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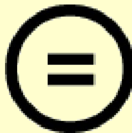

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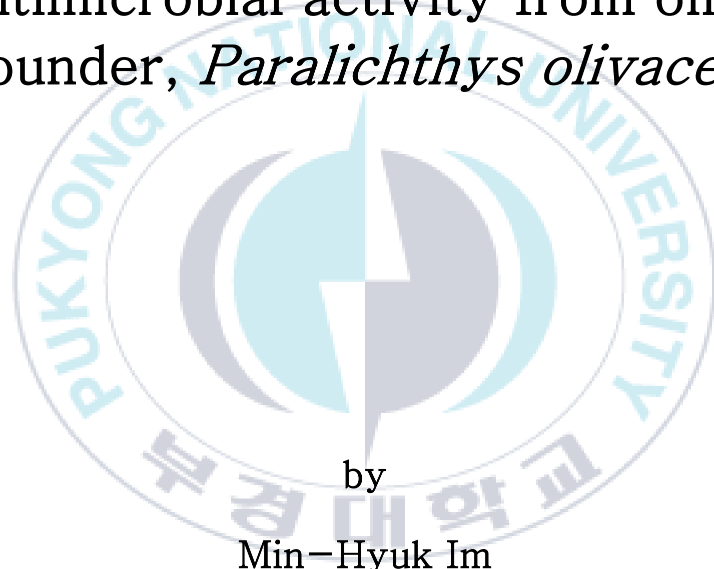
Thesis for the Degree of Master of Fisheries Science

Characterization of recombinant Liver–Expressed Antimicrobial Peptide–2 (LEAP–2) and its antimicrobial activity from olive flounder, *Paralichthys olivaceus*



by

Min–Hyuk Im

Department of Fisheries Biology
The Graduate School
Pukyong National University

February 2024

Characterization of recombinant Liver-Expressed Antimicrobial Peptide-2 (LEAP-2) and its antimicrobial activity from olive flounder, *Paralichthys olivaceus*

넙치(*Paralichthys olivaceus*) 유래 재조합 간 발현 항균 펩타이드-2의 항균활성 분석

Advisor: Prof. Jong-Myoung Kim

by Min-Hyuk Im

A thesis submitted in partial fulfillment of the requirements
for the degree of

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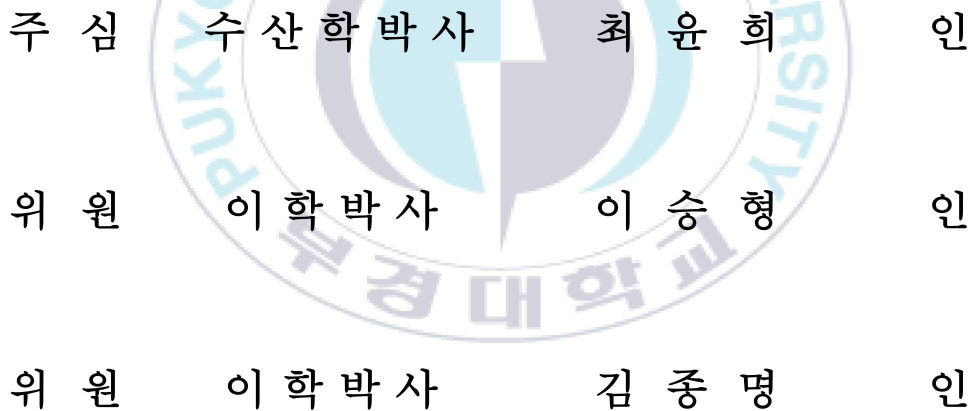

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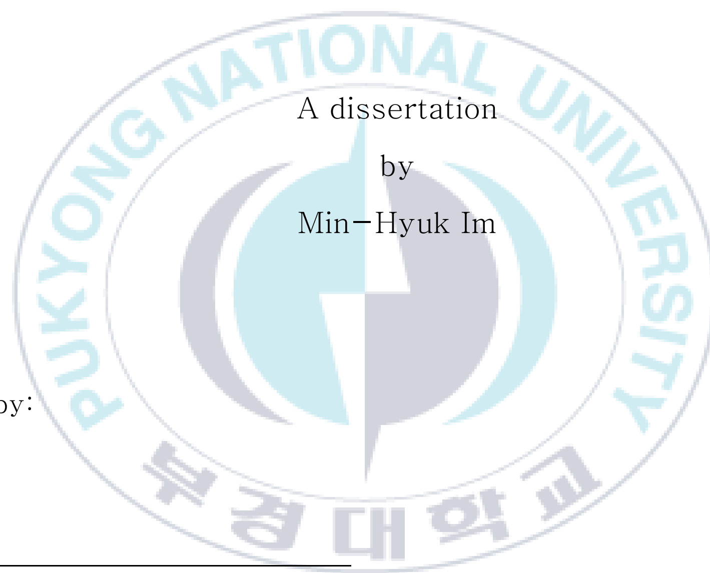
Characterization of recombinant Liver−Expressed Antimicrobial Peptide−2 (LEAP−2) and its antimicrobial activity from olive flounder, *Paralichthys olivaceus*



A dissertation

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February 2024

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Characterization of recombinant Liver−Expressed Antimicrobial Peptide−2 (LEAP−2) and its antimicrobial activity from olive flounder, *Paralichthys olivaceus*

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Abstract

Teleost belonging to a subclass of vertebrate families have an acquired immune system comparable to higher mammals in addition to innate immune system. Liver−expressed antimicrobial peptide−2 (LEAP−2) is a cysteine−rich peptide known to play an important role in the innate immune system of fish. In this study, RT−PCR was carried out to obtain the gene encoding LEAP−2 of olive flounder, *Paralichthys olivaceus*, and to analyze its tissue−specific expression pattern. The gene region encoding the except for signal peptide of LEAP−2 was amplified from cDNA synthesized using RNA extracted from liver of olive flounder and was cloned into the expression vector pET−44a$^{(+)}$ with his_6−tag at the C−terminus. The rOfLEAP−2 was expressed in *Escherichia coli* BL21 (DE3 codon plus) upon induction with IPTG. The rOfLEAP−2 was purified (from *E. coli*) by using nickel column and

were subject to various antimicrobial activity analysis against Gram-positive (*Bacillus subtilis, Lactococcus garvieae, Streptococcus parauberis*) and Gram-negative bacteria (*E. coli, Vibrio harveyi, Edwardsiella tarda*). The sequence of the gene encoding LEAP-2 from olive flounder was identified to has an open reading frame of 309 bp, which encodes a protein consisting of 102 amino acids. In tissue-specific expression analysis, LEAP-2 gene showed predominant expression in liver and lower expression in intestine. The purified rOfLEAP-2 showed distinguishable antimicrobial activity against all bacteria except *E. tarda,* tested. Upon testing in combination with ampicillin and LEAP-1, the result showed a synergistic effect against *B. subtilis* and *V. harveyi* as verified by the fractional inhibitory concentration index (FICI). The result from outer membrane permeability test performed using N-phenylnaphthalen-1-amine (NPN) showed a distinguishable outer membrane permeability by rOfLEAP-2. The result was also confirmed by scanning electron microscope assay (SEM) showing a disrupted surface morphology of *E. coli* upon incubation with rOfLEAP-2.

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Characterization of recombinant Liver−Expressed Antimicrobial Peptide−2 (LEAP−2) and its antimicrobial activity from olive flounder, *Paralichthys olivaceus*

Ⅰ. Introduction

Teleost such as olive flounder belonging to a subclass of vertebrate families have an acquired immune system comparable to higher mammals in addition to innate immune system. In addition, unlike land, teleost live in an environment that is always exposed to aquatic pathogenic bacteria, so innate immunity is very important for survival and growth of fish (Bergljot., 2006). Antimicrobial peptide (AMP) is known as one of the innate immune systems in living organisms. AMPs are ancient but powerful host innate defense molecules with cationic and amphipathic properties that are found in all forms of life including fish (Seo et al., 2012). In fish, AMPs can be classified into five groups: Hepcidin (also known as LEAP−1), Liver−expressed antimicrobial peptide−2 (LEAP−2), cathelicidin, defensins and piscidins. Among them, LEAPs have been found and studied in other animals including many fish species. Two forms of LEAP were isolated from human blood,

named LEAP-1 and LEAP-2 (Krause et al., 2000, Krause et al., 2003). LEAP-1 contains eight conserved cysteine residues that can form stable β-sheets, while LEAP-2 contains four highly conserved cysteine residues forming two disulfide bond. LEAP-2 sequences have been first cloned and reported in rainbow trout (Zhang et al., 2004). Some studies have demonstrated that LEAP-2 has selective antimicrobial activity against bacteria and fungi (Hocquellet et al., 2010, Li et al., 2014, Liu et al., 2010). The LEAP-2 has been studied in many species, including pigs, cattle, chickens, ducks, and fish (Bao et al., 2006, Bo et al., 2019, Hong et al., 2019, Yang et al., 2014, Zhang et al., 2018, Zhang et al., 2004). However, the antimicrobial activity and mechanism of olive flounder LEAP-2 are not clearly known.

Olive flounder (*Paralichthys olivaceus*) is an economically important aquaculture species with high values in Korea. However, as the water temperature has increased due to the effects of global warming, the risk of pathogenic bacteria is also increasing (Susan et al., 2023). Although the liver was an organ that received little attention in relation to the antimicrobial function of fish, recent, additional discoveries of liver-specific antimicrobial genes have begun to attract attention in relation to the use of antibiotics (Lee et al., 2014). Therefore, in this study recombinant LEAP-2 of olive flounder was analyzed to confirm

antimicrobial activity and mechanism against Gram−positive & Gram − negative bacteria in vitro. Also, synergistic effect with ampicillin (and LEAP−1 of olive flounder) was tested to evaluate its availability as a biomedicine.

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Ⅱ. Materials and Methods

1. RT–PCR & Bioinformatics analysis

1–1. RNA extraction

RiboEX^{TM} was used to extract total RNA. 100 mg of liver tissue was homogenized in 1 ml RiboEX^{TM} reagent. Homogenate was mixed with 200 μL of chloroform and incubated in ice for 10 min, followed by. Centrifugation at 12000 x *g*, 4℃, for 10 min. 400 μL of supernatant solution was separated and mixed with 400 μL of isopropanol followed by incubation in ice for 10 min. After incubation, the mixture was centrifuged at 12000 x *g*, 4℃, for 10 min. Then pellet was washed with 70% DEPC – treated ethanol and centrifuged at 7500 x *g*, 4℃, for 3 min Ethanol was removed and pellet was dried at room temperature for 10 min. Dried pellet was resuspended of 120 μL DEPC–treated water.

The quality and quantity of extracted total RNA were measured immediately using ASP–2680 spectrophotometer (ACTGen) at 260/280 nm absorbance.

1-2. cDNA Synthesis

To synthesize cDNA from total RNA M-MLV cDNA synthesis kit (Enzynomics, Korea) was used, followed by PCR reaction. PCR sample was prepared 20 μL volume containing cDNA template, specific primer (F/R), 2X TOP simple™ DyeMIX-Tenuto (Enzynomics, Korea). Specific primer was designed as a region corresponding to the mature peptide of the LEAP-2 gene. (Table 1). PCR was carried out initial denaturation at 95℃ for 5 min, with 30 cycle of denaturation at 95℃ for 30 sec, annealing for 50 sec at 55℃, and extension at 72℃ for 1 min. Then final extension was carried out at 72℃ for 5 min.

1-3. Tissue expression pattern analysis (LEAPs)

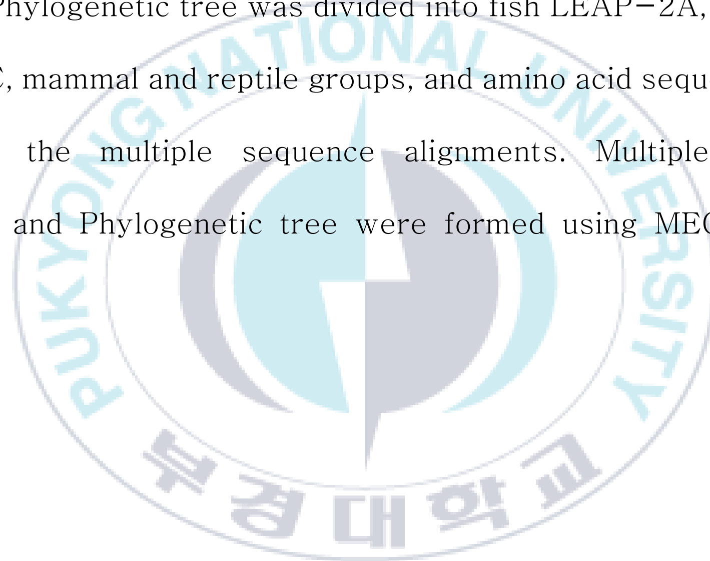
Tissues (eye, gill, liver, heart, kidney, stomach, intestine, spleen, skin, muscle, blood, gonad) were obtained from adult olive flounder which had weight of 70.9 g, length of 19.8 cm, height of 7.1 cm. The LEAP-1 and LEAP-2 genes were amplified by PCR using designed specific primer from NCBI (XM_020092771.1, XM_020105005.1). Beta actin gene of olive flounder was used as reference gene for quantification of cDNA. The RT-PCR condition was set as follows; initial denaturation at 95℃ for 5 min with 25 cycles of denaturation at 95℃ for 30 s, annealing at 55℃ for 50 s, and extension at 72℃ for 1 min. Then final extension was carried out at 72℃ for 5 min. The PCR product was identified 1.5% agarose gel electrophoresis followed by staining with ethidium bromide.

1-4. Bioinformatic analysis of olive flounder LEAP-2

The amino acid sequences of olive flounder LEAP-2 (OfLEAP-2) were aligned with those of LEAP-2 peptide from other organisms searched from NCBI GenBank database. The cleavage sites for the OfLEAP-2 signal peptide was predicted by signalP-6.0 program (https://services.healthtech.dtu.dk/services/SignalP-6.0/) and

OfLEAP−2 domains were analyzed by Expasy Prosite (https://prosite.expasy.org/).

Theoretical molecular weight of mature peptide region and isoelectric point (pI) was predicted using Expasy ProtParam tool (https://web.expasy.org/protparam/). The predicted protein 3D structure was analyzed by UniPro (https://www.uniprot.org/peptide-search). Phylogenetic tree was divided into fish LEAP−2A, LEAP−2B, LEAP−2C, mammal and reptile groups, and amino acid sequences were added to the multiple sequence alignments. Multiple sequence alignment and Phylogenetic tree were formed using MEGA−X 11.0 program.



Purpose	Target	Sequence (5'-3')	Product size(bp)
RT-PCR of LEAP-1	OF-LEAP-1	F: ATG AAG CGA TTC AGC ATT GCA G	267
		R: TCA GAA CTT GCA GCA GGG G	
RT-PCR & Sequence checking of LEAP-2	OF-LEAP-2	F: ATG CAG CAG AGA AGA AGT TTG G	306
		R: TCA GTA CTT CAC AGG CTC GG	
RT-PCR (Housekeeping gene)	OF- β -actin	F: GGA ATC CAC GAG ACC ACC TAC A	264
		R: CTG CTT GCT GAT CCA CAT CTG C	
Cloning into pET44a (+) vector	rOF-LEAP-2	F: A TAT CAT ATG GGT CCG CTG GCC	219
		R: T ATA CTC GAG GTC CTT CAC AGG	

Table 1. The list of primer sequence used for amplication in this study.

2. Production of recombinant OfLEAP−2−like protein

2−1. TA cloning

To prepare gene insert (for OfLEAP−2 expression), PCR reaction was carried out using a specific primer added with *Nde I*, *Xho I* sequence. PCR condition was performed as follows; initial denaturation at 95℃ for 5 min, with 30 cycles of denaturation at 95℃ for 30 sec, annealing at 55℃ for 50 sec, and extension at 72℃ for 1 min. Then final extension was carried out at 72℃ for 5 min. (The PCR product was electrophoresed in 1.5% agarose gel with ethidium bromide, followed by−add) purification using DNA gel extraction kit (Bionics, Korea). The PCR product was ligated cloned into TA vector (pTOP TA V2, Enzynomics, Korea). Transformation procedure was immediately performed using 100 μl *E. coli* (DH5α) as follows condition; heat−shock at 42℃ for 45 sec, then put in ice for 3 min, and incubation by adding 600 μl LB medium at 37℃ for 1 hr. Mixture was plated in agar LB medium including ampicillin (100 ug/ml) and incubation for overnight at 37℃.

2-2. Induction & Expression of rOfLEAP-2

Recombinant OfLEAP-2 was subcloned into pET-44a$^{(+)}$ for expression in *E. coli*. Vector and insert were respectively digested by restriction enzyme (*Nde I* / *Xho I*) in 1 X EZ buffer IV at 37℃ for 2 hr, followed by ligation using T4 DNA ligase (Enzynomics, Korea) at room temperature for 1 hr. Then recombinant pET-44a$^{(+)}$ vector was transformed into *E.coli* BL21 (DE3-CodonPlus-RP, Agilent Technologies). Transformant was cultured at 37℃ with shaking 200 rpm in LB (Luria - Bertani) broth to which ampicillin (f.c: 100 ug/ml) was added. Pre-cultured cells were diluted 1:100 in fresh LB broth containing ampicillin (100 ug/ml) and cultured at 37℃ with shaking 200 rpm until the cell density reached to about 0.6 of OD_{600}. The rROfLEAP-2 was induced by using 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37℃ with shaking 160 rpm for 3 hr. Cells were harvested with centrifugation at 6000 x *g*, 4℃ for 30 min. SDS-PAGE was carried out to identify rOfLEAP-2 expression followed by, staining with coomassie brilliant blue G-250. Western blotting analysis was conducted to detect the rOfLEAP-2 using anti-His_6 tag monoclonal antibody and anti-mouse IgG-HRP (Invitrogen). Visualization for detection of rOfLEAP-2 was performed by using ImageQuant LAS 500 (GE Healthcare, Buckinghamshire, UK).

2-3. Purification of rOfLEAP-2

Bacterial cells induced with IPTG were precipitated by using centrifugation at 6000 x *g*, 4℃ for 30 min. The pellet was resuspended by using 1 X LEW (lysis - equilibration - wash buffer, 50 mM NaH2PO4, 300 mM NaCl, pH 8.0) followed by repeated cycles of freeze - thawing. Lysates were sonicated on ice (5 min, 20% amplitude for 5 sec) and added with DTT (f.c: 2 mM) and $CaCl_2$ (f.c: 10 mM) followed by incubation for 3 min in ice. Upon centrifugation of the lysates at 10000 x *g*, 4℃ for 30 min, the supernatant (soluble fraction) was loaded on nickel column (Protino® Ni-TED) equilibrated with 1 X LEW. The column resin was washed with 1X LEW for three times and then rOfLEAP-2 was eluted by 1 X LEW containing 20 mM imidazole.

3. Antimicrobial susceptibility test

3-1. Ultrasensitive radial diffusion assay (URDA)

The URDA method (Lehrer et al., 1991) was used to measure antimicrobial activity of rOfLEAP-2. Gram-positive (*B. subtilis*, *L. garvieae*, *S. parauberis*) and Gram-negative (*E. coli*, *V. harveyi*) bacteria strains were cultured in TSB at 30℃ or 37℃ with shaking for overnight. Bacteria cells were diluted with 20 mM phosphate buffer containing 0.03% TSB for optimal OD_{600} which was measured by using a spectrophotometer. Bacterial resuspension 400 ul was added to 7.1 ml of underlayer gel (20 mM phosphate buffer, 0.03% TSB, 1% agarose). Mixture was plated to gridded petri dish and then hardened for 10 min at room temperature. Testing samples 10 ul were loaded for each well of 2.4 mm diameter premade in the plate followed by incubation for 3 hr at room temperature. Total 7.5 ml of overlayer gel (20 mM phosphate buffer, 6% TSB, 1% agarose) was overlaid and incubated for 7 hr at 30℃ or 37℃. Antimicrobial activity of samples was calculated as diameter size of clear zone, which represent the area bacteria did now grow. Ampicillin was used as positive control to compare with antimicrobial activity of rOfLEAP-2, and 1 X LEW (pH 8.0) and sterile water were used as negative control.

3-2. Colony counting method

In the previous study, LEAP-1 antimicrobial activity was tested by poue plating method (Kim et al., 2022). To identify antimicrobial activity of rOfLEAP-2 compared to that of LEAP-1, antimicrobial activity of rOfLEAP-2 was evaluated using a similar method to that of the previous study. *V. harveyi and S. parauberis* were cultured for 18 hr and then adjusted to 10^4 CFU/ml using spectrophotometer, followed by serial dilution in TSB medium containing 1% NaCl. Serial dilution of purified rOfLEAP-2 (21.6 uM, 10.8 uM, 5.4 uM, and 2.7 uM) mixed with 100 ul of bacterial suspensions was incubated for 3 h at 28℃. After incubation, the 100 ul of mixture was plated in TSA medium containing 1% NaCl and incubated for 24 hr at 30℃. Then viable colony was calculated and represented to antimicrobial activity (%).

3-3. Synergistic effect test (with ampicillin and LEAP-1)

To investigate synergy effect of rOfLEAP-2 combined with ampicillin or LEAP-1 derived from olive flounder, each material was serially diluted from MIC (Minimum inhibitory concentration) to 1/8 MIC. Gram positive and Gram-negative bacteria including fish pathogenic bacteria were used in this study. The MIC of rOfLEAP-2 combined with olive flounder LEAP-1 or ampicillin was measured by URDA method. Diluted rOfLEAP-2 (MIC to 1/8 MIC), LEAP-1 and ampicillin (MIC to 1/8 MIC) was mixed by concentration. After primary culture for 3 hr at room temperature, second incubation was carried out for 7 hr at 30℃ or 37℃. After checking the clear zone, antibiotic synergistic effect was calculated formula of by fractional inhibitory concentration index (FICI). FICI $\leq$ 0.5 was interpreted as synergy, 0.5 $<$ FICI $<$ 4.0 addictive or indifferent, FICI $>$ 4.0 as antagonism.

$$FICI = FIC_A + FIC_B = A / MIC_A + B/MIC_B$$

A = MIC of drug A in combination MIC_A = MIC of drug A alone

B = MIC of drug B in combination MIC_B = MIC of drug B alone

3-4. Outer membrane permeability test (NPN uptake)

Outer membrane permeability was measured by NPN (N-phenylnaphthalen-1-amine) uptake assay. *E. coil* (ML35) was overnight cultured at 37℃ in TSB. Cell was adjusted to OD_{600} ≒ 0.5 by using spectrophotometer followed by centrifugation at 10000 rpm, for 5 min. Upon washing the pellet with 5 mM HEPES buffer (5 mM KCN, 5 mM Glucose, pH 7.2) followed by centrifugation, cell was adjusted OD_{600} ≒ 0.5. NPN (0.5 mM) was added to 3 ml bacteria and reacted for 3 min, upon addition of 30 ul LEAP-2 and Pisicidin (100 ug/ml). Fluorescence intensity was independently measured after reaction for 1 minute and 3 minutes by using spectrofluorometer (HORIBA/Fluorolog-QM) at 350/420 nm wavelength.

3-5. Scanning Electron Microscope Assay (SEM)

Scanning electron microscopic analysis was carried out to determine whether antimicrobial activity of olive flounder LEAP-2 is due to impairment of bacterial surface morphology. For this, *E. coli* (ML35) was cultured in TSB medium to 0.6 of OD_{600}, to adjust the cell density to 10^6 CFU/ml. Total 150 ul of LEAP-2 peptide (200 ug/ml) was added in 3 ml bacterial suspension and incubated in 37 °C for 30 min. The cells were centrifuged at 1000 x *g* for 10 min. All centrifuge was carried out followed condition; 1000 x *g* for 10 min. For prefixation, 3 ml of 2% glutaraldehyde was used and reacted for 3 hours in ice before centrifugation. Then 0.1 M phosphate buffer (1M Na_2HPO_4, 1M NaH_2PO_4, pH 7.4) was used to wash. For post-fixation, 3 ml of 1% osmium tetroxide was added and incubated for overnight, 4 °C. After centrifuge and wash, 1 ml of acetone (10%, 30%, 50%, 70%, 90%, 100% concentration) was added and reacted for 10 min in room temperature for dehydration. Centrifugation was performed whenever acetone concentration was changed. Then, 1 ml of isoamyl acetate and freeze dryer was used to eliminate moisture completely. The *E. coli* cells were fixed for SEM assay following standard operating conditions. The surface morphology was observed with a JEOL/JEM-F200 SEM (USA)

following the manufacturer's instruction.

Ⅲ. Results

1. Tissue expression pattern & Bioinformatics analysis

Sequence encoding OfLEAP-2 consists of 309 bp encoding 102 amino acids including, a signal peptide (1-29 region), prodomain (30-57 region) and mature peptide (58-102 region). The RT-PCR results of LEAP-1 & LEAP-2 showed different expression pattern. The LEAP-1 transcript showed a relatively high level in the liver, heart, kidney, spleen and blood, but a lower level in eye, intestine, and skin. In contrast, a predominant expression of LEAP-2 was detected from liver and low a level in intestine but not from in other tissues (Figure 1). Predicted molecular weight and isoelectric points (pI) was 8,059 Da and 8.87 respectively. The multiple sequence alignment showed that four conserved cysteine and the N-terminal of mature peptides are composed of six highly conserved amino acids, "MTPLWR" in fish LEAP-2A and LEAP-2B group and "RSLLWR" in fish LEAP-2C, respectively. In the mature peptide region, there are conserved amino

acid sequence “RXGH” in fish LEAP−2A and LEAP−2B group (Figure 2). Phylogenetic tree revealed that olive flounder LEAP−2 belonging to LEAP−2A group (Figure 3). For OfLEAP−2 expression excluding signal peptide, DNA corresponding to cDNA of OfLEAP−2 was amplified by PCR. The Also PCR product was cloned into pET−44a$^{(+)}$ vector by using *Nde I* & *Xho I* restriction enzyme site (figure 4). The cloned recombinant olive flounder LEAP−2 sequence confirmed by using T7 promoter (Figure 5). The predicted protein 3D structure was analyzed by UniPro (https://www.uniprot.org/peptide−search) (Figure 6).

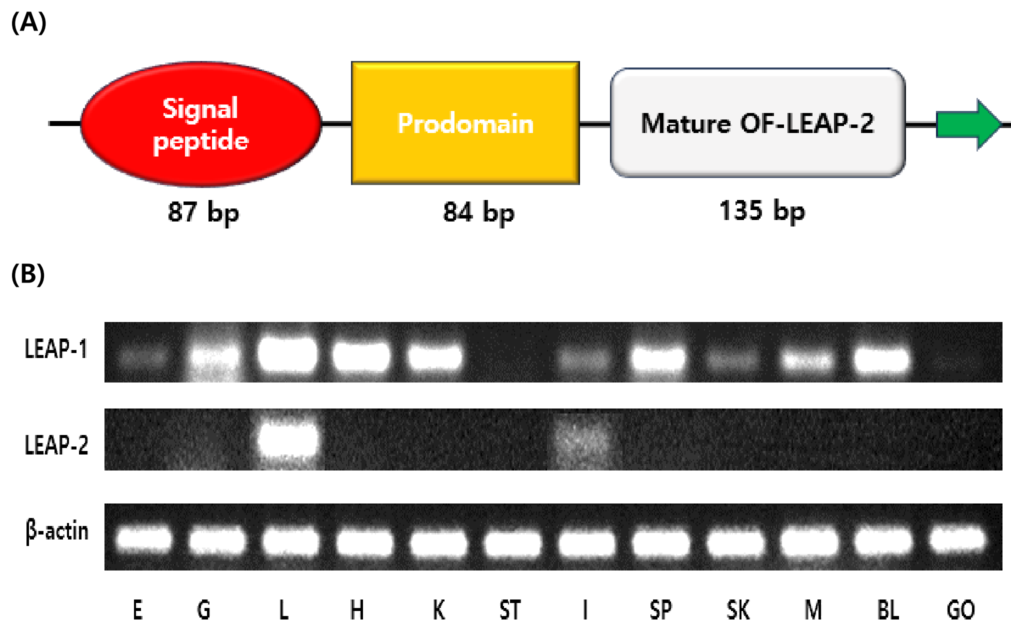


Fig. 1. (A) A schematic diagram of OfLEAP−2. (B) Tissue expression pattern of LEAP−1 and LEPA−2. E: Eye, G: Gill, L: Liver, H: Heart, K: Kidney, ST: Stomach, I: Intestine, SP: Spleen, SK: Skin, M: Muscle, BL: Blood, GO: Gonad.

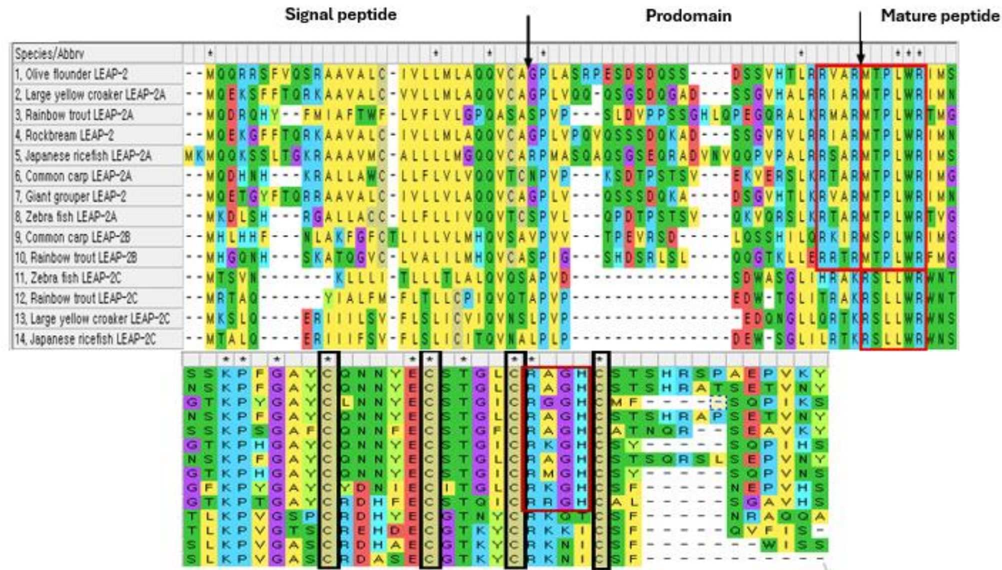


Fig. 2. Multiple alignment of the amino acid sequences of olive flounder LEAP−2 and its homologues. The predicted cleavage site for the signal peptide or mature peptide is represented with '↓'. The red box region indicates "RXXR", "MTPLWR", "RSLLWR", and "RXGH" motifs, respectively. Four conserved cysteine residues in the mature peptide are indicated by black square region. Accession numbers of sequences were included as ; *Paralichthys olivaceus* LEAP−2 (Olive flounder, ACB97648.1), *Larimichthys crocea* LEAP−2A (Large yellow croaker, AHY01375.1), LEAP−2C(AHY01377.1), *Oncorhynchus mykiss* LEAP−2A (Rainbow trout, NP_001117936.1), LEAP−2B (NP_001117937.1), LEAP−2C (ADN34603.1), *Oplegnathus fasciatus* LEAP−2 (Rock bream, AWO67306.1), *Oryzias latipes* LEAP−2A (Japanese rice fish, XP_004080006.1), *Cyprinus carpio* LEAP−2A (Common carp, AGK89728.1), LEAP−2B (AGK89729.1), *Epinephelus lanceolatus* LEPA−2A (Giant grouper, XP_033507724.1), *Danio rerio* LEAP−2A (Zebra fish, AAI62807.1) and LEAP−2C (NP_001373333.1.

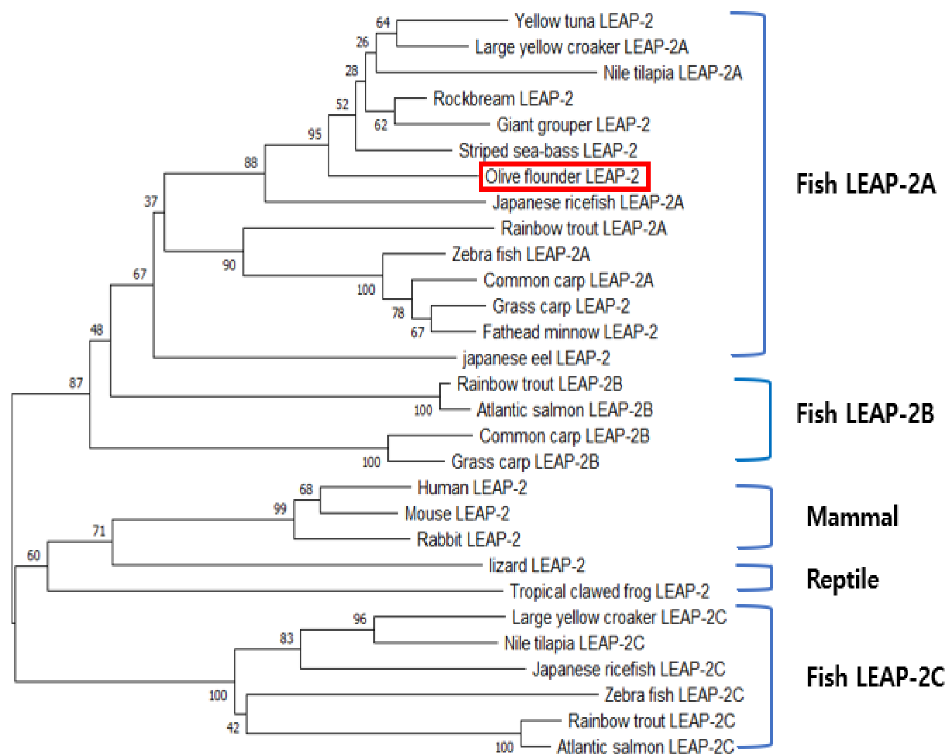
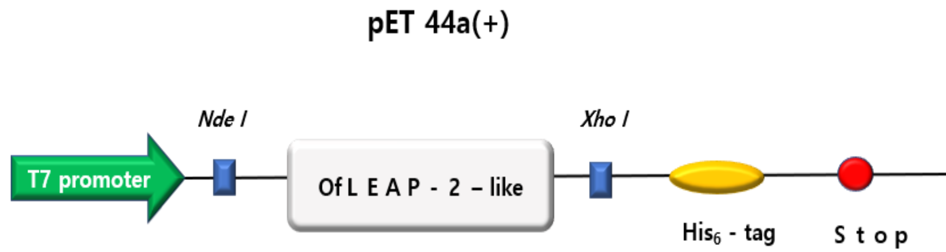


Fig. 3. Phylogenetic tree showing the evolutionary relationship of olive flounder LEAP−2 with other known LEAP−2s. The tree was made using the neighbour−joining method in MEGA11 program. Bootstrap values are indicated for each node (1000 replicates) and Taxonomic groups are indicated on the right. Accession numbers of sequences used under the sequence; *Paralichthys olivaceus* LEAP−2 (Olive flounder, ACB97648.1), *Larimichthys crocea* LEAP−2A (Large yellow croaker, AHY01375.1), LEAP−2C(AHY01377.1), *Oncorhynchus mykiss* LEAP−2A (Rainbow trout, NP_001117936.1), LEAP−2B (NP_001117937.1), LEAP−2C (ADN34603.1), *Oplegnathus fasciatus* LEAP−2 (Rock bream, AWO67306.1), *Oryzias latipes* LEAP−2A (Japanese rice fish, XP_004080006.1), *Cyprinus carpio* LEAP−2A (Common carp, AGK89728.1), LEAP−2B (AGK89729.1), *Epinephelus lanceolatus* LEPA−2A (Giant grouper, XP_033507724.1), *Danio rerio* LEAP−2A (Zebra fish, AAI62807.1) and LEAP−2C (NP_001373333.1), *Thunnus albacares* LEAP−2 (Yellowfin tuna, XP_044198358.1), *Oreochromis niloticus* LEAP−2A (Nile tilapia, XP_003457771.1), LEAP−2C(XP_013126243.1) , *Morone saxatilis* LEAP−2 (Striped sea−bass, XP_035531492.1), *Ctenopharyngodon*

Idella LEAP−2A (Grass carp, ACR54300.1), LEAP−2B (AGK89729.1) *Pimephales promeals* LEAP−2 (Fathead minnow, XP_039524703.1), *Anguilla japonica* LEAP−2 (Japanese eel, ALB07167.1), *Salmo salar* LEAP−2A (Atlantic salmon, XP_013994411.1), LEAP−2B (XP_013985003.1) , LEPA−2C (ADN34604.1), *Homo sapiens* LEAP−2 (Human, AAH70199.1), *Mus musculus* LEAP−2 (Mouse, NP_694709.1), *Oryctolagus cuniculus* LEAP−2 (Rabbit, ACK28141.1), *Zootoca vivipara* LEAP−2 (Lizard, XP_034961285.1) and *Xenopus tropicalis* LEAP−2 (Tropical clawed frog, NP_001106385.1).

(A)



(B)

(C)

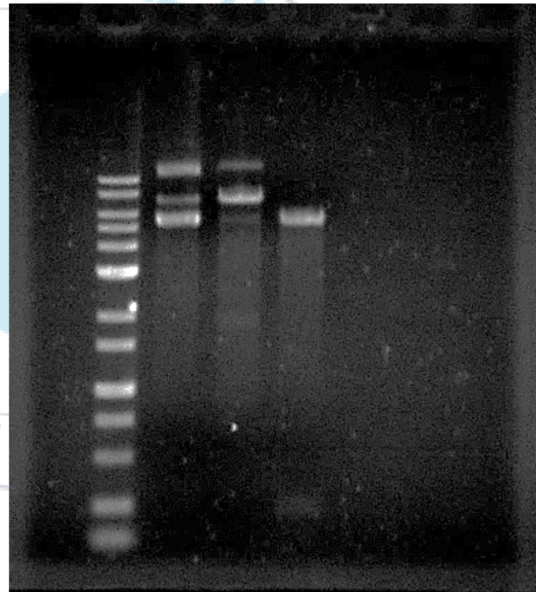


Fig. 4. (A) A schematic illustration the plasmid vector used for expression of recombinant OfLEAP−2. (B) PCR product of OfLEAP−2 cDNA. (C) pET−44a(+) vector (Lane 2) was digested with *Nde I* (Lane 3) and with *Nde I* and *Xho I* (Lane 4)

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ttcttctgaatattttgtttactttaagaaggagatatacatatg ggt ccg ctg gcc tct
                                          M   G   P   L   A   S
cgg ctg gag tcc gac tcc gac cag agt tca gac tca agc gtc cac acg ctg agg agg gtc
 R   L   E   S   D   S   D   Q   S   S   D   S   S   V   H   T   L   R   R   V
gct cgg atg acc ccg ctg tgg aga atc atg agc agt aaa cca ttc gga gcg tac tgc caa
 A   R  [M   T   P   L   W   R   I   M   S   S   K   P   F   G   A   Y   C   Q]
aac aac tac gag tgc tcc aca gga ctc tgc agg gcg ggt cac tgc tcc acc agc cat cgt
[N   N   Y   E   C   S   T   G   L   C   R   A   G   H   C   S   T   S   H   R]
tcc ccc gcc gag cct gtg aag tac ctc gag cac cac cac cac cac cac taa tgt taa tta
[S   P   A   E   P   V   K   Y   L   E] [H   H   H   H   H   H]  -
agttgggcgttcctaggctgataaaacagaatttgcctggcggcagtagcgcggtggtcc
cacctgaccccatgccgaactcagaagtgaaacgccgtagcgccgatggtagtgtggggt
ctccccatgcgagagtagggaactgccaggcatcaaataaaacgaaaggctcagtcgaaa
gactgggcctttcgttttatctgttgtttgtcggtgaacgctctcctgagtaggacaaatccg...
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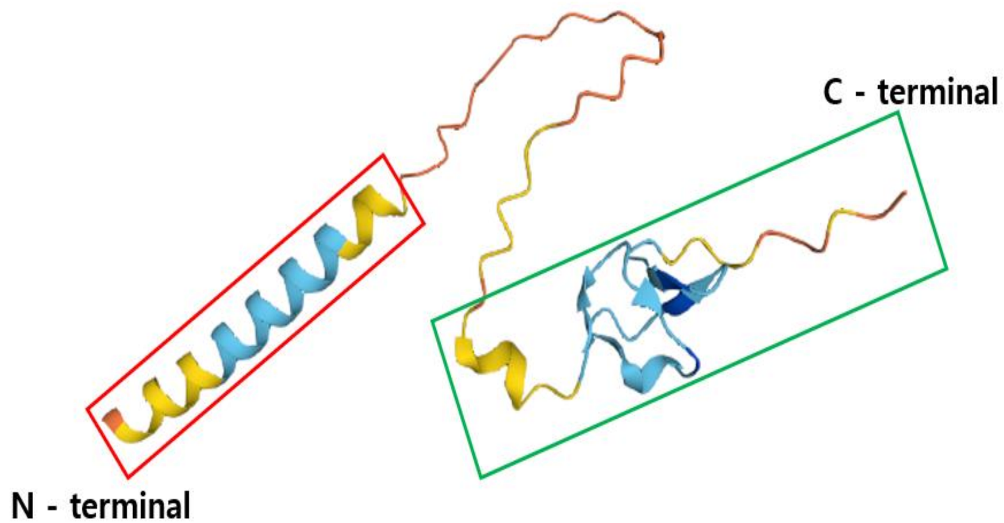
Fig. 5. Nucleotide and its coding sequence of the gene encoding recombinant olive flounder LEAP−2 cloned into pET−44a$^{(+)}$−vector. The green square represents mature peptide sequence, the black square indicates histidine tag



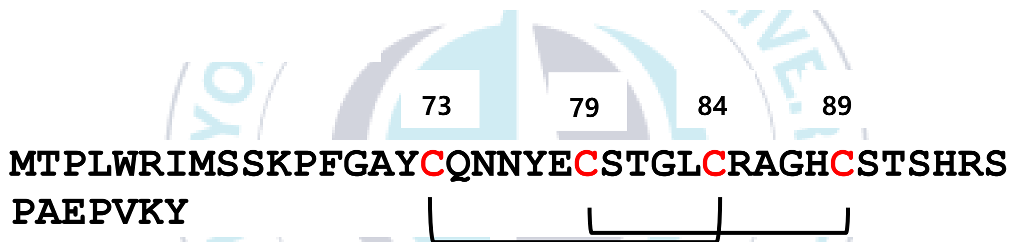
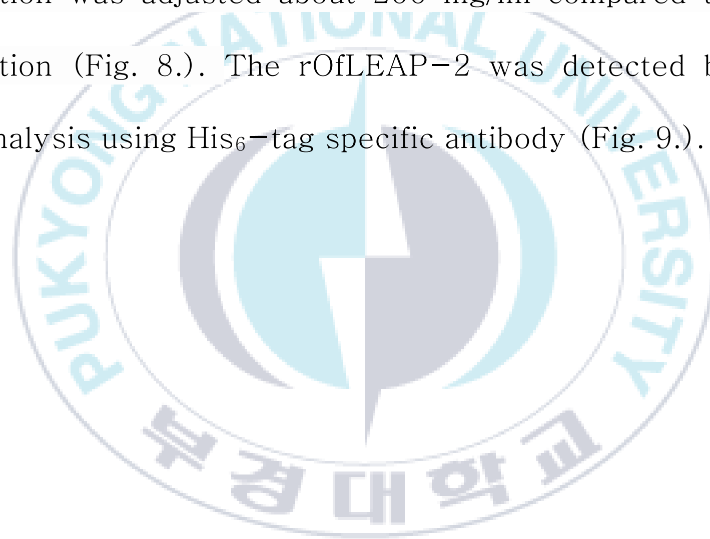


Fig. 6. The predicted 3D structure model of LEAP−2 protein from olive flounder, *Paralichthys olivaceus*. The red square region indicates signal peptide (1 – 29 amino acids), the green square represents mature peptide (58 – 102 amino acids) of OfLEAP−2. Four cysteine residues are shown in red letters and the predicted disulfide bonds are shown in nodes.

2. Purification of rOfLEAP-2 protein

Recombinant olive flounder LEAP-2 protein fused with his_6-tagging at the C-terminus was expressed from *E. coli* BL21 (DE3 codon plus) molecular weight of rOfLEAP-2 was estimated to be about 9.2 kDa size band in SDS-PAGE (figure 7). Purified recombinant protein concentration was adjusted about 200 mg/ml compared to the BSA concentration (Fig. 8.). The rOfLEAP-2 was detected by western blotting analysis using His_6-tag specific antibody (Fig. 9.).



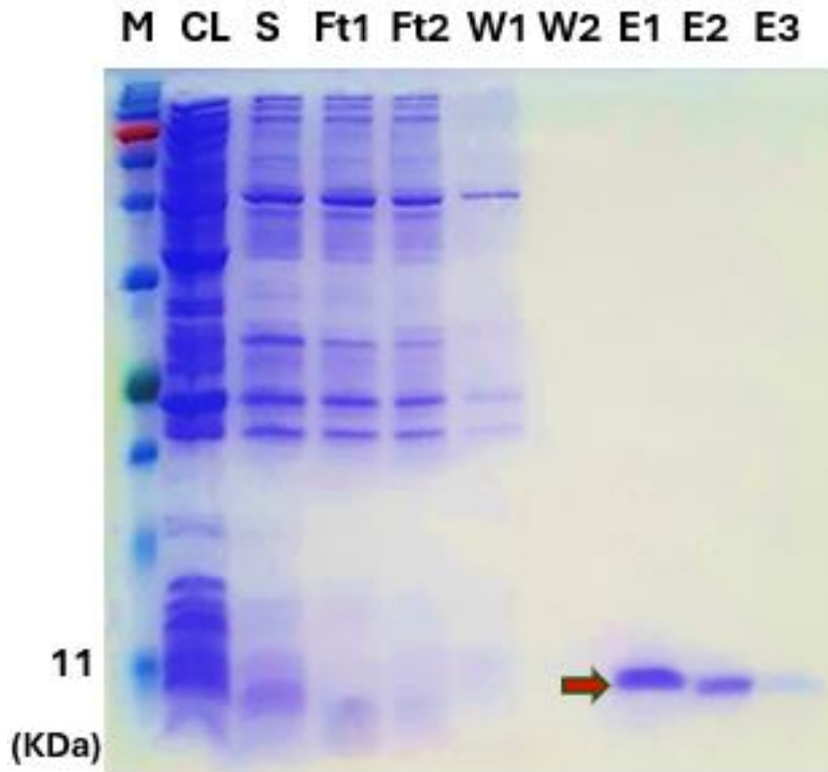


Fig. 7. SDS-PAGE (15% acrylamide gel) profiles of the fractions obtained during purification of recombinant olive flounder LEAP-2. CL: Cell lysate, S: Supernatant, Ft: Flow through, W: Wash, E: Elution.

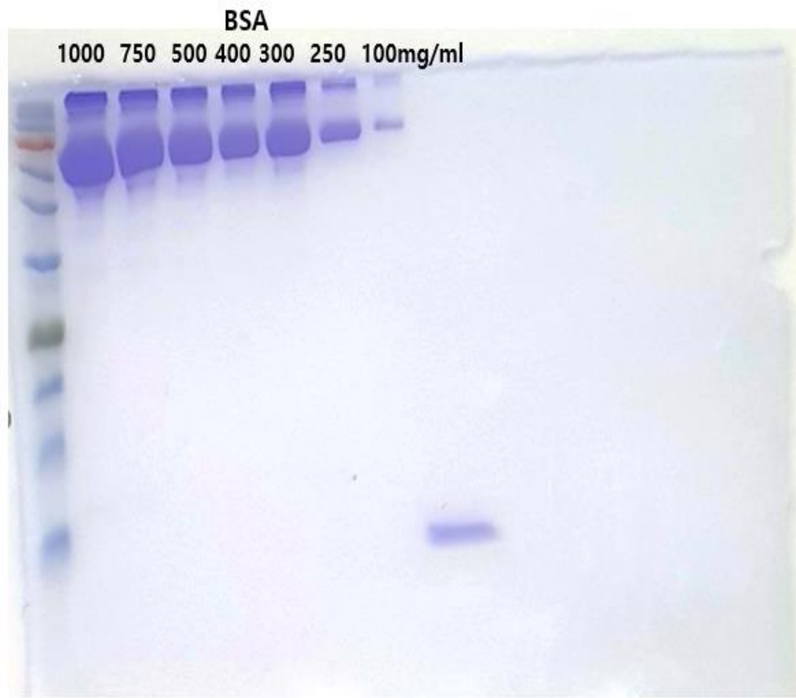


Fig. 8. SDS-PAGE profiles of recombinant olive flounder LEAP-2. Concentration of purified recombinant olive flounder LEAP-2 sample was compared with BSA.

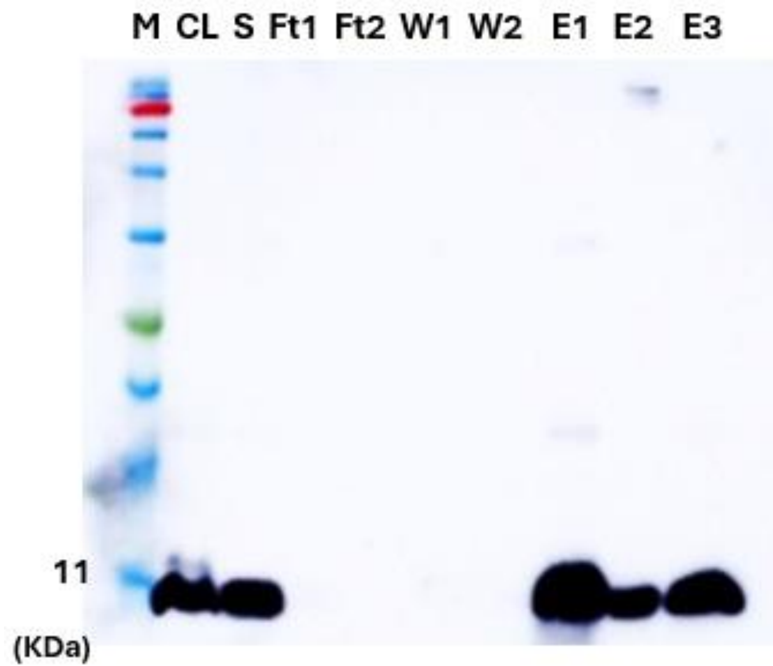


Fig. 9. Western blotting analysis of recombinant olive flounder LEAP−2 fused with His_6−tag in the fraction obtained from the purification. M: Marker, CL: Cell lysate, S: Supernatant, Ft: Flow through, W: Wash, E: Elution.

3. Antimicrobial activity

3-1. URDA

Antimicrobial activity of purified recombinant olive flounder LEAP-2 was tested by using ultrasensitive radial diffusion assay (URDA). Ampicillin (1.4 uM to 5.7 uM) was used as positive control together with 1 X LEW and autoclaved diluted water as negative controls. The rOfELAP-2 sample loaded from 2.7 uM to 21.6 uM showed antimicrobial activity against Gram-positive (*B. subtilis, S. parauberis, L. garvieae*) (Fig. 10.) and Gram-negative (*E. coli, V. harveyi*) bacteria (Fig. 11). The result showed the most sensitive antimicrobial activity of rOfLEAP-2 against *B. subtilis.* The minimum inhibitory concentration (MIC) exerted by 2.7 uM of the purified of recombinant olive flounder LEAP-2 was similar to the activity of range within 1.43 uM and 2.86 uM of ampicillin. The MIC for the tested bacteria were summarized as follows (Figure 12).

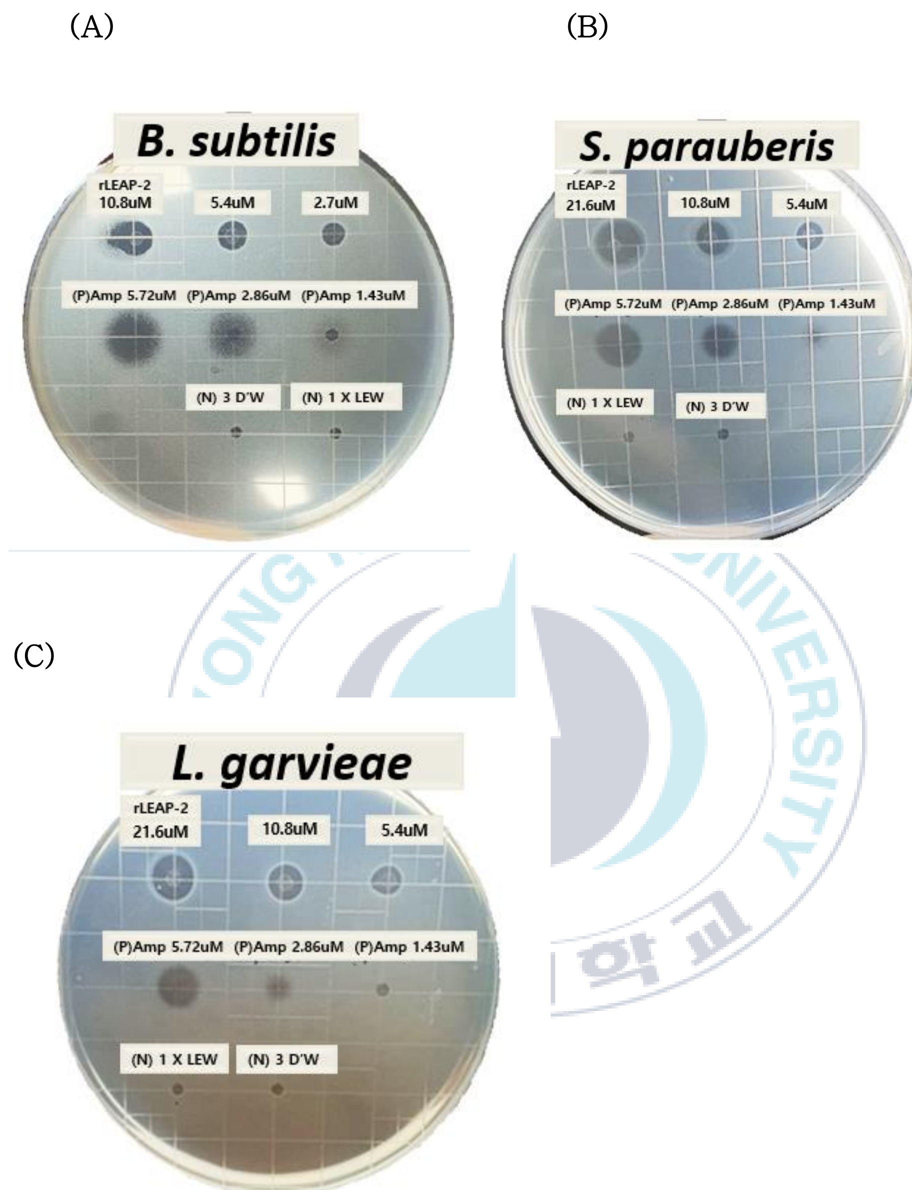


Fig. 10. Antimicrobial activity of purified recombinant olive flounder LEAP-2 against Gram positive bacteria. (A) *B. subtilis* (B) *S. parauberis,* (C) *L. garvieae,* (P) Positive control, and (N) Negative control. Wells diameter: 2.4mm

(A) (B)

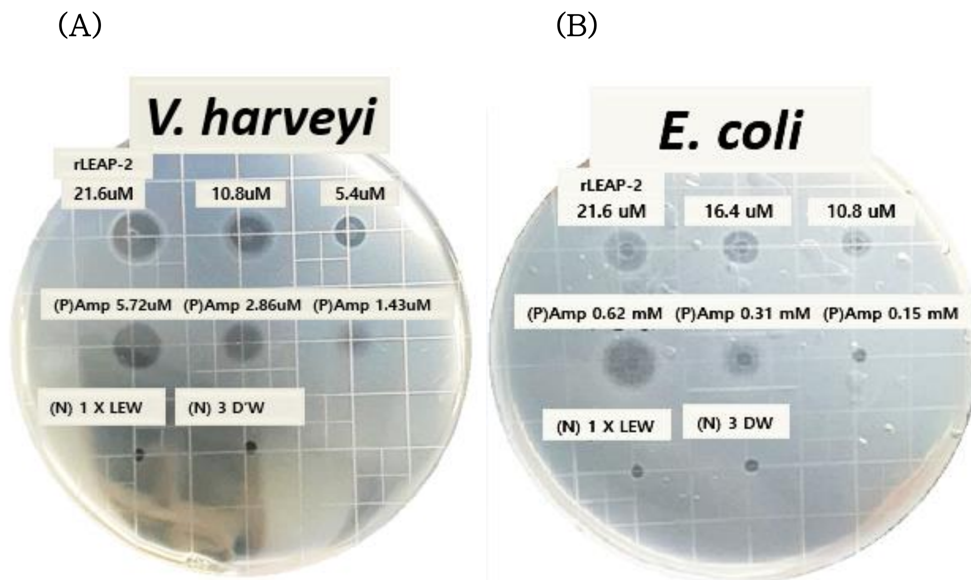


Fig. 11. Antimicrobial activity of purified recombinant olive flounder LEAP-2 against Gram negative bacteria. (A) *V. harveyi,* (B) *E. coli,* (P) Positive control, and (N) Negative control. Well diameter: 2.4 mm

Bacteria	Gram (+) / (-)	Culture medium	Culture temperature (℃)	MIC (ug/ml)
B. subtilis (KCTC1021)	(+)	TSB	37	25 ≤
S. parauberis (KCTC3651)	(+)	TSB (1% Nacl)	28	50 ≤
L. garvieae (KCTC5617)	(+)	TSB	30	50 ≤
V. harveyi (KCTC12724)	(-)	TSB (1% Nacl)	28	50 ≤
E. coli (ML35)	(-)	LB	37	50 ≤
E. tarda (KCTC12267)	(-)	LB	30	100 ≤

Table 2. Antimicrobial activities of olive flounder LEAP-2 *in vitro*.

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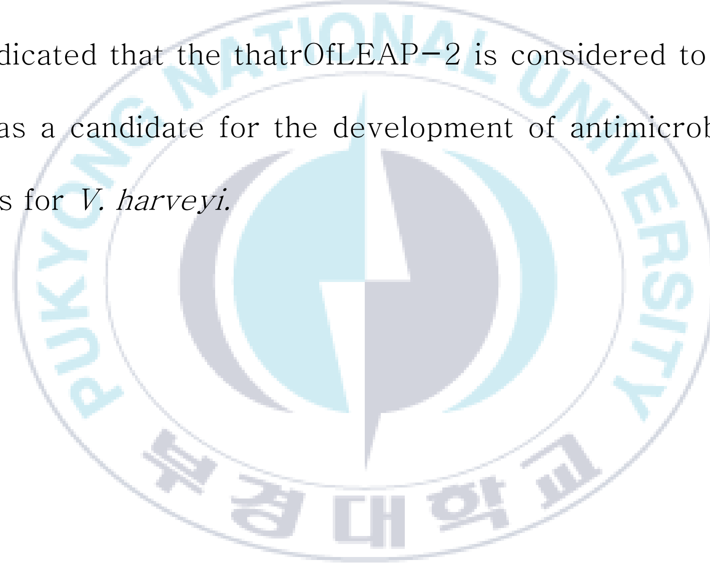
3-2. Colony counting method

Both OfLEAP-1 and rOfLEAP-2 showed antibacterial activity as confirmed by the ratio of decreased colonies of *V. harveyi* and *S. parauberis*. The result showed a higher antimicrobial activity of rOfLEAP-2 against *V. harveyi*, but a lower antimicrobial activity against *S. parauberis* as compared to those of LEAP-1 (Figure 12). T results indicated that the thatrOfLEAP-2 is considered to have more potential as a candidate for the development of antimicrobial peptide treatments for *V. harveyi*.



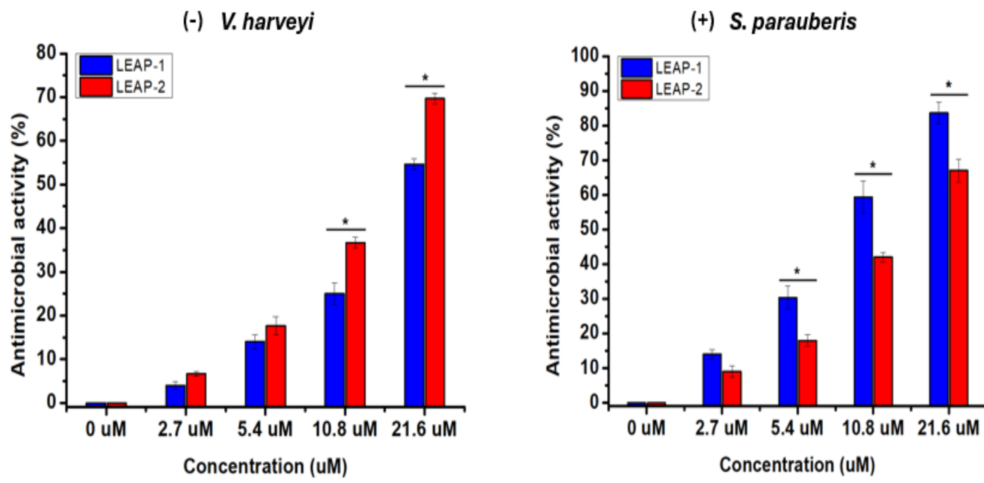


Fig. 12. Antimicrobial activity (%) was represented as the number of colonies decreased compared to the standard (10^4 CFU/ml). Blue graph : LEAP−1, Red graph : LEAP−2.

3−3. Synergistic effect analysis

Combination of recombinant olive flounder LEAP−2 and LEAP−1 and ampicillin showed a synergistic effect in antimicrobial activity against *V. harveyi* and *B. subtilis,* respectively. This was confirmed by the combinational usage of recombinant olive flounder LEAP−2 and LEAP−1 and ampicillin leading to FICI values of 0.375 against *V. harveyi* and 0.5 against *B. subtilis* (Figure 13). The recombinant LEAP−2 and tested materials (LEAP−1 and ampicillin) led to a lower minimum effective concentration for each agent when they were used in combination. Combined with the membrane−disrupted action of LEAP−2 the synergistic effects between LEAP−2 and drugs are likely attributed to the LEAP−2 mediated permeation of LEAP−1 and ampicillin from outer membrane to cytoplasmic targets.

Synergistic effect test of drugs and olive flounder LEAP-2 against Gram (+) & (-) bacteria					
Microorganism	**Drug combination**	**MIC**		**FIC**	**FICI**
		Alone	combination		
B. subtilis (+)	Ampicillin	0.5	0.125	0.25	0.5
	LEAP-2	25	6.25	0.25	
V. harveyi (-)	LEAP-1	62.5	7.8	0.125	0.375
	LEAP-2	50	12.5	0.25	

Fig. 13. The fractional inhibitory concentration index (FICI) was calculated for each combination using this equation: FICI = FICA + FICB, where FICA = MIC of drug A in combination/MIC of drug A alone, and FICB = MIC of drug B in combination/MIC of drug B alone. FICI of ≤0.5 was interpreted as synergy, 0.5 < FICI ≤ 1.0 as additive, 1.0 < FICI ≤ 4.0 as indifferent, and FICI > 4.0 as antagonism. Concentrations represent mean values of three independent experiments performed in duplicates.

3-4. Outer membrane permeability test (NPN uptake)

In order to test whether antimicrobial activity of rOfLEAP-2 is due to perturbation of membrane structure in the targeting bacteria, membrane permeability of the *E. coli* (ML35) was carried out by NPN uptake assay. This was analyzed by NPN that was excited at 350 nm and fluorescence intensity recorded at 420 nm. The result showed an increase in fluorescence upon treatments with LEAP-2 as consistent with a positive control, piscidin. Level of permeability by recombinant olive flounder LEAP-2 was estimated to be 68% cell permeabilization as compared to that of piscidin. (Figure 14). The results indicated that LEAP-2 represents antibacterial activity through binding with the outer membrane of bacteria.

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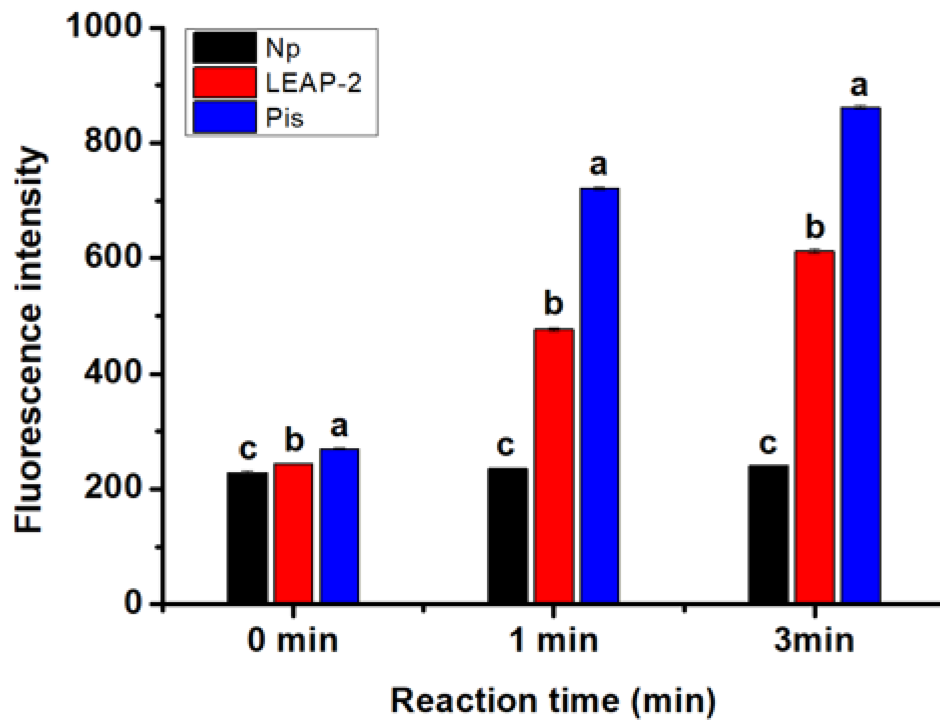


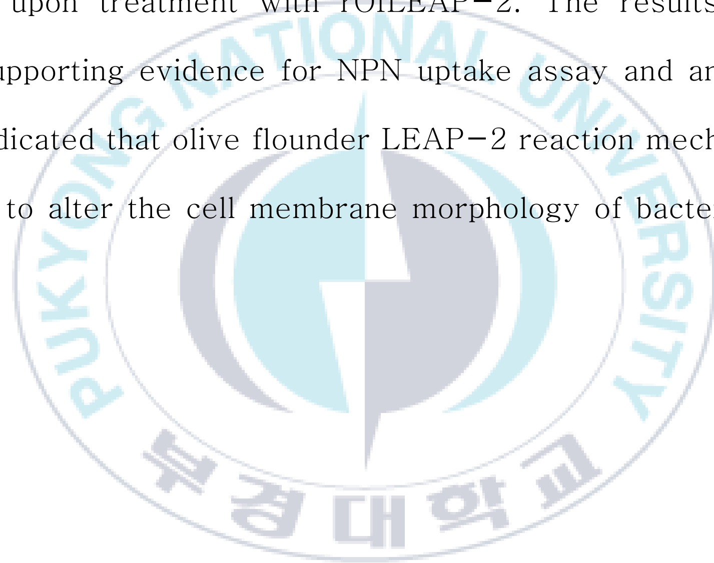
Fig. 14. NPN uptake assay of recombinant olive flounder LEAP−2 protein expressed from *E. coli*. The bacterial outer membrane permeability of rOfLEPA−2 was monitored for 3 min

Np: mixture of *E. coli* & NPN, Pis: Piscidin.

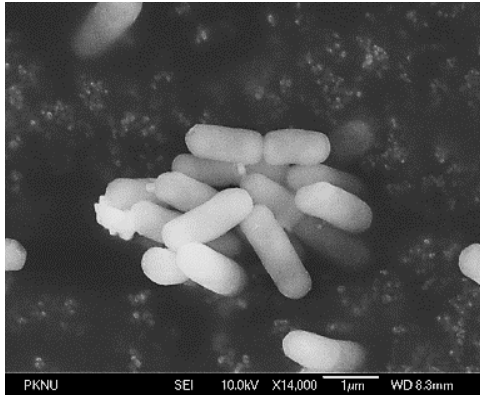
The rOfLEAP−2 and piscidin were used at concentration of 100 ug/ml

3-5. SEM

Scanning electron microscopic analysis was also carried out to further confirm NPN uptake assay results implicating an alteration in the structure of cell membrane by recombinant OfLEAP-2. The effect of damaging the cell membrane structure was tested by using *E. coli* (ML35). A clear impairment in the structure of membrane was observed upon treatment with rOfLEAP-2. The results providing further supporting evidence for NPN uptake assay and antimicrobial assays indicated that olive flounder LEAP-2 reaction mechanism was predicted to alter the cell membrane morphology of bacteria (Figure 15).



(A)



(B)

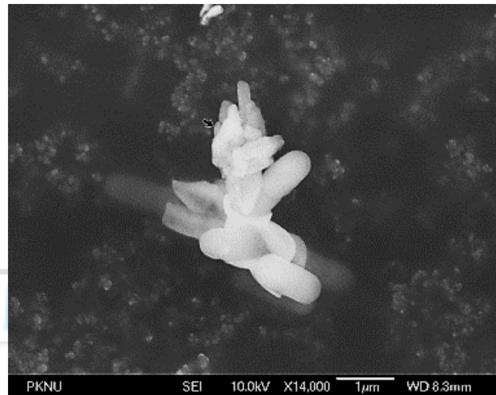


Fig. 15. Effects of olive flounder LEAP-2 on the surface morphology of *E. coli* (ML35). (A) No peptide group (control), (B) LEAP-2 peptide treated group.

Ⅳ. Discussion

Antimicrobial peptides (AMP) are one of the defense mechanisms used for life in nature, and research on them contributes to understanding environmentally friendly life phenomena in nature. New antibacterial peptides with high antibacterial properties that have been quickly explored and secured with the recent development of screening techniques are likely to be applied to the development of medical materials that imitate anticancer, and antiviral drugs, and biological functions in the future. In particular, marine life, unlike terrestrial life, is directly exposed to a large number of microorganisms present in the ocean, showing more interest in exploring and securing highly antibacterial peptides derived from new marine life that have not been known so far. As water temperature rises due to global warming, the risk of pathogenic bacterial infection in aquaculture organisms has been increasing. As a result, antibiotic use is increasing in fish farms, which poses a risk of antibiotic resistance and resistant bacteria. Therefore, it is necessary to develop alternatives to the next generation of antibiotics. In this study, we explored the function of recombinant olive flounder LEAP-2. The rOfLEAP-2 showed activity in a wide range of Gram-positive (*B. subtilis, S. parauberis, L. garvieae*) and Gram-negative (*E. coli, V. harveyi*) bacteria. Also, synergistic effect represented with ampicillin and LEAP-1 in *B. subtilis, V. harveyi.* In

NPN uptake assay, the rOfLEAP−2 showed outer membrane permeability 68% of piscidin. A significant difference was found between the 0 min groups, which is thought to be the result of the delay between the operation times of the fluorescent spectrometer for analysis of the sample. In view of these results, it is thought that synergy effects appeared through different mechanisms with ampicillin and LEPA−1. (Yue Chen et., al 2020). In fact, ampicillin inhibits cell membrane synthase, inducing cell death and LEAP−1 bind to transition metal necessary for sustain life, so induce apoptosis. Therefore, the recombinant olive flounder LEAP−2 has a potential to be developed as a biopharmaceutical in the future, and it is thought that additional research such as hemolytic activity and challenge experiments is needed to evaluate this.

Ⅳ. Abstract (Korean)

넙치(*Paralichthys olivaceus*) 유래 재조합 간 발현 항균 펩타이드-2의 항균활성 분석

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척추동물 계통에서 하위 분류에 속하는 진골어류는 상위의 고등 포유류에 비해 후천적인 면역 시스템을 가지고 있다. 간 발현 항균 펩타이드-2 는 어류에서 선천 면역에 중요한 역할을 하는 것으로 알려진 시스테인이 풍부한 펩타이드이다. 본 연구에서, 넙치의 LEAP-2 를 암호화하고 있는 유전자의 조직 별 발현 패턴을 확인하기 위해 RT-PCR 을 수행하였다. 넙치의 간에서 추출된 RNA 를 이용하여 합성한 cDNA 로부터 LEAP-2 의 신호 펩타이드를 제외한 나머지 영역을 암호화하는 유전자를 얻었다.

증폭된 LEAP-2 유전서열은 C 말단에 6 개의 히스티딘을 표지하는 발현벡터 pET-44a$^{(+)}$로 삽입되었으며 이후 재조합 LEAP-2 를 대장균(BL21 -DE3 codon plus)에서 IPTG 를 이용해 과발현시켰으며 SDS-PAGE 와 Western blotting 분석을 통해 발현 유무를 확인하였다. 재조합 LEAP-2 는 Ni-TED 컬럼을 사용하여 정제되었으며 어류의 병원성 수생균을 포함한 그람 양성(*B. subtilis, L. garvieae, S. parauberis*) 및 음성균(*E. coil, V. harveyi, E. tarda*)에 대해서 항균활성을 분석한 결과 정제된 재조합 넙치 LEAP-2 는

테스트균 중 *E. tarda* 를 제외하고 모든 시험 균에서 항균활성을 보였다. 또한 넙치의 재조합 LEAP-2 단백질과 ampicillin 및 넙치 유래 LEAP-1 과의 농도 별 조합에 대한 항균활성 분석 결과에서 각각 *B. subtilis, V. harveyi* 에 대해 시너지 효과를 나타내었으며 이는 분할 억제 농도 지수(FICI) 계산을 통해 검증되었다. 수생 균인 *V. harveyi* 와 *S. parauberis* 균에 대해서 LEAP-1 과 재조합 LEAP-2 의 항균활성 비교 결과 *V. harveyi* 균에서 재조합 LEAP-2 단백질이 LEAP-1 보다 더 높은 항균 활성을 나타내었다. 펩타이드의 작용 메커니즘을 조사하기 위해 세포 외막 투과성 테스트인 NPN uptake assay 를 실시한 결과는 강한 외막 투과성 항균 펩타이드로 알려진 Piscidin 대비 68%의 투과성 수치를 나타내었다. 세포막의 형태변화의 시각적 확인을 위해 대장균 시료를 전처리 후 주사전자현미경 분석법을 통해 세포막의 붕괴를 관찰한 결과, 넙치의 재조합 LEAP-2 항균활성은 세포막을 파괴시키는 기작에 기인함을 확인하였다. 본 연구의 결과는 넙치 유래의 LEAP-2 단백질이 병원성 세균에 대한 치료 물질로서의 가능성이 있는 것으로 생각되어진다.

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