydroxybenzotriazole (HOBT), and piperidine were purchased from Watanabe Chemicals Co. (Hiroshima, Japan). Trifluoroacetic acid (TFA), *m*-cresol, and 1,2-ethanedithiol were purchased from Wako Chemical Co. (Osaka, Japan). Thioanisole was purchased from Aldrich Chemical Co. (Milwaukee, USA) and *N,N*-dimethylformamide (DMF) was purchased from TEDIA (Ohio, USA). For the preparation of liposomes, 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phospho(1'-*rac*-glycerol) (DPPG), egg yolk L-α-phosphatidylcholine (EYPC), and egg yolk L-α-phosphatidylglycerol (EYPG) were purchased from Sigma (St. Louis, MO, USA). All other reagents used were of the highest grade avaible.

Synthesis and Purification of Peptides. The following antimicrobial polypeptides were used for antibacterial activity test: apidaecin Ib,[8] gramicidin S,[16] mastoparan B,[17] piscidin 1,[18] piscidin 4[19] and human histone H1. Gramicidin S was purchased from Sigma-Aldrich (St. Louis, MO, USA) and human histone H1 was purchased from New England BioLabs, Inc. (Ipswich, MA, USA). Other peptides were synthesized by using Fmoc chemistry with a Millipore Model 9050 peptide synthesizer (PerSeptive Biosystem, CA, USA) according to the manufacturer's protocol. After cleavage and side-chain deprotection using deprotection reagent [TFA: Thioanisole:*m*-Cresol:1,2-Ethanedithiol (5:1.7:0.6:0.9, v/v)] for 90 min at room temperature, the crude peptides obtained were dissolved in 5% acetic acid and purified by Sephadex G-25 (fine, 2.6 × 110 cm) column with 5% acetic acid. Then the peptides were further purified by a semi-preparative μBondapak C18 HPLC column (7.8 × 300 mm, 10 μm, 100 Å, Waters) in conjunction with a Waters 600 HPLC system and Waters 486 tunable absorbance detector. The solvent system was composed of H_2O containing 0.1% TFA (solvent A) and CH_3CN containing 0.1% TFA (solvent B) with a flow rate of 3.0 mL/min, and detected at 220 nm. After semi-purification, analytical RP-HPLC was also carried out by using a μBondapak C18 column (3.9 × 300 mm, 10 μm, 100 Å, Waters) with a flow rate of 1.0 mL/min. Fractions of the desired purity were pooled and lyophilized for experiments. The purity (> 95%) and molecular weights of synthetic peptides were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Voyager-DE^{TM} PRO spectrometer, Perseptive Biosystems, USA). The molecular weights of the synthetic peptides were as follows: apidaecin Ib; base peak, 2108.4, calcd. formular, $C_{95}H_{150}N_{32}O_{23}$, mastoparan B; base peak, 1612.1, calcd. formular, $C_{78}H_{138}N_{20}O_{16}$, piscidin 1; base peak, 2571.1, calcd. formular, $C_{122}H_{188}N_{38}O_{24}$, piscidin 4; base peak, 5313.9, calcd. formular, $C_{235}H_{360}N_{80}O_{63}$. They were dissolved in acidified water (0.01% acetic acid) to make stock solutions and then further diluted in the same solution for test.

Antibiotics. The following antibiotics were used for antibacterial activity test against *Edwardsiella* species: tetracycline (Sigma #T-3383), minocycline (Sigma #M-9511), gentamicin (USB #16051), kanamycin (BioShop #KAN201.5), streptomycin (Invitrogen #11860-038), ampicillin (Sigma #A-9518), and rifampicin (Sigma #R-3501). They were dissolved in acidified water to make stock solutions and then further diluted in the same solution for test.

Ultrasensitive Radial Diffusion Assay (URDA). The antibacterial activities of the peptides or antibiotics were assessed with an ultrasensitive radial diffusion assay against Gram-positive bacteria including *Bacillus subtilis* RM125, *Bacillus subtilis* KCTC1021, *Micrococcus luteus* ATCC9341, *Staphylococcus aureus* RM4220, and *Streptococcus iniae* FP5229, and Gram-negative bacteria including *Escherichia coli* D31, *Escherichia coli* KCTC1116, *Enterobacter cloacae* KCTC1685, *Shigella flexneri* KCTC2517, *Aeromonas hydrophila* KCTC2358, six *Edwardsiella* species, *Vibrio parahemolyticus* HUFP91, *Vibrio parahemolyticus* KCCM41664, and one yeast, *Candida albicans* KCTC7965, as described previously.[20] Briefly, they were incubated on Trypticase Soybean Agar (TSA) or Sabouraud Dextrose Agar (SDA) at the appropriate temperature (25 °C for fish pathogenic bacteria such as *A. hydrophila*, six *Edwardsiella* species, *S. iniae*, and two *V. parahemolyticus* species and 37 °C for others) for 16-18 h. One well-separated colony was picked with a wire loop and inoculated into 3 mL of Trypticase Soybean Broth (TSB) or Sabouraud Dextrose Broth (SDB) and incubated at the appropriate temperatures for 18 h to produce a moderately cloudy suspension. After overnight incubation, the bacterial and *C. albicans* suspension were diluted with sterile underlay gel solution without argarose to a McFarland turbidity standard of 0.5 (Vitek Colorimeter #52-1210, Hach, Loveland, Colorado) corresponding to ~10^8 CFU/mL of bacteria or 10^6 CFU/mL of *C. albicans*. One-half milliliter of diluted bacterial or *C. albicans* suspension was added to 9.5 mL of underlay gel containing 5×10^6 CFU/mL of bacteria and 5×10^4 CFU/mL of *C. albicans* in 10 mM phosphate buffer (pH 6.6) with 0.03% TSB or 0.03% SDB and 1% Type I (low EEO) agarose. The peptides or antibiotics were serially diluted 2-fold in 3 μL of acidified water (0.01% acetic acid, HAc) and each dilution was added to 2.5 mm diameter wells made in the 1 mm thick underlay gels. After incubation for 3 hr at either 25 °C or 37 °C, the bacterial or *C. albicans* 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 to the nearest 0.1 mm, using a vernier caliper (Mitutoyo Corporation, Japan). After subtracting the diameter of the well, the clearing zone diameters were expressed in units (0.1 mm = 1 U). The MECs (minimal effective concentrations, μg/mL) of the peptides or antibiotics were calculated as the X-intercept of a plot of units against the $\log_{10}$ of the peptides or antibiotics concentrations.[21] Piscidin 1, an α-helical AMP isolated from hybrid striped bass (*Morone saxatilis* × *M. chrysops*), was used as a positive control and 0.01% HAc was used as a negative control.

***Edwardsiella* Strains.** 5 strains of *E. tarda* including *E. tarda* H-4, *E. tarda* NUF251, *E. tarda* RE1, *E. tarda* RE2, and *E. tarda* RED2, isolated from diseased Japanese flounders, were kindly provided by Professor Jeong at Pukyong National University, Korea, and another *Edwardsiella* species, *E. ictaluri* KCTC12264, was purchased from Korean Collection for Type Cultures (KCTC).[22,23] Total 6 bacteria were used for anti-*Edwardsiella* species activity test with an URDA. All of bacterial strains were cultured on TSB containing 0.5% NaCl at 25 °C.

Killing Assays. To determine the killing activities of apidaecin Ib and antibiotic, the kinetic studies were performed using *E. tarda* H-4 at MECs, 5× MECs, and 10× MECs of them.[24] Overnight culture of *E. tarda* H-4 was diluted in TSB to a cell density of 10^6 CFU/mL and one milliliter of each suspensions were exposed to apidaecin Ib and antibiotic added to the specified final concentrations (1× MEC, 5× MEC, and 10× MEC) and incubated at 25 °C. At defined time intervals (0, 5, 10, 20, 40, and 60 min), equal aliquots were removed from each culture and serially diluted and 10 μL was plated onto TSA plates. The plates were incubated at 25 °C overnight, and the resulting colonies were counted. Percent killing was calculated as the proportion of live bacteria at a given time point following the addition of the peptide or antibiotic compared to the number of bacteria present prior to the addition of the peptide or antibiotic. Control cultures of each bacterium were incubated without any peptide or antibiotic and were assayed at time points corresponding to the times of assay of the test cultures to ensure that there was no spontaneous loss of viability.

Hemolytic Assay. The hemolytic activities of the peptides were determined by using human and fish red blood cells (RBCs). The RBCs were collected from heparin-treated blood by centrifugation at 4000 × g for 5 min and washed three times with 10 mM Tris-HCl buffer (pH 7.4/150 mM NaCl) to remove plasma and buffy coat and then resuspended in buffer to 3% hematocrit (2×10^8 cells/ml). 10 μL of peptides (the final concentration: 100 μg/mL - 3.13 μg/mL) was added to 90 μL of 3% hematocrit suspension in microcentrifuge tube and incubated for 60 min at 37 °C. After incubation, supernatants (70 μL) obtained from the centrifugation at 8000 × g for 10 min were placed in a microtiter plate and the absorbance was measured with an ELISA plate reader at 405 nm. Zero and 100% hemolysis were determined with the absorbance of buffer and 0.1% Triton X-100, respectively. The hemolysis percentage of peptides was calculated by the following formula:

$$\% \text{ Hemolysis} = [(Abs_{405nm} \text{ in the peptide solution} - Abs_{405nm} \text{ in buffer}) / (Abs_{405nm} \text{ in 0.1\% Triton X-100} - Abs_{405nm} \text{ in buffer})] \times 100$$

Salt Effect on the Anti-*Edwardsiella* Activities of Apidaecin Ib and Antibiotics. The effects of salt concentrations on the anti-*Edwardsiella* activities of apidaecin Ib and antibiotics were tested by determining their MECs in the presence of NaCl. NaCl was added to the underlay gel solution to obtain final NaCl concentrations of 86, 171, 256, 342 and 599 mM. Piscidin 1 was used as a positive control. MECs were calculated as describe above.

Preparation of Liposomes. For CD measurement, phospholipid liposomes were prepared as described previously.[25] Phospholipid [DPPC or DPPC-DPPG (3:1)] (20 mg, about 25 μmol) in chloroform (2 mL) was placed in a round-bottom flask. After evaporation of the solvent in nitrogen gas, residual film was dried under reduced pressure overnight. The dried lipid film was hydrated in 3 mL of 5 mM TES buffer (pH 7.4) containing 100 mM NaCl for 30 min with repeated vapor and mixing. To obtain the small unilamellar vesicles (SUVs), the suspension was sonicated to become clear at 50 °C for 1 hr using a TOMY SEIKO ultrasonic disrupter model UR-200P and afterwards diluted to 25 mL with the same buffer, in which lipid concentration was 1.1 μM. The obtained SUVs were used for the CD measurement.

For leakage experiment, large unilamellar vesicles (LUVs) [EYPC or EYPC-EYPG (3:1) and DPPC or DPPC-DPPG (3:1)] with encapsulated calcein were prepared by the extrusion method.[26] The desired mixtures of phospholipids were dried in glass tubes first under nitrogen and then lyophilized overnight to obtain lipid films. The dry lipid films were suspended in leakage buffer (20 mM TES buffer containing 150 mM NaCl, pH 7.4) and 70 mM calcein and then vortexed occasionally to disperse the lipids. The suspension was frozen-thawed in liquid nitrogen for ten cycles and extruded 10 times through a 0.1 μm polycarbonate membrane filters in an Avanti mini extruder apparatus (Avanti Polar Lipids, Inc., Alabaster, AL). After extrusion, unencapsulated calcein was removed from the LUVs with encapsulated calcein by gel filtration on an 18-cm Sepharose 4B column equilibrated with leakage buffer. Fractions containing LUVs were used for calcein release measurements. Leakage measurements were done at 25 °C and 41 °C, respectively.

Circular Dichroism (CD) Measurement. The circular dichroism spectra were recorded on a JASCO J-600 instrument with a personal computer (NEC PC-9801) using a quartz cell of 1-mm path length. Spectra were measured at 50 μM and 1 mM of peptide and lipid [DPPC liposomes and DPPC-DPPG (3:1) liposomes] concentrations and 50% (v/v) trifluoroethanol (TFE) in 5 mM TES buffer (pH 7.4) containing 100 mM NaCl. Four scans per sample were performed over the wavelength range 190-230 nm at 25 °C. The CD data were expressed as mean molar residue ellipticity (μ) expressed in deg·dmol^{-1}. To minimize scattering due to

liposomes, each CD spectrum of liposomes was subtracted from that of the peptides measured in the presence of liposomes.

Leakage of Calcein from Liposomes. To determine the kinetics of membrane perturbation by apideacin Ib and if there was a preferential targeting of membranes with certain compositions, we measured the abilities of apidaecin Ib and piscidin 1, as a positive control, to induce leakage of a fluorescent dye (calcein) from acidic liposomes including EYPC/EYPG (3:1, w/w) (natural phospholipids that contain unsaturated fatty acid chains) or DPPC/DPPG (3:1, w/w) (synthetic phospholipids that contain saturated fatty acid chains) liposomes and from neutral liposomes including EYPC or DPPC liposomes. Leakage of calcein from liposomes was determined by the procedure of Park *et al.*[26] A liposome susupension (25 μL, final concentration is 95 μM) containing encapsulated calcein and 50 μL of an appropriately diluted peptide solution in TES buffer were added to 20 mM TES buffer (pH 7.4) to give a final volume of 1.0 mL. The increase in the fluorescence of calcein, when leaking out of liposomes, was monitored at an emission wavelength of 520 nm with an excitation wavelength at 490 nm. The change in fluorescence intensity was measured for 3 min after the addition of peptide. Complete release of calcein was achieved by adding 10 μL of 10% (v/v) Triton X-100. The percentage of dye release was calculated as follows:

$$\text{Dye-leakage (\%)} = 100 \times (F - F_0)/(F_t - F_0)$$

Where F is the fluorescence intensity caused by the peptide and F_0 and F_t are those with buffer alone and with 0.1% Triton X-100, respectively.

Results and Discussion

Antimicrobial and Hemloytic Activity of Apidaecin Ib. Three isoforms of apidaecins, apidaecin Ia, Ib, and II, that were first isolated from lymph of honeybees show very similar antibacterial activity, while their concentrations in immune bee lymph differ greatly.[7] Apidaecin Ib is a major form which has up to 80-90% of the total contcentation and this is the reason for selection of apidaecin Ib among apidaecin isoforms.[9] Apidaecin Ib is composed of 18 amino acid residues containing 6 Pro residues (30%) and 3 Arg residues (17%) (Fig. 1(a)). Based on the presence of 6 Pro residues, it could be expected that apidaecin Ib conforms an unordered structure and exhibits the specific antibacterial spectrum rather than the broad one, which is shown by antimicrobial peptides conforming specific secondary structures such as α-helix or β-structure.[27]

The antimicrobial activity of apidaecin Ib was determined by measuring minimal effective concentrations (MECs) against bacteria and one yeast, *C. albicans*, using an ultrasensitive radial diffusion assay (URDA). Apidaecin Ib displayed strong antimicrobial activity only against several tested Gram-negative bacteria including *E. coli* (not *E. coli* D31), *E. cloacae,* and *S. flexneri* (MECs: 0.3-1.5 μg/mL),

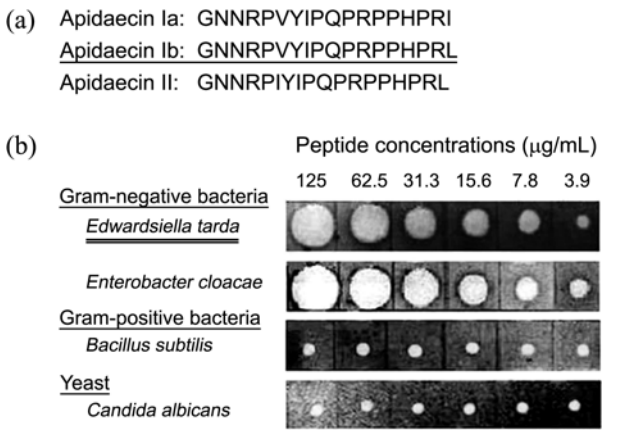

Figure 1. Amino acid sequences of apidaecin Ia, Ib, and II (a). Ultrasensitive radial diffusion assay of apidaecin Ib from 3.9 μg/mL to 125 μg/mL concentration against *E. tarda*, *E. cloacae*, *B. subtilis*, and one yeast, *C. albicans* (b).

but showed no activity against all tested Gram-positive bacteria including *B. subtilis*, *M. luteus*, *S. aureus*, and one yeast, *C. albicans* (MECs: > 125 μg/mL) (Fig. 1(b) and Table 1). Interestingly, among fish pathogenic bacteria such as *A. hydrophila*, *E. tarda*, *S. iniae*, and two *V. parahemolyticus*, apidaecin Ib showed potent antibacterial activity only against *E. tarda* (MEC: 3.2 μg/mL). However, piscidin 1, used as standard antimicribial peptide, showed broad spectrum antimicrobial activity against all tested bacteria and one yeast, *C. albicans*, but showed no significant activity only against *E. tarda* (MEC: > 125 μg/mL). The hemolytic activities of apidaecin Ib and piscidin 1 were tested using human and fish erythrocytes (Table 2). Although apidaecin Ib did not cause hemolysis of red blood cells (RBCs) up to

Table 1. Antimicrobial activities of apidaecin Ib and piscidin 1

Microbe	Gram	Minimal effective concentration (μg/mL)	
		Apidaecin Ib	Piscidin 1
B. subtilis PM125	+	> 125.0	4.5
B. subtilis KCTC1021	+	> 125.0	7.5
M. luteus ATCC9341	+	> 125.0	8.0
S. aureus RM4220	+	> 125.0	4.4
E. coli D31	-	> 125.0	3.8
E. coli KCTC1116	-	1.0	7.0
E. cloacae KCTC1685	-	0.3	6.0
S. flexneri KCTC2517	-	1.5	8.0
Fish pathogenic bacteria			
A. hydrophila KCTC2358	-	> 125.0	10.0
E. tarda H-4	-	3.2	> 125.0
S. iniae FP5229	+	> 125.0	6.5
V. parahemolyticus HUFP91	-	> 125.0	10.0
V. parahemolyticus KCCM41664	-	> 125.0	3.0
Yeast			
C. albicans KCTC7965		> 125.0	> 62.5

Table 2. Hemolytic activities of apidaecin Ib and piscidin 1 against human and fish erythrocytes

Concentration (μg/mL)	Hemolysis %			
	Apidaecin Ib		Piscidin 1	
	Human erythrocytes	Fish erythrocytes	Human erythrocytes	Fish erythrocytes
3.13	0.3	0.1	1.5	1.3
6.25	0.4	0.2	3.6	4.7
12.5	1.0	0.3	18.9	15.8
25	1.0	1.2	51.6	48.5
50	1.3	2.4	83.7	80.3
100	4.3	2.6	100	100

Table 3. Antibacterial activities of apidaecin Ib, gramicidin S, mastoparan B, piscidin 1, piscidin 4 and histone H1 against *E. tarda* H-4

Antimicrobial polypeptide	Minimal effective concentration (μg/mL)	Origin	Activity spectrum
Apidaecin Ib	0.2	Honeybee	Gram-(−)
Gramicidin S[a]	> 31.3	Bacterium	Gram-(±)
Mastoparan B[b]	> 125.0	Wasp	Gram-(±)
Piscidin 1[c]	>125.0	Fish	Gram-(±)
Piscidin 4[d]	> 125.0	Fish	Gram-(±)
Histone H1	> 62.5	Human	Gram-(±)

[a]Gramicidin S: cyclo-(Val-Orn-Leu-D-Phe-Pro)$_2$ [16]. [b]Mastoparan B: LKLKSIVSWAKKVL-NH_2 [17]. [c]Piscidin 1: FFHHIFRGIVHVGKTIHRLVTG [18]. [d]Piscidin 4: FFRHLFRGAKAIFRGARQGWRAHKVVSRYRNRDVPETDNNQEEP [19].

Table 4. Antibacterial activities of apidaecin Ib and piscidin 1 against *Edwardsiella* species

Microbe	Minimal effective concentration (μg/mL)	
	Apidaecin Ib	Piscidin 1
E. tarda H-4	3.2	> 125.0
E. tarda NUF251	3.6	> 125.0
E. tarda RE1	1.0	> 125.0
E. tarda RE2	0.6	> 125.0
E. tarda RED2	1.8	> 125.0
E. ictaluri KCTC12264	0.8	> 125.0

Table 5. Anti-*Edwardsiella* activities of apidaecin Ib and antibiotics against *E. tarda* H-4 and *E. ictaluri* KCTC12264

Therapeutic class	Antibiotic	Minimal effective concentration (μg/mL)	
		E. tarda H-4	*E. ictaluri* KCTC12264
Tetracyclines	Tetracycline	31.3	31.3
	Minocycline	> 125.0	31.3
Aminoglycosides	Gentamicin	5.0	1.3
	Streptomycin	17.0	2.8
	Kanamycin	6.0	1.4
β-lactams	Ampicillin	> 62.5	1.6
Other antibiotics	Rifampicin	> 125.0	125.0
Antimicrobial peptides	Apidaecin Ib	2.6	0.7
	Piscidin 1	> 125.0	> 125.0

100 μg/mL concentration, piscidin 1 caused considerable hemolysis even at 12.5 μg/mL. These results indicate that apidaecin Ib has *E. tarda* specific antibacterial activity without significant hemolytic activity.

Anti-*E. tarda* Activity of Antimicrobial Polypeptides (AMPPs). Up to date, little is known about the anti-*Edwardsiella* species activity of AMPPs. In this regard, to compare anti-*Edwardsiella* efficiency of apidaecin Ib with other AMPPs including gramicidin S, mastoparan B, piscidin 1, piscidin 4, and human histone H1, antibacterial activity was tested against *E. tarda* with an URDA (Table 3). Unlike apidaecin Ib (MEC; 0.2 μg/mL), all tested AMPPs did not show any significant activity (MECs; > 31.3 μg/mL) (Table 3). These results indicate that *E. tarda* is naturally resistant to some AMPPs but is espicially sensitive to apidaecin Ib.

Anti-*Edwardsiella* Species Activity of Apidaecin Ib. To know if apidaecin Ib have specific inhibitory property against other *Edwardsiella* strains or species, antibacterial activity was tested with 4 other isolates of *E. tarda* strains including *E. tarda* HUF251, *E. tarda* RE1, *E. tarda* RE2, and *E. tarda* RED2, and another *Edwardsiella* species, *E. ictaluri* KCTC12264 (Table 4). Apidaecin Ib exhibited potent antibacterial activity against all tested *Edwardsiella* species (MECs: 0.6-3.6 μg/mL), but piscidin 1 did not show any significant activity against all tested *Edwardsiella* species up to 125 μg/mL concentration. These results indicate that apidaecin Ib has *Edwardsiella* species-specific antibacterial activity.

Comparison of Anti-*Edwardsiella* Activity of Apidaecin Ib with Antibiotics. To compare anti-*Edwardsiella* activity of apidaecin Ib with some antibiotics including tetracycline, minocycline, gentamicin, kanamycin, streptomycin, ampicillin, and rifampicin, anti-*Edwardsiella* activity was tested with an URDA against *E. tarda* and *E. ictaluri*. In previous reports, all *Edwardsiella* species were naturally sensitive to several antibiotics such as tetracyclines, aminoglycosides, most β-lactam antibiotics, and quinolines, but resistant to macrolides, glycopeptides, rifampicin and fusidic acid.[12,28,29] Our results were also similar to these reports (Table 5). Apidaecin Ib and aminoglycosides including gentamicin, kanamycin, and streptomycin showed broad-spectrum potent activity against both strains (MECs; 0.7-17 μg/mL). The initial action site of apidaecin-type peptides have been known as the bacterial membrane through a non-poreforming mechanism involving stereospecificity and aminoglycosides have also been known to bind to LPS, components of bacterial outer membrane, and then disorganized it.[7,30] These reports indicate that their action sites are similar and act on the bacterial membrane. Tetracycline also showed potent activity against both bacteria (MECs; 31.3 μg/mL), but minocycline and ampicillin showed species-dependent activity: they have potent activity only against *E. ictaluri* (MECs; 31.3 or 1.6 μg/mL, respectively). Otherwise, rifampicin did not show

any significant activity against both strains (MEC; ≥ 125 μg/mL). Species-dependent activities might be largely attributed to the difference of outer membrane of *Edwardsiella* species and action mechanisms of antibiotics. The LPS pattern of *E. ictaluri* is different from that of *E. tarda*.[31]

Based upon our results, apidaecin Ib and aminoglycosides are the most effective antimicrobials for both *Edwardsiella* species. However, aminoglycoside antibiotics (e.g. gentamicin or streptomycin) induce ototoxicity and could be impaired neuromast function.[32] These results strongly suggest that apidaecin Ib is more suitable for treatment of *Edwardsiella* species infection in aquaculture than aminoglycosides and could replace antibiotics.

Salt Effect on the Anti-*Edwardsiella* Activity of Apidaecin Ib and Antibiotics. Although peptides exhibit significant *in vitro* activity against pathogenic bacteria, for many antimicrobial peptides, this activity appears to be lost under physiological salt and serum conditions (150 mM NaCl).[33] This indicates that mono or divalent ion could be worked as an inhibitor for antimicrobial activity of peptides. Some peptides show potent salt-insensitive antimicrobial activities (e.g. tachyplesins, polyphemusins, and piscidin 1) but some peptides show salt-sensitive antimicrobial activities (e.g. defensins, some cathelicidins, and magainins).[34-37] To determine the effect of salt on anti-*Edwardsiella* activity of apidaecin Ib and antibiotics, MECs were determined in the presence of several different NaCl concentrations (Tables 6 and 7). Although antibacterial activities of tetracycline, minocycline and ampicillin were not virtually affected by NaCl concentration, apidaecin Ib, gentamicin, kanamycin, and streptomycin were drastically inhibited in their activities from the presence of 171 mM NaCl (1.0%, w/v) or 256 mM NaCl (1.5%, w/v) against *E. tarda* or *E. ictaluri*. These results suggest that anti-*Edwardsiella* activity of apidaecin Ib is salt-sensitive and could be used under low salt condition such as fresh water (less than 1% NaCl concentration). Interestingly, *E. ictaluri* did not grow at 599 mM NaCl concentration (3.5%) equivalent to sea water indicating that

Table 6. Effect of NaCl on anti-*E. tarda* H-4 activities of apidaecin Ib and antibiotics

Antibiotic	Minimal effective concentration (μg/mL)				
	NaCl Concentration (mM)				
	86 (0.5%)	171 (1.0%)	256 (1.5%)	342 (2.0%)	599 (3.5%)
Tetracycline	31.3	31.3	31.3	31.3	31.3
Minocycline	> 125.0	> 125.0	> 125.0	> 125.0	> 125.0
Gentamicin	5.0	31.3	62.5	125.0	> 125.0
Streptomycin	17.0	125.0	> 125.0	> 125.0	> 125.0
Kanamycin	6.0	31.3	62.5	125.0	> 125.0
Ampicillin	> 62.5	> 62.5	> 62.5	> 125.0	> 125.0
Rifampicin	> 125.0	> 125.0	> 125.0	> 125.0	> 125.0
Apidaecin Ib	2.6	31.3	62.5	125.0	> 125.0
Piscidin 1	> 125.0	> 125.0	> 125.0	> 125.0	> 125.0

Table 7. Effect of NaCl on anti-*E. ictaluri* KCTC12264 activities of apidaecin Ib and antibiotics

Antibiotic	Minimal effective concentration (μg/mL)				
	NaCl Concentration (mM)				
	86 (0.5%)	171 (1.0%)	256 (1.5%)	342 (2.0%)	599 (3.5%)
Tetracycline	31.3	31.3	31.3	31.3	ND
Minocycline	31.3	31.3	31.3	31.3	ND
Gentamicin	1.3	3.3	16.0	125.0	ND
Streptomycin	2.8	31.3	62.5	> 125.0	ND
Kanamycin	1.4	6.0	31.3	125.0	ND
Ampicillin	1.6	1.7	2.5	3.8	ND
Rifampicin	125.0	> 125.0	> 125.0	> 125.0	ND
Apidaecin Ib	0.7	3.8	31.3	> 125.0	ND
Piscidin 1	> 125.0	> 125.0	> 125.0	> 125.0	ND

*ND: Not determined because *E. ictaluri* was not grow at this NaCl concentration

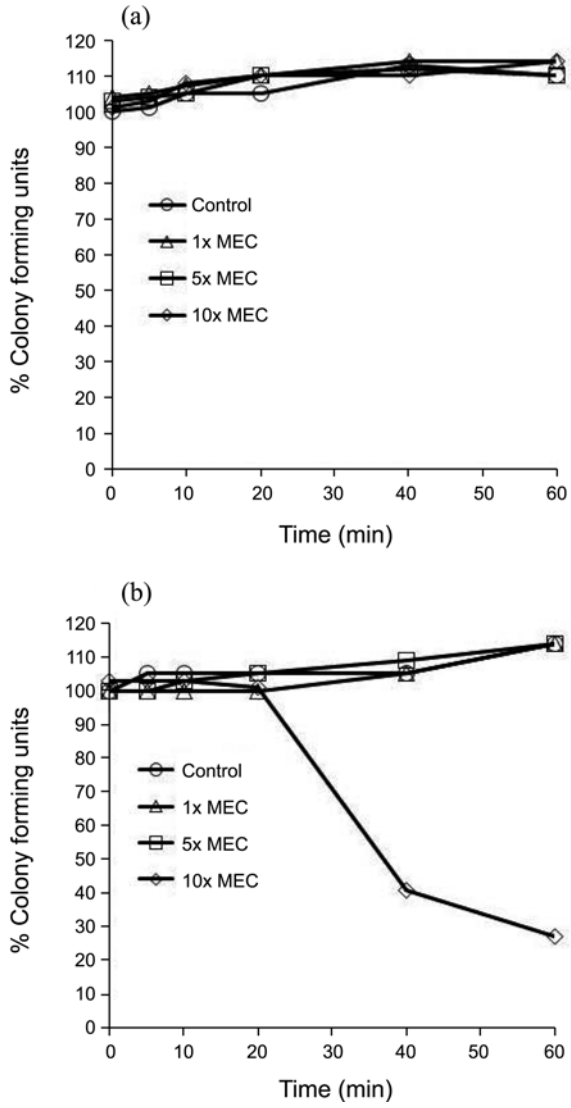

Figure 2. Bacterial killing kinetics of apidaecin Ib (a) and gentamicin (b) at different time intervals against *E. tarda* H-4: Control (without antimicrobial agents, ○), 1×MEC (△), 5×MEC (□), and 10×MEC (◇).

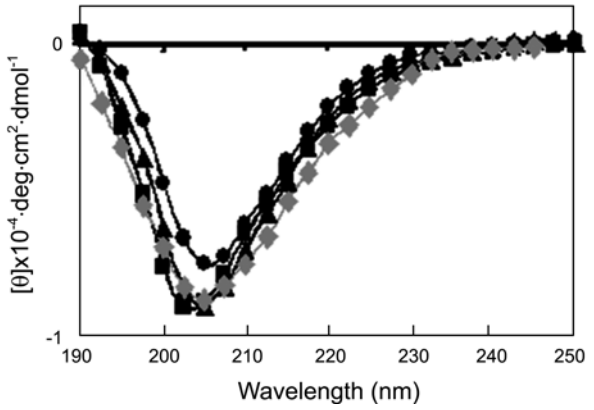

Figure 3. CD spectrum of apidaecin Ib in 10 mM TES buffer (■), DPPC (●), DPPC-DPPG (3:1) liposomes (▲), and 50% TFE (◆) at 25 °C. Peptide and lipid concentration were 50 μM and 1 mM, respectively.

its growth might be inhibited by high salt concentration. This result strongly supports the reason *E. ictaluri* is mostly affected to freshwater fish such as catfish. This result is similar to report by Waltman *et al.* (1986). Although *E. ictaluri* strains are tolerated at 1% (all the strains) and 1.5% (90% of the strains) NaCl, they did not grow in 2% or higher NaCl solutions.[38]

Killing Kinetic Studies of Apidaecin Ib and Gentamicin. To know the inhibition mode of apidaecin Ib and gentamicin, killing activities were tested against *E. tarda* H-4 at the MECs (2.6 μg/mL for apidaecin Ib and 5.0 μg/mL for gentamicin), 5 × MECs and 10 × MECs depending on the defined time intervals (Fig. 2). Apidaecin Ib did not kill *E. tarda* H-4 at all tested concentrations, but gentamicin showed killing activity only at the 10 × MECs from 20 min after it added. These results indicate that the inhibition mode of apidaecin Ib on *E. tarda* H-4 is a bacteriostatic process rather than bactericidial one, but gentamicin might be acted as a bacteriostatic or bactericidal agent depending on its concentration.

CD Spectra of Apidaecin Ib. To investigate whether the secondary structure of apidaecin Ib need or not for binding with membrane, the CD spectra was measured in TES buffer, DPPC, DPPC-DPPG (3:1) liposomes and in 50% TFE (Fig. 3). The overall band shapes of apidaecin Ib were unaffected by the tested environments and showed only a negative band in the vicinity of 205 nm, characteristic of an unordered conformation. This result indicated that apidaecin Ib did not conform specific secondary structure in membrane environments and it might need other factors such as sterospecificity for binding with membrane rather than specific secondary structure such as α-helix or β-sheet.[39] Based upon amino acid composition, apidaecin Ib contains 30% of Pro residues and it causes a bending of the peptide chain and inhibits the formation of the ordered secondary structures.

Leakage Ability of Apidaecin Ib. To know whether bacterial membranes could be preferentially targeted by apidaecin Ib, membrane perturbation was examined by measuring calcein released from both acidic liposomes including EYPC/EYPG (3:1) or DPPC/DPPG (3:1) and neutral liposomes including EYPC or DPPC (Fig. 4). Piscidin 1 showed strong lytic activity on both liposomes, but apidaecin Ib exhibited very weak leakage activity on both liposomes. These results suggest that apidaecin Ib do not perturbe the membrane bilayer and its action mechanism is quitely different from that of other lytic linear α-helical peptides such as piscidin 1. This is in agreement with the results obtained from the killing kinetic studies. Lacking the leakage ability might be due to the fact that proline-rich apidaecin Ib is unable to adopt specific secondary structures such as an α-helix or an amphipathic arrangement for preferrable membrane permeabilization.[40]

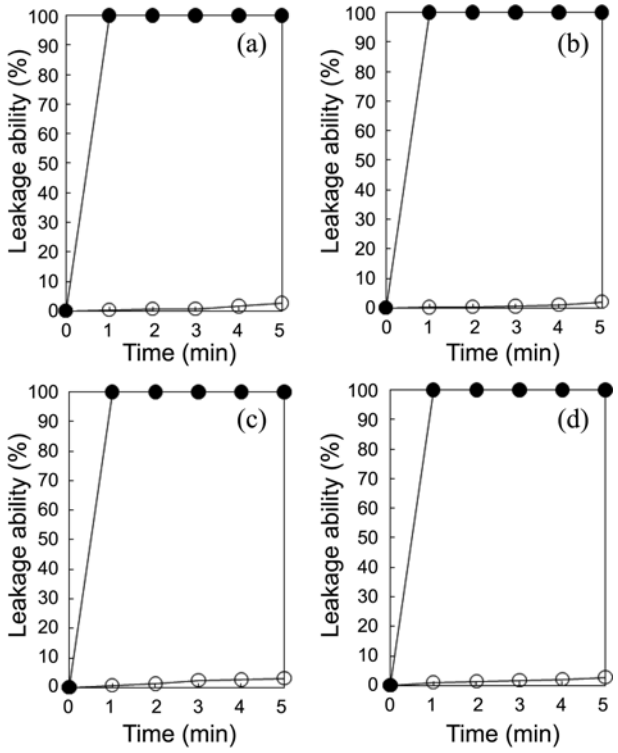

Figure 4. Release of encapsulated calcein from neutral liposomes including DPPC (a) or EYPC (b) and acidic liposomes including DPPC/DPPG (3:1) (c) or EYPC/EYPG (3:1) (d), measured for 3 min after the addition of 25 μM peptides: apidaecin Ib (○) and piscidin 1 (●).

Conclusion

In conclusion, the present study demonstrate that apidaecin Ib has *Edwardsiella* species-specific antibacterial activity through a bacteriostatic process without significant hemolytic activity, but its activity is salt-sensitive. These results suggest that apidaecin Ib promises a possible application as a new preventive alternative to conventional antibiotics in the treatment of *Edwardsiella* species infections in freshwater fish aquaculture.

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